PYRUVATE DEHYDROGENASE COMPLEX -
CORRELATION BETWEEN STRUCTURE AND
FUNCTION

by

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A thesis submitted in conformity with the requirements
for the degree of Doctor of Philosophy
Graduate Department of Biochemistry
University of Toronto

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Human PDH complex deficiency is an extremely heterogenous disease in its presentation. Very little correlation between clinical course and molecular characteristics has been established so far. In order to more accurately define the structure of the complex, mutational analyses were performed on its two components, PDH-E₁α and protein X. A series of PDH-E₁α deletion mutants was constructed and expressed in a cell line with zero PDH complex activity due to a null E₁α allele (the π₀ cell line). Sequential deletion of the C-terminus by 1, 2, 3 and 4 amino acids resulted in PDH complex activities of 100, 60, 30 and 14% compared to wild-type E₁α expressed in PDH complex deficient cells. In addition the somatic and the testis-specific PDH-E₁α human isoforms were determined to be biochemically equivalent. Furthermore, a series of PDH-E₁α histidine mutants (H63, H84, H92 and H263) was constructed and expressed in the π₀ cell line. H63 and H263 were shown to be essential for the PDH complex activity, while H92 was implicated in the PDH-E₁α₂β₂ heterotetramer formation and/or stability. A novel PDH-E₁α mutation R141L was characterized in a male patient and it was found in about 75% of the studied DNA derived from various tissues (fibroblasts, liver and muscle). This scenario would indicate a case of E₁α somatic mosaicism. The R141L mutation is a severe one and it must have occurred in
one of the E₁α alleles during early embryogenesis. To gain further insight into the nature and function of the domains of the human protein X, two artificially created variants, K37E and S422H as well as the wild-type were expressed in SV40-immortalized protein X-deficient and E₂-deficient human skin fibroblasts. The former mutant does not carry the lipoic acid moiety; the latter mutant was designed to investigate the possibility that protein X could exhibit an intrinsic transacetylase activity. None of the expressed protein X variants had an effect on the specific activity of the PDH complex, suggesting that the human protein X plays a purely structural role. A novel syndrome affecting multiple mitochondrial functions was described and localized by microcell-mediated transfer to chromosome 2p14-2p13.
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<td>adenosine diphosphate</td>
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<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
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<tr>
<td>b.p.</td>
<td>base pairs</td>
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<td>BCKDHC</td>
<td>branched chain alpha-keto acid dehydrogenase</td>
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<tr>
<td>E₃</td>
<td>dihydrolipoamide dehydrogenase</td>
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<td>GDG^{26/27}NN</td>
<td>thiamin diphosphate bindig motif. Three invariant amino acid residues, GDG are separated by either 26 or 27 variable residues from an invariant duet of asparagines (NN)</td>
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<td>ISP</td>
<td>iron-sulphur protein</td>
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<tr>
<td>$K_m$</td>
<td>rate constant</td>
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<td>polyacrylamide gel electrophoresis</td>
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<td>pyruvate dehydrogenase phosphatase</td>
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<td>RT</td>
<td>reverse transcriptase</td>
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<tr>
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<td>sodium dodecyl sulfate</td>
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<tr>
<td>TCA cycle</td>
<td>tricarboxylic acid cycle</td>
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<tr>
<td>ThDP</td>
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<tr>
<td>Ts</td>
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NOTICE

All references made to the amino acid sequence of either PDH-E₁α or BCKDH-E₁α are to the mature protein. Therefore to obtain residue numbers relative to the pre-cursor polypeptides add 29 residues.
CHAPTER 1

INTRODUCTION
1.1 WHAT ARE MITOCHONDRIA

Mitochondria are the cells' power sources. They are distinct organelles with two membranes. Usually they are rod-shaped, however they can be round or form a tubular reticulum. Mitochondria contain two membranes, separated by a space. The outer membrane limits the organelle. The inner membrane is organized with folds or shelves that project inward. These are called "crystalline mitochondrial". Inside the space enclosed by the inner membrane is the matrix which appears to be moderately dense with DNA, ribosomes, or small granules. The mitochondrial DNA codes for some of its essential protein components. Mitochondrial enzymes carry out many metabolically important chemical reactions. These include the TCA cycle, fatty acid oxidation, ATP synthesis, beginning of the urea cycle, non-essential amino acid synthesis, ketone bodies synthesis, Ca$^{2+}$ sequestration and many more (fig. 1.1).

1.2 2-OXO ACID DEHYDROGENASES – OVERVIEW

2-Oxo acid dehydrogenase complexes convert 2-oxo acids to the corresponding acyl-CoA derivatives and produce NADH and CO$_2$ in an irreversible reaction. Five members of this family are known at present, the pyruvate dehydrogenase complex (PDHC), the 2-oxoglutarate dehydrogenase complex (2-OGDHC), the branched-chain alpha-keto acid dehydrogenase complex (BCKDHC), the glycine cleavage system (GCS) and the acetoin dehydrogenase complex (ADHC). They function at strategic points in, usually aerobic, catabolic pathways and are therefore subject to stringent control. With the exception of GCS the constitution of these complexes is based on common principles where many peripheral components of the same type bind to a central core with octahedral (24 subunits) or icosahedral (60 subunits) symmetry. The molecular mass depends on the particular
Figure 1.1. Schematic drawing of a mitochondrion. Shown are various compartments and components of the Electron transport chain I-IV and the ATPase (marked in red). Also shown are the TCA cycle, the PDH complex, the import machinery (TOM, TIM23 and TIM22). Grey ellipse symbolizes various transporters for substances going in and out of the mitochondrion.
species and ranges from 0.7 to more than 10 MDa. Within this common architectural design there are many variations [Patel et. al 1996].

1.3 THE PYRUVATE DEHYDROGENASE COMPLEX

Pyruvate dehydrogenase complex (PDHC) converts pyruvate to acetyl-CoA by the combined action of three enzymes, pyruvate dehydrogenase (E₁), dihydrolipoamide transacetylase (E₂) and dihydrolipoamide dehydrogenase (E₃), thereby linking glycolysis with the TCA cycle (Fig. 1.2 A & B).

A
Fig. 1.2. Position of the pyruvate dehydrogenase complex (PDHC) within cellular metabolism (A). DHAP: dihydroxyacetone phosphate. GA-3-P: glyceraldehyde-3-phosphate. Reaction sequence of PDHC (B). Pyruvate dehydrogenase (E₁) uses thiamin diphosphate (ThDP) to carry out the oxidative decarboxylation of the substrate with formation of acetyl lipoic acid, covalently attached to the dihydrolipoamide transacetylase (E₃) component. The E₃-component catalyses the transfer of the acetyl group to coenzyme A. The resulting dihydrolipoyl group is then reoxidized by NAD⁺, catalyzed by dihydrolipoamide dehydrogenase (E₅).

Although the subunit organization of prokaryotic and eukaryotic PDH complexes is similar, the mechanisms involved in their regulation are relatively complex. Bacterial PDH complexes are regulated only by allosteric inhibition and activation (by ATP and AMP respectively) as well as by product inhibition. In contrast, the activity of most eukaryotic PDH complexes is regulated by a phosphorylation-dephosphorylation cycle carried out by endogenous regulatory enzymes: a PDH-specific kinase and a PDH-specific phosphatase [Reed 1974, Patel et. al. 1996]. Putative orthologs of the PDH kinase have been found in *S. cerevisiae*, *C. elegans*, *D. melanogaster* and in *A. thaliana* [Hunter et. al. 1997, Bateman et.
al. 1999]. Eukaryotic but not prokaryotic PDH complexes contain an additional component, protein X, whose role and origins will be discussed in section 1.7.

The probable evolutionary precursor of the PDH complexes is the pyruvate ferredoxin oxidoreductase seen in present day prokaryotic archaea bacterium. Archaea are organisms capable of living under the extreme conditions, which prevailed at the early stages of life evolution. Instead of using the PDH complex to metabolize pyruvate, they utilize a pyruvate ferredoxin oxidoreductase, which possesses a \( \alpha_2\beta_2 \) structure containing two iron-sulphur clusters and two thiamin diphosphates but no lipoic acid-containing \( E_2 \) and no flavin binding \( E_3 \). The thiamin diphosphate-binding motif is located on the alpha subunit while the beta subunit contains ferredoxin-type [4Fe-4S] cluster binding motif (CXXCXXCXXXCP). The reducing equivalents are transferred via a ferredoxin to cytochrome c and the acetyl residue is transferred coenzyme A. There are no significant homologies between the alpha or the beta subunits of pyruvate ferredoxin oxidoreductase and pyruvate dehydrogenase with the exception of thiamin diphosphate binding motif (see below). [Kerscher and Oesterhelt 1981, Kerscher and Oesterhelt 1982, Plaga et. al. 1992].

1.4 \( E_1 \)

The eukaryotic pyruvate dehydrogenase component (\( E_1 \)) catalyses the decarboxylation of pyruvate coupled to the reductive acetylation of the \( E_2 \)-bound lipoyl groups (fig 1.2B). It is a hetrotetramer of two \( \alpha \) and two \( \beta \) subunits with apparent molecular weights of 41 kDa and 36 kDa, respectively [Reed 1974]. Two isoforms of \( E_1\alpha \) exist in humans: the somatic isoform with the locus on the X chromosome and the testis-specific isoform with the locus on chromosome 4 (see section 1.6 below). \( E_1 \) requires thiamin
diphosphate (ThDP) and Mg$^{2+}$ ions as essential cofactors [Sable and Gubler 1982]. During the reaction pyruvate is rapidly decarboxylated and produces a thiamin diphosphate-bound enamine. The enamine then reacts with lipoamide to form a tetrahedral intermediate. Rearrangement results in the formation of acetyl dihydrolipoamide and thiamin diphosphate (ThDP). Due to the lack of structural data of this reaction scheme is still hypothetical, but recent model studies support this mechanism [Chiu et. al. 1996]. The rate of reductive acetylation of the lipooyl group has been measured by chemical quench rapid mixing experiments, using $^{14}$C pyruvate as substrate. The observed rate is comparable to the rate of the overall reaction, which suggests that reductive acetylation represents the rate-limiting step in the overall reaction [Danson et. al. 1978, Berg et. al. 1998].

The enzyme shows high specificity for its 2-oxo acid substrate and for the folded lipooylated lipoyl domain. Free lipoamide or lipoyl-containing peptides are very poor substrates for the acetylation reaction [Graham et. al. 1989].

Although much is known about ThDP catalysis (see below) very little structural information on the E$_1$ component is available. Amino acid sequence alignments of E$_1$ with other ThDP-binding enzymes, revealed that this subcomplex contains a β-turn-α-turn-β structural motif, GDG$^{26-27}$NN (in the primary sequence three amino acid residues, GDG, separated by 26 or 27 variant residues from two invariant NN residues) that is responsible for the binding of thiamine diphosphate and Mg$^{2+}$ cofactors [Hawkins et. al. 1989, Dyda et. al. 1993, Arjunan et. al. 1996, Lindquist et. al. 1992, Muller and Schulz 1993]. Partial information about the three dimensional structure was provided by the recently solved crystal structure of the heterotetrameric (α$_2$β$_2$) E$_1$, BCKDH complex from Pseudomonas putida which reveals a tightly packed arrangement of the four subunits with the β$_2$-dimer held
between the jaws of a ‘vise’ formed by the $\alpha_2$-dimer. A long hydrophobic channel, suitable to accommodate the $E_2$ lipoyl-lysine arm, leads to the active site, which contains ThDP [Aevärsson et. al. 1999]. Each heterotetramer contains two ThDP molecules that are positioned at the interface between the $\alpha$ and the $\beta$ subunits. Possible amino acid residues involved in the formation of the ThDP active site as well as the heterotetramer formation were investigated as a part of this thesis (see chapters 2 & 3). Results of this investigation as well as results obtained by other laboratories support the above-described structure [Seyda and Robinson 2000, Seyda et. al. 2000, Ali et. al. 1993, Brown et. al. 1997].

Alignments of $E_i\alpha$ and $E_i\beta$ primary sequences have indicated additional conserved sequences, one in $E_i\alpha$ that may be involved in subunit interaction with $E_i\beta$, one in $E_i\beta$ that may be involved in the binding of $E_2$, and others with no obvious function (for more details see section: mutational studies of the PDH-$E_i$, below) [Wexler et. al. 1991]. However, until a crystal structure is obtained for the $E_i$ of the pyruvate dehydrogenase complex, all structural predictions must remain speculative.

### 1.5 THIAMIN METABOLISM

In 1930, W. Langenbeck found that primary amines such as thiamin diphosphate can act as catalysts in the decarboxylation of 2-oxo acids [Langenbeck and Hutshenreuther 1930]. It is proposed that the coenzyme binds to the active side in the V-conformation in the tautomeric amidine structure. Groups which play essential roles in the catalytic mechanism of ThDP are the $C_2=N^+$ group of the thiazolium ring and the $4'$-amino pyridine ring system (fig. 1.3A) [Schellenberger 1982]. The $C_2=N^+$ group provides the real catalytic center of the cofactor. Its thermodynamic ($pK_a$) and kinetic properties (C-H dissociation rate), together
with the ability to function as a sink for mobile electrons give the thiazolium system a distinct advantage as a catalyst. Stetter [Stetter 1976] has described the type of charge reverse catalysis used by ThDP in detail. The initial driving power of the catalytic mechanism originates from the high nucleophilic potential of the C₂ carbanion (ylid form of ThDP) in contact with the electrons of the attached sulfur atom. A strategically placed histidine molecule (His 128β in fig 1.3 B) is thought to be very important in creation of the ylid form in the human E₁ [Chun and Robinson 1993]. The rate of catalysis is further increased by the interaction with strategically placed acidic and basic residues in the apoprotein [Schellenberger et. al. 1997, Kerr et. al 1997]. The 4' -amino pyridine ring system assists in the essential steps of the enzyme reaction. An abstraction of the N⁷⁺ proton by the protein (glu 59β anion – fig1.3 B- in hydrophobic surroundings) could transfer the negative charge to the 4'-positioned nitrogen, thus qualifying the latter to bring about the observed rate enhancement [Harris and Washabaugh, 1995a, Harris and Washabaugh 1995b, Washabaugh et al. 1994, Lobel et. al. 1996]. Pyruvate is added to the C₇-position of ThDP accompanied by the protonation of the 2-oxo group. The acetyl adduct is then eliminated from that position by the deprotonation of the corresponding α-hydroxy group of 2-(1-hydroxyethyl)-ThDP conjugate.

All ThDP binding enzymes require bivalent metal ions (mostly Mg²⁺ or Ca²⁺) for coenzyme binding (and therefore activity). The only function of the metal ion consists of complexing the pyrophosphate group to a conserved group of side chains in the pyrophosphate binding area of the different apoenzymes [Muller et. al. 1993]. All the documented X-ray crystal structures show the coenzyme placed at the interface between the subunits. By anchoring with the pyrophosphate group and the aminopyrimidine moiety in
Fig. 1.3. The chemical structure of thiamin pyrophosphate in its ylid form (A); Proposed mechanism of ThDP-assisted decarboxylation of pyruvate by human E₁ (B). Shown are residues postulated to participate in the catalysis (His₁₂₈β and Glu₅₉β) as well as residues involved in binding the Mg²⁺ cofactor and the pyrophosphate tail of the ThDP molecule.
different attached subunits, dimers are formed as the minimum functional units of ThDP enzymes [Muller et. al. 1993]. In this way, the coenzyme plays also a crucial role as linker group between neighboring subunits and consequently in the formation of the correct quaternary structures. As a result of this coenzyme linking, quaternary structures of ThDP enzymes are generally found to be very sensitive to pH, ionic strength and certain anions like $\text{SO}_4^{2-}$, $\text{PO}_4^{3-}$ or $\text{Cl}^-$ [Konig et. al. 1992]. Other molecular properties, like the aggregation of the tetramers to the active form oligomers also depend on these factors [Mucke et. al. 1996]. The fragility of the normal quaternary structures of ThDP enzymes may be one of the reasons that many of the early attempts to crystallize ThDP enzymes had failed.

Despite the depth of knowledge with respect to its role as an essential metabolic cofactor many of the genetic aspects of thiamin biosynthesis and metabolism remain unresolved. This is especially surprising since thiamin auxotrophs were amongst the earliest nutritional mutants isolated from a variety of organisms, including bacteria, fungi and plants [Beadle and Tatum 1941, Langridge 1994]. Higher eukaryotes are unable to synthesize their own cofactor and are therefore dependent on external sources as vitamin $\text{B}_1$ (thiamin). Some organisms such as $\text{S. cerevisiae}$ are capable of both de novo thiamin biosynthesis as well as acquisition from external sources, thereby presenting a model system for the analysis of both regulatory features and genetic contributors to the ThDP biosynthetic pathway. In addition, the availability of the complete yeast genome sequence, in combination with genetic and biochemical analyses, can be used for the identification and characterization of the full complement of ThDP-dependent enzymes in a eukaryotic organism.

Precursor product studies have shown that in all synthesizing organisms, including $\text{S. cerevisiae}$, thiamin is formed from two precursor molecules, 4-methyl-5-(β-hydroxymethyl)
thiazole (HET) and 2-methyl-4-amino-5-hydroxymethylpyrimidine (HMP) [Begley 1996, Estramareix et. al. 1996]. These are phosphorylated and then condensed together to form thiamin phosphate (ThP) which is subsequently converted to the enzyme cofactor thiamin diphosphate (ThDP). In yeast this final step occurs in two stages, hydrolysis to free thiamin followed by pyrophosphorylation (fig. 1.4).

![Fig 1.4. Schematic outline of thiamin metabolism in yeast. HET, hydroxyethylthiazole; HET-P, hydroxyethylthiazole phosphate; HMP, hydroxymethylpyrimidine; HMP-PP, hydroxymethylpyrimidine diphosphate; ThP, thiamin phosphate; ThDP, thiamin diphosphate.](image)

All of the enzyme activities responsible for carrying out these steps, with the exception of the phosphatase acting on ThDP, have now been characterized in cell-free extracts of S.
cerevisiae and in some cases the proteins have been purified and the corresponding structural genes identified [Brown GK 1970, Kawasaki 1993, Nosaka et. al. 1994, Camiener and Brown 1970, Nosaka et. al. 1999].

The origins of the two precursors, HET and HMP are not fully understood in any eukaryotic organism. This is largely because neither thiamin itself nor its pathway intermediates are accumulated to high levels, estimates are around 30 μg per gram dry weight of cells, thereby making it extremely difficult to distinguish any radiolabeled tracer incorporated specifically into thiamin from the overwhelming unincorporated background levels. However, it is clear that the biosynthetic pathways of prokaryotes and eukaryotes differ [Begley 1996, Estramareix et. al. 1996]. In yeast the most likely precursors of HET are 2-pentulose-5-phosphate, glycine and cysteine and of HMP, histidine and pyridoxine.

As already mentioned above, animals depend on external sources of thiamin as vitamin B₁, which is a water-soluble substance that can be taken into cells across the membrane via carrier-mediated processes [Rose 1988, Rindi 1992, Bettendorff and Winns 1994]. Inside the cells, thiamin is phosphorylated to ThDP. In diseases such as beriberi and Wernicke-Korsakoff syndrome, which are caused by thiamin deficiency, decreases in the activities of one or more of the ThDP-dependent enzymes are thought to account for the impairment of cell functions [Haas 1988]. Moreover, ThDP is the precursor for thiamin triphosphate that is suspected to have a specific role in neuronal activity [Cooper and Pincus 1979, Matsuda and Cooper 1981].

An important aspect of thiamin metabolism in mammals is the regulation of the intracellular levels of four thiamin forms (thiamin, thiamin monophosphate, thiamin diphosphate (ThDP), and thiamin triphosphate) over a narrow range. The main factors
involved in this regulation are membrane-associated thiamin transport systems and the cellular enzyme, thiamin pyrophosphokinase (TPK). The recent isolation of the TPK cDNA from mammalian cells should help elucidate of the thiamin metabolism in higher animals [Nosaka et. al. 1999].

A number of biochemical analyses suggest that several types of thiamin transport systems exist in mammals, and in several cells the driving force for thiamin uptake appears to be its phosphorylation to ThDP [Rose 1988, Rindi 1992, Bettendorff and Winns 1994]. Recently, the mammalian thiamin transport protein, THTR-1, was identified as the gene mutated in thiamin-responsive megaloblastic anemia [Lebay et. al. 1999, Fleming et. al. 1999, Diaz et. al. 1999].

1.6 \( E_1\alpha \) – THE COORDINATE REGULATION OF SOMATIC AND TESTIS-SPECIFIC ISOFORMS

The gene for the somatic isoform of the human \( E_1\alpha \) subunit of the PDH complex is linked to the X-chromosome (\( Pdha-1 \)). This poses some problem in spermatogenesis, where both X and Y chromosomes become transiently inactivated. The behavior of the X chromosome during and after completion of the meiotic division in spermatogenetic cells is still poorly understood. During the early-pachytene stage of the meiotic prophase of spermatogenesis, the X and Y chromosomes separate from the autosomes and form the sex body in which the sex chromosomes have a condensation pattern different from that of the autosomes [Sachs et. al. 1954, Solari 1974] and are transcriptionally inactive [Monesi 1965]. The function of the sex body is still unknown. It has been proposed that a regulatory gene product Xist and an orphan receptor gene product GCNF may be transcriptional regulators of
spermatogenesis [Bauer et. al. 1998, Goto et. al. 1998] however, all participants are yet to be discovered. After completion of the meiotic division, the X and Y chromosomes regain their ability to express genes, by as yet unknown mechanism [Nagamine et.al. 1990, Capel et. al. 1993, Conway et. al. 1994, Hendriksen et. al. 1995]. The inactivation of *Pdha-1* in spermatocytes is compensated by testis-specific expression of the autosomal gene *Pdha-2* (chromosome 4) [Brown RM et. al. 1989a, Dahl et. al. 1990], which is inactivated in somatic tissues [Brown RM et. al. 1990, Dahl et. al. 1990]. The demonstration of the X-chromosome localization of PDHA-1 resolved a number of questions concerning the variable phenotype displayed by patients with E$_1$ deficiency (and identified mutations in the E$_1$$\alpha$ subunit). Most patients show a broad range of neurological abnormalities, with the severity showing some dependence on the nature of the mutation in the E$_1$$\alpha$ gene. However, in females, the disorder is further complicated by the pattern of X-chromosome inactivation [Brown RM et. al. 1989b, Brown RM et. al. 1994, Dahl 1995]. The testis-specific gene expressed from chromosome 4 has no role to play in the X-linked deficiency, and no apparent functional differences have been reported for the two isoforms [Jeng et. al. 1998, Seyda et. al. 2000]. The equivalent PDH-E$_1$$\alpha$ genes have also been described in the mouse [Brown RM et. al. 1990 Fitzgerald et. al. 1992]. The mouse genes *Pdha-1* and *Pdha-2* map to chromosomes X and 19 respectively. Thus in most mammals, the existence of an autosomal variant of the E$_1$$\alpha$ subunit ensures that cellular energy requirements are maintained during spermatogenesis.

At the molecular level, little is known about the mechanisms involved in expression and silencing of E$_1$$\alpha$ isoforms in spermatogenesis. Even less is understood about the silencing of the testis-specific gene in somatic tissues. From an evolutionary perspective the
appearance of an autosomal form ensured the perpetuation of eutherians. A comparative analysis of the amino acid sequences of the X chromosome-linked somatic and autosomal genes in human and mouse indicates that both are highly homologous (95%). The testis isoforms, show comparatively less homology with each other (75%), and comparison between the somatic and the testis isoforms reveals 86% homology.

Both the human and mouse X-linked genes contain introns, whereas the testis autosomal isoforms are intronless. Some explanation about the origins of evolution of the two isoforms was provided by comparative genetic analysis between mammalian and marsupial homologues. Only one copy of the PDHA-1 gene containing introns is present in marsupials and is expressed in both testis and somatic tissues. This suggests that a common ancestor of marsupials and eutherians also had a single autosomal $E_1\alpha$ gene and that the second isoform arose after marsupials and eutherians diverged [Fitzgerald et. al. 1993]. The eutherian X-linked gene is therefore presumed to have arisen following translocation of the autosomal gene to the short arm of the X chromosome. The testis-specific autosomal intronless form has been selected for over time. Several theories exist to account for the appearance of this variant. One of these suggests that such genes represent functional retroposon [McCarrey and Thomas 1987]. Evidence supporting this arises from the identification of direct repeat sequences flanking both the human and mouse testis-specific $E_1\alpha$ coding region which is characteristic of retro-transposed genes (and pseudogenes) [Young et. al. 1998].

It has been shown that the appearance of the $Pdha-2$ mRNA correlates with the temporal disappearance of the somatic form following X-chromosome inactivation [Iannello and Dahl 1992]. Expression of $Pdha-2$ mRNA was initially detected at low levels in the
early lepotene spermatocytes but was significantly elevated at the pachytene spermatocyte stage. The translation of PDHA-2 transcript does not occur until post-meiosis [Iannello and Dahl 1992].

Transcriptional regulation of these genes involves specific molecular mechanisms, some of which are only now beginning to be understood. The 5' upstream core promoter region of Pdha-2 does not appear to harbor either TATA or CAAT boxes, although transcriptional initiation may be directed by a Sp1-dependent mechanism [Smale et. al. 1990, Berg et. al. 1993]. Several investigators have already reported potential candidate cis-elements and nuclear factors that may play an important role in modulating gene activity during spermatogenesis [Robinson MO et. al. 1989, Bunick et. al. 1990, Johnson et. al. 1991, Gebara and McCarrey 1992, Grimes et. al. 1992, Lim and Chae 1992, Mizuno et. al. 1992, Goto et. al. 1993]. In addition, regulation of Pdha-2 expression may be influenced by methylation, since eight CpG dinucleotides were found within the core promoter. All of these sites were shown to be methylated in an inactive promoter, whereas the active promoter was hypomethylated [Iannello et. al. 1997]. During spermatogenesis, the pattern of CpG methylation of the Pdha-2 gene varies according to the spermatogenic cell type. In pachytene spermatocytes, the promoter is hypomethylated, correlating well with the observed levels of Pdha-2 expression seen in those cells [Iannello et. al. 1997]. Based on these findings, it could be proposed that tissue specificity may occur as a result of methylation-mediated global inhibition and that one model for the onset of gene activation early in spermatogenesis may involve both hypomethylation and the availability of specific, functionally active testis-specific transcription factors.
Future research on the testis-specific isoform of PDH-E,α should provide important answers about its transcriptional regulation. Partial explanations may come from studies on the testis-specific isoform of phosphoglycerate kinase 2 (PGK-2), since it also possesses a similar profile of isotype switching and would require the same outcome of transcriptional regulation. PGK-2, like PDHA-2, is believed to have arisen through retroposition from its X-chromosome linked homologue, PGK-1, and is also an autosomal, intronless gene [Boer, et. al. 1987, McCarrey and Thomas 1987]. It would be interesting to investigate, whether both genes may be involved in some cases of male infertility where the cause has not been identified, since mitochondrial dysfunctions have been previously linked to decreased sperm mobility [Ruiz-Pesini et.al. 1998]. Another unanswered question about the PDHA-2 homologue is whether it is identically regulated by all of the isoforms of the PDH kinase and the PDH phosphatase. However perhaps the most relevant clinical question to be answered in the future is whether or not expression of PDHA-2 can be used as a PDHA-1 substitute in cases of PDHA-1 deficiencies.

1.7 E$_2$ AND PROTEIN X

The E$_2$ component (dihydrolipoamide transacylase) of the PDH complex forms a central, symmetrical core around which multiple copies of E$_i$ and E$_r$ are arranged. In all of the 2-oxo acid dehydrogenase complexes, the E$_2$ component has a multi-domain structure in which the independent folding units of the lipoyl domains, the E$_i$/E$_r$ binding domain and the catalytic domain are separated by a flexible linker of 20-40 amino acids (fig. 1.5A). The linker sequences are rich in proline, alanine and lysine which are characteristic of interdomain segments [Perham and Packman 1989].
species (fig. 1.5A). The lipooyl cofactor functions as an intermediate carrier to couple the activities of the different multienzyme components [Berg and de Kok 1997]. The reason for the variation in the number of lipooyl domains is unknown, but in the case of E. coli (three domains) it was shown in mutation studies that growth rates could be directly correlated with the number of lipooyl domains [Dave et. al. 1995]. In contrast, it was concluded that only the outermost lipooyl domain needs to be lipooylated to obtain full catalytic activity. The other

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**Fig. 1.5.** The domain structure of the acetyl transferase core enzyme from E. coli, mammals and yeast (A) and the mammalian/yeast protein X (B). The domains are separated by flexible linker region of 20-40 amino acids, indicated by zig-zagged lines. The lipooyl groups are covalently attached to lysyl residues in amide linkage, providing an additional flexible linker of 1.4 nm.

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domains are only required to extend the reach of the outermost domain and to improve the conformational mobility in order to facilitate the substrate transfer between the active sites [Guest et. al. 1997]. In a small number of prokaryotes a lipooyl domain was found at the N-
domains are only required to extend the reach of the outermost domain and to improve the conformational mobility in order to facilitate the substrate transfer between the active sites [Guest et. al. 1997]. In a small number of prokaryotes a lipoyl domain was found at the N-terminus of the E₁β subunit (Z. mobilis) and at the N-terminus of the E₃ component (A. eutrophus, N. meningitidis and Mycoplasma capricolum) [Hein and Steinbuchel 1994, Ala’Aldeen et al. 1996, Zhu and Peterkofsky 1996]. The role of lipoyl domains as part of either E₁β or E₃ components is not yet known but a participation in the overall reaction was suggested [Zhu and Peterkofsky 1996]. Multiple lipoyl domains in different complex components may provide extra lipoyl cofactors that could participate in catalysis and therefore improve the overall specific activity.

Several structures of lipoyl domains, obtained by expression of E₂ sub-domains, have been solved by NMR spectroscopy [Dardel et. al. 1993, Green et. al. 1995a, Berg et. al. 1996, Ricaud et. al. 1996]. All lipoyl domain structures, whether from PDHC or 2-OGDHC, show a very similar overall fold, called a β-barrel sandwich hybrid [Chothia and Murzin 1993]. The domain is formed by two very similar and almost parallel four-stranded antiparallel β-sheets connected by loops and turns (fig 1.6). At the far end of one of the sheets the lipoylated specific lysine residue is presented to the solvent in a β-turn connecting two successive strands [Mattevi et. al. 1992].

Between the lipoyl domain(s) and the catalytic domain a binding domain of only 36 amino acids is located. The crystal structure of a PDH complex between the subunit binding domain and the E₃ component from B. stearothermophilus shows that the site of interaction is so close to the E₃ dimer axis that only a single binding domain can interact with the E₃ dimer [Mande et. al. 1996]. The C-terminal part of the binding domain contains a number of basic
The C-terminal domain of E₂ contains the catalytic site and the information necessary to allow aggregation into the characteristic cubic core structure of the PDH complex, as indicated by electron microscopy and X-ray crystallography. This cubic core is made up of E₂ trimers, eight of which occupy the corners of the cube [Mattevi et. al. 1992b, Mattevi et. al. 1993]. A 3 nm long channel located at the trimer interface extends across its full length and forms the catalytic center. Lipoamide enters this channel from the outside. At the end of the channel the acetyl moiety carried by lipoic acid is transferred to a molecule of CoA by a mechanism which is not yet fully understood. Studies conducted using mutant catalytic domains identified three residues, histidine, aspartic acid and a serine that are crucial for catalysis [Hendle et. al. 1995]. Eukaryotic but not prokaryotic E₂ components contain an additional peptide, which also carries a lipoyl group, called protein X (fig. 1.5B) [DeMarcucci and Lindsay 1985, Jilka et. al. 1986, Behal et. al. 1989]. For yeast protein X it was shown that its lipoyl domain could function in the overall reaction [Rahmatullah et. al. 1990].

Much of what is known about protein X comes from studies in yeast and only limited information is available for mammalian analogues. Because yeast and mammalian protein X
Much of what is known about protein X comes from studies in yeast and only limited information is available for mammalian analogues. Because yeast and mammalian protein X sequences differ considerably, simple extrapolations from yeast to mammals may not be reliable [Behal et. al. 1989, Harris et. al. 1997].

The mammalian protein X (approximately 50 kDa), copurifies with, and is tightly bound to, E\textsubscript{2} [DeMarcucci et. al. 1985, Jilka et. al. 1986]. Limited proteolysis of the bovine protein X [Rahmatullah et. al. 1989, Neagle et. al. 1989] as well as sequence analysis of the yeast and human genes [Behal et. al. 1989, Harris et. al. 1997] indicate that protein X from both sources has only a single lipoyl domain and a peripheral subunit-binding domain, which are homologous with their counterparts in the yeast E\textsubscript{2} chain.

The human protein X shows significant sequence similarity to the yeast protein X only in amino-terminal, lipoyl-bearing domain (49% identity between residues 4-83). In this region, both proteins have the signature motif of a lipoyl-bearing domain. Beyond residue 83, however, very little sequence similarity is found for these proteins that have been assigned a similar function in their respective complexes [Harris et. al. 1997]. The yeast protein lacks a well-defined proline-alanine-rich domain that would readily identify a hinge region. On the other hand, a short sequence exists between residues 166 and 175 of human protein X that has significant homology to sequences within the E\textsubscript{1}/E\textsubscript{2}-binding domains of mammalian E\textsubscript{2} and protein X. Conservation of this sequence in both the human and the yeast polypeptides suggests that this motif may be particularly important for E\textsubscript{3} binding. However, it is known that E\textsubscript{1}-deficient PDH complex from beef heart cannot be reconstituted with yeast E\textsubscript{3} [Sanderson et. al. 1996]. This finding may reflect poor binding of yeast E\textsubscript{3} to the
mammalian protein X as a consequence of marked differences in sequence and therefore structure of the E₃-binding domains of yeast and mammalian protein X homologues.

The carboxyl-terminal region of yeast protein X shows no sequence similarity with human E₂ or human protein X. The active site motif DHRXXDG characteristic of transacetylase is not present in the yeast protein X and no evidence has been presented for acyltransferase activity with either the native or recombinant yeast protein X [Meang et. al. 1994, Meang et. al. 1996].

The remarkable differences between yeast protein X and mammalian protein X coupled with the remarkable similarity between mammalian E₂ and mammalian protein X raise interesting questions, not the least of which is whether anchoring E₃ to E₂ is its only function. Our group investigated the lipoyl-binding domain as well as the putative catalytic domain of the human protein X by transfection of various mutants into a cell line with no endogenous protein X expression. Our investigations (chapter 4) sought to determine whether lipoylation of protein X is not necessary for the activity of the PDH complex and whether this polypeptide probably has its own intrinsic activity.

1.8 LIPOIC ACID METABOLISM

The E₂ subcomplex has an absolute requirement for lipoylation in order to produce transacetylase activity. A set of proteins is involved in the post-translational attachment of the lipoyl group. In E. coli, two genes, lipA and lip B, were identified that encode, respectively, an enzyme that inserts sulphur atoms to produce the dithiolane ring (fig. 1.7) and a lipoyl-protein ligase required for the attachment of the lipoyl group to the E₂ subcomplexes to form 2-oxo acid dehydrogenases [Boom et. al. 1991, Morris et. al. 1995].
The *lip* A gene product was purified and characterized as an ATP-dependent protein ligase of 38 kDa, containing a 4Fe-4S cluster where the iron atoms are thought to be chelated by cysteines at the CXXXCXXX consensus sequence in the N-terminal part of the protein [Green et. al. 1995, Sanyal et. al. 1994]. In the reaction described below, lipoate synthase is thought to act as a substrate providing sulphur atoms (of its Fe-S centre) for incorporation.

![Chemical structure of octanoic acid and lipoic acid](image)

**Fig. 1.7. The last step of lipoic acid synthesis carried out by lipoate synthase.** Octanoic acid serves as a template for the addition of -SH groups from lipoate synthase that acts as a substrate. The Fe-S cluster in apolipoate synthase is thought to be reconstituted by NifS protein, a cysteine transferase (see text).

[Bui et. al. 1998]. It has been proposed that NifS, a cysteine transferase enzyme, in the presence of cysteine and Fe^{2+} can mediate the reconstitution of the Fe-S cluster in apolipoate synthase [Bui et. al. 2000]. Little is known about the mechanism of the reaction. From *in vitro* assays, it has been shown that it requires the presence of S-adenosylmethionine (AdoMet) and NADPH/flavodoxin reductase/flavodoxin as reducing agent [Ollagnier-de Choudens and Fontencave 2000]. This has led to the suggestion that lipoate synthase belongs to the family of Fe-S enzymes with radical-based mechanisms, such as the anaerobic
pyruvate formate lyase (PFL), the ribonucleotide reductase (RNR) systems and the lysine aminomutase [Kulzer et. al. 1998, Ollagnier et. al. 1997, Petrovich 1992]. In the proposed mechanism, the iron-sulphur center transfers a single electron from reduced flavodoxin for reductive cleavage of AdoMet to produce methionine and the 5'-deoxyadenosyl radical. The latter then abstracts a hydrogen atom of a specific C-H bond of the lipoate substrate with the resulting octanoate radical then being activated to incorporate a sulphur atom [Shaw et. al. 1998]. Recently, it has been shown that a special acyl carrier protein (ACP) is involved in the biosynthesis of the lipoic acid precursor octanoyl-ACP and in the transfer of lipoyl-ACP to the respective complexes, both in E. coli and in mitochondria [Wada et. al. 1997. Jordan and Cronan 1997]. The mammalian analogues of the bacterial lipoic acid synthetic pathway have not yet been identified. However, the human and bovine lipoic acid ligase gene been cloned [Fujiwara et al. 1997, Fujiwara et al. 1999].

1.9 E₃

The E₃ (dihydrolipoamide dehydrogenase) re-oxidizes the dihydrolipoic acid bound to a lysine residue in the transacetylase. It is a common component of all 2-oxo acid dehydrogenase complexes and it belongs to a family of flavoproteins known as the pyridine nucleotide-disulfide oxidoreductases [Reed 1974]. Other members of this family are glutathione reductase [Williams 1992], mercuric ion reductase [Fox and Walsh 1982], trypanothione reductase [Shames et. al. 1986] and thioredoxin reductase [Holmgren 1980].

The enzyme consists of two identical subunits with two active sites located at the subunit interface. Each monomer is composed of four domains: the FAD-binding domain, the NAD-binding domain, the central domain and the interface domain [Faure et. al. 2000].
The catalytic site involves some residues in the interface domain (this could explain why a dimer association is necessary for the activity of the protein) and consists of the isoalloxazine ring of FAD, a redox-active disulfide bridge in one monomer (1), and a histidyl residue in the other monomer (2) (Fig. 1.8A). The isoalloxazine ring separates the binding site of the dihydrolipoamide from the binding site of NAD\(^+\). The FAD molecule is in an extended conformation. The NAD\(^+\) cofactor binds on the re-side of the flavin [K. Maeda-Yorita et. al. 1994]. During catalysis, the enzyme shuttles between the oxidized (E\(_{ox}\)) and the two-electron-reduced (EH\(_2\)) state [Massey and Veeger 1961]. Because both the FAD and the disulfide can accept electrons a four-electron reduced state (EH\(_4\)) is also possible. Reduction to the EH\(_4\) state occurs only in cases where the redox potential of the flavin is relatively high. Such situation has consequences for catalysis because only the EH\(_2\) state is catalytically active. Lipoamide dehydrogenases from different species show differences in EH\(_2\):EH\(_4\) equilibria [Snoep et. al. 1993].

The catalytic cycle is shown in Fig 1.8 B. In the first step of the reductive half reaction, a histidine residue abstracts a proton from dihydrolipoamide, which subsequently attacks the disulphide bridge to form a mixed disulphide. Electrons are then transferred to FAD and subsequently to NAD\(^+\) [Benen et. al. 1991, Benen et. al. 1992].
1.10 REGULATION OF THE PYRUVATE DEHYDROGENASE COMPLEX

Regulation of the activity of the pyruvate dehydrogenase complex is of critical importance in all mitochondria-containing cells of higher eukaryotes. The complex has to be active when the complete oxidation of glucose is needed for the generation of energy, whereas its activity has to be severely suppressed when glucose is in short supply, and three carbon compounds are needed for gluconeogenesis. In addition, different tissues show preferences for different fuels. Consequently, the levels of PDH complex activity in the mouse decreases in the following order: brain > kidney > heart > adipose tissue > liver [Dey et. al. 1996]. Regulation of the activity of the complex is accomplished by two different mechanisms: end-product inhibition by acetyl CoA and NADH [Garland and Randle 1964] and by interconversion of its pyruvate dehydrogenase (E₃) component between active (non-
phosphorylated) form and inactive (phosphorylated) forms (fig. 1.9). Both control mechanisms may be interdependent in vivo since they often rely on the same metabolic effectors. End-product inhibition is a result of reductive acetylation of lipoate moieties with concomitant accumulation of Eᵢ-bound hydroxyethyl-ThDP carbanion, creating an impediment to further pyruvate decarboxylation [Randle 1983].

The physiologically more important regulation occurs by phosphorylation of three serine residues located on the α subunit (Eᵢα), catalyzed by pyruvate dehydrogenase kinase (PDK). Dephosphorylation is catalyzed by pyruvate dehydrogenase phosphatase (PDP). The physiological role of multi-site phosphorylation remains unclear but it may be a determinant of differentiation between short- and long-term PDH complex inhibition or, alternatively, a reflection of substrate specificity exhibited by different isoenzymes of the PDH kinase (see below). Using site-directed mutagenesis of the three phosphorylation sites of the human PDH-Eᵢ, expressed in E. coli, Korotchkina and Patel were able to demonstrate that mutation at site 1 (S263) but not at sites 2 and/or 3 (S271 and S203 respectively) decreased specific activity of Eᵢ and also increased $K_m$ values for ThDP and pyruvate [Korotchkina and Patel 1995]. They were also able to demonstrate that phosphorylation of each site resulted in a complete inactivation of the Eᵢ, however the rates of phosphorylation and inactivation were site-specific. A number of studies on pig heart [Sugden et. al. 1978, Sugden et. al 1979, Kerbey and Randle 1979] and bovine kidney [Sugden and Simister 1980] indicated that phosphorylation at sites 2 and 3, in addition to site 1, markedly inhibited the rate of PDH reactivation by phosphatase. These results are not in agreement with studies by Teague et. al. [Teague et. al. 1979] who found that the phosphoryl group on sites 2 and 3 did not significantly affects the rate of dephosphorylation at site 1 or the rate of reactivation of the
enzyme in bovine kidney. The physiological relevance of these findings is presently unknown. This is complicated by the fact that at the time of the studies the existence of PDH kinase and PDH phosphatase isoenzymes was not established. Furthermore, in all cases crude PDH kinase and PDH phosphatase preparations obtained from different animal tissues were used. These preparations could potentially contain more than one isoform of the kinase or phosphatase.

Interestingly, only sites 1 and 3 are conserved in yeast, C. elegans and D. melanogaster with significantly higher residue conservation around site 1. Site 2 does not seem to be conserved and is absent in yeast and worms (personal observations).

Mammalian tissues express four PDK isoenzymes [Bowker-Kinley et. al. 1998] and two pyruvate dehydrogenase phosphatase isoenzymes [Huang et. al. 1998]. These isoenzymes have unique regulatory properties and differ in their level of expression in different cell types, thereby establishing a means for tissue-specific control of the pyruvate dehydrogenase complex [Bowker-Kinley et. al. 1998, Huang et. al. 1998]. In general, the short-term kinase activity responds to the relative concentrations of the PDHC substrates NAD⁺, CoA-SH and the kinase product ADP (inhibition) and PDHC products NADH, acetyl CoA and kinase substrate ATP (activation). This adjusts flux through PDHC on a moment-to-moment basis together with more global control through product inhibition and pyruvate concentration.

Some determinants of effector control of PDK have been defined. Pyruvate and ADP have an effect through direct binding to PDK producing a synergistic inhibition [Hucho et. al. 1972, Pratt and Roche 1979]. ThDP also inhibits kinase activity as a result of binding at the catalytic site of E₁, thereby altering the conformational state of phosphorylation site(s).
[Butler et. al. 1977]. NADH and acetyl-CoA stimulate the kinase through the reduction and acetylation of lipoyl moieties. NAD\(^+\) and CoA-SH can reverse this effect, respectively. [Cate and Roche 1978 Roche et. al. 1989]. Figure 1.9 shows the control of the PDH kinase as well as the PDH phosphatase by various effectors.

Antisense expression of the Arabidopsis thaliana PDK cDNA led to marked elevation of PDHC activity in transgenic plants with increases ranging from 137% to 330% compared to controls. Immunoblot analyses performed with a monoclonal antibody to the PDH-E\(_1\)\(\alpha\) indicated that the increased PDHC activity was not the result of an increase in the level of PDH protein. The PDH complex from transgenic plants showed an expected reduced sensitivity to ATP-dependent inactivation compared to that observed with wild-type plants. A global effect was quite dramatic as transgenic plants with partially repressed PDK also displayed altered vegetative growth with reduced accumulation of vegetative tissues, early flower development enhanced growth rate and shorter generation time [Zou et. al. 1999].

In addition, there is also long-term regulation [Randle 1986] as revealed under stress conditions of disease in mammals. This involves the hyper-phosphorylated state of pyruvate dehydrogenase that develops under conditions such as starvation, diabetes, ischaemic heart disease, sepsis and lactic acidosis [Randle 1986]. In contrast to the short-term effects, long-term effects correlate with a stable increase in the activity of PDK. Recent evidence strongly suggests that short-term and long-term regulation of the PDHC activity may be carried out by different isozymes of PDK [Gudi et. al. 1995, Bowker-Kinley et. al. 1998]. Although the contributions of isozymes of PDH kinase to the regulation of the PDH complex activity have not been fully elucidated, it appears that ubiquitously expressed PDK2 is likely to contribute the most to short-term regulation, whereas the long-term regulation is largely carried out by
the inducible PDK4 [Bowker-Kinley et. al. 1998, Wu 1998]. Data from various laboratories indicates that expression of these two isoforms is subject to modulation by the nutritional and hormonal state of an animal (see below) [Harris et. al. 1997, Sugden et. al. 1998, Wu et. al. 1998]. For example, starvation and chemically induced diabetes increase the amount of PDH2 protein present in rat liver mitochondria [Harris et. al. 1997, Sugden et. al. 1998] and the amount of PDH4 protein present in rat heart mitochondria [Wu et. al. 1998]. Similarly, experiments conducted by Majer et. al. indicate the skeletal muscle levels of PDK2 and PDK4 mRNA decrease in response to insulin [Majer et. al. 1998]. Increased phosphorylation of the pyruvate dehydrogenase complex as a result of increased PDK4 expression and activity has been proposed to be important for suppression of glucose oxidation in the heart in starvation and diabetes [Wu et. al. 1998].

Kinase stimulation was also suggested to result from modification of the lipoate and/or lipoate domain (L2) on E2. This domain becomes specifically engaged in binding the kinase thereby attenuating its activity through a substantial range in response to modest changes in the mitochondrial redox state [Ravindran et. al. 1996].

Currently, further progress in studies on PDH kinase is tempered by the lack of understanding as to how this important enzyme functions. By specificity, PDK is a Ser-specific protein kinase [Linn et. al. 1969], however its sequence does not show any similarity with the rest of eukaryotic Ser/Thr-specific protein kinases other than bacterial histidine protein kinases [Popov et. al. 1993, Hanks and Quinn 1991]. Experiments performed by Bowker-Kinley and Popov suggest that the catalytic domain of PDK is likely to be folded similarly to the catalytic domains of the members of ATPase/kinase superfamily that include
molecular chaperone Hsp90, DNA gyrase B and histidine protein kinases [Bowker-Kinley and Popov 1999].

The reverse of PDH complex phosphorylation and subsequent activation is carried out by pyruvate dehydrogenase phosphatase (PDP). PDP is one of the few mammalian phosphatases residing within the mitochondrial matrix. It is a dimeric enzyme consisting of catalytic and regulatory subunits [Lawson 1993]. The enzymic activity of phosphatase depends on tissue type and the intramitochondrial concentrations of Mg\(^{2+}\), Ca\(^{2+}\), NADH and insulin (fig. 1.9) [Pettit 1972]. Two catalytic subunit isoenzymes, PDP1 and PDP2, and one common regulatory subunit, PDR have been identified to date [Huang et. al. 1998]. Both isoenzymes of PDP are loosely associated with the PDH complex but become complex-bound upon stimulation [Teague et. al. 1982]. They are homologous to the cytosolic phosphatases of the 2C family [Lawson 1993] and both are Mg\(^{2+}\)-dependent, but the sensitivity of PDP2 to Mg\(^{2+}\) ions is almost 10-fold lower. PDP1 is stimulated by Ca\(^{2+}\) ions, causing an increase in its \(V_{\text{max}}\). It has been postulated that binding of Ca\(^{2+}\) ions favourably affects the position of the active site, making it more accessible to substrate. PDP2 seems to be Ca\(^{2+}\) ion insensitive, instead being sensitive to the biological polyamine spermine, which, in turn, has no effect on the enzymatic activity of PDP1. Spermine is unlikely to be the physiologically relevant ligand for PDP2 but rather may mimic the effects of a presently unknown compounds or the regulatory subunit. Nevertheless, it is obvious that the physiological role of PDP2 in the regulation of mammalian PDH complex should be considerably different from that of PDP1 [Huang et. al. 1998].

Isoenzymes of PDP have a different pattern of tissue distribution. This may be correlated with different roles played by the PDH complex in different tissues. For example
in skeletal muscle PDHC is primarily a catabolic enzyme and is regulated by calcium ions. In the liver, PDHC is mainly an anabolic enzyme involved in the biosynthesis of lipids. In contrast, in adipocytes, PHDC is involved in insulin regulation of glycogen and fatty acid synthesis.

![Diagram of pyruvate dehydrogenase complex](image)

**Fig. 1.9.** The control of phosphorylation-dephosphorylation of pyruvate dehydrogenase by various effectors. After Reed (1974).

Experiments conducted by Huang et. al. seem to support these hypotheses. By immunoblotting it was shown that PDP1 is predominantly expressed in mitochondria from skeletal muscle, whereas PDP2 is much more abundant in the liver. Both isoenzymes are expressed in adipocytes, but the level of expression of PDP2 is relatively higher [Huang et. al. 1998]. These observations may provide a rationale for previous findings, which indicated
that for example total PDP in adipose tissue responds to Ca\(^{2+}\) stimulation only at low concentration of Mg\(^{2+}\) and disappears when the concentration of Mg\(^{2+}\) is raised [Rutter et. al. 1989]. As mentioned above PDP2 has a relatively low activity when the concentration of Mg\(^{2+}\) is low. Thus, it is likely that under these conditions the total PDP activity of adipose tissue primarily reflects the activity of PDP1, which is present in adipose mitochondria at low levels. At high concentrations of Mg\(^{2+}\) (i.e. when the level of intracellular ATP is low), the activity of PDP2 is high, and the total activity reflects the contribution of PDP2, which is abundant in these mitochondria and insensitive to Ca\(^{2+}\) ions. Additionally, since the PDH complex in adipose tissue is directly regulated by insulin, it may provide a missing link in the mechanism of insulin-stimulated adipocyte metabolism (It is not known at this time whether there is specificity for particular phosphorylation sites in long- or short-term regulation).

Isoenzymes of PDP in mammals appear to be different with respect to their tissue distribution, kinetic parameters, and regulation. Therefore, it is likely that they are responsible for different aspects of regulation of PDH complex activity. However, further studies are needed in order to precisely define the contributions made by each of them.

1.11 EXTRACELLULAR REGULATION OF THE PYRUVATE DEHYDROGENASE COMPLEX

Activation of a wide variety of plasma membrane receptors causes cascades of events designed to acutely adjust intracellular conditions to the outside environment. In a muscle or a nerve cell it is caused by neurotransmitter action [Katz and Miledi 1967], in ‘non-excitable’ cells such as hepatocytes the stimulus is caused by the binding of hormones to membrane receptor proteins [Woods, et. al. 1986, Rooney et. al. 1989]. A common mechanism used by
extracellular stimuli to exert control over cellular metabolism relies on mobilization of Ca\(^{2+}\) from intracellular stores and entry of Ca\(^{2+}\) from extracellular medium, resulting in an increase in the cytosolic Ca\(^{2+}\) concentration \(\left[\text{Ca}^{2+}\right]_c\). It has been proposed that ligand-stimulated increase in \(\left[\text{Ca}^{2+}\right]_c\) leads to elevations in mitochondrial Ca\(^{2+}\) \(\left[\text{Ca}^{2+}\right]_m\), and subsequent activation of Ca\(^{2+}\)-sensitive inramitochondrial dehydrogenases (including PDH complex), providing increased respiratory substrates for the electron transport system [McCormack et al. 1990, Hansford 1994].

Hormone-mediated Ca\(^{2+}\) mobilization is coupled to the phosphoinositide signaling pathway (the IP\(_3\) pathway) [Berridge 1993, Clapham 1995, Rooney and Thomas 1993, Thomas et al. 1992, Thomas et al. 1996]. IP\(_3\)-mediated mobilization of intracellular Ca\(^{2+}\) stores results in a large, rapid \(\left[\text{Ca}^{2+}\right]_m\) increase, which occurs in parallel with the \(\left[\text{Ca}^{2+}\right]_c\) increase invoked by the same hormone treatment. Mean \(\left[\text{Ca}^{2+}\right]_m\) increases are several fold higher than the changes observed in mean \(\left[\text{Ca}^{2+}\right]_c\), perhaps due to the sigmoidal kinetics of the mitochondrial Ca\(^{2+}\) uniporter [Rizzuto et al. 1992, Rizzuto et al. 1993, Rizzuto et al. 1994]. The frequency of \(\left[\text{Ca}^{2+}\right]_c\) spiking is determined by the strength of the extracellular stimulus. Higher agonist concentrations are associated with higher frequencies of these oscillations until eventually, at saturation hormone concentrations, the oscillations fuse to give a sustained high value of \(\left[\text{Ca}^{2+}\right]_c\) [Pralong et al. 1994, Hajnoczky et al. 1995]. Until recently, little was known about how the cell decodes these oscillatory signals into a final metabolic output. It has been demonstrated that the mitochondria are closely juxtaposed to the IP\(_3\), Ca\(^{2+}\)-release sites, such that IP\(_3\)-dependent Ca\(^{2+}\) release gives rise to localized, but short lived domains of high \(\left[\text{Ca}^{2+}\right]_c\) which are sensed by the mitochondrial Ca\(^{2+}\) uptake sites (fig. 1.10) [Robb-Gaspers et al. 1998a]. This was concluded after studies in permeabilized cells where
an increase to 1-2 µM caused slow mitochondrial Ca\(^{2+}\) accumulation, whereas Ca\(^{2+}\) release from the IP\(_3\)-sensitive stores in the presence of EGTA dramatically elevated [Ca\(^{2+}\)]\(_m\). Calcium enters mitochondria electrophoretically, driven by the large, internally negative, membrane potential [Mitchell 1979, Mitchell 1966]. The process has historically been referred to as a uniport and the carrier protein as a uniporter [Mitchell 1966, Mitchell 1979]. The uniporter protein remains ill-characterized and its gene(s) have not been cloned.

Calcium leaves the mitochondria in exchange for H\(^+\) (predominant mechanism in liver) or for Na\(^+\) (predominant mechanism in nerve and muscle) in a process, which has generally been considered to be electroneutral [Brand 1985].

Recently it has been discovered that the pyruvate dehydrogenase complex is strategically positioned near Ca\(^{2+}\) uptake sites, presumably to minimize the lag on the response [Capaldi – unpublished data]. Therefore, each large amplitude [Ca\(^{2+}\)]\(_m\) spike can be efficiently transferred into the mitochondrial matrix.

Although the rise of intramitochondrial [Ca\(^{2+}\)]\(_m\) levels is rapid, the [Ca\(^{2+}\)] efflux from the mitochondria appears relatively slow. The slow decay of mitochondrial NADH levels and ATP production following each [Ca\(^{2+}\)]\(_m\) spike results in a sustained increase in these parameters at higher [Ca\(^{2+}\)]\(_m\) spiking frequencies. As a consequence, Ca\(^{2+}\)-sensitive intramitochondrial targets integrate the oscillating cellular Ca\(^{2+}\) signals into a smooth continuous metabolic output from the mitochondria. In other words, there seems to be some sort of desensitization process that occurs with Ca\(^{2+}\) signaling, and it has been postulated that the sustained, post-stimulatory output from the PDH complex is actually attributable to changes in the levels of metabolites ADP/ATP and NAD\(^{+}\)/NADH rather than Ca\(^{2+}\) [Robb-Gaspers et. al. 1998b].
Fig. 1.10. Schematic diagram showing mitochondrial Ca\(^{2+}\) uptake sites in juxtaposition to the IP\(_{3}\)-sensitive Ca\(^{2+}\) release channel. PDH – pyruvate dehydrogenase complex, OGDH – 2-oxoglutarate dehydrogenase complex.

This comes from the fact that Ca\(^{2+}\)-mediated events enhance the supply of respiratory substrates and increase the potential for mitochondrial ATP production, concomitant with the activation of ATP utilizing pathways such as gluconeogenesis, lipogenesis and glycogenolysis.

Recently however, it has been demonstrated that in selected cell types that the recovery of [Ca\(^{2+}\)]\(_{m}\) to baseline values can be relatively fast due to the induction of a transient opening of the permeability transition pore, with a rapid efflux of accumulated Ca\(^{2+}\) into the cytosol [Ichas et al. 1997]. Thus the hormone-induced [Ca\(^{2+}\)]\(_{c}\) signal is much more potent.
and would not necessarily invoke desensitization in the PDH complex response permitting repeated waves of response.

It is now becoming clear that mitochondria are not just passive participants in cellular Ca$^{2+}$ signaling pathways. This organelle’s large capacity to accumulate Ca$^{2+}$, coupled with the strategic localization of Ca$^{2+}$ release channels with mitochondrial Ca$^{2+}$ uptake sites, offers the potential for the mitochondria to modulate the rate of rise and the magnitude of the Ca$^{2+}$ spike during a physiological stimulus. This behavior has been demonstrated in some experiments where greatly elevated cytosolic [Ca$^{2+}$], failed to elevate mitochondrial [Ca$^{2+}$]m [Rizzuto et. al. 1993]. How this behavior relates to the regulation of the PDH complex is largely unknown. It is quite possible that there are clusters of PDH complexes aggregated only near selected mitochondrial Ca$^{2+}$ uptake sites and PDHC activation occurs only if these specific sites are juxtaposed to the endoplasmic reticulum’s Ca$^{2+}$ release sites.

The flux control of oxidative phosphorylation at the pyruvate dehydrogenase complex level is designed as another stratum of control in addition to the classic view of Chance and Williams [Chance and Williams 1956] of energy production control by ADP as substrate for phosphorylation. Therefore it is easy to appreciate the complexity involved in the control of the cellular redox-state and the efficiency with which the system operates. What is quite puzzling is that different tissues employ or rely differentially on the two regulatory strata for control. For example in adrenal glomerulosa, pancreatic β-cells and hepatocytes, activation of pyruvate dehydrogenases by Ca$^{2+}$ outweighs any putative electron transport chain activation by ADP, in contra-distinction to what is seen in the heart [Scott et. al. 1994, White and Wittenberg 1993]. This was demonstrated by fluorescence measurement of changes in mitochondrial NAD reduction upon intracellular Ca$^{2+}$ increases. Clearly there is more to Ca$^{2+}$
activation of the PDH complex. It is tempting to implicate different PDH phosphatase isoforms in this differential behaviour, since one of them is clearly Ca\(^{2+}\)-nonresponsive. Additionally, there may be other regulatory factors that interact with the PDH complex. For example the classical acute hormonal effect on the activity of the PDH complex is its activation in adipose tissue by insulin [Hughes and Denton 1976]. Although the mechanism of this activation is unknown, it does not involve Ca\(^{2+}\) ions [Marshall et. al. 1984]. Insulin-induced generation of hydrogen peroxide has been suggested to be an alternative insulin-signaling pathway in fat cells [Krieger-Bauer and Kather 1992]. Alternatively, studies by Tan et. al. have shown that following insulin stimulation glucose can directly increase the level of PDH-E\(_{1}\alpha\) mRNA as a result of increased E\(_{1}\alpha\) transcription [Tan et. al. 1998]. Furthermore, a ChoRE-like sequence in the human E\(_{1}\alpha\) -100 b.p. promoter was found to be responsible for the effect of glucose on promoter activity. It has been suggested that this effect relates to a phosphorylation/dephosphorylation mechanism, possibly involving PP-1 dephosphorylation of transcription factor Sp1 [Daniel and Kim 1996, Tan et. al. 1998].

While the regulation of PDH complex activity in healthy subjects especially by insulin is well documented, less is known about functional changes in the PDH complex in diseased states such as in diabetes, obesity or heart disease. Recently, increased PDK activity has been implicated in the pathogenesis of insulin resistance and non-insulin-dependent diabetes mellitus (NIDDM) in obese subjects [Randle et. al. 1994a, Randle et. al. 1994b]. Measurements of PDK2 and PDK4 mRNA during hyperinsulinemic-euglycemic clamp studies in skeletal muscle biopsies derived from healthy subjects and of PDK2 in cell culture (cultured myocytes) indicated that both transcripts decrease in response to insulin. In contrast, in insulin-resistant individuals there is an insufficient downregulation of PDK
mRNA, which leads to impaired glucose oxidation followed by increased fatty acid oxidation [Majer et. al. 1998]. In some individuals with chronic diabetes, there is evidence for a concurrent cardiomyopathy, which is distinct from the vascular changes that may occur as disease complications [Regan et. al. 1977, Rodrigues and McNeill 1992]. Accompanying this is a profound inhibition of flux through the cardiac pyruvate dehydrogenase complex [Chatham and Forder 1993] possibly due to the general potentiation of the pyruvate dehydrogenase kinase activity seen in the diabetic heart [Kerbey and Randle 1981, Kobayashi and Neely 1983] or due to decreased phosphatase activity as a consequence of low values of \([Ca^{2+}]_m\) [Hansford and Zorov 1998]. In such cases, there could be therapeutic benefit in partial inhibition of the mitochondrial \(Na^+/Ca^{2+}\) exchanger or antiporter, to increase the gradient \([Ca^{2+}]_m/[Ca^{2+}]_c\) [Hansford 1994].

In diabetes-independent heart failure such as in chronic mechanical overload there is a decrease in the flux of glucose to acetyl CoA as hypertrophy develops leading to impaired substrate delivery to mitochondria consistent with the energy deprivation hypothesis [Seymour et. al. 1996, Ingwall 1993]. This decrease does not result from alterations in the availability of exogenous substrates or changes in the activity of the total amount of PDH enzyme complex [Seymour and Chatham 1997]. Thus the cause is yet to be identified, however there have been some suggestions that hormonal effects on cardiac muscles may be effected leading to a similar decline seen in obese diabetic patients (see above) [Seymour and Chatham 1997].

The impact on the pyruvate dehydrogenase complex of the vast number of insulin agonists/antagonists has remained poorly defined. These agonists/antagonists include growth hormone, cortisol, glucagon, catecholamines, thyroid hormones, sex steroids, noradrenaline,
etc. The role of glucocorticoids in oxidation of glucose via the PDHC system has only recently gained some interest, even though the involvement of adrenal steroids in glucose metabolism have been recognized since 1940s. In a recent study, it was reported that the activity of pyruvate dehydrogenase was decreased in blood mononuclear cells following an administration of a single-dose of methylprednisolone [Pellacani et al. 1999]. In addition, total pyruvate dehydrogenase (PDH) and insulin-stimulated PDH activities were decreased in fat pads from dexamethasone-treated rats compared to control values [Martin 1984, Begum 1984]. A putative glucocorticoid-responsive element was identified in the promoter of the E_1α subunit [Maragos et al. 1989].

In addition to effects seen with glucocorticoids, the PDH complex responds to thyroid hormone. For example, in hyperthyroidism, the rate of fatty acid oxidation in hepatocytes and cardiomyocytes is increased [Sugden et al. 1992, Holness et al. 1987] concomitant with the enhancement in PDK activity [Orfali et al. 1995, Sugden et al. 1996]. These effects are opposed by the addition of insulin to the culture medium and, in hepatocytes, by inhibition of mitochondrial fatty acid oxidation [Orfali et al. 1995, Sugden et al. 1996]. Studies by Priestman et al. have further demonstrated that hyperthyroidism increases PDK2 activity in the liver by increasing the amount of detectable protein. In contrast, hyperthyroidism increases the activity of cardiac pyruvate dehydrogenase kinase without changing in the tissue concentration of PDK2 – one of the major isoforms in the heart. Therefore it appears that the thyroid hormone regulation of PDH complex activity differs between these important tissues [Priestman et al. 1997].

In contrast to glucocorticoids and thyroid hormone, sex hormones such as estradiol and estrone have a stimulatory effect on the functioning of the PDH complex [Fraser et al.
Studies in diabetic mice have shown that development of cardiomyopathies can be prevented by 7-12 weeks estrone treatment, which decreases the level of phosphorylation of the pyruvate dehydrogenase in the heart by yet unknown mechanisms [Kuo et. al. 1986].

Many factors other than those described above are likely to be involved in the regulation of the PDH complex. Once our knowledge is augmented by the specific mechanisms implicated in glucose flux through this complex, we can start designing therapeutic agents and methods, allowing proper health maintenance and disease management. It is thus extremely important to continue innovative studies on the metabolic action of different hormones and/or metabolites and their effects on the PDH complex, since a great number of signaling pathways seem to cross at this important enzyme.

1.12 IMPORT INTO MITOCHONDRIA

Since the genome of mammalian mitochondria encodes only a handful of proteins, the biogenesis of the organelle depends on the coordinated import of nuclear-encoded precursor proteins from the cytosol. Therefore mitochondria contain an elaborate network of protein translocases in the outer and inner membrane along with a battery of chaperones and processing enzymes in the matrix and intermembrane space to mediate protein translocation. A mitochondrial protein is escorted through the cytosol by chaperones to the TOM complex (translocase of the outer membrane). After crossing the outer membrane, the import pathway diverges and one of two TIM complexes (translocase of inner membrane) are utilized, depending on the mitochondrial destination of the precursor (fig. 1.11) [Ryan and Jensen 1995, Schatz and Dobberstein 1996, Neupert 1997, Pfanner 1998].
Figure 1.11. Protein import pathways into mitochondria. Cytosolic proteins are imported through the TOM and then, depending on their destination, remain in the outer membrane (OM), intermembrane space (IMS), or engage the translocases of the inner membrane (IM). Precursors with an amino-terminal targeting sequence generally engage the TIM23 complex, whereas proteins that reside in the inner membrane, often lacking a targeting sequence, engage the TIM22 complex.
Each one of mitochondria-targeted polypeptides has its own biochemical identity and each requires different degree of assistance provided by the cellular components that mediate targeting. Some precursors are prone to misfolding or aggregation and are therefore assisted by chaperones such as Hsp70 and MSF to prolong their import competence. These chaperones can prevent and even reverse protein aggregation, and directly transfer precursors to the TOM complex [Mihara and Omura 1996, Hachiya et. al. 1995]. As the mRNA encoding a mitochondrial precursor protein leaves the nucleus it is bound by ribosomes that generate precursor polypeptides [Michael 2000]. These will eventually encounter a mitochondrion. Once a precursor engages the TOM complex on the mitochondrial surface, all subsequent precursors are likely to be imported co-translationally. Therefore within an eukaryotic cell a mixture of co-translational and post-translational protein import mechanism occurs. The relative kinetics of translation and import, as well as the stability of particular mRNAs, might effect the requirement for cytosolic factors that assist either the folding or import of newly-translated precursor polypeptides into mitochondria. In addition, recent evidence suggests that factors associated with active polysome complexes can assist protein import co-translationally [Wiedmann et. al. 1994].

The translocase of the outer membrane (TOM) consists of protein import receptors and the import channel. The receptors (Tom20, 22, 37, and 70, with the number indicating molecular weight of respective polypeptides) on the mitochondrial surface recognize targeting information on the precursor proteins, while components Tom40 and the small Tom proteins 5, 6 and 7 form the channel through which the translocating precursor passes [Lithgow 2000]. Whether the precursor protein has been released from the ribosome or remains in the process of translation, the amino-terminus interacts with the acidic receptor
components, Tom20 and Tom 22, to initiate translocation [Schatz 1997]. The additional receptor subunits Tom37 and Tom70 interact with other parts of the precursor and prevent aggregation [Hachiya et. al. 1995]. Nascent polypeptides are threaded through a 20 Å channel in the outer membrane translocase. This channel is most likely, formed by Tom40 [Schwaartz and Matouschek 1999, Hill et. al. 1998]. Evidence suggests that the receptors and the channel physically interact with each other and that Tom70 and Tom20 also feed precursor proteins into the translocase [Haucke et. al. 1996].

Most mitochondrial precursors contain an amino-terminal targeting sequence, but many proteins contain targeting and sorting information within the mature part of the protein. This information determines which TIM complex participates in their translocation across the inner membrane. Precursors with an amino-terminal targeting presequence follow the general import pathway [Pfanner and Meijer 1997, Kaldi and Neupert 1998, Horst et. al. 1997] and their import is mediated by the Tim17/Tim23 complex (referred to as TIM23) and the associated translocation motor. This translocation requires the presence of a membrane potential (Δψ) and ATP hydrolysis by mHsp70 on the matrix side [Pfanner and Meijer 1997, Kaldi and Neupert 1998, Horst et. al. 1997]. The TIM23 complex acts independently of the TOM complex although the two can be reversibly associated while a precursor is in transit [Horst et. al. 1995, Berthold et. al. 1995]. Tim17 and Tim23 form a 90 kDa complex in the inner mitochondrial membrane. The precursor is recognized by the negatively charged domain localized on Tim23 [Ryan et. al. 1994, Maarse et. al. 1994, Ryan and Jensen 1993, Dekker Et. al. 1993], with translocation initially being driven by the membrane Δψ. Further translocation is facilitated by ATP-dependent mechanism of the translocation motor located on the matrix side and composed of Tim44, mitochondrial hsp70 and mitochondrial GrpE.
To date, additional proteins in the TIM machinery have been identified, but their specific role in protein import has not been determined [Tokatlidis et. al. 1996, Arnold et. al. 1998, Kanamori et. al. 1997]. This pathway is utilized by all of the components of the pyruvate dehydrogenase complex.

Other inner membrane proteins lack a cleavable targeting sequence, carrying instead their targeting and sorting information internally within their amino acid sequence. These proteins are translocated through the inner mitochondrial membrane by another major import pathway designated TIM22 [Koehler et. al. 1998, Koehler et. al. 1999, Kerscher et. al. 1997, Sirrenberg et. al. 1998, Adam et. al. 1999]. Components of this pathway are located in the mitochondrial inner membrane and intermembrane space and form a 300 kDa complex. The principal members of this complex are Tim22, Tim54 and Tim18, all of which are integral membrane proteins. Tim18 may regulate assembly of the functional complex since its depletion yields a lower molecular weight complex [Kerscher et. al. 2000]. A family of small proteins in the intermembrane space (referred to as Tiny Tims) that bind and thread the nascent polypeptides emerging from the TOM complex aids the translocating effort of the principal complex. Five proteins, Tim8, Tim9, Tim10, Tim12 and Tim13, have been identified so far in yeast, all sharing a ‘twin CX3C’ motif, in which two cysteine residues are separated by three amino acids. Each cysteine motif is separated by 11-16 amino acids [Koehler et. al. 1998, Koehler et. al. 1999, Sirrenberg et. al. 1998, Adam et. al. 1999]. Small Tim proteins bind zinc and zinc binding is required for their function in vivo. The exact function of Tiny Tims is still uncertain. One possibility is that they act as chaperone-like
molecules to guide the precursor across the aqueous intermembrane space, yielding a soluble intermediate [Koehler et. al. 1998, Koehler et. al. 1999].

1.13 COMMERCIAL USES OF 2-OXO ACID DEHYDROGENASES

Enzymes are finding increasing usage as catalysts in pure and applied chemistry especially in delivering specific enantiomers in excess, in combination with high catalytic efficiency. Environmental applications also lend themselves to the use of enzymes as a mean of reducing the impact of hazardous compounds, organic solvents and other chemical waste. These applications are especially important in the field of carbohydrate chemistry, where the inherent multifunctionality of sugars present a problem for the organic chemist who has to use a plethora of protective groups in order to prevent unwanted reactions of the hydroxyl, keto or phosphate groups [Grueckhammer et. al. 1991, Gijsen et. al. 1996].

The potential of enzymes such as 2-oxo dehydrogenases to form chiral synthones with high enantioselectivity under mild conditions has led to increasing application of these biocatalysts in organic synthesis. The advantage of the use of these enzymes include the potential for both breaking and forming of C-C bonds and the forming of a ThDP-bound carbanionic intermediate (active aldehyde) [Drzua and Waldmann 1996, Kluger 1992, Csuk and Glanzer 1991]. Chiral α-hydroxy ketones are versatile building blocks for the organic and pharmaceutical chemistry, e.g. for the synthesis of vitamin E and antifungals. Another well-known example is (R)-phenylacetyl carbinol – a precursor of ephedrine. However, this type of synthetic application for such enzymes is often limited due to a narrow substrate range. These disadvantages may be overcome by specific ‘enzyme tailoring’.

Versatile tools are offered by modern molecular biology, including methods based on random mutagenesis as well as a rational site-directed mutagenesis approach. A well-known
example of the 'random' method is the directed evolution, which combines 'error-prone' PCR, exon shuffling with appropriate selection and screening parameters [Shao and Arnold 1996, Patten et. al. 1998].

The 'rational' approach requires detailed knowledge of mechanism, which might be investigated or altered by directed mutagenesis. An essential prerequisite is structural information as a basis for computer modeling to design appropriate mutants. A very successful example involves pyruvate decarboxylase (PDC), an enzyme with an important role in glycolysis and ethanol fermentation responsible for the non-oxidative decarboxylation of pyruvate to acetaldehyde (it can also utilize other 2-oxo acids) [Neuberg and Karczag 1911, Neuberg and Rosenthal 1913]. Pyruvate decarboxylase is perhaps the simplest ThPD dependent enzyme and in many ways analogous to the E₁ component of pyruvate dehydrogenase complex. With both enzymes decarboxylation is thought to be initiated by formation of the C2 carbanion of ThDP, which then forms a nucleophilic adduct with C2 of the substrate pyruvate – for details see section 1.4 [Breslow 1958, Konig 1992]. In the case of PDC the detailed investigations of the reaction mechanism performed by many groups has permitted the rational design of mutants tailored to elucidate the predicted function of certain amino acid residues as well as to alter the catalytic properties [Bruhn et. al. 1995, Pohl 1997]. As a result more than thirty compounds have been identified as substrates for the S. cerevisiae PDC [Iding et. al. 1998]. These substrates include 3-halopyruvate derivatives, α-Keto-butanoic acid, α-Keto-4,4-dimethylpentanoic acid, or α-Ketophenylglyoxylic acid to name a few (for an extensive review see Iding et.al. 1998). In the case of pyruvate dehydrogenase complex, it has been found that the yeast PDH can convert free acetaldehyde (by condensation with pyruvate) to acetoin [Alkonyi et al. 1976]. Pyruvate can also react
with succinic semialdehyde to form 5-keto-4-hydroxyhexanoic acid [Yokota and Sasajima 1986]. Kubasik et. al. showed the condensation reaction between pyruvate and glyoxylate catalyzed by PDHC to form acetol as the major product and 1,2-dihydroxy-4-ketovaleric acid as a minor product [Kubasik et al. 1972]. PDH is also capable of condensing pyruvate with aldoses to produce 1-deoxyketoses [Yokota and Sasajima 1986]. Up to now the investigation of the synthetic potential of α-keto acid-utilizing enzymes has been limited [Reynolds et. al. 1988, Weiss et al. 1988, Wilcocks and Ward 1992, Wilcocks et al. 1992, Prosen and Ward 1994]. However, as our structural knowledge about other ThDP-binding enzymes increases it becomes increasingly feasible to realize the potential of their application in chemical and pharmaceutical sciences. Pyruvate dehydrogenase is especially easy to appreciate in this respect since like PDC its natural substrate is pyruvate. Future endeavors should be made a lot easier with the application of recently-engineered ‘lab-on-a-chip’ processes which allows a high throughput molecular screening of either mutant proteins and/or their substrates using extremely small amounts of material.

1.14 MUTATIONAL STUDIES OF THE PDH-E₁

As already mentioned above, the mammalian E₁ is composed of two non-identical subunits α and β with a tetrameric structure (α₂β₂) and is regulated by reversible interconversion between an active nonphosphorylated form and inactive phosphorylated form. The available evidence shows that both subunits are probably critical for catalysis [Roche and Reed 1972, Stepp and Reed 1985, Ali et. al. 1993, Chun and Robinson 1993, Eswaran et. al. 1995, Aevarson et. al. 1999], even-though the GDG$^{26\alpha27\beta}$NN motif responsible for the binding of ThDP and Mg$^{2+}$ is located on the E₁α subunit.
Site-directed mutagenesis studies on PDH from *Z. mobilis* confirmed the importance of the ThDP fold in maintaining enzyme activity [Diefenbach et. al. 1992, Candy and Duggleby 1994]. In particular, substitutions other than D to G at position 440 led to an inactive protein, as did a N467G substitution. The sequence alignment of nearly twenty ThDP-dependent enzymes also succeeded in identifying the likely region for ThDP binding in the E₁ isolated from *E. coli* [Hawkins et. al. 1989]. Russell et. al. confirmed the importance of this region by showing that the PDH complex activities of site-directed variants (G231A, G231S, C259S, C259N and N258Q) were severely affected. Yi et. al. extended this study to demonstrate that these substitutions also affect the activation of the enzyme by ThDP-Mg²⁺ [Yi et. al. 1996].

In 1991 Wexler et. al. used alignment algorithms to identify several regions of the α and the β subunits that are highly conserved not only in the two subunits of E₁ from various species, but also in those of branched chain alpha-keto acid dehydrogenase complexes from both prokaryotic and eukaryotic species [Wexler et. al. 1991]. There were two common regions of extended homology as identified previously including the ThDP binding motif located between human E₁α amino acids 156-203, and a region encompassing phosphorylation sites 1 and 2 spanning amino acids 263-274 (fig. 1.12 A) [Wexler et. al. 1991]. Also identified was a previously unrecognized region of high homology located between amino acids 217-261 (fig. 1.12 A) of the human PDH-E₁ sequence. This region appears to be unique to α-keto acid dehydrogenases, which have distinct α and β subunits, as no such segment appears in *E. coli* PDH-E₁ [Stephens et. al. 1983] or the E₁ components of 2-oxoglutarate dehydrogenase complexes [Darlison et. al. 1984, Repetto and Tzagoloff 1989], which are dimers composed of only a single E₁ polypeptide. A point mutation,
R234G, in this region has been identified in several PDH-deficient patients. This mutation affects protein stability but not catalytic activity [Kerr et. al. 1988, Wexler et. al. 1990, Wexler et. al. 1992]. Interestingly, changes in R234 to G and sometimes to Q, are the most frequently reported E₁α mutations and are carried by the mothers with no ill-effect. They seem to produce deleterious effects only in boys while no female patient has been reported to date (one carrier mother was reported to have a low IQ) [Chun et. al. 1993, Awata et. al. 1994, Naito et. al. 1994, Chun et. al. 1995, Lissens et. al. 1996, Briones et. al. 1996, Wexler et. al. 1997]. Since previous studies have shown that α and β require each other’s presence for stability in PDH and BCDH complexes [Ho et. al. 1989b, Fisher et. al. 1989], this mutated amino acid residue may disrupt a critical site of interaction for the two subunits thereby rendering an unstable complex [Wexler et. al. 1991].

There is another region thought to participate in the human E₁ heterotetramer formation, localized at the C-terminus of PDH-E₁α [Seyda et. al. 2000]. This conclusion was based on experimental data presented in this thesis in chapter 2.

When E₁β subunits from different species are aligned, it becomes apparent that there is greater sequence homology between β than between α subunits (fig. 1.12 B) [Wexler et. al. 1991]. This phenomenon may explain why naturally occurring mutations are only detected in the E₁α subunit but not in the E₁β subunit. Four regions of E₁β have been identified. Region 1 spanning amino acid residues 25-59 (in the mature human E₁β sequence) was proposed to be one of the sites at which E₁β binds to E₂ (fig. 1.12 B) [Wexler et. al. 1991]. The involvement of E₁β in binding E₂ was suggested based on tryptic digestion studies that have shown that E₁β, but not E₁α, is protected when bound to the transacetylase (E₂). Region 2 spans amino acid residues 140-176 (fig. 1.12 B) and it displays 49% identity to
### Fig 1.12 A. Alignment of 6 E₆α sequences from *Bacillus stearothermophilus* - B. stearothermophilus; *Saccharomyces cerevisiae* - S. cerevisiae; *Schizosaccharomyces pombe* - S. pombe; *Arabidopsis thaliana* - A. thaliana; *Mus musculus* - M. musculus and *Homo sapiens* - H. sapiens. Amino acid residues are shaded if more than 4 residues are identical in a column and boxed if all residues are identical in a column. Serines 203, 264 and 271 (human sequence) are sites of phosphorylation.
Fig 1.12 B. Alignment of 6 β sequences from *Bacillus steathermophilus* - B. steathermophilus, *Saccharomyces cerevisiae* - S. cerevisiae, *Schizosaccharomyces pombe* - S. pombe, *Arabidopsis thaliana* - A. thaliana, *Mus musculus* - M. musculus and *Homo sapiens* - H. sapiens. Amino acid residues are shaded if more than 5 residues are identical in a column and boxed if all residues are identical in a column.
3-isopropylmalate dehydrogenase from *T. aquaticus*. Based on this sequence analysis, Wexler et al. suggested that this region may represent a site that is involved in the oxidative decarboxylation of pyruvate and other 2-oxo acid dehydrogenases [Wexler et al. 1991]. A conserved aromatic residue (Trp135 in the human mature polypeptide) very closely located to region 2 has been identified by Ali et al. as a functionally essential part of the enzyme, further supporting the proposed role of region 2 [Ali et al. 1995]. Region 3 (amino acid residues 230-266) and region 4 (amino acid residues 270-303) (fig. 1.12 B) have also been identified but possible functions have not been determined [Wexler et al. 1991].

In 1993 a model for the ThDP-binding site of human pyruvate dehydrogenase E, was proposed, implicating histidine residues in pyrophosphate coordination through hydrogen bonding [Chun and Robinson 1993]. Conserved histidine residues have been implicated in the geometry and catalytic mechanism of the ThDP-binding enzymes [Lindquist et al. 1992, Aevanono et al. 1999]. As part of this thesis a series of PDH-E,α histidine mutants (H63, H84, H92 and H263) (fig. 1.12 A) was investigated to determine if they were essential for the activity. Mutations introduced at H92 resulted in the absence of immunoreactive material for both the E,α and E,β subunits and may have impaired import or assembly of precursor peptides into the mature PDH complex (for further discussion see chapter 3) [Seyda and Robinson 2000].

1.15 EXPRESSION SYSTEMS

Continual advances in gene expression technology have allowed production of recombinant proteins to become a somewhat easier and more efficient process. Vector size has been reduced. Sequences that allow rapid detection and purification of recombinant proteins have been made available. New selectable markers have been introduced. With so
many systems available, the challenge is in choosing the one that is most suitable for answering specific biological questions [Invitrogen catalogue 2000]. When it comes to studies on the mammalian pyruvate dehydrogenase complex the preferred system so far has been bacterial expression, especially in *E. coli* [Russell et. al. 1992, Lawson et. al. 1993, Lessard and Perham 1994, Korotchkina and Patel 1995a,b, Yi et. al. 1996, Harris et al. 1997].

Mammalian, nuclear-encoded mitochondrial proteins are commonly expressed in *E. coli*. The major advantage of this system is the ability to rapidly produce protein in large quantities. *E. coli* grow at a very fast rate giving the opportunity to purify, analyze and use the expressed protein in a much shorter time. In addition, transformation of *E. coli* cells with the foreign DNA is easy and requires minimal amounts of material. These reasons explain the popularity of bacterial systems, however, *E. coli* and other bacteria do not contain mitochondria. Therefore mitochondrial polypeptides with cleavable signal sequence must be expressed as already mature proteins, as has been shown for dihydrolipoamide dehydrogenase (E$_d$). When expressed as the precursor protein the enzyme exhibits only residual activity compared to the mature form upon purification [Kim et. al. 1991]. Additionally, in order to achieve successful expression, one must avoid the usage of codons that are rarely used natively in bacterial hosts. These codons occur very infrequently in the coding sequences of bacterial proteins and are therefore not well tolerated by the host, potentially contributing to low yield. Another factor to consider is the potential toxicity of the foreign protein to the bacterium. In these cases there is a possibility of complete deletion or mutations introduced in the coding sequence of the heterologous protein brought about by the host [Arakawa and Timasheff 1987].
The expression of recombinant proteins in the reducing environment of *E. coli*’s cytoplasm very often leads to the formation of insoluble inclusion bodies, which contain unfolded proteins. This necessitates the development of refolding protocols, optimized for each molecule, to recover active material. One of the major problems in such processes is the formation of aggregates as a consequence of the interaction of hydrophobic patches on the surface of misfolded or partially folded proteins [Arakawa and Timasheff 1987, Verma et al. 1997]. Another problem posed by expression in bacteria is the fact that some proteins prefer to adopt an energetically stable state that is not their native state [Huston et al. 1991]. As a result it is difficult to assess the true impact of specific mutations on the overall function of a protein. Furthermore, since studies of regulatory mechanisms are limited to *in vitro* conditions true physiological relevance is not so easily interpreted since the composition of buffer systems as well as concentrations of proteins in a test tube may not necessarily represent conditions within the living cell.

Genes expressing the E₁α and E₁β chains of mammalian PDH complex as well as all other components of the mammalian BCDH complex have been overexpressed in *E. coli*. Coexpression of both subunits generates active E₁α₂β₃ heterotetramer, whereas mixing individually expressed subunits does not [Korotchkina et al. 1995b, Wynn et al. 1992]. This suggests that simultaneous expression of both subunits in the same cellular compartment is important for assembly into a functional E₁ component *in vivo*. Furthermore, chaperonins appear to be involved in promoting the assembly of the active tetrameric enzyme, as indicated by coexpression of the E₁α and E₁β genes in an *E. coli* strain deficient in chaperonins groEL and groES. In this mutant host both protein yield and recovery of the soluble fraction was significantly decreased as compared to a complemented system where
expression of the chaperonins was restored by use of a second vector [Wynn et. al. 1992]. Similarly, coexpression is required for the functional assembly of mammalian protein X with mammalian transacylase (E coli) but not the transacylase alone [Harris et. al. 1997]. It needs to be mentioned at this point that in order to assess functional viability of bacteria-generated mammalian proteins, information of the activity of the native protein purified from the original source counterpart must either be available or generated.

In spite of limitations there are advantages to the bacterial expression systems (aside from those already mentioned above). The ability to purify proteins to homogeneity as well as relatively high yields allow precise measurements of such parameters as $K_m$, $K_{cat}$, $V_{max}$ (assuming that polypeptides refold correctly) as well as accurate calculation of specific activity. Furthermore, the quantities that can be potentially generated allow for structural studies on the protein and/or protein complex by either X-ray crystallography and/or NMR.

An alternative to the bacterial expression systems are the yeast expression systems. The main advantages of yeast expression systems are related to their eukaryotic features. Unlike E. coli, yeast provide advanced protein folding pathways for heterologous proteins and, when yeast signal sequences are used. Proteins can be therefore secreted and processed correctly. Unlike mammalian expression systems, yeast can be rapidly grown on simple growth media. Proteins which accumulate as insoluble inclusion bodies in E. coli are often soluble when expressed in yeast [Ridder et. al. 1995]. However this system has not been used for studies of proteins from mammalian pyruvate dehydrogenase complexes presumably because expression of mammalian homologues in yeast knock-out strains do not generally produce functional complementation. Similarly, expression of mammalian homologues for the purpose of purification would probably require either expression of already mature
proteins in the cytoplasm or expression of yeast/mammalian fusion protein to assure proper import and/or processing of the recombinant protein in the mitochondria.

During the course of this thesis we used human cells to express human proteins. The main advantage of our mammalian cell expression lies in the fact that the signals for synthesis and processing of recombinant mammalian proteins are properly and efficiently recognized by the mammalian cell. Therefore we were able to express exogenous proteins in their precursor form. The system that we used was unique in that the endogenous counterparts of the exogenously expressed proteins (E, α and protein X) are not expressed due to naturally occurring null mutations [Seyda et. al. 2000, Seyda and Robinson 2000, chapter 5]. This allowed us to study mutant proteins in their natural environment and abolished the need for PDH complex purification. Furthermore, this system in conjunction with immunoblot and in vitro import experiments, allowed assessment of the impact of amino acid sequence changes on either specific activity, assembly and/or import into mitochondria [Seyda et. al. 2000, Seyda and Robinson 2000]. This is not permitted by the E. coli expression system. The main disadvantage of this system however, lies in the fact that we failed to restore the full activity of the pyruvate dehydrogenase complex using wild type polypeptides. Clearly, there must be some regulatory mechanisms that control temporal and/or spatial expression of different PDH complex components, which are not mimicked by expression from heterologous operon in our expression plasmids [Brown et. al. 1997, Seyda et. al. 2000]. Furthermore, although this system in conjunction with immunoblot studies allows approximation of changes in specific activity brought about by an engineered mutation, it does not allow accurate numerical calculation. The study of kinetic properties of expressed mutants is also limited.
In conclusion, we used the above described system and found it superior to the bacterial alternative in providing a convenient method as well as clear answers to the questions we have posed. Additionally, we were the first research group in the world to utilize it for the purpose of investigating artificially-generated mutants of the human $E_{1}\alpha$ and protein X.

1.16 THE METABOLON

Over the last 20 years, a considerable body of evidence supports the idea that sequential enzymes within a metabolic pathway interact with each other to form highly organized enzyme complexes called metabolons [Velot et. al. 1997, Robinson JB, Jr. et. al. 1987, Srere 1987]. Data from a number of experimental approaches support the metabolon concept in connection with the Krebs TCA cycle, demonstrating specific interactions between six of the eight sequential enzymes, including malate dehydrogenase (MDH), citrate synthase (CS), and aconitase (ACO) [Srere et. al. in 1997]. It was also demonstrated that five of the Krebs TCA cycle enzymes (fumarase, MDH, CS, ACO and isocitrate dehydrogenase) from *E. coli* can be isolated as a high molecular weight complex able to catalyze the sequential reactions leading from fumarate to $\alpha$-ketoglutarate [Barnes and Weitzman 1986]. Furthermore, it was found that many of the enzymes previously thought to exist in a soluble form in the mitochondrial matrix, bind to the inner surface of the mitochondrial inner membrane [Srere et. al. in *Channeling in intermediary metabolism* 1997]. One possible reason for the existence of these organized structures is to prevent the intermediates from escaping into solution where they may be sequestered by other enzymes for use in different metabolic pathways [Welch 1977, Spivey and Merz 1989]. Consequently, the close
proximity of enzymes responsible for catalyzing consecutive steps of a metabolic pathway may be used to increase the metabolic flow through the pathway, by assuring the channeling of the intermediate.

It has been demonstrated that the pyruvate dehydrogenase complex may also participate in the formation of a metabolon. First evidence came from studies by Glutz and Walter, who noticed that acetyl-CoA formed from pyruvate, is incorporated into citrate rather than into ketone bodies [Glutz and Walter 1975, Walter 1976]. Sumegi and Alkonyi presented data that indicated physical interaction between the PDH complex and citrate synthase. According to these data, citrate synthase binds to the isolated transacetylase core, but in the binding to the whole PDH complex the two other components are also involved [Sumegi and Alkonyi 1983]. Subsequently, Sumegi and Srere demonstrated that the PDH complex also binds to the NADH ubiquinone reductase (complex I) in a saturable way [Sumegi and Srere 1984]. If these findings are indeed true, it would mean that the PDH complex not only links glycolysis with the TCA cycle, but also physically tethers the TCA cycle to the electron transport chain resulting in a functionally logical enzyme organization, where the NADH production and NADH oxidation takes place in an efficient coupled manner. The physical binding of the pyruvate dehydrogenase complex to other mitochondrial proteins could also form a plausible explanation for roles of high homology regions with no apparent function observed in the primary sequences of PDH complex components [Wexler et. al 1991]. Mutagenesis of these areas should have measurable effects on pathways rather than individual reactions especially with respect to time resolution.

Step-by-step our knowledge of mitochondrial structure and function is becoming more complete. Part of the challenge at present is that of organizing and confirming the vast
array of data generated each day. Because of the key importance of the pyruvate dehydrogenase complex in health and disease an understanding of the integrated operation of this enzyme within metabolism could have a major impact on various aspects of health care. An integrated scientific approach will not only study the complex as a separate entity but also study it in the context of the mitochondrion, the cell, the tissue type and ultimately the organism.

The last chapter of this thesis probes into this integrative work and introduces the idea that the proper function of the pyruvate dehydrogenase complex depends on the activity of an as yet unidentified single gene component whose gene product maps to chromosome 2. This gene product links five important enzymes: the pyruvate dehydrogenase complex, the 2-oxo glutarate dehydrogenase complex, the branched chain alpha-ketoacid dehydrogenase complex, the glycine cleavage system and the ubiquinol cytochrome c reductase (complex III). As our knowledge of mitochondrial function increases, it is becoming more evident that a common type of post-translational modification interconnects activities of some mitochondrial enzyme complexes.

1.17 OBJECTIVES OF THE THESIS

During the course of this thesis, a number of investigations were launched in order to augment the scientific community’s knowledge about the structure and function of the mammalian (human) pyruvate dehydrogenase complex.

As an opening to this investigation, the C-terminus of PDH-E₅α was studied. This project was based on the availability of clinical, genetic and biochemical data from patients with naturally occurring E₅α C-terminal deletions who were the longest survivors with PDH
complex deficiency. Through a sequential deletion of a quartet of C-terminal amino acid residues a hypothesis was investigated that this part of the protein may be involved in the formation of a stable $E_1\alpha_2\beta_2$ heterotetramer (chapter 2). Chapter 2 also investigated the biochemical equivalence of the somatic and the testis-specific isoforms of PDH-\(E_1\alpha\). To aid our efforts in both cases, a PDH-\(E_1\alpha\)-deficient cell line (the $\pi^0$ cell line) was created for the purpose of exogenous plasmid expression of a number of variant $E_1\alpha$ cDNAs.

In the past, experiments based on crystallographic methods showed that in some ThDP-utilizing enzymes, histidine residues located on the $\alpha$ subunit (or its equivalent sequence) are involved in the formation of the ThDP-binding site. To determine whether the same holds true for the human PDH-\(E_1\alpha\), investigation of four histidine residues was performed using site-directed mutagenesis and expression of resulting variant cDNAs in the $\pi^0$ cell line (chapter 3).

The controversy surrounding the biological role of the mammalian protein X precipitated a series of experiments described in chapter 4. Two basic questions were posed:

1. Is lipoylation of the invariant lysine residue on protein X required for its activity?
2. Does protein X have any intrinsic transacylase capability?

To address these questions, two protein X mutants (K37E and S422H) were expressed and characterized in a protein X-deficient and an $E_2\beta$-deficient immortalized human skin fibroblast cell line.

During the course of this thesis, a discovery was made of the first male PDH-\(E_1\alpha\) somatic mosaic. Both genetic and biochemical investigations of this case were performed in order to determine the impact of this event on the overall PDH complex activity in the
individual carrying the mosaicism as well as to postulate the possible circumstances of its formation (chapter 5).

In a small number of patients with PDH complex deficiency, the disease does not stem from a primary defect in any one of the PDH complex components, but rather it presents as a secondary symptom caused by an as yet unknown factor. To identify this unknown factor fibroblast cell lines derived from patients suffering from a secondary PDH deficiency were subjected to microcell-mediated chromosome transfer coupled with genetic exclusion and deletion mapping techniques. The goal of this series of experiments was to find a specific chromosomal interval where the locational probability for the locus of the unknown factor is very high. This project is described in chapter 6.
CHAPTER 2

SEQUENTIAL DELETION OF C-TERMINAL AMINO ACIDS OF THE E$_1$$\alpha$ COMPONENT OF THE PDH COMPLEX LEADS TO REDUCED STEADY-STATE LEVELS OF FUNCTIONAL E$_1$$\alpha_2$$\beta_2$ TETRAMERS: IMPLICATIONS FOR PATIENTS WITH PDH DEFICIENCY.

Human PDH complex deficiency is an extremely heterogeneous disease in its presentation and clinical course. We have characterized novel mutations that affect the C-terminal portion of PDH-E$_1$$\alpha$ coding sequence. Although the molecular defects underlying these mutations are different, both effectively produce a stop codon prematurely three amino acids from the C-terminus. The clinical and biochemical consequences of these mutations are unusual in that the affected individuals are very long-term survivors with PDH complex deficiency despite having low (<20%) activity in skin fibroblasts. These findings prompted us to investigate the C-terminus of E$_1$$\alpha$ in greater detail. We constructed and expressed a series of PDH-E$_1$$\alpha$ deletion mutants in a cell line with zero PDH complex activity due to a null E$_1$$\alpha$ allele. Sequential deletion of the C-terminus by 1, 2, 3 and 4 amino acids resulted in PDH complex activities of 100, 60, 36 and 14% compared to wild-type E$_1$$\alpha$ expressed in PDH complex deficient cells. The immunodetectable protein was decreased by the same amount as the activity suggesting that the stability and/or assembly of E$_1$$\alpha_2$$\beta_2$ heterotetramer might depend on the intactness of PDH-E$_1$$\alpha$ C-terminus. In addition we compared the somatic and the testis-specific isoforms of E$_1$$\alpha$ and concluded that they are biochemically equivalent. *Hum. Mol. Genet.* (2000) 9, 1041-1048

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2.1 INTRODUCTION

Pyruvate dehydrogenase deficiency is a well-defined biochemical defect, which is clinically very heterogeneous. It is a major cause of primary lactic acidosis and Leigh disease in infants and young children, and in most cases is due to a defect in the \( E_1 \alpha \) gene [Robinson BH 1995a&b, Dahl et. al. 1992]. It may also present as a milder carbohydrate sensitive, intermittent ataxia syndrome [Robinson BH 1995a&b]. Despite the fact that there are two \( E_1 \alpha \) genes, one encoded on the X chromosome and one on chromosome 4, mutations in the somatically expressed X-linked \( E_1 \alpha \) gene are responsible for producing the well documented \( E_1 \) deficiency syndromes associated with the PDH complex [Robinson BH 1995a&b, Brown et. al. 1989, Brown et. al. 1990, Takakubo and Dahl 1992]. There is no expression of the chromosome 4 gene in tissues other than the testis [Takakubo and Dahl 1992]. Suggestions that the testis isoform could substitute for the X-linked coded \( E_1 \alpha \) protein if suitably expressed have been evaluated using a bacterial expression system with some success [Jeng et. al. 1998]. The testis \( E_1 \alpha \) gene product will integrate to produce a fully functional complex able to undergo phosphorylation and dephosphorylation as does the complex made with the X-linked \( E_1 \alpha \) gene product [Jeng et. al. 1998]. In this study we have used human skin fibroblasts with no endogenous \( E_1 \) activity (the \( \pi^0 \) cell line), as a vehicle to express both the somatic and the human testis-specific \( E_1 \alpha \) isoforms and compare their activities. In the past there have been reports that some mutations within the \( E_1 \alpha \) coding region which affect the C-terminus lead to a significant decrease in the \( E_1 \alpha \) as well as \( E_1 \beta \) immunoreactive material [Marsac et. al. 1997, Endo et. al. 1991, Endo et. al. 1989]. Two of these cases are female patients and the biochemical and protein expression data are hard to evaluate since one normally expressing \( E_1 \alpha \) allele is present and cell cultures are a mosaic
There is reduced expression in cell culture of $E_1\alpha$ protein in a case with one allele truncated by 8 amino acids [Endo et. al. 1991]. In a case of PDH deficiency in a 10 year old male with relatively mild symptoms of exercise intolerance and lactic acidemia, the $E_1\alpha$ mutation resulted in the addition of 33 amino acids with a residual PDHC activity of 27.4% [Endo et. al. 1989]. In these cases little systematic investigation has been carried out to determine whether the decreased amount of activity is due to decreased mRNA, decreased protein import into mitochondria or increased mitochondrial degradation of imported mutant protein.

Here we present results from three patients with mutations in the C-terminal portion of the $E_1\alpha$ coding region, who are very long-term survivors with PDH complex deficiency. We then compare these results with those of a study where four C-terminal amino acids of the $\alpha$ subunit of $E_1$ were sequentially deleted and resulting $E_1\alpha$ variants were expressed in a $\pi^0$ cell line.

2.2 MATERIALS AND METHODS

Enzymology and immunological analysis. Cultured skin fibroblasts were grown from skin biopsies in $\alpha$-MEM culture medium. Activity of the PDH complex in the native and dichloroacetate-activated state was determined in fibroblast extract by the method of Sheu et al. [Sheu et. al. 1981]. Western blotting was performed on mitochondrial extracts prepared by the method of Pitkanen et al. [Pitkanen et. al. 1996], using polyclonal antibody against porcine heart PDH complex antibody raised in rabbit [Ekong 1982].

RNA and cDNA preparation. Total RNA was extracted from cultured skin fibroblasts using TRIZol™ Reagent (Total RNA Isolation Reagent) from GIBCO BRL. First strand cDNA synthesis was carried out using total cellular RNA (20 $\mu$g) and an $E_1\alpha$-specific oligonucleotide $\alpha$G17’ (5’-TCTAGAATTCGTACAAACTGCATGCAATTAC-3’) with M-MLV reverse transcriptase (GIBCO BRL).

Amplification of DNA. DNA was amplified by PCR with cDNA as a template. 250
μM of each deoxynucleotide (dATP, dCTP, dGTP and dTTP), 10 μl of 10X PCR buffer (GIBCO BRL), 1.5 mM MgCl₂, oligonucleotide primer (1μg each), and 2 units of Taq polymerase (GIBCO BRL), in total reaction volume of 100 μl. PCR amplification [Saiki et. al. 1988] of the cDNAs extracted from primary cell lines was carried out as described previously [Chun et. al 1991]. Briefly, the total coding sequence of the E₁α subunit was amplified in two overlapping fragments, subcloned into pSP65 T-A cloning vector (Clontech) and sequenced using a series of α-specific primers. DNA was prepared from cultured skin fibroblasts of patients and control cell lines by a modified version of the method of Miller et al. [Miller et. al. 1988]. After 30 cycles of amplification under the conditions specified, the amplified fragments were visualized on ethidium bromide-stained gels, extracted and subcloned into pSP65. All DNA sequencing was performed by the Sanger dideoxy chain termination method [Sanger et. al. 1979] on double-stranded templates using a T7 polymerase sequencing kit (Pharmacia). For making E₁α deletion mutants, five microliters of the cDNA reaction mixture was used for PCR using E₁α-specific forward primer Kpn-F (5’-TTTGGTGACCTTGTGGAGTCGCCGCTGC-3’) and a series of reverse primers, each containing XhoI restriction site:

Xho-R (5’-TTTCTCGAGGAACACTGTCTGGTAGCC-3’) - for wild-type E₁α cDNA

E₁α-Δ1-R (5’-TTTCTCGAGTTAGACTTAAAC’ITGATCCACTGATTC-3’) - for Δ1 cDNA

E₁α-Δ2-R (5’-TTTCTCGAGTTARGACTTAAAC’ITGATCCACTGATGGC-3’) - for Δ2 cDNA

E₁α-Δ3-R (5’-TTTCTCGAGCTTAAACTTGATCCACTGATTGGC-3’) - for Δ3 cDNA

E₁α-Δ4-R (5’-TTTCTCGAGAAACTTGATCCACTGATTGGCACC-3’) - for Δ4 cDNA

The last four primers were designed so that a series of E₁α cDNA clones were made which were sequentially shorter by three nucleotides. In translation they produce a series of E₁α proteins sequentially shorter from the C-terminus by one amino acid.

The PCR conditions used were 94°C for 1 minute, 60°C for 1 minute and 72°C for 1.5 minutes for 35 cycles. The PCR product was then subcloned into pCR 2.1 (Invitrogen) using a TA CloningR Kit (Invitrogen).

Construction of the testis-specific variant of E₁α. Since the testis-specific gene is intronless, its sequence was obtained from genomic DNA by PCR. PCR primers were
designed with 5' restriction sites for easy cloning: forward primer Ts-F (5'-TTTGGTACCTGCCATCTACAGCACTCCGT-3') and reverse primer Ts-R (5'-TTTCTCGAGGCTCCTTGAGTTGGAGAACAC-3'). The PCR conditions used were 94°C for 30 sec, 58°C for 30 sec, and 72°C for 1 min 30 sec. The testis-E_{1α} was also first subcloned into pCR 2.1 to find an error-free clone (in comparison to the sequence published by Dahl [Dahl et. al. 1990], and then recloned into the KpnI/XhoI sites of pcDNA 3.1.

**DNA sequence analysis.** Plasmid clones containing PCR products cDNA were sequenced according to recommendations by the manufacturer (Amersham-Pharmacia Biotech) with the T7 Sequencing™ Kit. The reaction mixture was separated on an acrylamide gel. The entire coding region of the patient's cDNA was sequenced with the primers described [Dahl et. al. 1990].

**Cloning of E_{1α} variants into the expression vector.** Wild-type and mutant clones were digested with KpnI/XhoI and subcloned into the KpnI/XhoI site of pcDNA 3.1 (Invitrogen).

**Construction of π^{0} (PDHC-deficient) cell line.** A human female skin fibroblast cell line with a 7 b.p. deletion at base 931 (exon 10) in one of the E_{1α} alleles was transformed with SV40 large T antigen using a vector containing a large T antigen DNA (courtesy of Dr. John Dick, HSC, Toronto) by transfection. Transfection was carried out using the Superfect reagent (Qiagen) according to manufacturer's specifications on cells 18-22 hours after splitting at confluence of 60-80% in 6-well plates (35mm). 2 μg of column-purified (Qiagen) plasmid DNA was combined with 8 μl of the Superfect reagent in 150 μl of serum-free α-MEM. Cells were split 24-36 hours after transfection at a ratio of one 35 mm plate into five 100 mm plates. After 10-14 days transformed foci were cloned and assayed for PDHC activity. Clones that had zero PDHC activity, named π^{0}, were chosen for further characterization. In order to confirm the absence of the E_{1} protein, Western blot analysis was performed on mitochondria from the π^{0} cell line, the control cell line as well as the parental cell line. Twice the amount of protein was used for the π^{0} cell line than for the control or the parental cell lines to ensure that the experiment was not performed at the antibody's limit of immunoreactivity.

**Expression of wild-type and mutant E_{1α} variants in the π^{0} cell line.** All transfections
were performed as described above using the π₀ cell line and Qiagen-column purified DNA (either WT, mutant or vector alone) (Qiagen) which was linearized with PvuI. Two days post transfection cells were plated into 100mm plates and selection was applied at a concentration of 0.2 mg/ml G418 for 14-20 days until visible colonies could be detected. These colonies were cloned using cloning rings and PDHC activity was determined.

Quantitative PCR. RNA for quantitative PCR was isolated from transfected human skin fibroblasts using Total nucleic acid isolation kit (Qiagen). First strand cDNA synthesis was done as described above using oligo dT as a primer. A negative control was designed so that first strand synthesis was carried without MLV reverse transcriptase and used later in the PCR to assure that no plasmid DNA contamination gave false positive results. PCR was performed using a set of target primers as well as a set of internal control primers targeted against human phosphoglycerate kinase (PGK) (both sets of primers were present in the same PCR reaction). Primer sequences were the following target primers:

pcDNA 3.1F (5'-TTAATACGACTCACTATAGGGAG-3') and
pcDNA 3.1R (5'-AACTAGAAGGGCACAGTCGAGG-3')

internal control primers:
PGK-For (5'-CAGTTTGGAGCTCCTGGAAG-3')
PGK-Rev (5'-TGCAAATCCAGGGTGCAGTG-3')

PCR was carried out as described above using the following set of conditions: 94°C for 30 sec, 59°C for 30 sec, 72°C for 45 sec in total volume of 50 μl. The following number of cycles was performed: 18, 20, 22, 24, 26, 28, and 30. One PCR tube was set up per each set of cycles. Samples were run on a 2% agarose gel, in sequential order of the number of cycles and the amount of PCR product was quantitated by densitometry.

Cell culture. Untransfected, SV40-transformed human skin fibroblasts were grown in α-MEM supplemented with 15% Fetal Bovine Serum (FBS). Transfected SV40-transformed human skin fibroblasts were grown in α-MEM supplemented with 15% FBS supplemented with 0.2 mg/ml G418. Increased thiamin concentration in the medium was achieved whenever necessary by addition of 0.1 mg per 10 ml of medium.

Mitochondrial import studies. Mitochondria were isolated from rat hearts by the method of Takahashi and Hood [Takahashi and Hood 1996]. Full-length cDNAs encoding wild-type and variant Elα as well as human MnSOD were subcloned into pGEM 3Z.
Proteins were synthesized using TNT Coupled Reticulocyte Lysate System (Promega) using $^{35}$S-methionine as radioactive label (Amersham). Mitochondria were incubated with lysate containing the translated radiolabeled precursor proteins at 30°C for various periods of time, as indicated below. Final import reactions consisted of 40 μl (120 μg) of mitochondria, 5 μl of reticulocyte lysate and 2 μl of translation reaction. Glutamate was added as an additional respiratory substrate at a final concentration of 33 mM. Following incubation, mitochondria were recovered by centrifugation and washed twice with the import buffer. Pellets were resuspended in 15 μl of import buffer and 15 μl of 2X loading buffer (10% glycine (v/v), 80 mM SDS, 62.5 mM Tris-HCl, pH6.8, 5% 2-mercaptoethanol (v/v), and 10% Coomassie blue). Samples were denatured and electrophoresed through 12.5% SDS-polyacrylamide gel at 80V. Gels were fixed (30% methanol (v/v), 3% glycerol (v/v)), treated for 30 min with Amplify (Amersham), dried and exposed to film (Bio-Max, Kodak) at -80°C. Autoradiograms were quantified using densitometry.

2.3 RESULTS

Case Reports

Patient 1 & 2. These are twin brothers born in 1959 who were normal until 2 years of age when they started to have episodes of weakness, ataxia and hyperventilation. They were labelled as cerebral palsy in their early childhood. One twin appears to be more adversely affected than the other, though both have persevered with education at a school for the disabled. While they have learning difficulties, they are not mentally retarded. A recent admission for one twin (GF) after a collapse followed by dystonic movements revealed hypodensities in the internal capsule and basal ganglia on CT scanning. During this crisis he had an elevated blood lactate but this soon returned to normal. In cultured skin fibroblasts from the patients, overall PDH complex activity was 12.2% and 12.4% for the native complex (patient 1 and 2 respectively) and 13.8% and 14.6% for the DCA activated complex (patient 1 and 2 respectively). In cultured lymphoblasts from the patients, overall PDH
complex activity was 25.7% and 22.8% for the native complex (patient 1 and 2 respectively), and 32.5% and 25.7% for the DCA activated complex (patient 1 and 2 respectively).

**Patient 3.** This patient, now 39 years old, first diagnosed as PDH deficient by John Blass [Blass et. al. 1978] many years ago, was initially seen at 17 months for intermittent ataxia and dysarthria. He continued to have 5-6 attacks per year until his teens when the frequency decreased to 1-2 episodes per year. Attacks are usually precipitated by viral illness, stress, or anxiety and are associated with high blood pyruvate levels (0.4 mM). In addition to his intermittent symptoms, he has constant coarse bilateral hand tremor, dysarthria, and progressive Dupuytren's contractures in both hands. He also has persistent appendicular and truncal ataxia, which worsens during periods of metabolic decompensation. Vertigo, particularly pronounced when viewing rippling water, occurred in recent ataxic attacks. At ages 34 and 37 he suffered acute psychotic episodes requiring psychiatric inpatient care and antipsychotic medications, with paranoia, anxiety, memory loss and mutism. He has also been severely depressed. Despite his episodic illness the patient attended university, obtaining a degree in computer engineering. He works as a computer programmer. He has been treated with intermittent acetazolamide, intermittent prednisone and persistently with 300 mg/day of thiamine. In cultured fibroblasts from the patient, overall PDH complex activity was 14.7% for the native complex and 22.8% for the DCA activated complex.

Western blot analysis showed lowered PDH E1α and β immunoreactivity for all three patients, with about 15% and about 30% levels of the control present in fibroblasts and lymphoblasts respectively (Fig. 2.1A & B).
Figure 2.1. Western blot analysis of cultured skin fibroblasts (A) and lymphoblasts (B) derived from patients with E$_1$$\alpha$ C-terminal deletions. One hundred micrograms of total protein from mitochondria-rich fractions were separated on 12.5% SDS/polyacrylamide gel and were transferred to nitrocellulose. The filter was probed with an antibody against affinity-purified pig heart PDH complex raised in rabbits. Lanes are from left to right: control, patient 3 patient 1, patient 2.
Figure 2.2. Sequencing of plasmids containing PCR products of PDH-E\textsubscript{1}\(\alpha\) cDNA derived from patients with E\textsubscript{1}\(\alpha\) C-terminal deletions. Encircled base (A) indicates the site of C1163A substitution and boxed AAGT indicate the site of insertion @ bp 1159. Shown is a control, C and the affected males.

Characterization of the mutations. Patients 1 and 2 have an AAGT insertion at 1159 and patient 3 has a C1163T change. Both mutations effectively produce a stop codon prematurely a short distance from the C-terminus (Fig. 2.2).

Creation of the \(\pi^0\) cell line for E\textsubscript{1}\(\alpha\) expression. We transformed a human skin fibroblast cell line derived from a female patient with a mutation that nullified one of the PDH-E\textsubscript{1}\(\alpha\) alleles (see materials and methods). After transformation and cloning of individual foci, two types of clones were identified, those that had normal PDHC activity and those that had zero PDHC activity, renamed \(\pi^0\). Both types as well as the parental cell line (see
materials and methods) were probed with anti-total PDHC antibody by Western blotting. As shown in Fig. 2.3, neither E₁α nor E₁β subunits are expressed in the π₀ cell line, however the expression of the E₂ component of the PDH complex remains unaltered. Clones with normal PDHC activities also had normal expression of the PDH complex subunits. The parental cell line however showed substantial decrease in both the E₁α and E₁β protein levels. Densitometry analysis of protein peaks reveals that the amount of each of the E₁ subunits is about 10% of the control. This correlates with the 7.5 ± 2.3% residual activity in the parental cell line.

![Western blot analysis](image)

**Figure 2.3. Western blot analysis of the π₀ cell line as well as the parental and control cell lines.** Sixty micrograms for the control and the parental cell line, and one hundred micrograms for the π₀ cell line of total protein from mitochondria-rich fractions were separated on 12.5% SDS/polyacrylamide gel and were transferred to nitrocellulose. The filter was probed with an antibody against affinity-purified pig heart PDH complex raised in rabbit. C = control cell line, P = parental cell line.
Expression of C-terminus deletion mutants of E1α. To investigate the importance of the C-terminus of PDH-E1α for the stability and/or assembly of the E1αβ2 heterotetramer, we constructed a series of PDH-E1α deletion mutants where each mutant was sequentially shorter by one amino acid from the C-terminus. cDNA constructs corresponding to these E1α amino acid variants (Δ1, Δ2, Δ3, and Δ4) as well as the testis-specific isoform were subcloned into a mammalian expression vector and transfected into the π0 cell line. All PCR-generated E1α cDNA variants were digested with KpnI/XhoI, and subcloned into the mammalian expression vector pcDNA 3.1. The individual clones were sequenced to confirm that there were no PCR artefacts generated. The error-free clones were used in the transfection experiments.

The π0 cell line was transfected with cDNAs encoding the wild-type E1α, the testis-specific E1α as well as four different C-terminus deletion mutants of E1α: Δ1, Δ2, Δ3, and Δ4. Stably expressing clones were selected, subcloned, and analysed for expression of the E1α variant proteins by the total PDHC activity assay (Table 2.1) as well as by the binding of antibody directed against pig heart total PDH complex (Fig. 2.4). Levels of exogenous E1α mRNA expression were examined by quantitative PCR using PGK mRNA as an internal control (Fig. 2.5). This method was chosen to differentiate between the endogenous and exogenous E1α mRNA levels due to the fact that although the π0 cell line presents with a complete loss of enzyme activity and immunoreactive E1α protein the transcription and message production of the endogenous E1α remains unaltered.

Analysis of PDH complex activity assays. Complete recovery of activity upon post-transfectional wild-type E1α cDNA expression in π0 cell lines was not achieved (Table 2.1), and rates of only 55% compared with control were recorded. Expression of the testis-
specific \(E_1\alpha\) as well as \(\Delta 1\) variant resulted in a comparable restoration of activity – about 55%. When rates for \(\Delta 2\), \(\Delta 3\) and \(\Delta 4\) were recorded and averaged they were 33%, 20% and 8% respectively (Table 2.1). Therefore a specific pattern emerged from this part of the study, where total PDH complex activity decreased as the C-terminal residues were removed one by one from the \(E_1\alpha\) protein.

\[
\begin{array}{|c|c|c|c|}
\hline
\text{\(E_1\alpha\) VARIANT} & \text{PDH COMPLEX ACTIVITY} & \text{\(\%\) OF CONTROL} \\
& \text{nmol/min/mg} & \text{DCA ACTIVATED} & \\
& \text{NATIVE} & \text{DCA ACTIVATED} & \\
\hline
\text{control} & 0.98 \pm 0.14 [10] & 1.12 \pm 0.16 [10] & 100\% \\
\text{\(E_1\alpha\) cDNA} & 0.47 \pm 0.09 [5] & 0.61 \pm 0.06 [5] & 55\% \\
\text{testis-specific} & 0.53 \pm 0.10 [6] & 0.59 \pm 0.08 [6] & 54\% \\
\text{\(\Delta 1\)} & 0.49 \pm 0.11 [5] & 0.62 \pm 0.04 [5] & 55\% \\
\text{\(\Delta 2\)} & 0.34 \pm 0.04 [5] & 0.37 \pm 0.05 [5] & 33\% \\
\text{\(\Delta 3\)} & 0.19 \pm 0.04 [4] & 0.23 \pm 0.03 [4] & 20\% \\
\text{\(\Delta 4\)} & 0.10 \pm 0.03 [7] & 0.09 \pm 0.05 [7] & 8\% \\
\text{\(\pi^0\)} & 0.0 [11] & 0.0 [11] & 0 \%
\hline
\end{array}
\]

Table 2.1. Total PDH complex activities recorded and averaged for PDH-\(E_1\alpha\) variants transfected into the \(\pi^0\) cell line. Number of determinations for each clone are placed in brackets. \(\%\) of control is calculated using DCA activated values and given to enhance the clarity of the table.

\textit{Analysis of expression of variant \(E_1\alpha\) proteins.} To compare the rates of total PDH complex activities with the levels of \(E_1\alpha\) protein expression, Western blotting was performed using antibodies directed against total PDH complex. The levels of \(E_1\alpha\) expression for each clone were standardized to the corresponding levels of \(E_2\) (the core protein) by densitometry analysis and expressed as a ratio of \(E_1\alpha:E_2\). The ratio \(E_1\alpha:E_2\) for the SV 40-transformed
control cell line was taken as 1 (Fig. 2.4). Results of this experiment mirror results obtained from the activity assays such that progressively lower amounts of immunoreactive $E_1\alpha$ protein were detected as the C-terminal $E_1\alpha$ residues were removed. The amount of $E_1\alpha$ protein expressed for each mutant also correlates with the corresponding activity of the complex, perhaps indicating that the specific activities of mutant complexes are unaltered. The expressed levels of $E_1\beta$ progressively decrease as well, implying that $E_1\beta$ is incapable of autonomous existence without $E_1\alpha$ [Robinson BH 1995a, Kerr et. al. 1988, Robinson et. al. 1989]. The amount of protein detected for the testis-specific and wild-type $E_1\alpha$ were comparable, further supporting the hypothesis that these isoforms are interchangeable within the PDH complex.

Figure 2.4. Western blot analysis of the exogenous expression of the wild-type and variant PDH-$E_1\alpha$. Eighty micrograms of total protein from mitochondria-rich fractions were separated on 12.5% SDS/polyacrylamide gel and were transferred to nitrocellulose. The filter was probed with an antibody against affinity-purified pig heart PDH complex raised in a rabbit. The amounts of $E_1\alpha$ were normalized by densitometry analysis to the amounts of $E_2$ (PDH core protein) and expressed underneath the figure as a ratio of $E_1\alpha/E_2$. The ratio of $E_1\alpha/E_2$ for the control SV 40-transformed cell line was taken as 1.
Analysis of mRNA levels for exogenous Ela variants. Results for the quantitative PCR performed on clones expressing wild-type, Δ1, and Δ4 PDH-Ela are shown in Fig. 2.5. Densitometric analysis of band intensities showed that when compared to the internal control (PGK - upper band), the levels of exogenous mRNA transcribed from the CMV promoter of the transfected vectors were similar in each cell line. Comparable results were obtained for the remaining PDH-Ela clones (results not shown). Therefore these findings show that

![Figure 2.5](image_url)

**Figure 2.5.** Quantitative RT-PCR specifically designed to pick up the exogenous but not the endogenous Ela mRNA, performed on wild-type Ela (A), Δ1 variant (B) and Δ4 variant (C). The experiment was performed on 1 μg total RNA extracted from a single confluent 100 mm plate. The ratio of PGK to Ela was determined by densitometry and found to be about 1:2. Similar results were obtained for the remaining Ela variants. Number of PCR cycles performed is indicated on the top of each panel.
lower protein levels for E1α deletion mutants were not caused by lower levels of mRNA transcription.

Import of E1α variants into the mitochondrial matrix. To investigate whether lower levels of immunoreactive E1α deletion mutant proteins were due to deficient import into mitochondria, all PDH E1α variant proteins were synthesized in vitro and added to freshly prepared rat heart mitochondria. A mitochondrial protein MnSOD was used to assess the import competence of the system (data not shown). Results for this experiment are shown in Fig. 2.6. The 49 kDa precursor proteins were imported into mitochondria isolated from rat heart, being processed to the mature-size 42 kDa protein. By densitometric analysis, it was established that despite small differences in overall amount adhering to mitochondria, by comparing the amount of precursor E1α (p-E1α) to mature E1α (m-E1) there was no difference in precursor processing between wild-type E1α and any one of the investigated E1α deletion variants.

Figure 2.6. In vitro import of human precursor PDH-E1α. Full length precursor wild-type and variant E1α proteins were synthesized using TNT Coupled Reticulocyte Lysate System (Promega) with 35S-methionine as radioactive label (Amersham). Import into isolated rat heart mitochondria was for 10 and 30 min, as indicated. (-) mitos = in vitro translated wild-type E1α incubated without rat heart mitochondria; p-E1α = 49-kDa precursor, m-E1α = 42-kDa mature form.
2.4 DISCUSSION

We have shown that three patients with a long lived mild phenotype of PDH complex deficiency display a strong correlation between residual PDH complex activity in fibroblasts and lymphoblasts and the levels of the E1α and the E1β subunits as seen by immunoblot. E1 subunit levels of 12-15% seen in fibroblasts correlated with residual activities of the PDH complex of about 15%, and residual E1α levels of 25% seen in lymphoblasts correlate with enzyme activities of 26-32% for the PDH complex. These patients had E1α mutations, which effectively removed the 3 C-terminal amino acids (SerValSer) from the protein, exposing a lysine as the N-terminal residue. For the majority of patients investigated with PDHC - E1 deficiency, residual rates in fibroblasts and lymphoblasts are the same, whereas rates in heart, kidney and liver can vary in the same patient [Robinson 1995]. Thus the two-fold difference in activity and protein seen in lymphoblasts versus fibroblasts was due to different residual levels of mutant E1 in the complex presumably caused by differential rates of degradation. This conclusion that the missing C-terminal amino acids led to increased degradation of a potentially functionally active protein E1 component was further investigated by making mutant cDNA constructs coding for subunits missing 1, 2, 3 and 4 amino acids and expressing them in a π0 cell line totally devoid of PDH complex activity. The residual activity of the PDH complex again decreased with increasing number of amino acids removed, correlated strongly with the amount of immuno-titratable E1α and E1β subunits in the complex suggesting no change in specific activity of E1. This supports a mechanism in which truncations of E1α appear to bestow instability either on the E1αβ2 tetramer or alternatively on the whole assembled complex. The fact that E2 levels remain the same while the E1α and E1β are depleted, mitigates against a mechanism involving the whole complex. Similarly
differential degradation of $E_1\alpha$ mRNA species and possible failure of import of truncated $E_1\alpha$ protein were ruled out by examining mRNA levels and import respectively (Figs. 2.5 and 2.6). Missense mutations of $E_1\alpha$ with no change in protein length may also present in males as carbohydrate sensitive ataxia [Robinson BH 1995a&b]. In this case the change in activity may be due to $E_1$ protein degradation or more usually to a change in specific activity of the $E_1$ component [Robinson 1995, Kerr et. al. 1998, Robinson et. al. 1989, Chun et. al. 1995].

The present study showed that it is possible to complement the PDH enzyme defect caused by a faulty PDH $E_1\alpha$ gene by transfecting the cells with the normal PDH $E_1\alpha$ cDNA. However, normal levels of activity were never achieved, in stably transfected populations. Nevertheless, significantly higher values were recorded than has been previously reported by others for this type of transfection [Brown et. al. 1997]. A possible explanation is that overexpression of the $E_1\alpha$ subunit altered the proper stoichiometry of $E_1\alpha$ to $E_1\beta$ proteins and therefore interfered with the overall PDH complex assembly [Brown et. al. 1997]. Furthermore, it is known that the $E_1\alpha$ subunit alone has no demonstrable enzymatic activity and requires assembly with $E_1\beta$ in order to form an active $\alpha_2\beta_2$ heterotetramer. Otherwise, the proteins degrade, as seen in patients with PDH deficiency [Brown 1992, Chun et. al. 1991], and branched-chain $\alpha$-keto acid dehydrogenase deficiency [Fisher et. al. 1991, Zhang et. al. 1989]. Therefore, the overexpressed $E_1\alpha$ proteins may degrade at the time when there is no $E_1\beta$ protein available due to uncoordinated expression [Stankovics and Ledley 1993, Saijo et. al. 1996].

The influence of thiamin on patients with PDH complex deficiency has been controversial, with many patients having been treated from the time of diagnosis [Robinson
Thiamin diphosphate is thought to be bound by elements of both $E_1\alpha$ and $E_1\beta$ subunits, each $\alpha_2\beta_2$ heterotetramer has two ThDP molecules, one each bridging an $E_1\alpha$ and $E_1\beta$ to form an active site [Robinson and Chun 1993]. The patients here represent the two ends of the spectrum in that one pair of sibs were totally untreated because of late diagnosis while the other patients with the same mutation were treated from an early age. It would seem that the treated patients have fared better than the untreated.

These belong to a group of relatively long-lived, mildly affected males who have the presentation originally referred to by us and others as carbohydrate-sensitive ataxia [Robinson et al. 1987, Blass et al. 1971]. There are two further patients from the Maritime provinces of Canada who have the 1159 AAGT ins mutation [Chun et al. 1991]. These unrelated males are both now 13 years of age, attend special school and have bouts of ataxia which are carbohydrate and stress induced. So far these patients have no detectable CNS lesions and have been given thiamin supplements from an early age. Our conclusion is that this group of patients with apparently low residual fibroblast activities of PDH complex nevertheless have mild phenotypes because specific activity of $E_1$ is not altered by C-terminal truncation. Overall activity is governed by tissue specific degradation mechanisms allowing the brain to have the 30-40% normal activity necessary for normal function [Robinson BH et al. 1995].

Despite its importance, the molecular mechanisms governing the tight tissue-specific regulation of testis-specific isoform of the PDHC $E_1\alpha$ subunit remain poorly understood [Young et al. 1998]. Even more limited data have been published on the biochemical aspects of this isoform. Results obtained in this study show for the first time that the expression of the testis-specific $E_1\alpha$ isoform in a mammalian cell line produces a fully
functional PDH complex, supporting previous in vitro findings [Jeng et. al. 1998]. This shows that should a mechanism for inducing somatic expression of the testis-specific E1α isoform be found, correction of X-linked E1α defects might be feasible.

2.5 ACKNOWLEDGEMENTS

We thank MRC Canada for the financial support of this research project, and Dr. Ives of St. John's, Newfoundland for the cell lines on patients 1 & 2.
FUNCTIONAL EXPRESSION OF FOUR PDH-E\textsubscript{1}\textalpha\textalpha\textalpha\textbeta\textalpha RECOMBINANT HISTIDINE MUTANTS IN A HUMAN FIBROBLAST CELL LINE WITH ZERO ENDOGENOUS PDH COMPLEX ACTIVITY.

Conserved histidine residues have been implicated in the geometry and catalytic mechanism of the E\textsubscript{1}\textalpha proteins of the PDH complex. We constructed and expressed a series of PDH-E\textsubscript{1}\textalpha histidine mutants (H63, H84, H92 and H263) in a cell line with zero PDH complex activity due to a null E\textsubscript{1}\textalpha allele. Based on immunoblot and enzyme activity analyses, all introduced histidine mutations, with the exception of H92, affected the specific activity of the PDH complex. We showed that H63 and H263 are essential for the activity since mutations introduced at those sites, produced a PDH complex with near-zero activity. Mutations introduced at H84 only partially reduced activity, implying that H84 may play a less critical role in the PDH complex. Mutations introduced at H92 caused absence of immunoreactive material for both the E\textsubscript{1}\textalpha and E\textsubscript{1}\textbeta subunits and may have impaired import or assembly of precursor peptides into the mature PDH complex. Biochem. Biophys. Res. Comm. (2000) 270, 1068-1073
3.1 INTRODUCTION

In 1993 a model for the thiamin diphosphate-binding site of human pyruvate dehydrogenase E₁ was proposed. This model was constructed based on homologies between the published X-ray crystal structure of a ThDP-binding enzyme transketolase (from *Hanensula polymorpha*) and E₁ from human, *S. stearothermophilus*, *E. coli*, as well as *S. cerevisiae* pyruvate decarboxylase. [Chun and Robinson 1993]. According to this model, two histidine residues, H84 and H263, are required for diphosphate co-ordination through hydrogen bonding. To investigate the validity of this model, we mutated each of the two histidines to alanine, lysine or aspartate, and expressed the resultant E₁α variants in a transformed human skin fibroblast cell line with no endogenous E₁ activity. Because there are two additional histidine residues in the vicinity of H84, namely H63 and H92, we also introduced mutations at these positions (either aspartate or asparagine), and expressed thus created variants.

3.2 MATERIALS AND METHODS

*Enzymology and Immunological Analysis.* Activity of the PDH complex in the native and dichloroacetate-activated state was determined in fibroblast extracts by the method of Sheu [Sheu et. al. 1981]. Western blotting was performed on mitochondrial extracts prepared by the method of Pitkanen. [Pitkanen et. al. 1996], using polyclonal antibody against porcine heart PDH complex antibody raised in rabbit. [Ekong 1982].

*RNA and cDNA Preparation.* Total RNA was extracted from control cultured skin fibroblasts, using TRizolTM Reagent (Total RNA Isolation Reagent) from GIBCO BRL. First strand cDNA synthesis was carried out using total cellular RNA (20 µg) and an E₁α-
specific oligonucleotide αG17’ (5’-TCTAGAATTCTGACAAACTGCATGCAATTAC-3’)
with M-MLV reverse transcriptase (GIBCO BRL).

Amplification of DNA. DNA was amplified by PCR with cDNA as a template, 250 μM of each deoxynucleotide (dATP, dCTP, dGTP and dTTP), 10 μl of 10X PCR buffer (GIBCO BRL) 1.5 mM MgCl₂, oligonucleotide primer (1μg each), and 2 units of Taq polymerase (GIBCO BRL), in total reaction volume of 100 μl. PCR amplification [Saiki et al. 1988] of the cDNAs was carried out according to specifications below. The amplified fragments were subcloned into pCR2.1 cloning vector (Invitrogen) using a TA Cloning Kit (Invitrogen). DNA sequencing was performed using the Sequencing TM Kit (Amersham-Pharmacia Biotech). The reaction mixture was separated on an acrylamide gel. The entire coding region of the EIα cDNA was sequenced with the primers described elsewhere [Chun et al. 1993]. For synthesis of EIα Wild type and histidine constructs, five microliters of the cDNA reaction mixture was used for PCR carried out using EIα-specific forward primer Kpn-F (5’-TTTGGTACCTTGTGAGGAGTCGGCCGCTGC-3’), a reverse primer Xho-R (5’-TTTCTCGAGGAGAACACTGTCTGGTAGCC-3’) and a series of site-directed mutagenesis primers containing an appropriate base(s) change - see below - using a 1-step PCR method developed by Ling and Robinson [Ling and Robinson 1995]. PCR reaction conditions were the following, 20 cycles at conditions: 94°C for 30 sec, 57°C for 30 sec, 72°C for 30 sec, followed by one cycle at conditions: 94°C for 2 min, 65°C for 2 min, 72°C for 6 min, followed by 30 cycles at conditions: 94°C for 30 sec, 57°C for 30 sec, 72°C for 1 min 30 sec. Kpn-F and Xho-R primers were used at concentration of 500 ng/100μl and the mutagenic primers were at concentration of 30-80 ng/100 μl. Sequences of mutagenic primers were as follows:

E1A-63Asp-R  5’-ACCATCACACAAGTCACAGAAAAACAC-3’
E1A-63Asn-R  5’-ACCATCACACAATTACAGAAAAACAC-3’
E1A-84Ala-R  5’-GTGGATGGAGAGCGTCTGTGGGGTTG-3’
E1A-84Asp-R  5’-TGTGATGAGATCGTCGTGGGGGTTG
E1A-84Lys-R  5’-TGTGATGAGTTTGCTGTGGGGTTG-3’
E1A-92Asp-R  5'-AAAGTAAAGCCGTCAGCCCGGTAGG-3'
E1A-92Asn-R  5'-AAAGTAAAGCCATTAGCCCGGTAGG-3'
E1A-263Ala-F  5'-CCGTTACCACGGAGCCAGTATGAGT-3'
E1A-263Asp-F  5'-CCGTTACCACGGAGACAGTATGAGT-3'
E1A-263Lys-F  5'-CCGTTACCACGGAAAAAGTATGAGT-3'

**Cloning of E₁α variants into the expression vector.** Wild type and mutant clones were digested with KpnI/XhoI and subcloned into the KpnI/XhoI site of pcDNA 3.1 (Invitrogen).

**Construction of π₀ (PDHC-deficient) cell line.** The π₀ cell line was constructed as described in chapter 2.

**Expression of Wild Type and mutant E₁α variants in the π₀ cell line.** All transfections were carried out as described above using the π₀ cell line and Qiagen-column purified DNA (either WT, mutant or vector alone) (Qiagen) which was linearized with PvuI. Two days post transfection cells were plated into 100 mm plates and selection was applied at a concentration of 0.2 mg/ml G418 for 14-20 days. Single colonies were cloned and PDHC activity was determined. Expression from the permanently integrated pcDNA-E₁α constructs was stable and reproducible. Approximately 12 colonies per construct were selected and assayed.

**Detection of mRNA synthesis by the PCR method for H92 variants.** RNA for PCR was isolated from H92D- and H92N-transfected human skin fibroblasts using Total nucleic acid isolation kit (Qiagen). First strand cDNA synthesis was carried out as described above using oligo dT as a primer. A negative control was designed so that first strand synthesis was carried without MLV reverse transcriptase and used later in the PCR to assure that no plasmid DNA contamination gave false positive results. PCR was performed using simultaneously a set of target primers as well as a set of internal control primers targeted against human PGK. Primer sequences were the following target primers:
- pcDNA 2F (5'-GAGACCCAAGCTGGCTAGCG-3')
- pcDNA 2R (5'-CAACTCAATTCGGCGTACAGTCT-3')

**Internal control primers:**
PGK-For (5'-CAGTTTGGAGCTCCTGGAAG-3')
PGK-Rev (5'-TGCAAATCCAGGGTGCAGTG-3')

PCR was carried out as described above using the following set of conditions: 94°C for 30 sec, 59°C for 30 sec, 72°C for 45 sec in total volume of 50 μl for 30 cycles. Samples were ran on a 2% agarose gel, photographed, and the amount of PCR product was quantitated by densitometry.

Cell culture. Untransfected, SV40-transformed human skin fibroblasts were grown in α-MEM supplemented with 15% Fetal Bovine Serum (FBS). Transfected SV40-transformed human skin fibroblasts were grown in α-MEM supplemented with 15% FBS supplemented with 0.2 mg/ml G418.

Mitochondrial import of H92N and H92D mutants. Mitochondria were isolated from rat hearts by the method of Takahashi and Hood [Takahashi and Hood 1996]. Full-length cDNAs encoding Wild type and H92N and H92Q PDH-E1α variants were subcloned into pGEM 3Z. Proteins were synthesized using TNT Coupled Reticulocyte Lysate System (Promega) using [35S]-methionine as radioactive label (Amersham). Mitochondria were incubated with lysate containing the translated radiolabeled precursor proteins at 30°C for 40 min according to the method of Takahashi and Hood [Takahashi and Hood 1996]. Final import reactions consisted of 40 μl (120 μg) of mitochondria, 5 μl of reticulocyte lysate and 2 μl of translation reaction. Following incubation, 10 μl of trypsin (1.25 mg/ml) was added to selected reactions and digestion was carried out on ice for 10 min. Reactions were terminated with addition of 10 μl of soybean trypsin inhibitor (2.5 mg/ml). Mitochondria were recovered by centrifugation and washed. Pellets were resuspended and mixed with 2X 10% Coomassie blue loading buffer. Samples were electrophoresed through 12.5% SDS-polyacrylamide gel at 80V. Gels were fixed, treated for 30 min with Amplify (Amersham), dried and exposed to film (Bio-Max, Kodak) at -80°C. Autoradiograms were quantified using densitometry.
3.3 RESULTS

Site-directed Mutagenesis and expression of $E_1\alpha$ variants. Site-directed mutagenesis was used to investigate four histidine residues in PDH-E$_1\alpha$. H84 and H263 were replaced with alanine, aspartate or lysine, and H63 and H92 were replaced with either aspartate or asparagine. These E$_1\alpha$ variants were subcloned into the mammalian expression vector pcDNA 3.1 and transfected into the $\pi^0$ cell line to establish permanently expressing cell lines. Stably expressing clones were selected, subcloned, and analysed for expression of the $E_1\alpha$ variant proteins by the total PDHC activity assay (Table 3.1), as well as by the binding of antibody directed against pig heart total PDH complex (Figure 3.2).

Activity assay results. (presented in Table 3.1) Recovery of activity upon post-transfectional wild-type $E_1\alpha$ cDNA expression in the $\pi^0$ cell line gave a maximum rate of activity of 55% compared with un-manipulated control fibroblast cultures. Rates recorded for H63, and H263 mutants were $\leq$ 1%. Rates recorded for H92 mutants were zero. Rates recorded for H84 mutants were about 60% of the exogenously expressed wild type $E_1\alpha$, or 33% of the control PDH competent cell line.

Expression of $E_1\alpha$ variants in the $\pi^0$ cell line. To compare the rates of total PDH complex activities with the levels of $E_1\alpha$ protein expression, immunoblotting was performed using antibodies directed against total PDH complex. Results of the immunoblots for selected mutants are shown in Fig. 3.1. All mutants with the exception of H92D and H92N showed expression of both $E_1\alpha$ and $E_1\beta$ in comparable amounts. However, the total amount of $E_1\alpha$ protein (relative to the endogenously expressed E$_2$ protein) was less, in stable wild type $E_1\alpha$
transfectants compared to PDH competent controls confirming the enzymatic assay results.

<table>
<thead>
<tr>
<th>E₁α VARIANT</th>
<th>PDH COMPLEX ACTIVITY (nmol/min·mg cell protein⁻¹)</th>
<th>% OF CONTROL</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NATIVE</td>
<td>DCA ACTIVATED</td>
</tr>
<tr>
<td>control</td>
<td>0.98 ± 0.14 [10]</td>
<td>1.12 ± 0.16 [10]</td>
</tr>
<tr>
<td>π₀</td>
<td>0.0 [11]</td>
<td>0.0 [11]</td>
</tr>
<tr>
<td>E₁α cDNA</td>
<td>0.47 ± 0.09 [8]</td>
<td>0.61 ± 0.06 [8]</td>
</tr>
<tr>
<td>H63D</td>
<td>0.017 ± 0.009 [6]</td>
<td>0.012 ± 0.009 [6]</td>
</tr>
<tr>
<td>H63N</td>
<td>0.012 ± 0.007 [6]</td>
<td>0.015 ± 0.008 [8]</td>
</tr>
<tr>
<td>H84A</td>
<td>0.29 ± 0.08 [5]</td>
<td>0.37 ± 0.04 [5]</td>
</tr>
<tr>
<td>H84D</td>
<td>0.32 ± 0.07 [6]</td>
<td>0.035 ± 0.06 [6]</td>
</tr>
<tr>
<td>H84K</td>
<td>0.35 ± 0.06 [5]</td>
<td>0.034 ± 0.06 [5]</td>
</tr>
<tr>
<td>*H92D</td>
<td>0.0 [7]</td>
<td>0.0 [7]</td>
</tr>
<tr>
<td>*H92N</td>
<td>0.0 [7]</td>
<td>0.0 [7]</td>
</tr>
<tr>
<td>H263A</td>
<td>0.013 ± 0.009 [6]</td>
<td>0.009 ± 0.009 [6]</td>
</tr>
<tr>
<td>H263D</td>
<td>0.010 ± 0.008 [6]</td>
<td>0.013 ± 0.008 [6]</td>
</tr>
<tr>
<td>H263K</td>
<td>0.012 ± 0.007 [5]</td>
<td>0.010 ± 0.007 [5]</td>
</tr>
</tbody>
</table>

Table 3.1. Mean total PDH complex activities given ± SEM for controls, for π₀ cells and for PDH-E₁α variants transfected into the π₀ cell line. The number of determinations for each clone is given in brackets. Values are given for the native enzyme and for enzyme after activation by dichloroacetate (DCA). *These clones failed to produce any measurable ¹⁴CO₂ above background in the assay system used. Percentage values are calculated using DCA activated values and are given for the purpose of greater clarity of the table.
RT-PCR to determine the level of exogenous mRNA for H92 variants. As can be seen in Fig. 3.1, none of the H92 variants were able to produce detectable $E_1\alpha$ protein. To determine the underlying cause, quantitative RT-PCR was designed to determine the pattern of mRNA transcription for these mutants. As shown in Fig. 3.2, the amount of mRNA transcribed from the exogenous promoter for any one of the H92 variants appears to be comparable to the amount transcribed for the Wild type $E_1\alpha$ construct. Thus the failure to produce H92D and H92N variants was not due to absence and/or instability of exogenous mRNA.

Import of H92 variants into mitochondria. To investigate whether lack of immunoreactive material for H92 $E_1\alpha$ variants was caused by deficient import of protein precursors into mitochondria, $^{35}$S-labeled H92D and H92N were synthesised in vitro along with the wild-type $E_1\alpha$ and added to freshly prepared rat heart mitochondria. The 45 kDa $E_1\alpha$ precursor protein was imported into rat heart mitochondria, after cleavage to the mature-size 42 kDa protein (Fig. 3.3). The amount of precursor $E_1\alpha$ (p-$E_1\alpha$) and mature $E_1\alpha$ (m-$E_1\alpha$) were then compared before and after external trypsin addition. While the import-dependent cleavage appears to be indistinguishable from Wild type protein, it is apparent that there is less of the H92D or H92N mature variant proteins post-trypsin than the wild type $E_1\alpha$. This suggests that appropriate cleavage but poor incorporation of the H92 modified proteins occurs, implying that the processed product is degraded.
Figure 3.1. Western blot analysis of the plasmid expression of the wild-type and variant PDH-E₁α. Sixty micrograms of total protein from mitochondria-rich fractions were separated on 12.5% SDS/polyacrylamide gel and were transferred to nitro-cellulose. The filter was probed with an antibody against affinity-purified pig heart PDH complex raised in rabbit. The mitochondrial extracts present on the gel are from left to right: 1. control cultured skin fibroblasts, 2. π⁰, PDH-deficient cell line, 3. π⁰ cells transfected with wild type (WT) E₁α cDNA, 4. π⁰ cells transfected with H63D E₁α, 5. π⁰ cells transfected with H63N E₁α, 6. π⁰ cells transfected with H84A E₁α, 7. π⁰ cells transfected with H84K E₁α, 8. π⁰ cells transfected with H92D E₁α, 9. π⁰ cells transfected with H92N, 10. π⁰ cells transfected with H263D E₁α, 11. π⁰ cells transfected with H263K E₁α.
Figure 3.2. RT-PCR specifically designed to generate a signal from the exogenous but not the endogenous E$_1$α mRNA, performed on wild-type E$_1$α (WT), the π$^0$ cell line, as well as H92D and H92N E$_1$α variants. The experiment was performed using 1 μg total RNA extracted from a single confluent 100 mm plate as described in the methods section. The ratio of PGK to E$_1$α was determined by densitometry and found to be about 3:1 for all examined clones. Each lane represents the visualized PCR product. Lane 1. π$^0$ cells, Lane 2. π$^0$ cells transfected with H92D, Lane 3. π$^0$ cells transfected with H92N, Lane 4 π$^0$ cells transfected with wild-type E$_1$α, Lane 5. as for lane 4 but no reverse transcriptase used before amplification.
Figure 3.3. *In vitro* import of human precursor PDH-E₁α. Full length precursor wild-type and variant E₁α proteins were synthesized using TNT Coupled Reticulocyte Lysate System (Promega) with ³⁵S-methionine as radioactive label (Amersham). Import into isolated rat heart mitochondria was carried for 40 min, after which some reactions were subjected to trypsin digestion (10 μl of 1.25 mg/ml) for 10 min on ice. Soybean trypsin inhibitor (10 μl of 2.5 mg/ml) was used to stop the reactions. (-) = non trypsin treated reactions; (+) = trypsin-treated reactions; p-E₁α = 45 kDa precursor; m-E₁α = 42 kDa mature form.

3.4 DISCUSSION

In 1993 a model for the thiamin diphosphate-binding site of human pyruvate dehydrogenase E₁ was proposed based on the X-ray crystal structure of transketolase [Chun and Robinson 1993]. To investigate the validity of this model we created and expressed a number of PDH-E₁α variants that included H63, H84, H92 and H263. A transformed clonal fibroblast line from a female patient with PDH deficiency and a frameshift mutation in the coding sequence of the E₁α gene with zero endogenous PDHC activity was generated to permit analysis of expression of different PDH E₁α cDNA variants in a uniform genetic and biochemical background. The use of this transformed π⁰ line was necessary to enable
selection of stably transfected clones and generate the quantity of cells sufficient to allow repeated enzyme assays and other investigations such as Western blot, always using the same clonal population.

Based on the Western blot and the enzyme activity analyses, all introduced histidine mutations (with the exception of H92) affected the specific activity of the PDH complex. Such a conclusion is supported by the fact that the level of E1α present within each of the mutant complexes were comparable i.e. they were the same based on the E1α/E2 (the core) protein ratio.

We showed that mutations in His 263 decreased PDH complex activity to very low values (see Table 3.1). Such an outcome is not surprising, since this histidine residue is a conserved feature of the active site in all known heterotetrameric E1 proteins, and is a prime candidate for involvement in catalysis in all 2-oxo acid dehydrogenase multienzyme systems [Schneider and Lindquivist 1998, Wexler et. al. 1991]. It has been previously shown to be essential for the activity of rat branched-chain α-ketoacid dehydrogenase. [Hawes et. al. 1995a]. In mammalian PDH and BCKDH complexes, histidine 263 is located next to serine 262, which regulates the activity of the entire complex by phosphorylation/dephosphorylation. It has been proposed in the past that this serine-histidine pair in a concerted fashion polarises the α-carbonyl group of the substrate and therefore activates its decarboxylation. [Hawes et. al. 1995b].

Our results for any one of the H84 variants, (values recorded were about 60% of values obtained for the Wild type PDH-E1α and about 33% of the control cell line), imply
that H84 may play a less critical role in the PDH complex. The observed decline in the complex activity was more-likely caused by surface and/or packing distortion brought about by a missfitted residue rather than by alterations within the active centre. Additional support for this conclusion comes from amino acid alignment of various E₁ proteins performed by Wexler et. al. [Wexler et. al. 1991]. According to this alignment, H84 is not a conserved feature within the PDH or BCKDH complex families. Therefore this residue is probably not critically important to the overall function of the PDH complex.

On the other hand we showed that mutations introduced at the H63 residue, resulted in a nearly non-functional PDH complex. This finding stands in disagreement with our originally-proposed ThDP-co-ordinating model. However, Ali et. al. identified a neighbouring cysteine, cysteine 62, as a substrate for the thiol-specific NEM (N-ethylmaleimide) inactivation of the PDH complex. [Ali et. al. 1993]. The inactivation was prevented by thiamin diphosphate in combination with pyruvate but ThDP alone did not afford protection, suggesting that the co-factor does not directly interact with C62. This assumption can be further supported by the finding that in the PDH complex of E. coli, a corresponding cysteine residue is involved in the irreversible inactivation of the PDH-E₁ by 2-oxo-3-alkynoic acids with phenyl substituents at carbon 4 [Brown et. al. 1997]. This class of compounds acts as pyruvate analogue and therefore competes for the active centre. A recent paper by Aevarson et. al. proposes, that the active site in the E₁ of Pseudomonas putida BCKDH complex is connected to the surface by a long funnel-shaped channel that is lined by several hydrophobic residues from both the α- and the β-subunits. This channel forms a point-of-entry for the lipoate moiety of E₂ as well as a site of acyl transfer [Aevarsson et. al. 1999]. The bottom
end of this pocket hosts thiamin diphosphate and is lined with three residues. These residues are Phe-Tyr-Met in branched chain alpha-keto acid dehydrogenase complexes and Phe-Cys-His in PDH complexes. The histidine residue in the latter sequence corresponds to His63 in the human PDH-E1α. Therefore it is possible, that rather than being involved in ThDP co-ordination, H63 has a more defined role in the interaction of the E1 heterotetramer with the lipoate residue.

When PDH complex activity assays were performed on any one of the H92 variants, no significant restoration of activity was observed. Moreover, there was no immunoreactive material detected by Western blot analysis for E1α or E1β (Fig. 3.1). However, it was proven by the RT-PCR technique that the exogenous mRNA was synthesised from the CMV promoter (Fig. 3.2). The results of the in vitro mitochondrial import assay suggest that H92 variants may be import compromised as less mature protein was detected inside mitochondrial matrix for the H92D and H92N variants than for the Wild type E1α (Fig. 3.3).

It has been reported in the past that certain mutations in the E1α coding sequence affect the amount of E1α as well as E1β immunoreactive material present within the mitochondrial matrix. Most of them have been localized to the C-terminal portion of E1α [Naito et. al. 1994, Fujii et. al. 1996], however Marsac et al. reported a cysteine to a serine mutation at position 88 (C88S) in the mature sequence (cysteine 116 in the precursor sequence) that had a protein-decreasing effect on the E1 component. [Marsac et. al. 1997]. It is therefore possible that mutations introduced in amino acid residues around H92 impair interaction with chaperone protein post signal cleavage leading to improper folding. A third possibility exists
where the $E_1$ component assembly occurs simultaneously as the subunits are translocated across the mitochondrial membrane. Alternatively, mutations at or near H92 position may prevent proper linking of the $\alpha$ subunit with the $\beta$ subunit and as a result the unstable subcomplex is rapidly degraded. The reason for the difference in the level of protein expression between the transfection experiment (no immunoreactive material detected) and the import experiment (some $E_1\alpha$ protein present within the mitochondrial matrix) may be due to the difference in sensitivities between these two techniques.

3.5 ACKNOWLEDGEMENTS

We thank the Medical Research council of Canada (Grant MT-6573) for the support of this research project.
CHAPTER 4

EXPRESSION AND FUNCTIONAL CHARACTERIZATION OF THE HUMAN PROTEIN X VARIANTS IN SV40-IMMORTALIZED PROTEIN X-DEFICIENT AND E₂-DEFICIENT HUMAN SKIN FIBROBLASTS

To gain further insight into the nature and function of the domains of the human protein X (a pyruvate dehydrogenase complex component), we expressed the wild-type as well as two artificially created variants, K37E and S422H, in SV40-immortalized protein X-deficient and E₂-deficient human skin fibroblasts. The former mutant does not carry the lipoic acid moiety, the latter mutant was designed to investigate the possibility that protein X could exhibit an intrinsic transacetylase activity and use either its own catalytic centre or the catalytic centre of E₂. Similar experiments have been performed in the past using the S. cerevisiae expression system. However, lack of sequence similarity between the mammalian and the yeast protein X homologues, suggests they are not biochemically equivalent. Mutant cells transfected with the wild-type gene for protein X produced a PDH complex that exhibited about 50% overall activity of the control cells. None of the expressed protein X variants had an effect on the specific activity of the PDH complex, suggesting that the human protein X plays a purely structural role in the functioning of the pyruvate dehydrogenase complex. In press by Archives of Biochemistry and Biophysics.
4.1 INTRODUCTION

In the purified eukaryotic PDHC, an additional component, protein X exists [De Marcucci and Lindsay 1985, Jilka et al. 1989]. Since, similarly to E2 protein X can be acetylated by \(^{14}\)C pyruvate, it was thought to be a proteolytic fragment of E2. However, immunological and proteolytic analyses suggested that E2 and protein X are two different polypeptides, despite their similarities. It was further clarified that each PDHC contains 6 or 12 protein X subunits [De Marcucci and Lindsay 1985, Jilka et al. 1989, Rahmatullah et al. 1989, Neagle et al. 1989]. Since protein X is tightly associated with E2 and binds to E3, one of its proposed functions is to facilitate the interaction between these two subcomponents [Gopalakrishnan et al. 1989, Neagle and Lindsay 1991, McCartney et al. 1997]. Since protein X is acetylated \textit{in vivo}, it has also been suggested that it may be involved in transacetylation [Lawson et al. 1991a]. Recently it has been proposed that protein X serves a purely structural role and is not involved in the catalysis [Harris et al. 1997].

Although the role of protein X within the mammalian PDH complexes remains elusive, cells deficient in its expression retain only about 10-20% of the PDH complex activity [Marsac et al. 1993, Aral et al. 1997, Ling et al. 1998].

Protein X from \textit{S. cerevisiae} has been cloned and sequenced in 1989 by Behal et al. Its N-terminal lipoyl domain is similar to that of yeast E2 [Behal et al. 1989]. However, the sequence of the human homologue remained elusive for many years, finally determined in 1997 by Harris et al. [Harris et al. 1997]. The predicted amino acid sequence of the putative human protein X is similar to the published deduced amino acid sequence of yeast protein X for the first 117 amino acids. The remainder shares a very limited degree of similarity with
that of the yeast homologue. The human protein X also shares limited similarities with the human or eukaryotic E2 at its amino- and carboxy- termini.

Studies involving limited proteolysis, molecular genetics, $^1$H NMR spectroscopy as well as the alignment of human protein X with other eukaryotic E2 proteins clearly identified three domains, separated by two hinge regions, each rich in proline, glycine and alanine. The N-terminal domain forms a single lipoyl domain with high similarity to E2, the amphipathic middle segment proposed to bind E3 and/or E2 that shows low similarity to E2, and a C-terminal putative E3-binding and catalytic domain with high E2 similarity [Harris et. al. 1997, Lawson et. al. 1991a, Perham and Packman 1989, Guest et. al. 1989].

Studies involving protein X from either bovine or porcine sources employing Arg C proteolysis point to its structural role in binding the E3 component [Rahmatullah et. al. 1989, McCartney et. al. 1997, Sanderson et. al. 1996]. However, genetic experiments in S. cerevisiae indicate that the lipoyl domain of protein X can substitute, at least in part, for the lipoyl domain of E2 [Lawson et. al. 1991b].

To gain further insight into the nature and function of the domains in the human protein X, we expressed the wild-type as well as K37E and S422H variants in SV40-immortalized protein X-deficient and E2-deficient human skin fibroblast cell lines [Ling et. al. 1998].

4.2 MATERIALS AND METHODS

Enzymology and Immunological Analysis. Activity of the PDH complex in the native and dichloroacetate-activated state was determined in fibroblast extracts by the method of Sheu et al. [Sheu et. al. 1981]. Activity of dihydrolipoamide transacetylase was also
determined in fibroblast extracts by the method of Butterworth et al. [Butterworth et al. 1975]. Western blotting was performed on mitochondrial extracts prepared by the method of Pitkänen et al. [Pitkänen et. al. 1996], using polyclonal antibody against porcine heart PDH complex antibody raised in rabbit [Ekong 1982]. The amount of protein X expression in each sample was standardised with the amount of immunoreactive material for the 49 kDa subunit - one of the components of mitochondrial complex I.

**RNA and cDNA Preparation.** Total RNA was extracted from cultured skin fibroblasts using TRIzol™ Reagent (Total RNA Isolation Reagent) from GIBCO BRL. First strand cDNA synthesis was carried out using total cellular RNA (10 μg) and an oligo dT(22) reverse primer with M-MLV reverse transcriptase (GIBCO BRL).

**Amplification of DNA.** DNA was amplified in the PCR with cDNA as a template. 250 μM of each deoxynucleotide (dATP, dCTP, dGTP and dTTP), 10 μl of 10X PCR buffer (GIBCO BRL) 1.5 mM MgCl₂, oligonucleotide primer (1μg each), and 2 units of Taq polymerase (GIBCO BRL), in total reaction volume of 100 μl. The amplified fragments were subcloned into pCR2.1 cloning vector (Invitrogen) using a TA Cloning® Kit (Invitrogen). DNA sequencing was performed using the Sequencing TM Kit (Amersharn-Phmacia Biotech). The reaction mixture was separated on an acrylamide gel. The entire coding region of the protein X cDNA was sequenced with the primers described elsewhere [King et. al. 1998]. PCR amplification of the cDNAs was carried out according to specifications below [Saiki et. al. 1988]. For synthesis of the wild-type and variant protein X constructs 5 μl of the cDNA reaction mixture was used for PCR, carried out using the following outside primers: Xrep-KpnI-F (5'-CAGTGGTACCAGCC AGTGAGAAGGCCGTCAAA-3'), X-exp-2R (5'-TCTTAGTGCAGGCTAGCAATGTGATTTGATT-3') and the following site-directed mutagenesis primers: for K37E (5'-ATCTAAG GTAACCACAGCTTCGT3') and for S422H (5'-AAGTGACCACAGCTTGGTTG-3'), using a one-step PCR method developed by Ling and Robinson [Ling and Robinson 1995]. PCR reaction conditions were the following, 20 cycles at conditions: 94°C for 30 sec, 57°C for 30 sec, 72°C for 30 sec.
followed by one cycle at conditions: 94°C for 2 min, 65°C for 2 min, 72°C for 6 min, followed by 30 cycles at conditions: 94°C for 30 sec, 57°C for 30 sec, 72°C for 1 min 30 sec. Kpn-F and Xho-R primers were used at concentration of 500 ng/100μl and the mutagenic primers were at concentration of 30-80 ng/100 μl.

Cloning of Protein X Variants into the Expression Vector. Wild-type and mutant cDNA clones generated as described above were digested with KpnI/Xhol and subcloned into the KpnI/Xhol site of pcDNA 3.1 (Invitrogen).

Protein X and E2-Deficient Cell Lines. A skin fibroblast cell line homozygous for a 4 bp deletion in the putative mitochondrial targeting signal sequence, which results in a premature stop codon [Ling et. al. 1998], was transformed with SV40 large T antigen using a vector containing a large T antigen DNA (courtesy of Dr. John Dick, HSC, Toronto) by transfection. Similarly a fibroblast cell line with dihydrolipoamide transacetylase deficiency (only 50% of control activity) but no visible changes in E2 immunoreactivity was also transformed with SV40 large T antigen. Transfections were carried out using the Superfect reagent (Qiagen) according to manufacturer’s specifications on cells 18-22 hours after splitting at confluence of 60-80% in 6-well plates (35 mm). 2 μg of column-purified (Qiagen) plasmid DNA was combined with 8 μl of the Superfect reagent in 150 μl of serum-free α-MEM. Cells were split 24-36 hours after transfection at a ratio of one 35 mm plate into five 100 mm plates. After 10-14 days transformed foci were cloned and assayed for PDHC or transacetylase activity to confirm that there were no detectable changes resulting from transformation. In order to confirm the absence of protein X in the protein X-deficient cell line and confirm unaltered immunoreactivity of E2 in the E2-deficient cell line, Western blot analysis was performed on mitochondrial fractions from these cell lines (data not shown).

Expression of Wild-Type and Mutant Protein X Variants in the Protein X-Deficient and E2-Deficient Cell Lines. All transfections were carried out as described above using the Superfect reagent (Qiagen) and 2 μg of column-purified plasmid DNA linearized with PvuI. 48 hours post-transfection cells were passaged and subjected to G418 selection (0.2 mg/ml).
After 14-20 days G418-resistant colonies were cloned using cloning rings, grown and assayed for PDHC activity.

4.3 RESULTS AND DISCUSSION

In most lipoate-binding proteins a highly conserved consensus sequence ETDKA contains a lysine residue that serves as a point of attachment for lipoic acid through an amide bond [Fujiwara et. al. 1979, Reed and Hackert 1990, Perham 1991, Fujiwara et. al. 1992]. In order to determine whether the lipoate moiety on protein X is necessary for the proper function of the PDH complex we mutated this lysine residue to a glutamate (K37E). We also introduced a mutation in the S422 residue (S422H) located in the C-terminal putative catalytic centre of protein X. All known proteins with transacetylase activity contain a histidine residue in this position, which is thought to be involved in the transfer of an acetyl residue to coenzyme A. This catalytic histidine is invariably present in the highly conserved sequences DHRXXDG [Leslie et. al. 1988, Russel et. al. 1992, Griffin and Chuang 1990, Meng and Chuang 1994]. In the human protein X the corresponding sequence, spanning residues 421-426, is slightly altered and reads DSRXXDD. By introducing S422H mutation we wanted to test the possibility that protein X could exhibit an intrinsic transacetylase activity using either its own catalytic centre or the catalytic centre of E2.

We employed a human skin fibroblast cell line devoid of endogenous expression of protein X to investigate the above-described protein X cDNA variants. Unlike previous investigations involving mammalian protein X (either bovine or porcine), this system allows observations under conditions that most closely resemble the natural metabolic state of the PDH complex. Similar experiments have been performed in the past using the S. cerevisiae
expression system but the sequence dissimilarity between the mammalian and the yeast protein X homologues suggests they may not be biochemically equivalent. The use of transformed cell lines was necessary to enable selection of stably transfected clones and generate the quantity of cells sufficient to allow repeated enzyme assays and other investigations, always using the same clonal population. The present study showed that it is possible to correct PDH enzyme defect caused by the absence of endogenous protein X by transflecting the deficient cells with the normal protein X cDNA. Although this has not been accomplished before, normal levels of activity were not restored in transfected populations (Table 4.1). The system we have utilized gives stable transfectants expressing wild-type protein X with PDHC activities of the order of 50% of control values (Table 4.1). Both K37E and S422H mutants gave rates for the PDH complex similar to the wild-type protein and were also about 50% of control values (Table 4.1). Transcription of the wild-type as well as other protein X variants was driven by a cytomegalovirus (CMV) promoter, which usually generates a high mRNA titre. However, protein X is a constituent of a complex (PDHC), therefore a possible explanation is that its over-expression is not sufficient to mimic the likely co-ordinated in vitro transcription of mRNA of protein X. E2 and other PDH complex proteins and therefore interferes with the overall PDH complex assembly. It is known that the mammalian protein X subunit has no demonstrable transacetylase activity of its own (Lindsay - personal communication). Therefore, the over-expressed protein X may degrade at the time when there is no E2 protein available to form the core of the PDH complex due to uncoordinated expression. These findings were supported by the results of the Western blot analysis (Fig. 4.1) where the exogenously expressed protein X variants were
present in noticeably smaller amounts compared with the control. The reduced amount of protein X seen on the Western blot also provides an explanation for the reduced overall activity of the recombinant PDH complex. Since the ratio of the amounts of 49 kDa protein (internal protein loading control) to protein X are the same for all the variants we can conclude that expression of the X protein is not altered by the introduced mutations. As can be seen in Fig. 4.1, lane 5, the non-lipooylated protein X undergoes a significant upward shift in apparent mobility. This observation agrees with previous experiments performed on lipooyl domains of acetyltransferase components of α-ketoacid dehydrogenase complexes [Fujiwara et. al. 1996]. Since there is no observable difference in activity between the wild-type and the K37E mutant, it can be concluded that concordant with studies conducted in S. cerevisiae [Lawson et. al. 1991b], the lipooyl moiety is not essential for the human protein X function in the presence of functional E2.

<table>
<thead>
<tr>
<th>PROTEIN X VARIANT</th>
<th>PDH COMPLEX ACTIVITY (nmoles min(^{-1}) mg cell protein(^{-1}))</th>
<th>*% OF CONTROL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Native: 1.02 ± 0.06 [10] DCA Activated: 1.10 ± 0.05 [10]</td>
<td>100%</td>
</tr>
<tr>
<td>Protein X-deficient line</td>
<td>0.22 ± 0.02 [8]</td>
<td>20%</td>
</tr>
<tr>
<td>Protein X wild-type cDNA</td>
<td>0.52 ± 0.03 [7]</td>
<td>51%</td>
</tr>
<tr>
<td>K37E</td>
<td>0.57 ± 0.05 [8]</td>
<td>53%</td>
</tr>
<tr>
<td>S422H</td>
<td>0.55 ± 0.04 [7]</td>
<td>53%</td>
</tr>
</tbody>
</table>

Table 4.1 Mean total PDH complex activities given ± SEM for controls, for protein X-deficient cells and for protein X exogenous variants transfectected into the protein X-deficient cell line. The number of determinations for each clone is given in brackets. Values are given for the native enzyme and for enzyme after activation by dichloroacetate (DCA). *Percentage values are calculated using DCA activated values and are given for the purpose
of greater clarity of the table.

Figure 4.1 Western blot analysis of the plasmid expression of the wild-type and variant protein X peptides. 60 μg of total protein from mitochondria-rich fractions were separated on 12.5% SDS/polyacrylamide gel and were transferred to a nitrocellulose membrane. The filter was probed with an antibody against affinity-purified pig heart PDH complex raised in rabbit. The mitochondrial extracts present on the gel are from left to right: Lane 1 control cultured skin fibroblasts; Lane 2 protein X-deficient cell line; Lane 3 wild-type protein X; Lane 4 S422H variant; Lane 5 K37E variant.

<table>
<thead>
<tr>
<th>PROTEIN X VARIANT</th>
<th>PDH COMPLEX ACTIVITY (nmoles min⁻¹ mg cell protein⁻¹)</th>
<th>TRANSACETYLASE ACTIVITY (nmoles min⁻¹ mg cell protein⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NATIVE</td>
<td>DCA ACTIVATED</td>
</tr>
<tr>
<td>Control</td>
<td>1.19 ± 0.05 [6]</td>
<td>1.30 ± 0.09 [6]</td>
</tr>
<tr>
<td>E₂-deficient cell line</td>
<td>0.22 ± 0.04 [7]</td>
<td>0.27 ± 0.06 [7]</td>
</tr>
<tr>
<td>Protein X wild-type cDNA</td>
<td>0.20 ± 0.03 [6]</td>
<td>0.26 ± 0.03 [6]</td>
</tr>
<tr>
<td>K37E</td>
<td>0.24 ± 0.05 [6]</td>
<td>0.21 ± 0.05 [6]</td>
</tr>
<tr>
<td>S422H</td>
<td>0.22 ± 0.04 [6]</td>
<td>0.24 ± 0.04 [6]</td>
</tr>
</tbody>
</table>

Table 4.2 Mean total PDH complex activities as well as transacetylase (E₂) activities given ± SEM for controls, for protein X-deficient cells and for protein X exogenous variants transfected into the E₂-deficient cell line. The number of determinations for each clone is given in brackets. Values for the PDH complex are given for the native enzyme and for the enzyme after activation by dichloroacetate (DCA).
Since we were unable to detect significant differences in transacetylase activity for the S422H mutant in the protein X-deficient cell line, another experiment was carried out where this mutant, along with the K37E mutant and the wild-type protein X, were transfected into an E2-deficient cell line. We wanted to determine whether the E2-deficient background would be more suitable for detection of possible subtle differences in activities between mutants and wild-type protein X, and therefore make them more pronounced. Although the molecular defect of the E2-deficient cell line has not been identified so far, it retains about 20% of the total PDHC activity and about 50% of transacetylase activity (Table 4.2). Yeast protein X contains a lipoyl domain but it does not exhibit acetyltransferase activity [Yazdi et. al. 1994]. We recorded no differences in activities for the measured enzymes (Table 4.2) except for the slight increase in the PDH complex activity for the S422H mutant; the difference however was not significant. We therefore conclude that the S422H protein X has no endogenous transacetylase activity.

4.4 ACKNOWLEDGEMENTS

We thank the Medical Research Council of Canada for their support of this work.
CHAPTER 5

A CASE OF PDH-E₁α MOSAICISM IN A MALE PATIENT WITH SEVERE METABOLIC LACTIC ACIDOSIS.

We have characterized a novel mutation in a male patient that affects the coding sequence of PDH-E₁α gene and changes Arginine 112 to a Leucine. This nucleotide substitution was found in about 75% of the studied DNA (fibroblasts, liver and muscle), a scenario that would indicate a case of E₁α mosaicism in a male patient. When the mutant E₁α protein was expressed in human skin fibroblasts with zero endogenous PDH complex activity and E₁α protein expression, no significant restoration of activity was recorded in contrast to the Wild type cDNA, even though both Wild type and mutant protein levels were comparable. We concluded that the R112L mutation is a severe one and that it must have occurred in one of the E₁α alleles during early embryogenesis. Submitted for publication to Human Molecular Genetics.
5.1 INTRODUCTION

Because of Lyonization patterns of X chromosome in females as well as the crucial importance of PDHC in the central nervous system metabolism, E1α deficiencies are a problem both in males and females even though only one E1α allele in females carries the mutation. The majorities of mutations are germ-line and are not carried somatically by either parent. Most mutations are localised between exons 5 to 11, which constitute the latter half of the coding sequence [Robinson 1989, Dahl et al 1992]. In males they are mostly missense mutations. In females deletions and insertions which completely nullify one of the alleles are more common. [Wicking et. al. 1986, Ho et. al. 1986, MacKay et. al. 1986, Brown et. al. 1987, Robinson 1989b, Oldand DeVivo 1989, Dahl et. al. 1990, Kitano et. al. 1990, Brown et. al. 1990, Chun et. al. 1993, Hansen et. al. 1991]. In this study we report a case of E1α mosaicism in a male patient which probably resulted from de novo mutation in one of the E1α alleles during embryogenesis.

5.2 MATERIALS AND METHODS.

Enzymology and Immunological Analysis. Cultured skin fibroblasts were grown from forearm skin biopsy in α-MEM culture medium. Activity of the PDH complex in the native and dichloroacetate-activated state was determined in fibroblast extract by the method of Sheu [Sheu et. al. 1981]. Western blotting was performed on mitochondrial extracts prepared by the method of Pitkanen [Pitkanen et. al. 1996], using polyclonal antibody against porcine heart PDH complex antibody raised in rabbits [Ekong 1982].

RNA cDNA and genomic DNA preparation. Total RNA was extracted from cultured skin fibroblasts using TRIzolReagent (Total RNA Isolation Reagent) from GIBCO BRL. First strand cDNA synthesis was carried out using total cellular RNA (20 µg) and an E1α-specific oligonucleotide αG17' (5'-TCTAGAATTCGTACAAACTGCATGCAATTAC-3') with M-MLV reverse transcriptase (GIBCO BRL). Genomic DNA was isolated from fibroblasts, as well as liver and muscle samples by a modified version of the method of
Amplification of DNA. DNA was amplified by the PCR method, with cDNA or genomic DNA as template, 250 μM of each deoxynucleotide (dATP, dCTP, dGTP and dTTP), 10 μl of 10X PCR buffer (GIBCO BRL) 1.5 mM MgCl₂, oligonucleotide primer (1μg each), and 2 units of Taq polymerase (GIBCO BRL), in total reaction volume of 100 μl. Five microliters of the cDNA reaction mixture was used for PCR carried using Eₐα-specific forward primer Kpn-F (5'-TTTGGTACCTTGTGAGGAGTCGCCGCTGC-3') and reverse primer Xho-R (5'-TTTCTCGAGGAGAACACTGTCTGGTAGCC-3'). The PCR conditions used were 94°C for 1 minute, 60°C for 1 minute and 72°C for 1.5 minutes for 35 cycles. The PCR product was then subcloned into pCR 2.1 (Invitrogen) using a TA Cloning Kit (Invitrogen).

DNA Sequence Analysis. Plasmid clones containing PCR products amplified from the patient’s cDNA were sequenced according to recommendations by the manufacturer (Amersham-Pharmacia Biotech) with the T7 Sequencing Kit. The reaction mixture was separated on an acrylamide gel. The entire coding region of the patient’s cDNA was sequenced with the primers described elsewhere [Chun et al. 1993].

Cloning of the Wild Type and mutant Eₐα into an expression vector. Wild type and mutant clones were digested with KpnI/XhoI and subcloned into the KpnI/XhoI site of pcDNA 3.1 (Invitrogen).

Construction of π⁰ (PDHC-deficient) cell line – as described in chapter 2.

Expression of Wild Type and mutant Eₐα in the π⁰ cell line. All transfections were performed as described above using the π⁰ cell line and Qiagen-column purified DNA (either WT, mutant or vector alone) (Qiagen) which was linearized with Scal. Two days post transfection cells were plated into 100 mm plates and selection was applied at a concentration of 0.2 mg/ml G418 for 14-20 days until visible colonies could be detected. These colonies were cloned using cloning rings and PDHC activity was determined.

Tissue distribution of the mutation. The following primer was designed: E1A-Scal 5'-TATGTTTCTGCCATTTCCAGTACT-3' which includes a half restriction site for Scal. The other half Scal restriction site is created by the mutant but not by the wild type Eₐα sequence. Therefore only the mutant sequence is cut. The reverse primer used was 15-9611-R 5'-GGCCAAATCTAAAGCAGCACAC-3'. PCR conditions: 94°C for 1 minute, 54°C for 1
minute, 72°C for 25 seconds; 35 cycles in total volume of 100 μl. Buffer system, primer usage and DNA polymerase use were as described above. PCR products were subjected to digestion with Scal (Gibco BRL), separated on 2% agarose gel and gels were stained in ethidium bromide.

5.3 RESULTS

Clinical and biochemical findings. The patient was a male who died in the newborn period, following severe metabolic lactic acidosis with elevated blood and urine lactate and a lactate/pyruvate ratio of 8.08 (control 24). In cultured skin fibroblasts, overall PDH complex activity was significantly lowered (table 5.1 shows the results of native and DCA activated total PDH complex values recorded).

<table>
<thead>
<tr>
<th>CELL LINE</th>
<th>PDHC ACTIVITY (nmol/min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>native PDH complex</td>
</tr>
<tr>
<td>patient</td>
<td>0.25 ± 0.05 [6]</td>
</tr>
<tr>
<td>control</td>
<td>1.14 ± 0.12 [4]</td>
</tr>
</tbody>
</table>

Table 5.1. Total PDH complex activities recorded for the patient’s and control cell lines. Numbers of determinations for each cell line are placed in brackets.

Characterization of the mutation. We have isolated the proband's E₁α cDNA and subcloned it into PCR 2.1 vector (promega). Single clones were sequenced in the entire coding region and a G to T substitution at nucleotide 422 was found in six out of eight clones (fig 5.1). The rest of the coding region was identical to the published sequence [Dahl et. al. 1987]. This mutation, which results in a substitution of Arginine 112 to Leucine in the mature E₁α sequence, was confirmed by sequencing independently isolated clones carrying fragments of PCR-amplified genomic DNA sequences. A mosaic pattern of its occurrence
was confirmed. This base change was not found in the mother's genomic DNA derived from a blood sample.

*Tissue analysis of the mutation occurrence.* To determine the tissue distribution of mutated vs Wild type alleles of $E_1\alpha$ gene, we have extracted genomic DNA from samples of the proband's skin fibroblasts, liver and muscles as well as his mother's blood. We have subjected these samples to PCR analysis designed to create a ScaI digestion site in the mutant but not wild type sequences. Simultaneously we used a negative (100% wild type) and a positive (100% mutant) control. PCR fragments of 152 b.p. were obtained and subjected to a restriction digest with ScaI. Results are shown in fig. 5.2. As determined by densitometry analysis, about 75% mutant to 25% wild type ratios were observed for all three tissues.

*Expression of the $E_1\alpha$ cDNA harboring the R112L mutation.* To determine whether the R112L mutation was the cause of lower PDHC activity, we transfected $E_1\alpha$ cDNA harboring the G422T change, and expressed it in an immortalized human skin fibroblast cell line. This cell line has been engineered to express no endogenous $E_1\alpha$ protein and therefore exhibits absence of detectable endogenous PDHC activity (the $\pi^0$ cell line). After expression, the control cDNA was able to restore total PDH complex activity to values of approximately 60% of control, while the mutant cDNA gave no significant activity increase for all examined clones. Western blot analysis performed on mitochondrial preparations from transfected cell lines indicates that both the mutant and the Wild type proteins are expressed in comparable amounts (fig. 5.3). Therefore it can be concluded that the lack of restoration of PDH complex activity is caused by the defective mutant protein and not by a defect in exogenous protein expression or defective import of precursor protein into mitochondria.
Figure 5.1. Sequencing of plasmid clones containing PCR products amplified from the patient's cDNA. The circle indicates the site of G422T substitution. Shown are the control (C) and the affected male (P).
Figure 5.2. Tissue distribution of mutated vs wild-type alleles of PDH-E1α. Genomic DNA was extracted from the samples of proband’s skin fibroblasts, liver and muscle as well as his mother’s blood, and subjected to PCR designed to create a ScaI digestion site in the mutant but not wild type sequences. A negative (100% wild type) and a positive (100% mutant) control were also used. PCR fragments of 152 b.p. were digested with ScaI, ran on 2% agarose gel and stained with ethidium bromide. Fragment sizes created as a result of ScaI digest: 123 b.p. (lower band) and 29 b.p.(not shown).
Figure 5.3. Western blot analysis of the exogenous expression of the mutant R112L and Wild type PDH-E1α in the π0 cell line. Eighty micrograms of total protein from mitochondria-rich fractions were separated on a 12.5% SDS/polyacrylamide gel and were transferred to nitrocellulose. The filter was probed with an antibody against affinity-purified pig heart PDH complex raised in rabbits. Shown are also SV40 transformed normal skin fibroblasts (control) and the π0 cell line (π0).

5.4 DISCUSSION

The mutation presented in this case affects an arginine residue that is conserved among E1α proteins from yeasts, rat and human. It was found in a sporadic case with an unaffected mother, and thus represents a de novo mutation. The nucleotide substitution was only observed in about 75% of the studied DNA (fig 5.2). Such a result is puzzling because the E1α gene is X-linked, and a male patient should have only a single copy of the gene due to hemizygosity. Consequently the normal base G should not be detectable. Post-zygotic mutation in the E1α gene, absent in the germline, could constitute a possible explanation. One would then expect that only a fraction of the cells in the body would carry the mutation.

The proportion of Wild type to mutant allele-carrying cells may vary from one cell type to
another as a function of the stage of development at which the mutation occurred. From the restriction digest analysis it appears that, at least for tissues examined, the ratio of mutant to wild type DNA is about 75:25. Since all these tissues are derived from different germ layers ie. liver from the endoderm, and skin fibroblasts and muscles from the mesoderm, the mutation must have occurred very early in development before gastrulation. Although karyotyping was not performed for this cell line, one can rule out with reasonable certainty a scenario of chromosomal translocation where two copies of the E_lα gene were present in the male zygote: one on the X-chromosome, and the other on an autosomal chromosome carrying the translocated fragment. This defective autosome could be inherited either from the mother or the father. In such case one would expect results of the Sca I digest to be 50:50 wild type to mutant DNA.

X-linked pyruvate dehydrogenase complex deficiency is caused by a large variety of mutations in the E_lα gene. [Wicking et. al. 1986, Ho et. al. 1986, MacKay et. al. 1986, Brown et. al. 1987, Robinson 1989b, Old and DeVivo 1989, Dahl et. al. 1990, Kitano et. al. 1990, Brown et. al. 1990, Chun et. al. 1993, Hansen et. al. 1991, Chun et.al.1993, Chun et.al. 1995, Lissens et.al. 1996]. Usually only one or two patients are found in families. due to the severity of the disease. As in other X-linked diseases, patients are sometimes recognised without apparent carrier status in the mother. This is usually considered to result from a unique meiotic event, but a mitotic origin in early embryogenesis cannot be excluded. Indeed, in some cases of PDH complex deficiency [Dahl et. al. 1992, Chun et. al. 1993, Chun et. al. 1995, Briones et. al. 1996, Lissens et. al. 1996, Wexler et. al. 1997], as well as in some cases of other X-linked disorders such as Duchenne muscular dystrophy [Bakker et. al. 1987], ornithine transcarbamylase deficiency [Maddalena et. al. 1988], and hemophilia A
[Brocker-Vriends et. al. 1990], clear indications for a mitotic origin have been obtained by recurrence of new mutations in a sibship or by somatic mosaicism in the mother or in the patient.

PDH complex activity in skin fibroblasts was decreased to about 25-30% of normal, which might be explained either by a small amount of normal enzyme produced from the putative 25% of the normal gene or by a residual activity of the mutant enzyme, or both. Such a finding is often observed in enzymatic deficiencies with missense mutations, in contrast to nonsense or frameshift mutations, which give rise to null alleles and therefore to an absent protein or truncated protein with complete loss of function. [Matsuda and Tanase 1997, Shiloh 1997, Lahvis and Bradfield 1998]. However, when the mutant E₁α was expressed in the n⁰ cell line no significant restoration of activity was recorded in contrast to the Wild type cDNA, even though both Wild type and mutant protein levels were comparable. Therefore it appears that the R112L mutation is a severe one, despite the fact it does not lie in the conserved ThDP-binding motif GDGX²⁶²⁷NN. As mentioned above, this arginine residue is conserved in E₁α polypeptides from yeast, rat and human. Moreover, it is right next to a glycine residue that is conserved in Baccillus Stearothermophilus, yeast, rat and human. Therefore the protein resulting from this mutation may be disfunctional either because of improper folding or interference with catalytic centre of the PDH heterotetramer or both. Alternatively, it may interfere with the proper functioning of the conserved glycine residue, having nevertheless the same effect.

5.5 ACKNOWLEDGEMENTS

We thank the Medical Research Council of Canada for financial support of this project.
CHAPTER 6

A NOVEL SYNDROME AFFECTING MULTIPLE MITOCHONDRIAL FUNCTIONS, LOCATED BY MICROCELL MEDIATED TRANSFER TO CHROMOSOME 2p14 – 2p13

We have studied cultured skin fibroblasts from three siblings and one unrelated individual with fatal mitochondrial disease manifesting soon after birth. After incubation with 1 mM glucose, these four cell strains exhibited lactate/pyruvate ratios which were six times greater than those of controls. Upon further analysis enzymatic activities of the pyruvate dehydrogenase complex, the 2-oxoglutarate dehydrogenase complex, NADH cytochrome c reductase, succinate dehydrogenase, and succinate cytochrome c reductase were severely deficient. In two of the siblings the enzymatic activity of cytochrome oxidase was mildly decreased (~50%) as well. Metabolite analysis performed on urine samples taken from these patients revealed high levels of glycine, leucine, valine and isoleucine, indicating glycine cleavage system and branched-chain alpha-ketoacid dehydrogenase abnormalities. In contrast, fibroblast pyruvate carboxylase, mitochondrial aconitase and citrate synthase activities were normal. Immunoblot analysis of selected complex III subunits (core 1, cyt c1, and ISP) as well as the pyruvate dehydrogenase complex subunits revealed no visible changes in the levels of all examined proteins decreasing the possibility of an import and/or assembly factor being involved. In order to elucidate the underlying molecular defect, microcell-mediated chromosome fusion analysis was performed between the studies fibroblasts (recipients) and a panel of A9 mouse:human hybrids (donors) developed by Cuthbert et al. [Cuthbert, A.P., Trott, D.A., Ekong, R.M., Jezzard, S., England, N.L., Themis, M., Todd, C.M., and Newbold, R.F. et al. Cytogenet. Cell Genet. 71, 68-76 (1995)]. Complementation was observed between the recipient cells from both families and the mouse:human hybrid clone carrying human chromosome 2. These results indicate that the underlying defect in our patients is under the control of a nuclear gene, whose locus is on chromosome 2. A 5 cM interval has been identified so far as potentially containing the critical region for the unknown gene. This interval maps to region 2p14 – 2p13. Submitted for publication to American Journal of Human Genetics.

Contributions to this chapter:
Newbold R.B. Department of Biology and Biochemistry, Brunel University, Uxbridge, UK, Hudson T. Montreal General Hospital, Montreal, Quebec, MacKay N. Hospital for Sick Children, Toronto, Ontario, Susan Malaney Germain Institute of Medical Research, Darlinghurst, Australia, D. Gonzalez-Helphen, Departamento de Bioenergetica, Universidad Nacional Autonoma de Mexico, Mexico, D.F.
6.1 INTRODUCTION

Inherited defects in mitochondria where more than one enzyme is affected with low activity are relatively common in association with mtDNA, which is mutated, deleted or depleted [Zeviani et al. 1989, Brown and Wallace 1994, Bodnar et al. 1995, Grossman and Shoubridge 1996, Moraes et al. 1999]. However, defects with multiple enzymopathies, which are associated with defects in nuclear genes are rare and so far confined to abnormalities in import proteins [Jin et al. 1996, Jin et al. 1999, Koehler et al. 1999] or in the Lon proteases responsible for mitochondrial protein turnover [van Dijl et al. 1998].

Fatal neonatal infantile forms of the defects associated with primary lactic acidosis have been reported, including defects of the pyruvate dehydrogenase complex [Brown et al. 1988, Robinson et al. 1987, Robinson 1995a, Morten et al. 1998], the NADH-coenzyme Q reductase complex [Hoppel et al. 1987, Moreadith et al. 1984], the cytochrome oxidase complex [DiMauro et al. 1988, Lombes et al. 1989, Lombes et al. 1996] and the ubiquinol cytochrome c reductase complex [Valnot et al. 1999, Kennaway et al. 1988]. Here we report two cases in which cultured skin fibroblasts showed decreased activities of the pyruvate dehydrogenase complex, the 2-oxoglutarate dehydrogenase complex and the succinate cytochrome c reductase. Furthermore, metabolite urine analysis indicated possible deficiencies of branched chain alpha-ketoacid dehydrogenase as well as the glycine cleavage system. We have identified that this new syndrome is caused by a defect in a gene whose locus lies on chromosome 2p14-2p13 (84-89cM).
6.2 CASE REPORT

The three siblings

Three siblings, two boys and one girl (4142, 4143, 4144), born a few years apart to non-consanguineous parents after uncomplicated pregnancies presented with feeding difficulty, weakness, lethargy and decreasing responsiveness within a few days after birth. They were admitted to a hospital where laboratory investigation showed metabolic acidosis with elevated blood lactate (table 6.1).

<table>
<thead>
<tr>
<th></th>
<th>Blood pH</th>
<th>pCO₂</th>
<th>BASE EXCESS</th>
<th>BLOOD LACTATE</th>
</tr>
</thead>
<tbody>
<tr>
<td>4142 (male)</td>
<td>6.61</td>
<td>35</td>
<td>-33</td>
<td>N/A</td>
</tr>
<tr>
<td>4143 (female)</td>
<td>7.11</td>
<td>60</td>
<td>-12</td>
<td>18.5 mEq/L</td>
</tr>
<tr>
<td>4144 (male)</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
</tbody>
</table>

Table 6.1. Results of laboratory investigation performed on the three siblings.

Assisted ventilation was necessary because of respiratory failure. Urine organic acids analysis performed on one of the siblings (4143) showed a highly elevated urinary lactate with a secondary elevation of 2-hydroxybutyrate (5.5 mEq/ml creatinine by GC). Amino acids showed glycine, leucine, isoleucine, phenylalanine, alanine, valine and taurine above normal with the most significant elevation in glycine being 5.4 times elevated. All three died within a month after birth. A limited autopsy was done on another sibling (4142) and significant findings included pulmonary oxygen toxicity; pneumonitis; eosinophilic infarction of the right and left anterior papillary muscles; focal
tracheal mucosal denudation; focal intra-alveolar hemorrhage; focal renal hemorrhage; and white matter spongiosis consistent with a metabolic abnormality.

_Unrelated patient_

A male infant was born by spontaneous vaginal delivery at 33 weeks gestation as a product of _in vitro_ fertilization to a 30 year old G4, P0, extopic 3 mother. During pregnancy, the mother had gestational diabetes and pregnancy induced hypertension with proteinuria. Four months after birth he developed epileptic seizures as well as elevated levels of serum glycine (571 μM) and CSF glycine (28 μM). At six months of chronological age he was developmentally delayed and the frequency of seizures increased. Subsequently, he developed dilated cardiomyopathy and epileptic encephalopathy. He suffered from consistent vomiting, progressive lethargy, tachypnea, diaphoresis, respiratory distress, and hepatomegaly. At seven months he became acidotic (pH 7.14, bicarbonate 12, base excess -17). Initial lactate was 11.5 mM but increased to 19 mM in the course of few weeks. CSF glycine levels rose to 1039 μM and his condition progressively deteriorated. He died at eleven months of chronological age.

6.3 MATERIALS AND METHODS

_Cell lines_. Cell lines used in this study were obtained by skin biopsy of the forearm of patients after informed consent. Primary fibroblast lines were established from four patients (4142, 4143, 4144, and 11571) with a compound mitochondrial enzymes deficiency all of whom exhibited the same phenotype. Fibroblasts were grown in Eagles minimal essential medium (α-MEM) and 10% fetal calf serum, supplemented with glucose to a concentration of 10.5 mM. Lactate-to-pyruvate ratio measurements in confluent fibroblast cultures were carried out as described previously [Robinson et. al. 1985]. The primary fibroblast cell lines were transduced with a retroviral vector expressing the E6E7 region of type 16 human papilloma virus to extend their life span.
[Compton 1993] and grown in high glucose DMEM supplemented with 20% fetal bovine serum.

**Microcell-mediated chromosome transfer.** A panel of human:mouse monochromosomal hybrids [Cuthbert et al. 1995] was used as the source of normal human donor chromosomes. All 22 autosomes and the X chromosome were serially transferred into one of the patient cell lines by microcell-mediated chromosome transfer [Fournier 1981]. Briefly, donor cells were plated in α-MEM containing 10% fetal bovine serum and hygromycin B (400U/ml; ICN) 3 days before fusion. The medium was then changed to DMEM plus 20% fetal bovine serum and the cells were exposed to colchicine (0.06 μg/ml) for 48 hours to induce micronucleation. The cells were then collected by trypsinization and plated on plastic ‘bullets’ (custom-made from tissue culture plates to fit into 50-ml centrifuge tubes) coated with crosslinked concanavalin A (Sigma). Microcells were prepared by centrifugation at 34-37°C and 15,000 r.p.m. (SS34 rotor) in media containing cytochalasin B (10 μg/ml; Sigma), filtered through 8 μm and 5 μm filters, pelleted at 3000 r.p.m. on a benchtop centrifuge and resuspended in serum-free medium. The microcell suspension was added to the plate containing the recipient cells along with phytohaemagglutinin (100 μg/ml) and incubated for 20-30 min. Microcells were fused with 45% PEG plus 10% DMSO for 60 s, washed with serum-free medium and incubated in α-MEM plus 20% FBS. After 48-72 hrs, fused cells were selected in hygromycin B (100U/ml). Colonies were picked, expanded and analyzed 3-4 weeks later.

**Enzymology, spectrophotometric and Immunological Analysis.** Activity of the PDH complex in the native and dichloroacetate-activated state was determined in fibroblast extracts by the method of Sheu [Sheu et al. 1981]. Activity of the 2-oxoglutarate complex was determined in fibroblast extracts by the method [Hyland and Leonard 1983]. Activity of the NADH cytochrome c reductase was assayed in 0.1 M potassium phosphate (KPi) buffer, pH 7.0 containing 94 μM cytochrome c and 1mM azide. To start the reaction, 10 mM NADH was added, and reduction of cytochrome c was monitored at 550 nm. Cytochrome oxidase activity was assayed in 0.1 M potassium phosphate (KPi) buffer, pH 7.0. Cytochrome c was reduced with 3.2 mM ascorbate followed by dialysis against 0.1 M potassium phosphate (KPi) for 2 days, during which
the buffer was changed twice. Reduced cytochrome c (94 μM) was added to start the reaction and oxidation of cytochrome c was followed at 550 nm. Citrate synthase assay was performed as described in [Robinson et. al. 1987]. Succinate dehydrogenase activity was determined in mitochondria purified from skin fibroblasts by the method of Hatefi and Stiggall [Hatefi and Stiggall 1978]. Succinate cytochrome c reductase activity was determined in fibroblast extracts by the method of Yu and Yu [Yu and Yu 1982]. Aconitase activity was determined in mitochondria purified from skin fibroblasts by the method of Racker [Racker 1950]. Western blotting was performed on mitochondrial extracts prepared by the method of Pitkanen et. al. [Pitkanen et. al. 1996], using polyclonal antibody against porcine heart PDH complex antibody raised in rabbit [Ekong 1982], holo bc1 and ISP-bc1 against bovine heart and liver complex antibody raised in rabbit [Vazquez-Acevedo et. al. 1993].

Preparation of human liver mitochondria. Mitochondria were isolated from frozen post-mortem human livers using a method resulting from a combination of those described by Craig and Hood [Craig and Hood 1997] and Pitkanen et. al. [Pitkanen et. al. 1996]. Tissues were finely minced and then gently homogenized in buffer (50 mM HEPES pH 7.4, 70 mM sucrose, 220 mM Mannitol, 1 mM EGTA and 2 mg/ml fatty acid free BSA (Sigma-Aldrich, St. Louis, MO)). Following homogenization the mixture was centrifuged at 3000 x g for 10 min. The supernatant was then centrifuged at 15,000 x g for 10 min. The pellet was gently solubilized in homogenization buffer and centrifugation steps were repeated. The pellet was resolubilized and recentrifuged at 15,000 x g for a final time. Following this, the mitochondrial pellet was resuspended in buffer (0.34 M sucrose, 100 mM KCl, 10 mM Tris-Cl. 1 mM EDTA) at a concentration of 1 mg/ml protein.

Free radical production assay. Superoxide production was determined using the chemiluminescent dye of high quantum efficiency, lucigenin (10,10-dimethyl-9,9-biacridinium dinitrate; Sigma Chemical Co.) [Pitkanen and Robinson 1996]. The concentration of the working solution of lucigenin was 4.3 μM. The measurements were performed in buffer containing 70 mM sucrose, 220 mM mannitol, 2.5 mM potassium phosphate (KPi) pH 7.5, 2.5 mM MgCl₂, 0.5 mM EDTA, 0.1% BSA. Lucigenin was added to the buffer immediately prior to measurements. All measurements were made at
room temperature using a lumat model 9507 (Berthold, Wildebad, Germany) luminometer. The signal from the dye was integrated over a period of 30 s following the addition of substrate (2 mM succinate) and the background signal during this same period was subtracted from the sample signal. Mitochondria were pre-incubated with or without inhibitors in the measurement buffer for 1 min prior to addition of substrate. The substrate was added using the automatic injection system of the Lumat 9507, following the determination of the background signal over a 15 s period. Measurements were carried out in the presence or absence of either antimycin A (20 μg/ml final concentration), myxothiazol (10 μM final concentration) (Sigma-Aldrich, St. Louis, MO) or stigmatellin (20 μM final concentration) (Fluka Biochemicals, Ronkonkoma, N.Y.). These inhibitor concentrations were shown to fully inhibit enzyme activity [Raha et. al. 2000]. 10 μg of mitochondrial proteins was measured per each sample.

**Deletion and exclusion mapping.** Oligonucleotide primers for polymorphic microsatellite markers on chromosome 2 were obtained (Research Genetics and ACGT). Deletion mapping was done on DNA isolated from hygromycin B-resistant clones obtained following microcell fusion from the A9-2 monochromosomal hybrid line using Pure Gene kit. Exclusion mapping was carried out on the three affected siblings as well as the family of the unrelated individual. This family included the affected individual, his brother and both parents. Genomic DNA for this experiment was isolated from primary skin fibroblasts using total DNA isolation kit (Qiagen). PCR was performed using the Research Genetics protocol, AmpliTaq gold (PE Applied Biosystems) and [γ35S]-dATP. PCR products were run on 6% sequencing gels exposed O/N using BioMax film (Kodak) and developed. PCR was also performed as above in the absence of [γ35S]-dATP. Products were run on Supergel 250™ (Hellix); gels were stained with Cybr Green™ for 20 min and pictures were taken using a UV camera.
6.4 RESULTS

Skin fibroblast cultures were established pre-mortem. The most striking finding was the high production of lactate relative to pyruvate in skin fibroblasts incubated with 1 mM glucose for 1 h [Robinson et. al. 1987]. This ratio exceeded seven times the normal value (table 6.2). A very low activity of the pyruvate dehydrogenase complex was also recorded with values reaching at most 25% of the control in the dichloroacetate-activated state. Furthermore, breakdown of the activities of individual PDH complex components revealed a decreased activity of the first subcomplex, pyruvate dehydrogenase (E₁), while the activities of the remaining components, dihydrolipoamide transacetylase (E₂) and dihydrolipoamide dehydrogenase (E₃) were normal. Activities of the NADH cytochrome c reductase (complex I + III), succinate cytochrome c reductase (complex II + III), succinate dehydrogenase (complex II) and the 2-oxoglutarate dehydrogenase complex were also decreased reaching at most 35%, 41%, 43% and 28% of control values respectively. The activity of cytochrome c oxidase (COX) was decreased to about 50% of control values in two of the siblings (4143 and 4144). In contrast activity values for pyruvate carboxylase, mitochondrial aconitase as well as citrate synthase were normal. Urine organic acid analysis performed on one of the siblings (4143) showed a highly elevated urinary lactate with a secondary elevation of 2-hydroxybutyrate (5.548 mEq/ml creatinine by GC). Amino acids showed glycine, leucine, isoleucine, phenylalanine, alanine, valine and taurine above normal with the most significant elevation in glycine being 5.42 times elevated. There was also a significant increase in the CSF glycine levels in the unrelated patient.
<table>
<thead>
<tr>
<th>ENZYME ACTIVITY (nmoles/min/mg)</th>
<th>CONTROL</th>
<th>SIB 1 (4142)</th>
<th>SIB 2 (4143)</th>
<th>SIB 3 (4144)</th>
<th>UNRELATED PATIENT (11571)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PDHC (active)</td>
<td>0.98±0.14 [9]</td>
<td>0.16±0.04 [6]</td>
<td>0.22±0.05 [10]</td>
<td>0.05±0.02 [9]</td>
<td>0.21±0.04 [4]</td>
</tr>
<tr>
<td>PDHC (DCA activated)</td>
<td>1.16±0.16 [9]</td>
<td>0.13±0.03 [6]</td>
<td>0.25±0.06 [10]</td>
<td>0.04±0.02 [8]</td>
<td>0.36±0.06 [4]</td>
</tr>
<tr>
<td>E₁</td>
<td>1.41±0.21 [5]</td>
<td>0.48 [1]</td>
<td>0.63 [1]</td>
<td>0.36±0.07 [5]</td>
<td>N/A</td>
</tr>
<tr>
<td>E₃</td>
<td>7.2±0.5 [2]</td>
<td>9.3 [1]</td>
<td>7.3 [1]</td>
<td>7.2±0.6 [2]</td>
<td>N/A</td>
</tr>
<tr>
<td>OGDHC</td>
<td>1.5±0.3 [4]</td>
<td>0.20±0.04 [2]</td>
<td>0.40±0.05 [2]</td>
<td>0.12±0.05 [2]</td>
<td>0.42±0.06 [2]</td>
</tr>
<tr>
<td>NCR (I + III)</td>
<td>6.4±0.2 [5]</td>
<td>1.9±0.4 [4]</td>
<td>1.0±0.3 [4]</td>
<td>1.3±0.5 [3]</td>
<td>2.3±0.5 [3]</td>
</tr>
<tr>
<td>COX</td>
<td>5.2±0.7 [4]</td>
<td>5.0±0.3 [3]</td>
<td>2.2±0.5 [4]</td>
<td>2.7±0.5 [3]</td>
<td>4.9±0.5 [4]</td>
</tr>
<tr>
<td>SDH</td>
<td>16.5 ± 2.4 [4]</td>
<td>N/A</td>
<td>7.0 ± 0.2 [2]</td>
<td>N/A</td>
<td>6.9 ± 0.2 [2]</td>
</tr>
<tr>
<td>mitochondrial aconitase</td>
<td>4.0 ± 0.6 [2]</td>
<td>N/A</td>
<td>3.5 ± 0.6 [2]</td>
<td>N/A</td>
<td>3.2 ± 0.5 [2]</td>
</tr>
<tr>
<td>SCR</td>
<td>5.6±1.01 [2]</td>
<td>N/A</td>
<td>1.4±0.3 [2]</td>
<td>1.5±0.3 [2]</td>
<td>2.7±0.7 [2]</td>
</tr>
</tbody>
</table>

Table 6.2. Various mitochondrial enzyme activities of patients suffering from the multiple disorder. Activities were determined in cultured skin fibroblasts from each patient and a group of controls. Values are given as the mean of determinations (number given in parenthesis) + S.E.M. Individual determinations were made on separate cultures on different days and multiple control cell lines were used. PDHC – pyruvate dehydrogenase complex, E₁ – pyruvate dehydrogenase, E₂ – dihydrolipoamide transacetylase, E₃ – dihydrolipoamide dehydrogenase, OGDHC – 2-oxoglutarate dehydrogenase complex, NCR - NADH cytochrome c reductase (complex I + III), COX – cytochrome c oxidase, L/P ratio – lactate to pyruvate ratio, SDH - succinate dehydrogenase (complex II), SCR – succinate cytochrome c reductase (complex II + III).
To determine the chromosomal location of the gene defect in these patients, we rescued this phenotype by functional complementation with a normal human chromosome transferred into deficient cells by microcell-mediated chromosome transfer. A panel of stable human:mouse hybrid cell lines containing human chromosomes tagged with a selectable marker HyTK was used as a source of donor human chromosomes [Cuthbert et. al. 1995]. All 22 autosomes and the X chromosome were transferred, one at a time, into a patient fibroblast line (4143 – one of the three siblings) and the cells were selected in hygromycin B. 3-4 weeks post-fusion, surviving colonies were picked and PDH complex activity was measured on pooled colonies obtained for each individual chromosome transfers. Transfer of chromosome 2 (A9.2 human:mouse hybrid cell line) restored PDH complex activity to control levels (Table 6.3). This restoration was not observed with any other autosome or the X chromosome (Table 6.3). Transfer of chromosome 2 also restored the activity of 2-oxoglutarate dehydrogenase complex and the NADH cytochrome c reductase in that cell line (4143).

To confirm a common genotype in the selected patients, we transferred chromosome 2 into skin fibroblasts obtained from another affected sibling (4142) and the unrelated patient (11571). Additional clones were isolated from the latter patient. Similarly to the previous results, this chromosome restored activities of all assayed enzyme complexes (Table 6.4).

We also tested PDH complex activities in individual clones obtained from patients 4143 and 11571. Enzyme activity was restored in 23 of 25 independent clones derived from patient 4143 patient and 30 of 36 clones in cells derived from patient 11571 (data not shown).
<table>
<thead>
<tr>
<th>CHROMOSOME</th>
<th>NADH cytochrome c reductase ACTIVITY (nmole min⁻¹ mg cell protein⁻¹)</th>
<th>PDH COMPLEX ACTIVITY (nmole min⁻¹ mg cell protein⁻¹)</th>
<th>NATIVE</th>
<th>DCA ACTIVATED</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>2</td>
<td>5.98±0.34 [4]</td>
<td>1.12±0.09 [9]</td>
<td>1.5±0.145 [9]</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>-</td>
<td>0.21±0.04 [2]</td>
<td>0.24±0.06 [2]</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>1.99±0.71 [2]</td>
<td>0.23±0.07 [3]</td>
<td>0.23±0.04 [3]</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>1.87±0.45 [2]</td>
<td>0.25±0.06 [2]</td>
<td>0.19±0.04 [2]</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>-</td>
<td>0.19±0.02 [2]</td>
<td>0.29±0.05 [2]</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>-</td>
<td>0.22±0.05 [3]</td>
<td>0.20±0.03 [3]</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>2.08±0.43 [2]</td>
<td>0.30±0.06 [2]</td>
<td>0.31±0.04 [2]</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>10</td>
<td>-</td>
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<td>0.17±0.05 [2]</td>
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</tr>
<tr>
<td>11</td>
<td>-</td>
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<td>0.25±0.04 [2]</td>
<td></td>
</tr>
<tr>
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<td>0.23±0.05 [2]</td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>14</td>
<td>2.24±0.39 [2]</td>
<td>0.26±0.06 [3]</td>
<td>0.21±0.03 [3]</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>16</td>
<td>1.99±0.22 [2]</td>
<td>0.29±0.38 [3]</td>
<td>0.28±0.07 [3]</td>
<td></td>
</tr>
<tr>
<td>17</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>18</td>
<td>-</td>
<td>0.28±0.04 [2]</td>
<td>0.20±0.04 [2]</td>
<td></td>
</tr>
<tr>
<td>19</td>
<td>2.13±0.45 [2]</td>
<td>0.23±0.06 [2]</td>
<td>0.25±0.05 [2]</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>-</td>
<td>0.26±0.05 [2]</td>
<td>0.30±0.04 [2]</td>
<td></td>
</tr>
<tr>
<td>21</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>22</td>
<td>2.56±0.63 [2]</td>
<td>0.22±0.05 [2]</td>
<td>0.27±0.04 [2]</td>
<td></td>
</tr>
<tr>
<td>X</td>
<td>-</td>
<td>0.34±0.07 [2]</td>
<td>0.29±0.03 [2]</td>
<td></td>
</tr>
<tr>
<td>Patient 4143</td>
<td>1.02±0.25 [4]</td>
<td>0.22±0.05 [10]</td>
<td>0.24±0.05 [10]</td>
<td></td>
</tr>
<tr>
<td>control</td>
<td>6.56±0.58 [5]</td>
<td>0.98±0.08 [9]</td>
<td>1.21±0.14 [9]</td>
<td></td>
</tr>
</tbody>
</table>

Table 6.3 Functional complementation analysis of skin fibroblasts from a patient suffering from multiple mitochondrial enzyme disorder with a panel of human chromosomes. Pyruvate dehydrogenase complex and the NADH cytochrome c reductase (complex I + III) activities were measured after microcell-mediated transfer of single HyTK-tagged normal human chromosomes into fibroblasts from patient 4143 (one of the three siblings). Enzyme activities were measured on multiple pooled clones recovered after selection in hygromycin B. In a few cases (N/A) transfer of these chromosomes was not performed, since chromosome 2 corrected the phenotype in this and other lines (4144, 11571) before the procedure was carried out. (−) designates instances where activities of NADH cytochrome c reductase were not measured.
Table 6.4. Functional complementation analysis of selected enzyme complexes (the bc1 complex, the PDHC and the 2-OGDH) pre and post chromosome 2 transfer. Skin fibroblasts from two siblings (4143 and 4144) and the unrelated patient (11579) were used. Enzyme activities are shown for the affected cell lines, followed by the activity in the chromosome 2 hybrid cells obtained by microcell-mediated chromosome transfer. The values obtained for each enzyme complex measured and compared with control values from normal fibroblasts (control).

To narrow down the potential locus region on chromosome 2, we genotyped three affected siblings as well as the unrelated patient, his parents and his unaffected brother with a set of chromosome 2 microsatellite markers. In theory, affected family members should share common alleles at the locus of the gene defect, while being discordant at that locus with unaffected family members. This analysis resulted in isolation of two regions: one on the p arm of chromosome 2 and one on the q arm (Fig. 6.2). The region on the p arm extended between markers D2S1337 and D2S1790 (approximately 24 cM) and the region on the q arm extended between markers D2S439 and D2S407 (approximately 18 cM). During the course of this experiment we had to exclude the

<table>
<thead>
<tr>
<th>CELL LINE</th>
<th>NADH cytochrome c reductase ACTIVITY (nmoles min⁻¹ mg cell protein⁻¹)</th>
<th>PDH COMPLEX ACTIVITY (nmoles min⁻¹ mg cell protein⁻¹)</th>
<th>2-OGDH ACTIVITY (nmoles min⁻¹ mg cell protein⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4143</td>
<td>1.02±0.25 [4]</td>
<td>0.22±0.05 [10]</td>
<td>0.37±0.05 [2]</td>
</tr>
<tr>
<td>4143 x chr 2</td>
<td>5.98±0.33 [4]</td>
<td>1.12±0.09 [9]</td>
<td>1.04±0.10 [2]</td>
</tr>
<tr>
<td>4142</td>
<td>1.95±0.38 [4]</td>
<td>0.16±0.04 [3]</td>
<td>0.11±0.09 [2]</td>
</tr>
<tr>
<td>4142 x chr 2</td>
<td>6.62±1.05 [2]</td>
<td>0.91±0.09 [4]</td>
<td>1.13±0.16 [2]</td>
</tr>
<tr>
<td>11571</td>
<td>2.28±0.53 [3]</td>
<td>0.21±0.03 [4]</td>
<td>0.42±0.06 [2]</td>
</tr>
<tr>
<td>11571 x chr 2</td>
<td>4.56±0.58 [3]</td>
<td>1.11±0.09 [6]</td>
<td>1.29±0.13 [2]</td>
</tr>
<tr>
<td>control</td>
<td>6.56±0.58 [5]</td>
<td>0.98±0.08 [9]</td>
<td>1.34±0.13 [4]</td>
</tr>
</tbody>
</table>
family of the unrelated patient because the extremely high degree of consanguinity rendered the data obtained non-informative.

Chromosomes incorporated into the genome of recipient cells by microcell transfer often undergo deletions and rearrangements [Newbold and Cuthbert 1996, Leach et. al. 1989]. To further narrow the region containing the gene of interest, we used polymorphic microsatellite markers from chromosome 2 to map the regions that were incorporated into the genomes of complementing and non-complementing clones isolated after hygromycin B selection from patients 4143 and 11571. In functionally complemented clones, markers for the exogenous chromosome that are missing could be excluded from the region of interest. Similarly, in non-complemented clones informative markers that are present could be excluded as well. Analysis of 13 complementing clones and 1 non-complementing clone from patient 4143 and 30 complementing and 6 non-complementing clones from patient 11571 allowed the mapping of the gene defect to the region between markers A053XF9 to GATA-P132045 (approximately 5 cM) (Fig. 6.2). This analysis excluded the region on the q arm of chromosome 2 obtained by marker analysis in the three siblings.

To determine whether the genetic defect shared by the affected individuals had any impact on the amount of protein expressed for the deficient complexes, immunoblotting was performed using antibodies directed against total PDH complex, the holo bc₁ complex and bc₁ complex iron-sulfur protein (ISP). Results of the immunoblots are shown in Fig.6.1A (PDH complex), Fig. 6.1B (holo bc₁ complex) and Fig 6.1C (ISP). All investigated components are expressed in levels comparable to the control sample.
indicating that the disorder does not affect the import, assembly or stability of the investigated complexes.

Oxygen free radicals can be generated from two potential sites within bc1 complex [Raha et. al. 2000]. Inhibition with antimycin A prevents transfer of electrons from cytochrome b to Q₁, thereby increasing the concentration of Q⁰ semiquinone. This increase in the Q⁰ semiquinone species results in an increase in the free radical species since O₂ is derived by donation of an electron from Q⁰ to molecular oxygen [Raha et. al. 2000]. The antimycin A-dependent production of superoxide was observed for the control human liver mitochondria (Table 6.5), however liver mitochondria obtained from one of the siblings failed to produce any significant increase in free radical production upon antimycin A addition. Moreover, there was a statistically significant decrease in free radical production observed (Table 6.5). Addition of myxothiazol (100 μM) or stigmatellin (20 μM) to the liver mitochondria from either patient or control produced only a marginal (statistically non-significant) increase in the production of superoxides, above basal.

<table>
<thead>
<tr>
<th>INHIBITOR</th>
<th>*SUPEROXIDE FORMATION</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CONTROL</td>
</tr>
</tbody>
</table>

Table 6.5. Effect of inhibitor-dependent superoxide production in control and multi-complex deficient liver mitochondria. *Values were standardized to the activity of citrate synthase, a mitochondrial matrix marker.
Fig. 6.1. Western blot analysis of the endogenous expression of the pyruvate dehydrogenase complex (A), selected holo bc1 complex proteins (B), and the bc1 complex iron-sulphur protein ISP (C). 60 µg of total protein from mitochondria-rich fractions were separated on 12.5% SDS/polyacrylamide gel and were transferred to nitrocellulose. The filter was probed with an antibody against affinity-purified pig heart PDH complex raised in rabbit (A) or an antibody against holo bc1 complex or bc1-ISP raised in rabbit (B and C). The mitochondrial extracts present on the gels are from left to right: lane 1, control cultured skin fibroblasts, lane 2 and 3, skin fibroblasts from two affected siblings (4143 and 4144), lane 4, fibroblasts from the unrelated patient (11571). In panel C the sample for the unrelated patient is not present.
Fig 6.2. Ideogram of human chromosome 2 showing the results of exclusion and deletion mapping for the mitochondrial multienzyme deficiency phenotype. Microsatellite markers that were used in the analysis are indicated. The thick vertical lines to the right of the markers indicate regions that were obtained by either deletion or exclusion mapping.
6.5 DISCUSSION

Investigations of the cultured skin fibroblasts derived from the three siblings (4142, 4143, 4144) as well as the unrelated patient (11571) showed increased lactate-to-pyruvate ratio in the cells typical of a severe respiratory chain problem (Table 6.2) [Robinson et. al. 1985, Robinson et. al. 1990]. Deficient activities of NADH cytochrome c reductase, the pyruvate dehydrogenase complex, the 2-oxoglutarate dehydrogenase complex and succinate cytochrome c reductase suggested the presence of a defect, which was decreasing the activity of several mitochondrial enzymes. This was further confirmed by metabolite analysis in the blood pointing to a deficiency of the branched chain alpha-keto acid dehydrogenase complex and the glycine cleavage system. However, the activities of pyruvate carboxylase, mitochondrial aconitase, lipoamide dehydrogenase (E₃) and citrate synthase were normal. Immunoblot analysis performed on mitochondrial extracts derived from the four patient cells (Fig. 6.1) showed that the pattern of the selected subunits (PDH complex and bc₁ complex) was similar to the control cell line. This observation suggests that the examined polypeptides are correctly imported into and/or folded within the mitochondria and assembled into complexes. Previous studies indicated that a defect in mitochondrial import machinery presents with a lack of immuno-detectable proteins within the organelle [Jin et. al. 1999]. Similarly deficiencies in factors required for assembly of multi-subunit complexes produce at best only partially assembled complexes [Petruzzella et. al. 1998, Brasseur et. al. 1997]. This would indicate that the above-described genetic defect occurs at the posttranslational, and probably post-assembly level.
In both families there was no previous history of family members on the maternal side with symptoms associated with mitochondrial myopathy. Although mtDNA was not analyzed for pathogenic mutations the fact that the majority of the affected enzyme complexes have no mitochondrially-encoded components makes mitochondrial aberrations an unlikely cause.

In trying to pinpoint a genetic defect which would explain all of the above, we decided to conduct functional complementation analysis using microcell-mediated chromosome transfer. Our results clearly show that introduction of a normal chromosome 2 into the three cell lines with multiple mitochondrial enzyme deficiency can revert their phenotype and restore deficient enzyme activities. Evidence that phenotypic changes were not the mere consequence of manipulations associated with the chromosome transfer protocols but were truly controlled by chromosome 2-specific DNA sequences comes from two observations. First, similar changes were not seen when other chromosomes were transferred into the same recipient cells instead of a chromosome 2. Second, only a small (5cM) portion of the exogenous chromosome 2 in the hybrid cell line resulted in restoration of affected enzymes activities, further attesting to the association of these phenotypic properties with specific DNA sequences. This region when absent in hybrid cell lines produced clones that still exhibited mutant phenotype (2 clones of 4143 and 6 clones of 11571 – see results).

Our ultimate goal in this study was to better define the chromosomal region(s) associated with the disorder that caused multiple mitochondrial enzyme malfunctions, in order to facilitate the eventual isolation of the specific gene(s) controlling this disorder. This was achieved by obtaining subclones of chromosome 2 hybrids that did not regain
their affected enzyme activities. Examination of the integrity of the exogenous chromosome 2 in these clones showed that portions of it had been lost in each case. Similarly, examination of the integrity of the exogenous chromosome 2 clones that reverted the mutant phenotype showed that in these revertants showed that portions of it had been lost in each case. The smallest deletion, which could be defined by this approach extended from A053XF9 to GATA-P132045 (Fig. 6.2). Most of the known polymorphisms within this region were non-informative because the corresponding alleles on the exogenous chromosome were indistinguishable from those on the endogenous copies. This complicated attempts at further narrowing this candidate region.

Results from the measurement of bc1 complex (complex III) oxygen free radical production indicate that there is only a residual transfer of electrons from ubiquinone to the iron-sulphur center in the deficient cell line and confirm a deficiency in the activity of that complex. This reasoning is based on the observation that inhibition of electron flow between coenzyme b$_{562}$ and Q$^1$ with antimycin failed to produce any significant increase in reactive species production [Raha et. al. 2000]. Moreover, the amount of radicals produced with antimycin inhibition significantly fell when compared to the antimycin-free sample. This observation further supports the above hypothesis. A question that needs to be answered at this point is how can this hypothesis be compatible with the data from the Western blot suggesting the ISP to be fully assembled into the bc1 complex. Kapazoglou et. al showed that in a triple substitution mutant, C107S/H109R/C112S, replacing conserved residues involved in the ligation of the [2Fe-2S] centre of the Rieske protein it was able to assemble into the cytochrome bf complex in isolated chloroplasts
Cytochrome bf complex and mitochondrial bc1 complex share a high degree of topological and functional similarity especially when it comes to the Rieske protein [Soriano et. al. 1999]. Therefore a similar outcome can be predicted for the Rieske protein from mitochondrial bc1 complex.

In trying to determine the nature of the genetic defect underlying the above-described disorder a common factor or process involved in all the affected enzyme complexes is all important. All gathered data so far, points to an unknown factor that is somehow involved in metabolism of sulphur in the mitochondria and/or its supply to the affected complexes. Sulphur is an essential part of the lipoic acid, which is a cofactor shared by the PDH complex, the 2-oxoglutarate dehydrogenase complex, the branched chain alpha-keto acid dehydrogenase complex as well as the glycine cleavage system. Sulphur is also a part of respiratory complexes II and III in form of the iron-sulphur centers. In contrast, assembled sulphur-containing active centers are not a part of the pyruvate carboxylase or citrate synthase complexes.

The nearly-completed human genome project should provide sequence information for the isolated interval on chromosome 2, where the locus for the unknown gene is most likely located. Future work will determine whether the candidate gene product turns out to have either a sulphur transferase or cysteine desulphurase activity. Known sulphur transferase genes do not map near to chromosome 2p14-2p13.

6.6 ACKNOWLEDGEMENTS

We thank MRC Canada for the financial support of this research project.
CHAPTER 7

DISCUSSION AND FUTURE DIRECTIONS
7.1 COMPARISON OF PDHC WITH BCKDHC AND OGDHC

The pyruvate dehydrogenase complex is a member of the 2-oxo acid dehydrogenase complex family, which also includes the branched chain alpha-keto acid dehydrogenase complex (BCKDHC) and the 2-oxoglutarate dehydrogenase complex (OGDHC). Sequence comparison of subcomplexes E₁ and E₂, yield surprising results. While there is a great degree of homology between α and β subunits of PDH-E₁ and BCKDHE₁ from different species [Wexler et. al. 1991], no significant homology is detected with OGDH-E₁ other than the ThDP binding site [Koike et. al. 1992, Koike 1998]. Furthermore, while E₁ subcomplexes from PDH and BCKDH are heterotetramers of two α and two β subunits, the E₁ of OGDH is a homodimer [Pettit et. al. 1973]. In contrast, the E₂ components from all three complexes preserve a high degree of homology with the exception of the number of lipoic-binding domains [Koike 1998]. (Since all these complexes have the same E₃ subunit this discussion will exclude it.) Both PDHC and BCKDHC are subject to regulation by phosphorylation/dephosphorylation of specific serine residues, three in case of the PDHC and two in case of the BCKDHC [Roche and Patel 1989, Patel and Roche 1990, Behal et. al. 1993, Sugden and Holness 1994, Yeaman 1989, Harris et. al. 1986].

Recent application of the tools of molecular biology provided insight into the structure, regulation, and defects of the genes encoding various PDH complexes and BCKDH complexes as well as their component polypeptides. In this respect, the research for the OGDH complex is slightly lagging [Patel and Harris 1995]. Analysis of the available information gathered for each one of the complexes provides the means of finding common structural and regulatory motifs and facilitates a better understanding of them.
PDH complex deficiency is the most prevalent among the three complex deficiencies. As already mentioned in the introduction, it is extremely heterogeneous in its clinical manifestations, which are limited largely to the central nervous system. In contrast defects of the branched chain alpha-keto acid dehydrogenase complex as well as the 2-oxoglutarate dehydrogenase complex are a lot less prevalent – with the exception of some ethnic populations [Fisher et. al. 1991]. Inborn errors in the subunits of BCKDH complex result in Maple Syrup Urine Disease (MSUD) [Chuang et. al. 1991, Nobukuni Zhang et. al. 1991, et. al. 1992, Hauashida et. al. 1994, Matsuda et. al. 1990, Fisher et. al. 1991, Zhang et. al. 1989]. The resulting block produces large increases in concentration of the branched-chain amino acids and alpha-keto acids in the blood, tissues and urine of MSUD patients. The disease results in urine with a maple syrup odor, but also causes seizures, mental retardation, coma and death if not treated. Maple Syrup Urine Disease like PHD deficiency is genetically heterogeneous with the majority of mutations being in the coding regions of either the E1α or E2. Few cases of isolated primary deficiencies of the 2-oxoglutarate dehydrogenase complex have been reported in human. Major clinical features associated with this deficiency are neurological and muscular impairments. However, although all the component subunit genes for the complex have been cloned, no molecular defects have been identified so far [Kohlschutter et. al. 1982, Bonnefont et. al. 1992, Guffon et. al. 1993].

7.2 COMPARISON OF THE E1 FORMS

Since only the PDH-E1 and BCKDH-E1 exhibit significant regions of sequence identity among the 2-oxo acid dehydrogenase complex family, it is only logical to limit comparison to only these two members. Comparison of naturally occurring as well as
directed mutagenesis of the coding sequences of these two proteins allow mutual structural and functional extrapolations.

The majority of missense mutations in the BCKDH-E\(_1\)\(\alpha\) gene are located in the C-terminus, similarly to mutations in the PDH-E\(_1\)\(\alpha\), and they can be grouped into two clusters. One cluster is associated with the ThDP-binding site the other with the putative subunit interaction region [Chuang 1998]. Similarly to the PDH-E\(_1\), the \(\alpha\) and \(\beta\) components of BCKDH-E\(_1\) require co-expression for assembly into a functional \(\alpha_2\beta_2\) heterotetramer [Wynn et. al. 1998]. Furthermore, it has been assessed by expression of a recombinant BCKDH-E\(_1\) in GroES- and GroEL-deficient strain of \(E.\ coli\) that functional assembly of the heterotetramer requires these molecular chaperonins. The mitochondrial homologues are chaperonins Hsp60 and Hsp10, respectively [Wynn et. al. 1998]. There is no direct evidence that these chaperonins are involved in the \textit{in vivo} folding and assembly of the human PDH-E\(_1\), pyruvate dehydrogenase complexes, although complexes from \(T.\ flavus\) and \(B.\ stearothermophilus\) do not spontaneously renature from their separated components unless chaperonin Hsp60 is included to stimulate the reactivation of the enzyme complex [Witzmann and Bisswanger 1998].

While many disease-causing mutations are described for PDH-E\(_1\)\(\alpha\), limited biochemical and structural data associated with these mutations makes it difficult to assess their full impact on the PDH complex [Chun et. al. 1995, Naito et. al. 1994, Lissens et. al. 1996, Dahl et. al. 1992, Awata et. al. 1992]. Similarly, there is a very limited experimental data available for residues suspected of being structurally and/or functionally important - aside form the studies conducted on the \(E.\ coli\) and \(Z.\ mobilis\) proteins involving mutagenesis of the GDG\(^{1627}\)NN ThDP-binding motif [Diefenbach et. al. 1992, Candy and Duggleby 1994,
Yi et. al. 1996]. Furthermore, with the exception of the work by Korotchkina and Patel involving phosphorylation sites of the mammalian E₁ [Korotchkina and Patel 1995], no studies were conducted on the mammalian E₁α₂β₂ heterotetramer. Therefore, the work described in this thesis is a first attempt since 1995 to systematically dissect various aspects of the structure of the human E₁ in its natural cellular environment (chapter 2, 3 and 5).

Exogenous expression of mammalian E₁α is indeed difficult, however not impossible. Our efforts were largely aided by the construction of the π⁶ cell line (for the protocol see chapter 2). In 1997 Brown et. al. made a similar cell line for the purpose of establishing a reliable screening system for PDH-E₁α somatic mutations [Brown et. al. 1997]. The extremely low rates of activity recovered with the wild-type E₁α cDNA post-transfectionally in this cell line was probably the reason that this cell line was not used for functional studies of PDH-E₁α protein. Failure to produce sufficiently high PDH complex activity post-transfectionally was probably dictated by the absence of cloning procedure applied to each individual colony produced after selection with the antibiotic (G418). In our experiment we observed that some post-transfectional colonies, even-though antibiotic resistant, did not produce exogenous E₁α mRNA and therefore E₁α protein (personal observation). On average, the ratio of successful trasfections to non-successful transfections was 2 in 9 colonies. Therefore, by use of the clonal isolation of colonies we were able to establish a convenient and reliable system where results could be easily verified by repeated transfection of variant PDH-E₁α cDNAs.

In contrast to the field of the E₁ subcomplex of the PDHC, the corresponding subcomplex of the mammalian BCKDHC has been more extensively studied. This includes many naturally occurring mutations [Wynn et. al. 1998] as well as crystallographic studies on
the BCKDH complex from *P. putida* [Aevarsson et al. 1999]. Therefore the only way of referencing the work contained within this thesis is to compare it with findings available from the BCKDH complex. Human BCKDH-E,α mutations that affect the formation of a functional α₂β₂ heterotetramer are predominantly located in the last 35 amino acids of the C-terminus. These include F364C, Y368C and Y393N of the α polypeptide. Experiments performed by Wynn et al. indicate that this group of mutations does not only reduce the rate of the assembly of the mutant E,α with normal E,β but also prevents conversion of the dimeric αβ assembly intermediate into the stable heterotetramer [Wynn et al. 1998]. Furthermore, these mutant E,α proteins have higher propensity for proteolytic digestion compared with the wild type which would explain the markedly reduced levels of E,α and E,β subunits on the Western blot [Matsuda et al. 1990, Fisher et al. 1991]. Chapter 2 of this thesis described studies on the C-terminal residues of the human PDH-E,α. Similarly to the human BCKDH-E,α, the C-terminus of the human PDH-E,α is also involved in the heterotetramer formation [Seyda et al. 2000]. Sequential removal of the last four C-terminal amino acid residues (-KSVS) correlated with progressive decline in the amount of immunoreactive PDH-E,α and PDH-E,β proteins present in mitochondria. Crystal structure of the E₁ heterotetramer of *P. putida* BCKDH complex, supports involvement of the E₁α C-terminus in the heterotetramer formation [Aevarsson et al. 1999]. Interestingly, there is a lack of homology between the last 35 residues of the E₁α subunit of various 2-oxo acid dehydrogenase complexes [Wexler et al. 1991]. Clearly this is an example of evolutionary preservation of a functional domain without preservation of the amino acid sequence. The series of experiments described in chapter 2 made an important contribution the field of mammalian pyruvate dehydrogenase studies as they confirmed the suspected involvement of
the PDH-Eια C-terminus in the formation of a stable heterotetramer. In addition, these experiments delineated the critical number of residues required for the heterotetramer formation to the very last quartet of amino acid residues on the Eια subunit. Furthermore, they established a base for studies on the correlation between tissue-specific stability and activity of the Eι subcomplex and raised an intriguing hypothesis that the rate of degradation and/or turnover of the Eι subcomplex in the brain and the nervous system may be rather slow, since patients suffering from PDH-Eια terminal deletions (described in chapter 2) are the longest survivors with PDH complex deficiency [chapter 2].

Naturally occurring mutations in the coding sequence of the human BCKDH-Eια that are located in the ThDP binding motif include G204S, R220W and N222S. The two latter changes have no effect on the assembly of the mutant Eι heterotetramer as determined by pulse-chase labeling [Wynn et. al. 1998]. Instead they disrupt the pentameric coordination of the Mg2+ cation resulting in the inability of Eι to bind the pyrophosphate moiety of ThDP and therefore carry out its catalytic function [Wynn et. al. 1998]. Based on these results similar function can be ascribed to residues Eι194 and N196 of the human PDH-Eια. This is further supported by mutational analysis results for the corresponding N258Q of the PDH-Eι from E. coli, where this substitution affected the activation of the enzyme by ThDP-Mg2+ [Russell et. al. 1991, Yi et. al. 1992]. In contrast G204S impedes the assembly of the mutant Eια subunit with the normal Eιβ. Based on the crystal structure of the P. putida BCKDH-Ei, the region around G204 in the human BCKDH-Eια is involved in the formation of the interface between the two non-identical subunits of Eι [Aevarsson et. al. 1999]. Several naturally occurring mutations affecting the ThDP region in the human PDH-Eια have been reported as well. These are F176L, M181V, and P192L [Chun et. al. 1993, Dahl and Brown 1994, Chun
et. al. 1995, Hemalatha et. al. 1995]. They are exclusively present in males and produce a PDH complexes with a range of activities (1.6% to 35.5%) even with the same mutation [Chun et. al. 1993, Dahl and Brown 1994]. Therefore based on the available evidence I propose that the region spanning residues 176 to 192 in the mature form of human PDH-E,α is probably also involved in the αβ interface formation with a molecule of ThDP extending across this interface.

One of the most puzzling naturally occurring mutations in the PDH-E,α is R263G or sometimes R234Q (mature protein) [Chun et. al. 1993, Naito et. al. 1994, Awata et. al. 1994, Chun et. al. 1995, Lissens et. al. 1996, Briones et. al. 1996, Wexler et. al. 1997]. This mutation causes a lower amount of immunoreactive E,α and E,β and was therefore proposed to disrupt the formation of the α2β2 heterotetramer. A corresponding mutation (which would have been Y262 – see fig 1.12A) has not been identified in the human BCKDH-E,α, however a naturally occurring mutation T265R was identified in several patients [Chuang and Shih 1995, Cox et. al. 1997]. Experiments by Wynn et. al. involving recombinant mutant E,α and Wild type E,β proteins coexpressed in E. coli, strongly suggest that the T265 residue is important for proper folding of the E,α subunit as well as playing a role in subunit interaction [Wynn et. al. 1998]. Furthermore, both mutations R263G (PDH-E,α) and T265R (BCKDH-E,α) are located at the putative subunit-interaction site conserved between BCKDH and PDH E,α proteins, identified by Wexler et. al. [Wexler et. al. 1991].

Part of this thesis included studies of four PDH-E,α histidine residues, H63, H84, H92 and H263 (chapter 3) [Seyda and Robinson 2000]. Based on gathered evidence we hypothesized that H63 and H263 were crucial for catalysis without affecting the E,α2β2 heterotetramer stability and/or assembly. H263 corresponds to H292 in the human BCKDH-
E1α, which has been extensively studied by coexpression of recombinant E1 proteins in *E. coli* [Hawes et. al. 1995]. Similarly to human PDH-E1α studies, substitution of H292 to alanine (H292A) resulted in an inactive enzyme. Furthermore, H292A abolished the ability of BCKDH-E1 apoenzyme to reconstitute with thiamin diphosphate clearly serving a critical role in the binding of ThDP coenzyme [Hawes et. al. 1995]. Furthermore, it has been proposed in the past that H263 residue in concerted fashion with the neighboring S262 polarizes the α-carbonyl group of the substrate and therefore activates its decarboxylation [Hawes et. al. 1995b]. Based on these studies a similar role can be assigned to the H263 of the human PDH-E1α. This function would be common to all PDH-E1α and BCKDH-E1α subunits consistent with the levels of sequence homology observed for this residue [Wexler et. al. 1991].

In addition to H292, two additional neighbouring residues R288 and D296 were investigated in the human BCKDH-E1α [Hawes et. al. 1995]. The corresponding residues in the human PDH-E1α are R269 and D257. Given the high level of sequence conservation observed for both residues (100% for all examined subunits to date), their proposed functions in the BCKDH-E1α can be extrapolated to the α subunit of the human PDH. The positively charged R288 (R269) was proposed to serve as an anion binding site for the carboxyl group of α-keto acids. The negatively charged D296 (D257) was proposed to extract a specific proton or a critical hydrogen bonding for stabilization of a transition state structure [Hawes et. al. 1996].

The role of H63 is a lot more undefined. As one possibility H63 could be involved in coordination of the ThDP as proposed in chapter 3 [Seyda and Robinson 2000]. Alternatively, based on sequence alignment of the human PDH-E1α with BCKDH-E1α from
*P. putida*, whose X-ray crystal structure has been solved, H263 could be a part of three-residue sequence, F-C-H that lines the bottom of a pocket involved in the transfer of acetyl moiety to the lipoic acid moiety of E2 [Seyda and Robinson 2000].

During the original investigation of the human PDH-E,α histidine residues a surprising discovery was made about H92 (chapter 3) [Seyda and Robinson 2000]. When PDH complex activity assays were performed on any one of the H92 variants, no significant restoration of activity was observed. Moreover, there was no immunoreactive material detected by Western blot analysis for E₁α or E₁β (fig. 3.1, chapter 3). However, it was proven by the RT-PCR technique that the exogenous mRNA was synthesized driven by the CMV promoter (fig. 3.2 chapter 3). The results of the *in vitro* mitochondrial import assay suggest that H92 variants may be import compromised as less mature protein was detected inside the mitochondrial matrix for the mutants than for the wild-type H92 (fig 3.3, chapter 3). Nobukuni et. al. reported a R114W mutation in BCKDH-E,α of a Japanese patient with maple syrup urine disease. This mutation affected the α₂β₂ heterotetramer formation as shown by decreased amounts of immunoreactive E₁α and E₁β proteins [Nobukuni et. al. 1993]. This arginine residue is highly conserved among the BCKDH-E,α and PDH-E,α proteins and it corresponds to R90 of the human PDH-E,α sequence [Wexler et. al. 1991]. It is therefore possible that mutations introduced in amino acid residues around R90 and H92 in the human PDH-E,α (R115 and A117 in the human BCKDH-E,α) disturb interaction with chaperone protein(s) post signal cleavage leading to improper folding. Alternatively, mutations near these residues may prevent proper linking of the α subunit with the β subunit resulting in an unstable and rapidly degraded heterotetramer [Seyda and Robinson 2000].
The series of experiments described in chapter 3 constitute the first systematic study of the $E_1$ heterotetramer using a mammalian expression system and site-directed (SDS) mutagenesis of the human $E_1\alpha$ gene. However, in the case of PDH-$E_1\alpha$ variants described chapter 3, there is compelling evidence that the conclusions drawn for each histidine mutant are correct and that the utilized system was able to distinguish between non-critical as well as functionally and structurally critical residues. Firstly, only histidine residues (H63 and H263) that are conserved among different species abolished PDH complex activity when mutated. Secondly, the $E_1\alpha$ variants harboring a mutation at these conserved residues were properly imported and assembled judging from the western blot analysis and the *in vitro* import studies (Fig. 3.1 and 3.3). Mutations that affected functionally non-critical residue (H84) resulted in only a small decline in the overall complex activity and mutations that affected structurally critical histidine residue (H92) produced an import- and/or assembly-compromised $E_1\alpha$ polypeptide (fig. 3.1 and 3.3).

As a part of this thesis, a naturally occurring human PDH-$E_1\alpha$ mutation R112L (R141L in the precursor $E_1\alpha$) was described (chapter 5). This appears to be the first recorded case of male somatic mosaicism in the PDH-$E_1\alpha$ gene based on our investigation and assessment of biochemical presentations in the affected individual. When the mutant $E_1\alpha$ was expressed in the $\pi^0$ cell line no significant restoration of activity was recorded in contrast to the wild type cDNA, even though expressed wild type and mutant protein levels were comparable. Therefore it appears that the R112L mutation is a severe one, despite the fact it does not lie in the conserved TPP-binding motif $\text{GDGX}^{\text{Gly}}\text{NN}$. This arginine residue is conserved in $E_1\alpha$ polypeptides from yeast, rat and human (see chapter 5). Moreover, it is adjacent to a glycine residue that is conserved in *Baccillus Stearothermophilus*, yeast, rat
and human. Therefore the protein resulting from this mutation may be dysfunctional because of interference with catalytic center of the PDH heterotetramer. Alternatively, it may interfere with the proper functioning of the conserved glycine residue, having nevertheless the same effect. The region around R112 in the human PDH-E$_{1}\alpha$ corresponds to a region of $P. putida$ BCKDH-E$_{1}\alpha$ that forms a structurally conserved core between BCKDH-E$_{1}$ and transketolase form $S. cerevisiae$ [Aevarsson et. al. 1999]. However, since R112 is not conserved between PDH complexes and BCKDH complexes it is difficult to assess its exact function.

Further analysis of naturally-occurring and artificially produced missense mutations in either the human PDH-E$_{1}\alpha$ or the human BCKDH-E$_{1}\alpha$ points to mutations that are probably not critically important for the proper functioning of the E$_{1}$ proteins since they do not produce complexes of drastically low activity [Chun et. al. 1995, Lissens et. al. 1996, Marsac et. al. 1997, Matsuda et. al. 1995, Fujii et. al. 1994, Matthews et. al. 1994, Takakubo et. al. 1995]. The observed declines in the overall complex activity are more likely caused by surface and/or packing distortion brought about by misfitted residues rather than by alterations within the active center and/or subunit interaction interfaces. The effect of them is illustrated by changes to the H84 in the sequence of the human PDH-E$_{1}\alpha$ described in chapter 3 [Seyda and Robinson 2000].

The lack of recorded naturally occurring mutations in the N-terminus of the human BCKDH-E$_{1}\alpha$ and the scarcity of recorded mutations in the N-terminus of the human PDH-E$_{1}\alpha$ could indicate either one of two scenarios:

1. The N-terminus is not critical for the catalytic function and/or subunit binding and therefore can tolerate mutations which produce no phenotypic changes
2. The N-terminus is critical for catalytic function and/or subunit binding and therefore cannot tolerate mutations which when they occur are not compatible with life.

The N-terminus of the mature PDH-E,α and BCKDH-E,α polypeptides show little sequence conservation within the 2-oxo acid dehydrogenase complex family [Wexler et. al. 1991]. Sequence alignment by crystal structure merging of *P. putida* BCKDH-E,α and *S. cerevisiae* transketolase showed poor agreement between structures of their N-termini [Aevarsson et. al. 1999]. The first 77 amino acid residues of the mature BCKDH-E,α have no corresponding residues in the transketolase [Aevarsson et. al. 1999]. Residues 2-14 of the BCKDH-E,α form an extended tail embracing the other α subunit within the α2 dimer, but the majority of the N-terminal residues lie on the outside of the E,α molecule [Aevarsson et. al 1999]. Given the fact that the BCKDH complex and the PDH complex are more closely related than the BCKDH complex and transketolase, the N-terminus of the human PDH-E,α could perform a similar function to the N-terminus of *P. putida* BCKDH-E,α, although it is shorter by about 30 amino acid residues. Therefore it is possible that only a small fraction of the N-terminal amino acid residues of the human PDH-E,α serve a defined structural role with the majority of amino acid residues located on the outside of the E,α molecule and therefore are fairly tolerant of substitutions.

In summary, PDH-E,α molecule can be divided into functional motifs that are depicted in figure 1.7.

### 7.3 PROTEIN X

Chapter 4 explored some aspects of the human protein X of the PDHC. This lipooyl-bearing polypeptide is still a subject of considerable controversy. The one striking feature of protein X is the sequence dissimilarity between putative yeast and human orthologues [Harris
et. al. 1997]. Protein X orthologues from other organisms have not been isolated to date and searches of various genetic databases using either the human or the yeast sequences yield dihydrolipoamide transacetylase proteins (E₂) from different organisms rather than putative protein X [personal observation]. It has been generally assumed that all pyruvate dehydrogenase complexes of eukaryotes contain protein X even-though only the bacterial, yeast and mammalian PDH complexes have been studied at some detail. However, research shows that this polypeptide is absent in some worms such as P. equorum and A. suum since incubation of the complex with ¹⁴C-pyruvate results in the acetylation of only E₂ [Komuniecki et. al. 1992, Diaz and Komuniecki 1994]. Furthermore, it appears that in these organisms the subunit-binding domain of E₂ may be significantly larger or be flanked by larger than normal interdomain regions. An enlarged subunit-binding domain may be necessary to accommodate the additional binding of E₃ to the E₂ subunit in these complexes, in the absence of protein X [Komuniecki et. al. 1992]. The proposed function for the protein X so far has been that of binding E₃ to the PDH core (E₂) [Rahmatullah et. al. 1989, Neagle and Lindsay 1991, Lawson et. al. 1991]. Our results (chapter 4) further support this hypothesis, since mutant lipoate-less protein X produced a human PDH complex of the same activity as wild-type protein. However this role is not exclusive of its role in catalysis. Protein X is probably able to participate in the acetyl moiety transfer, however it is not necessary for this activity. A few lines of evidence do suggest this conclusion. Firstly, in yeast complexes where the lipoyl binding lysine residue of E₂ was replaced by arginine (K47R), the overall activity of the PDH complex was reduced by 88% [Lawson et. al. 1991]. The fact that about 12% of the overall activity was retained by the mutant PDH complex provides support for the proposal that the lipoyl domain of yeast protein X can substitute, at
Fig 7.1. Functional motifs of PDH-E₁α.
least in part, for the lipoyl domain of E2. Secondly, when the lipoyl-binding lysine was also changed to arginine (K43R) in the yeast protein X, and expressed in conjunction with the K47R mutant of E2, PDH complex showed no detectable activity in the overall reaction [Lawson et al. 1991]. Thirdly, mammalian and yeast PDH complexes contain only 6 or 12 protein X subunits [Jilka et al. 1986, Maeng et al. 1994, Sanderson et al. 1996]. It has been shown for the E. coli PDH complex, containing three lipoyl domains per E2 chain, that nearly half of the lipoyl domains can be removed by limited proteolysis without significant loss in overall complex activity [Berman et al. 1981, Stepp et al. 1981]. Therefore there is an apparent excess of lipoyl groups and thus the contribution of 6 or 12 additional lipoyl groups coming from protein X versus 120 and 60 from human and yeast E2 components respectively does not seem to make significant contribution.

Finally, it is quite intriguing that protein X is an exclusive component of PDH complexes and not other 2-oxo acid dehydrogenase complexes. However, Rice et al. found that the N-terminal region of the E1 component of mammalian 2-oxoglutarate dehydrogenase complex contains a sequence similar to protein X found to be involved in E1 binding [Rice et al. 1992].

7.4 MULTIPLE MITOCHONDRIAL ENZYME DISORDERS

Chapter 6 of this thesis explored a novel disorder resulting in combined deficiencies of key mitochondrial enzymes. Several laboratories from around the world have given similar reports in the past. Such disorders can be loosely divided into three groups: those affecting flavin-containing enzymes such as enzymes of β-oxidation pathway MCAD, LCAD and SCAD as well as complexes I and II of the respiratory chain [Antozzi et al. 1994], those
affecting import and/or turnover of mitochondrial proteins [van Dijl et. al. 1998, Koehler et. al. 1998], and those affecting enzymes containing sulphur and/or iron atoms [Drugge et. al. 1995, Rotig et. al. 1997, Sperl et. al. 1992, Desnuelle et. al. 1989, Robinson et. al. 1992, Hall et. al. 1993]. The latter group can be further divided into those affecting the assembly of the affected enzymes i.e. depressed or absent subunits of affected complexes [Robinson et. al. 1992, Hall et. al. 1993], and those with seemingly no impact on subunit assembly [Sperl et. al. 1992].

Some of these disorders have a clearly identified molecular defect such as Friedrich's ataxia caused by mutations in the frataxin gene, mitochondrial Lon protease disorder – impacting mitochondrial protein turnover – and the deafness/dystonia syndrome caused by a defect in the mitochondrial import machinery [Rotig et. al. 1997, van Dijl et. al. 1998, Koehler et. al. 1999]. However the majority of disorders have no known underlying molecular cause. The molecular defect of the disorder described in chapter 6 has been allocated to the region on chromosome 2p14-2p13 spanning an interval of approximately 5 cM. The next few months will be devoted to scanning this region for possible candidate genes as well as scanning the patients' DNA for mutations in these candidate genes.

The probable cause that combined deficiencies of mitochondrial enzymes, including the one described in chapter 6, are seemingly rare is that diagnostic laboratories offer limited number of available services, for example only the respiratory chain enzyme testing. Once a deficiency of a particular enzyme is found, further investigations are abandoned. The measurement of the PDH complex activity is somewhat complicated and is not carried out in most diagnostic laboratories. According to one report, combined enzyme deficiencies constitute about 5 to 10% of the patients with enzymatic defect in the mitochondrial energy
production [Sperl et. al. 1992]. Future research should discover more of these interesting cases, especially when their molecular causes are better understood. Therefore the results presented in chapter 6 can be categorized as pioneering work, setting precedence for future research involving regulatory and/or assembly factors critical for proper function of mitochondria. This work also implies the importance of biochemical analysis used to aid genetic methods in the process of novel gene(s) discovery.

7.5 FUTURE DIRECTIONS

Every day more and more information is available about the structure and function of the human pyruvate dehydrogenase complex. The challenge now lies in trying to integrate this information and achieve a full appreciation of this metabolically important complex and its role in the context of a cell as well as specific tissues.

When it comes to specific subcomplexes, $E_1$, $E_2$, and $E_3$, research efforts of various scientific groups in the past seem to have been concentrated the most on the $E_1\alpha$ subunit and to a lesser extent on $E_2$ and protein X. This leaves $E_1\beta$ and $E_3$ somewhat neglected. Therefore good functional studies utilizing a variety of reliable systems are required for all PDH complex components. Generation of high resolution X-ray crystal structures for a PDH complex from any species should provide the means of resolving a lot of the present unknowns, however not all questions can be answered by studying crystal-generated data. Therefore it is equally important to study various PDH complex components both in vitro and in vivo.

With respect to continuation of the work described in this thesis, the main effort should be placed in generating more $E_1\alpha$ and protein X mutants and their subsequent expression in the $\pi^0$ and protein X-deficient immortalized cell lines. However probably the
most challenging and interesting work would be the determination of the molecular basis underlying the multiple mitochondrial enzyme deficiency syndrome, which includes severe deficiency of the pyruvate dehydrogenase complex.
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