The Molecular Basis of Genetic Disorders Associated with Essential Elements Metabolism

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Cambridge
Scholars
Publishing

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This book first published 2024

Cambridge Scholars Publishing

Lady Stephenson Library, Newcastle upon Tyne, NE6 2PA, UK

British Library Cataloguing in Publication Data
A catalogue record for this book is available from the British Library

ISBN (10): 1-5275-5266-7
ISBN (13): 978-1-5275-5266-1

To

My Mentor, Prof. Ravindra Nath, and my Parents

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PREFACE

Spectacular and far-reaching advances have been made in the knowledge related to the significance of essential elements (micronutrients) in human health and disease. Advances and breakthroughs in molecular biology, biotechnology, and molecular diagnostics have allowed us to understand more about metabolism, regulation, and functions of the micronutrients which will ultimately lead to formulations of novel strategies against specific metal related genetic disorders.

Metabolic genetic disorders associated with metals are considered classic single-gene disorders. Strikingly, the latest development in next-generation sequencing and other technologies have discovered that the classical one gene-one enzyme paradigm is not always a fact. Now, it is very clear that multiple genes may lead to the same phenotype presentation or may influence the severity of diseases. Moreover, the presence of mutations in different domains of the same protein might produce a difference in phenotype. Therefore, effective therapeutics should be considered. New advancements in DNA manipulation via clustered regulatory interspaced short palindromic repeat (CRISPR)-Cas 9 technology could be a potential future therapy in genetic disorders. The chapters of this book are comprehensively written with special reference to the molecular genetics of essential element-related disorders, which are of utmost importance in the development of precise medicine.

Molecular identification and the characterization of mutations in genes resulting from a genetic disorder are of threefold significance: (1) They help us in understanding the molecular mechanism of the disease process to formulate the therapeutic strategy. (2) They provide a DNA-based confirmatory diagnosis asymptomatically. (3) The identification of the most common mutations in a genetic disorder are useful in prenatal/neonatal diagnosis. In fact, mutation analysis is the only way to distinguish the affected one from the carrier and help in genetic counseling which is the best way to eliminate the genetic disorder from the population because prevention is better than cure.

Increasingly, medical graduates and postgraduates must be equipped to integrate genetic knowledge into all areas of medicine as well as basic medical sciences in order to deliver a dimension of practice and patients care that has hitherto largely been the domain of a small breed of specialties. We have sought to provide a comprehensive basic text for those who seek a work of reference through which they can swim to calm waters, rather than one in which they will quickly drown.

To help the readers to achieve those learning goals, the text is succinctly written. Each chapter has a brief introduction of its own explaining the importance of the experiment and the historical context in which it was carried out. At the end of each chapter is a complete running summary and future perspectives to sustain enthusiasm as well as to motivate a desire to understand the basics in a comprehensive way.

In the next half-century, our understanding of how living organisms function at the molecular level, together with our ability to intervene, will expand in ways we are only beginning to perceive.

ACKNOWLEDGMENTS

I am deeply obligated to Dr. G.K. Khullar, Emeritus Professor, Department of Biochemistry, Post Graduate Institute of Medical Education and Research, Chandigarh, India, who developed rational thought and scientific temper in the budding stage of my science career as well as his continuous effort to bring forth this book in the present format.

We are grateful to the many colleagues, particularly Dr. Munish Kumar, Post Graduate Institute of Medical Education and Research, Chandigarh, India, Dr. Isha Singla, Maharishi Markandeshwar Institute of Medical Sciences and Research, Ambala, India, and Miss. Sonia Deswal at Maharishi Markandeshwar Institute of Medical Sciences and Research, Ambala, India, who generously contributed their time and effort to this project.

I particularly want to acknowledge Rebecca Gladders, Commissioning Editor, Cambridge Scholars Publishing, for her constant source of encouragement and discipline.

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1. Biological Significance and Genetic Disorders of Copper Metabolism

1.0 Introduction

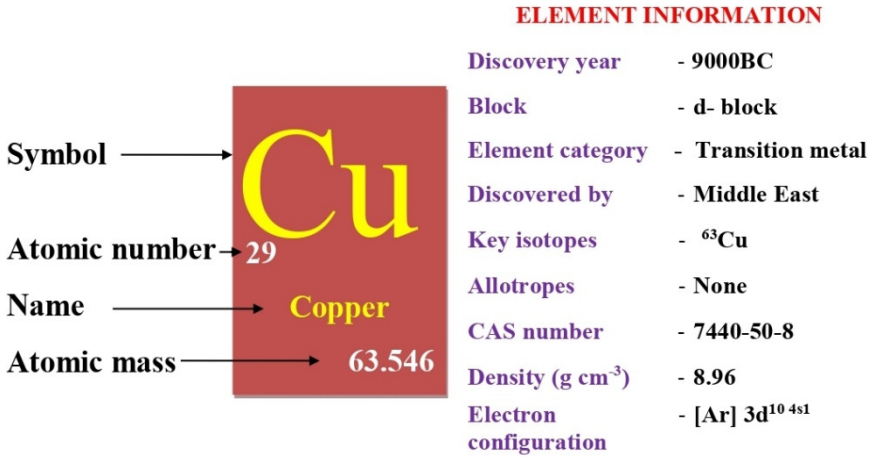

Figure 1.1 Physicochemical properties of copper (Cu).

Copper is an essential element that belongs to group IIb of the periodic table. Copper is found in the free metallic state in nature. The physicochemical properties of copper are depicted in Figure 1.1. Notwithstanding, copper is an indispensable trace element in the life processes of most animals and plants, being a cofactor of various enzymes as well as binding to many important molecules to maintain structural conformations. Proteins and bioorganic molecules allow copper to play a major role in biological functions. Notably, the essential nature of copper in animals was first demonstrated by Hart *et al.* in 1928 [1, 2]. Strikingly, excess free copper, either in body fluids or intracellular, is highly toxic owing to its redox nature. Therefore, nature has provided tight regulation to maintain fine copper homeostasis at both the cellular and organism level during the time course of the evolution of organisms. A little deviation from the homeostasis leads to various copper-associated disorders. These disorders include Wilson disease, Menkes disease, occipital horn syndrome, neuropathy, Alzheimer's disease, and Parkinson's disease. In view of these facts, a better understanding of copper metabolism is of utmost importance in pathogenesis, molecular diagnosis and therapeutic aspects of various congenital disorders of copper metabolism. Thereby,

there is a need to address the biochemistry of the proteins which are involved in human copper metabolism.

1.1 Copper-dependent major biological molecules

1.1.1 Cytochrome c oxidase (EC 1.9.3.1)

Cytochrome c oxidase (COX) or complex IV of the mitochondrial respiratory chain plays a fundamental role in the energy production of aerobic cells. COX is a multimeric enzyme located in the inner mitochondrial membrane which is of dual genetic origin. Homosapien COX consists of 13 subunits. Subunits 1, 2, and 3 are large, highly hydrophobic transmembrane proteins encoded by the mitochondrial genome which form the catalytic core of the enzyme and contain metal prosthetic groups. Copper and heme form a unique heme compound found exclusively in COX with three redox, viz a Cu_A center in subunit 2, and a heme α and a Cu_B- heme a3 binuclear center in subunit 1. The remaining subunits are small in size and are encoded by the nuclear genome. They are needed for the assembly and stability of the holoenzyme and for its dimerization.

Complex IV is the terminal enzyme of the electron transport chain, existing either as randomly scattered complexes or as a component of super complexes. NDUFA4 was previously assumed to be a subunit of complex I but recent biochemical data suggested it may be a subunit of complex IV, which is a monomer containing 14 subunits. Since NDUFA4 lies exactly at the dimeric interface in previously reported crystal structures of complex IV homodimer which preclude complex IV dimerization [3]. This complex contains three copper II ions, one magnesium ion, and one zinc ion, two heme α molecules, and several phospholipid molecules.

COX assembly and stability of fully congregated enzymes depend on the mitochondrial compartments of two partners of oxidative phosphorylation systems, the mobile electron carrier cytochrome c and the mitochondrial ATPase. It catalyzes the electron transfer from cytochrome c to di-oxygen in the final step of mitochondrial oxidative phosphorylation. The reaction occurs in concurrence with proton pumping from the matrix to intermembrane spaces resulting in the generation of a transmembrane proton gradient, which is subsequently used for the synthesis of ATP. Cytosolic copper is delivered to COX via copper chaperone CoX 17 [4]. The overall structure of COX subunits is explained in Figure 1.2 [3].

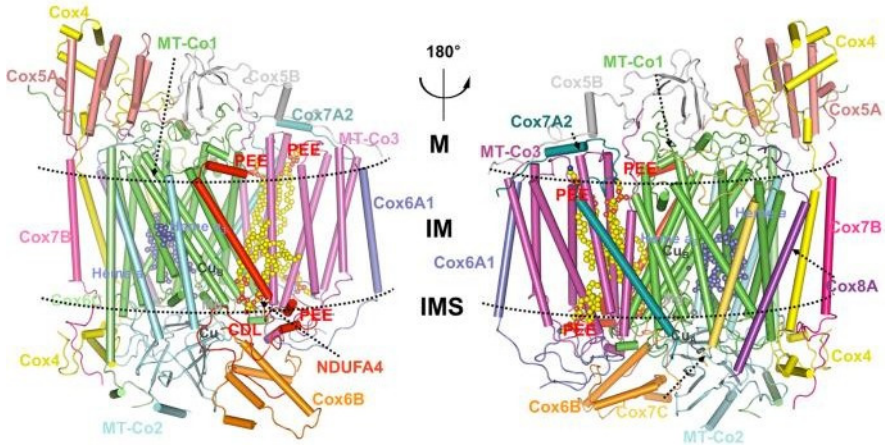

Figure 1.2 The overall structure of human COX. The subunits of COX are colored and labeled with text in the same colors. Spheres show the cofactors and phospholipids; Cardiolipin-CDL; Phosphatidylethanolamine-PEE. The two dashed lines show the transmembrane region. M-matrix; IM-inner membrane; IMS-intermembrane space [3].

1.1.2 Cu-Zn superoxide dismutase (EC 1.15.1.1)

Cu-Zn superoxide dismutase (SOD) belongs to the family of oxidoreductase known as superoxide dismutase (SOD). There are three isoenzyme forms of SOD in mammals, namely the cytosolic and extracellular, both containing Cu, and Zn at their active sites, and the mitochondrial SOD, which contains Mn at its active site. Cu-Zn SOD catalyzes the reaction between superoxide and water to yield oxygen and hydrogen peroxide.

Notably, most of the enzymes which produce and require superoxide are present in peroxisomes, for instance superoxide dismutase, catalase, and peroxidase. This reaction occurs in the following manner:

$$O_2^- + O_2^- + 2\,H^+ \xrightarrow{SOD} H_2O_2 + O_2$$

However, hydrogen peroxide (H_2O_2) is also highly toxic and needs to be detoxified. Therefore, cells use the enzyme catalase, a ubiquitous heme protein that further catalyzes the dismutation of hydrogen peroxide into water and molecular oxygen as follows:

$$2\,H_2O_2 \xrightarrow{\text{Catalase}} 2\,H_2O + O_2$$

Structurally, Cu-Zn SOD is a metalloprotein with a molecular mass of 62.913 kDa, containing both Cu and Zn ions in each subunit. The SOD protein is a dimer composed of two identical subunits that are oriented so that the two active sites are facing away from each other. Each subunit is composed of 151 amino acid residues. These amino acids are arranged in eight β-sheets and three exterior loops. Each subunit contains copper and zinc ions at the active site. Strikingly, the copper ion acts as an electron transfer center, whereas the zinc ion serves as a structural scaffold and contributes to the positive charge on the active site which attracts the negatively charged superoxide anion (Figure 1.3).

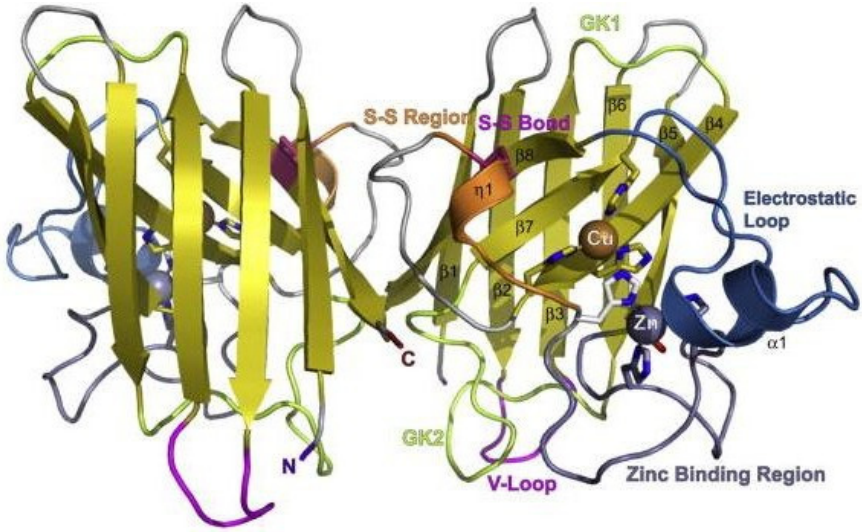

Figure 1.3 The structure of the SOD1 dimer shows the Cu-Zn SOD sequence conservation, fold, and structural and functional regions. The structural elements are coded with abbreviations: including V-loop—Variable loop; GK1 and GK2—Greek key loops 1 and 2; S-S—disulfide regions; N and C—termini. The metal-ligand residues are shown as sticks with the bridging histidine His61 in white [5].

Amyotrophic lateral sclerosis (ALS), is a disease commonly referred to as Lou Gehrig's disease and is due to the presence of mutation in the SOD1 gene. However, there is an urgent need to elucidate a better understanding

of how the spectrum of mutations in SOD causes ALS. It would be of great help to develop better diagnosis and potential therapeutics.

1.1.3 Dopamine β-hydroxylase (DBH) (EC1.14.17.1)

DBH is also known as dopamine β-monooxygenase. DBH is a copper-containing oxygenase that catalyzes the hydroxylation of the β carbon of a wide variety of phenylalanine derivatives using oxygen and ascorbate as cofactors. It belongs to a small unique class of Cu-containing hydroxylases and requires two Cu ions per unit for its activity [6, 7]. DBH is generally considered to be exclusively localized in the chromaffin granules of the adrenal medulla and sympathetic neurons of the central peripheral nervous system. It is present in the catecholamine storage vesicles, where it catalyzes the conversion of 3- and 4-dihydroxy phenylalanine (DOPA) to dopamine. Serum DBH is released from tissues with nor-adrenaline following stimulation. Reduced levels of serum DBH associated with trisomy 21 are found in Down syndrome patients. The human DBH sequence from the NCBI database with accession # PO9172 was searched for protein sequence blast. The primary structure of human DBH is 617 amino acids in length with 15 cysteine residues, of which 14 are involved in disulfide bond formation [8]. Cys154-Cys596, Cys232-Cys283, Cys269-Cys295, Cys390-Cys503, Cys394-Cys565, and Cys466-Cys488 are involved in intramolecular disulfide bonds whereas two cysteines at positions 528 and 530 are involved in intermolecular disulfide bonds. DBH is comprised of three major domains. The first major domain on the N-terminal side is the DOMON domain [7, 8]. This domain is situated from position 57 to 173 after signal sequencing. Following the DOMON domain is the copper type II ascorbate-dependent monooxygenase N-terminal domain located from position 359 to 526. The two monooxygenase domains generate the catalytic unit of the enzyme and each one binds to one Cu atom which is required for the enzyme activity. The structure of DBH is given in Figure 1.4.

EPR and EXAFS analysis of DBH protein revealed that coordination of two Cu atoms in the oxidized state produces Cu_A (His) 3 (H_2O) and Cu_B $(His)_2X$ (H_2O), where X is either the His or O- donor ligand or solvent (8). It has been shown that two subunits form a dimer connected through an interchain disulfide link between Cys528A-Cys528B and Cys530A-Cyst530B. The DOMON domain potentially promotes tetramerization in which the substrate dopamine and potential therapeutic inhibitors nepicastat are stabilized in the active site through multiple hydrogen bonding. The DBH gene is located on the long (q) arm of the chromosome

at position 34:2 (q34.2) from 133638372-133659329 and consists of 12 exons [UniProt kB-P09172(DOPA-Human)]. A number of SNPs are known to exist within the coding amino acid sequence of the enzyme [9, 10].

Eighteen of such SNPs were studied along with their neighboring residues at a 6 Å radius of the concerned SNP. In fact, the tetramer form is known to be the active form of DBH, whilst no activity was found in the dimer or monomer form. Hence, SNPs close to the active site at the interface region of the tetramer are most harmful to DBH activity. A better understanding of the effects of these SNPs on the enzyme activity can help with the rationale of drug configuration. Strikingly, polymorphisms in the DBH gene have been reported to be associated with neurological diseases such as Parkinson's disease, Alzheimer's disease, schizophrenia and attention deficit hyperactivity disorders, which have overlapping neurological symptoms with WD [11] and ortho-static hypotension.

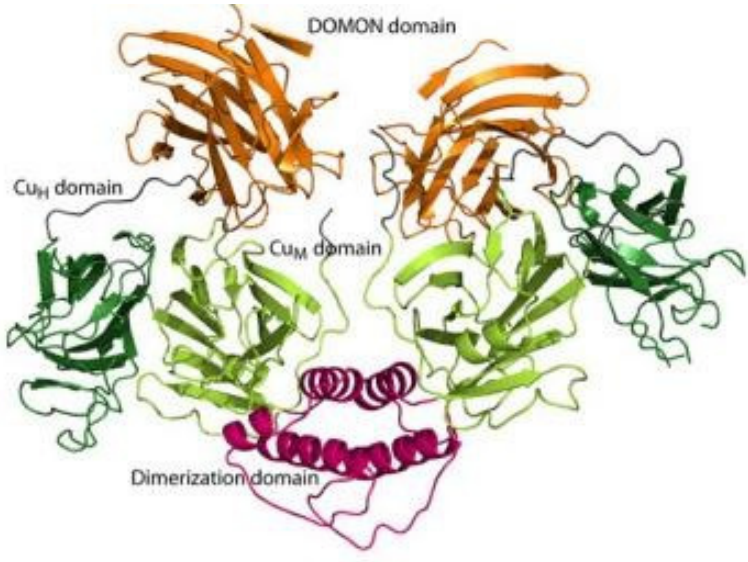

Figure 1.4 Structure of the human DBH dimer. The overall structure can be seen from two angles (at 90° to each other). Orange color—DOMON domain, dark green—Cu_H domain, light green—Cu_M domain, magenta—dimerization domain, gray—the interdomain regions [8].

1.1.4 Lysyl oxidase (EC 1.4.3.13)

Mammalian lysyl oxidases (LOX) are a family of copper-dependent amine oxidases, primarily involved in the remodeling of the extracellular matrix (ECM). They initiate covalent cross-linkage formation in elastin and collagen by oxidizing the peptidyl lysine of the proteins to amino adipic semialdehyde [12]. Depletion of copper using copper chelating agents from the preparations of lysyl oxidase results in the loss of enzyme activity. Hence, copper deficiency in humans leads to a reduction of cross-linking of elastin and collagen and thereby to faulty connective tissue as expressed in Menkes disease patients.

The LOX gene is mapped on the long arm of chromosome 5 with the genomic localization as 5q23.1 which consists of seven exons and six introns which are distributed across approximately 14.5 kb of genomic DNA. This gene encodes a member of lysyl oxidase family proteins by alternative splicing which generates five different LOX enzymes, namely LOX and LOX like 1-4. All these proteins are highly homologous in the catalytic C-terminal end in mammals. Notably, one of these variants is proteolytically processed to generate a regulatory pro-peptide and mature enzyme. The copper-dependent amine oxidase activity of this enzyme functions in the cross-linking of collagen and elastin while the pro-peptide plays a role in tumor suppression. Moreover, defects in this gene have been linked with a predisposition to thoracic aortic aneurysms and dissections [13].

Human lysyl oxidase is synthesized as a proenzyme of 396 amino acid residues, processed by bone morphogenetic protein-1 (BMP-1). As a result, an active form of LOX (M30KDa) and its N- terminal pro-peptide (147 residues) are produced [3]. LOX contains five disulfide bridges, a copper ion and a tyrosyl quinone cofactor. The cofactor is a quinone whose structure is derived from the cross-linking of the € amino group of a peptidyl lysine with the modified side chain of a tyrosine residue and it has been designated lysine tyrosyl quinone (LTQ) [14].

Human LOX mutations Q267P, S280R, M298R, and S348R also predispose to aortic aneurisms and dissections in humans [15-17]. Besides its role in ECM assembly and architecture, LOX is overexpressed under hypoxia and is involved in cancer progression and metastasis, particularly in breast cancer metastasis to the bone via the formation of a metastatic niche [18, 19]. Targeting LOX in fibrosis and cancer may thus be a new therapeutic approach in these diseases.