Characterization of a Novel Amino-Terminal Domain From A Copper Transporting P-Type ATPase Implicated in Human Genetic Disorders of Copper Metabolism

by

Michael DiDonato

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

© Copyright by Michael DiDonato 1999
The author has granted a non-exclusive licence allowing the National Library of Canada to reproduce, loan, distribute or sell copies of this thesis in microform, paper or electronic formats.

The author retains ownership of the copyright in this thesis. Neither the thesis nor substantial extracts from it may be printed or otherwise reproduced without the author’s permission.
This thesis is dedicated to the memory of

Michele DiDonato

and

Domenico Bettini
ABSTRACT

Characterization of a novel copper transporting P-type ATPase implicated in human genetic disorders of copper metabolism

Degree of Doctor of Philosophy, 1999.

Michael DiDonato

Graduate Department of Biochemistry, University of Toronto

The ~70 kDa copper binding domain from the Wilson disease copper transporting P-type ATPase was expressed and purified as a fusion to glutathione-S-transferase (GST). Following purification, a detailed study of the metal binding characteristics of the domain, as well as the structural consequences of metal binding, was carried out. In order to determine the metal binding specificity of the domain, immobilized metal ion affinity (IMAC) and $^{65}$Zn blotting analyses were performed. The results of these analyses indicate that the domain is able to bind a wide range of transition metals with varying affinities. The apparent order of affinity was found to be: Cu(I)/Cu(II) > Zn(II) > Ni(II) > Co(II). Copper bound to the domain is in the +1 oxidation state as assessed using the colorimetric copper(I) chelator bathocuproine disulfonic acid (BCS) and x-ray absorption near edge structure (XANES) spectroscopy. Neutron activation analysis (NAA) indicated a copper/protein stoichiometry of 6.5 copper atoms / mole of protein. In competitive $^{65}$Zn blotting experiments non-radioactive copper (Cu(I) or Cu(II)) was able to inhibit $^{65}$zinc binding to the domain in a sigmoidal manner, suggesting the presence of cooperativity. This sigmoidal inhibition pattern was not observed for any of the other metals tested. Detailed structural studies using circular dichroism (CD) spectroscopy indicate that significant changes in both secondary and tertiary structures take
place in the domain upon copper binding. Furthermore, the structural changes which occur upon the addition of zinc are significantly different from those which occur with copper. X-ray absorption spectroscopy (XAS) indicates that the copper atoms are ligated by two sulfur atoms in a distorted linear arrangement. The XANES spectra did not change significantly with incremental addition of copper to the domain indicating that all six binding sites may be structurally similar.
No amount of experimentation can prove me right; a single experiment can prove me wrong.

-Albert Einstein
Acknowledgments

I would like to thank all the people who, in one way or another, have helped me in my graduate career over the past five years. First and foremost I would like to thank my supervisor Dr. Bibudhendra Sarkar for allowing me the freedom to pursue my interests in his lab and for all his help and guidance over the years. I would also like to thank all the members of my committee, Dr. David MacLennan, Dr. Robert Morris and Dr. Diane Cox for all their suggestions, help and encouragement. I could not have reached this point without the help and assistance of my lab-mates both past and present. To Suree, thank you for always being there as a friend and for your constant stream of suggestions and solutions to my problems, you have been a true role model. Many thanks to Tokameh who shared with me both the pain and exhilaration of research during our tour of duty in the lab. you were missed over the last year. I would also like to thank Nira and Loretta for all their help and assistance over the years. To all the members of the Sarkar Lab (past and present) Suree, Tokameh, Mike, Negah, Lakshmi, Andrew, Damiano, Xuefeng and Jin-you, thank you for making the lab a great place to work. I will miss those lab lunches and the crosswords.

I must also take this opportunity to thank the person who has had the most influence on my life, my wife Grace DeSantis. She has helped in more ways than I can say and has been the rock which has anchored me in the storm. She has always been there with her support and has never tired of hearing my depressing research stories. Her intelligence and perseverance has always been an inspiration to me.

I would also like to thank all my friends for their help, support and friendship over the years. Thank you to Nana and Natalie first for teaching me molecular biology and for making the Pulleyblank lab a great place, but more importantly for your friendship and
support through the "rough" times in grad school. To Voula, thanks for all the QC on my 
data presentation (it really paid off in Edmonton!), for drafting me into the BGSU and for 
enlightening me on the "purpose" of glycerin soap. To Dina, thanks for all the spanakopita 
and dolmathakia and for always having interesting stories to tell me. To Randy, thanks for 
getting me involved with Aliquotes and giving me an excuse to put up a web site, it was great 
while it lasted.

To everyone else on the third floor who I've missed, thanks for making my stay at 
Sick Kids interesting and fun, you will all be missed. Lastly I would like to thank my family 
for always supporting my interests and encouraging me to strive for higher goals. I love you 
all very much.
# Table of Contents

ABSTRACT iii  
ACKNOWLEDGEMENTS vi  
TABLE OF CONTENTS viii  
LIST OF TABLES xii  
LIST OF FIGURES xiii  
ABBREVIATIONS xv

## CHAPTER 1

**Introduction**

<table>
<thead>
<tr>
<th>Section</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td>Metals in Biological Systems</td>
<td>2</td>
</tr>
<tr>
<td>1.2</td>
<td>Form and Function of Copper in Biological Systems</td>
<td>4</td>
</tr>
<tr>
<td>1.2.1</td>
<td>Types of copper</td>
<td>4</td>
</tr>
<tr>
<td>1.2.2</td>
<td>Important copper-containing enzymes in humans</td>
<td>7</td>
</tr>
<tr>
<td>1.3</td>
<td>Copper Homeostasis in Humans</td>
<td>12</td>
</tr>
<tr>
<td>1.3.1</td>
<td>Copper uptake and circulation</td>
<td>13</td>
</tr>
<tr>
<td>1.3.2</td>
<td>Membrane transport of copper</td>
<td>18</td>
</tr>
<tr>
<td>1.3.3</td>
<td>Intracellular copper trafficking</td>
<td>21</td>
</tr>
<tr>
<td>1.3.4</td>
<td>Excretion of copper</td>
<td>25</td>
</tr>
<tr>
<td>1.4</td>
<td>Cation Transporting ATPases</td>
<td>29</td>
</tr>
<tr>
<td>1.4.1</td>
<td>Types of cation transporting ATPases</td>
<td>31</td>
</tr>
<tr>
<td>1.4.1.1</td>
<td>P-type ATPases</td>
<td>31</td>
</tr>
<tr>
<td>1.4.1.2</td>
<td>V-type and F-type ATPases</td>
<td>37</td>
</tr>
<tr>
<td>1.4.2</td>
<td>Menkes and Wilson Disease Copper Transporting ATPases</td>
<td>41</td>
</tr>
<tr>
<td>1.5</td>
<td>Human Genetic Disorders of Copper Metabolism</td>
<td>50</td>
</tr>
<tr>
<td>1.5.1</td>
<td>Menkes disease</td>
<td>50</td>
</tr>
<tr>
<td>1.5.1.1</td>
<td>Genetics of Menkes disease</td>
<td>50</td>
</tr>
<tr>
<td>1.5.1.2</td>
<td>Clinical and Biochemical findings</td>
<td>54</td>
</tr>
<tr>
<td>1.5.1.3</td>
<td>Current treatments</td>
<td>59</td>
</tr>
<tr>
<td>1.5.1.4</td>
<td>Animal models</td>
<td>61</td>
</tr>
</tbody>
</table>
Table of Contents (cont'd)

1.5.2  Wilson disease 63
  1.5.2.1 Genetics of Menkes disease 63
  1.5.2.2 Clinical and Biochemical findings 66
  1.5.2.3 Current treatments 70
  1.5.2.4 Animal models 73

1.6  Project Rationale 78

CHAPTER 2

Materials and Methods

2.1  DNA Preparation 82
  2.1.1 Cell lines 82
  2.1.2 Preparation of competent BL21(DE3) and DH5α Cell 82
  2.1.3 Transformation of competent BL21(DE3) and DH5α Cell 82
    2.1.3.1 Preparation of glycerol stocks 83
  2.1.4 Purification of plasmid DNA 83
  2.1.5 DNA agarose gel electrophoresis 84
  2.1.6 Purification of DNA restriction fragments from agarose gels 84
  2.1.7 DNA Sequencing 84
  2.1.8 Restriction enzyme digests and ligation reactions 85

2.2  Construction of Expression Vectors 85
  2.2.1 Construction of pGEX-WCBD 85
  2.2.2 Construction of pGEX-WCBD(P) 86

2.3  Expression and Purification of GST-WCBD and GST-WCBD(P) 89
  2.3.1 Buffer and reagent preparation 89
  2.3.2 Expression of GST-WCBD and GST-WCBD(P) 89
  2.3.3 Purification of GST-WCBD and GST-WCBD(P) 90
  2.3.4 SDS-PAGE 91
  2.3.5 Protein quantitation 92
  2.3.6 N-Terminal Amino Acid Sequencing 92
  2.3.7 Amino Acid Analysis 93

2.4  Removal of GST from GST-WCBD and GST-WCBD(P) 93
  2.4.1 Thrombin cleavage of GST-WCBD 93
Table of Contents (cont'd)

2.4.1.1 Optimization of thrombin cleavage conditions 94
2.4.2 PreScission™ cleavage of GST-WCBD(P) 94

2.5 Characterization of the Metal Binding Properties of WCBD 95
2.5.1 Immobilized Metal Ion Affinity Chromatography (IMAC) 95
2.5.2 ⁶⁵Zn(II) Blotting Assay 95
  2.5.2.1 Effect of pH on ⁶⁵Zn(II) Blotting Assay 96
  2.5.2.2 Effect of DTT on ⁶⁵Zn(II) Blotting Assay 96
2.5.3 Competition ⁶⁵Zn(II) Blotting Assay 96
2.5.4 Stoichiometry of metal binding 97
2.5.5 Neutron Activation Analysis (NAA) 97

2.6 Characterization of the Copper Binding Sites 98
2.6.1 Preparation of apo-protein 98
2.6.2 Preparation of Samples For XAS and CD analysis 99
  2.6.2.1 Preparation of Dry XAS Samples 99
  2.6.2.2 Preparation of Liquid XAS and EPR Samples 100
2.6.3 EXAFS and XANES Analysis of Cu-GST-WCBD 100
2.6.4 Oxidative release of copper from GST-WCBD 101

2.7 Conformational Analysis of GST-WCBD and WCBD 101
  2.7.1 Analysis of protein structural changes upon metal binding 101
  2.7.2 Estimation of secondary structure content 103

CHAPTER 3

Metal Binding Properties of the N-Terminal Copper Binding Domain from the Wilson Disease Copper Transporting P-Type ATPase (WND)

3.1 Introduction 105

3.2 Results 107
  3.2.1 Expression and purification of GST-WCBD, GST-WCBD(P), and WCBD 107
  3.2.2 Metal binding characteristics of GST-WCBD and WCBD 110
    3.2.2.1 Immobilized metal ion affinity chromatography (IMAC) 110
    3.2.2.2 Neutron activation analysis (NAA) 112
    3.2.2.3 ⁶⁵Zinc blotting analysis 112
Table of Contents (cont'd)

3.2.2.4 Competition $^{65}$Zinc blotting analysis 116

3.3 Summary and Discussion 118

CHAPTER 4

Structural Characterization of the Copper Binding Domain
And Its Metal Binding Sites

4.1 Introduction 122

4.2 Results
   4.2.1 X-ray absorption spectroscopy (XAS) analysis of GST-WCBD 124
   4.2.2 Circular dichroism analysis of GST-WCBD and WCBD 127
   4.2.3 Estimation of secondary structure content 133
   4.2.4 Oxidative release of copper from GST-WCBD 134

4.3 Summary and Discussion 137
   4.3.1 Hypothetical model for the function of the N-terminal copper
   binding domain of ATP7B in vivo 145

CHAPTER 5

General Discussion and Future Directions

5.1 General Discussion 152

5.2 Future Directions 154

5.3 Literature Cited 158
List of Tables

Table 1.1  Function of Some Biologically Important Metal Ions  3
Table 1.2  Physiological Role of Copper-Containing Enzymes in Humans  5
Table 1.3  Developmentally Important Copper-Containing Enzymes in Humans  8
Table 1.4  Dietary Modifiers of Intestinal Copper Uptake in Humans  14
Table 1.5  Metal Content of Rat Bile  26
Table 1.6  Metal Transporting Proteins Containing N-terminal HMA Domains  45
Table 1.7  Symptoms of Classical Menkes Disease and Occipital Horn Syndrome (OHS)  55
Table 1.8  Typical Copper Measurements in Normal Adults and Menkes Disease Patients  57
Table 1.9  Features of Menkes Disease in Humans and the Mottled Mouse Mutants  62
Table 1.10  Typical Copper Measurements in Normal Adults and Wilson Disease Patients  69
Table 1.11  Comparison of Biochemical Findings in Wilson Disease and its Animal Models: Toxic Milk Mouse and Bedlington Terriers  74
Table 2.1  CD Parameters Used in the Conformational Analysis of GST-WCBD and WCBD  102
Table 4.1  Secondary Structure Estimation for Metal Titrated WCBD and GST-WCBD Using SELCON and K2D  135
Table 4.2  CD Transitions of Aromatic Side Chains  141
List of Figures

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td>Intestinal and Cellular Copper Uptake Pathways</td>
<td>19</td>
</tr>
<tr>
<td>1.2</td>
<td>Transport Mechanism of SERCA and Na⁺/K⁻ ATPases</td>
<td>32</td>
</tr>
<tr>
<td>1.3</td>
<td>Topology of Type I and Type II ATPases</td>
<td>34</td>
</tr>
<tr>
<td>1.4</td>
<td>Comparison of Wilson (WND), Menkes (MNK) and Rat (rWND) Copper Transporting ATPases</td>
<td>43</td>
</tr>
<tr>
<td>1.5</td>
<td>Proposed Structure for Wilson and Menkes Copper ATPases</td>
<td>44</td>
</tr>
<tr>
<td>1.6</td>
<td>Phylogenetic Relationship Between HMA Containing Proteins</td>
<td>47</td>
</tr>
<tr>
<td>1.7</td>
<td>Genomic Structure of the Disease Gene (ATP7A)</td>
<td>52</td>
</tr>
<tr>
<td>1.8</td>
<td>Genomic Structure of the Wilson Disease Gene (ATP7B)</td>
<td>64</td>
</tr>
<tr>
<td>2.1</td>
<td>Parental and Modified GST Fusion Expression Vectors</td>
<td>87</td>
</tr>
<tr>
<td>2.2</td>
<td>GST-WCBD(P) Expression Vector with PreScission™ Cleavage Site</td>
<td>88</td>
</tr>
<tr>
<td>3.1</td>
<td>Effect of Copper on BL21(DE3) Growth Following Induction</td>
<td>108</td>
</tr>
<tr>
<td>3.2</td>
<td>Summary of Typical Purification of WCBD and GST-WCBD</td>
<td>109</td>
</tr>
<tr>
<td>3.3</td>
<td>Immobilized Metal Ion Affinity Chromatography (IMAC) of GST-WCBD</td>
<td>111</td>
</tr>
<tr>
<td>3.4</td>
<td>Effect of pH on the Binding of ⁶⁵Zn</td>
<td>114</td>
</tr>
<tr>
<td>3.5</td>
<td>Effect of Reducing Agents on the Binding of ⁶⁵Zn</td>
<td>115</td>
</tr>
<tr>
<td>3.6</td>
<td>Competition of ⁶⁵Zn Binding to WCBD</td>
<td>117</td>
</tr>
<tr>
<td>4.1</td>
<td>Normalized Copper XANES Spectra For GST-WCBD with Various Amounts of Copper</td>
<td>125</td>
</tr>
<tr>
<td>4.2</td>
<td>EXAFS Spectra For GST-WCBD with Various Amounts of Copper</td>
<td>126</td>
</tr>
<tr>
<td>4.3</td>
<td>CD Spectra of GST-WCBD Titrated with Copper</td>
<td>129</td>
</tr>
<tr>
<td>4.4</td>
<td>CD Spectra of GST-WCBD Titrated with Zinc</td>
<td>130</td>
</tr>
</tbody>
</table>
List of Figures (cont'd)

Figure 4.5  CD Spectra of WCBD Titrated with Copper  131
Figure 4.6  CD Spectra of WCBD Titrated with Zinc  132
Figure 4.7  Oxidative Release of Bound Copper From WCBD  136
Figure 4.8  Hypothetical Model for the Function of WND in vivo  148
Abbreviations

\( \beta \)-Me \( \beta \)-Mercaptoethanol
BCS Bathocuproinedisulfonic acid
BSA Bovine serum albumin
CAPS 3-(cyclohexylamino)-1-propanesulfonic acid
cDNA Complimentary deoxyribonucleic acid
DNA Deoxyribonucleic acid
DTT Dithiothreitol
EDTA Ethylenediaminetetraacetic acid
EPR Electron paramagnetic resonance
ERDBD Estrogen receptor DNA binding domain
EXAFS Extended X-ray absorption fine structure
GST Glutathione-S-Transferase
HMA Heavy metal associated domain
IPTG Isopropyl \( \beta \)-D-Thiogalactopyranoside
LB Luria-Bertani Broth
NAA Neutron activation analysis
OMIM Online Mendelian Inheritance in Man
PAGE Polyacrylamide gel electrophoresis
PMSF Phenylmethylsulfonyl fluoride
RT Room temperature
RT-PCR Reverse transcription polymerase chain reaction
SDS Sodium dodecyl sulfate
SOC Save Our Cells (medium)
TAE Tris-Acetate-EDTA Buffer
TCA Trichloroacetic acid
TCEP Tris(2-carboxyethyl)phosphine
WCBD Wilson Disease Copper Binding Domain
XANES X-ray absorbance near edge structure
XAS X-ray absorption spectroscopy
CHAPTER 1

Introduction
1.1 Metals in Biological Systems

Metals play a vital role in the proper function of many biological processes and are therefore integral to the maintenance of life. It is believed by some, that the origin of life itself was aided in part by the presence of various metals in the primordial environment of the early Earth (Ochiai, 1995; Österberg, 1995). The role of metal ions in biological systems has evolved to include important roles in transport, signaling, catalysis and structure-promotion. The biological functions of some selected metal ions are summarized in table 1.1. There are many attributes of metal ions which make them especially useful in biological systems. Two of the most important are complex formation and redox properties. When metal ions form complexes, the properties of the metal as well as the ligand are altered. The simplest example of this is the alteration of the pKₐ of water upon the addition of metal. When iron(III) is introduced into an aqueous solution the pKₐ of the bound water ligands is dramatically altered and is substantially lower than that of the bulk water, 2.5 versus 15.6 (Burgess, 1978). This property can also be observed with other ligands and is integral to the function of proteins which contain a metal factor.

In these cases the metal ion may promote electrophilic activation, charge neutralization, and the creation of a more effective nucleophile. Alternatively, the metal ion may help establish the proper substrate geometry, or activate the substrate by imposing a distortion or strain (Phipps, 1995). In many cases the binding of a metal ion to a protein can lead to the stabilization of a particular conformation which may be significantly different than what would be observed in the absence of metal (Glusker, 1991). The redox properties of certain metal atoms also play an important part in biological systems since the various oxidation states of the metal usually have different properties and provide
### Table 1.1
Function of Some Biologically Important Metal Ions

<table>
<thead>
<tr>
<th>Metal</th>
<th>Biological Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium</td>
<td>Charge carrier; osmotic balance</td>
</tr>
<tr>
<td>Potassium</td>
<td>Charge carrier; osmotic balance</td>
</tr>
<tr>
<td>Magnesium</td>
<td>Structure, hydrolase; isomerase</td>
</tr>
<tr>
<td>Calcium</td>
<td>Structure, signaling; charge carrier</td>
</tr>
<tr>
<td>Vanadium</td>
<td>Nitrogen fixation; oxidase</td>
</tr>
<tr>
<td>Chromium</td>
<td>Involvement in glucose tolerance and metabolism</td>
</tr>
<tr>
<td>Molybdenum</td>
<td>Nitrogen fixation; oxidase; oxo transfer</td>
</tr>
<tr>
<td>Tungsten</td>
<td>Dehydrogenase</td>
</tr>
<tr>
<td>Manganese</td>
<td>Photosynthesis; oxidase; structure</td>
</tr>
<tr>
<td>Iron</td>
<td>Oxidase; dioxygen transport; electron transfer, nitrogen fixation</td>
</tr>
<tr>
<td>Cobalt</td>
<td>Oxidase; alkyl group transfer</td>
</tr>
<tr>
<td>Nickel</td>
<td>Hydrogenase; hydrolase</td>
</tr>
<tr>
<td>Copper</td>
<td>Oxidase; dioxygen transport; electron transfer</td>
</tr>
<tr>
<td>Zinc</td>
<td>Structure; hydrolase</td>
</tr>
</tbody>
</table>

Adapted from (Lippard and Berg, 1994).
an additional control point for modulation of the proteins' activity. Many metalloenzymes carry out complex redox reactions which are critical to the maintenance of life. These are usually two electron reactions which often involve group transfer as well. Examples of these types of reactions are the addition and removal of oxygen from a substrate.

Although a critical component of many enzymes, most metal ions are only required in trace amount. An excess of metal ions, especially those with redox properties such as copper and iron, pose a potential threat to the organism. As a result, many organisms have had to evolve specific and efficient methods to regulate both the uptake and efflux of metal ions from their cells in order to avoid the toxic effects of metal accumulation and the equally toxic effects of metal deficiency. The result is a whole host of proteins, and control mechanisms which are only now becoming well understood.

1.2 Form and Function of Copper in Biological Systems

1.2.1 Types of Copper

Copper is an essential element which is present in the active sites of many enzymes carrying out a variety of functions (Table 1.2). Most of biological copper exists bound to proteins and these copper binding sites are divided into 3 main types based on their spectroscopic properties. Type 1 copper is not normally found in humans and consists of a single copper atoms ligated by four ligands in a distorted tetrahedral arrangement. This type of copper is characterized by an intense absorption (ε = 3000 - 5000 M⁻¹ cm⁻¹) at ~ 625 nm, imparting a blue color to the protein, and an unusually small hyperfine splitting constant in its EPR spectra (Solomon et al., 1976). The intense blue color of these proteins has led to
<table>
<thead>
<tr>
<th>Physiological Role</th>
<th>Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Radical Scavenging</td>
<td>Cu/Zn Superoxide Dismutase</td>
</tr>
<tr>
<td></td>
<td>Metallothionein</td>
</tr>
<tr>
<td></td>
<td>Ceruloplasmin</td>
</tr>
<tr>
<td>Metal Transport</td>
<td>Metallothionein</td>
</tr>
<tr>
<td></td>
<td>Ceruloplasmin</td>
</tr>
<tr>
<td></td>
<td>Albumin</td>
</tr>
<tr>
<td></td>
<td>ATOX1†</td>
</tr>
<tr>
<td></td>
<td>Ccs1†</td>
</tr>
<tr>
<td></td>
<td>Cox17†</td>
</tr>
<tr>
<td>Electron Transport</td>
<td>Cytochrome oxidase</td>
</tr>
<tr>
<td>Ferrooxidase Activity</td>
<td>Ceruloplasmin</td>
</tr>
<tr>
<td></td>
<td>Ferroxidase II</td>
</tr>
<tr>
<td>Adenosine and Homocysteine Synthesis</td>
<td>Adenosylhomocysteinase</td>
</tr>
<tr>
<td>Blood Coagulation</td>
<td>Factor V</td>
</tr>
<tr>
<td></td>
<td>Factor VII</td>
</tr>
</tbody>
</table>

*†Recently identified intracellular copper chaperones.*
this type of copper also being referred to as 'blue copper'. The type 1 copper center can accommodate the geometry of both copper(I) and copper(II). The second type of copper centers are referred to as 'normal' copper since their spectroscopic properties are similar to those of many low molecular weight copper complexes. This site is characterized by a hyperfine coupling constant as detected by EPR, which is considerably larger than for type 1 copper and a weak absorption in the UV/Vis spectrum. Type 2 copper centers usually consist of a distorted square planar arrangement of ligands and are practically colorless (Harris, 1995). The usual oxidation state for copper in this site is +2. The third type of copper differs from the first two in that it is a binuclear site. Type 3 copper sites exist as a Cu(I)-Cu(I) couple to which oxygen is able to bind bridging the two copper atoms. The binding of oxygen as a stable peroxide species results in the oxidation of the copper pair from the +1 to the +2 oxidation state (Solomon, 1988). The Cu(II)-Cu(II) form of the protein would be expected to have an EPR spectrum since Cu(II) is a d⁹ system; however, the proximity of the two copper atoms (~ 3.5 Å (Volbeda and Hol, 1988)) results in spin coupling abolishing the EPR spectra. The oxygenated type 3 copper site is characterized by a strong absorption at 330 nm (Harris, 1995).

Although the copper sites described above are the most common of those found in biological systems, another class of copper site which is composed of a type 3 and type 2 site has also been described. This trinuclear site is found in all multicopper oxidases described thus far and is composed of a triangular arrangement of copper atoms with the binuclear type 3 copper site at the base and a type 2 copper site at the apex (Allendorf et al., 1985; Spira-Solomon et al., 1986). Lastly, mono copper sites based on copper in the +1 oxidation state have sometimes been referred to as type 4 copper. However, since copper is in the +1
oxidation state, with a d$^{10}$ closed-shell system, it is spectroscopically inactive and hence not easily detected. In order to characterize these types of copper centers, techniques such as magnetic circular dichroism and various types of XAS must be employed.

1.2.2 Important Copper Containing Enzymes in Humans

There are many developmentally important enzymes in humans which require copper to function properly (Table 1.3). A deficiency of copper in the body will lead to a decreased activity of these enzymes with serious consequences. It is this scenario which is present in Menkes disease, where many of the observed phenotypes can be traced back to a deficiency in copper-requiring enzymes. Among the most important copper-containing enzymes in humans are cytochrome c oxidase, ceruloplasmin, lysyl oxidase, and Cu/Zn superoxide dismutase (SOD).

Ceruloplasmin is a large, secreted, multicopper serum glycoprotein which carries approximately 95% of all copper circulating in the body (Frieden, 1986). The crystal structure of holoceruloplasmin has been solved and shows that the holoprotein contains six copper atoms, two type 1 sites, type 2 and 3 sites and a type 4 site (Zaitseva et al., 1996). The type 3 and type 2 sites are arranged in trinuclear cluster which is similar to that found in laccase and imparts ferroxidase activity on the protein (Rydén, 1988). The protein is synthesized in hepatocytes and copper incorporation takes place early in the secretory pathway possibly in the golgi or rough endoplasmic reticulum, with N-glycosylation not being required for copper loading (Sato and Gitlin, 1991). Although the level or availability of copper has no effect on the synthesis or secretion of the protein, apoceruloplasmin lacking oxidase activity is unstable and rapidly degraded once secreted (Gitlin et al., 1992). The
### Table 1.3
Developmentally Important Copper-Containing Enzymes in Humans

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Function</th>
<th>Consequence of Deficiency</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytochrome oxidase (EC 1.9.3.1)</td>
<td>Electron transport chain</td>
<td>Muscle weakness, neurological effects, hypothermia</td>
</tr>
<tr>
<td>Superoxide dismutase (EC 1.15.1.1)</td>
<td>Free radical scavenger</td>
<td>Implicated in amyotrophic lateral sclerosis (ALS)</td>
</tr>
<tr>
<td>Tyrosinase (EC 1.14.18.1)</td>
<td>Melanin synthesis</td>
<td>Loss of pigmentation</td>
</tr>
<tr>
<td>Dopamine-β-hydroxylase (EC 1.14.17.1)</td>
<td>Catecholamine production</td>
<td>Neurologic effects</td>
</tr>
<tr>
<td>Lysyl oxidase (EC 1.4.3.13)</td>
<td>Cross-linking of collagen and elastin</td>
<td>Arterial abnormalities, loose skin and joints</td>
</tr>
<tr>
<td>Ceruloplasmin (EC 1.16.3.1)</td>
<td>Ferrooxidase, possible copper transporter</td>
<td>Anemia, secondary copper deficiency</td>
</tr>
</tbody>
</table>
precise function of ceruloplasmin in the body is not known, although roles in copper transport (Campbell et al., 1981; Dameron and Harris, 1987; Hilton et al., 1995; Hsieh and Frieden, 1975; Marceau and Aspin, 1973) and iron metabolism (Frieden, 1986; Harris, 1995a; Roeser et al., 1970), due to its ferroxidase activity, have been proposed.

The trinuclear copper cluster is responsible for activating oxygen during the catalytic cycle of the protein and residues in both the amino and carboxy terminus of the protein provide essential ligands for the trinuclear copper center (Zaitseva et al., 1996). Studies have shown that ceruloplasmin plays a key role in the mobilization and oxidation of iron stores and its subsequent incorporation into transferrin (Osaki and Johnson, 1969). This is supported by the observation that animals deprived of dietary copper have little or no circulating holoceruloplasmin and are unable to release iron into the plasma from tissue stores (Roeser et al., 1970). Mutations in the ceruloplasmin gene have been linked to aceruloplasminemia, a disorder characterized by progressive neurodegeneration of the retinal and basal ganglia (Harris et al., 1998). The mutations characterized so far have been shown to abolish amino acids in the carboxy terminus which are critical for the formation of the trinuclear copper cluster (Daimon et al., 1995; Harris et al., 1996; Harris et al., 1995; Okamoto et al., 1996; Takahashi et al., 1996; Yoshida et al., 1995). This correlates with the finding of little or no oxidase activity in the plasma of affected individuals (Harris et al., 1998).

Lysyl oxidase is an extremely important enzyme, particularly during development, since it is responsible for the formation and function of connective tissue throughout the body (Linder, 1991). Lysyl oxidase is a secreted protein of about 32 kDa in size with one tightly bound copper atom in the +2 oxidation state, ligated by four ligands (at least three of which
are nitrogen) in a distorted tetrahedral environment (Gacheru et al., 1990). The enzyme functions by catalyzing the oxidative deamination of lysine and hydroxylysine side chains and oxidizing the ε-amino carbon to an aldehyde to form allysine. The resulting aldehyde groups serve as the center for a cascade of spontaneous reactions involving the side chains of non-oxidized lysine residues which results in the formation of inter- and intramolecular crosslinks which stabilized the collagen and elastin fibers (Linder and Hazegh-Azam, 1996). Like other copper oxidases, lysyl oxidase uses molecular oxygen and 6-hydroxy dopa to carry out the oxidative deamination with the release of ammonia and peroxide.

Lysyl oxidase is widely expressed and is especially abundant in skin, veins, arteries and other structures containing large amounts of connective tissue (eg. cartilage). It is often isolated as four isoforms each with a molecular weight of 32 kDa. The enzymes are synthesized as a pro-polypeptide of 50 kDa which is post-translationally modified by proteolysis and glycosylation to produce the final mature form of 32 kDa (Romero-Chapman et al., 1991; Trackman et al., 1992; Trackman et al., 1990). Little is known about how the enzyme is regulated; however, many studies have pointed to copper as an important regulator of lysyl oxidase. Early reports on the effects of dietary copper deficiency on lysyl oxidase activity indicated that depressed copper levels resulted in decreased lysyl oxidase activity in both chick aorta and lung (Harris, 1986; Harris et al., 1974). The lower lysyl oxidase levels resulted in the proportion of salt extractable or non-crosslinked collagen to increase in these tissues. Furthermore, the authors observed that feeding copper deficient animals trace amounts of copper or adding it to cultures of aortic tissue resulted in the immediate restoration of lysyl oxidase activity (Harris, 1976; Rayton and Harris, 1979). More recent studies in rats have shown that even though copper deficiency results in a depression of lysyl
oxidase activity, the steady state levels of lysyl oxidase mRNA are unaffected (Rucker et al., 1996). These results suggest that copper does not seem to be involved in the regulation of lysyl oxidase synthesis even though it is required for activity.

Superoxide dismutase (SOD) is a 32 kDa homodimeric protein which usually contains one mole of copper and one mole of zinc per monomer. Manganese and iron variants of the enzyme also occur, especially in bacteria (Linder and Hazegh-Azam, 1996). The copper binding site in copper-zinc SOD is type 2, and the copper atom is coordinated by four histidine residues, one of which serves as a bridging ligand between the copper atom and the zinc atom. The copper and zinc sites are located at the bottom of a long narrow channel where the substrate diffuses into the active site. Mutation of one of the histidine residues surrounding the copper to a cysteine results in the creation of a stable copper site closely resembling that of type 1 copper (Lu et al., 1992). Although the activity of SOD requires the presence of two essential metals, copper and zinc, its expression in many tissues is only regulated by copper (Harris, 1992a; Harris, 1992). Copper-zinc SOD is present in the cytoplasm of eukaryotic cells where it is responsible for the dismutation of superoxide (O$_2^-$) to hydrogen peroxide (H$_2$O$_2$) and O$_2$ through the cyclic reduction and oxidation of the bound copper atom (McCord and Fridovich, 1969). Biochemical and immunological studies have indicated that the enzyme is located in peroxisomes (Dhaunsi et al., 1992; Keller et al., 1991).

It has also been shown that SOD is a critical enzyme in antioxidant defense. Oxygen exposure has been shown to increase SOD activity as well as mRNA levels in both human endothelial cells and yeast (Bermingham-McDonogh et al., 1988; Linder, 1991). This correlates well with the finding that transgenic mice with elevated SOD activity have
increased resistance to reperfusion injury after stroke, which in many cases is ascribed to peroxidative processes (Maitre et al., 1993). Genetic analysis has shown that 10-15% of familial amyotrophic lateral sclerosis (FALS) cases are linked to mutations in the SOD1 gene (Deng et al., 1993; Rosen et al., 1993).

Many of the point mutations in the SOD1 gene lead to a mutant protein which is still able to bind copper and has normal superoxide scavenging activity (Corson et al., 1998). These results suggest that loss of superoxide scavenging activity is not the primary cause of FALS, indicating that FALS may be a result of a gain-of-function associated with these mutations (Gurney et al., 1994; Ho et al., 1998; Reaume et al., 1996; Ripp et al., 1995; Wong et al., 1995). The most promising theory puts forth the idea that these mutations lead to a less restrictive active site, allowing access of other substrates which could lead to aberrant chemistry at the copper atom. These substrates may include hydrogen peroxide (H$_2$O$_2$) and peroxynitrite (ONOO$^-$) which have been shown to react with copper-zinc SOD in vitro to oxidize substrates and catalyze the nitration of phenolic groups, respectively (Beckman et al., 1993; Hodgson and Fridovich, 1975; Ischiropoulos et al., 1992; Wiedau-Pazos et al., 1996). The toxic products produced by these aberrant reactions may be the cause of FALS; however, further work is required to confirm these hypotheses.

1.3 Copper Homeostasis in Humans

To avoid the toxic effects of copper excess or deficiency, an efficient method for the uptake, distribution and excretion of copper is needed. Although many aspects of copper transport have been studied for over the past 30 years, until recently little was known about the molecular aspects of copper trafficking within the body. The recent discovery of the
Wilson and Menkes copper transporting ATPases, together with the identification of copper chaperones, has given us an unprecedented understanding of the mechanisms behind copper homeostasis. These discoveries and those to follow will be critical in developing new treatments for disorders of metal metabolism.

1.3.1 Copper Uptake and Circulation

Copper uptake in humans is confined mainly to the stomach and small intestines. Although the overall mechanism of copper uptake in the intestines is not understood, several studies have identified several key features of the system. It has been demonstrated in several studies that copper uptake in the intestines is inversely related to the amount of copper ingested (Turnlund et al., 1989; Turnlund et al., 1985). Thus with very high copper diets, the absorption of copper in the intestines can be as low as 12% of maximum. A theoretical absorptive maximum of 65% of ingested copper has been suggested, but, with typical diets the actual copper uptake is about 40% (Wapnir, 1998). These observations have strengthened the view that intestinal copper uptake plays a central role in the regulation of overall copper absorption. Intestinal copper absorption is influenced by many dietary factors such as pH, vegetable fibers, carbohydrates, and fats (table 1.4). The effect of pH on copper absorption has been studied in the rat and it was found that the rate of copper absorption in the ileum is higher at pH 5.8 or 7.8 than at pH 6.3 – 7.3 (Wapnir and Stiel, 1987).

Sodium has also been suggested to be involved in the uptake of copper by the intestinal mucosa. Perfusion studies using rat ileum and jejunum found that the rate of copper uptake was significantly decreased when sodium was omitted from the perfusate
Table 1.4
Dietary Modifiers of Intestinal Copper Uptake in Humans

<table>
<thead>
<tr>
<th>Modifier</th>
<th>Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fibre</td>
<td></td>
</tr>
<tr>
<td>Hemicellulose</td>
<td>↓</td>
</tr>
<tr>
<td>Carbohydrates</td>
<td></td>
</tr>
<tr>
<td>Fructose</td>
<td>↓</td>
</tr>
<tr>
<td>Glucose polymers</td>
<td>NK</td>
</tr>
<tr>
<td>Fats</td>
<td></td>
</tr>
<tr>
<td>Long-chain fatty acids</td>
<td>NK</td>
</tr>
<tr>
<td>Medium-chain fatty acids</td>
<td>NK</td>
</tr>
<tr>
<td>Protein</td>
<td></td>
</tr>
<tr>
<td>High-protein diet</td>
<td>↑</td>
</tr>
<tr>
<td>Excess amino acids</td>
<td>±</td>
</tr>
<tr>
<td>Organic acids</td>
<td></td>
</tr>
<tr>
<td>Ascorbic acid</td>
<td>±</td>
</tr>
<tr>
<td>Polybasic amino acids</td>
<td>↑</td>
</tr>
<tr>
<td>Divalent cations</td>
<td></td>
</tr>
<tr>
<td>(Zinc, Iron, Tin, Molybdenum)</td>
<td>↓</td>
</tr>
</tbody>
</table>

NK = Not Known
A similar result was also observed when amiloride was used to inhibit the function of the sodium-proton antiporter and the sodium-calcium exchanger (Wapnir, 1991). Although these results suggest a link between intestinal copper uptake and sodium, the nutritional and biological significance of such a link is unknown. The effect of carbohydrates, especially fructose, has been studied extensively in rats. The effect of high fructose diets in rats can lead to copper uptake insufficiency and these effects seem to be both species- and sex-dependent (Fields et al., 1984; Reiser et al., 1983). This effect has also been observed in humans where high fructose diets can lead to decreased copper status (Reiser et al., 1985). The widespread use of fructose-containing sweeteners and the finding that copper sufficiency is closely linked to cholesterol metabolism makes these studies especially relevant (Klevay, 1987).

The effect of amino acids on intestinal copper absorption is varied and is directly linked to their concentration in relation to copper. One study in humans found that subjects whose diet was supplemented with methionine had a net copper absorption which was nearly double that of unsupplemented controls (Kies et al., 1989). Conversely, an excess of dietary amino acids such as histidine and cysteine can have serious consequences on copper uptake. In perfusion studies using rats, excess dietary histidine has been shown to cause increasing losses of copper through the urine and to reduce copper uptake significantly (Harvey et al., 1981). These observations probably stem from the ability of histidine to chelate copper, thereby reducing its bioavailability, or competing for binding with the intestinal transport proteins. A similar effect has also been observed for cysteine. In addition to being an effective chelator for copper, cysteine is also able to reduce copper from the divalent to monovalent state, having a deleterious effect on copper uptake (Baker and Czarnecki-
Maulden, 1987). Other divalent cation such as zinc, ferrous iron, and stannous tin can act in a competitive manner to decrease intestinal copper uptake (Hall et al., 1979; Pekelharing et al., 1994; Van Campen and Scaife, 1967; Yu et al., 1994a).

The transport of copper from the intestinal lumen to the intestinal mucosa is a carrier-mediated process involving at least one saturable component (Crampton et al., 1965; Turnlund et al., 1989). Although the identity of the intestinal copper transport protein is not known, the Menkes disease copper transporting ATPase is a strong candidate in light of the Menkes phenotype and the strong expression of the protein in the intestinal mucosa (Chelly et al., 1993; Mercer et al., 1993; Vulpe et al., 1993). Once copper enters the blood stream it becomes bound to a variety of proteins. More than 90% of copper is bound to ceruloplasmin, a large multicopper serum glycoprotein; however, this copper is tightly bound and considered non-exchangeable (Linder and Hazegh-Azam, 1996). The remaining 5 – 10% is exchangeable and is bound to albumin, and amino acids as low molecular weight complexes. Several careful and detailed studies revealed that the main copper-amino acid complex in the serum was in the form of a 1:2 copper-histidine complex (Neumann and Sass-Kortsak, 1967; Sarkar and Kruck, 1966). Further studies also revealed that histidine could form a ternary complex with albumin and copper (Lau and Sarkar, 1971; Sarkar and Wigfield, 1968). The copper binding site on albumin has been very well characterized (Iyer et al., 1978; Laussac and Sarkar, 1980; Laussac and Sarkar, 1984). The copper binding site on albumin is at the amino terminus and involves the first three residues (Asp-Ala-His). Copper is ligated in a pentacoordinate arrangement using the amino-terminal nitrogen, the carboxyl side chain of aspartic acid, two intervening peptide nitrogens, and an imidazole nitrogen from a histidine
residue in the third position. The four nitrogen ligands are in a square-planar arrangement with the carboxyl oxygen occupying the axial position.

Copper has also been observed to bind to another large serum protein found in rats known as transcuprein (Linder, 1991; Weiss and Linder, 1985). Since this protein has yet to be cloned and has not been fully characterized, conclusions cannot be drawn regarding its biological function (Barrow and Tanner, 1988; Goode et al., 1989; Tsai et al., 1992). It is clear that albumin is responsible for carrying the majority of newly absorbed copper ions in the bloodstream. Approximately 40 μg of copper are required to fill all the high affinity albumin copper sites in 1 ml of human serum, but the amount that actually binds in vivo is far less (Linder and Hazegh-Azam, 1996). Although albumin is an important transporter of copper in the bloodstream, its role in the uptake of copper by various tissues remains unknown. When albumin is completely absent, as is the case in analbuminemia, there does not seem to be any disruption in the distribution of copper (Vargas et al., 1994). Although albumin levels in the nagase rat are 4000 times lower than normal, intravenously injected $^{67}$Cu entered the liver at least as fast in these animals as in normal animals and its reappearance in ceruloplasmin seemed to be accelerated. These findings suggest that albumin may be playing a passive role in copper transport, acting as a reservoir for newly absorbed copper. This view is strengthened by the observation that copper uptake by hepatocytes is inhibited by albumin and no cell surface receptors for albumin have been found on these cells (Ettinger et al., 1986). It is likely that albumin-bound copper must exchange with other components, which then facilitate its uptake into cells. Transfer of copper between albumin and transcuprein has been shown, as has exchange between albumin and histidine (Lau and Sarkar, 1971; Sarkar and Wigfield, 1968; Tsai et al., 1992; Weiss and
Linder, 1985). The former may be important in rodents while the latter is more important in humans.

1.3.2 Membrane Transport of Copper

There are at least two pathways by which cells take up copper from the bloodstream (fig. 1.1). The first pathway involves copper release and uptake from ceruloplasmin. Ceruloplasmin has been shown to be able to donate copper to cells as well as to intracellular, copper-containing proteins (Campbell et al., 1981; Dameron and Harris, 1987; Hsieh and Frieden, 1975; Marceau and Aspin, 1973). The uptake of copper from ceruloplasmin is believed to occur via interaction with a cell surface ceruloplasmin receptor, releasing ceruloplasmin bound copper. The released copper is then reduced by a cell surface reductase and enters the cell through an energy-independent transport protein. Although the nature of the uptake protein has not been established conclusively, hCTR1 has been proposed to be the high affinity copper uptake protein in humans (Zhou and Gitschier, 1997). This protein was initially discovered by its ability to complement the yeast high affinity copper uptake mutant, ctr1. The human CTR1 protein shares 29% identity with its yeast counterpart; however, it is much smaller in both the intracellular and extracellular metal binding domains (Zhou and Gitschier, 1997). A second protein, hCTR2, which is similar to hCTR1 was discovered through database searching and both genes are expressed in all tissues. The exact role of these proteins in the uptake of copper remains to be established.
Figure 1.1 Intestinal and Cellular Copper Uptake Pathways. Copper is absorbed from the gastrointestinal tract and enters the exchangeable pool. During uptake, copper is reduced to Cu(I) by a hypothetical membrane reductase and is absorbed by the cell through a passive transporter, possibly hCTR1. In the ceruloplasmin (Cp) pathway, copper is released from Cp through interaction with its receptor, followed by reduction and uptake. Once inside the cell, copper becomes bound to glutathione (GSH) and various copper chaperones (ATOX1, Ccs1, Cox17). The Wilson disease ATPase is positioned in the trans-golgi membrane where it pumps copper into the excretory pathway for incorporation into cuproproteins.
Putative ceruloplasmin receptors have been identified in a variety of tissues and cell types; however, those which have been isolated, differ in size and composition (Barnes and Frieden, 1984; Gordon et al., 1987; Kataoka and Tavassoli, 1984; Kataoka and Tavassoli, 1985; Omoto and Tavassoli, 1990; Orena et al., 1986; Stern and Frieden, 1993; Stevens et al., 1984). These differing proteins may represent different components of the ceruloplasmin receptor, all of which have some affinity for ceruloplasmin. Studies on the uptake of ceruloplasmin-bound copper by human erythrocyte cells revealed that ceruloplasmin itself was not internalized when copper was taken up by the cell (Percival and Harris, 1990). This study also found that the addition of ascorbate strongly stimulated uptake of copper while the addition of cuprous chelators inhibited it. In addition, sulfhydryl-modifying reagents were also found to inhibit uptake, implicating cysteine residues in the copper uptake process. Taken together these results suggest that copper is taken up in the monovalent rather than the divalent form (Harris, 1991; Percival and Harris, 1989).

The second pathway involves uptake from non-ceruloplasmin-bound copper such as that bound to histidine, albumin, and other low molecular weight complexes (Harris and Sass-Kortsak, 1967; Sass-Kortsak et al., 1967). As is the case for the ceruloplasmin pathway, uptake of copper does not involve internalization of its ligands (Ettinger et al., 1986; van den Berg and van den Hamer, 1984). Studies on the kinetics of copper uptake by cultured hepatocytes support a system which is carrier-mediated, energy-independent and saturable (Ettinger et al., 1986; McArdle et al., 1988; Schmitt et al., 1983; Stockert et al., 1986; Weiner and Cousins, 1980). Uptake via this pathway can also be inhibited by other metals such as zinc or cadmium, reflecting the similar coordination preferences of these metals, or suggesting that the protein involved is a general metal carrier (Ettinger et al., 1986;
Schmitt et al., 1983). As has been mentioned previously, histidine and albumin have different effects on the uptake of copper. In the absence of albumin, at ratios less than 1:1 with copper, histidine has no effect on copper uptake. At higher concentrations, histidine acts as a weak competitive inhibitor of copper uptake in hepatocytes, but, in the presence of albumin, stimulates the uptake of copper (Darwish et al., 1984). Studies on the uptake of copper by placental cells shows that histidine stimulates copper uptake in the presence of serum while albumin inhibits it (Mas and Sarkar, 1992). These findings and the observation that analbuminemia in humans and animals does not result in a disturbance of copper uptake strongly suggest that histidine plays a central role in the uptake of copper from the bloodstream.

1.3.3 Intracellular Copper Trafficking

Once copper is taken up by the cell, it enters a series of complex pathways which are only now becoming understood (fig 1.2). Much of the absorbed copper is immediately excreted from the cell via pathways which will be discussed in the following section. Both copper and copper proteins are distributed throughout the cell and are present in all cellular organelles including lysosomes, mitochondria, endoplasmic reticulum, nucleus and the cytosol (Vulpe and Packman, 1995). Examples include metallothionein (MT) in the nucleus, cytosol, and lysosomes (Janssens et al., 1984a; Janssens et al., 1984), cytochrome c oxidase in the mitochondria (Kodama et al., 1989), lysyl oxidase in the golgi and secretory organelles (Kuivaniemi et al., 1986), and superoxide dismutase (SOD) in the cytosol and possibly peroxisomes (Crapo et al., 1992). For many years the mechanism by which copper was delivered to various proteins and cellular compartments was not understood. Recently a
family of small cytosolic proteins has been identified which are responsible for the delivery of copper to specific cellular targets. These proteins have come to be known as copper chaperones. All of these proteins were initially identified in yeast and their human orthologues were identified by complementation studies.

The copper chaperone family currently includes three members: ATX1/human ATOX1 (formerly known as HAH1) (Hung et al., 1998; Klomp et al., 1997; Lin et al., 1997), Lys7/human Ccs1 (Culotta et al., 1997; Gamonet and Lauquin, 1998), and Cox17/human Cox17 (Amaravadi et al., 1997; Beers et al., 1997; Glerum et al., 1996). Each of these proteins delivers copper to a different copper containing protein. ATX1 delivers copper to CCC2 (the yeast homologue of WND), lys7 delivers copper to superoxide dismutase and Cox17 delivers copper to the mitochondria for incorporation into cytochrome c oxidase. A direct interaction between these chaperones and their target proteins has only been demonstrated in two cases. ATX1 and the amino-terminal domain of CCC2 have been shown to interact using yeast two-hybrid analysis (Pufahl et al., 1997). In the case of Ccs1 an in vitro interaction with SOD was demonstrated using fusion proteins and an in vivo interaction was demonstrated by co-immunoprecipitation (Casareno et al., 1998). Although it is known that Cox17 is responsible for delivering copper to the mitochondria, no specific target has yet been identified. A possible target of Cox17 that has been proposed is Sco1 (Glerum et al., 1996). Sco1 is a mitochondrial protein which has been shown to be critical for the proper assembly of cytochrome c oxidase and has been localized to the inner mitochondrial membrane (Buchwald et al., 1991; Krummeck and Rodel, 1990; Petruzella et al., 1998; Schulze and Rodel, 1988). Despite these suggestive results, a direct interaction between Cox17 and Sco1 has not been demonstrated in vivo or in vitro.
In most cases the structure of the chaperone is very similar to its target. The primary structure of Atxl is very similar to the amino-terminal metal binding repeats in CCC2 (Pufahl et al., 1997). Atxl also contains one copy of the metal binding motif, Met-Thr-Cys-Xaa-Xaa-Cys, which is most likely involved in metal ligation and is present in two copies at the amino terminus of CCC2 (Yuan et al., 1995). Similarly, the primary structure of Ccs1 bears a striking similarity to the carboxy-terminus of SOD. Since SOD is known to homodimerize, this similarity has lead to the idea that the chaperone dimerizes with SOD to facilitate the transfer of copper between the two proteins. (Casareno et al., 1998; Culotta et al., 1997). Since the cellular target of Cox17 is unknown, no comparisons can be made regarding similarity of structure. The copper binding site of Cox17 has been characterized spectroscopically. The protein was found to contain two copper atoms in a binuclear cuprous-thiolate complex which is substantially more labile than similar clusters in other proteins such as metallothionein (Srinivasan et al., 1998).

Glutathione is also another major chelator of copper in the cytoplasm; however, its function may be more important in the case of copper overload than under normal conditions. Studies on copper-loaded hepatoma cells have indicated that over 60% of cytosolic copper exists as the copper-glutathione complex (Freedman et al., 1989). The copper-glutathione complex can also donate copper to various proteins. In vitro studies have shown that the copper-glutathione complex can donate copper to apo-SOD, apo-metallothionein, and apo-ceruloplasmin (Ciriolo et al., 1990; Ferreira et al., 1993; Musci et al., 1996).

Metallothioneins are small molecular weight (6.5 kDa) metal binding proteins which have several distinguishing features. They have a high metal binding capacity (7 – 10 g atoms/mol); 30% of the amino acids are cysteine yet the native protein has no disulfide
bonds, are devoid of aromatic residues, and exhibit a substantial amount of polymorphism (Cousins, 1985). Metallothionein was originally isolated as a cadmium and zinc binding protein, but is usually found naturally as a zinc binding protein (Kagi and Vallee, 1960). Indeed many of the biological functions of metallothionein seem to revolve around zinc metabolism (Cousins, 1985). It has been suggested that metallothioneins act as a reservoir for zinc, regulating its intracellular availability. In support of this notion, the reversible exchange of zinc between metallothionein and the estrogen receptor (2 zinc finger protein) has been demonstrated, as has the removal of zinc from zinc finger-containing transcription factors such as Sp1 and TFIIB (Cano-Gauci and Sarkar, 1996; Zeng et al., 1991a; Zeng et al., 1991).

Metallothionein synthesis can be induced by a variety of factors including heavy metals, physical stress, glucocorticoids, and heat shock. Metallothionein is able to bind other heavy metals such as copper, cadmium, iron, and mercury (Kagi and Schaffer, 1988). Although the level of metallothionein induction by zinc is greater than that by copper, metallothionein has a higher binding affinity for copper than zinc (Lipsky and Gollan, 1987). It is unlikely that metallothionein plays a central role in the intracellular trafficking of copper, and its primary role in relation to copper may be one of detoxification and storage. Many studies have shown that copper-metallothionein accumulates in the lysosomes of copper-loaded animals, probably as a protective measure of the hepatotoxic effects of excess copper (Gooneratne et al., 1980; Johnson et al., 1981; Verity et al., 1967). The copper stored in metallothionein can be made available to other proteins either following metallothionein degradation in lysosome (Mehra and Bremner, 1983) or by exchange via complexation with glutathione (Freedman et al., 1989). Yeast strains which have had their metallothionein-like
gene knocked out are able to thrive on normal media and maintain normal levels of copper enzymes, but are intolerant to copper stress. Expression of mammalian metallothionein in these strains restores their ability to grow in high copper media, supporting the notion that metallothioneins play a major role in copper detoxification and are not central to normal intracellular copper metabolism (Thiele et al., 1986). It is likely that a similar scenario takes place in mammalian cells.

1.3.4 Excretion of Copper

The efficient excretion of excess copper is an integral component of proper copper metabolism, and the liver plays an important part in this process. Upon being absorbed by the liver, copper may be returned to circulation by incorporation into ceruloplasmin, incorporated into copper containing enzymes, stored within metallothionein, or excreted into the bile. Urinary copper excretion is negligible under normal physiologic conditions since most of the circulating copper is either bound to ceruloplasmin or confined within erythrocytes, and little or no copper permeates the glomerular capillaries (Owen Jr, 1964). The small amount of ingested copper that does appear in the urine most likely originates from the amino acid-bound fraction of plasma copper and the dissociation of the copper-albumin complex. In the overall scheme of copper homeostasis, urinary excretion does not appear to play an important role. However, cupruria does occur in the later stages of pathological conditions which affect primary copper homeostatic mechanisms such as biliary copper excretion, copper storage and ceruloplasmin synthesis.

Biliary excretion is an important mechanism for the excretion of many trace metals (table 1.5) and accounts for ~80% of the copper leaving the liver (Evans, 1973). The
Table 1.5
Metal Content of Rat Bile

<table>
<thead>
<tr>
<th>Metal</th>
<th>Concentration (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Manganese</td>
<td>5.4 ± 0.8</td>
</tr>
<tr>
<td>Iron</td>
<td>18.5 ± 1.5</td>
</tr>
<tr>
<td>Copper</td>
<td>27.5 ± 3.0</td>
</tr>
<tr>
<td>Zinc</td>
<td>5.5 ± 0.08</td>
</tr>
<tr>
<td>Molybdenum</td>
<td>3.6 ± 0.3</td>
</tr>
<tr>
<td>Vanadium</td>
<td>1.9</td>
</tr>
<tr>
<td>Chromium</td>
<td>2.1</td>
</tr>
<tr>
<td>Strontium</td>
<td>1.3 ± 0.8</td>
</tr>
<tr>
<td>Lead</td>
<td>2.8 ± 0.7</td>
</tr>
</tbody>
</table>

Adapted from (Dijkstra et al., 1996).
exact mechanism of copper excretion into the bile is not known; however, three potential routes can be distinguished: i) lysosomal exocytosis, ii) a glutathione-dependent route (via glutathione conjugate transporter), and iii) secretion by a copper transporting ATPase. The idea that lysosomes are the source of copper which is excreted into the bile stems from a large number of studies which have shown that large amounts of copper are stored within hepatic lysosomes under certain physiologic conditions (Mohan et al., 1995; Sternlieb, 1989). In a kinetic study where $^{64}$copper was administered orally to a single Wilson disease patient the specific activity of hepatic lysosomes was similar to that found in the bile (Sternlieb et al., 1973). This finding, together with the observation that lysosomes could normally release their contents into the bile by exocytosis (De Duve and Wattiaux, 1966; LaRusso and Fowler, 1979), strengthened the idea that lysosomes are the primary source of biliary copper. Studies in copper-loaded rats have also shown an association between biliary copper output and the activity of lysosomal enzymes in bile (Gross et al., 1989) and the authors suggest that exocytosis of lysosomal contents into bile represents a major mechanism for biliary copper excretion under these conditions. Much of the copper stored in lysosomes has been found to exist as a complex with a polymerized form of metallothionein (Gross et al., 1989).

Glutathione (GSH) has been implicated in the biliary excretion of copper since the early eighties when it was shown that administration of diethylmaleate (DEM) to rats lead to a strong reduction in the biliary excretion of copper, concomitant with a decrease in hepatic GSH levels (Alexander and Aaseth, 1980). The inhibitory effect of DEM is also seen with intravenously administered copper following DEM treatment. DEM is able to form conjugates with GSH, leading to a lowering of available hepatic and biliary GSH. Analysis
of bile fluids from rats after copper administration revealed that copper in the bile is conjugated to GSH (Alexander and Aaseth, 1980). In studies with normal rats it was noticed that intravenously administered copper was eliminated in a biphasic manner composed of a rapid phase immediately after injection, followed by a slow gradual phase (Nederbragt, 1989). In normal rats the rapid phase is inhibitable with DEM, but the slow phase is not, suggesting different mechanisms for each process (Houwen et al., 1990). This has been confirmed in the mutant GY rat which, despite doubled hepatic GSH levels, is unable to secrete GSH into the bile. In these animals, the rapid phase is absent; however, the slow phase as well as secretion of endogenous copper do not seem to be affected (Dijkstra et al., 1990; Houwen et al., 1990).

These studies strongly suggest that an adequate supply of intracellular GSH is required for the proper excretion of both endogenous copper and intravenously administered copper into bile. However, the rapid phase of biliary copper excretion is only present when GSH is able to be secreted into the bile. In light of these findings, it seems probable that the rapid phase involves transport of copper-GSH complexes into bile (fig. 1.2) and this would most likely occur through the canalicular multispecific organic anion transporter (cMOAT) (Lee et al., 1997). This finding has been further elaborated by studies of dietary copper overload in mutant GY rats. In these studies, it was shown that GY rats fed high copper diets accumulated copper in their livers to the same extent as control animals and had the same rate of biliary copper excretion (Dijkstra et al., 1990; Houwen et al., 1990). This implies that GSH and cMOAT are not involved in this process, leading the authors to suggest that there is another saturable copper transport system in the rat liver.
The Wilson disease copper transporting ATPase (WND) has been implicated as this saturable component in the rat liver. Although some have demonstrated ATP dependent transport in isolated rat liver plasma membranes (Dijkstra et al., 1995), localization studies have not identified the presence of the Wilson disease ATPase on the canalicular membrane. However, WND may still play an important part in biliary copper excretion. Localization studies have shown that WND resides in the trans-golgi under steady-state conditions, but undergoes a reversible, copper-specific translocation to a cytosolic vesicular compartment under elevated copper concentrations (Hung et al., 1997). These vesicles could be lysosomes; however, ATP dependent copper transport in pure lysosomal membranes has not been evaluated. The ATPase would pump copper into this vesicle, which could then fuse with the canalicular membrane and dump its contents into the bile. In this case, GSH has been postulated to reduce copper(II) to copper(I), leading to the formation of copper(I)-GSH, which could donate copper(I) to WND. The copper chaperone, ATOX1, would also be involved in the donation of copper to WND, in addition to the copper(I)-GSH pathway. This step may be critical in the efficient transport of copper by this ATPase, as has been demonstrated for bacterial copper transporting ATPases (Solioz and Odermatt, 1995). In either case, it is clear that GSH plays a central role in the biliary excretion of copper.

1.4 Cation Transporting ATPases

Cation transporting ATPases (also known as ion motive ATPases) are responsible for the translocation of ions across biological membranes and are critical for the maintenance of ion gradients, proper intracellular ion composition and ion resistance. These membrane bound pumps are found in bacteria, plants, fungi and animals and function by using the
energy obtained from the hydrolysis of ATP to move the ion across the membrane. The known ATPases have been classified into three major groups; P-type, V-type, and F-type (F$_1$F$_0$-type) ATPases. P-type ATPases are distinguished from the other two types by the formation of a phosphorylated intermediate during the transport cycle. V- and F-type ATPases are structurally and genetically related; however, they have different mechanisms of action. F-type ATPases are normally run in reverse using an ion gradient to generate ATP. An example of this is the ATP synthase, located in the inner mitochondrial membrane, which uses the H$^-$ gradient generated from oxidative respiration to drive the synthesis of ATP from ADP (Fillingame, 1997). In contrast to F$_1$F$_0$ ATPases, V-type or vacuolar ATPases function exclusively as proton pumps using the hydrolysis of ATP to drive the reaction. V-type ATPases are involved in energizing intracellular compartments of the vacuolar system providing an electrochemical gradient for the transport of solutes and acidifying several of these compartments (Gluck, 1992).

Recently, two genes which are defective in two human genetic disorders of copper metabolism (Menkes and Wilson disease) have been shown to encode copper transporting P-type ATPases (MNK and WND respectively). These were the first heavy metal transporting ATPases identified in humans. They are part of a larger family of heavy metal transporters found in bacteria and yeast, responsible for the transport of a diverse set of metals, including copper, cadmium, zinc, mercury and arsenic (Silver, 1996). Although similar in many respects to other P-type ATPases, the heavy metal ATPases have some unique features, the functions of which are beginning to be elucidated. The following sections will provide a brief outline of the structural features of each type of ATPase noting similarities and differences.
1.4.1 Types of Cation Transporting ATPases

1.4.1.1 P-Type ATPases

Characteristic features of P-type ATPases which distinguish them from the other two types (V and F) are the formation of a phosphoenzyme intermediate, cycling between two distinct forms (E₁ and E₂) and inhibition by vanadate (Moller et al., 1996). The mechanism of action of two extensively studied P-type ATPases the Na⁺/K⁺-ATPase (Glynn and Karlish, 1990) and the sarco(endo)plasmic reticulum Ca²⁺-ATPase (SERCA) (MacLennan et al., 1997a) are depicted in figure 1.2. Generally, this mechanism can be represented by a four step reaction E₁ → E₁P → E₂P → E₂ → E₁ and is referred to as the Post-Albers scheme (Post et al., 1972). In the first step, a phosphorylated intermediate (E₁P), with bound cations, is formed by the reaction of the enzyme with MgATP. This is a high energy intermediate which can be dephosphorylated with ADP reforming ATP. Following phosphorylation of the protein, structural changes occur which lead to the translocation of the bound ions across the membrane. This in turn results in the conversion of the phosphorylated enzyme to the low-energy E₂P form which is unreactive with ADP. In this state, the affinity of the enzyme for its respective ions is low. The final steps in the reaction involve removal of the phosphate group by hydrolysis and reformation of the E₁ state. In many cases reforming the E₁ state involves the transfer of other ions, K⁺ for the Na⁺/K⁺-ATPase and H⁺ for SERCA (Levy et al., 1990; Yu et al., 1993). The vanadate ion (VO₄³⁻) is a potent inhibitor of P-type ATPases. It is a transition state analogue of phosphoryl transfer reactions because it readily adopts a pentacovalent bipyramidal geometry. Its inhibitory action most likely stems from its ability to form a stable transition state complex with the ATPase (Cantley et al., 1978).
Figure 1.2 *Transport Mechanism SERCA and Na⁺/K⁺ ATPases*. A SERCA mechanism. Calcium and ATP bind in the cytoplasm resulting in phosphorylation of the E₁ enzyme (1) which undergoes a conformational change to the E₂ state (2) releasing calcium into the lumen (3), the enzyme is then dephosphorylated and the E₁ state is reformed (4). B Na⁺/K⁺ mechanism. Sodium and ATP bind in the cytoplasm resulting in phosphorylation of the E₁ enzyme (1) which rapidly changes conformation to E₂ (2). A series of reactions then occur, which results in the release of sodium and binding of potassium outside the cell, followed by dephosphorylation of the E₂ enzyme (3). The dephosphorylated E₂ enzyme undergoes another conformational change back to E₁, with the release of potassium into the cytoplasm.
The P-type ATPases can be divided into two main groups based on the type of ion transported, either heavy-metal (Type I) or non-heavy-metal (Type II) (figure 1.3). All heavy metal transporting ATPases have similar hydropathy profiles which are significantly different from those of non-heavy metal transporting ATPases. Members of the type I group include bacterial copper and cadmium ATPases (Nucifora et al., 1989; Odermatt et al., 1993), the nitrogen fixation protein fixI (Kahn et al., 1989), the human copper transporting ATPases (MNK and WND) (Bull et al., 1993; Tanzi et al., 1993; Vulpe et al., 1993) and many others. All members of this family have between 6 and 8 transmembrane segments with only one pair of transmembrane segments on the carboxy-terminal side of the cytoplasmic ATP binding domain. They also contain a highly hydrophobic region, immediately preceding the TGES/A conserved motif, which probably gives rise to two additional transmembrane segments in this region. This is in contrast to members of the type II family which contain only one pair of transmembrane segments in this region (Lutsenko and Kaplan, 1995). However, the most striking and interesting feature of the type I ATPases is the presence of a large amino-terminal cytosolic domain which contains between one and six copies of the metal binding repeat GMTCXXC or M/HXXMDHS/GXM (Bull and Cox, 1994; Petrukhin et al., 1994; Silver et al., 1993). The variable number of these repeats leads to a great diversity of molecular weights among members of the type I family of ATPases.

In contrast to type I ATPases, the type II, non-heavy metal transporting ATPases only have one pair of transmembrane segments before the TGES energy transduction motif. Members of this family have a catalytic subunit which consists of 8 to 10 transmembrane segments and are approximately 100 kDa in size (Lutsenko and Kaplan, 1995). The type II ATPases can be further subdivided into two groups IIA and IIB. Type IIA
Figure 1.3  Topology of Type I and Type II P-Type ATPases. Type I (heavy metal) and Type II (non-heavy metal) P-type ATPases. White blocks represent core structure, gray blocks represent putative or additional transmembrane segments. Conserved motifs are indicated, TGES (energy transduction), DKTG (phosphorylation site), GDGXD (hinge region), SEHPL (unknown function, type I only), GMTCXXC or M/HXXMDHS/GXM (metal binding, n = 1-6).
ATPases have a heterodimeric structure consisting of a catalytic \( \alpha \)-subunit and a glycosylated \( \beta \)-subunit. Examples of type IIA ATPases include the Na\(^+\)/K\(^-\) and H\(^+\)/K\(^-\) ATPases which exchange intracellular Na\(^+\) or H\(^+\) for extracellular K\(^-\) (Glynn and Karlish, 1990; Sachs et al., 1989). Type IIB ATPases function as monosubunit proteins transporting divalent cations into intracellular compartments or into the extracellular milieu. Members of this family include the calcium ATPases of the plasma membrane and sarco(endo)plasmic reticulum and the H\(^+\)-ATPases of fungi and plants (Moller et al., 1996). The bacterial inducible, high affinity K\(^-\) uptake ATPase (KdpK\(^-\)-ATPase) is somewhat different from both type I and type II ATPases. Like the type II ATPases, this protein has only one pair of transmembrane segments before the TGES motif and has no metal binding sites at the amino-terminus. However, it is also missing two transmembrane segments at the carboxy-terminus which make it shorter than other type II proteins and, in this respect, it resembles the type I proteins. Unlike the heavy metal transporters which function as monomers (Nucifora et al., 1989; Tsai and Linet, 1993), the KdpK\(^-\)-ATPase is active as a multisubunit complex. The complex consists of three subunits, A, B, and C, which contain 557, 682, and 189 amino acids, respectively (Hesse et al., 1984). The phosphorylation site is located in subunit B, while subunits A and C come together to form the transmembrane channel (Altendorf et al., 1994; Buurman et al., 1995).

The core structure of P-type ATPases contain many conserved motifs. Among these are the TGES, DKTG, and GDGXDN motifs. The TGES motif is located in the cytoplasmic loop between transmembrane segments 2 and 3, which is sometimes referred to as region B (Moller et al., 1996). This region has been implicated in energy transduction based on site directed mutagenesis of the TGES residues and proteolytic cleavage experiments at residues.
close to this motif in the SERCA ATPase (Clarke et al., 1990; Imamura and Kawakita, 1989; Juul et al., 1995; le Maire et al., 1990). These mutagenesis and cleavage experiments resulted in proteins which retained calcium binding and phosphorylation capacity, but were not able to transport calcium. Based on these results, it has been hypothesized that modifications in this region prevent the conformational change from the E\(_i\)P form to the E\(_2\)P form, leading to an accumulation of E\(_i\)P with bound calcium (Moller et al., 1996).

The DKTG motif is located in the large cytoplasmic loop between transmembrane segments 4 and 5 and is referred to as domain C. The DKTG motif is critical to the function of the ATPase, since it contains the conserved aspartate residue which is phosphorylated in the transport cycle of the protein. Mutation analysis in the SERCA ATPase has shown that both the aspartate and neighboring lysine residue are needed for phosphorylation to take place and cannot be replaced by any other residues (Maruyama and MacLennan, 1988). Furthermore, mutation of these residues or transposition of the aspartate and lysine residues (DK → KD) results in loss of phosphorylation and calcium transport (Maruyama and MacLennan, 1988). These results suggest that the lysine residue may be part of a stereospecific, acid-base catalyzed transfer of phosphate from ATP to the aspartate residue.

The GDGXDN 'hinge' motif is located at the end of domain C just before it enters the membrane again with transmembrane segment 5 and is referred to as region J. This region is predicted to be highly alpha-helical and has been predicted to form part of the 'stalk' of the ATPase (Moller et al., 1996).

The hinge motif also contains two aspartate residues which seem to be required for phosphorylation, as indicated by chemical modification and mutagenesis studies on both the Na\(^+\)/K\(^+\) and SERCA ATPases (Lane et al., 1993; Ohta et al., 1986; Vilsen et al., 1991).
Modification studies in the Na\(^+\)/K\(^+\) ATPase also indicate that this region may interact closely with the ATP binding region of the protein (Ohta et al., 1986). The SHEPL motif is only present in heavy metal transporting P-type ATPases in domain C, just after the phosphorylation site. The histidine in this motif is critical for proper function of the protein since mutation of this residue to serine in the Wilson disease copper ATPase leads to the Wilson disease phenotype (Petrukhin et al., 1993). Although no specific function has yet been ascribed to this motif in the metal ATPases, the analogous region in SERCA and plasma membrane Ca-ATPases is a site of regulation through interaction with phospholamban (James et al., 1989; Toyofuku et al., 1993) and calmodulin (Falchetto et al., 1991) respectively.

1.4.1.2 V-type and F-type ATPases

V-type (or vacuolar) ATPases are structurally related to F-type ATPases and hence will be considered together in this section. V- and F-type H\(^+\) ATPases differ from the P-type ATPases in two important ways. First, V- and F-type ATPases are large multisubunit complexes, in contrast to P-type ATPases which usually function as monosubunit proteins. Second, although they utilize the energy of ATP hydrolysis, V- and F-type ATPase do not utilize a phosphoenzyme intermediate (Nelson and Taiz, 1989). V-type ATPases pump protons from the cytoplasm to the lumen of vacuoles and other cellular organelles such as lysosomes, endoplasmic reticulum, golgi bodies and secretory granules. As such, they are closely involved in the maintenance of cellular pH and energizing intracellular compartments (Finbow and Harrison, 1997). Since the V-type ATPase does not use a counterion, its activity is electrogenic, creating an electrical potential difference (\(\Delta\Psi\)) across the membrane. This membrane potential is then used to drive solutes and other ions across the organelle.
membrane, as occurs in the accumulation of neurotransmitters in synaptic vesicles and amino acids in the vacuole lumen (Nelson, 1994). The acid pH conditions created by the pumping of protons into vacuoles is used for other functions as well (Mellman, 1992). In lysosomes, low pH is needed to activate acid hydrolases and the dissociation of receptor-ligand complexes (eg. Fe(III) from transferrin) (Brown et al., 1983). V-type ATPases are also closely involved in the trafficking within the endosomal / lysosomal compartment. Bafilomycin A1, a specific inhibitor of V-type ATPases, causes a decrease in the rate of receptor cycling (Johnson et al., 1993). It has since been found that acidification is a necessary step in the formation of endosome carrier vesicles, which are intermediates between early and late endosomes (Clague et al., 1994).

F-type ATPases (also known as F1F0-ATP synthases) function in reverse when compared to V-type ATPases. Whereas V-type ATPases use the hydrolysis of ATP to drive the creation of a membrane potential, F-type ATPases use a membrane potential to drive the synthesis of ATP from ADP. In animal cells, the enzyme is located in the inner mitochondrial membrane where it uses the proton gradient established by oxidative respiration to drive the synthesis of ATP. Closely related F-type ATPases are found in the thylakoid membrane of plant chloroplasts, and in the plasma membrane of bacteria such as E. coli. In plants, the enzyme functions in electron transport-driven photophosphorylation, while in bacteria the pump can operate reversibly depending on the nutritional status of the cell (Fillingame, 1997). Under aerobic growth conditions, a proton gradient is created across the plasma membrane by electron transport systems by pumping protons from the cytoplasm to the outside of cell. The proton gradient is then used to generate ATP and is also utilized in proton-coupled nutrient transport and flagellar rotation (Fillingame, 1997). Under anaerobic
conditions, the proton gradient is generated by the hydrolysis of glycolytically derived ATP by the F$_1$F$_0$-ATPase, pumping protons from the cytoplasm to the outside of the cell. All three F$_1$F$_0$-ATPases (mitochondrial, chloroplast, and bacterial) are structurally and mechanistically related, however they are regulated differently (Harris, 1995; Mills et al., 1995; Walker, 1994).

The F-type ATPases are composed of two structurally and functionally distinct sectors termed F$_1$ and F$_0$. The catalytic sector F$_1$ extends from the surface of the membrane as a knob-like projection. The F$_1$ sector can be removed from the membrane as a water soluble protein capable of hydrolyzing ATP (Fillingame, 1997). The F$_1$ sector is connected to the membrane bound F$_0$ sector via a narrow stalk. When the F$_1$ sector is not present, the F$_0$ sector can mediate the passive transport of protons across the membrane. Once properly assembled, the complex mediates the reversible coupling of ATP hydrolysis to proton transport. The bacterial F$_1$F$_0$-ATPases are composed of eight types of polypeptides in the following stoichiometry: $\alpha_3\beta_3\gamma\delta\epsilon$ (F$_1$) and $a_1b_2c_{12}$ (F$_0$) (Fillingame, 1997). The $\alpha_3\beta_3\gamma$ portion of F$_1$ from beef heart mitochondria has been crystallized and structural analysis shows a symmetrical hexameric arrangement of alternating $\alpha$ and $\beta$ subunits (55 kDa each) around a central core (Abrahams et al., 1994). The ATP binding sites are located within the $\beta$ subunit at the $\alpha\beta$ subunit interface. The $\gamma$ subunit (30 kDa) is located in the center of the hexamer and protrudes partially from the bottom of the complex. The three dimensional arrangement of the subunits in the F$_0$ domain is unknown.

The V-type ATPases, like their F-type counterparts, are composed of a catalytic V$_1$ sector and a membrane bound V$_0$ sector. The V$_1$ sector can be removed using a variety of methods, including chaotropic agents, alkaline carbonate, or nitrate and MgATP (Kane et al.,
1989; Lai et al., 1988; Xie and Stone, 1988). Unlike the F₀ sector, there is no evidence to suggest that the V₀ sector can passively transport protons in the absence of V₁ (Finbow and Harrison, 1997). The subunits in V₁ are designated A – G and range in size between 116 kDa and 13 kDa (Finbow and Harrison, 1997). Three of these, A, B, and C, have sequence similarity with α, β, and γ from the F₁F₀-ATPase the others having no obvious relationship. The stoichiometry of the V₁ sector is similar to that of the F₁ sector, possessing a pseudo-6-fold symmetry axis. The V₀ sector is composed of three different subunits, two of which are transmembrane (c and a large glycoprotein) and one which is hydrophilic, but tightly bound to the integral membrane components (Finbow and Harrison, 1997). Electron microscopy has shown that there are 6 c subunits in the V₀ sector, which are arranged symmetrically (Holzenburg et al., 1993). The stoichiometry of the other major subunits is the same as in the F₁F₀-ATPase (Nelson, 1992).

The mechanism by which V-type ATPases function is poorly understood at the moment; however, the recent crystal structure of the F₁ sector has allowed a putative mechanism to be proposed for the F-type-ATPases. Based on their structural similarities, one would predict the mechanism of action to be quite similar for V-type ATPases. The proposed mechanism is based on a rotating or alternate binding change mechanism. Several biochemical studies have revealed that ADP + P_i is converted into ATP at two or all three of the highly cooperative binding sites without major input of energy (Boyer, 1993; Boyer et al., 1973). However, the newly formed ATP molecule remains tightly bound and substrate binding at another site is coupled to product release at the currently active site. This latter process requires a major input of energy, which is derived from conformational changes linked to proton flux through the F₀ subunit. A series of elegant experiments have shown
that hydrolysis of ATP at the three catalytic sites in the $\alpha_3\beta_3$ hexamer drives the $\gamma$ subunit around in three steps (Duncan et al., 1995; Noji et al., 1997; Sabbert et al., 1997; Zhou et al., 1996).

In ATP synthesis, this process would operate in reverse, where conformational changes in the $F_0$ subunit resulting from proton flux would be translated to the $\gamma$ subunit. The conformational changes generated by proton binding at the $c$ subunits in the $F_0$ sector are transmitted to the $\gamma$ subunit in the $F_1$ sector via the $\varepsilon$ subunit. Crosslinking studies have shown that the $\varepsilon$ subunit extends the entire length of the stalk region (Aggeler and Capaldi, 1996; Zhang and Fillingame, 1995). The translocation of 4 protons leads to a 120° rotation in the $\gamma$ subunit, releasing one ATP molecule. In this way, the torque generated in the $F_0$ subunit by the binding of a proton is transmitted to the $\gamma$ subunit, forcing it to rotate in the center of the $\alpha_3\beta_3$ hexamer, releasing newly synthesized ATP.

1.4.2 Menkes and Wilson Disease Copper Transporting ATPases

The Menkes (MNK) and Wilson (WND) disease proteins are copper transporting P-type ATPases which have recently been identified in humans (Bull et al., 1993; Chelly et al., 1993; Mercer et al., 1993; Tanzi et al., 1993; Vulpe et al., 1993). Sequence analysis of these proteins indicate that they belong to a subfamily of eukaryotic cation transporting P-type (Type I) ATPases (see section 1.4.1.1) which is closely related to bacterial heavy metal ATPases (Bull et al., 1993; Chelly et al., 1993). The Menkes and Wilson proteins share a high degree of homology with each other. The overall sequence identity between the two proteins is 57%, however; this figure rises to 79% and higher in the phosphatase, transduction-phosphorylation, ATP and metal binding domains (Bull et al., 1993). Despite
their similarities, the MNK and WND proteins do have some distinct differences. The greatest of these is a 78 amino acid deletion in WND between the first and second metal binding domains (fig. 1.4). The significance of this deletion in the function of the proteins is not yet known. Interestingly, the rat orthologue of the WND gene is missing metal binding region 4, but the overall spacing and size of the amino-terminal metal binding domain is intact (fig. 1.4) (Wu et al., 1994).

The MNK and WND ATPases are type I, P-type ATPases having several features which distinguish them from other members of the P-type ATPase family. Some of these were discussed in section 1.4.1.1, including an additional pair of transmembrane segments immediately before the TGES motif and the absence of four transmembrane segments at the carboxy-terminus, in comparison with type II P-type ATPases (see fig. 1.3). In addition to these differences, the MNK and WND ATPases have a larger amino-terminal domain and several unique amino acid motifs which are conserved in bacterial heavy metal transporters (fig 1.5) (Bull et al., 1993; Silver et al., 1993; Tanzi et al., 1993; Vulpe et al., 1993). The large amino-terminal domain in the MNK and WND protein contains six copies of a metal binding sequence. The repeat is referred to as the heavy metal-associated (HMA) domain and has the following consensus sequence: [LIVN]-x(2)-[LIVMFA]-x-C-x-[STAGCDNH]-C-x(3)-LIVFG]-x(3)-[LIV]-x(9,11)-[IVA]-x-[LVFYS]. The highlighted cysteine residues are strictly conserved in all proteins which contain the repeat and they are most likely involved in the ligation of the metal atom.

The HMA has been found at the amino-terminus of a wide variety of heavy metal ATPases from various organism involved in the transport of copper, zinc, cadmium and mercury (table 1.6). The phylogenetic relationship between the proteins listed in table 1.6 is
Figure 1.4 Comparison of Wilson (WND), Menkes (MNK) and Rat (rWND) Copper Transporting ATPases. WND contains a large deletion (~78 amino acids) between metal binding repeats 1 and 2. rWND is missing the fourth metal binding repeat but overall spacing is intact. Vertical bars represent transmembrane segments, Td, transduction domain, Ch/Ph, channel phosphorylation domain, ATP, ATP binding domain.
Figure 1.5 Proposed Structure for Wilson and Menkes Copper ATPases. A Proposed structure for the Menkes (MNK) and Wilson (WND) disease copper ATPases based on sequence comparison with other cation transporting ATPases. B Alignment of metal binding domains from WND with other heavy metal transporters. ATX1 copper chaperone from *S. cerevisiae*, ATOX1 human homologue of ATX1, CopA copper ATPase from *E. hirae*, MerP mercury transport protein from *P. Aeruginosa*, CadA cadmium ATPase from *S. Aureus*. Conserved cysteine residues are bolded. CPC and SEHPL motifs are unique heavy metal transporting ATPases.
Table 1.6
Metal Transporting Proteins Containing Amino-Terminal HMA Domains

<table>
<thead>
<tr>
<th>Protein</th>
<th>Organism</th>
<th>Function</th>
<th>HMA</th>
<th>Swiss-Prot AC #</th>
</tr>
</thead>
<tbody>
<tr>
<td>YBAR</td>
<td><em>E. Coli</em></td>
<td>Cu-Transporting ATPase</td>
<td>1</td>
<td>P78245</td>
</tr>
<tr>
<td>MNK</td>
<td>Human</td>
<td>Cu-Transporting ATPase</td>
<td>6</td>
<td>Q04656</td>
</tr>
<tr>
<td></td>
<td><em>C. Elegans</em></td>
<td>Cu-Transporting ATPase</td>
<td>2</td>
<td>O17537</td>
</tr>
<tr>
<td>Rat</td>
<td>Cu-Transporting ATPase</td>
<td>6</td>
<td>P70705</td>
<td></td>
</tr>
<tr>
<td>Mouse</td>
<td>Cu-Transporting ATPase</td>
<td>6</td>
<td>P97422</td>
<td></td>
</tr>
<tr>
<td>WND</td>
<td>Human</td>
<td>Cu-Transporting ATPase</td>
<td>6</td>
<td>P35670</td>
</tr>
<tr>
<td>Rat</td>
<td>Cu-Transporting ATPase</td>
<td>6</td>
<td>Q64535</td>
<td></td>
</tr>
<tr>
<td>Mouse</td>
<td>Cu-Transporting ATPase</td>
<td>6</td>
<td>Q64446</td>
<td></td>
</tr>
<tr>
<td>Sheep</td>
<td>Cu-Transporting ATPase</td>
<td>6</td>
<td>O46518</td>
<td></td>
</tr>
<tr>
<td>CCC2</td>
<td><em>S. cerevisiae</em></td>
<td>Cu-Transporting ATPase</td>
<td>2</td>
<td>P38995</td>
</tr>
<tr>
<td>SYNA</td>
<td><em>Synecococcus 7942</em></td>
<td>Cu-Transporting ATPase†</td>
<td>1</td>
<td>P37385</td>
</tr>
<tr>
<td>CopA</td>
<td><em>E. Hirae</em></td>
<td>Cu-Transporting ATPase</td>
<td>1</td>
<td>P32113</td>
</tr>
<tr>
<td>CopP</td>
<td><em>H. Pylori</em></td>
<td>Cu Ion Binding Protein</td>
<td>1</td>
<td>Q48271</td>
</tr>
<tr>
<td>ATOX1</td>
<td>Human</td>
<td>Copper Chaperone</td>
<td>1</td>
<td>O00244</td>
</tr>
<tr>
<td>ATX1</td>
<td><em>S. cerevisiae</em></td>
<td>Copper Chaperone</td>
<td>1</td>
<td>P38636</td>
</tr>
<tr>
<td>CUA-1</td>
<td><em>C. Elegans</em></td>
<td>Cu-Transporting ATPase</td>
<td>3</td>
<td>O17737</td>
</tr>
<tr>
<td>ZntA</td>
<td><em>E. Coli</em></td>
<td>Zn-Transporting ATPase</td>
<td>1</td>
<td>P37617</td>
</tr>
<tr>
<td>ZiAA</td>
<td><em>Synecocystis Sp. PCC 6803</em></td>
<td>Zn-Transporting ATPase</td>
<td>1</td>
<td>Q59998</td>
</tr>
<tr>
<td>CadA</td>
<td><em>B. Firmus</em></td>
<td>Cd-Transporting ATPase†</td>
<td>1</td>
<td>P30336</td>
</tr>
<tr>
<td></td>
<td><em>S. Aureus</em></td>
<td>Cd-Transporting ATPase†</td>
<td>2</td>
<td>P37386</td>
</tr>
<tr>
<td>FixI</td>
<td><em>R. Meliloti</em></td>
<td>Nitrogen Fixation</td>
<td>1</td>
<td>P18398</td>
</tr>
<tr>
<td>PacS</td>
<td><em>Synecocystis Sp. PCC 6803</em></td>
<td>Cation Transp. ATPase</td>
<td>1</td>
<td>P37329</td>
</tr>
<tr>
<td>CtpA</td>
<td><em>M. leprae</em></td>
<td>Cation Transp. ATPase</td>
<td>1</td>
<td>P46839</td>
</tr>
<tr>
<td>CtpB</td>
<td><em>M. leprae</em></td>
<td>Cation Transp. ATPase</td>
<td>1</td>
<td>P46840</td>
</tr>
<tr>
<td>MerA</td>
<td><em>P. Aeruginosa</em></td>
<td>Hg(II) Reductase</td>
<td>1</td>
<td>P00392</td>
</tr>
<tr>
<td>MerP</td>
<td><em>P. Aeruginosa</em></td>
<td>Hg(II) Transport Protein</td>
<td>1</td>
<td>P04131</td>
</tr>
</tbody>
</table>

*The fourth HMA domains in these proteins are non-functional.
†Probable function based on primary sequence analysis.
shown in figure 1.6. When the HMA domain in the CopA protein (E. hirae copper ATPase) is compared to each of the HMA domains in the WND protein, the degree of similarity rises from 11% identity for the amino-terminal most HMA domain to 40% identity for the carboxy-terminal most HMA domain (Petrukhin et al., 1994). The identity between the carboxy-terminal most HMA domains of MNK and WND is 67%. These observation suggest that the evolution of the large amino-terminal domain in the MNK and WND proteins proceeded by gene duplication involving the 'splicing' in of each metal binding domain to the ATPase core, with the carboxy-terminal most domains being the latest additions (Petrukhin et al., 1994).

The MNK and WND proteins, as well as all bacterial heavy metal transporters, contain two unique amino acid sequences which are not present in non-heavy metal transporters, these being the CPC motif in the sixth transmembrane segment and the SEHPL motif following the phosphorylation site. Particularly interesting is the SEHPL motif which is absolutely conserved in MNK, WND and bacterial copper ATPase CopA (Odermatt et al., 1993; Petrukhin et al., 1993; Tanzi et al., 1993). This strict evolutionary conservation suggests that this region is of functional importance, a notion which is strengthened by the observation that mutation of the histidine residue in this motif to a serine leads to the Wilson disease phenotype (Thomas et al., 1995).

Although no specific function has been attributed to this motif in the heavy metal ATPases, the corresponding region in the sarco(endo)plasmic reticulum Ca-ATPase (SERCA) is the site of interaction with phospholamban (James et al., 1989; Toyofuku et al., 1993). Phospholamban is an endogenous, membrane bound inhibitor of SERCA which interacts with the calcium free form of the ATPase and lowers its apparent affinity for
Figure 1.6 Phylogenetic Relationship Between HMA Containing Proteins. Protein sequences obtained from the Swiss-Prot database were aligned using ClustalX with the Blossum 30 amino acid weighting matrix. The phylogenetic tree was calculated using ClustalX. See table 1.6 for Swiss-Prot accession numbers for each protein.
calcium. As calcium concentrations rise and more of the enzyme is converted to the calcium bound form, phospholamban is released from the ATPase. Phospholamban could also be released from the ATPase through phosphorylation of phospholamban by cAMP-dependent protein kinase A (Toyofuku et al., 1993). In the plasma membrane calcium ATPases, the analogous region was shown to interact with a synthetic calmodulin binding domain (Falchetto et al., 1991). The interaction of calmodulin with the plasma membrane calcium ATPase has been shown to result in an approximate 20-fold increase in the affinity for calcium (Larsen et al., 1981; Larsen and Vincenzi, 1979). In Na\(^+\)/K\(^+\) ATPases this region is susceptible to proteolytic digestion in the presence of potassium, but resistant to digestion when sodium is present (Jorgensen and Farley, 1988). These findings suggest that, in these ATPases, this region undergoes conformational changes during the transport cycle of the protein. The amino-terminal metal binding domains could play a regulatory role in addition to binding copper. In the absence of copper, the metal binding domain may interact with the SEHPL containing segment; once copper binds, conformational changes would release this interaction, activating phosphorylation of the enzyme and starting the transport cycle.

As compared to non heavy-metal transporting P-type ATPases, the WND and MNK proteins have two less transmembrane segments at the carboxy-terminus. In both the SERCA and Na\(^+\)/K\(^+\) ATPases the carboxy-terminal transmembrane segments are intimately involved in the cation translocation process (Clarke et al., 1989; Karlish et al., 1990). In addition it is interesting to note that the KdpK\(^+\)-ATPase in E. coli also lacks these carboxy-terminal transmembrane segments and requires other subunits to form a fully functional complex (see section 1.4.1.1). These observations suggest the possibility that the WND and MNK proteins require a second, unidentified subunit. This type of arrangement is seen in the
cadmium efflux system of S. aureus in which the components of the cadmium resistance mechanism (cadA and cadB) reside on two separate plasmids (Nucifora et al., 1989). The CPC motif present in the sixth transmembrane segment is also strictly conserved between the MNK, WND and bacterial heavy metal transporting ATPases. The proline residue in this motif is conserved in all P-type ATPases and has been shown to be critical in the formation of high affinity calcium binding sites in the SERCA ATPase (Vilsen et al., 1989). It is likely that the conserved cysteines in this motif are involved in binding the copper atom transiently as it moves through the membrane.

The WND and MNK proteins also have some important structural differences when compared to closely related bacterial heavy metal transporting ATPases. First, the MNK and WND proteins contain six copies of the HMA domain at their amino-terminus, while only one or two copies are present in the analogous prokaryotic proteins (Bull et al., 1993; Tanzi et al., 1993). Second, the WND and MNK proteins contain a long carboxy-terminal hydrophilic fragment which is similar to that found in the plasma membrane calcium ATPase and the H\(^+\) ATPase in plants (Pardo and Serrano, 1989). Lastly, the length of the sequence between the phosphorylation site to the hinge domain is 70 – 80 amino acids longer than in the analogous bacterial ATPases, but substantially shorter than in the corresponding region of other eukaryotic ATPases. As discussed above, this region in eukaryotic calcium ATPases is the site of interaction with phospholamban and calmodulin, two proteins which regulate the function of these ATPases. In contrast, the malarial calcium ATPase is lacking this sequence and, in its place, has an additional 100 – 200 amino acids which are not found in vertebrates, suggesting that this ATPase has its own regulatory protein (Kimura et al., 1993). By analogy, it has been suggested that the additional 70 – 80 amino acid residues carboxy-
terminal to the SEHPL motif in WND and MNK may reflect the unique regulatory requirements of these proteins (Petukhin et al., 1994).

1.5 Human Genetic Disorders of Copper Metabolism

Although trace amounts of many transition metal elements are needed to sustain life, amounts in excess of what is needed are usually detrimental. Therefore, precise control over the free concentrations of these trace metals is essential to maintain good health. This is especially important for redox active metals such as iron and copper which, if present in excess, can enhance free radical generation and lead to cellular damage (Britton, 1996). In humans, the two major genetic disorders of copper metabolism are Wilson and Menkes disease which are characterized by an excess and a deficiency of copper respectively. Recent advances in the cloning of the genes responsible for these disorders has led to several major insights into copper transport and intracellular trafficking.

1.5.1 Menkes disease

1.5.1.1 Genetics of Menkes Disease

Menkes disease (OMIM 309400) is a genetic disorder of copper metabolism characterized by an impairment in the absorption of dietary copper and a severe disturbance in the intracellular transport and trafficking of copper (Danks et al., 1973; Herd et al., 1987). Menkes and coworkers noted in their original study that the disorder seemed to be confined to males and was inherited in a sex-linked recessive manner (Menkes et al., 1962). The incidence of Menkes disease is approximately 1/300,000 live births (Tümer and Horn, 1997b). Early work by a number of researchers mapped the disease locus to band Xq13.3
(Tümer et al., 1992b; Tümer et al., 1992a; Verga et al., 1991). Using this early genetic work as a guide, the Menkes disease gene (ATP7A) was isolated independently by three groups using positional cloning techniques (Chelly et al., 1993; Mercer et al., 1993; Vulpe et al., 1993). The gene was found to contain 23 exons which span approximately 150 kb of genomic DNA (fig. 1.7) (Dierick et al., 1995; Tümer et al., 1995). The exons range in size from 77 to 726 bp, with introns ranging from 60 to 196 kb (Dierick et al., 1995). All splice sites were found to conform to the GT-AG rule, except for the donor site of intron 9, which precedes an exon which is alternatively spliced (Dierick et al., 1995). The Menkes gene (ATP7A) shares some similarity with Wilson disease gene (ATP7B). Following the exon coding for the fifth metal binding domain (exon 5 in ATP7A and exon 3 in ATP7B), the coding region of both the Menkes and Wilson proteins are virtually identical (Tümer et al., 1995). However, significant differences do occur at the 5' end; where the first four metal binding domains in the Wilson disease gene are contained in one exon, those of the Menkes disease gene extend over three exons (Dierick et al., 1995).

The Menkes gene encodes a 1500 amino acid protein which is predicted to be a copper transporting P-type ATPase. The predicted protein has a high degree of similarity to that encoded by the Wilson disease gene (see section 1.4.2). The main difference is the presence of a 78 amino acid insertion between metal binding domains 1 and 2 in the Menkes protein which is not present in the Wilson disease protein. Northern blot analysis has shown that the Menkes disease protein is expressed in all tissues except the liver, which correlates well with the observed clinical and biochemical features of the classical Menkes phenotype (Chelly et al., 1993; Vulpe et al., 1993). Analysis of numerous Menkes cases has revealed that approximately 15-20% of the mutations found in the Menkes disease gene consist of
Figure 1.7 Genomic Structure of the Disease Gene (ATP7A). Vertical lines indicate the positions of the introns, the numbers indicate the positions of the exons. Adapted from (Tümer and Horn, 1997b).

- Transmembrane segments
- Cu - Copper binding repeats
- PD - Phosphatase domain
- C - Conserved CPC motif
- D - Phosphorylation domain
- A - ATP binding domain
insertions or deletions of varying sizes (Chelly et al., 1993; Mercer et al., 1993; Vulpe et al., 1993). In a study of 41 unrelated patients presenting with the classical form of Menkes disease, 19 out of 41 mutations were due to insertion or deletions (Tümer et al., 1997a). The two largest of these deletions are a 14 bp deletion in exon 10 and a 10 bp deletion in exon 22. These deletions and others are predicted to alter the reading frame of the gene, leading to the introduction of a stop codon and premature truncations of the protein. Alternatively, these mutations may lead to the skipping of subsequent mutation-bearing exons, resulting in an internally deleted protein which may retain some function as has been reported for a 2 bp deletion in exon 7 (Das et al., 1994).

The study by Tümer et al. (Tümer et al., 1997a) also discovered that approximately half of the mutations they identified were located in exons 7-10 and half of these mutations affected exon 8 alone. Furthermore, five of the twelve point mutations identified in another series also affect exon 8 (Das et al., 1994). Exon 8 encodes a region of the protein between metal binding domain 6 and the first transmembrane segment of the protein. Although no specific function has been attributed to this region in the protein, it may play a role in the precise localization of the metal binding domain relative to the ATPase core. The importance of this region of the protein is demonstrated by the finding that individuals carrying mutations in exon 8 present the classical severe form of Menkes disease (Tümer et al., 1997a). In contrast, only two mutations have been found in the corresponding region of the Wilson disease gene (Figus et al., 1995). The vast difference in the frequency of exon 8 mutations between the two genes could indicate that, despite their similarities, these proteins may have significant differences in their mechanism of action.
1.5.1.2 Clinical and Biochemical findings

Menkes disease was originally described by John Menkes and coworkers in 1962 during their study of five patients from the same family who all died before three years of age (Menkes et al., 1962). Patients affected by the classical severe form of the disease originally described by Menkes and coworkers are unable to absorb copper and hence suffer from the effects of copper deficiency. As observed by Menkes, Menkes disease has a very early age of onset with untreated patients usually dying before the age of three. The patients in the original study were all delivered normally and had normal birthweights. However, in the weeks following birth, several phenotypes were observed which have now become the hallmarks of Menkes disease. It was noted that all patients gained very little weight despite a normal diet, and all had hair abnormalities either from birth or developing soon thereafter. These abnormalities consisted of hair that was coarse and brittle and was white in color due to depigmentation. Microscopic examination of individual hair fibers revealed several gross abnormalities, they were either twisted (pili torti), fractured at various intervals (trichorrhexis nodosa), or of varying caliber (monilethrix). However, despite these abnormalities, the level of sulfhydryls in the hair were found to be normal (Menkes et al., 1962). The presence of these hair abnormalities in Menkes patients led to the disorder also being referred to as kinky-hair syndrome.

Other case studies in the years following Menkes' initial description of the disorder expanded the list of clinical symptoms (Table 1.7) and have identified a moderate form, a late onset form, and a mild allelic form (occipital horn syndrome, OHS) of the disorder
## Table 1.7
Symptoms of Classical Menkes Disease and Occipital Horn Syndrome (OHS)

<table>
<thead>
<tr>
<th>Connective Tissue Symptoms</th>
<th>Classical Menkes</th>
<th>OHS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tortuous vessels</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Skeletal changes</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>Bladder diverticulae</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>Loose skin</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Loose joints</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Neurologic Symptoms</th>
<th>Classical Menkes</th>
<th>OHS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mental retardation</td>
<td>++</td>
<td>+ / -</td>
</tr>
<tr>
<td>Convulsions</td>
<td>++</td>
<td>-</td>
</tr>
<tr>
<td>Hypothermia</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Feeding difficulties</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Muscle tone changes</td>
<td>+</td>
<td>ND</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Other Symptoms</th>
<th>Classical Menkes</th>
<th>OHS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Facial dysmorphism</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Abnormal hair</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Hypopigmentation</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Laboratory Findings</th>
<th>Classical Menkes</th>
<th>OHS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum copper</td>
<td>↓↓</td>
<td>↓</td>
</tr>
<tr>
<td>Serum ceruloplasmin</td>
<td>↓↓</td>
<td>↓</td>
</tr>
<tr>
<td>Intracellular copper</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Life Expectancy</th>
<th>Classical Menkes</th>
<th>OHS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Life expectancy</td>
<td>&lt;3 years</td>
<td>NK</td>
</tr>
</tbody>
</table>

Adapted from (Tümer and Horn, 1997b).

*Life expectancy for OHS patients is not known. All known OHS patients are still alive with the oldest being 52. ND = Not Determined, NK = Not Known.*
(Danks, 1988; Danks et al., 1972; Horn et al., 1995; Peltonen et al., 1983; Procopis et al., 1981). Although most patients with OHS have only mild deficiencies, several cases with more severe symptoms such as psychomotor retardation and seizures have been described (Mentzel et al., 1999; Wakai et al., 1993). The patients were also found to have extremely low levels of copper in the serum, brain, and liver, and extremely low ceruloplasmin levels. Typical copper measurements from Menkes patients are summarized in table 1.8. Attempts to correct the problem by oral administration of copper failed; however, intravenous injection of copper was able to normalize ceruloplasmin levels and copper clearance from the serum was also normalized (Danks et al., 1972). This and other evidenced strengthened the idea that the basic biochemical defect was a severely compromised ability to absorb intestinal copper, compounded by disturbances in the intracellular transport and trafficking of copper (Danks et al., 1973; Herd et al., 1987). Cultured fibroblasts from Menkes patients have been found to accumulate copper in the form of copper metallothionein and metallothionein induction in cultured muscle cells from these patients is elevated relative to normal cells (Beratis et al., 1978; Chan et al., 1978; Goka et al., 1976; Herzberg et al., 1990; Onishi et al., 1980; Riordan and Jolicoeur-Paquet, 1982).

The clinical symptoms of classical Menkes disease can be attributed to a deficiency of developmentally important cuproenzymes such as lysyl oxidase, tyrosinase, cytochrome c oxidase, dopamine β hydroxylase, Cu/Zn superoxide dismutase and amine oxidase (table 1.3) (Danks, 1995; Menkes, 1988). Lysyl oxidase is an essential enzyme needed for the crosslinking of connective tissue; a deficiency in this enzyme would lead to weakened connective tissue, which could lead to the connective tissue defects, such as arterial rupture,
Table 1.8
Typical Copper Measurements in Normal Adults and Menkes Disease Patients

<table>
<thead>
<tr>
<th></th>
<th>Menkes Patient (3-12 Months)</th>
<th>Normal Infant (3-12 Months)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum ceruloplasmin OD units/ml</td>
<td>&lt;0.08</td>
<td>&gt;0.25</td>
</tr>
<tr>
<td>Serum ceruloplasmin mg/L</td>
<td>&lt;50</td>
<td>&gt;200</td>
</tr>
<tr>
<td>Serum copper (µM)</td>
<td>&lt;6</td>
<td>&gt;12</td>
</tr>
<tr>
<td>Liver copper (µg/g dry weight)</td>
<td>10 – 20</td>
<td>70 – 140</td>
</tr>
<tr>
<td>Duodenal copper (µg/g dry weight)</td>
<td>50 – 80</td>
<td>7 – 29</td>
</tr>
</tbody>
</table>

Adapted from (Danks, 1995).
observed in Menkes patients (Danks, 1995; Kuivaniemi et al., 1982; Peltonen et al., 1983). The absence of tyrosinase could lead to the hair depigmentation observed in Menkes patients and low levels of cytochrome c oxidase can result in temperature instability and seizures (Bankier, 1995). Examination of the cuproenzyme status in hepatocytes from murine models of Menkes disease reveals that they have normal activity, although the level of copper in the liver is severely depressed (Phillips et al., 1986). This suggests that intracellular copper transport in the liver is normal in Menkes patients.

The biochemical effects of Menkes disease suggest that the body is in a condition of copper deficiency. Therefore, the Menkes ATPase would be expected to be present in the intestinal mucosa where it would function to transfer copper from the intestine into the bloodstream. In Menkes disease, the ATPase is non-functional, resulting in an accumulation of copper in the intestines in the form of copper metallothionein, which is lost from the body when these cells are sloughed off leading to a global copper deficiency. Recent studies using immunofluorescence techniques have localized the Menkes ATPase to the golgi apparatus under normal copper concentrations (Petris et al., 1996; Yamaguchi et al., 1996). It has also been shown that under elevated copper conditions, the ATPase undergoes a reversible, copper dependent relocalization from the golgi to the plasma membrane (Petris et al., 1996). Therefore, under normal copper conditions, the ATPase functions to pump copper into the golgi for incorporation into cuproenzymes or to pump copper into the body from the intestinal mucosa. When elevated copper conditions are encountered, the ATPase is translocated to the plasma membrane where it functions to expel copper from the cell. These observations explain why fibroblasts from Menkes patients have normal copper uptake activity, but are not able to expel copper to avoid copper accumulation.
1.5.1.3 Current Treatments

Since the main problem in Menkes patients is a global deficiency of copper, treatments have concentrated on restoring normal copper levels in the body by the administration of copper. However, since intestinal absorption of copper is extremely low in Menkes patients, the copper complexes must be administered parenterally. Some early complexes which were evaluated for treatment purposes included copper chloride, copper sulfate, copper EDTA, and copper albumin. However, it was found that these complexes are unable to produce significant clinical improvement especially in regards to neurodegeneration (Garnica, 1984). The only copper complex which has been used successfully to treat Menkes disease is copper-histidine (Sarkar, 1980). Copper-histidine is a normal component of human serum and has been shown to play an important role in the transport and uptake of copper (Mas and Sarkar, 1992; Sarkar, 1981; Sarkar and Kruck, 1966). Detailed copper uptake studies conducted in vitro have suggested that copper uptake by brain tissue is mediated by complexation to histidine and have also suggested that the copper-histidine complex is able to cross the blood-brain barrier (Barnea et al., 1990; Barnea and Katz, 1990; Hartter and Barnea, 1988; Katz and Barnea, 1990).

Patients treated with subcutaneous injections of copper-histidine have shown significant clinical and biochemical improvement (Kreuder et al., 1993; Sarkar et al., 1993; Sherwood et al., 1989). The best outcomes have occurred in patients in which treatment was started as early as possible. These case studies have shown a normalization of serum copper, ceruloplasmin, dopamine and norepinephrine levels after three months of treatment (Kreuder et al., 1993). The normalization of dopamine and norepinephrine levels indicate that normal
dopamine β-hydroxylase levels may have been restored. The severity of the mutation in the two longest lived Menkes patients who have responded very well to copper-histidine treatment has been confirmed by DNA sequence analysis. It has been discovered that these two patients harbor mutations in the Menkes disease gene which totally abolish the production of a functional ATPase. In the first patient a mutation in exon 4 introduces a premature stop codon in the third metal-domain, resulting in the production of a polypeptide without the ATPase 'core'. In the second patient the mutation occurs in exon 12, again leading to the introduction of a stop codon upstream of the phosphatase domain. This mutation produces a polypeptide which contains the first four transmembrane segments, but lacks the remainder of the ATPase 'core' (Tümer et al., 1996).

Despite significant clinical and biochemical improvements in these patients, connective tissue disorders continue to persist, indicating that lysyl oxidase levels are not being restored by copper-histidine injections. Alternatively, the persistence of these disorders may reflect the slow turnover of connective tissues. It is also likely that copper presented in the copper-histidine complex is not available for incorporation into lysyl oxidase. Studies carried out in brindled mice, a murine model for Menkes disease, have shown that subcutaneous injections of copper(I) in an alkyl polyether / sebacic acid solution are able to increase dramatically the level of lysyl oxidase in these animals (Royce et al., 1982). These findings suggest that the oxidation state of copper may influence its ability to penetrate certain intracellular compartments. Hence the treatment of Menkes patients could be enhanced by providing copper both in the +1 and +2 oxidation state, possibly using copper-histidine in conjunction with another agent.
1.5.1.4 Animal Models of Menkes Disease

Animal models are indispensable tools for the study of human disorders since they permit studies which would otherwise be impossible in humans. For Menkes disease the main animal models are the mottled mutations in mice (Danks, 1977; Danks, 1986; Danks and Camakaris, 1983; Hunt, 1974; Xu et al., 1994). A comparison of the symptoms found in these animals with Menkes patients is shown in Table 1.9. The series of mutants, mottled (Mo), dappled (Mo<sup>dp</sup>), tortoiseshell (Mo<sup>tn</sup>), brindled (Mo<sup>br</sup>), viable brindled (Mo<sup>vbr</sup>), blotchy (Mo<sup>blt</sup>), macular (Mo<sup>ml</sup>) and pewter (Mo<sup>new</sup>) are usually considered allelic and display extensive phenotypic variability. These series of mutations were identified in the 1950s as X-linked mutations which led to patchy coat color in females and were lethal in males. In hemizygous males the displayed phenotypes include connective tissue disorders in blotchy and viable brindled, prenatal lethality in dappled and tortoiseshell, severe neurologic disease in brindled and macular, and isolated coat color defects in pewter (Levinson et al., 1994).

Early studies on the brains from brindled males revealed that copper levels were significantly decreased (Hunt, 1974). This led to the proposal that a deficiency of copper was the primary defect in these animals and the diverse phenotypes arose from secondary deficiencies in copper-requiring enzymes (Darwish et al., 1983). This idea was further strengthened by the finding that cultured cells from many organs, except the liver, had defects in copper export, which resulted in the cytosolic accumulation of copper (Darwish et al., 1983; Packman, 1987; Sayed et al., 1981). Defective export and accumulation of copper are also found in cultured fibroblasts from Menkes patients (Beratis et al., 1978; Chan et al., 1978; Goka et al., 1976; Onishi et al., 1980). The mouse orthologue of the human Menkes
Table 1.9
Features of Menkes Disease in Humans and the Mottled Mouse Mutants

<table>
<thead>
<tr>
<th></th>
<th>Menkes Disease</th>
<th>Brindled Mouse</th>
<th>Blotchy Mouse</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inheritance</td>
<td>X-linked</td>
<td>X-linked</td>
<td>X-linked</td>
</tr>
<tr>
<td>Hair deformity</td>
<td>++</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Hair keratin disulfide deficiency</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Arterial abnormalities</td>
<td>++</td>
<td>+ /–</td>
<td>+</td>
</tr>
<tr>
<td>Lysyl oxidase deficiency</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Life expectancy</td>
<td>1-2 years</td>
<td>13-15 days</td>
<td>6-18 months</td>
</tr>
<tr>
<td>Liver copper</td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
</tr>
<tr>
<td>Kidney copper</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
</tr>
<tr>
<td>Intestinal copper</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
</tr>
</tbody>
</table>

Adapted from (Danks, 1995).
gene has been cloned, and resides on a region of the mouse X-chromosome which is syntenic to the region where the human Menkes gene is located (Cecchi and Avner, 1996; Levinson et al., 1994; Mercer et al., 1994).

### 1.5.2 Wilson disease

#### 1.5.2.1 Genetics of Wilson Disease

Wilson disease (OMIM 277900) is an autosomal recessive disorder of copper transport which was first described by Kinnear Wilson in 1912 (Wilson, 1912). Wilson disease is characterized by the toxic accumulation of copper in various tissues but primarily the liver, kidney, and brain. The frequency of the disease is approximately 1/100,000 but seems to be more prevalent in Japan, Sardinia, and China (Reilly et al., 1993; Sternlieb, 1990; Tümer and Horn, 1997b). Early genetic studies assigned the Wilson disease gene to a single locus by linkage to the esterase D gene (Bull et al., 1993; Frydman et al., 1985) while later studies placed the gene close to a cluster of polymorphic markers in the q14.3 band of chromosome 13 (Bowcock et al., 1987; Bowcock et al., 1988; Yuzbasiyan-Gurkan et al., 1988). Following these studies, the Wilson disease gene (ATP7B) was cloned independently by two groups (Bull et al., 1993; Tanzi et al., 1993). The Wilson disease gene consists of 22 exons (fig. 1.8), with exon 21 being expressed in the liver and exon 22 being expressed in the kidney (Cox, 1995; Petrukhin et al., 1994; Thomas et al., 1995). The exons range in size from 77 bp to 2355 bp (Petrukhin et al., 1994). The ATP7B transcript is expressed at a high level in the liver and kidney, with a lower level being detected in the lung and placenta (Bull et al., 1993). The pattern of expression correlates well with the observed clinical and biochemical features of the disease (see section 1.5.2.2). The gene is predicted to encode a
Figure 1.8 Genomic Structure of the Wilson Disease Gene (ATP7B). Vertical lines indicate the positions of the introns, the numbers indicate the positions of the exons.

- Transmembrane segments
- Cu - Copper binding repeats
- PD - Phosphatase domain
- C - Conserved CPC motif
- D - Phosphorylation domain
- A - ATP binding domain
copper transporting P-type ATPase which has many features in common with other cation
transporting P-type ATPases as well as several bacterial heavy metal transporters (see section
1.4.2).

In contrast to the Menkes gene, most of the mutations in the Wilson disease gene do
not involve large insertions or deletions. Missense mutations in the Wilson disease gene
have been found in all exons, with the exception of exons 1-5, which encode the six amino-
terminal metal binding motifs, and exon 21, which encodes the carboxy-terminal tail of the
protein (Shah et al., 1997; Thomas et al., 1995). Many other mutations in the Wilson disease
gene, including small insertion/deletions, nonsense, frameshift, and splice site mutations have
been found and are summarized elsewhere (Chuang et al., 1996; Loudianos et al., 1996;
Nanji et al., 1997; Shimizu et al., 1995; Thomas et al., 1995). From these studies, it is clear
that the most common missense mutation in many populations is His1069Gln which is
present in about 38% of homozygous Wilson disease patients. The mutation occurs in a
conserved loop motif (SEHPL), which is adjacent to the conserved phosphorylation motif
DKTG. No specific function has yet been attributed to this motif, but it seems critical for the
correct function of the heavy metal ATPases, as it is absolutely conserved in the Wilson,
Menkes, and CopA (bacterial copper ATPase) proteins (Odermatt et al., 1993; Petrukhin et
al., 1993; Tanzi et al., 1993).

The high frequency of the His1069Gln mutation has permitted a genotype/phenotype
correlation for this mutation. In one study, patients homozygous for the His1069Gln
mutation were found to have a later average age of onset as compared to the heterozygous
patients, 20 years versus 15 years (Shah et al., 1997). In addition, all homozygotes were
found to have an equal incidence of hepatic and neurologic symptoms. The His1069Gln
mutation was also found at high frequency in a study conducted on Mediterranean populations (Figus et al., 1995). This study also found that the His1069Gln is always associated with the same haplotype suggesting that it most likely arose from an ancient mutational event (Figus et al., 1995). The molecular basis of the adverse effects of the His1069Gln mutation has recently been investigated. Recent studies have shown that transfection of the Wilson cDNA containing the His1069Gln mutation into mouse fibroblasts gives rise to a protein with a 5-fold shorter half-life, which is mislocalized to the endoplasmic reticulum (Payne et al., 1998a). The authors also noted that the mislocalization was temperature-sensitive and suggest that misfolding followed by degradation constitutes the molecular basis of the defect in these Wilson patients.

1.5.2.2 Clinical and Biochemical findings

Wilson disease results from the accumulation of toxic levels of copper in various organs due to a severe deficiency in the excretion of copper from the body. Wilson disease displays extensive clinical heterogeneity and has a variable age of onset which can range from the age of 8-9 years to the mid-50's. The progression of the disease can be roughly divided into three distinct clinical phases. In the first phase, copper begins to accumulate in the cytoplasm of hepatocytes due to impaired biliary excretion of copper (Strickland et al., 1973). In the second phase, as more copper is absorbed, the cytosolic concentration of copper rises resulting in the induction of large amounts of metallothionein and to the chelation and storage of excess copper (Hunziker and Sternlieb, 1991). The high levels of cytosolic copper in hepatocytes eventually leads to necrosis, resulting in the release of large amounts of copper into the bloodstream. In the final stage of the disease, increasing amounts
of copper begin to accumulate in other organs such as the brain, kidney and cornea. The sudden influx of large amounts of copper into the bloodstream results in oxidative damage to erythrocyte membranes, leading to hemolytic anemia (Dobyns et al., 1979; Mowat, 1994). Wilson disease patients can also be divided into one of three groups, depending on what combination of symptoms are present. Patients may either display hepatic symptoms, neurologic symptoms, or both.

The deposition of copper in the cornea gives rise to dull-yellow Kayser-Fleischer (K-F) rings which are a diagnostic hallmark of Wilson disease (Scheinberg and Sternlieb, 1984). The presence of K-F rings has been found in 95% of all Wilson disease patients and in all patients which display neurologic disorders (Danks, 1995). As a result of its prevalence in the majority of patients, K-F rings have become a diagnostic hallmark of the disease in the latter stages. The deposition of copper in the brain during the latter stages of the disease correlates with the finding that patients with an early age of onset usually display predominantly hepatic symptoms, while those with a later stage of onset display predominantly neurologic symptoms (Strickland et al., 1973; Vulpe and Packman, 1995). The list of symptoms relating to hepatic dysfunction is extensive and varied. These include jaundice, asymptomatic cirrhosis, subacute hepatitis (non-A or B) and chronic aggressive hepatitis (Mowat, 1994). The neurologic symptoms in Wilson disease patients can include deteriorating coordination, tremors, dysarthria, drooling, personality changes, dementia, slurring of speech, rigidity and behavioral problems (Mowat, 1994; Strickland and Leu, 1975). The neurologic symptoms in Wilson disease can be attributed to cerebellar or extrapyramidal involvement or a combination of both (Dobyns et al., 1979).
The biochemical findings in Wilson disease patients are consistent with a disturbance in the excretion of copper and, primarily, a defect in the biliary excretion of copper (Strickland et al., 1973). Typical copper measurements in Wilson disease patients are summarized in table 1.10, along with those in normal adults. Urinary copper levels in Wilson disease patients are elevated due to the accumulation of copper in the kidneys, which leads to renal dysfunction in the form of albuminuria and renal rickets (Walshe, 1989). Since incorporation of copper into ceruloplasmin is also impaired in Wilson disease, there is a greater proportion of copper bound to albumin and low molecular weight complexes in the serum; however, the overall serum concentration of copper is lowered (Barrow and Tanner, 1988). Recent immunohistochemical studies have shown that the Wilson disease ATPase is localized to the trans-golgi network under steady-state conditions (Hung et al., 1997; Nagano et al., 1998). Furthermore, as also observed for the Menkes disease ATPase, these studies have shown that the ATPase undergoes a rapid, copper dependent relocalization from the golgi to a cytosolic vesicular compartment, as well as to the plasma membrane.

The function of the ATPase in the trans-golgi is most likely to transport copper from the cytoplasm into this compartment for incorporation in secreted, copper-containing proteins such as ceruloplasmin. This idea correlates with the finding of low ceruloplasmin levels in Wilson disease patients. The relocalization of the ATPase to a vesicular compartment or the plasma membrane in response to elevated copper levels would lead to efflux of copper from the cell, either via the bile (through the fusion of vesicles with the canalicular membrane) or across the plasma membrane (Dijkstra et al., 1996; Hung et al., 1997). There is also one recent report which has localized the Wilson disease ATPase to the mitochondria, where it
Table 1.10
Typical Copper Measurements in Normal Adults and Wilson Disease Patients

<table>
<thead>
<tr>
<th></th>
<th>Wilson Disease</th>
<th>Normal Adult</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum ceruloplasmin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>OD units/ml</td>
<td>0 – 0.25</td>
<td>0.25 – 0.49</td>
</tr>
<tr>
<td>mg/L</td>
<td>0 – 200</td>
<td>200 – 400</td>
</tr>
<tr>
<td>Serum copper (µM)</td>
<td>3 – 10</td>
<td>11 – 24</td>
</tr>
<tr>
<td>Urinary copper (µg/24 hours)</td>
<td>100 – 1000</td>
<td>40</td>
</tr>
<tr>
<td>Liver copper (µg/g dry weight)</td>
<td>200 - 3000</td>
<td>20 – 50</td>
</tr>
</tbody>
</table>

Adapted from (Danks, 1995).
has been suggested to provide copper for incorporation into cytochrome oxidase; however, this has yet to be confirmed by others (Lutsenko and Cooper, 1998).

1.5.2.3 Current Treatments

Treatments for Wilson disease patients have concentrated on the use of chelators to mobilize copper from the centers of accumulation and promote its excretion from the body. The first chelator used widely for the treatment of Wilson disease was BAL (2,3-dimercaptopropanol) (Denny-Brown and Porter, 1951). In this initial study of five patients, the authors observed a significant reversal of tremors and rigidity following treatment with BAL. However, these improvements were only observed after longterm use and were less significant in patients with more acute cases of the disease (Cartwright et al., 1954; Matthews et al., 1952). Furthermore, patients had to deal with painful intramuscular injections and often side effects. These concerns led to the development of D-penicillamine which improved upon BAL in every respect (Walshe, 1956). It could be administered orally, is less toxic and more effective than BAL. The standard dose of D-penicillamine for adults is 500 mg given twice daily, with close monitoring of urinary copper. On this dose, urinary copper excretion can increase to between 4 and 5 mg per day (Sass-Kortsak, 1975).

Treatment with D-penicillamine usually lasts for a lifetime and produces significant clinical and biological improvements in a large number of cases. In some patients, neurologic symptoms may disappear, liver function may return to normal, and Kayser-Fleischer rings usually fade and then disappear (Sass-Kortsak, 1974). However, despite its vast improvement over BAL, some severe side effects have been reported for D-penicillamine as well. The most common side effects in the early stages of treatment include
fever, anorexia, pyridoxine deficiency, and a mild depression of leukocyte and platelet counts (Mowat, 1994; Sass-Kortsak, 1975; Scheinberg, 1968). In very few cases, serious toxic effects may include systemic lupus erythematosus, and Goodpasture's syndrome (Mowat, 1994; Sass-Kortsak, 1975). D-penicillamine has also been linked to changes in the crosslinking of collagen and elastin, leading to serious skin and connective tissue disorders (Iozumi et al., 1997; Narron et al., 1992; Pasquali-Ronchetti et al., 1989). There are also at least two cases on record of asymptomatic Wilson disease patients experiencing severe neurological deterioration soon after initiation of D-penicillamine therapy (Brewer et al., 1994; Glass et al., 1990). There is also a danger in interrupting D-penicillamine treatment once started. There are several cases on record in which patients have stopped taking their tablets and irreversible liver damage has developed within two years (Scheinberg et al., 1987; Walshe and Dixon, 1986).

A number of other chelating agents have been developed and used successfully in cases where patients develop toxic reactions to D-penicillamine or treatment is interrupted. These include trientine (triethylenetetramine dihydrochloride) (Scheinberg et al., 1987; Walshe, 1982), tetrathiomolybdate (Brewer et al., 1994a; Walshe, 1984), and zinc therapy (Alexiou et al., 1985; Hoogenraad, 1998; Hoogenraad et al., 1979; Hoogenraad et al., 1978; Van Caillie-Bertrand et al., 1985). Trientine may be a viable alternative to D-penicillamine in some cases; no toxic side effects have been observed in 20 patients using the drug for up to 13 years (Scheinberg et al., 1987; Walshe, 1982). Trientine has also been employed successfully in one female patient who had previously been on D-penicillamine. Trientine was administered during pregnancy without adverse affects on the fetus (Devesa et al., 1995). Trientine may be an effective treatment when D-penicillamine has been withdrawn;
however, commercial availability of the compound is still a problem. In addition, trientine and D-penicillamine seem to act on different pools of copper. D-penicillamine is able to mobilize copper from the liver, but competes relatively poorly for copper bound to albumin (Sarkar et al., 1977). In contrast, trientine cannot mobilize copper from the liver, but is effective in competing for copper bound to albumin (Sarkar et al., 1977). Both drugs are able to mobilize copper from the kidneys, promoting its removal through urinary excretion.

Tetrathiomolybdate is another chelator with great potential which has been used to treat Wilson disease. It is an extremely potent chelator of copper which can cause severe copper deficiency in normal rats within 1 week and is, therefore, not suited for long-term treatment of Wilson disease (Brewer et al., 1991; Mason et al., 1989; Walshe, 1989). However, tetrathiomolybdate can be used for initial decoppering followed by maintenance with zinc. At the very least, tetrathiomolybdate is a possible alternative in cases where toxicity to D-penicillamine develops. Unlike other chelating agents which promote the mobilization and excretion of copper through the urine, zinc therapy promotes fecal copper excretion. Oral zinc therapy is thought to promote copper excretion by inducing the synthesis of metallothionein in the intestine, thereby blocking the absorption of copper (Hoogenraad, 1998; Hoogenraad et al., 1979; Hoogenraad et al., 1978). Although zinc has a greater ability to induce metallothionein synthesis than does copper, metallothionein has a higher affinity for copper than zinc (Lipsky and Gollan, 1987). As a result the newly synthesized metallothionein sequesters copper in the upper intestine, preventing its transport into the portal circulation. The sloughing off of intestinal cells into the lumen results in the elevated copper levels observed in the feces of zinc-treated Wilson patients (Brewer et al., 1983; Lipsky and Gollan, 1987). Patients with acute liver failure and encephalopathy are
usually beyond the help of chelation therapy. In these cases liver transplantation may provide the only hope for survival. The results of 57 Wilson disease patients who underwent liver transplantation showed that there was a 72% survival rate after the first year, with 4 patients requiring retransplantation (Mowat, 1994; Schilsky et al., 1994).

1.5.2.4 Animal Models

Several animal models for Wilson disease have been identified and all display clinical and biochemical symptoms which are very close to those found in human Wilson disease patients. These animal models include the toxic milk (tx) mouse (Rauch et al., 1986; Rauch and Wells, 1995), the Long Evans Cinnamon (LEC) rat (Li et al., 1991) and bedlington terriers (Su et al., 1982; Twedt et al., 1979). The biochemical features of these animals, as compared to Wilson disease, are summarized in table 1.11. The toxic milk mouse and the LEC rat display very similar clinical and biochemical features. The toxic milk mutation was first recognized as an autosomal recessive trait which was lethal to the pups of affected females due to a severe copper deficiency in their milk (Rauch, 1983). In affected females, copper accumulates in the liver, leading to histological changes and cirrhosis in the absence of any neurological effects. It has been shown that the excess copper which accumulates in the toxic mice is in the form of copper metallothionein (Koropatnick and Cherian, 1993; Rauch et al., 1986).
Table 1.11
Comparison of Biochemical Findings in Wilson Disease and its Animal Models: Toxic Milk Mouse and Bedlington Terriers

<table>
<thead>
<tr>
<th></th>
<th>Wilson Disease</th>
<th>Bedlington Terrier</th>
<th>Toxic Milk Mouse</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hepatic copper accumulation</td>
<td>++</td>
<td>+++</td>
<td>++</td>
</tr>
<tr>
<td>(µg/g dry weight)</td>
<td>200 – 3000</td>
<td>2000 – 12000</td>
<td>300 – 400</td>
</tr>
<tr>
<td>Reduced biliary copper excretion</td>
<td>++</td>
<td>+</td>
<td>NK</td>
</tr>
<tr>
<td>Reduced copper incorporation into ceruloplasmin</td>
<td>+++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>Liability to hemolytic crisis</td>
<td>+</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>Chronic liver cell damage</td>
<td>++</td>
<td>+ / −</td>
<td>++</td>
</tr>
<tr>
<td>Copper overflow to other tissues</td>
<td>++</td>
<td>+ / −</td>
<td>+ / −</td>
</tr>
</tbody>
</table>

Adapted from (Danks, 1995).
Analysis of metallothionein mRNA levels in the liver of these animals shows that they are normal in younger tx mice and highly increased in adult tx mice (Mercer et al., 1992). This finding suggests that metallothionein regulation is normal in these animals and excess copper is leading to the induction of metallothionein. Morphological examination of livers from adult tx mice has revealed a pattern of damage which is significantly different from that observed in Wilson patients and has led to some doubts as to the validity of the tx mouse as a model for Wilson disease (Biempica et al., 1988). However, recent studies have mapped the tx mutation to chromosome 8 where it has been shown to reside in the murine orthologue of the human Wilson disease gene confirming that the tx mouse is, indeed, a valid model for Wilson disease (Rauch and Wells, 1995; Reed et al., 1995; Theophilos et al., 1996). Interestingly, the fourth metal binding domain in the mouse and rat orthologues of the Wilson disease gene has been altered such that one or both of the conserved cysteine residues are no longer present (Theophilos et al., 1996; Wu et al., 1994).

The LEC rat is probably the most used and hence best understood model of Wilson disease. This inbred rat strain was established from a closed colony of normal Long-Evans parental rats through successive generations of sibmating (Yoshida et al., 1987). LEC rats suffer from spontaneous hepatitis, hemolytic anemia and jaundice at approximately 4 months of age, followed by death in 50% of animals due to fulminant hepatitis (Li et al., 1991; Yoshida et al., 1987). The surviving 50% suffer from chronic hepatitis and usually go on to develop liver cancer (Masuda et al., 1988; Powell, 1994; Sawaki et al., 1990; Yoshida et al., 1987). In contrast to Wilson disease, neurological abnormalities resulting from copper accumulation in the brain are rarely seen in LEC rats. As in Wilson disease, LEC rats accumulate copper in the liver, but serum ceruloplasmin activity is severely depressed (Li et
al., 1991a; Schilsky et al., 1994a). Genetic analysis has shown that the mutation causing the spontaneous hepatitis in LEC rats resides in a gene on chromosome 16 which is the rat orthologue of the Wilson disease gene (Muramatsu et al., 1994; Wu et al., 1994). The mutation is predicted to give rise to a premature stop codon leading to termination of the protein prior to the ATPase 'core'. Recently, a novel splice variant of the Wilson disease gene in rats has been identified (Borjigin et al., 1999). This variant is expressed in the brain and eye and its activity is apparently regulated by the circadian system. The functional significance of this variant has yet to be identified.

The bedlington terrier model is probably the least understood and, therefore, the least used of the Wilson disease animal models. Copper toxicosis (CT) in bedlington terriers is an autosomal recessive disorder characterized by a defect in the biliary excretion of copper, leading to copper accumulation in the liver and to cirrhosis (van de Sluis et al., 1999). Despite its similarity to Wilson disease, CT in bedlington terriers does have some significant differences. Bedlington terriers with CT do not show neurological abnormalities, Kayser-Fleisher rings are absent and ceruloplasmin levels are observed to be normal (Hunt et al., 1986; Twedt et al., 1979). Genetic analysis has failed to locate mutations in the ATP7B gene and no linkage has been shown to esterase D (ESD) or retinoblastoma (RB), two genes which are tightly linked to ATP7B in humans and mice (Reed et al., 1995; Scheffer et al., 1992; Yuzbasiyan-Gurkan et al., 1993). However, acute hepatitis in the LEC rat is also not linked to RB, suggesting that not all mammalian species share a syntenic relationship between RB and ATP7B (Sasaki et al., 1993). Taken together these results suggest that CT in bedlington terriers may involve a gene which is not homologous to the Wilson disease gene in humans. In support of this possibility, recent mapping studies have determined that the CT locus in
bedlington terriers maps to a region on dog chromosome 10 which is syntenic to a region on chromosome 2 in humans, thereby eliminating ATP7B as a possible candidate gene (van de Sluis et al., 1999). In lieu of these observations, the exact cause of CT in bedlington terriers remains unknown.
1.6 Project Rationale

The discovery of the genes responsible for Menkes and Wilson diseases, and the copper transporting ATPases they encode, has advanced our understanding of intracellular copper trafficking. These two ATPases are the first members (in humans) of a rapidly growing class of heavy metal transporting ATPases which have recently been discovered in a variety of organisms. Although similar to other cation transporting P-type ATPases, the heavy metal ATPases have several features which distinguish them from non-heavy metal transporting ATPases (see section 1.4). Among these are the amino acid motifs (CPC and SEHPL) which are critical for proper function of the ATPase, but are absent in the non-heavy metal ATPases. The other unique and probably most interesting feature of the heavy metal ATPases is the presence of a large amino-terminal domain which contains between 1 and 6 copies of the heavy metal associated (HMA) domain. The MNK and WND proteins both contain six HMAs at their amino-terminus, while those from bacteria and lower animals contain between 1 and 2 copies.

Comparison of the amino acid sequence from the HMAs in the MNK and WND proteins with that from bacterial proteins clearly indicates that the human HMAs are derived from their bacterial counterparts (Petrukhin et al., 1994). However, one obvious difference between the human proteins and their bacterial counterparts is the number of HMAs present. Therefore, one of the underlying objectives of this work is to determine the significance of having six HMAs in the human protein when only one or two seem to be sufficient in the bacterial and yeast proteins. Several biochemical studies have hinted at the possibility that the HMA domains may have roles in the ATPase additional to binding metals. Mutational and functional studies have shown that the HMAs in the WND and MNK proteins are not
functionally equivalent and that not all are needed for the transport of copper to occur (Forbes et al., 1999; Hung et al., 1997; Payne and Gitlin, 1998). Furthermore, in vivo subcellular localization studies using fluorescent antibodies have shown that both the MNK and WND proteins reside in the trans-golgi network and both undergo translocation to the plasma membrane and a cytosolic vacuolar compartment under elevated copper concentrations (Hung et al., 1997; Nagano et al., 1998; Petris et al., 1996). This unique ligand-mediated response has led to the hypothesis that the amino-terminal domain in the MNK and WND proteins serves as a 'sensor' for cytoplasmic concentration of copper which, in turn, could regulate the activity of the ATPase.

Both the HMAs and the large amino-terminal metal binding domain in the MNK and WND proteins are novel protein entities which have not been characterized in any other known protein. A thorough understanding of their function in the context of the whole ATPase can only be gained following the characterization of the metal binding sites as well as the structural dynamics of the amino-terminal domain as a whole. With this objective in mind, the WCBD was systematically characterized, starting with the cloning, expression and purification of the domain. Once purified, the WCBD was analyzed by determining i) its metal binding characteristics, ii) its relative affinity for copper, iii) the structural effect of metal binding, and iv) the structural properties of the copper binding sites. The results from such an analysis may then be combined from functional and mutational studies carried out in systems using the entire ATPase. In the absence of a three dimensional structure, the metal binding and structural dynamics studies will provide a wealth of information on this novel and previously uncharacterized domain. These studies will increase our knowledge of the role of this domain in the ATPase and could lead to the development of new pharmaceutical
agents for the treatment of Menkes and Wilson diseases. Furthermore, these studies will also point the way to deciphering the structure and function of other heavy metal transporting ATPases by providing a model for the structure and function of the large amino-terminal metal binding domain.
CHAPTER 2

Materials and Methods
2.1 DNA Preparation

2.1.1 Cell lines

Two separate cell lines were used for the maintenance of plasmid DNA and for the expression of GST fusion proteins. *E. coli* strain DH5α (*supE44 ΔlacU169 (φ80 lacZΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1*) was used for the maintenance and production of plasmid DNA for the construction of the GST expression vector constructs, since they were found to lead to higher purity plasmid preps (Hanahan, 1983). *E. coli* strain BL21(DE3) (*hsdS gal (λcIts857 ind1 Sam7 nin5 lacUV5-T7 gene 1)*) were used for the expression of GST fusion proteins as recommended in the GST Gene Fusion System Handbook (Amersham-Pharmacia Biotech) (Studier and Moffatt, 1986).

2.1.2 Preparation of competent BL21(DE3) and DH5α Cells

Preparation of competent BL21(DE3) and DH5α cells was carried out using the calcium chloride method (Sambrook *et al.*, 1989). Briefly, cells were grown in LB broth (1% w/v peptone, 0.5% w/v yeast extract, 1% w/v NaCl) to an *OD*$_{600}$ of 0.4-0.6. The cells were then cooled on ice for 10 minutes and harvested by centrifugation for 10 minutes at 3000 x g, 4°C. The pellets were resuspended in ice-cold 0.1 M CaCl$_2$. The cells were then centrifuged as above and resuspended in 0.1 M CaCl$_2$, 10% glycerol (2 ml / 50 ml original culture) and stored at −70°C.

2.1.3 Transformation of competent BL21(DE3) and DH5α Cells

Calcium chloride competent cells were transformed by the heat-shock method as described in the TA Cloning Kit (Invitrogen). Briefly, 50 μl of competent cells were thawed
on ice and mixed with 1 µl DNA (purified plasmid or from a ligation reaction), 2 µl 0.5 M β-Me and incubated on ice for 30 minutes. Following this incubation, the cells were heat-shocked for 30 seconds in a 42°C waterbath and returned to ice for 2 minutes. 450 µl of prewarmed SOC media (2% w/v peptone, 0.5% w/v yeast extract, 0.05% w/v NaCl, 20 mM glucose, 2.5 mM KCl, 10 mM MgCl₂) was added to the cells and left to recover for 1 hour at 37°C before they were selected on LB-ampicillin (100 mg/L) agar plates.

2.1.3.1 Preparation of glycerol stocks

A single positive clone of BL21(DE3) transformed with either pGEX-WCBD or pGEX-WCBD(P) was inoculated into 5 ml LB-ampicillin and grown overnight with shaking at 37°C. To 1 ml of the overnight culture was added 0.5 ml of sterile glycerol stock solution (65% v/v glycerol, 0.1 M MgSO₄, 0.025 M Tris-HCl, pH 8.0) was added. The mixture was split into aliquots (250 µl) and stored at −70°C until needed for expression. Glycerol stocks prepared in this manner were stable for >1 year if kept at −70°C.

2.1.4 Purification of plasmid DNA

Small scale preparation of plasmid DNA was carried out using the QIAprep Miniprep Kit (QIAGEN) which uses the modified alkaline lysis method of Birnboim and Doly (Birnboim and Doly, 1979). Transformed E. Coli were grown overnight in 5 ml of LB broth supplemented with ampicillin (100 mg/L). The following day, cells were harvested by centrifugation (5 minutes, 3000 x g, 4°C) and processed as specified in the QIAprep miniprep handbook. Double stranded plasmid DNA was quantitated using its absorbance at 260 nm (1.0 OD₂₆₀ unit = 50 µg/ml double stranded DNA) (Sambrook et al., 1989).
2.1.5 *DNA agarose gel electrophoresis*

Gel electrophoresis was used to determine the size and purity of purified plasmids and for the separation of different-sized DNA fragments prior to purification. Agarose slab gels using the TAE buffer system were used (Sambrook *et al.*, 1989). All 1% agarose gels were prepared in 0.5X TAE buffer containing 1 μg/ml ethidium bromide and were run at a constant voltage (100 V) until adequate separation was achieved. Ethidium bromide stained nucleic acid bands were visualized using ultraviolet light.

2.1.6 *Purification of DNA restriction fragments from agarose gels*

DNA fragments were separated by agarose gel electrophoresis and purified using the Sephaglas™ BandPrep Kit (Amersham-Pharmacia Biotech), which is a modification of the NaI method (Vogelstein and Gillespie, 1979). Purified DNA fragments were quantified using their absorbance at 260 nm and used without further purification in subsequent procedures.

2.1.7 *DNA Sequencing*

Double-stranded plasmid DNA was sequenced by the dideoxy sequencing method (Sanger *et al.*, 1977) using the T7 Sequencing™ Kit (Amersham-Pharmacia Biotech) according to the manufacturer's directions. The purity of the plasmid preparations was checked prior to sequencing by measuring the OD (260 nm / 280 nm) ratio and ensuring that it was at least 1.8.
2.1.8 *Restriction enzyme digests and ligation reactions*

Restriction and modifying enzymes used in these reactions were obtained from Amersham-Pharmacia Biotech and New England Biolabs (NEB). The reactions were carried out under the conditions specified by the manufacturer, using the buffers supplied. All ligation reactions were carried out using T4 DNA ligase from NEB. The vector and insert were ligated at a 1:1, 1:3, 1:5, and 1:10 molar ratio starting with 50 ng of vector and the corresponding amount of insert calculated using equation 1.

\[
X \text{ ng insert} = \frac{(Y \text{ bp insert})(50 \text{ ng vector})}{(\text{size in bp of vector})}
\]

The value obtained for \(X\) from this equation gives the amount of insert for a 1:1 molar ratio. Multuples of \(X\) (3X, 5X and 10X) were used for the other ratios. Generally 400 units of ligase (NEB unit definition) were added to each ligation reaction, total reaction volume was 20 \(\mu\)l. Ligation reactions between fragments of DNA with compatible ends were carried out overnight at 16\(^\circ\)C. Usually, 1 \(\mu\)l of the ligase reaction was used to transform bacteria and the remainder was stored at -20\(^\circ\)C.

2.2 *Construction of Expression Vectors*

2.2.1 *Construction of pGEX-WCBD*

The 2-kilobase cDNA encoding the Wilson disease protein copper binding domain (WCBD), was produced in two fragments from total human liver RNA using reverse transcriptase-polymerase chain reaction (RT-PCR). This reaction was performed by J.R.
Forbes in the laboratory of Dr. D.W. Cox. Primers were designed to create a *BamHI* restriction site at the 5' end and a *SalI* site at the 3' end. The two cDNA fragments overlapped at an *NsiI* restriction site. The primers used for the 5' fragment were (5' side) 5'-TATCGGATCCATGCCTGAGC-AGGAGA-3' (5'-BamHI), and (3' side) 5'-AACTTTAAAATTTCCAGGTGG-3' (OL-1); 3' fragment (3' side) 5'-ACTTGTGCAGCTGCTTATTTCCATTTTG-3' (3'-SalI), and (5' side) 5'-GGAATGCATTGTAAGTCTTGGCG-3' (OL-2). A stop codon (bold) was inserted just before the *SalI* site; restriction sites are underlined. The two fragments were digested with *BamHI* and *NsiI* or *SalI* and *NsiI*, respectively, and following purification from an agarose gel, ligated into the *BamHI* and *SalI* sites of the GST expression vector pGEX-4-T-2 (Amersham-Pharmacia Biotech, Inc) to create the expression vector pGEX-WCBD (Fig. 2.1). This vector contains a thrombin cleavage site between the GST moiety and the WCBD. The fidelity of the construct was confirmed by dideoxy sequencing.

### 2.2.2 Construction of pGEX-WCBD(P)

The WCBD DNA insert was excised from pGEX-WCBD using *BamHI* and *SalI*, purified on a 1% agarose gel and inserted into the *BamHI* and *SalI* sites of the GST fusion expression vector pGEX-6P-2 (Amersham-Pharmacia Biotech, Inc) to create the expression vector pGEX-WCBD(P) (Fig. 2.2). This vector contains a PreScission™ (human rhinovirus 3C protease) cleavage site between the GST moiety and the WCBD (Walker *et al.*, 1994). The fidelity of the construct was confirmed by dideoxy sequencing.
Figure 2.1. Parental and Modified GST Fusion Expression Vectors. A. parental GST fusion expression vector, B GST-WCBD expression vector containing ~ 2 Kb WCBD cDNA insert, C thrombin protease recognition site located between GST and WCBD. Cleavage by the protease occurs at the Arg - Gly bond. D thrombin 5 and 3 amino acid consensus sequences. P1 and P2 are hydrophobic amino acids, P3 and P4 are nonacidic amino acids, P1' or P2' are Gly.
Figure 2.2. GST-WCBD(P) Expression Vector with PreScission™ Cleavage Site. A GST-WCBD(P) expression vector with PreScission™ protease cleavage site between GST and WCBD. B PreScission™ protease recognition site. Cleavage occurs at the Gln - Gly peptide bond.
2.3 Expression and Purification of GST-WCBD and GST-WCBD(P)

2.3.1 Buffer and reagent preparation

All buffers and reagent solutions were prepared using purified distilled water with a conductivity of at least 18 M. Deionized urea was used in all buffers requiring it and was prepared as follows: approximately 100 – 150 g of AG501-X8 (D) mixed bed resin (BIO-RAD) was added to 2 L of 8 M urea in distilled water and stirred overnight at RT; the resin was then removed from the solution by vacuum filtration. All buffers not containing reducing agents (DTT or β-Me) were argon purged for at least 30 minutes at RT prior to use. Vinyl, powder free gloves (Baxter Healthcare Corp) and clear plastic pipette tips were used in all manipulations to avoid metal contamination. Residual metal contamination in dialysis tubing was removed prior to use by boiling the tubing for approximately 1 to 2 hours in 5 to 6 changes of distilled water.

2.3.2 Expression of GST-WCBD and GST-WCBD(P)

An aliquot of a glycerol stock containing BL21(DE3) transformed with either pGEX-WCBD or pGEX-WCBD(P) was inoculated into 100 ml of LB supplemented with ampicillin (100 µg/ml) and grown overnight at 37°C with shaking. Following overnight growth, the culture was divided in two and diluted into 2 x 1.5 L fresh LB supplemented with ampicillin and 100 µM CuSO₄ (Tris-buffered, pH 7.0) in 2.8 L Fernbach flasks. The cultures were grown with shaking at 37°C until midlog phase was reached (A₆₀₀ of 0.6-0.8), at which point they were induced with 0.1 mM IPTG for 3-4 hours. Following the induction period, the cells were harvested by centrifugation at 4420 x g for 15 minutes, at 4°C. The supernatant
was poured off and the bacterial pellets were either stored at $-20^\circ$C overnight or lysed immediately.

### 2.3.3 Purification of GST-WCBD and GST-WCBD(P)

The bacterial pellets were resuspended in lysis buffer (20 mM Tris-HCl, pH 8.0, 130 mM NaCl, 1 mM EDTA, 5 mM DTT, 10% glycerol, 1 mM PMSF, 1% Triton X-100 and 0.1 mg/ml lysozyme) and lysed by two cycles of freeze-thaw. The lysate was centrifuged at 118,000 x g for 45 minutes, at $4^\circ$C. The soluble fraction was decanted and stored at $4^\circ$C. Fusion protein which localized to inclusion bodies was solubilized and refolded, essentially as described previously (Dombroski et al., 1992). Briefly, inclusion bodies were solubilized by homogenization in solubilization buffer (50 mM Tris-HCl, pH 8.0, 6 M Urea, 8 mM DTT, 1 mM EDTA) and the resulting suspension was centrifuged at 118,000 x g for 45 minutes at $4^\circ$C. Solubilized fusion protein was refolded by extensive dialysis against refolding buffer (50 mM Tris-HCl, pH 8.0, 20% glycerol, 5 mM DTT, 1 mM EDTA). Refolded fusion protein was combined with the soluble fraction. The resulting mixture was made 1% in Triton X-100 and applied to a glutathione Sepharose 4B column previously equilibrated with TEND-T (20 mM tris-HCl pH 8.0, 130 mM NaCl, 1 mM EDTA, 5 mM DTT, 1% Triton X-100). The column was washed extensively with TEND-T followed by TEND (same as TEND-T but without Triton X-100). The fusion protein was eluted from the column with TEND-U (20 mM Tris-HCl, pH 8.0, 130 NaCl, 1mM EDTA, 5 mM DTT, 6 M Urea). Protein elution was monitored by $OD_{280}$ readings and samples from each fraction were analyzed by SDS-PAGE.
Fractions containing fusion protein were combined and applied to a Q-sepharose fast flow column (anion exchange), previously equilibrated with TEND-U at a flow rate of 2.2 ml / minute, collecting 5 ml fractions. After sample application, the column was washed extensively with TEND-U and OD$_{280}$ reading were taken on each fraction to monitor protein content. When OD$_{280}$ readings returned to baseline values, the fusion protein was eluted with a linear salt gradient (130 – 250 mM NaCl). Peak fractions were analyzed by SDS-PAGE and those containing fusion protein were pooled and refolded as described above.

2.3.4 SDS-PAGE

SDS-PAGE was performed on a mini slab gel apparatus (Hoefer Scientific Instruments) in the buffer system developed by Laemmli (Laemmli, 1970). To visualize the fusion protein as well as the WCBD, we prepared 10% separating gels overlaid with 4% stacking gels. The reduction of disulfide bonds and the incorporation of SDS were achieved by mixing protein samples with an equal volume of 2X loading buffer containing 0.72 M β-Me, boiling for 5 minutes, and immediately loading onto the gel. Gels were run at a constant voltage (175 V) until separation was achieved, as determined from the mobility of the dye front. Following electrophoresis, the gel was stained in a 0.125% w/v solution of Coomassie Brilliant Blue R-250 dissolved in 40% v/v methanol, 10% v/v acetic acid, 50% distilled water, with shaking for 15 minutes at RT. The gel was destained by incubation in 10% v/v methanol, 10% v/v acetic acid, 80% distilled water, with shaking overnight. Destained gels were photographed using the UVP gel documentation system in conjunction with UVP-Grab-IT™ software version 1.57b (DiaMed Lab Supplies). Molecular weights of the proteins were
estimated by comparison with wide range molecular weight marker run in parallel (200 – 6 kDa, NOVEX Mark12 Standards).

2.3.5 Protein quantitation

Quantitation of the amount of fusion protein or WCBD was carried out using the BCA Protein Assay Kit (Pierce). The assay was carried out as outlined in the instruction manual for the standard protocol. The concentration of the protein sample was determined by comparison to a standard curve derived from BSA (0 – 50 µg, run in duplicate). Results from this assay agreed very closely to those obtained by amino acid analysis.

2.3.6 N-Terminal Amino Acid Sequencing

N-terminal amino acid sequencing was carried out on the purified WCBD to confirm its identity. The samples were run on 10% SDS-PAGE gels and electroblotted onto PVDF membranes (BIORAD). Electroblotting was carried out in a Hoefer electroblot transfer apparatus (Amersham-Pharmacia Biotech) at a constant current of 0.5 A for 45 minutes using CAPS transfer buffer (10 mM CAPS pH 11.0, 10% v/v methanol) cooled with circulating water. Following blotting, the membrane was washed in two changes of transfer buffer and then stained with 0.1% w/v Coomassie Brilliant Blue R-250 dissolved in 50% v/v methanol, 50% distilled water for 1-2 minutes. The membrane was then destained with 50% v/v methanol, 10% v/v acetic acid, 40% distilled water for 5 minutes and then washed with several changes of distilled water for 5-10 minutes. The membrane was air dried and submitted for N-terminal sequence analysis. Seven cycles of automated amino acid sequencing was carried out on a Porton Instruments Gas-Phase Micro Sequencer model P1.
with online PTH amino acid analysis using a Hewlett-Packard HPLC, model 1090L. N-terminal sequencing reactions were carried out using Edman degradation (Matsudaira, 1989).

2.3.7 Amino Acid Analysis

Purified fusion protein was analyzed for total amino acid content to confirm the identity of the protein and to determine concentration. The fusion protein was hydrolyzed with hydrochloric acid (6 M) for 48 hours and the amino acids were reacted with phenylisothiocyanate (PITC) to form phenylthiohydantoin (PTH) amino acid derivatives. The PTH-amino acid derivatives were analyzed and quantitated by HPLC in comparison with a standard mixture of PTH-derivitized amino acids (Smith, 1997). Fusion protein concentration was determined by dividing the concentrations of PTH-Leu, PTH-Ala, PTH-Ile by 79, 66, and 59 respectively (corresponding to the molar amounts of each residue present in the fusion protein) and calculating the average of the values obtained.

2.4 Removal of GST from GST-WCBD and GST-WCBD(P)

2.4.1 Thrombin cleavage of GST-WCBD

Thrombin cleavage of GST-WCBD was either carried out on the column or on the purified fusion protein in solution. For cleavage of the column-bound fusion protein, 10 units of human thrombin (Sigma-Aldrich) / mg of fusion protein was added to 1 bed volume of TEND (20 mM Tris-HCl, pH 8.0, 130 mM NaCl, 1 mM EDTA, 5 mM DTT) and applied to the column. The reaction mixture was incubated at RT for 30-45, minutes followed by elution of pure WCBD in the same buffer. Thrombin was inhibited by the addition of PMSF.
to a final concentration of 1 mM. For cleavage of purified fusion protein in solution, 10 units of human thrombin / mg of fusion protein was added to the protein solution and the reaction was incubated for 30-45 minutes. After the incubation period, thrombin activity was inhibited by the addition of PMSF (1 mM final concentration) and the mixture was applied to a glutathione affinity column. The flow-through which contained the purified WCBD was collected.

2.4.1.1 *Optimization of thrombin cleavage conditions*

Thrombin cleavage conditions for GST-WCBD were optimized in the following manner to minimize the non-specific degradation of the WCBD. To a 2 ml solution of the purified fusion protein (0.48 mg / ml) were added 9.5 cleavage units of human thrombin and the reaction mixture was incubated at RT. Samples (50 μl) were taken out every 30 minutes for the first 4 hours and then every hour thereafter and mixed with an equal volume of 2X loading buffer containing 0.5 mM PMSF. The samples were boiled for 5 minutes and analyzed on 15% SDS-PAGE slab gels.

2.4.2 *PreScission™ cleavage of GST-WCBD(P)*

Removal of GST from GST-WCBD(P) was carried out on the purified fusion protein in refolding buffer (50 mM Tris-HCl, pH 8.0, 1 mM DTT, 20% glycerol). Briefly, 1 unit of PreScission™ protease (Amersham-Pharmacia Biotech) / mg fusion protein was added to the protein solution and the reaction mixture was incubated at 5°C for 16-48 hrs. The progress of the cleavage reaction was monitored by removing aliquots several time intervals and analyzing them by SDS-PAGE. Once the reaction was judged complete, the mixture was
passed over a glutathione affinity column to remove free GST and the protease (the protease is supplied as a fusion with GST). The flow-through contained the purified WCBD.

2.5 Characterization of the Metal Binding Properties of WCBD

2.5.1 Immobilized Metal Ion Affinity Chromatography (IMAC)

Samples of the purified fusion protein were dialyzed against IMAC buffer (20 mM NaH₂PO₄, pH 7.0, 0.5 M NaCl) with or without 6 M urea and applied to chelating sepharose fast flow columns (Amersham-Pharmacia Biotech) charged with either Co(II), Ni(II), Zn(II), Cu(II), Fe(II) or Fe(III) as per the manufacturer's instructions. Elution of the fusion protein from the matrix was achieved either by lowering the pH to 6.0 or 4.0 or by the sequential addition of chelators (EDTA, imidazole, BCS). Samples of each fraction were analyzed on 10% SDS-PAGE gels.

2.5.2 ⁶⁵Zn(II) Blotting Assay

⁶⁵Zn(II) blotting analysis was performed essentially as described previously (Schiff et al., 1988) with the following modifications. Samples of GST-WCBD, WCBD, GST and estrogen receptor DNA binding domain (ERDBD) were subjected to SDS-PAGE and electroblotted onto nitrocellulose membranes (BIORAD). Electroblotting was carried out in a Hoefer electroblot transfer apparatus (Amersham-Pharmacia Biotech) at a constant current of 0.3 A for 1.5 hours using CAPS transfer buffer (10 mM CAPS, pH 11.0) cooled with circulating water. Following electroblotting, the membranes were equilibrated in metal binding buffer (100 mM Tris-HCl, pH 7.0, 50 mM NaCl, 1 mM DTT) for 2 hours at RT with shaking. The membranes were then probed with 10 μCi of ⁶⁵ZnCl₂ (30 μM final Zn(II))
concentration) in 20 ml metal binding buffer, without DTT unless otherwise noted, for 1 hour at RT, with shaking. The membranes were then washed twice (15 minutes each) with the same buffer. The membranes were then wrapped in plastic wrap and exposed to Biomax MR film (Kodak) for 22 hours at −70°C with intensifying screen. Following autoradiography, the film was developed using a M35A X-OMAT processor (Kodak).

2.5.2.1 Effect of pH on $^{65}$Zn(II) Blotting Assay

The effect of pH on the binding of zinc to the domain was assessed using the $^{65}$Zn(II) blotting assay. The assay was carried out as described above, except that the pH of the metal binding buffer was varied from 6.5 to 9.0 in steps of 0.5 pH units (6 separate blots). The experiment was carried out using GST-WCBD, WCBD, GST and ERDBD.

2.5.2.2 Effect of DTT on $^{65}$Zn(II) Blotting Assay

The effect of DTT on the binding of zinc to the domain was assessed using the $^{65}$Zn(II) blotting assay. The assay was carried out as described above, at either pH 8.0 or pH 6.5, under the following conditions: in the presence of 1 mM DTT during the addition of metal, in the complete absence of DTT throughout the assay; and in the presence of 1 mM DTT only during the equilibration step (standard assay conditions). The experiment was carried out using GST-WCBD, WCBD, GST and ERDBD.

2.5.3 Competition $^{65}$Zn(II) Blotting Assay

The standard $^{65}$Zn(II) blotting assay was carried out as described above, using purified WCBD, except that the membranes were probed with $^{65}$Zn(II) in the presence of a
non-radioactive competitor metal at concentrations ranging from 0.01 mM to 1 mM. Blots probed with \( ^{65}\text{Zn(II)} \) in the absence of a competitor metal were used as controls. The signals from the autoradiograms were quantified using the gel-plotting macros in the NIH Image 1.61 software package. The percent zinc bound was calculated by dividing the signal obtained in the presence of a competitor to that obtained in its absence (taken as 100% zinc binding). In some instances the strips were stained with 0.1% w/v Amido Black dissolved in 45% v/v methanol, 10% v/v acetic acid, 45% distilled water for 1-2 minutes, followed by destaining in several changes of 45% v/v methanol, 10% v/v acetic acid, 45% distilled water. Stained membranes were analyzed to confirm that equal amounts of protein were present in each lane.

2.5.4 Stoichiometry of metal binding

The copper binding capacity of the domain was evaluated by NAA. Briefly, urea-denatured fusion protein was dialyzed against refolding buffer containing copper (20 mM Tris-HCl, pH 8.0, 10% glycerol, 0.1 mM DTT, 0.1 mM CuCl\(_2\)). Following dialysis, the protein concentration was determined using the BCA protein assay (Pierce), and the samples were then dialyzed extensively against 1% formic acid (pH 3.0) and lyophilized over a 4 day period on a Freezemobile 25XL lyophilizer (Virtis). Approximately 1.225 mg of the lyophilized fusion protein (0.0129 μmol) was submitted for copper determination by NAA.

2.5.5 Neutron Activation Analysis (NAA)

Neutron activation analysis was performed at the Slowpoke reactor facility at the University of Toronto. In general samples analyzed for copper were irradiated for 2 hours
using a neutron flux of $1.0 \times 10^{12}$ neutrons/cm$^2$/s. Samples were counted after a 3 hour delay for 600 seconds per sample. The amount of copper is each sample was determined by comparing the amount of $^{65}$Cu ($t_1 = 5.1$ minutes, 1039 keV) in the sample to an external standard using the comparator method. Samples analyzed for copper and zinc were irradiated for 16 hours using a neutron flux of $2.5 \times 10^{11}$ neutrons/cm$^2$/s. Samples were counted after a 6 hour delay for 1000 seconds per sample. The amount of copper and zinc in each sample was determined by comparing the amount of $^{64}$Cu and $^{69m}$Zn ($t_1 = 12.8$ hours, 511 keV and $t_1 = 13.8$ hours, 438.7 keV respectively) in the sample to an external standard using the comparator method (Hancock, 1976; Hancock, 1984; Hancock, 1978).

2.6 Characterization of the Copper Binding Sites

2.6.1 Preparation of apo-protein

Metal removal from the fusion protein as well as the WCBD was achieved as follows: A solution containing the fusion protein or the WCBD was made 0.5 M in β-Me and incubated at $4^\circ$C for 6-8 hrs. Following this incubation, the proteins were rapidly precipitated by the addition of TCA to a final concentration of 10%, followed by centrifugation at 3000 x g for 15 minutes at $4^\circ$C. The protein pellet was resolubilized in 0.5 M Tris base, 6 M Urea, 0.5 M β-Me with shaking at $4^\circ$C overnight. After resolubilization, the protein was again precipitated with TCA and resolubilized in 0.5 M Tris, 6 M Urea. The protein was then refolded by dialysis against modified refolding buffer (50 mM Tris-Acetate, pH 8.0, 20% glycerol) and then refolding buffer without glycerol. The protein concentration was determined by the BCA protein assay (Pierce) and then analyzed for metal content by NAA.
2.6.2 Preparation of Samples For XAS and CD analysis

Fusion protein and WCBD containing various amounts of copper or zinc were prepared as follows. DTT was added to samples of apo-GST-WCBD or apo-WCBD to a final concentration of 1 mM and incubated on ice for 30 minutes. The required amount of copper or zinc (using CuSO₄ and ZnSO₄) was then added to achieve the desired final molar ratio of metal/protein and incubated at RT for at least 30 minutes. A 15 fold molar excess of metal was usually used to achieve full reconstitution of the protein with metal. The unbound metal and DTT were then removed by extensive dialysis against 25 mM Tris-Acetate pH 8.0 (25 mM Tris-HCl pH 8.0 for CD samples). The protein concentration was again confirmed by the BCA protein assay and metal content was assessed by neutron activation. All dialysis buffers were purged with argon before use and dialysis was performed in sealed containers. Typical concentrations of apo-fusion protein used in this procedure were between 1 and 5 mg/ml. In some cases an alternative procedure for the addition of copper or zinc to the apo-protein was used to eliminate the possible interference of exogenous sulfur-containing compounds. In this method, the protein sulhydryls were reduced by the addition of a 1:1 molar ratio (with respect to cysteine concentration) of TCEP. Copper was then added to the apo-protein in the presence of 1 mM ascorbic acid. Ascorbic acid was omitted during the addition of zinc to the apo-protein.

2.6.2.1 Preparation of Dry XAS Samples

Dry XAS samples were prepared as described above (2.6.2), except that the samples were dialyzed against 25 mM Ammonium Acetate, pH 7.5 after incubation with the metal.
Following protein concentration and metal content determination, the samples were lyophilized by first flash freezing them using a dry ice / acetone bath and then lyophilizing them for 4 days on a Freezemobile 25XL lyophilizer (Virtis). After lyophilization, the samples were shipped to Dr. Que's lab at the University of Minnesota for XAS analysis.

2.6.2.2 Preparation of Liquid XAS and EPR Samples

Liquid samples for XAS and EPR were prepared as described above (2.6.2), except that the apo protein solutions were more concentrated (16 mg/ml) and, after incubation with metal the protein was dialyzed against 25 mM Tris-Acetate, pH 8.0, 30% glycerol. Empty EPR tubes and XAS sample holders were obtained from Dr. Que's lab and filled with 0.75 ml and 0.2-0.3 ml of sample, respectively. The XAS sample was frozen by placing the holder on top of a piece of dry ice while the EPR sample was flash frozen using liquid nitrogen. The filled tubes and sample holder were then shipped back to Dr. Que's lab on dry ice for analysis.

2.6.3 EXAFS and XANES Analysis of Cu-GST-WCBD

EXAFS and XANES analysis were carried out by Hua-Fen Hsu in Dr. Que's lab, as follows. X-ray absorption spectra were collected between 8799 eV and 9726 eV at beamline X9B of the National Synchrotron Light Source (NSLS) at Brookhaven National Laboratory. The protein data were collected in fluorescence mode \( \left( A_{\text{exp}} \left( C_f/C_0 \right) \right) \) at 10-20 K by using a flat Si(220) double-crystal monochromator and a 13-element Ge detector. The monochromator was calibrated using the edge energy of copper foil at 8979.0 eV. Data extraction and analysis was performed as previously described (Scarrow et al., 1987; Shu et
al., 1995). To fit the EXAFS contribution from copper scatterers, the empirical amplitude reduction factor A and the shell-specific edge ΔE were extracted from the XAS data of crystallographically characterized model complexes, [Cu(SC6H5)3]2- and [Cu(Im)4]2+.

2.6.4 Oxidative release of copper from WCBD

To samples of copper reconstituted fusion protein (1.8 mg/ml, 18.4 μM) were added either hydrogen peroxide (H2O2) or ceric ammonium nitrate (Ce(NH4)2(NO3)6) to a final concentration of 250 μM and the reaction was incubated at RT for 2 hours. One sample did not receive any oxidizing agents and was used as the control. The samples were then dialyzed extensively against 25 mM Tris-Acetate, pH 8.0, at 4°C and an aliquot from each sample was taken (1.53 mg protein) and submitted for copper determination by NAA.

2.7 Conformational Analysis of GST-WCBD and WCBD

2.7.1 Analysis of protein structural changes upon metal binding

Samples for CD analysis containing either copper or zinc were prepared as described in section 2.6.2 and analyzed on a Jasco J-720 spectropolarimeter. For analysis of changes in secondary structure, samples were loaded into a 0.1 cm path length CD cell and spectra were recorded from 300 - 190 nm. For analysis of changes in tertiary structure, samples were loaded into a 2 cm path length CD cell and spectra recorded from 400 - 250 nm. In both cases, spectra were recorded using the parameters listed in table 2.1. The spectra were
Table 2.1
CD Parameters for the Conformational Analysis of
GST-WCBD and WCBD

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Band Width</td>
<td>1.0 nm</td>
</tr>
<tr>
<td>Sensitivity</td>
<td>10 mdeg</td>
</tr>
<tr>
<td>Resolution</td>
<td>0.2 nm</td>
</tr>
<tr>
<td>Response</td>
<td>1 second</td>
</tr>
<tr>
<td>Speed</td>
<td>50 nm/second</td>
</tr>
<tr>
<td>Accumulation</td>
<td>4</td>
</tr>
</tbody>
</table>
corrected for the contribution of the buffer and noise reduced and the data were converted to molar ellipticity using the software supplied with the spectrometer. Molar ellipticity values per residue were calculated by dividing the molar ellipticity by either 867 for GST-WCBD or 649 for the WCBD.

2.7.2 Estimation of secondary structure content

Secondary structure content estimation of both the WCBD and the fusion protein reconstituted with various amounts of copper and zinc was carried out using the SELCON (self consistent method) and K2D (neural network) programs (Andrade et al., 1993; Bohm et al., 1992; Merelo et al., 1994; Sreerama and Woody, 1994; Sreerama and Woody, 1993). For the SELCON program, CD data between 260 - 200 nm and the secondary structure assignments and protein data base of W. C. Johnson Jr., containing 33 proteins, were used (Johnson, 1990). Parameters for the SELCON programs were as follows:

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CONST1 (limit for sum of fractions</td>
<td>sum fk - 1.0)</td>
</tr>
<tr>
<td>CONST2 (limit for fractions, fk &gt;)</td>
<td>-0.15</td>
</tr>
<tr>
<td>NITR (max number of iterations for self-consistency)</td>
<td>10</td>
</tr>
<tr>
<td>CONVRG (convergence criteria for sel-consistency)</td>
<td>0.005</td>
</tr>
</tbody>
</table>

For the K2D program, CD data between 240 and 200 nm was used. The SELCON program was used to predict α-helix, β-sheet, β-turns, and other conformations, while the K2D program was used to calculate α-helix, β-sheet and random conformations. The K2D program was found to give very good estimates of β structure and this value was used in conjunction with the values from SELCON to estimate overall protein secondary structure content (Greenfield, 1996). These and other programs for the estimation of secondary structure from CD spectra are available at the following website http://www2.umdnc.edu/cdrwjweb/index.htm. I gratefully acknowledge the assistance of Dr. Norma J. Greenfield in the use and application of these programs.
CHAPTER 3

Metal Binding Properties of the N-Terminal Copper Binding Domain from the Wilson Disease Copper Transporting P-Type ATPase (ATP7B)

3.1 Introduction

In many ways the presence of copper in biological systems is a double edged sword. Although trace amounts of copper are absolutely required to sustain life, an excess or a deficiency of copper can lead to serious biochemical defects (Danks, 1995; DiDonato and Sarkar, 1997). Many aspects of copper transport have been studied in the past, however in recent years, key discoveries have been made which have allowed us to start dissecting the detailed mechanism of intracellular copper trafficking and copper homeostasis. The greatest of these discoveries has been the isolation of the gene responsible for both Menkes (Chelly et al., 1993; Mercer et al., 1993; Vulpe et al., 1993) and Wilson (Bull et al., 1993; Tanzi et al., 1993) diseases, two major genetic disorders of copper metabolism in humans. The discovery of these genes and the putative copper transporting P-type ATPases they encode, have opened up new avenues of research in the copper transport are1.

Understanding the mechanism of action of these proteins in vivo will be a necessary first step in understanding the disease mechanism. These ATPases represent a new class of human heavy metal transporting ATPases and have several interesting features. Although they share a great deal of homology with each other as well as with other cation transporting ATPases, they do have some features which are not present in other ATPases (Bull et al., 1993; Tanzi et al., 1993; Vulpe et al., 1993). Among these are a conserved Cys-Pro-Cys motif in transmembrane six, a conserved Ser-Glu-His-Pro-Leu motif in a cytoplasmic loop of unknown function and a large amino-terminal region containing six copies of the heavy metal-associated domain (HMA). The consensus sequence for the HMA, which spans the complete domain is: [LIVN]-x(2)-[LIVMFA]-x-C-x-[STAGCDNH]-C-x(3)-[LIVFG]-x(3)-[LIV]-x(9,11)-[IVA]-x-[LVFYS]. The HMA has also been found in the metal binding
domains of various bacterial heavy metal transporting proteins (Odermatt et al., 1994; Phung et al., 1994). The two cysteine residues (bold) are strictly conserved and are most likely involved in metal ligation.

The presence of the large amino-terminal copper binding domain in the Menkes and Wilson proteins raises many questions as to its function. The repeated copper-binding motif represents a totally new and uncharacterized protein and its copper-binding site. Although it is likely that the two conserved cysteine residues in the motif participate in the ligation of copper, nothing further is known about this motif or its function in vivo. To begin to characterize this motif and the copper binding sites, we have developed a bacterial expression system for the production of the entire amino-terminal metal binding domain of the Wilson disease protein (ATP7B) fused to glutathione-S-transferase (GST).
3.2 Results

3.2.1 Expression and purification of GST-WCBD, GST-WCBD(P), and WCBD

The 2 kbp cDNA encoding the entire amino-terminal metal binding domain (residues 1-649, ~70 kDa in size) of the Wilson disease ATPase was produced, cloned and expressed as described in sections 2.2 and 2.3. As illustrated in figure 3.1, E. coli transformed with the expression plasmid pGEX-WCBD exhibited slightly better growth following induction in media supplemented with increasing amounts of copper when compared to E. coli transformed with the parental expression vector pGEX-4T-2. This indicates that the expressed fusion protein may be offsetting the damaging effects of excess copper on the bacteria by binding it and lowering its free concentration.

A typical purification of WCBD, GST-WCBD, and GST-WCBD(P) is shown in figure 3.2. Approximately 70% of the expressed fusion protein was found in inclusion bodies, while 30% remained soluble. Fusion protein localized to inclusion bodies was recovered by solubilization and refolding from urea. Using a combination of glutathione affinity chromatography, and denaturing ion exchange chromatography the fusion protein was obtained in a >90% pure form. The identity of the protein was confirmed by amino-terminal sequencing.

Treatment of the purified fusion protein eluted from the glutathione affinity column with 6 M urea in the absence of reducing agents with BCS, gave rise to a reddish-orange color (λ_{max} = 480 nm) indicative of the Cu(I)(BCS)_{2} complex (Chen et al., 1996). This suggests that the domain binds copper in the +1 oxidation state and that copper is being incorporated into the protein during its production in bacteria. Absorption spectra of the purified fusion protein in the visible and UV regions do not show any features which would
Figure 3.1 *Effect of Copper on BL21(DE3) Growth Following Induction.* Growth of BL21(DE3) transformed with either A pGEX-4T-2 or B pGEX-WCBD. Tris-buffered copper was added to the media at the indicated final concentration at time of induction. At the times indicated, OD$_{600}$ readings were taken on aliquots of cells, diluted 10 fold.
Figure 3.2 Summary of Typical Purification of WCB and GST-WCBD. GST-WCBD was expressed and purified as described in section 2.3. A. purified WCB eluted from glutathione-sepharose 4B resin following 45 minutes cleavage of GST-WCBD with thrombin on the column. B. GST-WCBD eluted with urea from glutathione affinity resin following solubilization and refolding from inclusion bodies. C. purified GST-WCBD eluted from Q-sepharose FF anion exchange resin. Proteins derived from the modified vector pGEX-WCBD(P) were expressed and purified using the same procedure except, cleavage was carried out using the PreScission protease.
indicate the presence of copper in the +2 state, further suggesting the presence of Cu(I). The metal bound to the domain appears to be fairly strong since it is able to withstand denaturing, refolding and the presence of reducing agents (10 mM glutathione, 5 mM DTT). Metal binding under these conditions has also been observed for the estrogen receptor DNA binding domain (2 zinc finger protein having 4 cysteines in each finger) where a combination of low pH, urea, DTT and chelating agents are needed to remove the bound metal (Conte et al., 1996).

3.2.2 Metal binding characteristics of GST-WCBD and WCBD

3.2.2.1 Immobilized metal ion affinity chromatography (IMAC)

To investigate the metal binding properties of the domain, both immobilized metal ion affinity chromatography (IMAC) and $^{65}$Zinc binding assays were carried out using the fusion protein and the cleaved domain. The results of IMAC using the purified fusion protein are presented in figure 3.3. The experiment was run both under non-denaturing and denaturing conditions in the absence of reducing agents with nearly identical results. GST alone did not bind to uncharged metal columns and had some weak, non-specific interactions under non-denaturing conditions on metal charged columns. The fusion protein did not bind to columns charged with either Fe(II) or Fe(III). The other transition metals showed varying affinities for the domain. Based on the sequential elution conditions used, the apparent order of affinity for the different transition metals for the domain was: Cu(II)>>Zn(II)>Ni(II)>Co(II).

The variation in affinity may reflect the inability of the protein metal binding sites to adopt the preferred conformation for that metal atom. Interestingly, the fusion protein could
Figure 3.3 Immobilized Metal Ion Affinity Chromatography (IMAC) of GST-WCBD. Samples (S) of the fusion protein were applied to chelating sepharose FF columns charged with the indicated metal under non-denaturing conditions. Protein which did not bind to the column came off in the flow through (FT). Bound protein was eluted by sequential treatment with low pH (6.5 - 4.0), increasing amounts of imidazole (100 - 500 mM), EDTA and BCS. No binding was observed to columns charged with iron (+2 or +3). Very similar results were obtained using denaturing conditions.
only be released from the Cu(II)-charged column by the addition of the specific copper chelator BCS. Also, elution of the fusion protein with BCS was accompanied by the formation of the reddish-orange Cu(I)(BCS)$_2$ complex, indicating that copper was bound to the domain in the +1 oxidation state. Considering that the column was charged with Cu(II) in the absence of reducing agents, this observation suggests that Cu(II) atoms may be reduced to Cu(I) upon binding to the domain.

3.2.2.2 Neutron activation analysis (NAA)

To determine the stoichiometry of metal binding to the domain, neutron activation analysis was used to quantitate the amount of copper bound to GST-WCBD following reconstitution with excess metal during refolding. A sample (1.225 mg) of GST-WCBD (~96 kDa, 0.0129 µmol) was analyzed by NAA and found to contain 0.089 ± 0.005 µmol of copper. This analysis revealed that 6.5 – 7.3 moles of copper are bound per mole of fusion protein. Similar ratios were obtained if refolded apo-fusion protein was reconstituted with copper. The amount of copper bound was found to be the same for both GST-WCBD and WCBD, indicating that GST does not bind any copper and that the domain is able to bind 6 – 7 copper atoms. These results are consistent with similar observations made during the characterization of Cox17, which was also expressed as a fusion to GST (Srinivasan et al., 1998).

3.2.2.3 $^{65}$Zinc blotting analysis

In order to determine if WCBD is able to bind zinc and to further investigate the metal binding properties of WCBD, a $^{65}$zinc blotting assay was employed. Preliminary
experiments indicated that both GST-WCBD and WCBD were able to bind zinc under the experimental conditions used. To determine the effect of pH on zinc binding, the assay was carried out over a range of pH values from 6.5 – 9.0; these results are summarized in figure 3.4. Both GST-WCBD and WCBD are able to bind zinc over the entire pH range evaluated. Progressive loss of zinc binding activity is only seen at the upper end of the pH range and may be related to the pKₐ of the ligating atoms. GST alone did not bind appreciable amounts of zinc, while the ERDBD (positive control) seemed to be much more sensitive to variations in pH. The effect of reducing agents (DTT) on the binding of zinc was also examined. These results are summarized in figure 3.5. The ⁶⁵zinc binding assay was carried out at pH 6.5 and 8.0 in either the absence or presence of DTT or just equilibrating the blot with DTT and then removing it before addition of ⁶⁵zinc. In the absence of DTT, zinc binding activity was not observed at either pH value. However, strong zinc binding was observed when DTT was used in the equilibration step only or if it was present throughout the assay. When present throughout the assay, DTT was able to decrease the amount of non-specific binding dramatically, as observed at pH 8.0. This is most likely due to the ability of DTT to chelate non-specific and weakly bound zinc atoms. The requirement for DTT pretreatment suggests that cysteine residues are directly involved in metal ligation and that a free sulfhydryl is required for metal ligation. This supports the notion that the two cysteine residues in the HMA domain, strictly conserved in each of the metal binding domains of ATP7B, as well as many bacterial heavy metal transporters, are crucial for metal ligation (O'Halloran, 1993; Silver et al., 1989).
Figure 3.4 Effect of pH on the Binding of $^{65}$Zinc. Samples of GST-WCBD (F), WCBD (C), GST (G), and the estrogen receptor DNA binding domain (E) were subjected to $^{65}$zinc blotting analysis as described in section 2.5.2. In each case, blotting was performed in metal binding buffer at the indicated pH in the presence of 1 mM DTT.
Samples of GST-WCBD (F), WCBD (C), GST (G), and the estrogen receptor DNA binding domain (E) were subjected to zinc blotting analysis, as described in section 2.5.2, at two pH values (6.5 and 8.0). M, molecular weight markers. To test the effects of reducing agents, DTT was either omitted from the assay (-DTT), included only during the equilibration step (+DTT/Eq), or included throughout the assay (+DTT).
3.2.2.4 Competition \textit{^{65}Zinc blotting analysis}

The ability of WCBD to bind copper and other transition metals was investigated using a competition \textit{^{65}zinc blot}. The results of this analysis are presented in figure 3.6. All competition \textit{^{65}zinc blots} were carried out using purified WCBD and DTT was present only in the equilibration step. Several other transition metals were able to compete successfully with zinc for binding to the domain. In particular, Cd(II), Au(III), and Hg(II) were found to have the highest affinities for the domain relative to zinc. Both Mn(II) and Ni(II) were not able to compete with zinc for binding to the domain when present in concentrations from 0.01 to 0.1 mM. Blotting with \textit{^{63}Ni(II)} directly instead of \textit{^{65}Zn(II)} did not give rise to a signal on the autoradiograph indicating lack of binding to the domain. Co(II), Cr(III), and Fe(III) were the weakest competitors of the transition metals tested. The binding of zinc also seemed to be specific, since a large excess of both Mg(II) and Ca(II) did not compete at all for zinc binding. Results with copper were very dramatic and unique compared to the other metals studied. At low concentrations, copper was able to decrease zinc binding by about 30%. However, as the concentration of copper was raised, the affinity of copper seemed to increase rapidly. This pattern was reproducible and independent of whether copper was presented in the +1 or +2 oxidation state, suggesting that the domain has similar affinities for both Cu(I) and Cu(II). The behavior of copper as a competitor for zinc binding was similar to that observed for the cooperative binding of ligands to proteins, indicating a possible cooperative binding mechanism for copper which was not observed for the other transition metals tested.
Figure 3.6 *Competition of Zn Binding to WCBD.* Samples of purified WCBD were subjected to competition Zn blotting analysis as described in section 2.5.2. In each case the final Zn concentration was 30 μM (~10 μCi). A, competition of Zn binding with various transition metals. All competitor metals were added as the chloride salt at the indicated concentration. Ni(II), Mn(II), Mg(II), and Ca(II) showed no affinity for the domain relative to Zn. B, competition of Zn binding with Cu(I) and Cu(II). Cu(II) was presented as CuCl₂ while Cu(I) was presented as tetrakis(acetonitrile)copper(I) hexafluorophosphate. This complex was prepared in an argon-purged solution of 2% acetonitrile.
3.3 Summary and Discussion

We have expressed the amino-terminal metal binding domain of ATP7B as a fusion to GST and studied its metal binding properties. We have shown that the domain binds approximately 6 moles of copper per mole of protein regardless of whether GST is present or absent. Using IMAC, we have been able to determine that the domain is able to bind a variety of different transition metals with the apparent order of affinity: Cu(I)/Cu(II)>Zn(II)>Ni(II)>Co(II) under both denaturing and native conditions. Specific binding of metalloproteins with internal metal binding sites to IMAC columns under denaturing conditions has also been observed for troponin T, which contains four repeated metal binding motifs (Jin and Smillie, 1994). In this assay, we did not observe binding of the domain to columns charged with either Fe(II) or Fe(III). This could indicate that the domain had no affinity for iron or that the metal binding sites were unable to ligate iron while it was bound to the IMAC matrix, possibly due to steric effects.

The domain bound very strongly to columns charged with copper, as expected, and could only be released with the specific copper chelator, BCS. Upon elution from the column, the presence of Cu(I) in the protein was indicated by the formation of the reddish-orange Cu(I)(BCS)$_2^-$ complex, a surprising result considering that the column was charged with Cu(II) and non-reducing conditions were used. This observation suggests that the domain is able to reduce copper from the +2 to +1 oxidation state upon binding. This could occur through the oxidation of a pair of cysteines in one domain followed by the binding of copper to a reduced pair of cysteines in another domain. This gives rise to the possibility that the domain could bind both Cu(I) and Cu(II) in vivo prior to transport. The oxidation state of
the transported copper is not yet known, however there is one report which suggests that it may be in the Cu(II) state (Bingham et al., 1996).

An in depth study of the metal binding properties of the domain using $^{65}$zinc blotting techniques was performed. The results from this analysis have confirmed and expanded upon those obtained in the IMAC experiments with two exceptions. Although the fusion protein bound to IMAC columns charged with Ni(II), Ni(II) was not able to compete successfully with zinc for binding to WCBD in competition $^{65}$zinc blotting experiments. This situation could arise if the affinity for zinc was much greater than that for nickel, in which case nickel would continuously be competed away from the protein by zinc. This was tested by blotting with $^{63}$nickel directly instead of $^{65}$zinc. Binding of $^{63}$nickel to WCBD was not observed, indicating that either the domain is not able to bind nickel or that the affinity for nickel is extremely low. The converse situation was observed for iron, where binding was not observed to iron-charged IMAC columns, but iron was able to act as a competitor for zinc binding in competition blotting experiments.

The reasons for these discrepancies are not clear and they may be due to the inherent differences in the two methods used. In IMAC, the metal is immobilized on a matrix and the protein must bind to the metal while it is bound to the matrix; in the blotting experiments, the metal must bind to protein which is immobilized on nitrocellulose. In IMAC, the protein is, in essence, 'sharing' the metal with the matrix, with some ligands coming from the matrix and others from the protein in a ternary form. Bearing this in mind, the results from the blotting assays may more accurately reflect the binding properties of the domain, since all ligands originate from the protein itself. The binding of transition metals other than copper to the WCBD has been observed by another laboratory (Lutsenko et al., 1997), and are in
agreement with what has been found in our studies. However, we have used two independent methods and a wider range of metals than was used in the study by Lutsenko et al. (1997) to assess the metal specificity of the WCBD.

The unique characteristics observed for copper as a competitor may have important consequences for the function of the domain in vivo. Although the blotting experiments cannot be used to establish the presence of cooperative copper binding conclusively, they are very suggestive. The binding of zinc to the domain is not surprising since the Gly – Met – Thr – Cys – Xaa – Xaa – Cys motif is also present in the bacterial zinc transporting ATPase, ZntA (Rensing et al., 1997). What is not clear is how the specificity for copper is conferred on the ATPase. If cooperative copper binding is taking place, it may be the basis of the specificity. A cooperative binding model for copper would require structural changes to take place upon successive metal binding, which would bring the ligating residues to the appropriate geometry for copper binding through tertiary folding of the protein. We have studied the structural changes which occur in the WCBD using circular dichroism spectroscopy and these results are presented in the following chapter.
CHAPTER 4

Structural Characterization of the Wilson Disease Copper Binding Domain And Its Metal Binding Sites
4.1 Introduction

The HMA domain is found in proteins from a wide range of organisms which are involved in the transport of a variety of heavy metals, including zinc, copper, cadmium and mercury. In most lower organisms, one or two copies of the HMA are present; in higher organisms five or six copies are common. Although it is commonly known that these domains are involved in ligating the metal atom prior to transport, little is known about the structure of these domains and how the same domain can serve to ligate diverse metal atoms. Furthermore, information regarding inter-domain interactions in multiple HMA domain proteins (Menkes and Wilson disease ATPases) is practically nonexistent. The structural information that is available is derived from studies on proteins with only one copy of the HMA or from a single HMA excised from a protein containing multiple copies (Gitschier et al., 1998; Pufahl et al., 1997).

The NMR solution structure of the fourth HMA domain from the Menkes disease protein complexed with silver(I) revealed linear coordination of the metal by the conserved cysteine residues (Gitschier et al., 1998). Silver is known to prefer linear coordination and it could be possible that the binding of silver to the domain may force linear coordination on the metal binding site. Therefore, it is not clear if the conformation this domain adopts in the presence of silver would be similar to that adopted in the presence of copper(I), which could bind in either a linear, trigonal, or tetrahedral fashion. Additionally, the authors found that the conformation of the apo-protein was essentially the same as the holoprotein. An XAS study of the yeast copper chaperone ATX1, which contains one copy of the HMA, revealed that copper ligation could be either linear or trigonal with all sulfur ligands (Pufahl et al., 1997). In this system, it was proposed by the authors that both conserved cysteines and a
methionine were involved in ligating the copper atom. Recently, a report has appeared from an EXAFS study of the full amino-terminal metal-binding domain of the Menkes protein fused to maltose binding protein (MBP). The authors of this report propose a completely linear ligation model involving only the conserved cysteine residues and also suggest a copper – copper interaction (Ralle et al., 1998). However, this report does not give any details regarding the copper:protein ratio or the purity of the sample, which would have allowed useful conclusions to be draw from the data.

A recent study of the second metal binding domain from the Menkes disease protein has shown that one domain does not undergo structural changes upon metal binding (Harrison et al., 1999). The study of a single metal binding domain from either the Menkes or Wilson disease protein can help in understanding the sub-structure of the intact domain; however, these studies cannot reveal any information on how the entire domain may function in vivo. It has been assumed that the conserved cysteine residues in the HMA repeats are the only residues involved in ligating the metal atom. However, analysis of the primary sequence of the Wilson disease protein reveals six additional cysteine residues, evenly distributed among the six HMA domains, which may play a role in metal ligation. Furthermore, other potential ligating residues such as histidine are also present in abundance in the amino-terminal metal-binding domain. Single domain studies or studies involving single HMA proteins would not be able to determine the potential function of these or other residues with regards to metal ligation. In order to address some of these questions, we have begun a detailed structural study of the copper binding sites in the WCBD, using XAS (in collaboration with Dr. Lawrence Que), in addition to analyzing the structural changes which take place upon metal binding to the WCBD, using circular dichroism spectroscopy.
4.2 Results

4.2.1 X-ray absorption spectroscopy (XAS) analysis of GST-WCBD

A detailed analysis of the copper binding sites was carried out using XAS techniques in collaboration with Dr. Lawrence Que, Jr. and Hua-Fen Hsu at the University of Minnesota. Since the WCBD contains six copper binding sites, XAS techniques carried out on the fully reconstituted domain (6 bound copper atoms) will give structural information which is averaged over the six sites. To address the question of whether each copper binding site is the same structurally, the XAS analysis was carried out on the fusion protein containing either 2, 3 or 5 copper atoms. The copper x-ray absorption near edge structure (XANES) spectra for all three samples are shown in figure 4.1. All three spectra are very similar and exhibit a feature at 8983 eV which is consistent with the $1s \rightarrow 4p$ transition of Cu(I) ions (Kau et al., 1987). The intensity of the transition at 8983 eV is correlated with the geometry around the copper atom. Tetrathedrally coordinated copper(I) atoms have low intensity transitions, while linear, two coordinate sites, have well resolved, high intensity transitions. The observed transition for the WCBD is weaker than that of diagonal compounds but stronger than that for trigonal compounds.

The extended x-ray absorption fine structure (EXAFS) spectra of the three samples are shown in figure 4.2. The EXAFS spectra for all the samples are very similar both in $k$-space and $r'$-space (fig. 4.2 A and B), which is consistent with the results of the XANES spectra of the same samples. The $r'$-space spectra of all samples exhibit a prominent feature centered around $r' = 1.8 \text{ Å} (r \sim r' + 0.4 \text{ Å})$ from a shell of sulfur scatterers. The best fit for the first coordination sphere was obtained assuming a single shell of two sulfur atoms at 2.17-2.19 Å. A single shell with one or three sulfur atoms or splitting the shell into one sulfur and
Figure 4.1 Normalized Copper XANES Spectra For GST-WCBD with Various Amounts of Copper. XANES spectra were taken from lyophilized samples of GST-WCBD containing either 2 (2 Cu), 3 (3 Cu), or 5 (5 Cu) atoms. The amount of copper was quantified using neutron activation analysis. Cu(SR)$_2$: [N(C$_3$H$_7$)$_4$]Cu(SC$_{10}$H$_{13}$)$_2$; Cu(SR)$_3$: [(C$_6$H$_5$)$_4$P] [Cu(SC$_6$H$_5$)$_3$].
Figure 4.2 *EXAFS Spectra For GST-WCBD with Various Amounts of Copper.* 2 Cu (solid line), 3 Cu (dotted line), 5 Cu (dash-dotted line). A EXAFS in k-space, B EXAFS in r'-space \((r - r' + 0.4 \text{ Å})\), C r'-space EXAFS for 5 Cu sample overlaid with that from Cu(SR)₂ (dot) and Cu(SR)₃ (diamond).
one low Z atom subshell at 2.17 - 2.19 and 1.9 - 2.1 Å respectively, resulted in poorer fits. The r'-space spectra of all samples also exhibit a minor peak at r' = 2.3 Å which is not present in the spectra of the model compounds [Cu(SC\textsubscript{10}H\textsubscript{13})\textsubscript{2}]\textsuperscript{+} and [Cu(SPh)\textsubscript{3}]\textsuperscript{2-}. A good fit for this feature could not be obtained using either sulfur or copper atoms.

4.2.2 Circular dichroism analysis of GST-WCBD and WCBD

The conformational dynamics of WCBD upon binding of metal were studied using circular dichroism spectroscopy. Spectra of samples with increasing amounts of either copper or zinc were recorded in the secondary structure region (250 – 200 nm) and the aromatic region (250 – 400 nm). The spectra of the fusion protein and the WCBD alone reconstituted with either copper or zinc are presented in figures 4.3, 4.4, 4.5 and 4.6. The results obtained for the fusion protein are very similar to those of the WCBD alone, with some exceptions. Addition of copper to the apo-fusion protein induced an increase in secondary structure (fig. 4.3 A). The magnitude of the increase was the greatest on going from the apo state to the 2:1 state and from the 2:1 to 4:1 state. Going from 4:1 to 6:1 or 8:1 did not result in any further significant secondary structure changes. Significant changes were also observed in the aromatic region (fig. 4.3 B). The changes in this region paralleled those observed in the secondary structure region with the largest changes occurring between apo and 2:1 and from 2:1 to 4:1. A large difference is also observed on going from 4:1 to 6:1, which is not observed in the secondary structure region. No significant changes were observed on going from 6:1 to 8:1.

The changes observed upon the addition of zinc to the apo-fusion protein are very different from those obtained with copper (fig. 4.4). Although the addition of zinc to the
apo-protein does cause an initial increase in secondary structure, additional molar equivalents seems to cause an alternating increase and decrease in secondary structure (fig. 4.4 A). This pattern is not observed for copper, suggesting that the binding of zinc induces a structure which is significantly different from that with copper. The differences between copper and zinc are also observed in the aromatic region (fig. 4.4 B) where the pattern of changes is significantly different from what is observed with copper. Substantial changes from the apo-protein spectra are only observed on going from apo to 4:1 and from 4:1 to 8:1. The spectra of the apo-fusion protein and the 2:1 and 6:1 zinc reconstituted protein are virtually identical. These observations, like those in the secondary structure spectra, suggest that the binding of zinc to the domain leads to a very different structure from that obtained with copper. This finding may have significant implications for the metal selectivity of the pump in vivo.

The titration of the WCBD alone with copper produced results which were similar to those observed with the fusion protein with some exceptions (fig. 4.5). A general increase in secondary structure was observed upon progressive addition of copper. The magnitude of the change was greatest between the apo form and the 2:1-form and to a lesser extent from the 4:1-form to the 6:1-form (fig. 4.5 A). In contrast to the fusion protein spectra, addition of copper equivalents above 6:1 resulted in an apparent loss of secondary structure in the domain. These differences could result from the presence and absence of GST, which may be influencing certain structural changes in the fusion protein. The changes in the aromatic region are also similar to those observed in the fusion protein, with some differences (fig. 4.5 B). The largest changes are observed on going from apo to 2:1 and from 2:1 to 4:1; however, in contrast to the fusion protein, only small changes were observed on
Figure 4.3 CD Spectra of GST-WCBD Titrated with Copper. The fusion protein was reconstituted with copper to the indicated ratio and CD spectra recorded. A. Secondary structure region, B. Aromatic region.
Figure 4.4 CD Spectra of GST-WCBD Titrated with Zinc. The fusion protein was reconstituted with zinc to the indicated ratio and CD spectra recorded. A. Secondary structure region, B. Aromatic region.
Figure 4.5 CD Spectra of WCBD Titrated with Copper. The WCBD was reconstituted with copper to the indicated ratio and CD spectra recorded. A. Secondary structure region, B. Aromatic region.
Figure 4.6 CD Spectra of WCBD Titrated with Zinc. The WCBD was reconstituted with zinc to the indicated ratio and CD spectra recorded. A. Secondary structure region, B. Aromatic region.
going from 4:1 to 6:1. There is also a significant change on going from 6:1 to 8:1, which was not observed in the fusion protein.

Titration of WCBD alone with zinc produced a pattern of conformational changes (fig. 4.6) which were different from that of copper and resembled those obtained from the addition of zinc to the apo fusion protein. The initial addition of zinc resulted in an apparent loss of secondary structure. The addition of increasing amounts of zinc produced an alternating increase and decrease in the amount of secondary structure present (fig. 4.6A). This pattern was significantly different from that obtained with copper and resembled the pattern found upon the addition of zinc to the fusion protein. The aromatic region (fig. 4.6B) also displayed features which were different from those obtained with copper. With the copper titrations, an initial increase at 260 nm was observed from the apo to 2:1 reconstituted protein. At higher ratios this increase subsided and was replaced with an increase in the 290 nm range. For the zinc samples, there was only a small increase at 260 nm for the addition of 2 equivalents of zinc and at higher ratios no increases in the 290 nm range were observed. These results suggest that the binding of zinc by the WCBD results in a conformation which is significantly different from that with copper.

4.2.3 Estimation of Secondary Structure Content

The secondary structure content of the WCBD with various copper ratios (0, 2, 4, 6, 8 metal atoms / mol protein) and of the fusion protein with zinc and copper (0, 2, 4, 6, 8 metal atoms / mol protein) were estimated as described in section 2.7.2. The results of this analysis are presented in table 4.1. Each of the different metal ratios represents a separate protein
sample prepared from the same apo stock. Metal concentrations were confirmed by NAA, and protein concentrations were confirmed using the BCA protein assay.

4.2.4 Oxidative release of copper from GST-WCBD

The ability of oxidizing agents to liberate bound copper from the fusion protein was evaluated and these results are presented in figure 4.7. The results indicated that only hydrogen peroxide was able to effect the release of approximately 2 copper atoms from the domain after a 2 hour incubation. Addition of the oxidizing agent, ceric ammonium nitrate, caused the protein to precipitate with no apparent loss of copper.
Table 4.1
Secondary Structure Estimation for Metal Titrated WCBD and GST-WCBD Using SELCON and K2D

<table>
<thead>
<tr>
<th>Metal : Protein Ratio</th>
<th>Apo</th>
<th>2:1</th>
<th>4:1</th>
<th>6:1</th>
<th>8:1</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cu-WCBD</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>α-Helix (%)</td>
<td>13</td>
<td>17</td>
<td>16</td>
<td>19</td>
<td>9</td>
</tr>
<tr>
<td>β-Sheet&lt;sup&gt;†&lt;/sup&gt; (%)</td>
<td>41(32)</td>
<td>30(28)</td>
<td>34(26)</td>
<td>29(25)</td>
<td>40(31)</td>
</tr>
<tr>
<td>β-Turns (%)</td>
<td>19</td>
<td>22</td>
<td>18</td>
<td>22</td>
<td>18</td>
</tr>
<tr>
<td>Other (%)</td>
<td>31</td>
<td>30</td>
<td>30</td>
<td>26</td>
<td>31</td>
</tr>
<tr>
<td><strong>Zn-WCBD</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>α-Helix (%)</td>
<td>15</td>
<td>18</td>
<td>14</td>
<td>16</td>
<td>18</td>
</tr>
<tr>
<td>β-Sheet&lt;sup&gt;†&lt;/sup&gt; (%)</td>
<td>27(22)</td>
<td>30(22)</td>
<td>29(21)</td>
<td>35(26)</td>
<td>29(22)</td>
</tr>
<tr>
<td>β-Turns (%)</td>
<td>18</td>
<td>21</td>
<td>18</td>
<td>19</td>
<td>20</td>
</tr>
<tr>
<td>Other (%)</td>
<td>34</td>
<td>30</td>
<td>29</td>
<td>27</td>
<td>28</td>
</tr>
<tr>
<td><strong>Cu-GST-WCBD</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>α-Helix (%)</td>
<td>16</td>
<td>29</td>
<td>30</td>
<td>38</td>
<td>30</td>
</tr>
<tr>
<td>β-Sheet&lt;sup&gt;†&lt;/sup&gt; (%)</td>
<td>37(21)</td>
<td>12(14)</td>
<td>11(13)</td>
<td>24(20)</td>
<td>11(15)</td>
</tr>
<tr>
<td>β-Turns (%)</td>
<td>26</td>
<td>20</td>
<td>21</td>
<td>15</td>
<td>21</td>
</tr>
<tr>
<td>Other (%)</td>
<td>21</td>
<td>39</td>
<td>38</td>
<td>23</td>
<td>39</td>
</tr>
<tr>
<td><strong>Zn-GST-WCBD</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>α-Helix (%)</td>
<td>14</td>
<td>18</td>
<td>14</td>
<td>25</td>
<td>16</td>
</tr>
<tr>
<td>β-Sheet&lt;sup&gt;†&lt;/sup&gt; (%)</td>
<td>31(28)</td>
<td>24(13)</td>
<td>35(27)</td>
<td>14(11)</td>
<td>28(17)</td>
</tr>
<tr>
<td>β-Turns (%)</td>
<td>17</td>
<td>17</td>
<td>19</td>
<td>18</td>
<td>18</td>
</tr>
<tr>
<td>Other (%)</td>
<td>25</td>
<td>31</td>
<td>25</td>
<td>43</td>
<td>27</td>
</tr>
</tbody>
</table>

<sup>†</sup>Values in brackets indicate β-Sheet content estimated by the K2D program, all others are from SELCON.
Figure 4.7 Oxidative Release of Bound Copper From WCBD. Copper reconstituted fusion protein was incubated for 2 hours in the presence of the indicated oxidizing agent. Remaining bound copper was quantified by NAA. †This compound caused the protein to precipitate.
4.3 Summary and Discussion

The XAS studies of the WCBD with sub-stoichiometric amounts of copper have provided a wealth of detailed structural information. The XANES spectra (fig. 4.1) further confirm the previous observation that copper bound to the WCBD is in the +1 oxidation state by the presence of a feature at 8983 eV. This feature is attributed to a 1s $\rightarrow$ 4p transition which is characteristic of Cu(I) atoms. The intensity of this transition in the spectra of WCBD is weaker than that for linear copper thiolate complexes. This observation suggests a distorted, two coordinate geometry around the copper atom. This distortion may arise from protein conformational effects or the presence of a weak interaction from a third ligand. There is some evidence of the latter due to the presence of the minor peak at $r' = 2.3 \text{ Å}$ in the EXAFS spectra. Good fits for this feature could not be obtained with either copper or sulfur atoms and, therefore, the identity of this scatterer remains undetermined.

This feature is not present in the spectra of the model compounds but is observed in the EXAFS spectra of the Menkes protein (Ralle et al., 1998). The authors of this paper suggest the possibility that this feature arises from the formation of copper clusters in the domain. Although this is a definite possibility, the study by Ralle et al. is not detailed enough to draw this conclusion. The XAS studies presented in this thesis are more detailed since various ratios of copper were used. The results show that the feature at $r' = 2.3 \text{ Å}$ increases in intensity as greater amounts of copper are added. This could lend support to the idea that copper clusters are formed as more copper becomes bound to the domain; however, the low intensity of this peak indicates that this is not a major form of the protein in these samples. In preliminary experiments using samples which were reconstituted with copper in the presence of TCEP and ascorbic acid, this feature is of greater intensity. The intensity of
the peak also rises as increasing amounts of copper are bound to the domain. Interestingly, this peak is also a major feature in the spectra of non-lyophilized samples prepared in the presence of DTT. It is possible that the domain can adopt multiple conformations under different conditions. Further studies will be needed to establish the identity of the scatterer which gives rise to this feature conclusively.

Fitting of the EXAFS data indicate that the first coordination sphere consists of two sulfur atoms with a Cu-S distance of 2.17 - 2.19 Å. This distance is much shorter than those in trigonal Cu(I)-thiolate complexes and is slightly longer than those of linear Cu(I)-thiolate complexes (Coucouvanis et al., 1980; Fujisawa et al., 1998; Koch et al., 1984). Trigonal Cu(I)-thiolate complexes have Cu-S bond distances of 2.27 - 2.38 Å, whereas linear Cu(I)-thiolate complexes have Cu-S bond distances of 2.14 - 2.15 Å in structurally characterized model complexes. The Cu-S bond distances in the WCBD are similar to those found in the Menkes protein (2.17 Å) but are much shorter than those found in the yeast copper chaperone ATX1 (2.25 Å), which has only one HMA domain (Pufahl et al., 1997). This implies that the copper sites in the WCBD are similar to those in the Menkes disease protein but significantly different than that in ATX1. These observations indicate that the presence of multiple HMA domains have an influence on the copper coordination environment. In light of these findings, studies based on a single domain from the WCBD or those without carefully characterized metal stoichiometries cannot provide an accurate representation of the domain as a whole.

The structure dynamics of the WCBD (and GST-WCBD) upon titration with either copper or zinc were studied using circular dichroism spectroscopy. Both the secondary structure region (200 - 270 nm) and the aromatic region (250 - 350 nm) were analyzed for
structural changes upon metal binding. The conformational changes which were observed upon metal binding were similar for both the WCBD and GST-WCBD proteins (for the same metal) with only minor differences. In the secondary structure region of proteins titrated with copper (figs. 4.3, 4.5, panel A), the general observation is that the ellipticity increases upon binding of greater amounts of copper. This increase suggests an increase or stabilization of secondary structures relative to the metal free (apo) protein. This was observed in both the fusion protein and the WCBD alone. In both cases, the magnitude of the change is greatest between the apo form and the 2:1 copper-form. However, in the WCBD, another significant increase takes place on going from 4 to 6 copper atoms and this is not observed in the fusion protein. Additionally, adding more than the stoichiometric amount of copper to the WCBD resulted in an apparent destabilization of secondary structure relative to the apo-protein. This phenomenon was not observed in the fusion protein. These differences are most likely the result of the presence of GST, which is able to form dimers in solution. We have observed that, during purification, the fusion protein is substantially more soluble and less prone to aggregation than the WCBD alone. Furthermore, addition of excess copper to concentrated solutions of the apo-WCBD usually results in the formation of a precipitate, a phenomenon which is not observed with the fusion protein. Although precipitate formation was not observed in the CD samples, soluble aggregates of the WCBD may have been formed at the higher copper ratios, leading to the apparent loss of secondary structure observed in the CD spectra of the WCBD with an 8:1 copper:protein ratio.

The changes in the aromatic region were also very similar between the fusion protein and the WCBD. The changes which occurred in the aromatic region spectra upon addition of copper paralleled those in the secondary structure region. The largest changes in spectral line
shape were observed on going from the apo-protein to the 2:1 copper form and again from the 2:1 form to the 4:1 copper form. It is interesting to note that, in the WCBD, the 2:1 and 4:1 copper forms seemed very similar in secondary structure but their tertiary structure spectra were significantly different. A similar phenomena was also observed in the fusion protein. This would seem to indicate that, although the secondary structure remains relatively constant, the relative orientation of secondary structure elements within the domain are changing, depending on how much metal is bound. These tertiary structure changes could be the basis of cooperativity in the domain where binding of copper to one site leads to a modification of binding properties at another site. Cooperativity of copper binding to the WCBD was suggested by competition zinc blotting experiments using copper.

The spectra in the aromatic region arise from the $\pi-\pi^*$ electronic transitions from the aromatic side chains of tyrosine, tryptophan, and phenylalanine residues. However, because these residues contain complete or partial planes of symmetry, the transition arise entirely from interaction with neighboring groups (Wingfield and Pain, 1996). A detailed interpretation of the spectra in this region is extremely difficult, since many of the transitions overlap (table 4.2). In proteins which contain several of each kind of aromatic amino acid (each in a different environment), deconvolution of the spectra to isolate contributions from each residue is nearly impossible in the absence of either a three dimensional structure or detailed mutational studies. Despite these difficulties, we can observe several patterns in both the fusion protein and the WCBD, which occur upon copper binding. In going from the apo to 2:1 copper form a decrease (more positive) in ellipticity occurs in the 250 to 270 nm region. When copper is added to a 4:1 ratio, the ellipticity in this region increases while the ellipticity in the 270 – 290 nm region decreases. The former region contains mainly
Table 4.2
CD Transitions of Aromatic Side Chains

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Electronic Transitions</th>
<th>Peak Wavelengths (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenylalanine</td>
<td>$^1L_b$</td>
<td>262, 268</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>$^1L_a$</td>
<td>far UV</td>
</tr>
<tr>
<td></td>
<td>$^1L_b$</td>
<td>277, 283</td>
</tr>
<tr>
<td></td>
<td></td>
<td>268, 289 (shoulders)</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>$^1L_a$</td>
<td>266, ~295 – 303$^2$</td>
</tr>
<tr>
<td></td>
<td>$^1L_b$</td>
<td>285, 292, 305$^3$</td>
</tr>
</tbody>
</table>

$^1$Data from (Strickland, 1974).

$^2$May be seen as a low intensity peak or shoulder from a Trp in a nonpolar environment.

$^3$Unusual peak observed in lysozyme.
phenylalanine and tyrosine contributions, while the latter region contains mainly tryptophan contributions (Wingfield and Pain, 1996). It is clear that the largest changes in the spectra (both line shape and intensity) occur between the 2 and 4 copper forms of the protein. This indicates that a significant tertiary structure change is taking place after the first few coppers are bound, possibly reflecting the cooperative binding of copper after the first few are bound. This correlates well with the competition zinc blotting studies (see section 3.2.2.4). At lower copper concentrations zinc binding was reduced in a linear fashion and this was followed by a sharp transition at high copper concentrations, which is indicative of cooperative binding.

The disulfide bond can also make significant contributions to the ellipticity in CD spectra because it does not contain an inherent plane of symmetry. The disulfide bond has a broad band of ellipticity in the 240 – 350 nm range and can augment or diminish the signal from tyrosine and tryptophan residues (Wingfield and Pain, 1996). This further complicates the interpretation of the spectra in this region. In cases where the disulfide makes a significant contribution to the spectra, its presence is confirmed by ellipticity above 320 nm. This feature is present in both the fusion protein and the WCBD when they were titrated with copper. It is most prominent in the apo form, since without copper and reducing agents the 18 cysteine residues in the domain will readily oxidize to form disulfide bonds. Furthermore, this feature decreases as the amount of copper increases, since the cysteine residues are now participating in metal ligation and are unavailable for disulfide formation. When six copper atoms are bound, the ellipticity above 320 nm is nonexistent, suggesting that no disulfides are present. The domain contains twelve conserved cysteine residues which are located in six HMA domains (2 per domain) and are involved in ligating the metal. In addition, another six cysteine residues are evenly interspersed between the HMA domains. It is not known
whether these form disulfides or are free in the native domain. The CD results indicate the absence of disulfides in the fully copper-reconstituted state, indicating that either these cysteines remain reduced or that they too are involved in metal ligation. However, at this point we do not have any evidence to support one view over the other.

In contrast to the spectra obtained with copper, those obtained from the titration with zinc are very different in both the secondary structure and aromatic regions (figs. 4.4 and 4.6). The addition of zinc to both the apo fusion protein and the apo WCBD resulted in a strange pattern of alternating increase and decrease of ellipticity which is in stark contrast to the progressive increase in ellipticity observed for the copper samples. The same was true for the aromatic region, where the pattern of changes observed were not as significant and did not resemble those for the proteins titrated with copper. In particular, the ellipticity above 320 nm, which is indicative of the presence of disulfide bonds, did not decrease in a linear fashion, as was observed with copper. Although we saw a decrease on going from the apo to the 2 and 4 copper forms, no additional increase was observed on going from 4 to 6 or 8 coppers. These observations suggest that although zinc is able to bind to the domain (as seen in IMAC and zinc blotting) it is not able to induce the same structure in the domain as copper; this may be an important factor for the function of this domain in vivo. Differences in the conformation of the domain with zinc and copper may be due to the different geometrical preferences of each metal. While zinc predominantly forms tetrahedral complexes, copper can adopt conformations ranging from linear to distorted tetrahedral. This distinction may play a role in conferring specificity of metal binding to the domain.

Two computer programs SELCON and K2D were used to estimate the secondary structure content of the fusion protein and the WCBD with various amounts of copper and
zinc (table 4.1). The SELCON program (Sreerama and Woody, 1994; Sreerama and Woody, 1993) uses CD data between 260 and 200 nm to predict α-helix, β-structure, and β-turns in globular proteins. The program starts by arranging the proteins in the database in order of increasing RMS difference from the CD spectrum to be analyzed and the spectra which are most dissimilar are deleted. An initial guess of the unknown protein is made and this conformation is included in the basis set, which is deconvoluted using singular value decomposition (SVD). The secondary structure of the unknown protein is then calculated. The solution replaces the initial guess and the process is repeated until self-consistency is attained. The K2D is a neural net program (Andrade et al., 1993; Merelo et al., 1994) which uses a technique called proteinotopic mapping to calculate α-helix and β-structure using CD data between 240 and 200 nm. It has been found to give the best estimate of β-sheet content using this data range and was used in conjunction with the SELCON program (Andrade et al., 1993; Merelo et al., 1994).

The general trend in the WCBD titrated with copper was an increase in α-helix content (~6%), a decrease in β-sheet content (~10%), no change in the β-turn content and a slight decrease in 'other' conformations (~2%). In contrast, titrating the WCBD with zinc produced a fluctuation in the secondary structure content which followed the patterns seen in the spectra. Both the trend and magnitude of changes were substantially different from those produced with copper, further supporting the idea that these metals induce very different conformations in the domain upon binding. The trends for the fusion proteins with either copper or zinc were similar to those for the GST free domains. The absolute amount of each secondary structure element was higher than for the WCBD alone due to the contributions of GST to the spectra. The effect of copper binding seems to be one of stabilizing or increasing
α-helical content at the expense of β-sheet content. The binding of copper could rigidify the secondary structure around the metal binding site, stabilizing secondary structural elements which, in the absence of metal, adopt a different conformation. Alternatively, stabilization could be achieved by interaction of distant parts of the domain which are brought into close proximity by the binding of copper.

These studies have shown that copper is bound fairly strongly to the domain; however, at some point, the copper must be removed from the domain. We have investigated the possibility that copper may be removed by oxidation. After incubation of copper WCBD with either hydrogen peroxide or ceric ammonium nitrate, only 2 copper atoms were liberated from the domain with hydrogen peroxide. Ceric ammonium nitrate resulted in the precipitation of the protein; however, when copper was measured in the dialyzed precipitate, all six copper atoms were still present. These results indicate that, at least with hydrogen peroxide, it is possible to release some of the bound copper. The other copper atoms may be located in regions inaccessible to the oxidizing agent (buried in the core) or involved in structures which are resistant to oxidation. These results suggest the possibility that copper could be transported in the +2 oxidation state. There is at least one study where ATP-dependent transport of copper(II) has been detected in the golgi apparatus of rat hepatocytes (Bingham et al., 1996). Further research into the form of the transported copper, as well as the kinetics of copper binding to the amino-terminal domain, is needed.

4.3.1 Hypothetical Model for the Function of the WCBD in vivo

The current work has provided insights into the mechanism by which metal is bound to the WCBD and the structural changes which take place upon copper binding. Functional
studies, both in cultured cells and yeast model systems have also provided important insights into the role of the amino-terminal metal-binding repeats in the context of the whole ATPase. Immunohistochemical studies in HepG2 cells have shown that, under normal, steady-state conditions, the WND protein is localized in the membrane of the trans-golgi (Hung et al., 1997). However, if the concentration of copper was elevated in the media, a protein translocation event was observed which resulted in the appearance of the WND protein in the plasma membrane as well as an unidentified cytoplasmic vacuolar location (Hung et al., 1997). Furthermore, this translocation event was reversible and dependent on copper, since removal of copper from the media resulted in the WND protein recycling back to the trans-golgi. This phenomena has also been observed for the MNK protein and it is likely that these proteins continuously recycle between the trans-golgi and the plasma membrane under steady state conditions; elevated copper concentrations may shift the equilibrium toward plasma membrane localization (Nagano et al., 1998; Petris et al., 1996).

It has also been shown that not all of the metal binding repeats are needed to mediate the transport function of the pump (Forbes et al., 1999). Using a yeast complementation assay, Forbes et al. (1999) were able to demonstrate that the mutation of metal repeats 1 through 4 did not affect the transport activity of the pump. Mutation of repeats 1 through 5 resulted in an approximate 5% drop in transport activity, while mutation of all six domains totally abolished transport activity. This is in contrast to what has been found for the MNK protein. In MNK, sequential mutation of the metal binding repeats does not result in a significant loss of transport activity until the third motif is mutated (Payne and Gitlin, 1998). This suggests that the metal binding repeats in the MNK protein are not functionally equivalent and that motif three may play a central role in the binding and/or transport of the
metal. In the WND protein the data suggest that the metal binding repeats closer to the membrane are more important than those at the amino-terminus. The metal binding sites in the WND protein are also not functionally equivalent, since swapping the amino-terminal three domains with the three domains closer to the membrane does not result in a functional protein (Forbes et al., 1999).

Taken together, these results can be used to construct a hypothetical model of how the amino-terminal metal-binding domain functions in the context of the whole ATPase (fig. 4.8). In this model, the WND protein would reside in the trans-golgi membrane under normal physiological conditions where it would function to pump copper from the cytoplasm into the trans-golgi lumen. This copper could then be incorporated into secreted copper-containing enzymes such as lysyl oxidase and ceruloplasmin. When unoccupied with copper, the amino-terminal metal-binding domain interacts with the other cytosolic loops of the ATPase (possibly the SEHPL motif region), preventing the transport of copper through the pump. The binding of copper (most likely to the metal repeats closer to the transmembrane segments) triggers a conformational change in the metal binding domain which disrupts or changes its interaction with the cytosolic loops of the ATPase. This conformational change could also promote the phosphorylation of the enzyme, initiating the transport cycle and allow copper to be pumped into the trans-golgi lumen.

When the cytoplasmic concentrations of copper rise (as in copper overload), the additional metal binding repeats would become progressively occupied with copper leading to further conformational changes in the domain. These conformational changes would somehow trigger the translocation of the WND (and MNK) proteins from the trans-golgi to both the plasma membrane and a cytoplasmic vacuolar compartment. At the plasma
Figure 4.8 Hypothetical Model for the Function of WND in vivo. Under normal physiological conditions, the WND protein is localized in the trans-golgi network (TGN). When not occupied with copper, the metal binding domain interacts with the cytosolic loops of the ATPase. Binding of copper to the metal repeats closest to the transmembrane segments results in a conformational change which stimulates the phosphorylation of the ATPase and initiation of copper transport into the TGN lumen. Under conditions of elevated cytoplasmic copper, all of the metal binding repeats become occupied with copper resulting in further conformational changes and the translocation of WND from the TGN to the plasma membrane (PM). At the PM, the WND protein would pump copper from the cytoplasm into the extracellular space, lowering the cytoplasmic concentration of copper. As intracellular copper levels decrease, the metal binding repeats become progressively unoccupied and WND is recycled back to the TGN. A similar mechanism could also take place with the MNK protein.
membrane the WND and MNK proteins would be involved in pumping copper out of the cell in an effort to lower the cytoplasmic concentrations of copper. As the intracellular levels of copper decrease, the metal binding domain would become progressively unoccupied leading to the recycling of WND back to the trans-golgi membrane. The nature of the cytoplasmic vacuolar compartment has not been analyzed. A recent study has shown that these cytoplasmic vesicles are localized towards the hepatocyte canalicular membrane and may provide a route for the excretion of copper into the bile (Schaefer et al., 1999). It is possible that this compartment could be a lysosome, since copper pumped into lysosomes could then be deposited into the bile through fusion with the canalicular membrane. If true, this would explain why biliary copper excretion in Wilson disease patients is severely compromised. This point will have to be clarified by future studies.

Several details of the mechanism presented in figure 4.8 still remain to be elucidated. For example, it is not known whether the copper atoms bound to the amino-terminal domain are actually transported through the ATPase or if copper atoms from the cytoplasm are transported. In either case, it seems clear from several studies of the WND and MNK proteins that not all the metal binding repeats are needed to allow transport activity (Hung et al., 1997; Nagano et al., 1998; Petris et al., 1996). If copper bound to the amino-terminal domain is transported, these observations suggest that copper is only transported from a few of the metal binding sites (those closer to the membrane in WND, and those closer to the amino-terminus in MNK). In this case, the other repeats could be functioning in a regulatory role, acting as 'sensors' of intracellular copper concentration. If the amino-terminal bound copper atoms are not transported, then the entire domain could be playing a role in regulating the activity of the protein. The oxidation state of the transported copper atom is also not
known. Although we have shown that copper bound to the amino-terminal domain is in the +1 oxidation state (DiDonato et al., 1997), some evidence suggests that copper is transported in the +2 oxidation state (Bingham et al., 1996). If transported in the +2 oxidation state, the amino-terminal bound copper would have to be oxidized prior to transport through the membrane. This may be mediated by the protein itself or by an external oxidizing agent such as hydrogen peroxide.
CHAPTER 5

General Discussion and Future Directions
5.1 General Discussion

The results presented in this thesis represent the first detailed characterization studies of the copper binding domain from the Wilson disease copper transporting ATPase. These studies have shown that the WCBD can bind a variety of transition metals in addition to copper with varying affinities. This finding is intriguing since it leads to the question of whether these other metals are transported by the WND ATPase as well. This question has not yet been addressed directly and will be an important topic for future research. However, we do know that the primary defect in Wilson disease is one of impaired copper efflux from the liver, leading to copper overload, which suggests that the main purpose of the WND protein is to transport copper. Therefore, in the cytoplasm of the hepatocyte, what mechanism does the protein employ to ensure specificity for copper? It is possible that the protein never has an opportunity to interact with other metals in vivo due to their localization in different cellular compartments. In recent years, a series of copper chaperones have been identified, which are responsible for targeting copper to specific intracellular targets (Valentine and Gralla, 1997). Similar proteins could exist for other transition metals to facilitate their delivery to specific cellular compartments.

Alternatively, the metal binding domain itself could be responsible for conferring metal specificity on the protein. Both the $^{65}$zinc blotting studies and the circular dichroism studies have shown that the binding of copper to the WCBD may have some unique properties which could result in specificity for copper as the transported ion. The competition $^{65}$zinc blotting studies have shown that a cooperative binding mode may exist for copper, but not for the other metals. The structural changes in the domain induced by copper binding are also very different from those induced by the binding of zinc. In the intact
protein, the binding of copper to the amino-terminal domain could trigger specific structural changes which lead to the initiation of the transport cycle. Zinc would not be able to induce the same conformation in the amino-terminal domain and, hence, the transport cycle could not be initiated. XAS studies have shown that copper is ligated by two sulfur atoms in a distorted linear arrangement. Zinc would not be able to bind to the domain in the same manner since it prefers tetrahedral coordination and it is likely that the binding of zinc would force the domain into a non-native conformation, an idea which is supported by our CD zinc titration results.

The detailed structural data presented in this thesis, together with in vivo and in vitro work of other groups, has allowed the formulation of a hypothetical model for the function of the WND protein in vivo and the role the WCBD plays in this mechanism (fig. 4.8). In this model, the translocation of the protein, which is observed at elevated copper concentrations, is mediated by conformational changes resulting from copper binding to the amino-terminal domain. However, recent studies have indicated that this may not occur in the Menkes protein. When the cysteine residues in HMA domains 1, 6, or 1-3 were mutated to serine in the Menkes protein, the protein was still able to relocate to the plasma membrane in response to elevated copper (Strausak et al., 1999). However, if HMAs 4-6 or 1-6 were mutated, the protein did not respond to elevated copper concentrations. Further analysis revealed that the first four HMAs could be eliminated without loss of copper induced relocation; mutation of the remaining two HMAs abolished its response to copper. Although the study by Strausak, et al. (1999) evaluated the effects of these mutations on copper induced relocation, they did not measure copper transport activity. An earlier report has shown that all transport activity
is lost when the cysteine residues in the first three HMA domains of the Menkes protein are mutated (Payne and Gitlin, 1998).

These observations are in sharp contrast to similar studies performed on the Wilson protein, which show that the first five HMA domains can be mutated without loss of copper transport activity (Forbes et al., 1999). Taken together, these studies illustrate that the HMA domains are not functionally equivalent and that the amino-terminal domains of the Menkes and Wilson proteins do not function in similar manners. In light of this, the results of the study by Strausak, et al. (1999) cannot be extrapolated easily to the Wilson disease. Similar studies on the Wilson disease protein will be needed to answer these questions.

5.2 Future Directions

The expression and characterization of the amino-terminal copper binding domain from the Wilson disease copper transporting ATPase has given us invaluable information on the possible function of this domain in the context of the entire protein. However, despite the detailed analysis which has been carried out thusfar, several important questions concerning the function of this domain in the transport of copper through the ATPase remain to be addressed. Among these is the mechanism by which copper is delivered to the ATPase and the subsequent steps which allow it to be transported across the membrane. The discovery of a family of cytoplasmic copper chaperones has provided new avenues of research in this area. Much of what is known about the function of the copper chaperones has been derived from complementation studies in yeast. The yeast copper chaperone ATX1 has been shown to interact with the amino-terminal domain of ccc2, the yeast orthologue of WND, by yeast two-hybrid analysis (Pufahl et al., 1997). The association of ATX1 and ccc2 was also shown
to be dependent on the presence of copper. Recently, the human orthologue of ATX1 has been isolated and is known as ATOX1 (formerly known as HAH1) (Klomp et al., 1997). Due to the similarity between the ATX1 and ATOX1 proteins, an interaction between ATOX1 and the amino-terminal domain of the Wilson disease protein has been assumed to exist, but has not been demonstrated experimentally.

In order to address the question of how copper is delivered to the domain in vivo, an association between recombinant ATOX1 and GST-WCBD will be demonstrated in vitro and then characterized to determine the mechanism by which copper is transferred from ATOX1 to the WCBD. The basis of the assay would consist of forming the complex in vitro and then isolating it using glutathione affinity chromatography. Truncation and site-directed mutagenesis studies will then be employed to isolate the region of the proteins which are critical for complex formation and metal transfer. Experiments with the truncated protein will establish if the chaperone is delivering copper to a specific HMA domain or has no specificity for individual HMA domains. Together, these studies will help to define the functional significance (if any) for each HMA repeat in the copper binding domain. The $K_D$ values for copper binding to either the WCBD or ATOX1 are also unknown. Solution ligand binding experiments will be used to determine the dissociation constant for copper bound to either the WCBD or ATOX1.

Another question, which is currently not addressed in the literature, is how copper is transported across the membrane by the ATPase. Our studies have shown that once copper is bound to the domain it is fairly resistant to oxidation and remains bound in the presence of excess reducing agents, denaturants, and chelating agents such as EDTA. These observations indicate that copper is bound tightly to the WCBD and lead to the question of how copper
can be removed from the WCBD once bound. We have investigated the possibility that copper may be released by oxidation during the transport cycle. Prolonged exposure of the copper loaded WCBD to excess hydrogen peroxide resulted in the release of only two copper atoms. In the intact protein, interactions between the amino-terminal domain and other parts of the ATPase could also facilitate its release from the WCBD and its translocation across the membrane. To further analyze this possibility, experiments must be conducted using the entire ATPase. The Wilson disease ATPase could be expressed in a mammalian system and isolated in vesicles in a functional form. Using this preparation, detailed transport studies could be performed. If inverted vesicles could be prepared, such that copper is pumped into the vesicle, the oxidation state of the transported copper could be determined by examining the copper in the vesicles after the assay is complete. The oxidation state of the copper inside the vesicles could easily be determined by electron paramagnetic resonance (EPR).

The structural studies presented in this thesis have shown that significant structural changes take place in the domain upon metal binding. Although both copper and zinc are able to bind to the domain, the structures they induce upon binding are very different. This difference could be the basis for the specificity of the ATPase. The HMA domain is found in many proteins which bind a variety of metals (table 1.6), suggesting that it may be a general metal binding motif. Studies on zntA, a zinc transporting ATPase in bacteria containing one HMA domain, have shown that this protein can transport cadmium as well as zinc, two metals with similar ligation geometries (Rensing et al., 1997). Our competition $^{65}$zinc blotting studies have suggested that the binding of copper to the domain may have some degree of cooperativity and that this property is unique to copper. In light of this
observation, we propose that the cooperativity observed for copper binding may contribute to the specificity of the ATPase. To further examine the role of the metal binding domain and the possible role it plays in conferring specificity to the ATPase, a chimeric protein could be created containing the copper binding domain from the WND protein and the ATPase "core" of zntA. The ability or inability of this chimera to transport copper could help confirm the hypothesis that the WCBD is playing a part in determining the specificity of the transported metal.

Although these experiments will give a very good understanding of how copper is transferred and bound to the domain, a complete understanding of the function of this domain will require a detailed three-dimensional structure. For this reason, the three-dimensional structure of the WCBD will be determined by x-ray crystallography. NMR will also be used to determine the structure of a truncated form (~35 kDa) of the domain, which contains only HMA domains 4, 5, and 6, since biochemical studies have indicated that these are of greater importance to the function of the Wilson disease protein than the others (Forbes et al., 1999). These structural studies, coupled with ongoing XAS experiments using copper and zinc reconstituted proteins, will allow us to better define the role of this novel domain in the overall mechanism of the ATPase.
5.3 Literature Cited


ALS-linked SOD1 mutation causes motor neuron disease characterized by vacuolar degeneration of mitochondria. Neuron 14, 1105-1116.


