STRUCTURAL CHARACTERIZATION OF F-TYPE AND V-TYPE ROTARY ATPASES BY SINGLE PARTICLE ELECTRON CRYOMICROSCOPY

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A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy

Department of Biochemistry
University of Toronto

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Abstract

Adenosine triphosphate (ATP) is the molecular currency of intracellular energy transfer in living organisms. The enzyme ATP synthase is primarily responsible for ATP production in eukaryotes. In archaea and some bacteria, ATP is synthesized by V-ATPase that is related to ATP synthase both in structure and function. Both of these enzymes are reversible rotary motors capable of catalyzing ATP synthesis or hydrolysis. The rotation of the central rotor, which is powered by the flow of proton (or sometimes sodium ion) down the electrochemical gradient through the membrane-bound F_o/V_o region, leads to the chemical synthesis of ATP in F_1/V_1 region. The F_1/V_1 region, on the other hand, can catalyze ATP hydrolysis, which in turn leads to proton (or sodium) pumping across the membrane through rotation of the central rotor in the opposite direction. This thesis describes structure determination of both the intact F-type and V-type enzymes using single particle electron cryomicroscopy (cryo-EM), with the aim of better understanding their overall architecture, subunit organization and the mechanism of proton translocation.
Our cryo-EM structural analysis on the F-type ATP synthase from *Saccharomyces cerevisiae* uncovered the arrangement of subunits a, b, c, and the two dimer-specific subunits e and g within the membrane-bound region of F$_{o}$. A model of oligomerization of the ATP synthase involving two distinct dimerization interfaces was proposed.

The rotor-stator interaction within the membrane-bound region of both enzymes is responsible for proton translocation. Our cryo-EM structures of the V-ATPase from *Thermus thermophilus* reveal that the interaction between the rotary ring (rotor) and the I-subunit (stator) is surprisingly small, with only two subunits from the ring making contact with the I-subunit near the middle of the membrane. Furthermore, the spatial arrangement of transmembrane helices resolved in subunit I can form two passageways that could provide proton access through the membrane-bound region and is consistent with a two-channel model of proton translocation.
Acknowledgments

The completion of this thesis would not be possible without the guidance and support from my supervisor, Dr. John Rubinstein. I want to thank John for his encouragement and inspirations over the past five years. John has helped every one of us in the group to stay productive. I want to thank my supervisory committee members, Dr. Gil Prive and Dr. Morris Manolson. I also gratefully acknowledge OGS and CIHR for funding this research.

It was a great pleasure working in the Rubinstein lab. I am truly indebted to all members of the lab for their help in each and every aspect of science. I specially thank Stephanie Bueler for her assistance in the ATP synthase purification; Lindsay Baker for her advice in computation; Dr. Samir Benlekbir, Nawaz Pirani and Janna Tuhman for their motivation and support.

This thesis is dedicated to my parents as well as all my friends. You have helped me to stay positive during difficult times.
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<th>Description</th>
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<tbody>
<tr>
<td>3D</td>
<td>three-dimensional</td>
</tr>
<tr>
<td>ADP</td>
<td>adenosine diphosphate</td>
</tr>
<tr>
<td>AIDS</td>
<td>acquired immunodeficiency syndrome</td>
</tr>
<tr>
<td>AMP</td>
<td>adenosine monophosphate</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>BN-PAGE</td>
<td>blue native polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>CA</td>
<td>correspondence analysis</td>
</tr>
<tr>
<td>CCF</td>
<td>cross-correlation function</td>
</tr>
<tr>
<td>CoQ</td>
<td>coenzyme Q</td>
</tr>
<tr>
<td>Cryo-ET</td>
<td>electron cryotomography</td>
</tr>
<tr>
<td>CTF</td>
<td>contrast transfer function</td>
</tr>
<tr>
<td>DCCD</td>
<td>N,N’-dicyclohexylcarbodiimide</td>
</tr>
<tr>
<td>DDM</td>
<td>n-dodecyl-β-D-maltoside</td>
</tr>
<tr>
<td>EM</td>
<td>electron microscopy</td>
</tr>
<tr>
<td>EMDB</td>
<td>electron microscopy data bank</td>
</tr>
<tr>
<td>ETC</td>
<td>electron transport chain</td>
</tr>
<tr>
<td>FRET</td>
<td>Foster resonance energy transfer</td>
</tr>
<tr>
<td>FSC</td>
<td>fourier shell correlation</td>
</tr>
<tr>
<td>HDL</td>
<td>high density lipoprotein</td>
</tr>
<tr>
<td>HIV</td>
<td>human immunodeficiency virus</td>
</tr>
<tr>
<td>HPLC</td>
<td>high performance liquid chromatography</td>
</tr>
<tr>
<td>NADH</td>
<td>nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>NARP</td>
<td>neurogenic muscle weakness, atxia, retinitis pigmentosa</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>NMR</td>
<td>nuclear magnetic resonance</td>
</tr>
<tr>
<td>NTP</td>
<td>nucleoside triphosphate</td>
</tr>
<tr>
<td>OSCP</td>
<td>oligomycin sensitive conferral protein</td>
</tr>
<tr>
<td>PCA</td>
<td>principal component analysis</td>
</tr>
<tr>
<td>Pmf</td>
<td>proton motive force</td>
</tr>
<tr>
<td>PMSF</td>
<td>phenylmethanesulfonylfluoride</td>
</tr>
<tr>
<td>PPI</td>
<td>inorganic pyrophosphate</td>
</tr>
<tr>
<td>RCT</td>
<td>random conical tilt</td>
</tr>
<tr>
<td>RHCC</td>
<td>right-handed coiled coil</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>SNR</td>
<td>signal-to-noise ratio</td>
</tr>
<tr>
<td>TBT-Cl</td>
<td>tributyltin chloride</td>
</tr>
<tr>
<td>TEM</td>
<td>transmission electron microscopy</td>
</tr>
<tr>
<td>TM</td>
<td>transmembrane</td>
</tr>
<tr>
<td>V-ATPase</td>
<td>vacuolar-type ATPase</td>
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1 CHAPTER 1: INTRODUCTION

1.1 Bioenergetics

1.1.1 ATP

Adenosine triphosphate (ATP) is the primary cellular energy currency and is essential for driving numerous biological reactions to maintain life. In humans, ATP is constantly being produced and utilized with an average turnover of 50-75 kg per day (Okuno et al., 2011). The chemical structure of ATP consists of an adenosine moiety and three phosphate groups. The hydrolysis of ATP to adenosine diphosphate (ADP) releases 46-57 kJ/mol of energy. The amount of energy liberated is essentially the Gibbs energy change for the reaction with respect to its displacement from equilibrium (Nicholls and Ferguson, 2002). In the cell, the ATP hydrolysis reaction is highly exergonic because the concentration of ATP is maintained two to three orders of magnitude higher than the concentration of ADP (Nicholls and Ferguson, 2002). ATP hydrolysis is usually enzymatically coupled to endergonic physiological reactions. In some reactions, such as fatty acid oxidation and nucleoside triphosphate (NTP) biosynthesis, ATP is also converted to adenosine monophosphate (AMP) and pyrophosphate (PPI), with PPI rapidly being hydrolyzed by inorganic pyrophosphatase (Voet et al., 2002).

1.1.2 Formation of ATP

The production of ATP can be accomplished by three different processes in the cell: substrate-level phosphorylation, oxidative phosphorylation (or photophosphorylation) and through homeostasis of intracellular adenine nucleotide concentration maintained by the enzyme adenylate kinase. Substrate-level phosphorylation, which involves direct transfer of a phosphoryl group to ADP to yield ATP, occurs both in glycolysis as well as in the citric acid cycle. For example, human erythrocytes rely exclusively on this process to generate their energy supply. The ubiquitous enzyme adenylate kinase plays an important role in keeping the balance between ATP and ADP concentration in different intracellular compartments; it catalyzes the reversible phosphoryl exchange reaction between two molecules of ADP to yield ATP and AMP.
thus is responsible for *de novo* ATP synthesis (Walker *et al.*, 1982). The majority of ATP, however, is synthesized by means of oxidative phosphorylation within mitochondria in eukaryotes or at the plasma membranes of prokaryotes. Oxidative phosphorylation describes the process in which the production of ATP is coupled to the transfer of electrons from electron donors to electron acceptors through a series of redox reactions. In mitochondria, the electron donors are NADH and FADH$_2$ and the ultimate electron acceptor is molecular oxygen. Both NADH and FADH$_2$ are products of the citric acid cycle. The redox reactions are carried out by four different protein complexes (Complex I, II, III and IV) embedded in the inner membranes of mitochondria, which are collectively known as the electron transport chain (ETC). Complex III and IV are also commonly referred to as the cytochrome bc1 and the cytochrome c oxidase, respectively. Each of these respiratory complexes contains various redox centers with increasing affinities for electrons. The sequence of electron transfer across the electron transport chain begins with the oxidation of NADH and the transfer of electrons to coenzyme Q (CoQ or ubiquinone) by complex I. CoQ also serves to collect electrons from FADH$_2$ via Complex II. The subsequent transfer of electrons from reduced CoQ to cytochrome C is mediated by Complex III. Complex IV, the terminal oxidase of the ETC, reduces molecular oxygen to water. With the exception of complex II, each of the respiratory complexes translocates protons across the inner membrane into the intermembrane space of mitochondria as electrons shuttle through, with a total of 10 protons being translocated per influx of two electrons. The resulting electrochemical gradient (also known as the proton motive force, pmf) is the driving force for ATP synthesis catalyzed by the enzyme called F$_1$F$_o$-ATP synthase (Boyer, 2002). ATP synthesis also occurs in aerobic bacteria, although their respiratory systems exhibit great diversity (Poole and Cook, 2000). Nevertheless, aspects such as the utilization of quinones for oxidation and the existence of a membrane-bound ATP synthase are conserved. Green plants and some bacteria, such as phototrophic bacteria, can synthesize their ATP by photophosphorylation, a light-driven reaction process analogous to oxidative phosphorylation in which ATP is synthesized by the chloroplast ATP synthase (CF$_1$CF$_o$ complex) (Richter *et al.*, 2005).
1.1.3 Structure of ATP synthase

The F₁F⁰-ATP synthase (also commonly referred to as the ATP synthase) is the smallest, electrically-driven nanomotor known in biology (Noji et al., 1997). It is a membrane-bound multisubunit complex consisting of two rotary motors with a total molecular weight of at least 500 kDa. In yeast ATP synthase, one of these rotary motors is called F₁ (α₃β₃γδε), a soluble portion of the enzyme, which contains the catalytic sites for ATP synthesis (Fig. 1.1A and Fig.1.2). The subcomplex α₃β₃ forms a hexamer with a central cavity that allows for the penetration of the γ rotor shaft (Abrahams et al., 1994). Subunits δ and ε are localized to the base of the rotor shaft and together with γ they form the foot of the central stalk (γδε) (Gibbons, 2000). Rotation of the central stalk within the α₃β₃ cavity causes conformational changes in the three catalytic sites located at the α/β interfaces leading to ATP synthesis. If isolated in vitro, the F₁ motor behaves as an ATPase and is called the F₁-ATPase. F₀, the other rotary motor, can be structurally classified into a peripheral stalk and a membrane-bound region (Fig. 1.1A). The peripheral stalk is made up of single copies of subunits b, d, h (F₆ in bovine) and the oligomycin sensitivity conferral protein (OSCP), whereas the membrane-bound region contains subunits a, c, e, f, g, i (sometimes called j), k, and 8 (also known as A6L in the mammalian enzyme) and a portion of subunit b that transverses the membrane (Dickson et al., 2006). Among the F₀ subunits, only subunit c is currently known to present in multiple copies. A total of 10 copies of subunit c in yeast are found to associate into a ring structure that interacts with the foot of the central stalk in the intact complex (Stock et al., 1999). Interestingly, the copy number of c-subunits in the ring has been experimentally studied in different organisms and is found to vary from 8-15 among bacterial, yeast, plant and mammalian ATP synthases (Watt et al., 2010; Meier et al., 2005; Murata et al., 2005a; Stock et al., 1999; Mitome et al., 2004; Toei M et al., 2007; Matthies et al., 2009; Vollmar et al., 2009; Pogoryelov et al., 2009). Sequence analysis of c-subunits, however, suggests that probably all higher eukaryotes (vertebrates and invertebrates) possess a c₈-ring (Watt et al., 2010). The c-ring has been experimentally shown to rotate relative to other F₀ subunits, driven by proton translocation across the membrane (Nishio et al., 2002). Subunit b of the peripheral stalk forms a long, continuous helix and extends into and possibly transverses the membrane twice (Walker and Dickson, 2006). This hydrophobic domain of subunit b likely interacts with other subunits within the membrane-bound region, as a result, the membrane-bound region is connected to the α₃β₃ hexamer through the peripheral stalk and the
central stalk. Other than the structure of c-ring, the exact architecture for the membrane-bound region is not known due to the absence of high-resolution atomic structures for the region.

In bacterial and chloroplast ATP synthases, the F₁ region is composed of the subunits αβγδε similar to the F₁ region in the eukaryotic ATP synthase (Fig. 1.1B). The subunit δ, however, belongs to the peripheral stalk, which also includes either two identical copies of b or b-like polypeptides (b and b'; I and II in chloroplast) (Stock et al., 2000). Subunit δ functionally replaces OSCP, with which it also shares a similar structure (Carbajo et al., 2005; Wilkens et al., 1997). In addition, the membrane-bound region of these enzymes is much simpler than the eukaryotic enzyme and only contains subunit a and the c-ring.

![Fig. 1.1. Schematic diagrams of subunit arrangement in ATP synthase. (A) Yeast ATP synthase. The locations of subunits 8, f, i/j and k are not known. The arrangement of subunits e and g are known from this work and are coloured in gray. (B) Bacterial ATP synthase. The F₁ and the Fₙ motors are shown separately. The central rotor is coloured red and the stator is colored blue. Note the difference in subunit nomenclature between the yeast and the bacterial ATP synthases. This figure is modified from (Lau et al., 2008; Okuno et al., 2011). The adaptation of this figure is by permission of the copyright holder(s).](chart)
1.1.4 Enzymatic mechanism of ATP synthase

Synthesis of ATP by ATP synthase is powered by proton translocation across the membrane. During oxidative phosphorylation, the unidirectional movement of protons from the intermembrane space into the mitochondrial matrix is determined by the difference in the electrochemical potential on either side of the membrane, which is maintained by the ETC. It has been proposed that protons flow through a proton pathway in the enzyme formed by subunit a and the c-ring, which in turn causes a rotation of the c-ring and the central stalk as a single entity relative to the rest of the complex (Fig. 1.1B) (Sambongi et al., 1999). ATP synthase can also function as a proton pump by hydrolysis of ATP, either \textit{in vitro} or \textit{in vivo}, the reverse of the ATP synthesis reaction. In fact, some anaerobic bacteria require an F-type ATP synthase to maintain internal pH homeostasis or pmf across the membrane, or both (Slonczewski et al., 2009). Facultative anaerobes such as Escherichia coli (E. coli), where the F-type ATP synthase normally functions to generate cellular ATP, can utilize ATP to pump protons under anaerobic conditions (Cingolani and Duncan, 2011). Using single molecule experiments, Noji et al. were able to directly observe the unidirectional rotation of the \(\gamma\)-subunit in F\(_1\)-ATPase upon ATP hydrolysis proving the rotary mechanism of ATP synthase (Fig. 1.3A) (Noji et al., 1997). Recently, direct observation of the c-ring rotation in the intact enzyme was made possible by reconstituting the enzyme in liposomes (Duser et al., 2009) and in lipid nanodiscs (Ishmukhametov et al., 2010), avoiding the use of detergent for solubilization of the complexes that rendered the enzyme uncoupled in earlier experiments (Fig. 1.3B) (Sambongi et al., 1999). The direction of the rotation of the central rotor, when viewed from F\(_o\) towards F\(_1\), is counterclockwise during ATP hydrolysis and clockwise during ATP synthesis.

1.1.4.1 The binding-change mechanism

The binding-change mechanism for ATP synthase was first proposed by Paul Boyer and was later supported by a series of X-ray crystallographic studies on the F\(_1\)-ATPase carried out by the group of John Walker. The first atomic structure of the bovine F\(_1\)-ATPase published in 1994 revealed that the three catalytic sites, which are formed predominantly by residues from the \(\beta\)-subunits, are in different conformations depending on the bound nucleotides (Fig. 1.2A).
Fig. 1.2. X-ray crystal structure of the αβγ. Subunits α, β and γ subunits are shown in yellow, green and red, respectively. (A) The catalytic interfaces, which are located at the α-β interface, are indicated with red circles. Each site binds AMP-PNP, ADP, or is empty and is designated as βTP, βDP, or βE, respectively. The noncatalytic interfaces are indicated with blue circles. All of the noncatalytic sites bind AMP-PNP. (B) Conformational states of β subunits and the catalytic sites, shown in side-view configuration. βE has an open conformation. Both βTP and βDP have a closed conformation entrapping a nucleotide within the binding pocket. This figure is modified from (Okuno et al., 2011). The adaptation of this figure is by permission of the copyright holder(s).
Fig. 1.3. Single molecule studies of F$_1$-ATPase and ATP synthase using dark-field microscopy (A) Observation of F$_1$ rotation. The β-subunits (green) were attached to the Ni-NTA glass surface through N-terminal histidine tags. A 40-nm gold bead was attached to the biotinylated γ-subunit (red) through streptavidin and biotinylated BSA. (B) ATP synthase was bound to the surface in a similar fashion as in (A) with an avidin-coated nanorod attached via a biotinylated subunit-c. This figure is modified from (Kinosita et al., 2004; Ishmukhametov et al., 2010). The adaptation of this figure is by permission of the copyright holders.

(Abrahams et al., 1994). One site contained AMP-PNP (an ATP analogue) (β$_{TP}$), the second site contained ADP (β$_{DP}$), whereas the third site was empty (β$_{E}$). Both the β$_{TP}$ and the β$_{DP}$ sites adopted closed conformations, dictated by a large ternary change to the β-subunit that involves an inward movement of its C-terminal helical region (Fig. 1.2B). The asymmetry manifested in the α$_3$β$_3$ hexamer is likely a result of the interactions between the α$_3$β$_3$ hexamer and the central stalk, since the crystal structure of the yeast F$_1$-ATPase in its nucleotide-free state was shown to adopt a similar overall structure to the nucleotide-bound bovine F$_1$-ATPase (Kabaleeswaran et al., 2009). These structural features are in full agreement with binding-change mechanism, which explains how the unidirectional interconversion of the three catalytic sites can be driven by
rotation of the central shaft. Although the crystal structures of F<sub>1</sub>-ATPases have provided strong structural support to the binding-change mechanism, much of its kinetic and mechanochemical properties have been determined using single-molecule studies. During ATP hydrolysis, the enzyme was found to rotate the γ-subunit within the α<sub>3</sub>β<sub>3</sub> cylinder in 120° steps in the F<sub>1</sub>-ATPase (Yasuda et al., 1998). For one complete revolution of the γ-subunit, three molecules of ATP are hydrolyzed (or synthesized in F<sub>1</sub>F<sub>0</sub>-ATP synthase). The 120° step was later resolved to be composed of a 80° and a 40° substep (Shimabukuro et al., 2003). Binding of ATP and cleavage of ATP was further demonstrated to induce the 80° substep and the 40° substep, respectively. The conformation preceding the 40° substep is therefore named the catalytic dwell state; this conformation was recently correlated with the conformation seen in the 1994 'reference structure' of the bovine F<sub>1</sub>-ATPase as well as with all subsequent crystal structures of F<sub>1</sub>-ATPase (Okuno et al., 2008). To date, a crystal structure representing the F<sub>1</sub>-ATPase in the ATP-waiting dwell state, the conformation prior to ATP binding, has not been determined. Nevertheless, the motions of the C-terminal domain of the β-subunits have been directly demonstrated to be the driving force of the central stalk rotation, that is, the mechanism by which chemical events are transferred to mechanical work (Fig. 1.2B) (Masaike et al., 2008). The importance of the C-terminal domain motions of the β-subunits is also in line with a study that showed the rotor-stator contacts at the bottom entrance of the α<sub>3</sub>β<sub>3</sub> hexamer is sufficient to produce torque without the penetrating portion of the γ-subunit (Furuike et al., 2008).

### 1.1.4.2 Proton translocation mechanism

Pmf-driven proton translocation through the membrane-bound region of F<sub>0</sub> generates rotation of the c-ring and the central stalk. The term ‘central rotor’ has been used to refer to the c-ring-central stalk subcomplex in the intact enzyme. The c-ring and the central stalk are probably tightly associated with each other to prevent slippage (Tsunoda et al., 2001). However, some flexibility was also localized to the contact of central stalk to the c-ring, an observation used to explain why the enzyme is capable of coping with c-rings of different sizes in different organisms (Sielaff et al., 2008; Wachter et al., 2011). During rotation of the central rotor, the catalytic α<sub>3</sub>β<sub>3</sub> hexamer is restrained by the peripheral stalk; its role has been attributed to resisting the torque generated by the central rotor (Junge et al., 1997; Dickson et al., 2006).
Because of the lack of structural insights into the intact membrane $F_0$ region at the atomic level, knowledge of the exact molecular mechanism of proton translocation by ATP synthase is limited. The most broadly accepted model of proton translocation is called the two-channel model, which was proposed almost two decades ago (Vik and Antonio, 1994; Junge et al., 1997). This model assumes that the hydrophobic stator subunit contains two aqueous half channels that allow protons to flow through (Fig. 1.4A). The two half channels are offset to one another and each is connected to different sides of the membrane (Elston et al., 1998). In between the two half channels, there exists a positively charged residue from subunit a to block the direct flow of protons from one side of the membrane to the other (Fig. 1.4B). This positively charged residue has been identified as an arginine (Arg) residue that is highly conserved among rotary ATPases and is absolutely critical for enzymatic function (Cain and Simoni, 1989; Hatch et al., 1995; Valiyaveetil and Fillingame, 1997; Vik et al., 1998). The conserved Arg residue is assumed to interact with the rotor sites through another conserved glutamic acid/glutamate (Glu) or aspartic acid/aspartate (Asp) residue found in the c-subunits of the rotor ring critical for proton transport. A crystal structure of the $c_{15}$-ring from *Spirulina platensis* F-type ATP synthase revealed a symmetric ring structure formed by two concentric rings of transmembrane helices (TM) derived from the c-subunits (Pogoryelov et al., 2009). Each c-subunit folds into a hairpin structure with an N-terminal TM and a C-terminal TM, when assembled together, producing an inner and an outer ring. Moreover, the C-terminal helices are orientated further away from the center of the ring than the N-terminal helices so that the conserved Glu residues can electrostatically interact with the conserved Arg residue from subunit a.

According to the model, protons from the acidic reservoir gain access to one of the charged Glu residues from a subunit c at the a-c interface, which are found approximately at the center of the ring, *via* the first half channel. The neutralization of one of the conserved Glu residues by the proton allows the ring to rotate out of the a-c interface into the lipid bilayer through Brownian motion. Rotation of the ring consequently brings a c-subunit bearing a protonated Glu from the lipid bilayer back into the a-c interface. The $pK_a$ of the Glu is thus lowered under the influence of the Arg residue, facilitating its deprotonation. The exit of the proton to the basic reservoir is thought to occur *via* the second half channel. Hence, the direction of the ring rotation is biased by the direction of the pmf. This model is compatible with several of the published X-ray crystal structures of the ring complexes within the rotary ATPase family.
(Junge and Nelson, 2005), although no physical channels can be identified from the ring itself (Fig. 1.5A). Instead, the channels may be contained within subunit a, or formed at the a-c interface, or both (Junge and Nelson, 2005). A corollary from the two-channel model is that the conserved Glu residues must be able to interact with the conserved Arg when undergoing deprotonation/reprotonation at the a-c interface. The Glu residues seen in the X-ray crystal structures determined at acidic pH adopt a 'proton-locked' conformations thought to resemble to the conformations when they are embedded in the lipid bilayer during catalysis (Fig. 1.5B and Fig. 1.4C) (Pogoryelov et al., 2009). To convert to an 'open' conformation, two possibilities have been postulated: the swiveling of the outer helix of subunit c (Rastogi and Girvin, 1999; Vincent et al., 2007) and rotameric reorientation of the glutamate side chain in the presence of a locally hydrated environment at the a-c interface (Pogoryelov et al., 2010). The swiveling of the subunit c helix, which would involve a large backbone rearrangement, seems unlikely because the intrinsic steric restraints imposed by the compactness of the ring seen in the X-ray crystal structure. Minor backbone rearrangement of subunit c in the presence of the stator, however, cannot be ruled out. Finally, the possibility of proton binding to the rotor ring as hydronium ion has been invoked (Boyer, 1988; Preiss et al., 2010). The X-ray crystal structures of the F-type ATPase rotor rings from cyanobacteria (Pogoryelov et al., 2009) and chloroplast (Krah et al., 2010) appear to contradict this hypothesis. Conversely, a bound water molecule was found in the proton-binding pocket of the X-ray crystal structure of the rotor ring isolated from an alkaliphilic bacteria and could favour this hypothesis (Vollmar et al., 2009; Preiss et al., 2010).

1.1.5 P/O ratio

The P/O ratio is defined as the number of ATP molecules synthesized by oxidative phosphorylation for each pair of electrons entering the ETC. The P/O ratio is an important value to be determined since it contributes to the understanding of the energy balance of the cell. Unfortunately, this value is difficult to be measured experimentally (Ferguson, 2010). The fact that the P/O ratio could be a non-integer value has been slowly accepted after the X-ray crystal structure of the yeast F₁-c₁₀ structure was solved, because P/O ratio can be directly inferred from the β/c stiochiometric ratio in the enzyme and is directly related to the H⁺/ATP ratio. For reasons
presently not understood, different species have evolved to have different rotor ring sizes so the bioenergetic cost of making an ATP molecule is also different among these species. While

Fig. 1.4. Model of the proton translocation mechanism. (A) The hypothetical two-channel model for the generation of rotation by proton flow through the membrane-bound region of ATP
synthase. The red line indicates the proton path. The blue dots represent the conserved Glu residues. (B) A more detailed depiction of the proton translocation mechanism of (A), viewed from F$_1$. A proposed rotor/stator interface, which involves three c-subunits, is shown. (C) A different proposed rotor/stator interface, which involves two c-subunits, is shown. The conserved Arg residue from subunit a is thought to interact with one of the conserved Glu residues from the c-subunits at any given time and minor conformation changes to the Glu sidechains are required to switch from the proton-locked state to the open state. This figure is modified from (Stock et al., 1999; Pogoryelov et al., 2009, 2010). The adaptation of this figure is by permission of the copyright holders.

Fig. 1.5. X-ray crystal structure of the rotor ring from the sodium-driven ATP synthase from *Ilyobacter tartaricus*. (A) Overall structure of the ring in ribbon representation. Each c-subunit is shown in a different colour. The sodium ions are represented as blue spheres. Detergent molecules inside the ring are shown as red and gray spheres. (B) Architecture of the sodium ion binding pocket formed by residues from two neighbouring c-subunits. The center of the bound sodium ion is represented by a blue sphere. This figure is modified from (Meier et al., 2005). The adaptation of this figure is by permission of the copyright holders.
inferring the P/O ratio from the size of the rotor ring is an attractive approach and is considered reliable by most investigators, the result is, however, is tentative. In fact, using a minimal chemiosmotic system in vitro, the $\text{H}^+/\text{ATP}$ ratios for two different ATP synthases with significant different rotor sizes (the $c_{10}$ ring in *E. coli* and the $c_{14}$ ring in spinach) have been experimentally determined to be identical, having a value of 4 in both cases (Steigmiller *et al.*, 2008). The discrepancy between the $\text{H}^+/\text{ATP}$ ratios derived from the $\beta/c$ stoichiometric ratio and from experiment suggested uncertainties yet to be settled in the field of bioenergetics.

1.1.6 Regulatory mechanism of ATP synthase

$\text{IF}_1$, the natural inhibitor of ATP synthase found in the mitochondria, can inhibit the ATP hydrolytic activity of ATP synthase by binding to a catalytic interface (Cabezon *et al.*, 2003). The result of this binding is to arrest the conformational changes of the catalytic sites thereby stopping the rotation of the central stalk. $\text{IF}_1$ may play an important role in limiting ATP consumption during cellular ischaemia (Gledhill and Walker, 2006). In bacteria and chloroplast, instead of $\text{IF}_1$, the $\varepsilon$-subunit is responsible for the inhibition of the ATPase activity of the enzyme (Cingolani and Duncan, 2011; Nowak and McCarty, 2004). The C-terminal domain of $\varepsilon$ can adopt either a 'contracted' conformation in the presence of ATP or an 'extended' conformation in the absence of ATP. Subunit $\varepsilon$ is an ATP sensor: its binding to ATP stabilizes the 'contracted' conformation, presumably when the cellular ATP concentration is high (Kato-Yamada, 2005). The ATPase activity of $\text{F}_1$ is inhibited when $\varepsilon$ adopts the 'extended' conformation, with its helical C-terminal domain penetrating into the $\alpha_3\beta_3$ cavity and inserting into the catalytic interface (Cingolani and Duncan, 2011). The binding modes for both $\text{IF}_1$ and the C-terminal domain of $\varepsilon$ were suggested to be closely related and a pmf is able to relieve their inhibitory effects on the enzyme (Feniouk *et al.*, 2007). Therefore, both of these inhibitors block the ATPase reaction and favour the ATP synthesis reaction.
1.1.7 ATP synthase and disease

A number of rare genetic mutations linked to primary defects in ATP synthase have been identified (Kucharczyk et al., 2009). These defects are all maternally inherited and are associated with subunit a from the Fₒ region, which is mitochondrially encoded. The most commonly found single amino acid mutation, Leu156 to Arg/Pro, manifests as NARP syndrome (Neurogenic muscle weakness, Atxia, Retinitis Pigmentosa) (Holt et al., 1990). At a higher mutation load, when heteroplasmy exceeds 90%, the disease presents as Leigh syndrome (Loeffen et al., 1998). Given the fundamental role of ATP synthase in the cell, diseases caused by ATP synthase defects are mostly severe and often fatal. Both reduced ATP production and accumulation of incomplete ATP synthase assemblies have been observed in these patients (Houstek et al., 2006). So far, no mutations have been identified in the subunit A6L (Houstek et al., 2006), the other mitochondrially encoded subunit.

The biosynthetic pathway of mammalian ATP synthase is currently not well understood. However, at least five nuclear-encoded assembly factors have been discovered (Schon et al., 2001; Houster et al., 2006). Mutations affecting two of these assembly factors, the human orthologues of Atp11p and Atp12p (yeast chaperones for F₁-ATPase biogenesis), were shown to lead to a significant overall decrease (<10-30% of control) in ATP synthase content in cells (Houster et al., 1999, Vojtiskova et al., 2004). Human Atp11p and Atp12p interact with the β-subunit and the α-subunit, respectively (Ackerman, 2002). However, it has been observed that even cells with 90% reduction in ATP synthase content can survive if the energy demand placed on them is not too high (Houster et al., 2004). Instead, the main factor that contributes to the pathogenesis of the disease seems to be related to the up-regulated Reactive Oxygen Species (ROS) production when ATP synthase is deficient and the membrane potential Δψ is high in the cell (Huttemann et al., 2008). ROS are produced predominantly by complex I and complex III (Finkel and Holbrook, 2000; Stefanatos and Sanz, 2011).

Under ischemic conditions, when cells are deprived of oxygen, the pmf across the mitochondrial inner membrane collapses and cellular ATP is produced by glycolysis. Consequently, the pH of both the cytosol and the mitochondrial matrix drops leading to the activation of the natural ATP synthase inhibitor protein, IF₁. A single important histidine residue (49 in the bovine protein) is responsible for the conversion of tetrameric, non-active IF₁ to
dimeric, active IF$_1$ (Cabezon et al., 2000). Active, dimeric IF$_1$ can bind two ATP synthase complexes simultaneously because the N-terminal regions responsible for the inhibitory effect on ATPase activity are arranged on opposite ends of the dimer. The tetramerization of IF$_1$ presumably masks the N-terminal regions of the protein, rendering it inactive. IF$_1$ may also play an important role in cancer. The expression and activity of IF$_1$ have been observed in tumor cells, where the presence of IF$_1$ may be required to preserve ATP in the anoxic necrotic centers of tumors (Green and Grover, 2000; Gledhill and Walker, 2005).

According to a growing number of reports (Martinez et al., 2003; Scotet et al., 2005; Vantourout et al., 2010), various subunits of the ATP synthase have been unexpectedly identified at the surface of cells and tumors: $\alpha$, $\beta$, $\gamma$, b, d, OSCP as well as the inhibitor protein IF$_1$. These studies collectively indicated that a complex similar or identical to the mitochondrial ATP synthase is present on the cell surface in an orientation with the F$_1$ projecting outside of the cell. Furthermore, the $\beta$-chain of F$_1$-ATPase has been specifically shown to bind a major protein component of high density lipoprotein (HDL), apoA-1, with high affinity ($10^{-9}$ M) determined by Biacore, leading to the hypothesis that these cell surface ATP synthases (ecto-ATP synthases) may be involved in the regulation of HDL in blood plasma (Martinez et al., 2003). Subsequent experiments additionally showed that apoA-1 stimulates the ATPase activity of cell surface ATP synthase through direct interaction (Martinez et al., 2000), and that extracellular ADP stimulates HDL endocytosis (Jacquet et al., 2005). Currently, whether or not ecto-ATP synthase can synthesize ATP is still under debate and the route by which ATP synthase localized to the cell surface is not known (Vantourout et al., 2010). Further experiments are needed to elucidate the potential functions of ecto-ATP synthase.

1.1.8 Supermolecular organization of ATP synthase

Mitochondrial cristae form a unique combination of simple tubular structures and more complex lamellar structures that emerge from the inner membrane through cristae junctions. Limited research has been devoted to the investigation of the mechanism of cristae formation in the mitochondria. ATP synthase has been shown to contribute to the formation of cristae morphology through dimerization and oligomerization (Giraud et al., 2002; Paumard et al., 2002).
It was first shown in yeast that detergent solubilization of mitochondria using mild detergents such as Triton X-100 and digitonin can produce ATP synthase dimers, which can be separated from the monomeric forms on a blue-native PAGE (BN-PAGE) gel (Fig. 1.6A) (Arnold et al., 1998). Moreover, these dimeric ATP synthases include three small additional subunits: e, g and k, which are absent in the monomeric forms thus are consequently called the dimer-specific subunits (Fig. 1.6B). These subunits were found to belong to the membrane-bound region (Arnold et al., 1998). Subunits e is known to be an integral membrane protein that spans the membrane once with an N\_in-C\_out orientation (Tokatlidis et al., 1996; Arnold et al., 1997; Yao et al., 2008). Subunits g and k have also been shown to behave as integral and peripheral membrane protein, respectively (Arnold et al., 1998). Deletion of subunits e, g and k does not affect the enzymatic activities of ATP synthases. However, mutant yeast strains lacking subunits e and g display altered cristae morphology, establishing a link between ATP synthase dimerization and generation of cristae morphology (Fig. 1.7) (Paumard et al., 2002). Subsequent studies were able to further isolate larger ATP synthase oligomers; these enzymatically active oligomers were built up of dimers as indicated by native PAGE (Fig. 1.6C) (Wittig and Schagger, 2005; Strauss et al., 2008). Therefore, ATP synthase is expected to dimerize \textit{via} two distinct interfaces. Subunit b, together with other hydrophobic F\_o subunits, may form the second dimerization interface (Wagner et al., 2010; Gavin et al., 2005). The phenomenon of ATP synthase oligomerization has also been extended to mammalian mitochondria including human mitochondria (Wittig et al., 2010).

The first clear picture of the supramolecular organization of ATP synthase oligomers was obtained using the technique of cryo-electron tomography (cryo-ET) from minimally disrupted inner membrane fractions of bovine and rat liver mitochondria, revealing the ATP synthase dimer ribbons as ubiquitous feature \textit{in vivo} (Fig. 1.8A) (Strauss et al., 2008). Recently, imaging of close-to spherical mitochondria in their native state has been made possible by applying dual-axis cryo-ET (Fig. 1.8B) (Dudkina et al., 2010). In this study, similar dimer ribbons of ATP synthase were also found in the electron tomograms of intact mitochondria. ATP synthases are preferentially localized to regions of membrane with high local curvature. An interpretation of these results is that the dimerization/oligomerization process induces membrane curvature (Strauss et al., 2008, Velours et al., 2009). Although the nature of the dimerization/oligomerization interfaces and the exact mechanism of this process remains to be
determined, low-resolution structures obtained from averaging of subtomographic volumes as well as from negative stain electron microscopy (EM) showed that dimers of ATP synthase particles interact via the region where subunits responsible for dimerization are expected to be found (Fig. 1.8.C and D) (Minauro-Sanmiguel et al., 2005; Couoh-Cardel et al., 2010; Dudkina et al., 2005). The role of ATP synthase oligomerization in the mitochondria is currently speculative. Some of the possible roles include simply increasing the surface area of the inner membrane, acting as a proton trap (Strauss et al., 2008), promoting IF1 binding (Wittig et al., 2010), and promoting efficient ATP synthesis by diminishing the rotational drag of the central rotor (Buzhynskyy et al., 2007).

Fig. 1.6. Detection of ATP synthase dimers and oligomers on native-PAGE. (A) ATP synthase dimers were extracted from yeast mitochondria at low Triton concentration (0.6g Triton/g of protein) and separated by blue native-PAGE (BN-PAGE). (B) Subunit compositions of the dimeric and monomeric forms of yeast ATP synthase were analyzed in a second dimension by
Tricine-SDS-PAGE following (A). Subunits e, g and k were identified by N-terminal protein sequencing and Western blotting after silver staining of the gel. (C) Separation of digitonin-solubilized (8 mg digitonin/mg protein) extracts of beef heart mitochondria on a clear-native PAGE showing a characteristic ladder of bands. ATP synthase oligomers were stained using an in-gel ATPase assay. This figure is modified from (Arnold et al., 1998; Strauss et al., 2008). The adaptation of this figure is by permission of the copyright holders.
Fig. 1.7. Mutant yeast cells devoid of subunits e and g show abnormal mitochondrial morphology. Thin yeast cell sections were stained and imaged by transmission electron microscopy. Arrows indicate abnormal mitochondria. (A) Wild-type. (B) Mutant devoid of subunit i. (C and D) Mutants devoid of subunit g. (E and F) Mutants devoid of subunit e. Scale bar represents 0.5 µm. This figure is modified from (Paumard et al., 2002). The adaptation of this figure is by permission of the copyright holders.
**Fig. 1.8.** *In situ* analyses of mitochondrial ATP synthases by cryo-electron tomography (cryo-ET). (A) Surface renderings of ATP synthase dimer ribbons in a tubular crista vesicle and in round vesicles from rat liver and bovine heart mitochondria, respectively. (B) Dual-axis cryo-ET of a mitochondrion from *Polytomella*. (C) Surface renderings of the cristae from dual-axis cryo-ET revealing row-like distribution of *Polytomella* ATP synthase F1 headpieces (pink). (D) Average of tomographic sub-volumes of ATP synthase dimers as seen in (C). Symbol ‘S’ represents the peripheral stalk. The scale bar is 10 nm. (D) Average of negatively stained single particles of dimeric ATP synthase isolated from *Polytomella* mitochondria using only side-views. This figure is modified from (Strauss *et al.*, 2008; Dudkina *et al.*, 2010). The adaptation of this figure is by permission of the copyright holders.
1.1.9 Respirasome

The concept of supramolecular organization of membrane protein complexes in the inner membrane of mitochondria is not only limited to ATP synthase but also applies to ETC complexes within the oxidative phosphorylation system (Schagger and Pfeiffer, 2000; Devenish et al., 2008). The classical model for the organization of ETC complexes envisions these complexes are randomly moving in the inner mitochondrial membrane to carry out their functions (Hackenbrock and Chazotte, 1986; Lenaz and Genova, 2007). This model is known as the random diffusion model or the fluid model. Conversely, the alternative solid model proposes that all respiratory complexes are organized into bigger structures necessary for efficient electron transport (Lenaz and Genova, 2007). Early evidence in support of the solid model was derived exclusively from electrophoretic co-migration of different respiratory complexes in BN-PAGE or density gradient centrifugations (Schagger and Pfeiffer, 2000; Krause et al., 2004; Eubel et al., 2004). These observations were based on solublized respiratory complexes with mild detergents, raising concerns that the identified supercomplexes are merely detergent artifacts, even though some of these supercomplexes were stable enough to be isolated for negative stain EM studies (Acin-Perez et al., 2008). The demonstration of these supercomplexes as bona fide components of the ETC was thoroughly addressed in a recent study by Acin-Perez et al., in which these supercomplexes were also tested for functions and temporal regulation (Acin-Perez et al., 2008). Furthermore, based on their findings, the authors proposed an alternative model, the plasticity model, that include both aspects of the fluid and the solid models. The plasticity model proposes that even though each of the individual respiratory complexes, with the possible exception of complex I, can move freely within the inner membrane and retain their functions, a portion of them can be occasionally found to associate with other complexes. The diversity of association between the respiratory complexes may be explained by the need for the cell to modulate the kinetic behaviour of the ETC in response to physiological changes.

1.1.10 Physiological roles of V-ATPase

The vacuolar-type ATPase (V-ATPase) is a multisubunit enzyme complex that is generally similar to the F-type ATP synthase in numerous ways, both in structure and function.
However, the F- and the V-ATPase are classified into two distinct superfamilies based on the differences in subunit sequences as well as structural features in the peripheral and central stalk regions, which are unique to each class of enzymes. The physiological role of V-ATPase in eukaryotes is to pump protons across membranes by harnessing the energy from ATP hydrolysis. V-ATPase is found in the endomembranes of almost all eukaryotic cells, including the membranes of lysosomes, endosomes, Golgi membranes, clathrin-coated vesicles and several types of secretory granules (Nishi and Forgac, 2002). The internal pH of these compartments is regulated by V-ATPase for important processes such as receptor-mediated endocytosis, protein sorting and degradation, post-translational modification as well as coupled transport of small molecules (Forgac, 1999; Stevens and Forgac, 1997; Bowman and Bowman, 2000). V-ATPases also reside in the plasma membranes of some specific cell types. For instances, the enzyme is present in the plasma membranes of epididymal cells, renal intercalated cells, osteoclasts and is crucial for the sperm maturation and activation, urine acidification and bone resorption, respectively (Brown and Breton, 2000; Li et al, 1999; Brisseau et al, 1996).

V-ATPases are also found in archaea and sometimes in bacteria (Yokoyama and Imamura, 2005). In archaea, V-ATPase (sometimes called A-ATPase) functions mostly as an ATP synthase by catalyzing the reverse of the proton pumping reaction. All archaea sequenced to date possess this type of ATPase/synthase (Lolkema et al, 2003). Although the F-type enzyme is more commonly found in bacteria as the ATPase/synthase, V-ATPase is sometimes found to substitute the F-ATPase as an ATP synthase, such as in Thermus thermophilus (Yokoyama et al, 1990, 1994), or it may act as a sodium ion pump, such as in Clostridium fervidus (Lolkema et al, 2003). Interestingly, the coexistence of F-type and the V-type ATPase in a single bacterium has also been discovered, as in Enterococcus hirae (Heefner and Harold, 1982; Murata et al, 2005b; Muench et al, 2011) and in three species of archaea (Sumi et al, 1992; Muller and Gruber, 2003; Muench et al, 2011). All V-ATPases originated from the archaeal kingdom (Lolkema et al, 2003). Bacteria likely have acquired V-ATPase from archaea through horizontal gene transfer during evolution (Gogarten, 1992; Hilario and Gogarten, 1998). Phylogenetic analysis of the stalk subunits and the operon structures among the ATPases/synthases found in bacteria and archaea strongly suggested that the bacterial V-ATPase and the A-ATPase represent the same enzyme (Lolkema et al, 2003). Although V-ATPases found in bacteria and archaea are more distantly related to the eukaryotic V-ATPase, a number of subunits share significant sequence
identity between these three kingdoms of life. The eukaryotic V-ATPase, however, contains additional subunits not present in bacterial and archaeal V-ATPases.

1.1.11 Structure of V-ATPase
1.1.11.1 Bacterial V-ATPase

From a structural point of view, the bacterial/archaeal V-ATPase (Fig. 9A) is a simpler version of the eukaryotic V-ATPase (Fig. 9B). Since the structure of the bacterial and the archaeal enzymes are highly similar, except for subunit nomenclature, for the purpose of this introductory chapter, the description of the archaeal V-ATPase will be excluded. By analogy to the F-ATPase, bacterial V-ATPase is made up of two major regions, the $V_1$ and the $V_o$ regions (Fig. 1.9A). The $V_1$ region consists of the catalytic $A_3B_3$ hexamer and the DF subcomplex of the central stalk. The $V_o$ region includes the remaining subunits with the stoichiometry of $C Elliot G_2I_12$. This region can further be subcategorized into the peripheral stalks and the membrane-bound region. The bacterial V-ATPase contains two peripheral stalks, each of them is a heterodimeric right handed coiled coil (RHCC) structure, a novel protein fold never observed before (Lee et al., 2010). In the membrane-bound region, there exists the rotary L-ring and the amphipathic I-subunit. The hairpin structure of subunit L is similar to subunit c of the F-type ATP synthase. Like the F-type ATP synthase, the copy number of ring subunits seem to vary among different species. Subunit I, on the other hand, is structurally distinct from any of the hydrophobic subunits of the F-type enzyme. In addition to its membrane-spanning domain, subunit I has a soluble, rod-shaped domain that horizontally links the two peripheral stalks (Bernal and Stock, 2004; Lee et al., 2010). The other remaining $V_o$ component is subunit C (similar to subunit d in eukaryotic V-ATPase). Subunit C is a funnel-shaped protein with three domains and has an overall distorted three-fold symmetry (Iwata et al., 2004; Numoto et al., 2004). Its crystal structure did not reveal a membrane anchor region in the protein; rather, it is believed that subunit C sits in between the L-ring and the DF subcomplex, forming part of the central stalk.
1.1.11.2 Eukaryotic V-ATPase

About half of the subunits in the mammalian V-ATPase have isoforms that are responsible for tissue-specific expression and targeting to different cell types and intracellular compartments (Muench et al., 2011). By contrast, only one of the subunits, subunit a, of the yeast V-ATPase has two isoforms (Vph1p and Stv1p) (Manolson et al., 1992, 1994; Kawasaki-Nishi, 2001b). Vph1p is specifically localized to vacuoles, whereas Stv1p is localized to the Golgi or the endosomes (Jefferies et al., 2008a). A significant feature unique to the eukaryotic V-ATPase is the presence of three peripheral stalks in the complex (Fig. 1.9B) (Diepholz et al., 2008; Zhang et al., 2008; Muench et al., 2009). Eukaryotic V-ATPases also contain three additional subunits that are not found in the bacterial V-ATPase: subunits C, H and e. Subunit C, which has no homolog in the bacterial V-ATPase, also links two peripheral stalks together and is functionally equivalent to the soluble domain of subunit I as well as subunit a of the bacterial and the eukaryotic V-ATPase, respectively (Drory et al., 2004). Subunit C may have an additional role of triggering the reversible disassembly/assembly of the complex in vivo in response to changing cellular conditions, as suggested by its intrinsic flexibility and the difference in affinities between the head and the foot domain for the peripheral stalks (Kane and Smardon, 2003; Drory et al., 2004). From cross-linking and docking studies, subunit H has been tentatively localized to the interface between the catalytic A₃B₃ hexamer and the membrane-bound region (Lu et al., 2002; Fethiere et al., 2005; Diepholz et al., 2008). It has been shown that subunit H participates in silencing the ATPase activity of the V₁ region (Jefferies and Forgac, 2008b) (see section 1.1.13). The role of subunit e is currently unknown (Compton et al., 2006). This small hydrophobic subunit of 8.4 kDa probably localizes to the membrane-bound region of Vₒ and is essential for the enzyme's activity. Finally, some mammalian cell types and non-mammalian tetrapods express an accessory Vₒ subunit, Ac45. Ac45 may have a regulatory role in the calcium-dependent peptide secretion process and osteoclastic bone resorption mediated by V-ATPase (Feng et al., 2008; Jansen et al., 2008).
Fig. 1.9. Structural models of the V-ATPase. (A) Bacterial V-ATPase showing the presence of two peripheral stalks and its simpler architecture. The solid lines indicate the approximate boundaries of the lipid bilayer. (B) Eukaryotic V-ATPase showing the presence of three peripheral stalks and additional stator subunits. Note the difference in subunit nomenclature between the enzymes. This figure is modified from (Lau and Rubinstein, 2010; Muench et al., 2011). The adaptation of this figure is by permission of the copyright holders.

Only three subunits share sequence similarities between the F-type and the V-type ATPase/synthase (A and β; B and α; L and c) (Yokoyama and Imamura, 2005). A crystal structure of the intact *T. thermophilus* V₁-ATPase showed that the long coiled-coil region of
subunit D is also structurally homologous to the γ-subunit of F-type ATP synthase, despite no apparent sequence similarity (Numoto et al., 2009).

1.1.12 Enzymatic mechanism of V-ATPase

V-ATPase is expected to catalyze ATP hydrolysis via the same general mechanism as the F-type ATP synthase. Indeed, ATP synthesis by the *T. thermophilus* V-ATPase reconstituted into proteoliposomes could be driven by ΔpH and Δψ, the two constituents of the pmf (Toei et al., 2007). Furthermore, in the presence of ATP, relative rotation of the central rotor (DFCL subcomplex in bacterial V-ATPase) with respect to the A3B3 hexamer in the intact complex was directly demonstrated with single molecule studies (Nakano et al., 2008; Furuike et al., 2011).

*In vitro*, the V₁ portion can be isolated from the bacterial V-ATPase complex (Nakano et al., 2008). Similar to F₁-ATPase, the V₁ region also possesses ATPase activity. The catalytic mechanism in V₁-ATPase appears to be slightly different from F₁-ATPase, even though the general principles of the binding-change mechanism still apply (Imamura et al., 2005; Furuike et al., 2011). While V₁ only appeared to dwell at every 120° irrespective of ATP concentration as determined by the three catalytic sites (Fig. 10A), the 120° step is further separated into a 80° and a 40° substep in F₁-ATPase (Fig. 10B) (Ariga et al., 2007). Each of the dwells should be comprised of at least two other catalytic events in addition to ATP binding; one of these is probably ATP hydrolysis.

The proton translocation mechanism of V-ATPase is much less well studied than the F-ATPase. The X-ray crystal structure of the rotor ring of the *E. hirae* sodium ion-driven ATPase, the only atomic structure currently available for the membrane-bound region of V₀, suggested that the proton translocation mechanism may be conserved among F-/V-type ATPases (Murata et al., 2005a). It has also been proposed that the hydrophobic stator subunit I also contains two half channels for proton access (Murata et al., 2005).
**Fig. 1.10.** Rotational schemes of the V₁ and the F₁ motors. The catalytic sites are represented by blue (V₁) or green (F₁) circles and the orientation of the γ is represented by arrows. (A) A possible rotational scheme of V₁. The motor pauses at every 120° irrespective of ATP concentration. ATP binding and ATP cleavage appears to occur at approximately the same angle. ADP and/or phosphate release may also occur at the same angle. (B) Rotational scheme of F₁. Each 120° step is composed of a 80° and a 40° substep. The timings for ADP and phosphate release as well as ATP cleavage have been determined in F₁. This figure is modified from (Imamura et al, 2005). The adaptation of this figure is by permission of the copyright holders.

1.1.13 Regulatory mechanism of V-ATPase

Bacterial V-ATPase hydrolyzes ATP at a very low rate in comparison to F₁-ATPase due to its intrinsic susceptibility to entrapment of Mg-ADP (in the absence of Pi) in the catalytic site leading to inhibition (Nakano et al, 2008). In fact, preincubation of the enzyme with ADP can completely abolish its ATPase activity, suggesting that the inactivation by ADP is essentially irreversible. Kinetic analyses also estimated that the $k_{on}$ for ADP is ~13-fold higher than the $k_{on}$ for ATP during ATP hydrolysis. This ADP-induced inactivation does not occur under ATP synthesis conditions. Since the biological role of V-ATPases found in bacteria is ATP synthesis, these unique enzymatic properties may have evolved to avoid wasteful ATP consumption when the pmf is low. Even though ADP inhibition is also observed with F-type ATP synthase, the enzyme appears to be less sensitive to this type of inactivation as ADP can be more readily expelled from the catalytic site by thermal fluctuations (Hirono-Hara et al, 2005). The effect of ADP inhibition of F₁-ATPases from different organisms, however, has not been explicitly
explored at their physiological temperatures. In one study, the effect of ADP inhibition on the *Bacillus PS3* thermophilic F$_1$-ATPase was found to be greatly enhanced at higher temperatures, consistent with the physiological role of this enzyme (Furuike *et al.*, 2008). In bacteria, the ε-subunit has been demonstrated to play a significant role in stabilizing the ADP-inhibited form of F$_1$ (Cingolani and Duncan, 2011). Interestingly, the activity of intact eukaryotic V-ATPase, an ATP-driven proton pump, can also be inhibited by ADP (Kettner *et al.*, 2003). The significance of this mechanism is currently not known.

Various regulatory mechanisms have been identified for the eukaryotic V-ATPase. The best-characterized mechanism involves the reversible association/dissociation in response to changes in cellular environment (*e.g.* Glucose concentration in yeast) (Kane and Smardon, 2003; Diakov and Kane, 2010). The dissociated V$_1$ region cannot hydrolyze ATP, possibly due to inhibition conferred by subunit H. Unlike the bacterial V$_o$ region, the eukaryotic V$_o$ by itself does not translocate protons (Zhang *et al.*, 1992; Yokoyama *et al.*, 2003).

### 1.1.14 V-ATPase and disease

Enveloped viruses enter the cell *via* clathrin-mediated endocytosis into the endosomes. These enveloped viruses utilize intracellular V-ATPase to enter the cell (Gruenberg and van der Goot, 2006). For example, influenza virus has a coat protein called hemagglutinin (HA) that, when exposed to the acidic pH maintained by V-ATPase in the endosome, undergoes a conformational change and exposes its membrane insertion domain (Gruenke *et al.*, 2002). HA then bridges the viral and the endosomal membranes, and oligomerizes to form a fusion pore that results in the release of viral RNA into the host cytoplasm. Bacterial toxins, such as anthrax toxin and diphtheria toxin, also enter the cells in a similar fashion and are therefore V-ATPase dependent (Gruenberg and van der Goot, 2006).

Distal renal tubular acidosis is a disease in which acid accumulation in the bloodstream as a result of kidney failure caused by malfunction of the plasma membrane V-ATPase of α-intercalated cells. Either mutations in subunit B or the a4 isoform of subunit a specific for renal cells have been linked to this inherited disease (Karet *et al.*, 1999; Smith *et al.*, 2000; Ochotny *et al.*, 2006). A second example of genetic disease linked to plasma membrane V-ATPase defect is
osteopetrosis, which is due to mutations in the a3 isoform of subunit a localized to the mature osteoclasts that normally function to degrade bone by secreting acid (bone resorption) (Frattini et al., 2000; Ochotny et al., 2006). Osteopetrosis is a form of rare bone disease in which bones are overly dense. Conversely, the a3 isoform of subunit a of V-ATPase can be intentionally inhibited by drug to reduce bone resorption to treat osteoporosis (Niikura et al., 2004). One existing inhibitor, FR167356, can specifically target the a3 isoform of subunit a thus discriminating osteoclast V-ATPase from lysosomal V-ATPase (Niikura et al., 2004). This potent inhibitor has the potential for clinical applications. V-ATPase is also present on the surface of some tumor cells, therefore, is expected to play a role in acidifying the extracellular medium (Sennoune et al., 2004). Tumor metastasis is well known to be triggered by low extracellular pH since several proteases (e.g., Cathepsins) activated by low pH may be involved in the digestion of extracellular matrix proteins crucial for the invasion process (Gocheva and Joyce, 2007). Finally, V-ATPase is a therapeutic drug target for human immunodeficiency virus (HIV) infection (Ayana, 2007). Subunit H of V-ATPase has been shown to bind both the HIV-encoded protein, NEF (Lu et al., 1998), as well as AP2 (Geyer et al., 2002) in vitro and in vivo. NEF also binds and internalizes CD4 (Greyer et al., 2002), the cellular receptor critically linked to host immune responses, and the decreased CD4 expression has been suggested to contribute to the pathogenesis of acquired immunodeficiency syndrome (AIDS). V-ATPase may act as an adapter to bridge the NEF and the AP2 complexed with clathrin to elicit the internalization of CD4 through endocytosis.

1.1.15 Evolution of the F-/V-ATPase and the flagellar motor

The sequences of subunits A and B are highly conserved between the bacterial/archaeal and the eukaryotic V-ATPases (~50% sequence identity), but are much less conserved with the homologous F-ATPase subunits β and α (~25% sequence identity) (Gogarten et al., 1992). Subunit A from all V-ATPases also contains a so-called non-homologous region that is absent in the catalytic subunits of the F-ATPases. Therefore, it is widely believed that the F- and the V-ATPases diverged early in evolution from a common ancestor. The existence of catalytic and noncatalytic subunits has also been proposed to be a result of gene duplication in the last
common ancestor of eubacteria, archaea and eukaryotes carrying an ATPase with six identical catalytic subunits.

The proteolipid ring subunit from the eukaryotic V-ATPases has four transmembrane helices, while most proteolipids from bacterial/archaeal V-ATPases have two. The first two and the last two transmembrane helices of the eukaryotic V-ATPases also share low sequence similarity with the F-ATPase proteolipid, which has two transmembrane helices (Hilario and Gogarten, 1998). As the two halves of the eukaryotic proteolipids are more closely related to each other than to the bacterial/archaeal proteolipids, the bigger size of the eukaryotic proteolipid is very likely to be derived from gene duplication and fusion events that occurred some time along the eukaryotic cell lineage after its divergence from the archaeal cell lineage. The bigger proteolipids from some archaeal and bacterial V-ATPases (i.e., Methanococcus and Enterococcus) may be explained by a second gene duplication/fusion event independent of the evolution of the eukaryotic proteolipid, and in the latter case, in conjunction with a horizontal gene transfer event from the archaeal domain into the bacterial domain (Hilario and Gogarten, 1998).

FliI, a component of the export apparatus of the bacterial flagellum, is an ATPase and is known to self-assemble into a homohexameric ring (Fig. 1.11, A and B) (Imada et al., 2007). It shares significant sequence homology (~23% identity) as well as structural similarities with each of the catalytic and noncatalytic subunits of the F-/V-ATPases (Gogarten et al., 1992). The X-ray crystal structure of another component of the export apparatus, FliJ, was recently determined (Fig. 1.11, C and D) (Ibuki et al., 2011). In addition to its structure being remarkably similar to the γ- and D-subunit of the F-type and the V-type ATPases, respectively, it was confirmed by cryo-EM to penetrate into the FliI homohexamer. These analyses indicated that the bacterial flagellum and the F/V-ATPases are evolutionarily related and FliI is possibly evolved from the common ancestor that also gave rise to the catalytic and noncatalytic subunits of F/V-ATPases. Evidence has also been put forward to establish an evolutionary link between the type III secretion system, the bacterial flagellum, and the F/V-ATPases (Macnab, 2003; Minamino et al., 2008).
Fig. 1.11. Structural similarities between components of the bacterial flagellum and the F-/\textit{V}-
ATPase. (A) Overlay of the FliI X-ray crystal structure (cyan) and the $\beta_{\text{DP}+\text{Pi}}$ subunit (red) of F$_1$-
ATPase (“half-closed” conformation). (B) A hexamer model of FliI was built based on the ‘1994’ bovine F$_1$-ATPase reference structure (Abrahams \textit{et al.}, 1994). (C) X-ray crystal structure of FliJ. (D) X-ray crystal structure of the \(\gamma\) subunit of bovine F$_1$-ATPase. This figure is modified from (Imada \textit{et al.}, 2007; Ibuki \textit{et al.}, 2011). The adaptation of this figure is by permission of the copyright holders.
1.2 Single particle cryo-EM

Electron cryomicroscopy (cryo-EM) of single particles has become one of the standard techniques for the three-dimensional (3D) structural determination of macromolecular assemblies, which are often either too large or too heterogeneous to be studied by either X-ray crystallography or nuclear magnetic resonance (NMR) spectroscopy. By preserving the specimen in vitreous ice, cryo-EM can study biological complexes in near native states. Since the pioneering work by the Dubochet and colleagues on the EM imaging of viruses in 1984 (Adrian et al., 1984), tremendous progress has been made in the field thanks to advances in almost all aspects of the process including specimen preparation, instrumentation, imaging conditions and computational methods. To date, cryo-EM has been able to achieve atomic resolution for a number of structures with inherent high symmetries, allowing ab initio atomic model building based on the cryo-EM maps alone (Zhang et al., 2010; Ge and Zhou, 2011). For biological complexes with low symmetry (or no symmetry) and/or lower mass, owing to less information content per particle image, the current achievable resolution is limited to ~4 Å (Cong et al., 2010). The study of membrane proteins by cryo-EM still remains one of the most challenging tasks as a result of conformational heterogeneity and the presence of detergent micelles in the image background giving rise to poor image contrast. The electron microscopy databank (EMDB) currently holds about 200 subnanometer resolution map depositions determined by single particle analysis. Of these, only five are from membrane protein structures (either alone or in complex with a soluble binding partner). Continuous improvement in the technique with focuses on specimen preparation and hardware development will likely allow routine atomic resolution structure determination by cryo-EM in the near future.

1.2.1 Image formation and contrast

Electrons can interact with the specimen either elastically or inelastically. Elastic scattering of electrons by the specimen involves no loss of energy and result in deflection of the incident electrons with a wide angle distribution. This type of scattering provides high-resolution information in the image. Inelastically scattering of electrons by the specimen, on the
other hand, involves loss of energy and the deflected electrons have a very narrow angle distribution. These electrons only give rise to low-resolution information. The total amount of scattering that occurs depends on the density and thickness of the specimen. The probability of the electrons interact with the specimen, also termed the 'scattering-cross sections', decreases with increasing accelerating voltage for both elastic and inelastic scattering events. The ratio of the elastic to inelastic scattering cross-sections, however, remains nearly constant at any practical accelerating voltage. For relatively thin specimen (<100 nm), it is expected that multiple scattering events do not occur for the elastically scattered electrons.

The total contrast in the image is derived from both amplitude contrast and phase contrast. Amplitude contrast arises from loss of electrons that are elastically scattered outside of the objective aperture of the microscope. Amplitude contrast is important for the overall density distribution of the image, but only contributes a few percent of total image contrast because protein specimens are made up of light atoms (carbon, nitrogen, oxygen and hydrogen atoms) that scatter electrons weakly. Commonly used values for amplitude contrast range between 3-10%, depending on the accelerating voltage of the microscope and the nature of the specimen (Yonekura et al., 2006). Ribosomes, consisting of both nucleic acid and protein, produces higher amplitude contrast because nucleic acids scatter more electrons than protein due to the higher atomic number of phosphorus.

The main source of contrast in transmission electron microscopy (TEM) of frozen-hydrated protein specimen is phase contrast, which is generated from the interference between the elastically scattered and unscattered beams. When scattered electrons are focused by the objective lens onto the image plane, the image is in exact focus and has only minimal phase contrast at high-resolution due to lens aberrations. Defocusing the microscope, which brings the image plane above the ideal image plane, is necessary to obtain image contrast at low-resolution by changing the path lengths for the rays and thus producing phase differences.

Because of the applied defocus and imperfections in the microscope, the resulting images are modified by the point-spread function, or equivalently in Fourier space, the contrast transfer function (CTF). The CTF modulates the sign of the phase and the amplitude of the Fourier component of the image and is a function of defocus (Fig. 1.12). The effects of the CTF on the image can be generally described as a high-pass filtration with several reversals and suppression
of contrast at different spatial frequency (inverse of resolution) zones. Therefore, in real space, different features in the image would appear to be enhanced or suppressed. In addition, at some spatial frequencies, no contrast is transferred so some information in the image is lost completely. In addition to the CTF, the amplitudes at high spatial frequencies of the image are also dampened due to imperfectly coherent illumination, lens current fluctuations and chromatic aberration, imperfect detectors, radiation damage and intrinsic molecular flexibility of the biological specimen, as well as specimen charging and beam-induced motion (see section 1.17) (Saad et al., 2001). All of these factors that contribute to the contrast loss at high resolution can be represented by an envelope function, \( E(s) \), which has the form of a simple Gaussian function,

\[
E(s) = e^{-Bs^2/4}
\]

where \( B \) denotes the experimental B-factor and \( E(s) \) is a function of spatial frequency \( s \). In a modern electron microscope equipped with a field emission gun, the effects of the first two factors (partial coherence and lens fluctuations) are quite small. Beam-induced motion of the specimen is by far the most serious degradation factor that leads to contrast loss (Glaeser et al., 2011).

Thus, the Fourier transform of an electron micrograph can expressed as follows,

\[
M(s, \theta) = F(s, \theta)CTF(s)E(s) + N(s, \theta)
\]

where \( s \) and \( \theta \) represents polar coordinates in Fourier space. \( F(s, \theta) \) is the structure factor and is a 2D slice through the 3D Fourier transform of the object. \( N(s, \theta) \) is an empirical noise function that encompasses many factors such as ice thickness, inelastic scattering, multiple scattering events and detector noise. \( N(s, \theta) \) cannot be directly subtracted from the image because it represents random fluctuations. When examining the quality of an electron micrograph, it is useful to compute the rotationally averaged 2D-power spectrum (square of the intensities),

\[
M_{rot}(s) = F_{rot}(s)CTF(s)E(s) + N_{rot}(s)
\]
where $M_{rot}(s)$ is the 1-D power spectrum computed either from the whole electron micrograph or from the set of individual particle images extracted from one micrograph. A typical plot of the rotationally averaged 1D power spectrum is shown in Figure 1.13B. It is possible to determine $F_{rot}(s)$, $CTF(s)$, $E(s)$ and $N_{rot}(s)$ from several power spectra computed from different micrographs of different defocuses. Both the CTF and the envelope functions must be corrected accurately during the image processing step in order to generate a reliable 3D reconstruction (Fig. 1.14). Furthermore, since signal-to-noise ratio (SNR) approximately equals to $F_{rot}(s)CTF(s)E(s)/N_{rot}(s)$ (Saad et al., 2001; Ludtke and Chiu, 2002), the quality of the images can be examined prior to image processing by plotting SNR versus spatial frequency.
Fig. 1.12. Dependence of the contrast transfer function (CTF) on defocuses. (A) CTF only for three defocus values: 1.5 µm (____), 2.0 µm (- - -), and 2.5 µm (- - - -). (B) CTF in combination with the envelope function. The envelope function degrades the amplitudes at high spatial frequencies. The defocus values are the same in (A). This figure is modified from (Penczek et al., 1997). The adaptation of this figure is by permission of the copyright holders.

1.2.2 Radiation damage

At present, the exact molecular mechanism of radiation damage on the specimen from inelastic scattering is not clearly understood but certainly involves ionization of the specimen, bond rupture and the formation of free radical species (Chen et al., 2008; Bammes et al., 2010; Baker et al., 2010). Bond rupture causes expansion or shrinkage of the protein complex, which can be a local or long-range effect. Ionization causes the build-up of positive charges on the specimen that can also induce stress and movement of the molecule. The movement of the specimen during the exposure leads to image blurring, a phenomenon known as the beam-induced motion. Using liquid nitrogen as the cryo-protectant in cryo-EM has been experimentally shown to increase the dose tolerance of the specimen by up to 10-fold compared to performing the imaging at room temperature (Hayward, 1979; Jeng and Chiu, 1984). The benefit of lower temperature is thought to be due to a 'cage effect', which traps molecular fragments liberated by radiolysis, preventing them from migrating through the specimen and causing further damage. Still, the total electron exposure is fundamentally limited by the specimen to about 30e/Å² before ‘bubbling’ occurs in the specimen, which is mainly caused by radiolysis of water molecules and formation of pockets of hydrogen gas – a severe form of radiation damage (Chen et al., 2008). The images recorded by this unavoidable 'low-dose imaging' are intrinsically noisy and must rely on averaging to retrieve the high-resolution signal. Therefore, the limiting factor in the resolution of cryo-EM structures is the image contrast and not the resolving power of modern instrument, which is on the order of 2 to 3 Å.
Fig. 1.13. Image power spectrum. (A) An example of an average 2D power spectrum computed from individual particle images boxed out from one micrograph. (B) Rotationally averaged 1D power spectrum computed from (A). The “ripples” indicates the signal above the noise level. This figure is modified from (Liu et al., 2010). The adaptation of this figure is by permission of the copyright holders.

Fig. 1.14. The combined effect of CTF and envelope function on an image. (A) A computed projection of a GroEL reconstruction. (B) The same image in (A) but with CTF and the envelope function applied. (C) A typical single particle image from a raw micrograph aligned to the projection in (A). (D) An average of 48 aligned particle images with corrections of the CTF
and the envelope function applied. This figure is modified from (Ludtke and Chiu, 2002). The adaptation of this figure is by permission of the copyright holders.

1.2.3 2D image analysis

Macromolecular complexes embedded in vitreous ice can adopt different orientations, appearing as different views seen in an electron micrograph. Particle images boxed out from electron micrographs are corrupted by random noise and cannot be reliably interpreted without computer-assisted 2D image analysis. The aim of 2D image analysis is to group particle images that show similar views together, but which differ from each other by an in-plane rotation as well as translational (xy) shifts – a process called classification. Usually particle images are aligned to one another by reference-free alignment prior to classification or alignment can be done through classification (as described later in multireference alignment). Particle images within each group (or class) can then be averaged together to generate a class average with improved SNR. The resulting class averages are generally used to examine specimen heterogeneity (conformational and/or compositional) and determine whether the specimen tends to adopt preferred orientations on the EM grid. The former condition poses a serious limitation to the practically achievable resolution in the 3D structure or even hampers one's ability to obtain a reliable reconstruction at all. The latter occurs when some parts of the molecule interact preferentially with the air-water interface, which is hydrophobic (Schmidt-Krey and Rubinstein, 2011). Preferred orientations of the specimen, which result in insufficient coverage of 3D orientation space, may present problems later on in map construction. Class averages are also sometimes used for initial model generation to bootstrap the refinement process.

1.2.3.1 Classification

There exist two types of classification approaches: supervised and unsupervised classification. Supervised classification describes the grouping of particle images according to their similarity through the use of two or more references or templates. The process relies on the use of the cross-correlation function and simultaneously involves 2D image alignment. This reference-based alignment technique, which is described in the next section, also forms the basis
of the 3D model refinement procedure called projection matching. One obvious disadvantage of this method is that the references are usually not known \textit{a priori} and the subjective choice of the references used can influence the final outcome. The method also fails if the SNR of the image drops below a certain threshold. In these cases, class averages would simply look like the references even if the particle images within the class are completely dissimilar to the reference, a phenomenon known as reference or noise bias. The danger of reference or noise bias, that is, the emergence of the signal entirely from reinforcement of noise, has been well documented and experimentally shown (Fig. 1.15) (Shaikh \textit{et al}, 2003; Stewart and Grigorieff, 2004). Some strategies have been developed to reduce this effect, but currently none can entirely eliminate this problem.

![Fig. 1.15. Experimental demonstration of the reference/noise bias problem in cryo-EM. Images containing only random noise were aligned once to a noise-free reference (see section 1.2.3.2 on 2D alignment and section 1.2.4.2 on 3D projection matching). A cross-section through the 3D reconstructions derived from aligned noise images using different common similarity measures: (A) phase residual, (B) linear correlation coefficient and (C) weighted correlation coefficient.](image-url)
(D) A cross-section through the noise-free reference. This figure is modified from (Stewart and Grigorieff, 2004). The adaptation of this figure is by permission of the copyright holders.

By contrast, unsupervised classification involves no preexisting references or templates to guide the grouping process. Although this type of classification can be done with raw data, due to its low SNR, a data reduction step, the multivariate data analysis, is usually carried out before the classification. Two almost equivalent strategies employed in the multivariate data analysis are principle component analysis (PCA) and correspondence analysis (CA) with PCA being more sensitive to scaling of the data. Both PCA and CA analyze the inter-image variations and decompose the data set into mutually distinct components on the order of their significance with minimal loss of information. After PCA or CA, classification can be carried out in a space with much lower dimensionality compared with raw images (i.e. with the initial N x N space dictated by images containing N x N density values).

One of the most commonly used unsupervised classification techniques is K-means clustering (Fig. 1.16). This technique is an iterative procedure that only requires a user-defined number of K classes. First, individual particle images (K seeds) are randomly drawn from the data set. Partitions are then created based on the seeds and every element is assigned to its nearest seed. Finally, a new seed for each of the subsets created by the partitions is re-calculated from the center of gravity of the resulting cluster. This procedure is repeated until convergence is reached. Because this technique does not guarantee finding the global optimum, K-means clustering should be repeated multiple times with different choice of seeds (Frank, 2006). As the number of classes is already fixed at the beginning of the process, different number of classes should always be tried in order to obtain the best solution.
Fig. 1.16. Schematic diagram of K-means clustering. From left to right. From among the image, represented by points in the 2D space here, three are picked at random as “seeds”. These seeds define the first partition of the set. Then, centers of gravity are computed for each subsets created by the partition. These centers define an improved partition, taking place of the seeds. The whole process is iterated several times until convergence is reached. This figure is modified from Wikipedia (http://en.wikipedia.org/wiki/K-means_clustering#cite_note-hamerly-8).

1.2.3.2 Alignment

Supervised classification involves aligning every particle image in the set to each of the available templates or references. For each image, the three parameters, x- and y- shifts and the in-plane rotation angle are usually determined exhaustively by computing the normalized (linear) cross-correlation coefficient (CC),

\[ CC(X, Y) = \frac{\sum_{i=1}^{N \times N} x_i y_i}{\sqrt{\sum_{i=1}^{N \times N} x_i^2 \sum_{i=1}^{N \times N} y_i^2}} \]

where \( X \) and \( Y \) are a particle image and a reference of dimensions \( N \times N \), respectively. The \( x_i \) and \( y_i \) denotes the pixel values of \( X \) and \( Y \). The maximum value in the search determines the image orientation with accuracy limited by the SNR.

When references or templates are not available, one can perform one of the available reference-free alignment algorithms to align image sets that are either homogeneous or heterogeneous. One approach is outlined here following (Penczek et al., 1992).

The first part of the algorithm is to find an approximate, 'initial' average without using a specific reference image:

1. Pick any two images randomly from the set
2. Align the two images
3. Generate a global average of these two images after they are aligned
4. Pick a third image randomly from the set and align it to the global average
5. Update the global average by including the third image
(6) Repeat steps 4 to 5 until all images are included from the set

Since this procedure is somewhat dependent on the sequence in which the images selected, the first part of this algorithm is usually repeated one more time with a different random order of image selection to mitigate such bias and the second global average is added to the first, creating the 'initial' average.

The second part of the algorithm is to carry out iterative refinement of this 'initial' average:

1. In a reverse sequential order, one image is removed from the set to create a 'subaverage'
2. Align the removed image \( p \) to the 'subaverage'
3. Update the 'initial' average with the new orientations of \( p \) found in step 2
4. Repeat steps 1 to 3 until no single image changes its position.

To obtain class averages from a set of particle images exhibiting different views, it is almost always useful to utilize both supervised and unsupervised classification, in conjunction with both reference-based and reference-free alignment strategies. An example of such an approach is multireference alignment (van Heel and Stoffler-Meilicke, 1985). Multireference alignment can be performed on any datasets as long as the dataset is large enough so that at least some of the similar views of the particle images occur in similar in-plane orientations, provided that the particle images can be centered in their boxes or the 'particle picking accuracy' is relatively high as a result images are more or less well-centered initially. Briefly,

1. Particles are classified using K-means clustering (unsupervised classification), with or without data reduction
2. Class averages are generated
3. Class averages are aligned to each other using reference-free alignment
4. All particles are aligned to the class averages by reference-based alignment to form a new,
aligned image set

(5) Steps 1 to 4 are repeated until no changes in the appearance of the class averages. The final results can be justified by comparing the class averages with the aligned raw particle images.

1.2.4 3D image processing

The projection theorem states that the Fourier transform of a 2D projection from the molecule corresponds with a central 2D slice through the 3D Fourier transform of the molecule (Fig. 1.17). Following the projection theorem, a 3D structure can therefore be recovered entirely from its 2D projections (views) (Dover et al., 1980). If a sufficient number of the 2D projections are acquired so their corresponding central slices cover the angular space in the 3D Fourier transform of the molecule, its structure can be recovered to a given resolution by calculating the inverse Fourier transform. The relationship between the finite number of projections needed and the achievable resolution is given by (Crowther et al., 1970, 1971)

\[ N = \frac{\pi D}{d} \]

where \( N \) is the number of equispaced projections, \( D \) is the diameter of the molecule and \( 1/d \) represents the given resolution.

1.2.4.1 Initial model generation

The 3D reconstruction process consists of two stages, the initial model generation stage and the refinement stage. Depending on the dataset, the initial model may or may not be critical. A low quality initial model may be sufficient if the molecule of interest contains some kind of symmetry and the quality of the data is relatively high. In this case, a random density (usually a Gaussian blob) may be used as a starting model for the refinement stage. One caveat is that the handedness of the final model is ambiguous and must be determined by other means. Another caveat is that unless some external standards are available to validate the result, it is impossible to determine if the refinement has converged onto the correct structure when such an initial
model is used. The use of a random blob as a starting model has been experimentally demonstrated to be a suitable strategy for some molecules with or without internal symmetry (Lau and Rubinstein, 2010; Ludtke et al., 2005; Yan et al., 2007; Sanz-Garcia et al., 2010). Alternatively, an initial model can also be a low-pass filtered X-ray or cryo-EM structure, provided that the identical structure or a similar structure already exists. This is useful, for example, when one aims to determine a structure in its ligand-bound state using its ligand-free state as the initial reference. Another way to obtain an initial model is by single particle cryo-electron tomography, an increasingly popular technique which has been traditionally used only

Fig. 1.17. Illustration of the projection theorem. This figure is obtained from Dr. Richard Henderson, MRC, Laboratory of Molecular Biology.
for large complexes such as viruses, but has been recently proved feasible for complexes even with mass of only 150 kDa (Sandin et al., 2004).

The random-conical tilt (RCT) reconstruction method (Radermacher et al., 1986, 1987a, 1987b) is considered the most general method for obtaining an initial model for small and asymmetric specimen. The method is also robust enough to deal with specimens that are heterogeneous in nature, although it can be difficult and time-consuming to implement. RCT is based on the fact that many macromolecules adopt some preferred orientations on the grid or that the preferred orientations can be induced by adsorbing the macromolecules onto a thin layer of continuous carbon. In the experiment, the specimen field is recorded twice, first with a tilted specimen stage (~60-70º) and second with an untilted specimen stage (0º). As any subset of the identical particle images in the untilted image form a conical rotation series with an in-plane rotation angle, classification and alignment is first performed on the particles in the untilted images to determine the relative in-plane orientations of each identical particle image within each class. After identifying the corresponding particles in the tilted images, for each class, these tilted particles are sorted in their correct order in the rotation series using their in-plane rotation angles as determined previously. Finally, particles within each class are combined to generate a 3D map, leading to many different reconstructions, each with missing information in Fourier space due to the inability to tilt the specimen stage to 90º. These maps must therefore be merged together using one of a variety of techniques. In the case of specimen heterogeneity, structures in different conformations can be sorted at this stage. The 3D maps that come out from the random-conical tilt method are always crude; they serve as initial models for the subsequent refinement procedure.

1.2.4.2 3D refinement and reconstruction

During refinement, roughly uniform projections covering the entire angular space are generated from the existing 3D reference with a user-specified angular spacing (step size). Each of the particle images is then matched exhaustively to each of these projections by computing the normalized CCF in order to determine its three Euler angles and two shift values, a process
known as projection matching, which is a form of supervised classification. After the five parameters are found for each particle image, a new 3D map is built, which then acts as the reference model in the second round of refinement. The procedure is repeated until resolution no longer improves. The angular step size is usually decreased incrementally as a means to improve model resolution. Rather than building a map directly from particle images, a variation of this approach is to include a class-averaging step, where a class average is first generated from the grouped particle images for each projection direction and reconstruction is built from the class averages instead (Ludtke et al., 1999). Maps can be constructed in Fourier space using the projection theorem with simultaneous CTF correction using the Fourier interpolation method, as incorporated in the program called Frealign (Grigorieff, 2007). Here, the correction of CTF is performed implicitly during the reconstruction step.

1.2.5 Resolution assessment

In single particle cryo-EM, resolution estimation is generally based entirely on internal consistency of the data. At the end of the refinement, the data set is typically divided into two halves and two separate maps are built each from one half of the data. Resolution is then measured by the Fourier shell correlation (FSC), the normalized cross-correlation as a function of spatial frequency between the two maps in Fourier space (Saxton and Baumeister, 1982; van Heel and Schatz, 2005):

\[
FSC_{12}(r) = \frac{\sum_{\ell \in r_i} F_1(r) \cdot F_2^*(r)}{\sqrt{\sum_{\ell \in r_i} F_1^2(r) \cdot \sum_{\ell \in r_i} F_2^2(r)}}
\]

\(F(r)\) is the complex “structure factor” at position \(r\) in Fourier space. Symbol “\(^*\)” denotes complex conjugate and \(r_i\) represents each resolution shell.

Although the entire curve is informative, for the sake of simplicity, single values are always chosen based on different thresholds to report the resolution of a structure. The choice of the threshold continues to be a controversial topic in the field (van Heel and Schatz, 2005). The most commonly used threshold is FSC=0.5 (Bottcher et al., 1997). A simple relationship holds
between FSC and the SNR for two half models (Frank and Al-Ali, 1975; Liao and Frank, 2011), which is

\[ SSNR = \frac{2FSC}{1-FSC} \]

SSNR represents the spectral SNR, which is the SNR as a function of spatial frequency. As a result, when FSC=0.5, the SNR of the 3D reconstruction drops to the value of 2. A threshold of FSC=0.143 was recently introduced based on a measure of map interpretability in X-ray crystallography (Rosenthal and Henderson, 2003). It is worth noting that the FSC curve itself is subject to various sources of errors (Penczek, 2010), such as improper CTF correction, poor choices of angular step size, reference or noise bias, nonuniform distribution of data in angular space, to name a few. Therefore, resolution assessment in cryo-EM should always be treated as an estimate rather than an absolute measure. Instead, the resolution of the reconstruction can be directly inferred from the resolvability of the density map when the resolution of the reconstruction is sufficiently high (<10Å) (Zhou, 2008; Chiu et al., 2005).
1.3 Thesis objectives

The goal of this project is to further our understanding of the rotational mechanisms of F-type ATP synthase and the closely related V-type ATPase through structure determination. Despite great efforts, no atomic structure of either an intact F-/V-type complex has been obtained thus far using protein X-ray crystallography. To this end, we exploited the approach of single particle cryo-EM to obtain these structures to low and intermediate resolution, currently providing the most detailed information on the architecture and subunit arrangement of these multisubunit complexes that cannot be afforded by other structural methods.

In the first part of the project, *S. cerevisiae* was chosen as our model system for studying the F-type ATP synthase because the enzyme can be genetically modified to allow inclusion of a hexahistidin tag for rapid purification. A ~24 Å resolution cryo-EM reconstruction of the yeast ATP synthase was determined, allowing direct comparison to the existing 3D structure of the bovine ATP synthase at a lower resolution, determined previously by the same technique. The differences in subunit composition between the yeast and bovine enzyme preparations after detergent extraction from mitochondrial membranes permitted the localization of subunits responsible for dimerization of ATP synthase.

Our structural study using the single particle approach was later expanded to include the V-ATPase from the eubacterium *T. thermophilus*, which forms the second part of the project. Owing to its simpler subunit composition, this enzyme is ideal for characterizing the basic components within the membrane-bound region necessary for proton translocation to occur. The resulting reconstruction revealed that the interaction of the proteolipid ring with the hydrophobic stator is mediated via a remarkably small area of contact.

The final part of the project is a continuation on the structure determination of the *T. thermophilus* V-ATPase with resolution improvement. By increasing the size of the data set and optimizing various aspects of the procedure, the resolution was significantly improved to beyond 10 Å, capable of revealing secondary structural elements. To the best of our knowledge, for the first time, all of the predicted transmembrane helices of the hydrophobic stator subunit were identified. The arrangement of these transmembrane helices could form the proposed proton channels.
2 CHAPTER 2: CRYO-EM STRUCTURE OF THE YEAST ATP SYNTHASE

The material presented in this chapter forms part of the following publication:


Contributions:

J.L.R designed and supervised the research. W.C.Y. purified the protein, performed the microscopy, activity assay and thin-layer chromatography as well as carried out the image analysis. L.A.B. provided the bovine map and calculated the difference map. J.L.R. modified the computer softwares. W.C.Y. and J.L.R. interpreted the data and wrote the manuscript.

Acknowledgments:

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2.1 Overview

The ATP synthase from \textit{S. cerevisiae} is a ~600 kDa enzyme consisting of a total of 17 different subunits. Subunits $\alpha$, $\beta$, $\gamma$, $\delta$ and $\varepsilon$ belong to the $F_1$ region, while the remaining subunits belong to the $F_0$ region. In the membrane, other than the ring of c-subunits that forms the central domain of $F_o$, the other hydrophobic $F_o$ subunits (a, e, f, g, i/j, k, 8 and the N-terminal portion of b) probably reside in a second, peripheral domain. Four of these subunits (e, g, i/j and k) are not essential to the function of the enzyme. Moreover, the association of subunits e, g and k with the rest of $F_o$ is weak and these proteins can be selectively removed by treatment with detergents (Arnold \textit{et al}, 1998).

ATP synthase forms dimers and larger oligomers in its native environment (Strauss \textit{et al}, 2008). Oligomerization of ATP synthase is necessary for the formation of mitochondrial cristae (Giraud \textit{et al}, 2002; Paumard, 2002). Both subunits e and g were initially shown to be crucial for formation of stable dimers, as deletion of subunits e and g led to an aberrant, onion-like cristae morphology. Further, subunit e was demonstrated to form homodimers in the mitochondria by cross-linking and affinity purification experiments (Brunner \textit{et al}, 2002). The incorporation of subunit g into the monomeric ATP synthase depends on subunit e, suggesting that they interact in the intact complex (Arselin \textit{et al}, 2003). The formation of ATP synthase dimers is a dynamic process: dimerization can be inhibited by phosphorylation of subunit g at a serine residue (serine 62) located in a region outside of the transmembrane domain (Reinders \textit{et al}, 2007). Stability of ATP synthase dimers, on the other hand, is maintained by the presence of the i- and k-subunits (Wagner \textit{et al}, 2010). In addition, using Fluorescence resonance energy transfer (FRET), the proximity of two b-subunits has also been demonstrated \textit{in vivo} in the absence of subunits e and g (Gavin \textit{et al}, 2005). Since oligomerization of ATP synthase requires two distinct dimerization interfaces, it is likely that one of these interfaces may involve subunits e and g, while the second is formed by subunits b of the peripheral stalks from two monomeric complexes.

Although much is known about the structure and arrangement of subunits in the $F_1$-ATPase and the peripheral stalk region from available X-ray crystal structures of these subcomplexes, only the structure of subunit c in the $F_o$ membrane-bound region is known (Watt \textit{et al}, 2010; Meier \textit{et al}, 2005; Murata \textit{et al}, 2005a; Stock \textit{et al}, 1999; Mitome \textit{et al}, 2004; Toei M
et al., 2007; Matthies et al., 2009; Vollmar et al., 2009; Pogoryelov et al., 2009). Here, we present a 3D reconstruction at 24 Å resolution of intact ATP synthase from the yeast *S. cerevisiae* determined by cryo-EM. The map allowed for the identification of subunit b, the ring of c-subunits, and more tentatively subunit a, within the membrane-bound region. Biochemical analysis of the enzyme preparation used to obtain this map revealed that the complex lacked subunits e, g, and k. Comparison of this map to a map of the bovine ATP synthase, which contains subunits e and g (Rubinstein et al., 2003), suggested where these proteins are likely to be located in the intact complex. The resulting 3D model of subunit arrangement in yeast ATP synthase can now be used as a framework for studying the mechanism of the enzyme and the nature of its oligomerization.

### 2.2 Materials and Methods

#### 2.2.1 Yeast strain

The *S. cerevisiae* strain USY006 encoding a His$_6$-tag at the N terminus of subunit β of ATP synthase was a gift from Professor D. Mueller, Rosalind Franklin University of Medicine and Science, Department of Biochemistry and Molecular Biology. The codons for Ala-Ser (GCT-AGC) were added in front of the codons for the six histidine residues to preserve the proteolytic processing site of mitochondrial protein, and the mature β-subunit consists of Ala-Ser-His$_6$ at its N-terminus as previously confirmed by primary sequence analysis (Mueller et al., 2004).

#### 2.2.2 Purification of ATP synthase from yeast *S. cerevisiae*

Eleven litres of YPEG medium (1 % [w/v] yeast extract, 2 % [w/v] peptone, 3 % [v/v] glycerol and 2 % [v/v] ethanol) were inoculated with a 200 mL overnight culture and yeast were grown at 30°C in a Microferm Fermentor (New Brunswick Scientific, Edison, NJ, USA) for 48 hrs until OD$_{600}$ reached a plateau (stationary phase). Thorough aeration is critical to obtaining high yields. Cells (~200 g) were then harvested by centrifugation at 4000 g for 15 mins. After washing cells in chilled water with several cycles of resuspension-centrifugation, cells were
resuspended in lysis buffer (100 mM Tris-HCl, 60 mM sorbitol, 5 mM EDTA, 5 mM \(\varepsilon\)-amino-\(n\)-caproic acid, 5 mM \(p\)-aminobenzamidine, 0.2% [w/v] bovine serum albumin, 0.001% [w/v] phenylmethyl sulphonyl fluoride [PMSF]; pH 8.0) prior to cell breakage. Cell lysis was carried out on ice in the cold room with a bead beater (3 x 1 min intervals with 1 min rest time in between). When necessary, the pH of the lysate was adjusted to 7.5 using Tris base. To collect the mitochondrial membrane fraction, cell debris was pelleted at 5500 g for 30 min followed by 47800 g for 20 min. The resulting membrane fraction may be stored at -80°C.

Mitochondria were lysed by homogenizing in 75 mL of phosphate buffer (50 mM sodium phosphate, 5 mM \(\varepsilon\)-amino-\(n\)-caproic acid, 5 mM \(p\)-aminobenzamidine, 0.001% [w/v] PMSF; pH 9.0). After stirring for an additional 30 min, membranes were collected by ultracentrifugation at 265000 g for 20 min. Mitochondrial membranes were subsequently solubilized with 1% (w/v) dodecyl maltoside (DDM) for 30 min in buffer A (50 mM sodium phosphate, 300 mM sodium chloride, 0.05% [w/v] DDM, 15 mM imidazole, 10% [v/v] glycerol, 5 mM \(\varepsilon\)-amino-\(n\)-caproic acid, 5 mM \(p\)-aminobenzamidine, 0.001% [w/v] PMSF; pH 7.4). Solubilization was carried out by adding dry detergent powder directly into a stirring solution. To remove insoluble material, solubilized fraction was centrifuged at 265000 g for 20 min.

Purification of the enzyme was carried out with affinity and gel filtration chromatography. The supernatant containing solubilized protein after centrifugation was loaded onto a 5 mL Ni-NTA column (HisTrap, GE Healthcare) equilibrated with buffer A. Contaminants were removed by washing the column extensively in buffer A followed by buffer B (buffer A containing 80 mM imidazole). The complex was eluted with buffer C (buffer A containing 300 mM imidazole). Purified proteins were buffer-exchanged into ATP synthase buffer (20 mM Tris-HCl, 100 mM sodium chloride, 0.05% [v/v] Brij-35, 50 mM sucrose, 2 mM magnesium sulfate, 10% [v/v] glycerol, 0.001% [w/v] PMSF; pH 8.0) using gel filtration (Superose 6; GE Healthcare). The final purified protein was concentrated to 3 mg/ml using a 100 kDa cut-off concentrator, dialyzed against cryo buffer (10 mM Tris-HCl, 10 mM sodium chloride, 0.05% [v/v] Brij-35, 2 mM magnesium sulfate; pH 8.0) and immediately flash-frozen with liquid nitrogen for storage at -80°C.
2.2.3 ATPase assay

ATPase activity was measured with an ATP-regenerating system (Nakano et al., 2008). A range of concentrations of purified enzyme was added into an assay mixture (50 mM Tris-Cl, 100 mM KCl, 2 mM MgCl2, 0.05% [w/v] DDM, 1 mM phosphoenolpyruvate, 0.2 mM nictodinamide adenine dinucleotide (NADH), 50 ug/ml pyruvate kinase, 50 ug/ml lactate dehydrogenase, and Mg-ATP at various concentrations; pH 8.0) and the rate of ATP hydrolysis at 25°C was monitored continuously by following the rate of NADH oxidation indicated by the decrease in absorbance at 340 nm. Oligomycin sensitivity, which is directly related to enzyme stability, was monitored by incubating the enzyme with oligomycin for 10 min prior to the assay, with a final concentration of 5 μg/mL.

2.2.4 Cryo-EM specimen preparation and image acquisition

For stability, the enzyme was incubated with 50-fold and 20-fold molar excesses of ADP and sodium azide, respectively, on ice prior to grid preparation. Specimen grids were prepared with a Vitrobot (FEI Company, Eindhoven, Netherlands). Four microlitres of purified proteins was applied to a home-made holey carbon grid at 100% relative humidity, after blotting of excess solution (8s blot time), the grid was plunge-frozen in a liquid ethane bath. Image acquisition was preformed with a FEI Tecnai F20 electron microscope equipped with a field emission gun and operating at 200 kV, 50 kx magnification, and with defoci between 4 and 6 μm. Films were digitized with an Intergraph Photoscan densitometer (Intergraph, Huntsville, AL) using a 7-μm step size. Pixels were then averaged 4 x 4 to give an effective pixel size of 5.6 Å. For hand determination, alignment parameter optimization, and map validation, a pair of micrographs was obtained from the same area of a specimen grid with the microscope goniometer set to +15° for the first micrograph and -15° for the second (Rosenthal and Henderson, 2003) (see Appendix 1).
2.2.5 Map construction and refinement

Defocus values for the micrographs were determined automatically with the program CTFFIND3 (Mindell and Grigorieff, 2003). A total of 6904 particle images were selected interactively with Ximdisp (Crowther et al., 1996) from 103 micrographs. Alignment of particle images and multivariate data analysis were carried out with SPIDER (Frank et al., 1996). Determination of the Euler angles for selected class averages was performed with ROTAN (Baker and Rubinstein, 2008) and an initial model was calculated with FREALIGN (Grigorieff, 2007). This method is suitable when the particle images predominately adopt side views on the EM grid (see Appendix 2).

The initial map was refined in three stages. First, the 6904 particles were classified to generate 125 class averages. These class averages were then aligned to the initial map with FREALIGN and used to construct an improved map. Next, the 6904 particle images were subjected to 25 rounds of refinement using a modified version of FREALIGN that performed exhaustive projection matching in the angular range $\psi \in \{0, 360°\}$, $\theta \in \{60, 120°\}$, and $\phi \in \{0, 360°\}$ with a linear cross-correlation function. The angular sampling of the three Euler angles was gradually reduced from 15° to 5° and the search was carried out in the resolution range 300 to 40 Å. In the final stage of map refinement, FREALIGN v7 was used for 12 rounds of refinement, optimizing the choice of alignment parameters with the 50 tilt pairs of particle images and the free-hand test (Rosenthal and Henderson, 2003) whenever resolution between rounds ceased to improve. For construction of the final map, a threshold (THRESH = 53.0) was applied to exclude the 464 particles with the lowest cross-correlation values to the reference.

2.2.6 Rigid-body fitting of atomic models and calculation of difference maps

Rigid-body fitting of the atomic model of the yeast $F_1$-c$_{10}$ subcomplex (Stock et al., 1999) into the cryo-EM map was carried out using colores within the Situs package (Chacon and Wriggers, 2002). For the peripheral stalk subcomplex, automated fitting and manual fitting gave similar results, but manual fitting while simultaneously following biochemical constraints, such as keeping the hydrophilic region of the subunit b out of the membrane region, gave rise to the
most probable docking of the peripheral stalk subcomplex. Fitting of the NMR model of the N-terminal domain of bovine OSCP (Carbajo et al., 2007) into the map and optimization of the fitting of the peripheral stalk were performed manually within the UCSF Chimera package from the Resource for Biocomputing, Visualization, and Informatics at the University of California, San Francisco (supported by NIH P41 RR-01081) (Goddard et al., 2007). To calculate a difference map, the bovine and yeast maps were first aligned manually and then the alignment was refined with USCF Chimera. The alignment was based on the positions of the central and peripheral stalks in order to avoid bias due to differences in the shape of the bovine and yeast F$_1$ and F$_o$ regions. Each map was scaled to a corresponding level, a threshold applied at the same value, and filtered to 24 Å. The yeast map was then subtracted from the bovine map and the resulting difference map filtered to 24 Å. Cryo-EM maps and atomic models were visualized and images were rendered with UCSF Chimera. Cross sections of the map and movies were prepared with ImageJ (Abramoff et al., 2004).

2.3 Results

2.3.1 Isolation and characterization of ATP synthase from *S. cerevisiae*

ATP synthase with hexahistidine tags at the N termini of the β-subunits was isolated from the yeast strain USY006. The resulting preparation of the enzyme appeared monomeric by negative-stain electron microscopy (Fig. 2.1A). The subunit composition of the complex was determined by mass spectrometry, comparison to purified F$_1$-ATPase (Fig. 2.2A), and comparison to preparations of enzyme from yeast strains with modified ATP synthase subunits (Rubinstein and Walker, 2002; Rubinstein et al., 2005; Bueler and Rubinstein, 2008). Subunits α, β, γ, δ, ε, a, b, c, d, f, and h were clearly identified (Fig. 2.2A). No evidence was found for the presence of subunits e, g, or k in the preparation despite use of a gel system optimized for their identification (Fig. 2.2B) (Arnold et al., 1998). The absence of subunits e, g, and k is consistent with these subunits being required for dimerization and selectively removed by many detergents, including dodecyl maltoside and Brij-35 in which we carried out our purification (Arnold et al., 1998). Thin-layer chromatography (results not shown) did not detect the presence of any
mitochondrial inner membrane lipids in the preparation, suggesting complete delipidation of the complex during purification.

The ATPase activity of the complex was determined to be $2.7 \mu$mol min$^{-1}$ mg$^{-1}$ by \textit{in vitro} assay, which is comparable to previous measurements for the purified yeast enzyme (Arselin et al, 1996). The coupling of $F_1$ and $F_0$ activities in this preparation was analysed by measuring the sensitivity of the ATPase activity to oligomycin, a specific inhibitor of proton translocation through $F_0$. The preparation in Brij-35 demonstrated 65% sensitivity to oligomycin, while enzyme prepared in DDM did not show oligomycin sensitivity. The sensitivity in Brij-35 is the same as for enzyme isolated in Triton X-100 after addition of phosphatidylcholine (Arselin et al, 1996).

\textbf{A}

\textbf{B}

\textbf{Fig. 2.1.} EM of the yeast ATP synthase. (A) Negatively stained EM. Particles in stain appear intact and monodispersed. (B) Cryo-EM. An image of yeast ATP synthase rapidly frozen on grids coated with a perforated carbon film. Particles tended to present side views with both the $F_1$ and the $F_0$ regions of the complex visible simultaneously. The scale bar represents 500 Å.
Fig. 2.2. SDS-PAGE of purified ATP synthase. Lanes containing molecular weight standards are labeled “MW”. Subunits were identified by their molecular weight, detection of tryptic fragments by mass spectrometry, comparison to yeast F$_1$-ATPase, and comparison to ATP synthase with engineered subunit fusions. (A) A Coomassie-stained 12 % total acrylamide (2.6 % cross-linker) tris-glycine SDS-PAGE gel of yeast ATP synthase and F$_1$-ATPase. (B) SDS-PAGE of yeast ATP synthase with a Coomassie-stained 16 % total acrylamide (3% cross-linker) tricine SDS-PAGE with 6 M urea. This gel system was found to be effective for identifying the smaller subunits of ATP synthase. The apparent high molecular weight contaminating bands visible predominantly in this gel system were identified as gel artefacts caused by subunits a and c. Tryptic fragments from these bands identified by LC-MS/MS were derived predominantly from the nearby alpha and beta subunits. However, MALDI-MS was able to identify the N-terminal SPLDQFEIR peptide from subunit a. Bands in these positions are known to arise from homo and hetero-oligomers of the hydrophobic subunits c and a, which are difficult to identify by mass spectrometry (Dr. John Walker, MRC, Mitochondrial Biology Unit, personal communication).

2.3.2 3D structure of the yeast ATP synthase

To avoid artefacts derived from images of protein in stain, purified ATP synthase was plunge-frozen on grids coated with a perforated carbon film and imaged by cryo-EM (Fig. 2.1B).
EM grids coated with perforated carbon film were used because bovine ATP synthase on continuous carbon film was previously shown to present a limited set of side views insufficient for 3D model building (Rubinstein et al., 2003) As observed previously with the bovine enzyme, most particles presented side views, probably due to the limited thickness of the ice layer in combination with detergent monolayers at the air–water interfaces (Rubinstein et al., 2003). A small fraction of particles appeared to be top views of ATP synthase or disrupted F₁ or Fₒ particles. Because top views could not be reliably distinguished from disrupted complexes, these particles were not included in the image analysis.

**Fig. 2.3.** 2D image processing. (A) Some individual particle images. (B) Class averages from 6904 individual particle images. Reliable side views of the complex were selected from class averages and are shown in parts i to vi. In all of these views, the F₁ and Fₒ regions of the complex could be clearly resolved, as could the central stalk of the complex. In some of the views, the peripheral stalk of the complex could also be seen. The scale bar represents 100 Å.

A total of 6904 particle images were selected for alignment and classification by multivariate data analysis. From 20 class averages, six reliable side views (Fig. 2.3B), each believed to be related to the others by a single rotation about the long axis of the complex, were identified using criteria specified earlier (Rubinstein et al., 2003). It is apparent without further
processing that the peripheral domain of the yeast $F_o$ observed in these class averages is significantly smaller than the peripheral domain of the bovine $F_o$.

Attempts to build an initial 3D map of this enzyme using a common lines strategy (where every side view shares the same common line) or using a random starting map have led to the construction of maps with no peripheral stalk (Chen et al., 2004; Mellwig and Bottcher, 2001), two peripheral stalks (Bottcher et al., 2000; Bottcher and Graber, 2000), or a very thin peripheral stalk that could not accommodate the now known crystal structures (Mellwig and Bottcher, 2003). These difficulties prompted our group to develop the rotational analysis method (Rubinstein et al., 2003; Baker and Rubinstein, 2008) (Appendix 2). The method allows calculation of the single angle describing the orientation of a side view about the long axis of the complex by measuring the displacement of asymmetric features from the central line of each class average. The relative orientations of the six class averages were determined by rotational analysis (Baker and Rubinstein, 2008), and an initial 3D map of the yeast ATP synthase was constructed and refined (Fig. 2.4A). The absolute hand of the map as shown was determined to be correct by the free hand test (Rosenthal and Henderson, 2003) (Appendix 1). The presence of a 6.4° phase residual difference between the correct and incorrect hand in the hand-determination test also validates the overall accuracy of the 3-D map (Rubinstein et al., 2003; Rosenthal and Henderson, 2003) (Appendix 1). The resolution of the map was determined to be 24 Å, as assessed by Fourier shell correlation (FSC) with the 0.143 criterion (Rosenthal et al., 2003) (Fig. 2.5). With the 0.5 criterion (Bottcher et al., 1997) for the FSC the resolution was measured at 34 Å. All of the features discussed below were visible in maps built to either resolution, although more distinctly in the higher-resolution map and, consequently, the figures presented here show the 24 Å resolution map. Although this map appears significantly different from earlier maps of the chloroplast (Mellwig and Bottcher, 2003), rat liver (Chen et al., 2004), and E. coli (Bottcher et al., 2000) enzymes, it shares many similarities with the bovine enzyme map (Rubinstein et al., 2003), suggesting a common architecture for mitochondrial ATP synthases.
**Fig. 2.4.** Three-dimensional map of the yeast ATP synthase and docking of atomic models.  
(A) A surface rendered view of the yeast ATP synthase map clearly shows the F₁, F₀, central stalk, and peripheral stalk regions of the complex. The peripheral stalk has a left-handed curvature with a kink near the bottom of F₁ as it bridges the gap between F₁ and F₀.  
(B) Atomic models of yeast F₁-c₁₀ subcomplex, a fragment of the bovine peripheral stalk, and the N-terminal domain of bovine OSCP were docked into the EM map. The OSCP N-terminal domain (PDB ID 2BO5) is blue and the components of the bovine peripheral stalk model (PDB ID 2CLY) are F₆ in green, b in magenta, and d in orange. The F₁-c₁₀ complex is coloured in grey. The F₁-c₁₀ atomic model was constructed by combining the yeast F₁-ATPase model (PDB ID 2HLD) with E.coli c-subunits (PDB ID 1A91) based on the yeast F₁-c₁₀ crystal structure (PDB ID 1Q01). The scale bar represents 50 Å.  
(C) Transverse sections of F₀ are shown both as contour plots and as colour gradient images. In the contour plots, the lowest density contour is drawn in green while the highest density contour is drawn in red. In part i, a ring-like density can be seen with a nearby density derived from the peripheral stalk just below the membrane surface. In part ii, nearer to the centre of the membrane region, an additional density contacts both the peripheral stalk density and the ring. The scale bar represents 50 Å.
Using rigid-body fitting, we docked the atomic model of the yeast $F_1$–c$_{10}$ subcomplex (Stock et al., 1999), bovine $b_{79-183}d_3-123F_{6(3-70)}$ subcomplex (Dickson et al., 2006), and the N-terminal domain of the bovine OSCP (Carbajo et al., 2005) into the yeast map (Fig. 2.4B). The orientation of the peripheral stalk subcomplex was chosen to be consistent with the density and also maintain the hydrophilic residues of the b-subunit above the membrane region and accommodate the known interaction of the N-terminal domain of OSCP with the N termini of the $\alpha$-subunits (Carbajo et al., 2007). With the exception of a few helices from the $\alpha$- and $\beta$-subunits that protruded from the density, the atomic models fit well into the EM map. Docking of the atomic models gave insight into subunit interactions within the complex. From the map it is possible to distinguish the catalytic $\alpha/\beta$ interfaces, which are short, flattened surfaces, from the noncatalytic interfaces, which are longer, flattened surfaces (Abrahams et al., 1994). Consistent
with the bovine ATP synthase map, the peripheral stalk binds to the surface of a noncatalytic α/β interface (Rubinstein et al., 2003). The peripheral stalk begins at the apex of F₁ and has a left-handed curvature where it spans the gap between F₁ and the membrane. This left-handed curvature matches the structure of the peripheral stalk subcomplex in 3-D crystals, but is inconsistent with the right-handed curvature that would be expected from a flexible peripheral stalk under strain during either ATP synthesis or ATP hydrolysis. The atomic model of the peripheral stalk fits into the EM map closer to the membrane surface than in a previous docking into the map of the bovine enzyme (Dickson et al., 2006). The docking presented here is also consistent with a later published crystal structure of the bovine F₁-ATPase in complex with the peripheral stalk subcomplex (Rees et al., 2009).

2.3.3 Location of subunits a, b, and c in the Fo region

Inspection of cross sections through the cryo-EM density map gave insight into the location and arrangement of subunits. Confidence in this approach was established by comparing transverse sections through the F₁ region (i.e., sections at right angles with the long axis of ATP synthase) to the known crystal structure of F₁-ATPase (Kabaleeswaran et al., 2006)(Fig. 2.6). Transverse sections through the density of the Fo region of the complex allowed the identification of the two separate domains in the membrane region separated by a region of low density (Fig. 2.4C). The c₁₀-ring (78 kDa), including side-chain density, occupies almost all the central membrane domain. Therefore, the peripheral membrane domain is likely to contain the hydrophobic subunits a, f, i, and 8 as well as the two transmembrane helices of subunit b (total mass 65 kDa). From a cross section just below the membrane surface of F₀, a ring-like density can be clearly observed at a position below the central stalk (Fig. 2.4C, part i). The ring has a diameter of 50 Å and corresponds to the expected location and dimensions of a ring of 10 c-subunits (Stock et al., 1999). In the same cross section, a density corresponding to the peripheral stalk can be observed adjacent to the c-ring where the peripheral stalk enters the membrane. The only peripheral stalk subunit that enters the membrane is subunit b, and, consequently, this density can be assigned to the b-subunit. Slightly deeper into the membrane, density corresponding to a third feature appears in contact with both the c-ring and the peripheral subunit
b density. This feature appears clockwise of subunit b (when viewed from $F_1$ towards the membrane) and can probably be assigned to the a-subunit, which is the largest membrane-bound subunit and is known to interact strongly with the c-subunit ring. These features give the overall arrangement of the most important subunits in the $F_o$ motor of ATP synthase.

**Fig. 2.6.** Cross-sections through the $F_1$ region of the cryo-EM map are consistent with the known crystal structures and dynamics of $F_1$. (A) An area of low density is found in the $F_1$ region just below the OSCP. There are no $\gamma$-subunit atoms in this region of the crystal structure. (B) $F_1$ has a uniform density approximately 50 Å below its apex. The crystal structure shows that $\gamma$ is in close contact with all of the $\alpha$- and $\beta$-subunits in this cross-section, explaining the inability to resolve the proteins. (C) Closer to the middle of $F_1$ diffuse low density is again visible. The disappearance of the $\gamma$-subunit density in this cross-section is likely the result of incoherently averaging of the different rotational positions of the curved protein in this region of the map where it is expected to make large movements during rotation. (D) Near the bottom of $F_1$, the $\gamma$-subunit of the central stalk can be seen penetrating into the $\alpha_3\beta_3$ hexamer. The $\gamma$-subunit and pairs of $\alpha$- and $\beta$-subunits are well separated in this cross-section of the crystal structure. The scale bar represents 50 Å
2.3.4 Comparison to the bovine ATP synthase structure: locations of subunits e and g

The bovine ATP synthase structure determined previously included subunits e and g (Rubinstein et al., 2003). The F_o membrane region of the yeast map resembled the bovine F_o region except with a smaller envelope for the density in the peripheral membrane domain (Fig. 2.7, A and B). The yeast map and a bovine map were scaled and aligned, and a difference map was calculated (Fig. 2.7C). When the F_o regions of the maps are aligned, the F_1 regions do not align well and, consequently, this portion of the difference map is not shown. A substantial density, located in the larger peripheral domain of the bovine F_o region, was present in the bovine map but not in the yeast map. Only a small amount of density at the edge of F_o was present in the yeast map but not in the bovine map. These differences cannot be explained by differences in bound detergent because both specimens were solubilized with DDM and imaged in Brij-35. Furthermore, both the yeast and the bovine preparations were entirely delipidated (Buchanan and Walker, 1996), so the difference in size of these domains must be attributed to the known difference in subunit composition of the two preparations of enzyme. The bovine F_o had an additional volume of ~15 % in the peripheral domain, which agrees well with the difference expected due to the presence of single copies of subunits e and g, which together represent ~14 % of the total mass of intact F_o in yeast. Further confirmation of the difference in the size of the peripheral domains of the bovine and yeast preparations has been obtained by constructing maps of affinity-purified bovine ATP synthase (Baker and Rubinstein, 2008) and a map of the yeast enzyme in the presence of ATP (Baker, Bueler, and Rubinstein, unpublished results). Cross-linking studies suggest that subunits e and g are in close association with each other (Belogrudov et al., 1996; Saddar and Stuart, 2005) and that subunit g interacts with subunit b within the complex (Soubannier et al., 1999). Our comparison of the bovine and yeast F_o structures and subsequent deduction of the location of subunits e and g places the two
Fig. 2.7. Comparison and difference maps of the yeast and bovine F<sub>o</sub> regions. Comparison of a bovine map and the yeast ATP synthase map revealed a significantly larger F<sub>o</sub> region in the bovine enzyme. (A) A view of the yeast map. (B) An equivalent view of the bovine map. (C) An overlay of the F<sub>o</sub> region from both maps and a difference map. The semitransparent grey surface represents the yeast map and the mesh represents the bovine map. Density present in the yeast enzyme but not the bovine enzyme is shown in red and density present in the bovine enzyme but not the yeast enzyme is shown in green. The scale bars represent 50 Å proteins distal from the c-ring and in close proximity to subunit b. A model of subunit arrangement in the F<sub>o</sub> region of the complex is given in Figure 2.8.

2.4 Discussion

Our observed location for subunit b at the periphery of the c-ring suggests that both subunits a and b interact closely with each other and together they interact with the ring of c-subunits. The interaction of the a-subunit with the c-ring is thought to form the proton-conducting passage through F<sub>o</sub> (Fillingame, 1997). The interaction of subunit b with the c-ring has been proposed following the characterization of an ATP synthase complex from Bacillus strain PS3 lacking subunit a that was able to assemble but which did not exhibit ATP hydrolysis
activity (Ono et al., 2004). The proximity of subunit b to the c-ring in our map is compatible with its forming a structure that guides c-ring rotation. However, in the model, subunit a is notably closer to the c-ring than subunit b, consistent with

![Diagram of the yeast F_o region](image)

**Fig. 2.8.** A model of the yeast F_o region. The F_o region is shown as a slice through the map of the complex. The c-subunits, which form a ring of helical hairpins, are shown as space-filling structures (PDB ID 1A91) within the corresponding ring of density. Density abutting the c-ring probably corresponds to the a-subunit and in close proximity, counterclockwise (viewed from F_1 towards F_o) is the b-subunit (both circled with a broken yellow line). The difference in F_o structure between the yeast and bovine maps, which differ in subunit composition, gives the location of the e and g subunits (depicted by the green mesh). The scale bar represents 50 Å

subunits a and c having more extensive interactions than subunits b and c. This finding explains the observation that a complex of subunits a and b together could be more readily isolated from bacteria than a complex of subunits b and c (Stalz et al., 2003)
Dimerization of ATP synthase is thought to have an important role in efficient energy transduction. The positioning of subunits e and g in the model suggests that dimers of the ATP synthase mediated by these subunits must be arranged with the peripheral stalks towards the centre of the dimer and the c-rings towards the outside. Although subunits e and g were the first identified as responsible for governing dimerization of ATP synthase, further oligomerization of the complex is necessary to produce the rows of dimers seen in mitochondria from paramecium (Allen et al., 1989), yeast (Buzhynskyy et al., 2007) and bovine heart (Strauss et al., 2008). This oligomerization would require an additional dimerization interface in the complex, as a single interface would form a self-limiting oligomer. In addition to subunit i, the location of which has not been defined by our model, subunits b and h of the peripheral stalk have been proposed to fill the role of the second dimer interface in ATP synthase (Paumard et al., 2002; Fronzes et al., 2006). In the model presented here, the position of the e- and g-subunits would enable b–b or h–h interaction after dimerization via subunits e and g (Fig. 2.9).

The direction of curvature of the peripheral stalk structure in this map of the ATP synthase is remarkable and suggests that the peripheral stalk may be a mobile structure. Rotation of the central rotor (viewed from F₁ towards F₀), either counterclockwise driven by F₀ during ATP synthesis or clockwise driven by F₁ during ATP hydrolysis, would apply the same strain to the peripheral stalk (Del Rizzo et al., 2006). A flexible peripheral stalk would be expected to adopt a right-handed curvature under the strain. However, in the map presented here of the inactive complex, the observed curvature was left-handed, as it is in the earlier bovine map. This curvature could imply that the peripheral stalk is a rigid structure that can resist the strain applied by rotation of the rotor. Alternatively, the peripheral stalk may deform under strain in order to store elastic energy (Cherepanov et al., 1999). This movement would have significant implications for a dimerization interface mediated by peripheral stalk subunits. This map of yeast ATP synthase at 24 Å resolution now provides a structural framework for investigating dimerization, peripheral stalk elasticity, and the architecture of the F₀ motor.
2.5 Conclusions

The cryo-EM structure of the ATP synthase from *S. cerevisiae* shows similar features to an earlier cryo-EM structure of the bovine enzyme but with important differences. It resolves the internal structure of the membrane region of the complex, especially the membrane-embedded subunits b, c, and a. Comparison of the yeast ATP synthase map, which lacks density from the dimer-specific subunits e and g, with a map of the bovine enzyme that included subunits e and g indicates where these subunits are located in the intact complex. The resulting map has allowed for construction of a model of subunit arrangement in the F$_o$ motor of ATP synthase that dictates how dimerization of the complex via subunits e and g might occur.

![Fig. 2.9. A model of ATP synthase oligomerization. (A) The dimerization interface mediated by subunits e and g is indicated with a green arrow. The dimerization interface mediated possibly by subunits b is indicated with a blue arrow. (B) A different view of (A).](image-url)
3 CHAPTER 3: STRUCTURE OF INTACT THERMUS THERMOPHILUS V-ATPASE BY CRYO-EM REVEALS ORGANIZATION OF THE MEMBRANE-BOUND $V_0$ MOTOR

The material presented in this chapter forms part of the following publication:


Contributions:

J.L.R. designed and supervised the research. W.C.Y. purified the protein, performed the activity assay and microscopy, as well as carried out the image analysis. W.C.Y. and J.L.R. interpreted the data and wrote the manuscript.

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3.1 Overview

The F-type and the V-type ATPases/synthases are evolutionarily related but differ in the details of subunit composition and arrangement. Similar to the F-type enzyme, V-ATPase uses a rotary catalytic mechanism where proton translocation through the membrane-bound $V_o$ region generates a torque on a rotor subcomplex that drives ATP synthesis in the $V_1$ region.

V-ATPases found in archaea and eubacteria have a simpler subunit composition and architecture than the eukaryotic V-ATPases. The V-ATPase from the eubacterium *Thermus thermophilus* is composed of nine different subunits with a stoichiometry of $A_3B_3CDE_{2}FG_{2}IL_{12}$. There are significant differences in the subunit composition of the V-ATPase and the F-type ATP synthase. For example, where the eukaryotic $F_1$ catalytic region consists of $\alpha_3\beta_3\gamma\delta\epsilon$, the $V_1$ catalytic region of either eukaryotic or prokaryotic V-ATPases consists of $B_3A_3DF$ with no equivalent of $\delta$ or $\epsilon$. In fact, the long coiled-coil helices and the $\alpha/\beta$ domain of $D$- and the $F$-subunits, respectively, are found together in the single subunit $\gamma$ of $F_1$ (Dr. Ken Yokoyama, Waseda University, Department of Physics, personal communication). Comparison of the central stalks of the F- and V-type enzymes shows that the central stalk from $V_1$ has a smaller foot (Numoto et al., 2009) than $F_1$ (Gibbons et al., 2000) and this may be required for interacting with subunit C, an adaptor protein that has no counterpart in the ATP synthase (Iwata et al., 2004). Instead of a single peripheral stalk, as found in ATP synthase, V-ATPases contain more than one peripheral stalk. The catalytic $A$-subunit of V-ATPase is homologous to the ATP synthase $\beta$-subunit; however, it also contains an additional nonconserved region that may be involved in the dissociation mechanism that regulates the proton pumping activity of V-ATPases in eukaryotes (Shao and Forgac, 2004). The transmembrane region of V-ATPase is similar to prokaryotic ATP synthase in that it is made up of a single, hydrophobic stator subunit in addition to the proteolipid ring. This hydrophobic stator subunit, called subunit I in the *T. thermophilus* V-ATPase, shares no structural similarity to the corresponding hydrophobic stator subunit $a$ of $F_o$ and also has a soluble N-terminal domain that interacts with the peripheral stalks (Kawasaki-Nishi et al., 2001a).

Previously determined low-resolution maps of V-ATPase from negatively stained specimen generally share a similar overall architecture (Bernal and Stock, 2004; Diepholz et al., 2008; Vonck et al., 2009; Wilkens et al., 2004; Zhang et al., 2008). Most eukaryotic enzyme
structures show three peripheral stalks with the archaeal and eubacterial structures more clearly showing two peripheral stalks. Additional support for this difference in the number of peripheral stalks comes from mass spectrometry and antibody labelling experiments (Esteban et al., 2008). Because these earlier models were derived from negative stain data, they do not give reliable information about the internal structures of the complexes. A cryo-EM map of the V-ATPase from Manduca sexta also clearly showed three peripheral stalks for the eukaryotic enzyme. The model, although obtained from unstained protein in ice, did not show the central rotor penetrating into the A$_3$B$_3$ hexamer and did not resolve significant internal features in the membrane region of the complex (Muench et al., 2009).

Crystal structures have been determined for a number of isolated subunits and subcomplexes from bacterial and archaeal V-ATPases. A high-resolution crystal structure of the A$_3$B$_3$ complex (Maher et al., 2009) and a low-resolution crystal structure of the A$_3$B$_3$DF complex (Numoto et al., 2009), both from T. thermophilus, revealed a structure similar to the F$_1$ region of F-ATPase. A crystal structure of the central stalk subunit C was also determined from the same organism (Iwata et al., 2004). For the peripheral stalk, structures of the globular C-terminal domain of subunit E from Pyrococcus horikoshii OT3 (Lokanath et al., 2007) as well as the almost complete EG subcomplex from T. thermophilus (Lee et al., 2010) V-ATPase have been determined by X-ray crystallography. The only membrane-embedded component of the V$_o$ region for which the atomic model is available is the proteolipid K-ring of the V-ATPase from E. hirae (Murata et al., 2005a).

In this chapter we present a 16 Å resolution 3D map of the intact T. thermophilus V-ATPase from cryo-EM of single particles. The quality of the map allows the various subunits and subcomplexes to be resolved. Asymmetric features in the map suggest that the enzyme relaxes to a single overall conformation when not performing ATP synthesis or hydrolysis. Most significantly, the map also reveals the arrangement of subunits in the machinery that couples proton translocation across the membrane to rotation of the rotor subcomplex.
3.2 Materials and Methods

3.2.1 *T. thermophilus* strain

The HB8 strain was purchased from ATCC (No. 27634) and was used without modification.

3.2.2 Purification of V-ATPase from *T. thermophilus*

Ten litres of medium containing 0.8 % (w/v) polypeptone, 0.4 % (w/v) yeast extract and 0.2 % (w/v) sodium chloride were inoculated with a 50 mL overnight culture and the bacteria were grown at 70 °C in a fermentor for 12-16 hours (end of log phase). Cells were harvested by centrifugation at 5000 g for 30 mins. Harvested cells were lysed in lysis buffer (50 mM Tris-Cl, 5 mM magnesium chloride, 0.001 % [w/v] PMSF; pH 8.0) using a Continuous Flow Disruptor (Avestin, Inc.), and membranes were collected by ultracentrifugation at 100000 g for 30 min. Membranes were washed in lysis buffer with 3-5 cycles of resuspension-centrifugation, followed by detergent solubilization at room temperature in Buffer A (50 mM Tris-HCl, 5 mM magnesium chloride, 150 mM sodium chloride; pH 8.0) containing 1 % (w/v) DDM for 30-min. After removing insoluble material by ultracentrifugation, the fraction containing solubilized membrane proteins was loaded onto a HiPrep Q-Sepharose column (GE Healthcare) previously equilibrated with Buffer A containing 0.02 % (w/v) DDM, and V-ATPase was eluted with a linear sodium chloride gradient (180-280 mM). Individual fractions containing V-ATPase identified by SDS-PAGE were pooled, concentrated, and purified further by size exclusion chromatography with a Superdex 200 column (GE Healthcare) in Buffer A containing 150 mM sodium chloride. Aliquots of purified protein were concentrated to 2.5 mg/ml and frozen in liquid nitrogen for storage at -80 °C.

3.2.3 ATPase assay

The activity of purified V-ATPase was determined using an ATP-regenerating system, as described in Section 2.2.3. To determine the N,N'-dicyclohexylcarbodiimide (DCCD) sensitivity of ATP hydrolysis activity, the enzyme (1 µM) was preincubated with 100 µM DCCD in 20 mM
Tris-Cl (pH 8.0), 1 mM MgCl₂, 0.1 M NaCl, and 0.02% (w/v) DDM for 1 hour at 25 degrees C prior to the assay. Purified V-ATPase showed > 95% DCCD sensitivity in ATPase assays.

3.2.4 Cryo-EM specimen preparation, image acquisition, map construction and refinement

Specimen preparation and image acquisition were performed as described in section 2.2.4, with minor modifications. Non-inhibited enzyme was used for grid preparation. Specimen was prepared on Quantifoil perforated specimen support grids (Quantifoil Micro Tools GmbH) with long blot time (15-20 s). Specimens were images using an exposure of ~25 electrons/Å² with defoci between 3 and 5 µm. Initial model was generated using the ROTAN procedure (Baker and Rubinstein, 2008) (see Appendix 2) and refinement was performed directly using raw particle images with FREALIGN (Grigorieff, 2007), omitting the class average step. The resolution range used in the refinement was 150-20Å. Out of the ~20000 particle images collected, ~16000 particle images with highest cross-correlation values were included in the final reconstruction.

3.2.5 Volume segmentation, modeling, atomic model fitting and map visualization

Segmentation was performed manually with qsegment in EMAN (Ludtke et al., 1999). A comparative model of the L₁₂-ring was generated with MODELLER (Sali and Brundell, 1993). Automated and semiautomated rigid-body fitting was carried out with Situs colores (Chacon and Wriggers, 2002) and with UCSF Chimera (Goddard et al., 2007), respectively. Map visualization and image rendering were performed with UCSF Chimera (Goddard et al., 2007).
3.3 Results

3.3.1 Protein purification, imaging, and model construction

V-ATPase was purified from wild-type *T. thermophilus* HB8 by conventional chromatographic techniques (Bernal and Stock, 2004). SDS–PAGE and negative stain EM were used to confirm that the enzyme preparation was consistent with previously characterized preparations (Bernal and Stock, 2004). Protein was frozen on perforated carbon film-coated EM grids and imaged by cryo-EM (Fig. 3.1A). A dataset of 19,825 particle images was collected and subjected to multivariate data analysis to generate high signal-to-noise ratio class average images (Fig. 3.1B). Class averages that represented *bona fide* views of the complex were selected using the mirror-pair logic described earlier (Rubinstein et al., 2003). From these class averages, it is apparent without further analysis that in the *T. thermophilus* enzyme the V\(_1\) and V\(_o\) regions are connected by a central stalk and two peripheral stalks. The relative orientation of the views shown in the class averages was determined by rotational analysis (Baker and Rubinstein, 2008) (see Appendix 2), and an initial 3D map was calculated from these views and refined by projection matching in Fourier space (Grigorieff, 2007) (Fig. 3.2A). The final EM map had a resolution of 21 and 17 Å as determined by Fourier shell correlation with the 0.5 and 0.143 criteria (Rosenthal et al., 2003), respectively, and 18 and 16 Å as determined by Fourier neighbor correlation with Rmeasure (Sousa and Grigorieff, 2007), using the 0.5 and 0.143 criteria, respectively (Fig. 3.3). All of the features discussed below were apparent at any of these resolutions but most distinctly so at 16 Å, and consequently the figures rendered here are for a map built to 16 Å. The map has a clear handedness and the absolute hand of the map, which was inconsistent between previous published models (Bernal and Stock, 2004; Diepholz et al., 2008; Vonck et al., 2009; Wilkens et al., 2004; Zhang et al., 2008; Muench et al., 2009), was found to be correct as represented here by using the free hand test (Rosenthal and Henderson, 2003) (see Appendix 1).

3.3.2 Overall 3D structure

The 3D map has several features in common with previously determined maps of bacterial and archaeal V-ATPases in negative stain (Bernal and Stock, 2004; Vonck et al., 2009),
and some features in common with maps of the eukaryotic enzyme. Surface renderings of the map clearly show the $V_1$ and $V_o$ regions, the peripheral stalks, and the central stalk (Fig. 3.2A). Transverse sections through the map reveal

**Fig. 3.1.** Electron cryomicroscopy of *T. thermophilus* V-ATPase. (A) Samples of purified V-ATPase complex were rapidly frozen on a perforated carbon film coated grid and imaged at liquid nitrogen temperature. Side views of V-ATPase complexes were readily apparent and had the dumbbell shape typical for this complex. Examples of individual particles are circled in red. (B) Class averages were generated from the dataset of particle images and averages for which there existed a mirror pair image were selected as *bona fide* views of the complex. Two peripheral stalks and a central stalk were seen in some averages. One of the peripheral stalks is indicated with a yellow cross and the other with a red cross. Some class averages show a peripheral stalk overlapping in projection with the central stalk. In two averages, the two peripheral stalks overlap in projection with each other and here the peripheral stalks are marked with an orange cross.

subunit boundaries and domain structures of individual subunits, as well as a layer of bound detergent surrounding the membrane-embedded region of the complex (Fig. 3.4). The different densities in the map were sufficiently resolved to allow segmentation of the entire complex into regions corresponding to three A-subunits, three B-subunits, a subcomplex consisting of subunits D and F, the C-subunit, the ring of L-subunits, a subcomplex consisting of subunits E, G, and I,
and the ring of density surrounding the $V_0$ region that is almost certainly derived from the micelle of DDM used to keep the complex soluble (Fig. 3.2B). The appearance of the detergent micelle is consistent with the 1.19 g/ml density of DDM (Trimmins et al., 1988), versus the 0.94 g/ml density of vitreous ice (Mishima et al., 1985) and the generally used density of ~1.36 g/ml for protein (Rubinstein, 2007). The thickness of this detergent micelle is ~25 Å, which is in good agreement with the known radius of a nonhydrated DDM micelle (Bamber et al., 2006). Detergent has been observed previously in single particle cryo-EM maps, usually as blurring at the periphery of membrane proteins (e.g., Menetret et al., 2007), but to our knowledge the map presented here shows by far the most distinct delineation observed to date between

![Fig. 3.2. Three-dimensional model of the V-ATPase. (A) A surface rendered side view of the final 3D map. (B) Individual segmented subunits or subcomplexes of the V-ATPase are shown inside the cryo-EM density map (semitransparent gray). Two of the three bulges in $V_1$ that allowed for identification of the A-subunits are indicated by arrows. The map was segmented into the A-subunits (yellow) and B-subunits (red), the C-subunit (light blue), a central rotor consisting of subunits D and F (dark blue), the L-ring (pink), the stator subcomplex consisting of subunits I, E, and G (green), and a micelle of the bound detergent (dark gray). The segmented densities are displayed at a slightly higher threshold than the map to accentuate the molecular boundaries.](image)

protein and a detergent micelle in a cryo-EM structure of a membrane protein complex. To interpret the map further, we performed automated rigid-body fitting of available crystal
structures and a comparative model of the L_{12} - ring, either directly into the whole density map or into individual segmented densities.

![Fourier shell correlation plot](image)

**Fig. 3.3.** Fourier shell correlation plot. The resolution of the map was determined by Fourier shell correlation from two half models (solid line) and by Fourier neighbor correlation with \( R_{\text{measure}} \) converted to Fourier shell correlation (broken line). The final EM map had a resolution of 21 Å and 17 Å as determined by Fourier shell correlation with the 0.5 and 0.143 criteria, respectively, and 18 Å and 16 Å as determined by Fourier neighbour correlation with \( R_{\text{measure}} \) using the 0.5 and 0.143 criteria, respectively.

### 3.3.3 The \( \text{V}_1 \) region

Subunits A and B are homologous to the \( \beta \)- and \( \alpha \)-subunits of ATP synthase, respectively, and almost certainly share the same functions. Subunit A differs from \( \beta \) due to the existence of an N-terminal nonhomologous region that appears as a large protrusion in the EM map (Fig. 3.2B). A crystal structure of the \( \text{A}_3\text{B}_3\text{DF} \) complex from *T. thermophilus* fits unambiguously into the \( \text{V}_1 \) region of the map (Fig. 3.5A). Although all three segmented A-subunits and all three segmented B-subunits can be overlaid with high fidelity when extracted from the map as monomers (Fig. 3.5C), the three different AB heterodimers have different structures within the
intact complex (Fig. 3.5B). These structural differences appear to reflect different conformational states with one AB heterodimer (labeled I) taking on a relatively ‘open’ conformation while the

**Fig. 3.4.** Cross-sections of the map clearly resolve internal features that could be used to segment the map. These cross-sections reveal: (i) The extent of penetration of the central rotor into the A$_3$B$_3$ hexamer and domains of subunits A and B; (ii) Different conformations of the different AB heterodimers; (iii) The membrane-bound L-ring, the I-subunit, and a layer of detergent; and (iv) The threefold pseudosymmetry of subunit C in the central rotor. The scale bar represents 50 Å.
Fig. 3.5. The V₁ region of the map. (A) Map segments and crystal structures are shown for subunits A (Yellow), B (Red), and D (Blue). The map segment for the peripheral stalks is shown in green. The peripheral stalks can be seen to be in contact with B-subunits. The AB heterodimers were assigned labels of I, II, and III. (B) One of the AB heterodimers appears to be in a different conformation from the others and does not overlay well. Specifically, whereas pairs II (semitransparent solid) and III (mesh) both seem to adopt similar ‘closed’ conformations (i), pair I (mesh) can be seen to adopt a more ‘open’ conformation than pair II (semitransparent solid) (ii). The scale bar represents 25 Å. (C) and (D) Despite the fact that the different AB
heterodimers appear to be in different conformations, the individual A subunits (yellow) and B subunits (red) could be overlaid with high fidelity, suggesting that the differences in the heterodimers arises due to a different relative orientation of the A and B subunits in the pair rather than errors in segmenting the individual subunits. (C) Overlay of the subunit A monomers: (i) Subunit A from pair II (semitransparent) with subunit A from pair III (mesh), (ii) Subunit A from pair II (semitransparent) with subunit A from pair I (mesh), subunit A from pair III (semitransparent) with subunit A from pair I (mesh). (D) Overlay of the subunit B monomers: (i) subunit B from pair II (semitransparent) with subunit B from pair III (mesh), (ii) Subunit B from pair II (semitransparent) with subunit B from pair I (mesh), subunit B from pair III (semitransparent) with subunit B from pair I (mesh).

other two heterodimers (labeled II and III) adopt more ‘closed’ conformations. The similarity of the individual A- and B-subunits rules out the possibility that these differences arose from segmenting errors and suggests that the majority of the conformational differences between the different AB pairs can be represented as a rigid-body motion of an A-subunit relative to a B-subunit. These differences in conformation match precisely the different conformations of AB heterodimers seen in a crystal structures of the V$_1$-ATPase (Numoto et al., 2009). That conformations of the AB heterodimers within the V$_1$ region can be observed, rather than being averaged as one would expect if the population of V-ATPase molecules had three chemically equivalent but structurally distinct positions for the rotor, suggests that all the rotor subcomplexes were in a single, lowest energy position when the specimen was frozen. Subunit D constitutes the majority of the central stalk of V-ATPase and can be seen to penetrate deeply in the A$_3$B$_3$ hexamer, unlike what was described for the V-ATPase from M. sexta (Muench et al., 2009) and consistent with the low-resolution crystal structure of the complex (Fig. 3.6A). Like the different AB heterodimers, the central rotor appears to have a single distinct conformation, allowing visualization of a structure that is consistent with the distantly related $\gamma$-subunit of bovine ATP synthase (Fig. 3.6B). The curved structure of subunit D results in it having different interactions with the three different AB heterodimers.
3.3.4 The stator subcomplex: subunits E, G and I

The two peripheral stalks of *T. thermophilus* V-ATPase consist of heterodimers of subunits E and G (Esteban *et al.*, 2008) that bind to the large cytosolic N-terminal domain of the membrane-bound subunit I. Together, these three different subunits form the stator subcomplex of the eubacterial enzyme. In the cryo-EM map presented here, the three different subunits could not be resolved from each other, and all are presented as a single map segment (Fig. 3.2B). The two peripheral stalks are each connected to a B-subunit at the top of V₁ and to a horizontal rod-like density at the end nearer the membrane (Fig. 3.2A and Fig. 3.5A). Unlike the peripheral stalk of the F-type ATP synthase, neither V-ATPase peripheral stalk runs along a catalytic/noncatalytic subunit interface (Lau *et al.*, 2008; Rubinstein *et al.*, 2003) but rather remains in contact solely with a noncatalytic B-subunit (Fig. 3.5A). Consistent with earlier
models of the eubacterial (Bernal and Stock, 2004) and archaeal (Vonck et al., 2009) V-ATPases, the peripheral stalks in our model are nearly parallel to each other and to the long-axis of the complex. This arrangement is different from the peripheral stalk of the F-type ATP synthase (Lau et al., 2008; Rubinstein et al., 2003), which wraps around the long-axis of the complex and the eukaryotic V-ATPase (Diepholz et al., 2008; Muench et al., 2009), where the peripheral stalks have a noticeable curvature. While consistently observed, the role of this bending of the peripheral stalks in some F- and V-type ATPases is not known but could relate to the storage of elastic energy in this part of the assembly.

Inconsistent with previous EM data for the eukaryotic V-ATPase, no portion of either stator contacts the C-subunit of the central rotor (Muench et al., 2009), and therefore would not impede rotation of the rotor. The bar that links the two peripheral stalks lies parallel to where the surface of the membrane would be. This density is expected to arise from the N-terminal domain of the 74 kDa subunit I. The two peripheral stalks have nearly identical structures near V₁ but clearly have different interactions with subunit I. Understanding how two identical EG subcomplexes can have different interactions with the same I-subunit will require higher-resolution information about this region of the complex. In eukaryotes, V₁ biosynthesis includes assembly of the E- and G-subunits (Forgac, 2007; Kane, 2006), offering three potential binding sites for peripheral stalks. In comparison, disruption of T. thermophilus V-ATPase leaves the peripheral stalks with V₀ (Yokoyama et al., 2000), suggesting that in this complex the two peripheral stalk binding sites in V₀ are higher affinity than the three potential binding sites in V₁. The presence of two high-affinity binding sites offers a possible explanation for how the T. thermophilus V-ATPase can assemble with just two peripheral stalks when there are three potential binding sites in V₁.

3.3.5 Subunit C of the central stalk and its interaction with the L-ring of the V₀ region

The coupling of the DF rotor of V₁ with the membrane-bound L-ring of V₀ occurs via the rotor subunit C. This C-subunit is homologous with the eukaryotic V-ATPase d-subunit, not the eukaryotic V-ATPase C-subunit. The T. thermophilus crystal structure of subunit C has threefold
pseudosymmetry and an overall funnel shape (Iwata et al., 2004). This pseudosymmetry is clearly visible in the EM map (Fig. 3.4 iv and Fig. 3.7A) with both a concave and a convex face apparent for the subunit (Fig. 3.7B). The preservation of the pseudosymmetry after averaging images could occur only if the map represents particles that have stopped in, at most, three different positions. The L-ring density and C-subunit densities are oriented such that they

**Fig. 3.7.** Subunit C and its interaction with the L-ring. (A) Shown from the V$_1$ side of the complex with the A- and B-subunits as semitransparent surfaces, the noncatalytic B-subunits (Red) appear to be in register with the domains of the C-subunit (Blue). (B) The C-subunit map segment (Semitransparent Blue), shown with the docked crystal structure, penetrates only slightly into the L-ring (Semitransparent Pink) and sits at an angle, putting the convex side of the subunit in contact with an edge of the ring. A comparative model for the L-ring, generated from the E.coli ATP synthase subunit c, was docked into the map.

...do not share the same symmetry axis. This asymmetric positioning of the C-subunit atop the L-ring (Fig. 3.7B) further supports the hypothesis that the map represents an average sampled from images of particles that have been trapped with the rotor in a single position. The L-subunit is known to form a dodecameric ring (Toei et al., 2007). Automatic docking of a comparative model of the L$_{12}$-ring positioned the ring with the connecting loops of the transmembrane helices of the L-subunits facing towards the V$_1$ region (Fig. 3.7B and Fig. 3.8D), as expected from a crystal
structure of the $F_{1\cdot c_{10}}$ complex from \textit{S. cerevisiae} ATP synthase (Stock et al., 1999). The convex face of the C-subunit can be seen to penetrate only slightly into the L-ring, with the narrowest point of subunit C contacting one edge of the L-ring (Fig. 3.7B). This positioning is somewhat different than has been modeled previously (Murata et al., 2005). With this orientation, the cytoplasmic loops of the L-subunits would interact with subunit C, thereby coupling the central stalk to the membrane-bound ring. The concave face of subunit C provides a socket to accommodate the DF subcomplex. The three domains of subunit C each align with a B-subunit of \textit{V}_1 (Fig. 3.7A) relating the orientations of subunits in \textit{V}_1 with the orientations of subunits in \textit{V}_0. The reversible dissociation of \textit{V}_1 from \textit{V}_0 that occurs in eukaryotes (Kane, 1995; Sumner et al., 1995) leaves the C-subunit homologue bound to \textit{V}_0 while the central DF rotor remains with \textit{V}_1 (Kane, 1995). The alignment of features in \textit{V}_1 and \textit{V}_0 could facilitate reassociation of the two subcomplexes by simultaneously allowing recoupling of the central rotor and the peripheral stalks.
Fig. 3.8. Subunit I and its interaction with the L-ring. (A and B) Surface rendered views of the L-ring (Pink) and I-subunit (Green) show that the two subunits interact minimally near the middle of the membrane region. The numbers ‘1’ and ‘2’ designate the ‘small’ and ‘large’ membrane-bound domains of subunit I, respectively. (C) The L-ring and I-subunit are contained within a detergent micelle (Semitransparent Dark Gray) that also includes large regions probably filled with lipids. (D) The map segment for the L-subunits is of reasonable size to accommodate the comparative model of the L-subunits is of reasonable size to accommodate the comparative model of the L_{12}-ring. The scale bar represents 25 Å and applies to all parts of the figure.
Fig. 3.9. Model for rotation generation in $V_o$. (A) During ATP synthesis, protons from the periplasmic side of the membrane are delivered to a charged Glu 63 residue by the periplasmic half-channel (domain 2), neutralizing it so that it can face the lipid bilayer. A second, cytoplasmic, half-channel counterclockwise from the periplasmic half-channel allows proton translocation into the cytoplasm and results in a negative charge on a Glu 63 residue that attracts it to the Arg 563 residue. (B) The peripheral stalks are optimally arranged to exert a force on the membrane-bound I-subunit perpendicular to a line drawn between the two stalks. This force, if applied to counter an attraction between a conserved negatively charged Glu 63 residue from a L-subunit and the positively charged Arg 563 residue of the I-subunit, would be eccentric on the rotor and would cause rotation of the L-ring. The map segment corresponding to the peripheral stalks and I-subunit is shown in green with the surface coloured blue at the contact point between subunit I and the L-ring to represent the positive charge of the conserved Arg 563 residue. The conserved Glu 63 residues are shown as space-filling representations. The Glu 63 residues that are expected to be uncharged are shown in gray whereas the Glu 63 residues that are expected to be negatively charged are shown in red. The scale bar represents 25 Å.

3.3.6 Protein interactions and arrangement in the $V_o$ region

The final segment in the membrane region of the complex corresponds to the C-terminal domain of subunit I. The map reveals that this region of subunit I has a defined domain structure in the membrane. Near the cytoplasmic side of the membrane-bound region, subunit I enters the membrane as a single density. Closer to the middle of the membrane, the subunit splits into two
domains that are separated by ~20 Å in cross-sections near the periplasmic side of \( V_o \). The smaller of these two domains appears to be an extension along the original trajectory of the peripheral stalk into the membrane whereas the second, larger, domain is found clockwise of the first domain when viewed from \( V_o \) to \( V_1 \) (Fig. 3.8A). It can also be seen from cross-sections and from the segmented density (Fig. 3.4 iii and Fig. 3.8, B and C) that there is only a small area of contact between subunit I and the ring of L-subunits. This contact occurs near the center of the membrane between the larger second domain of subunit I and the ring. The conserved Glu 63 residue of the automatically docked L-ring structure (Fig. 3.9A) is located at precisely the same position along the long-axis of the map as the contact point between subunit I and the ring. A previous ~24 Å resolution map of the yeast ATP synthase (Lau et al., 2008) suggested a small contact area between the proteolipid ring and the equivalent of the I-subunit in that enzyme. However, in the earlier map, the lower resolution and presence of several other membrane proteins in the complex prevented 3D analysis of the interaction. In the V-ATPase map, there are several gaps in the \( V_o \) region of the map that are not occupied by either subunit I or the L-ring. The L-ring itself also appears to have a central pore that goes from the cytoplasmic side to the periplasmic side of the membrane region. These gaps are almost certainly filled with lipids. X-ray crystallography of the \( E. hirae \) V-ATPase rotor (Murata et al., 2005) found that the ring contained bound lipids, whereas atomic force microscopy of 2D crystals of \( Ilyobacter tartaricus \) c-rings (Meier et al., 2001) similarly showed a plug that was probably composed of lipids (Meier et al., 2005). The hydrophobic tails of lipids have densities less than ice and would therefore appear as empty space in a cryo-EM map, consistent with what is observed here.

3.4 Discussion

The cryo-EM structure of the V-ATPase presented here reveals the most detailed model to date for any intact F-type ATP synthase or V-ATPase. At the current resolution, the two peripheral stalks have nearly identical structures and conformations. The apparent differences in conformation of the three AB heterodimers within the complex, the asymmetric positioning of the C-subunit atop the L-ring, and the curved nature of the central stalk and its asymmetric interactions with the A- and B-subunits suggest that the V-ATPase particles were imaged with the asymmetric rotor stopped in a single position. The binding change mechanisms postulates
three chemically equivalent catalytic sites that are made structurally distinct due to their different interactions with the central rotor (Boyer, 1997), and therefore that there should be three energetically equivalent positions for the rotor. This discrepancy suggests that the realization of the binding change mechanism in V-ATPase involves some degree of chemical nonequivalence to the three catalytic sites and that there exists a single lowest energy state.

The most important outstanding questions about the V-ATPases and the related F-type ATP synthase concern the mechanism of proton translocation through the membrane and the coupling of this activity to rotation of the rotor. In the T. thermophilus enzyme during ATP synthesis, proton translocation occurs from the periplasmic to the cytoplasmic side of the membrane and causes a clockwise rotation of the rotor when viewed from V₀ to V₁ (Imamura et al., 2003). Because the postulated half-channel from the periplasmic side of the membrane would provide protons to neutralize a Glu 63 residue of a L-subunit so that it can enter the lipid bilayer, one would expect the periplasmic half-channel to be clockwise of the cytoplasmic half-channel when viewed from V₀ to V₁ (Junge et al., 1997, 2005). The small area of contact between subunit I and the L-ring occurs on the larger of the two membrane-bound domains of subunit I, which is displaced clockwise from the rest of the subunit when the complex is viewed from V₀ to V₁. Therefore, it is possible that the larger membrane-bound domain of subunit I makes up the putative first half-channel of the Brownian ratchet model, and protons are supplied to the glutamate residues of the L-subunits via this domain. If the model is correct, the contact between subunits I and L, although small, would have to be sufficient to contain a terminus from both the cytoplasmic and periplasmic half-channels (Fig. 3.9A).

The asymmetry with which the membrane-bound components of the complex are arranged is also notable. The small contact area between subunit I and the L-ring suggests that the two subunits are held together by the peripheral stalks, not by an interaction between I and the L-ring. In turn, the two peripheral stalks must be held in place by their interactions with V₁. Any force attempting to displace subunit I from L would be countered by an opposing force applied by the peripheral stalks. The peripheral stalks are optimally arranged to counter forces attempting to push them away from or pull them towards V₁. Both peripheral stalks pushing away from or pulling toward V₁ would exert a force on subunit I perpendicular to a line drawn between the stalks (Fig. 3.9B). Therefore, from Figure 3.9B, it is apparent that the peripheral
stalks are optimally arranged to apply a force that is eccentric on the L-ring. It is well known that application of an eccentric force on a rotor will cause it to turn, and this principle powers all man-made motors. An attractive force between the positively charged Arg 563 residue of subunit I and a negatively charged Glu 63 residue of a L-subunit counterclockwise of the contact point (when viewed from $V_o$ to $V_1$), would cause the rotor to turn with a clockwise direction, as expected from the known clockwise rotation direction of the rotor during ATP synthesis (Imamura et al., 2003). Therefore, this eccentric force could be the basis for torque generation in $V_o$.

3.5 Conclusions

The V-ATPase from *T. thermophilus* was chosen in our study for cryo-EM structure determination due to its inherent stability. Its simpler subunit composition also facilitates the interpretation of the membrane-bound region, which is important for proton translocation. Our cryo-EM map of the V-ATPase was of sufficient quality to allow for segmentation, thereby delineating the molecular boundaries of subunits A and B, the three-fold pseudosymmetric C-subunit, the DF subcomplex of the central stalk, the L-ring, the stator subcomplex consisting of subunits I, E, and G, and a micelle of bound detergent. Furthermore, the architecture of the $V_o$ region shows a remarkably small area of contact between the I-subunit and the ring of L-subunits and is consistent with a two-channel model for proton translocation.
4 CHAPTER 4: SUBNANOMETER RESOLUTION STRUCTURE OF THE INTACT T. THERMOPHILUS H+-DRIVEN ATP SYNTHASE REVEALS THE ARRANGEMENT OF ITS TRANSMEMBRANE HELICES

The material presented in this chapter forms part of the following publication:


Contributions:

W.C.Y. and J.L.R. designed the research. J.L.R. supervised the research. W.C.Y. purified the protein and performed the microscopy. J.L.R. wrote the computer softwares. W.C.Y. and J.L.R. carried out the image analysis, interpreted the data and wrote the manuscript.

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4.1 Overview

The 16 Å low-resolution cryo-EM structure of the V-ATPase presented in chapter three unexpectedly adopted a unique conformation. Specifically, in that structure, the central rotor is arrested in a specific orientation relative to the stator. Conformational differences among the catalytic sites in the asymmetric A₃B₃ hexamer were observed, with no signs of rotational averaging or blurring of the V₁ region. On the basis of this observation, we propose that a large population of the purified complexes are structurally homogeneous so high-resolution structural analysis would be feasible.

In order to improve the resolution of a cryo-EM model, both the quality of images (SNR) and/or the accuracy of the orientation determination of the particle images can be improved. The accuracy of the orientation determination is also directly dependent on the quality of the particle images. Map degradation from misaligned images and images with poor high-resolution contrast can be represented by an overall temperature factor $B_{\text{overall}}$, which explains the loss of information at high resolution. Here, $B_{\text{overall}} = B_{\text{experimental}} + B_{\text{computational}}$. $B_{\text{experimental}}$ has already been discussed in the Introductory section 1.2.1. The number of particle images required to achieve a certain resolution is proportional to $e^{B/2d^2}$, where $d$ is the resolution (Å) and $B$ denotes the overall temperature factor $B_{\text{overall}}$ here. Therefore, our goal was to decrease the $B_{\text{overall}}$ so that a high-resolution map could be calculated with a reasonable number of particle images. Several strategies that led to resolution improvement of the map are described in this chapter.

How protons are translocated across the membrane by V-ATPase is not known. A model was proposed almost two decades ago to explain the mechanism of proton translocation and ring rotation. This model necessitates the existence of two non-collinear, aqueous half-channels formed at the rotor/stator interface. To date, no high-resolution structures of either the complete Fₒ/Vₒ region have been determined, thus structural evidence supporting the two-channel model is lacking. Our previously published 3D structure of the V-ATPase bearing an intact transmembrane domain uncovered the nature of the interaction between the rotor and the stator at low resolution. Here we report a new reconstruction of the *T. thermophilus* V-ATPase determined to subnanometer resolution.
4.2 Materials and Methods

4.2.1 Protein purification, grid preparation, and imaging

*Thermus thermophilus* HB8 was grown and the H⁺-driven ATP synthase purified by following an identical procedure to the one described in section 3.2.2. Grid preparation and imaging were performed as previously described except an electron exposure of ~18 electrons/Å² was used instead. This electron exposure optimizes the SNR at spatial frequencies relevant for refinement. Images were acquired with defocus values between 2.5 and 4.5 µm. For improved image quality, the acquired images were screened and only those that showed high contrast from thin ice layers, no noticeable drift, and oscillation of the contrast transfer function beyond 10 Å resolution were selected for further analysis.

4.2.2 3D map construction and segmenting

The previously published cryo-EM map of the *T. thermophilus* V-ATPase (Lau and Rubinstein, 2010) was filtered to 30 Å resolution and used as an initial model for refinement of the entirely new data set. The new dataset, obtained with a lower electron exposure from specimens in a thinner ice layer than used previously, showed CTF oscillations to a higher resolution than the original dataset. Initial particle orientations were determined by projection matching with Frealign (Grigorieff, 2007) using information out to 20 Å resolution. The accuracy of particle orientations were refined further with a new program (refine_fspace), ultimately using information out to 10.5 Å resolution. Refine_fspace performs projection matching in Fourier space while allowing continuous constrained optimization of the Euler angles and shifts with a simplex minimization algorithm. Prior to projection matching with a normalized correlation coefficient, image Fourier transforms were multiplied by the CTF and the map projections by the square of the CTF. To avoid influencing the measurement of resolution with noise bias, the highest spatial frequency used during refinement was kept below the resolution limit of the map.
From 46105 particle images, the top ~90% (42075) with the best cross correlation coefficients at their determined orientations were selected and the final 3D map was calculated by sinc function interpolation in Fourier space. The resolution of the final map was assessed by Fourier shell correlation (FSC) and Fourier Neighbor correlation (Sousa and Grigorieff, 2007) with the 0.143 (Rosenthal et al., 2003) and 0.5 (Bottcher et al., 1997) thresholds. Fourier components were sharpened with an inverse B-factor of 750 Å² and weighted for the signal-to-noise ratio with a filter (Rosenthal and Henderson, 2003). Segmentation was performed automatically using Segger (Pintilie et al., 2010), semi-automatically (Baker and Rubinstein, 2011) and manually using EMAN qsegment (Ludtke et al., 1999).

4.2.3 Model building and fitting

The PDB-IDs for atomic models used to interpret the 3D cryo-EM map were 3A5C [A3B3DF complex (Numoto et al., 2009)], 3K5B [EG complex (Lee et al., 2010)], 1R5Z [subunit C (Iwata et al., 2004)], and 1U7L [subunit C of the S. cerevisiae V-ATPase (Drory et al., 2004)]. A comparative model of the L-subunit in its proton-locked conformation was built with Phyre2 (Kelley and Sternberg, 2009) using the two C-terminal transmembrane helices of the NtpK subunit of the sodium-driven V-ATPase from Enterococcus hirae [PDI-ID 2BL2 (Murata et al., 2005a)] as the template. This template was identified automatically by Phyre2 (Kelley and Sternberg, 2009) with 99.9% confidence and represents 90% coverage of subunit L. An atomic model of the L₁₂-ring was constructed in Situs (Chacon and Wriggers, 2002). Rigid-body fitting of atomic models into the cryo-EM map was done with UCSF Chimera (Goddard et al., 2007) and flexible fitting of subunit C into the map was performed with IMODFIT (http://chaconlab.org/imodfit/index.html). All figures were rendered with UCSF Chimera (Goddard et al., 2007).
4.3 Results and Discussion

4.3.1 Structure of the membrane extrinsic region

In order to investigate how the structure of the *T. thermophilus* ATP synthase allows its rotary mechanism, we imaged the detergent solubilized intact enzyme by cryo-EM and calculated a 3D map to ~10 Å resolution (Fig. 4.1A and Fig. 4.3). This resolution was sufficient to observe α-helical elements in the structure. Achieving this level of resolution required optimization of the specimen preparation and imaging conditions, and development of a new map refinement algorithm. Fig. 4.1A shows a surface rendered view of the refined 3D map. The available crystal structures of individual subunits and subcomplexes of the enzyme were fitted into the map with remarkably good agreement to generate a pseudoatomic model of almost all of the extramembranous part of the enzyme (Fig. 4.1, B and C). A cross-section through the bottom of the V₁ region of the map shows that the two α-helices that make up each of the two peripheral stalks can be resolved (Fig. 4.2A, arrows), as can densities that correspond to α-helical elements in subunits of the assembly with more complex folds, such as the A-subunits (Fig. 4.2A, circled in yellow). The various subunits of the enzyme could be identified in the map, producing map segments that matched available crystal structures with high fidelity (Fig. 4.2, B-D). If the protein particles imaged in the cryo-EM experiment occupied all possible rotational states of the central rotor, then the 3D map would be expected to show the average conformation of each of the catalytic A and B subunits. However, docking the A₃B₃DF crystal structure (Numoto *et al.*, 2009) into the map revealed that the enzyme was arrested in a single rotational state (Lau and Rubinstein, 2010), explaining why we were able to extract nanometer-resolution information when averaging many different particles to calculate the map. This arrest in a single rotational state is clearly shown by fitting of the DF-rotor subcomplex into its corresponding map segment (Fig. 4.2D). Had the particles existed in multiple rotational states, then the density corresponding to the DF-subcomplex would appear as a rotational average of the structure of these subunits. The crystal structure of the A₃B₃DF subcomplex showed that two of the AB-subunit pairs took on closed or narrow conformations, designated AₐBₐ and Aₐ′Bₐ′, while the third adopted a wide open conformation, designated AₐBᵦ (Numoto *et al.*, 2009). From the position of the D- and F-subunits, it is apparent that the AₐBₐ pair, which corresponds to the αTPβTP pair in mitochondrial F₁-ATPase (Abrahams *et al.*, 1994), is positioned at the A/B
interface between the two peripheral stalks. Because we did not take any action to stop the rotor in one rotational state, the existence of this unique position suggests that in the intact complex the observed state has a lower energy than other positions of the rotor.

Fig. 4.1. 3D map and pseudoatomic model of the complete *T. thermophilus* ATP synthase. (A) A surface view of the 3D map showing the V₁ and V₀ regions. (B) and (C) The 3D cryo-EM map with fitted crystal structures and segments corresponding to individual subunits. Atomic models of the subunit A (yellow), B (red), C (cyan), D (blue), E (purple), F (orange), G (beige) were fitted into the map and subunits are labeled with their name. Surface rendered segments from the cryo-EM map are shown for subunits where atomic models are not available, such as the L₁₂-ring (magenta), subunit I (green), and residues of subunit D missing from its crystal structure (blue). The outer surface of the overall map is shown in semi-transparent grey. The excess space in the membrane region of the map is filled by the detergent micelle, which is not shown for clarity. The scale bar represents 25 Å.
Fig. 4.2. Map resolvability. (A) A cross-section through the lower part of the catalytic $V_1$ region of the map shows that the individual $\alpha$-helices that make up the two E-subunits (indicated with purple arrows) and two G-subunits (indicated with beige arrows) can be resolved. One of the A-subunits is circled in yellow to show that $\alpha$-helices can be seen in all subunits. Segments derived from the cryo-EM map (semi-transparent grey) agree well with the secondary structure for subunits of known structure, such as (B) subunit A, (C) the peripheral stalk subcomplex, and (D) the DF-subcomplex. The crystal structure of the D-subunit is missing residues 56-132 at the base of the segment and the C-terminal 18 residues at the top of the segment, and the density corresponding to these missing residues is indicated with blue arrows. The scale bar corresponds to 25 Å.
Fig. 4.3. Resolution assessment. The Fourier Shell Correlation curve (solid line) indicates a resolution of 9.7 Å with the 0.143 criterion and 11 Å with the 0.5 criterion while the Fourier Neighbor Correlation curve (dashed line) indicates a resolution of 10.3 Å with the 0.143 criterion and 11.7 Å with the 0.5 criterion. All of the features described in this manuscript were apparent at all of these resolutions, but most distinctly so at 9.7 Å resolution. Therefore, the map was filtered to 9.7 Å before rendering figures.

Contacts between the V$_1$ region of the complex and the peripheral stalks exclusively involve the B-subunits of V$_1$ and E-subunits of the stalks, and are mostly between the N-terminal $\beta$-barrels the B-subunits and the C-terminal $\alpha/\beta$-domains of the E-subunits. The N-terminal region of both subunits E and G in both of the peripheral stalks interact with the N-terminal region of subunit I. The funnel-shaped C subunit, which links the DF central stalk to the L$_{12}$-ring and has been crystallized in isolation (Iwata et al., 2004), is significantly more open in the intact enzyme than in the crystal structure so that it can accept the DF subunits into its central cavity (Fig. 4.4). The three pseudo-symmetric domains of the C-subunit can be differentiated in the map showing that interaction of the C-subunit and the L$_{12}$-ring primarily involves the N-terminal domain of subunit C, relying heavily on the N-terminal $\alpha$-helix of subunit C and the C-termini of the inner helices in the L$_{12}$-ring. The N-terminal soluble region of subunit I has been proposed to
have a structure similar to a *S. cerevisiae* V-ATPase subunit not found in the *T. thermophilus* enzyme (Lee et al., 2010), but for which there is a crystal structure available (Drory et al., 2004). The cryo-EM map shows that the N-terminal region of subunit I consists of an elongated helical bundle flanked by two domains, consistent with the structure of the yeast protein (Fig. 4.5A). The atomic structure of this subunit, however, only fits well into the EM segment after allowing for flexibility in the fitting algorithm (Fig. 4.6).

![Fig. 4.4. Conformation of subunit C and its interaction with the L-ring.](image)

(A) Side and (B) Top views comparing rigid body fitting of the C-subunit crystal structure (red) to flexible fitting of the crystal structure (cyan) into the corresponding map segment. Arrows indicate regions of major difference between the two atomic models. (C) and (D) Flexible fitting of the C-subunit gives it a more open conformation that allows it to accommodate the segment for the DF-subcomplex (dark blue) into its central cavity. (E) A side view of the C-subunit, and the segment corresponding to the L_{12}-ring, show that subunit C sits asymmetrically on the ring with its N-terminal α-helix (indicated by a cyan arrow) mediating most of the contact with the L-subunits. The scale bar represents 25 Å.
Fig. 4.5. The map segment corresponding to the N-terminal domain of subunit I (A) showed an elongated structure with remarkable similarity to the crystal structure of the *S. cerevisiae* subunit C (B), which is not found in the *T. thermophilus* enzyme. The fold recognition program Phyre2 automatically identified the N-terminal domain of subunit I and the *S. cerevisiae* subunit C as having similar folds. The scale bar represents 25 Å.

Fig. 4.6. Fitting of subunit C of yeast V-ATPase into the map segment of the hydrophilic domain of subunit I of the *T. thermophilus* V-ATPase. Rigid-body fitting (A and B) and flexible
fitting (C and D) of the subunit C atomic model into the map segment. (A and C) A side view along the same direction as in Fig. 4.1. (B and D) A top view of (A and C).

4.3.2 Overall structure of the membrane-bound $V_o$ region

The map provides the first detailed insight into how the I-subunit and L$_{12}$-ring fit together to allow the generation of rotation (Fig. 4.7A). Cross sections through the detergent-embedded region of the map reveal two concentric rings of densities, with the outer ring consisting of twelve well-resolved densities (Fig. 4.7, B-E). These twelve densities undoubtedly correspond to the outer helices of the L$_{12}$-ring, where each L-subunit consists of a helical hairpin. While the number of subunits in the proteolipid ring of rotary ATPases have been determined previously by structural analysis of isolated ring subcomplexes and other partial assemblies (Murata et al., 2005a; Meier et al., 2005; Pogorvelov et al., 2009; Stock et al., 1999), this map presents the first determination of the ring-structure in an intact rotary ATPase providing insight into how the I-subunit affects the structure of the L$_{12}$-ring. This information is necessary to assess the likelihood of proposed catalytic models in which the L-subunits undergo conformational changes when they contact the I-subunit (Fillingame et al., 2003). In the intact enzyme, the L-subunits in contact with the I-subunit do not noticeably break the twelve-fold symmetry of the dodecameric ring (Fig. 4.7), refuting models of proton translocation that require major conformational changes in the rotor. As shown previously (Lau and Rubinstein, 2010), the detergent used to keep the enzyme soluble, dodecylmaltoside, has a density higher than that of ice and is visible surrounding the transmembrane region of the complex (Fig. 4.7B-i, white bars). Features in the detergent micelle, which we expect to be mostly unstructured, may represent noise that is enhanced by construction of the map to nanometer resolution. A smaller density is visible within the center of the L$_{12}$-ring (Fig. 4.7B-i, yellow circle) and probably corresponds to a detergent or lipid plug observed by atomic force microscopy of isolated rings (Meier et al., 2001).
4.3.3 Structure of subunit I and its interaction with the L-ring

The map reveals for the first time the structure of the membrane-bound C-terminal region of subunit I. Proton translocation facilitated by the I-subunit is essential for driving rotation of the L\textsubscript{12}-ring, but until now there was no structural information available for subunit I or its equivalent subunit from any rotary ATPase. Here we observe eight transmembrane densities that can be attributed to α-helices in the C-terminal region of subunit I (Fig. 4.7A). This number of transmembrane helices is consistent with an experimentally tested topology map of the \textit{S. cerevisiae} V-ATPase α-subunit (Wang \textit{et al.}, 2008), with which the I-subunit sequence aligns.

\textbf{Fig. 4.7.} The membrane-bound region of the enzyme. (A) A view of the map segments for subunit I (\textit{green}) and the L-subunits (\textit{magenta}) shows an L\textsubscript{12}-ring and densities consistent with eight transmembrane helices for subunit I. Blue arrows indicate multiple contacts between the N- and C-terminal regions of subunit I. Dashed lines indicate the positions of cross sections through the cryo-EM density map parallel to the lipid bilayer surface (i), as well as the map segments truncated at the same height as the cross sections (ii). In the map cross sections, several of the outer helices of the L\textsubscript{12}-ring are indicated in each section (red arrows) and the densities arising from transmembrane helices of the I-subunit are outlines (\textit{green}). The white bars in part b-i indicate the detergent micelle and the yellow circle indicates a detergent or lipid density in the centre of the L\textsubscript{12}-ring. Some of the more tilted helices are most easily resolved in sections through membrane region that are not parallel to the membrane surface. (B) Near the cytoplasmic surface of the bilayer, subunit I is well separated from the L\textsubscript{12}-ring. (C) Approximately half way through the membrane, two helices from subunit I contact the outer helix of an L-subunit, labeled “12”, at a point that likely forms the mid-membrane end of the
cytoplasmic proton half-channel (circled in red). (D) A slice ~6 Å further towards the periplasm shows that helices from subunit I contact a different L-subunit, forming the mid-membrane end of the periplasmic half-channel (circled in blue). (E) Near the periplasmic surface of the bilayer the I-subunit is separated from the L_{12}-ring. The scale bars correspond to 25 Å.

well (Fig. 4.8). Although we can trace the complete trajectory of the eight transmembrane densities of subunit I, our inability to resolve the helices in the tightly packed inner ring of the L_{12}-oligomer means that we cannot rule out the possibility that we have missed a transmembrane helix in our analysis of subunit I. At the cytoplasmic surface of the membrane region, the N- and C-terminal regions of subunit I appear to be attached in more than one place (Fig. 4.7, A, B-ii, blue arrows). These connections show that there must be protein-protein interactions between the N- and C-terminal regions of subunit I involved in keeping the two regions rigidly attached. Within the membrane, subunit I divides into two clusters of helices: one that is mostly perpendicular to the membrane, and another cluster that contains highly tilted helices adjacent to the L_{12}-ring. The first cluster contacts a single L-subunit near the middle of the membrane region (Fig. 4.7C, circled in blue in part ii) while the second cluster contacts the adjacent L-subunit a small distance further towards the periplasm (Fig. 4.7D, circled in red in part ii).

4.3.4 Putative proton half-channels and the proton translocation mechanism

Proton translocation in rotary ATPases is thought to involve protonation and deprotonation of conserved mid-membrane glutamic acid or aspartic acid residues in the outer helices of the ring-forming subunits. In the L-subunit from the *T. thermophilus* ATP synthase, this conserved protonatable residue is Glu63. The contact of the I-subunit with two different L-subunits places the two L-subunits in distinct chemical environments and establishes the conditions necessary for a two half-channel model (Junge *et al.*, 1997; Pogoryelov *et al.*, 2009), with one L-subunit exchanging protons with the periplasm and one L-subunit exchanging protons with the cytoplasm. One cluster of four transmembrane helices in the I-subunit could conduct protons from the periplasm to the mid-membrane Glu63 residue of an L-subunit (Fig. 4.9A, blue dashed circle). The other cluster of four transmembrane helices in subunit I could conduct protons from the mid-membrane Glu63 residue of an L-subunit to the cytoplasm (Fig.
4.9A, red dashed circle). Crystal structures of other membrane proteins indicate that four transmembrane helices

**Fig. 4.8.** Sequence alignment between *T. thermophilus* ATP synthase subunit I, and *S. cerevisiae* V-type ATPase subunit a. The transmembrane helices identified in (Leng et al., 1999) and (Wang et al., 2008) are outlined in black. The soluble N-terminal region of the subunits are shown in blue while the membrane-bound C-terminal region is shown in black. The critical conserved arginine residue (Arg 563) is indicated with a red arrow.
are indeed sufficient for forming a proton pore, with available examples showing proton (Stouffer et al., 2008) and sodium (Gonzales et al., 2009) conducting pores composed of four and three transmembrane helices, respectively. Therefore, the arrangement of helices found in the cryo-EM map is consistent with a half-channel mechanism for proton translocation (Junge et al., 1997). In this model, protons flow from the periplasmic half-channel to the Glu63 residue of the L-subunit in contact with the periplasmic half-channel (the L-subunit with an outer helix labeled “1” in Fig. 4.5, B-D). Protonation of this Glu63 neutralizes the negative charge of the residue and allows the clockwise rotation (viewed from the cytoplasm) that places the Glu63 in the hydrophobic environment of the lipid bilayer. With the negative charge neutralized, Glu63 can assume the proton-locked conformation seen in a crystal structure of the *Spirulina platensis* *c*₁₅*-ring* (Pogoryelov et al., 2009) (Fig. 4.9B). In this conformation, both oxygen atoms in Glu63

![Fig. 4.9. Model for proton translocation. (A) A view of subunit I shown parallel to the membrane. The location of the L₁₂-ring is indicated by a semi-transparent magenta rectangle. Protons (yellow spheres) entering the periplasmic half-channel in subunit I, the probable location of which is circled (dashed blue line), are conducted to the center of the lipid bilayer where they neutralize the Glu63 residues on the L-subunits. The ring rotates clockwise (viewed from the cytoplasm to the periplasm) during ATP synthesis, bringing the protonated Glu63 residue of an L-subunit back into contact with the I-subunit at the cytoplasmic half-channel, the probable location of which is circled (dashed red line). (B) Viewed from the cytoplasm, two half-channels in subunit I are depicted as clusters of green cylinders. A comparative model of the L₁₂-ring depicts the conserved Glu63 residues in the proton-locked conformation seen in the crystal.](image-url)
structure of the *Spirulina platensis* c₁₅-ring when not in contact with subunit I, and in an open conformation, as seen in DCCD-modified crystal structure of the *S. plantensis* ring when in contact with subunit I. Protons are indicated by yellow spheres. Deprotonated Glu63 residues are shown with two red oxygen atoms while protonated Glu63 residues are shown with one red oxygen atom and one yellow oxygen atom. The conserved Arg563 residue is likely to reside near the cytoplasmic half-channel where it causes a drop in the pKa of the Glu63 residue and subsequent deprotonation.

are involved in hydrogen bonding with other residues in the L₁₂-ring and the polar Glu63 residue is tucked into the crevice between L-subunits away from the lipid environment. The directionality of the rotation is explained by a Brownian ratchet mechanism where random thermal rotational fluctuations are biased to go in the correct direction by the direction of the proton motive force across the membrane (Junge *et al.*, 1997). The rotation of the L₁₂-ring brings an L-subunit bearing a protonated Glu63 (on the helix labeled #12 in Fig. 4.7, B-D) out of the lipid environment and into contact with the cytoplasmic half-channel of subunit I. Subunit I contains a conserved and essential arginine residue (Arg563) of unknown function (Cain and Simoni, 1989; Kawasaki-Nishi *et al.*, 2001a). It has been postulated that Arg563 causes a decrease in the pKa of the Glu63 residue on the L-subunit in contact with the cytoplasmic half-channel, thereby causing it to lose its proton to the channel (Pogoryelov *et al.*, 2010). Arg563 may also stabilize the deprotonated Glu63 by forming a salt bridge (Meier *et al.*, 2005). Either or both roles suggest that Arg563 is most likely to be located at the mid-membrane end of one of the half-channels.

At both the cytoplasmic and periplasmic limits of the membrane-embedded region of the map (Fig 4.7, B and E) all helices from subunit I are well separated from the helices of the L₁₂-ring, making it highly unlikely that an entire half-channel could be made at the interface of helices from the I- and L-subunits (Steed and Fillingame, 2009). The observation that the two L-subunits in contact with the I-subunit are immediately adjacent to each other indicates that after a Glu63 residue is deprotonated by the cytoplasmic half-channel it is immediately reprotonated by the periplasmic half-channel with a single 30° rotational step of the rotor. The small contact area between the I-subunit and L₁₂-ring suggests that these membrane-bound components rely on the two peripheral stalks to hold them together in the precise arrangement necessary for the complex’s biological activity. This minimal contact is consistent with an important but fragile
interaction that might easily be disrupted by non-physiological conditions such as those needed to make 3D crystals, thus helping to explain why the membrane region of this class of enzyme has not been crystallized, despite significant efforts by many research groups. Knowledge of the precise residues involved in proton translocation will require construction of cryo-EM maps to higher resolution or formation of well-ordered crystals of this hitherto refractory protein complex. At subnanometer resolution, the complete structure of the \textit{T. thermophilus} H\textsuperscript{+}-driven ATP synthase indicates the overall framework by which the energy stored in a transmembrane proton motive force is converted into rotation in rotary ATPases.

4.4 Conclusions

By optimizing image quality and the image processing procedure, a reconstruction of the \textit{T. thermophilus} V-ATPase at subnanometer resolution was obtained. The cryo-EM map at significantly higher resolution shows that the rotary ring contains twelve L-subunits and that the I-subunit has eight transmembrane helices. In the middle of the membrane, the helices from the I-subunit make contacts with two different L-subunits in the ring, giving rise to the minimal rotor-stator interaction previously seen in the lower-resolution structure. The transmembrane helices of subunit I form bundles that could serve as half-channels across the membrane, with the first half-channel conducting protons from the periplasm to the L-ring and the second half-channel conducting protons from the L-ring to the cytoplasm. Our structure lends strong support to the current mechanistic model of proton translocation.
CHAPTER 5: OVERALL CONCLUSIONS AND FUTURE DIRECTIONS

5.1 Overall conclusions

Every organism needs ATP to survive. ATP stores free energy within cells to drive numerous processes such as metabolic reactions, synthesis of macromolecules, active transport, signalling and muscle contraction. In eukaryotes, the majority of cellular ATP is produced by the enzyme called ATP synthase through oxidative phosphorylation in mitochondria. Synthesis of ATP is driven by unidirectional proton translocation across the inner membrane determined by the pmf. In archaea and some bacteria, a different enzyme called V-ATPase is utilized for ATP synthesis. V-ATPase is also found in almost all eukaryotic cells, but instead, it functions as an ATP-driven proton pump. The F-type and the V-type ATPase/synthase are evolutionarily-related and they share a similar overall architecture. Details of subunit composition and arrangement, however, are quite different among these two enzymes. It is known that translocation of protons across the membrane-bound region of F\textsubscript{o}/V\textsubscript{o} causes the rotation of the central rotor that subsequently leads to ATP synthesis in the F\textsubscript{i}/V\textsubscript{i} region. Extensive studies have been carried out to investigate the catalytic mechanism of F\textsubscript{i}-ATPase and much of the details of its reaction scheme are now mostly understood. The mechanochemical cycle of V\textsubscript{i}-ATPase, on the other hand, was shown to have marked differences to that of F\textsubscript{i}-ATPase (Imamura et al., 2005). In either type of enzyme, the vital question of how proton translocation is coupled to ring rotation remains to be answered. To elucidate the mechanism of proton translocation, we determined the structures of these multisubunit complexes in their intact forms using the technique of single particle cryo-EM.

The cryo-EM map of the yeast ATP synthase revealed the locations of subunits a, b, c, e and g within the membrane-bound region. From the map, subunits a and b appear to have an intimate interaction. Subunit a is also found much closer to the c-ring than subunit b, suggesting a closer interaction between subunit a and the c-ring. Whether or not subunit b actually interacts with the c-ring is currently not known due to the low resolution of the map. Comparison of an available bovine map to the yeast map low-pass filtered to the same resolution permitted the assignment of the locations of the two dimer-specific subunits e and g, since our purification protocol for the yeast enzyme selectively removed these accessory subunits. The deduced
locations of these subunits, which are known to interact with each other, allowed us to map out one of the dimerization interfaces of ATP synthase. The transmembrane portion of subunit b of the peripheral stalk can form the second dimerization interface, as it is not occluded by subunits e and g on the peripheral end of the membrane-bound region. In addition to subunit arrangement, the peripheral stalk adopts a left-handed curvature in our structure. At present, the significance of this curvature is not fully understood but can be correlated with the role of a deformable peripheral stalk for transient energy storage during catalysis.

In V-ATPase, the peripheral stalk does not insert into the membrane in a similar manner to subunit b of ATP synthase, hence, the membrane-bound region only contains the single stator subunit, the I-subunit. At least for V-ATPase, the I-subunit alone is sufficient for keeping the association with the rotor ring while allowing for ring rotation. Subunit I is significantly different from subunit a of the ATP synthase because it is about twice the size of subunit a, with an additional hydrophilic N-terminal sector. Our 16 Å resolution cryo-EM map of the *T. thermophilus* V-ATPase showed that the C-terminal, membrane-spanning portion of subunit I is a two-domain structure. Examining the cross-sections of the map revealed that, near the center of the membrane, the interaction between subunit I and the rotary L-ring (homologous to c-ring of ATP synthase) is surprisingly small. The contact occurs between one of the domains and the L-ring. A similar type of interaction was observed between subunit a and the c-ring in the structure of the yeast ATP synthase, however, the lower resolution and the presence of other small subunits in the membrane prevented a detailed analysis of the rotor-stator interaction. According to the current working model of proton translocation, the small contact would contain both the ends for the cytoplasmic and periplasmic aqueous half-channels proposed to be found within subunit I.

The membrane extrinsic region of the V-ATPase map contains two peripheral stalks, each of them connecting the top of a single B-subunit to the hydrophilic sector of subunit I. Whereas the single peripheral stalk in ATP synthase has a noticeable curvature, these two peripheral stalks adopt a near-parallel conformation relative to the long axis of the complex, a conformation identical to one later determined independently by X-ray crystallography. We performed docking experiments and made comparison of the quaternary structures of the AB heterodimers produced by segmentation. One of the AB heterodimers was found in an 'open' conformation
relative to the other two AB heterodimers. The central stalk also appears to adopt a single conformation, engaging in different interactions with the three AB heterodimers, consistent with the binding-change mechanism. Together, these observations suggested that the enzyme was arrested in a single, lowest energy rotational state. A large ternary movement of the C-terminal region of the β-subunit of ATP synthase, which is equivalent to subunit A in V-ATPase, is involved in the open-to-close transition of the βE site. An overlay between the 'open' and the 'closed' AB pairs reveals that this open-to-close transition in V-ATPase rather involves a quaternary rearrangement at the interface between subunits A and B instead.

In an effort to gain detailed insights of the membrane-bound region responsible for proton translocation in the V-ATPase, a subnanometer resolution structure of the intact *T. thermophilus* V-ATPase was subsequently determined. Numerous rod-like densities corresponding to α-helices were identified unambiguously throughout different regions of the map, consistent with other structures determined to this intermediate resolution range. The map quality also allowed flexible fitting of X-ray crystal structures of subunits into their corresponding density to be performed reliably. Shown by our fitting analysis, subunit C was found to adopt a conformation in the intact complex distinct from the conformation determined separately in the crystal of the isolated subunit. This change of conformation allows the C-subunit to accommodate the DF subcomplex of the central stalk within its central cavity. The hydrophilic sector of subunit I has a structure related to a subunit unique to the eukaryotic V-ATPase, which was shown to possess intrinsic deformability by other studies.

With our ability to resolve secondary structure, the stiochiometry of the L-ring in the intact enzyme was confirmed to be twelve, as found previously in a projection map determined by electron crystallography of the ring in isolation. The dodecameric symmetry of the L-ring also seems to be preserved even when interacting with the stator subunit I. Eight transmembrane helices in the form of two clusters were identified in subunit I. These two clusters of helices made up the two domains seen at lower-resolution, with the diameter of the inner pores sufficiently large for fulfilling their roles in conducting protons. The existence of these hypothetical half-channels in subunit I awaits future experiments. At the center of the membrane, subunit I contacts only two L-subunits from the ring, enriching our understanding of nature of the seemingly small interaction between the rotor and the stator.
5.2 Future directions

5.2.1 Nucleotide occupancy of the catalytic sites of the purified V-ATPase

The 3D reconstruction of the V-ATPase does not provide any information about the nucleotide occupancy of the catalytic sites. X-ray crystal structures of the nucleotide-free and nucleotide-bound \textit{T. thermophilus} V\textsubscript{1}-ATPase showed an almost identical conformation (Numoto \textit{et al}, 2009), strongly suggesting that the central stalk is at least as important as the nucleotide in the formation of the asymmetric quaternary structure of the AB pairs. The nucleotide-bound structure was determined in the presence of Mg-ADP, and indeed, significant peaks were observed in the electron density difference map at two of the catalytic sites, which were interpreted as ADP moieties. These two catalytic sites adopted ‘closed’ conformations, whereas the catalytic site without nucleotide adopted the ‘open’ conformation. The ATPase activity of the nucleotide-bound V\textsubscript{1}-ATPase is inhibited by ADP, as supported by an ATPase assay in the same study. V-ATPase has a strong tendency to lapse into an ADP-inhibited state even at low ADP concentration and harsh EDTA/heat treatment is necessary to remove most of the ADP once it has bound to the complex (Imamura \textit{et al}, 2004; Nakano \textit{et al}, 2008). Depending on the conditions that the enzyme was purified in, it was possible to produce either the nucleotide-free or the ADP-inhibited forms of the enzymes (Nakano \textit{et al}, 2008; Numoto \textit{et al}, 2009). Therefore, although the X-ray crystal structure of the V\textsubscript{1}-ATPase fit well into our cryo-EM map in one unique orientation with respect to the peripheral stalks, a separate assay is needed to resolve the catalytic competency of our enzyme preparation. In our previous study, we showed that our preparation had some activity and was sensitive to the inhibitor DCCD in ATPase assays (Lau and Rubinstein, 2010). The observed activity, however, could represent the residual activity of ADP-inhibited complexes. To resolve this issue, the specific activity of the purified complexes should be determined. By taking into account the difference in the rates of activity between V\textsubscript{1} and V\textsubscript{1}V\textsubscript{o} (V\textsubscript{1}V\textsubscript{o} hydrolyzes ATP at rate ~6x slower than V\textsubscript{1} [Furuike \textit{et al}, 2011]), this determined value can be compared to a previously reported value derived from a nucleotide-free preparation of V\textsubscript{1} (< 0.1 mol ADP per mol of enzyme), where the nucleotide occupancy was determined by high performance liquid chromatography (HPLC) (Yokoyama \textit{et al}, 1998). The nucleotide occupancy of our preparation can thus be approximately deduced from the ratio between the experimental and the theoretical specific activity values.
5.2.2 Elucidation of the proton channels

Various inhibitors are known to specifically target V-ATPase (Huss and Wieczorek, 2009). However, in the absence of structural data, the exact binding-sites of most of these inhibitors are enigmatic, although some are known to target the ring subunit (Huss and Wieczorek, 2009). There exists one inhibitor, tributyltin chloride (TBT-Cl), that was found to react with subunit a of the F-type ATP synthase noncovalently with relatively high affinity (Ki=200 nM), potentially by acting as a proton channel blocker (von Ballmoos et al., 2004). Significantly, TBT-Cl was shown to abolish proton permeability of V_o reconstituted in liposome (Takeda et al., 2009), therefore it must also target the V_o region, likely via the same mechanism as in the F-type enzyme. At present, the locations of the proton channels in V-ATPase remain speculative. By determining a cryo-EM structure of the enzyme inhibited with TBT-Cl to intermediate resolution followed by calculating a difference map with our apo structure, the TBT-Cl density in its binding site within subunit I could be revealed. This strategy was utilized to elucidate the binding site of a potent inhibitor SCH28080 of similar size to TBT-Cl (TBT-Cl MW = 325.51; SCH28080 MW = 277.33) in the gastric H+,K+-ATPase (Abe et al., 2011). The binding site of TBT-Cl should coincide with the location of at least one of the proton half channels. Moreover, the residues important for TBT-Cl binding can be identified using a photoaffinity labeling strategy with a radioactive TBT-Cl derivative [diazirinedibutyltin chloride (DDBT-Cl)] (von Ballmoos et al., 2004), followed by in-gel proteolytic digestion and peptide mapping by matrix-assisted laser desorption ionization/time of flight (MALDI-TOF) mass spectrometry (Wu et al., 2005). To further confirm the results, enzymes bearing mutations within the putative proton channels can be produced by mutagenesis and then be tested for proton permeability with a luciferin/luciferase assay system (Toei et al., 2007), provided that the mutations are not detrimental to the organism and do not affect the assembly of the enzyme complex. If successful, this information together with the experimental cryo-EM map may be incorporated as experimental constraints for ab initio membrane protein modelling for high-resolution structure prediction of the I-subunit.
5.2.3  Structure determination of a catalytic intermediate of V-ATPase

During ATP hydrolysis, binding of ATP to the open site drives the open-closed transition in the A and B subunits and the rotation of the central stalk. Unlike the F-type ATP synthase, the central stalk of V-ATPase rotates in 120° steps in the presence of ATP without adopting the 80°-40° reaction scheme (see Introductory section 1.1.4.1). In F₁, ATP binding and cleavage occurs before and after the 80° substep, respectively. Presumably, the binding and cleavage of ATP in the two catalytic sites of V-ATPase occurs approximately at the same angle. The conformation seen in our V-ATPase reconstruction must represent one of these ATP-waiting dwell states between the successive 120° steps, or a state close to it, in which the open catalytic site is ready to bind a molecule of ATP. This open site can be found at the catalytic interface between subunit A and the subunit B that does not bind to a peripheral stalk. In the absence of a pmf or saturating ATP concentration, when the open site does not bind ATP, the conformations of three catalytic sites are likely to be influenced by the peripheral stalks in their most relaxed structural state. The X-ray crystal structure of the peripheral stalk, which fits well into our cryo-EM map, probably exists in this relaxed state (Lee et al., 2010). In the cryo-EM map of the intact complex, this relaxed state adopted by the peripheral stalks would also exist unless external rotational force is imposed onto the stator during ATP synthesis or hydrolysis. The asymmetry of A$_3$B$_3$ is also transmitted to the central stalk through their tight coupling, explaining why the central rotor adopts a unique rotational orientation in our structure.

Determining the structure of a catalytic intermediate of the V-ATPase would be of exceptionally value because it will reveal the structural changes to the complex associated with nucleotide binding or hydrolysis that are directly engaged in generating torque, such as rotation of the central rotor in response to closing of the open site in the V$_1$ region. Other passive structural changes, such as any potential movement of the peripheral stalks or other parts of the stator, would also be revealed. To accomplish this task, a cryo-EM structure of the T. thermophilus V-ATPase in complex with AMP-PNP could be determined. AMP-PNP, a non-hydrolyzable ATP analogue, is a commonly used inhibitor for crystallization of F$_1$-ATPase and can bind the catalytic sites, but possibly with slightly lower affinity (Lobau et al., 1997). Previous studies on the F-type ATP synthase have shown that the three catalytic sites are not chemically equivalent; they have significantly different binding affinities for ATP (or AMP-
PNP) (Menz et al., 2001; Lobau et al., 1997), which are dictated by the relative position of the central stalk within the catalytic hexamer. For example, in the case for *E. coli* ATP synthase, the Kd varies from 15 $\mu$ M to 0.5 $\mu$ M to 0.2 nM as each site goes through the three states from 'open' to 'loose' to 'tight', respectively (Lobau et al., 1997). The interconversion between these sites with different binding affinities is in accord with the binding change mechanism. This same phenomenon also applies to V-ATPase, although the binding affinities for each of catalytic sites have not been determined for this enzyme. By incubating the nucleotide-free V-ATPase with excess AMP-PNP (mM concentration), all three catalytic sites would be expected to be filled with the nucleotide. By analogy to F$_1$-ATPase, it is likely that the energy from ATP binding is solely responsible for driving rotation of the central rotor without assistance from cleavage of ATP (Adachi et al., 2007), and ATP hydrolysis likely takes place first followed by ATP binding at the dwell angle (Imamura et al., 2005). Therefore, the closing of the open site upon binding to AMP-PNP may in principal drive the rotation of the central rotor by $\sim$120°, where the rotor must stop at a position before the next ATP-waiting dwell angle, as the transformation to the next ATP-waiting dwell state is not possible without nucleotide release and cleavage at the other two sites. Thus, complete inhibition of the enzyme by AMP-PNP should produce a structurally homogeneous preparation amenable for cryo-EM study.
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Appendix 1

Hand determination

The absolute hand of a 3D reconstruction obtained only using untilted data, which are 2D projections of the 3D object, is arbitrary. The absolute hand can be directly determined from the map if the map is refined to sufficient resolution so that the molecular features can be interpreted with an X-ray crystal structure at high resolution. For a lower resolution map, however, an additional approach must be employed that requires the collection of tilt data to independently determine the absolute hand of the 3D map. This approach is called the free hand test. Briefly, a few areas of the micrographs are recorded twice at two goniometer angles. A pair of the micrographs imaged at two tilt angles relative to the electron beam is called a tilt pair. The individual particle images identified in the first micrograph in a tilt pair should be related to their corresponding particle images in the second micrograph by the tilt angle. After aligning the individual particle images identified to the existing 3D model, the orientations of the particle images in the second micrographs can be predicted. Projections are then generated from the 3D model based on these predicted orientations and compared to the particle images identified in the second micrographs by the use of a similarity measure, i.e., a phase residual. If the model is of correct hand, the projections should agree with the particle images of the second micrographs after rotating by the tilt angle used in the experiment. Conversely, the projections will agree with the particle images after rotation by the negative of the tilt angle if the model is of opposite hand.
Fig. A1.1. Hand determination for the yeast ATP synthase map. A data set of 50 particle image pairs with a goniometer tilt of 30° between them was used to determine the absolute hand of the 3D model. The plot shows the average phase residual of all of the second particles in the tilt pairs at different applied rotations after the first particles has been aligned to the model. This plot is called a free hand plot. The minimum of the plot is labeled with a star symbol. The expected minimum from the applied goniometer rotation was at (0°, -30°) if the model had the correct hand while a model of incorrect hand was expected to produce a minimum at (0°, 30°). The phase residual difference between (0°, -30°) and (0°, 30°) was 6.4°. The plot indicated that the hand of the model depicted was correct. The magnitude of the phase residual difference and the distance of the minimum position away from the expected position can also be used to optimize alignment parameters as well as to test the validity of the 3D model.
Fig. A1.2. Hand determination for the *T. thermophilus* map. A dataset of 50 particle image pairs with a goniometer tilt of 30° between them (from +15° to -15°) was used to determine the absolute hand of the 3D map, optimize the alignment of particles during model refinement, and objectively test the validity of the map. The free hand plot shows the average phase residual of all of the second particles in the tilt pairs at different applied rotations after the first particles had been aligned to the map. The minimum of the plot is labeled with a star symbol. The expected minimum from the applied goniometer rotation was at (0°, -30°) if the model had the correct hand while a model of incorrect hand was expected to produce a minimum at (0°, 30°). The phase residual difference between (0°, -30°) and (0°, 30°) was 14.9°, indicating that the hand of the model depicted was correct.
Appendix 2

The rotational analysis (ROTAN)

The ROTAN analysis is used to obtain an initial 3D model where most of the particles adopt side views on the EM grid. Side views differ from each other by a rotational angle about an axis in the plane of the grid. We call this axis as the tilt axis. The orientation of any individual particle can be described by three Eulerian angles, $\psi$, $\theta$, $\phi$. By following a convention, for simplicity, the $\psi$ and $\theta$ are taken as 90º for a side view and the relative orientation between two side views are described by the angle $\phi$.

In this approach, class averages must be first generated by methods of classification. The class averages are also side views. Then, 2D difference images are obtained by subtracting each class average from the average of all of the other class averages. The resulting difference images allow identification of asymmetric features, that is, features that appear in every difference image in a series and have the same y-positions but different x-positions in each image. It is easy to see that the displacement of each feature from the central line of each class average, $a$, can be related to the angle $\phi$ by $a = r \cos \phi$, where $r$ is the distance between the asymmetric feature and the rotation axis of the 3D complex (Fig. A2.1). When more than one asymmetric feature is identified within each class average, these features can be related to each other by an additional rotational angle $\delta$. Therefore, the displacement for each asymmetric feature in an image $j$ can be described by the following formula:

$$a_{ij} = r_i \cos (\phi_j + \delta_i)$$

$a_{ij}$ can be determined by assigning markers to the difference images. The values of $r_{ij}$, $\phi_j$ and $\delta_i$ can be determined by minimization of an error function. The values of $\phi_i$ give the relative angles between the class averages, which can be used to build an initial 3D map. The validity of this approach can be tested by plotting the values of $a_i/r_i$ against $(\phi_j + \delta_j)$ for each marker, which should roughly follow a cosine curve.
Fig. A2.1. The rotational analysis algorithm. Class averages corresponding to side views are related to each other by a rotation, $\phi_j$, about the long axis (tilt axis) of the complex. The asymmetric features in the structure each have some distance from the rotation axis of the complex, $r_i$, and some angular offset from an arbitrarily defined starting position, $\delta_i$. 
Determination of the initial 3D map of the yeast ATP synthase

Fig. A2.2. (A) The difference images. These difference images are shown in parts i to vi and correspond to the views in Fig 2.3 parts i and vi. On the asymmetric features, fiducial markers were plotted and subjected to rotational analysis. (B) The results of rotational analysis provided the orientations for each of the views in Fig 2.3. The quality of the fit of the views to the hypothesis that each class average is a side view about a single rotation axis was tested by plotting the ratio of the measured marker positions \((a_i)\) from part A to the calculated radii \((r_i)\) for the marker features versus the sum of the calculated viewing angle \((\phi_j)\) for each view and offset angle \((\delta_i)\) for each marker. The class averages in parts i and vi were assigned Euler angles of \(\psi = 90^\circ\), \(\theta = 90^\circ\) and \(\varphi = 231, 210, 95, 56, 42,\) and \(268^\circ\), respectively. Pairs of class averages that were approximate mirror images of each other were assigned \(\varphi\) angles approximately \(180^\circ\) apart, supporting the accuracy of the rotational analysis. The views fit the rotation hypothesis with a coefficient of determination \(R^2\) of 0.9944.
Fig. A2.3. From each view in Fig. 3.1, the positions of the markers for the two different peripheral stalks were subjected to rotational analysis. The class averages were assigned Euler angles of $\psi = 90^\circ$, $\theta = 90^\circ$, and $\phi = 204, 248, 286, 307, 341, 29, 75, 113, 133 \text{ and } 166^\circ$, respectively. Pairs of class averages that were approximate mirror images of each other had $\phi$ angles approximately $180^\circ$ apart (i.e., $175, 173, 173, 174, \text{ and } 175^\circ$) supporting the accuracy of the analysis. The views fit the rotation hypothesis with a coefficient of determination (R2) of 0.984.