Copper Proteins and Copper Enzymes

Volume I

Editor

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PREFACE

These volumes of *Copper Proteins and Copper Enzymes* are intended to describe the contemporary spectroscopy and other biophysical chemistry now being applied to copper proteins in order to determine the structures of their active sites. Several chapters of the treatise describe the functional understanding which is emerging from the new work. The authors are all major contributors to research progress on copper proteins and the volumes will be found to be definitive and authoritative.

The subject, copper proteins and copper enzymes, is a very lively one and is best considered in the broadest biological and chemical contexts as it continues to develop. Copper itself, born in the dust of the cosmos and comprising perhaps 0.007% of the earth's crust, occurs in the biosphere as about two dozen families of copper proteins which serve to transport O_2, to activate it toward reaction with organic molecules, and to transfer electrons between donors and acceptors. The families of copper proteins include the azurins, plastocyanins, metallothioneins, superoxide dismutases, ceruloplasmins, laccases, ascorbate oxidases, cytochrome *c* oxidases, monoamine oxidases, diamine oxidases, galactose oxidase, hexose oxidase, urate oxidases, polyphenol oxidases, phenol *o*-hydroxylases, *p*-coumarate 3-monooxygenase, dopamine β-monooxygenase, lysine protocollagen oxygenase, quercetin 2,3-dioxygenase, and the arthropodan and molluscan hemocyanins. The number of types of copper-binding domains in these proteins appears to be very limited, perhaps three or four (blue type-1 mononuclear copper sites; type-2 mononuclear copper sites; diamagnetic binuclear type-3 copper sites), and it is probable that there are very few evolutionary prototypes from which the existing families sprung.

The structures of the copper-binding domains are being rapidly worked out, as these volumes will demonstrate. However, the overall three-dimensional protein structures are in general not known, although progress is being made. The three-dimensional structures will represent major opportunities to understand the chemical biology of these proteins because their functional properties almost certainly depend upon the presence of structural domains other than the copper-binding ones; e.g., the affinity of the copper sites for oxygen may be strongly affected by the kind and disposition of amino-acid residues. In the case of hemoglobin, almost 90 abnormal hemoglobins are known to arise from amino-acid substitutions, which show altered O_2 affinities. Since the K_m (O_2) of copper enzymes is an adaptive property, it is likely that the structures of the active sites will vary in space even though the ligands remain the same for each type.

Another interesting problem against which the information in these volumes should be weighed lies in the fact that each of the functions served by copper proteins is also served by families of iron-, heme-, and flavin-containing proteins. Why then was copper selected when other prosthetic groups were available?

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He was born in Louvain in 1920 and educated there. He received his doctorate in physical chemistry (with Professor J. C. Jungers) from the Katholieke Universiteit in 1942. He was trained in protein chemistry as a Research Assistant and Senior Research Assistant of the National Fund for Scientific Research (Belgium) at the Laboratorium voor Biochemie in Louvain under Professor P. Putzeys. As a Graduate Fellow of the Belgian American Educational Foundation he was a Research Fellow in Physical Chemistry at the Department of Physical Chemistry, Harvard Medical School, Boston, Mass. (Professor E. J. Cohn, Professor J. T. Edsall, Professor J. L. Oncley).

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His major research interests, which included milk and barley proteins, are focused now on copper proteins, mainly on the structure, function, and biosynthesis of hemocyanins.

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COPPER PROTEINS AND COPPER ENZYMES

René Lontie

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Volume I

Chapter 1

INTRODUCTION

René Lontie

Copper is an essential trace element, although nearly all organisms have access to only very minute amounts. Notable exceptions are bacteria involved in the leaching of copper from low-grade ore[1] and the "copper flowers" in Central Africa.[2] These species raise an interesting problem, as they have to cope with otherwise toxic amounts of the metal.

Several books and conferences have been devoted to trace elements[3-7] and to copper and copper proteins.[8-18] Many chapters on copper and copper proteins are also to be found, e.g., in more general treatises and proceedings of symposia,[19-23] like in serial publications.[24-28]

Four chapters describe spectroscopic techniques which have contributed so much to the understanding of the active sites: electron paramagnetic resonance, nuclear magnetic resonance, X-ray absorption, and resonance Raman. Absorption and circular dichroic spectra are presented in the chapters on the individual proteins.

The blue oxidases contain three types of copper: "blue" (meaning strongly blue), "non-blue" (weakly colored), and diamagnetic pairs,[25] respectively named, type 1, type 2, and type 3. Most authors also use these designations for proteins with only one type. A different nomenclature is advocated in Chapter 3 of Volume I.

The polypeptide chains show a great diversity in size from the smaller ones of the small blue proteins and the copper-thioneins to those of the gastropodan hemocyanins, which are among the largest in nature. Special precautions are needed with the longer polypeptide chains in order to avoid proteolytic cleavage during their isolation, as shown for molluscan hemocyanins and ceruloplasmin (Chapter 6 of Volume II and Chapter 2 of Volume III).

Several chapters illustrate the great effort which went into the determination of amino-acid sequences, like those in Volume I on the small blue proteins, in Volume II on Cu/Zn-superoxide dismutases, hemocyanins, and tyrosinase, and in Volume III on ceruloplasmin, cytochrome *c* oxidase, and metallothioneins.

Conformations were only determined at a high resolution for two small blue proteins and for bovine Cu/Zn-superoxide dismutase (respectively, Chapters 6 and 7 of Volume I, and Chapter 2 of Volume II).

The first steps in the study of the biosynthesis of copper proteins are also presented: the isolation of mRNA's for hemocyanins and for ceruloplasmin (Chapter 6 of Volume II and Chapter 2 of Volume III) and the consideration of copper-thioneins as possible copper donors (Chapter 5 of Volume III).

While the type-1 proteins appear as a rather homogeneous group, the type-2 enzymes (Chapters 1, 2, 4, and 5 of Volume II) show a great diversity in reactivity. Type-3 copper in the blue oxidases (Chapters 1 to 3 of Volume III), by the presence of type-1 and type-2 copper, differs also quite much from the isolated type-3 sites in hemocyanins and tyrosinase (Chapters 6 and 7 of Volume II).

It has not been possible to cover all the copper proteins and copper enzymes. Typical

representatives are missing, like urate oxidase (uricase) (EC 1.7.3.3)[29,30] and nitrite reductase (EC 1.7.99.3).[31,32]

Copper does not seem essential for some enzymes, which have been claimed to contain this metal. A crystalline preparation of ribulose 1,5-bisphosphate carboxylase (EC 4.1.1.39) from tobacco did not seem to contain appreciable amounts of copper,[33] in contrast with an earlier report on a preparation from spinach.[34] With parsley the ribulose 1,5-bisphosphate oxygenase seemed a separate enzyme, which contained copper.[35] Indoleamine 2,3-dioxygenase did not contain significant amounts of copper, as a copper-rich protein was eliminated in the last step of the purification.[36] With L-tryptophan 2,3-dioxygenase (EC 1.13.11.11) of *Pseudomonas acidovorans*, which contains protoheme IX, it was shown that copper was not essential for the catalytic activity, although some preparations contained firmly bound copper.[37]

While this heme enzyme did not need copper, soluble guanylate cyclase from bovine lung contained 1 mol of copper and 1 mol of heme (ferroprotoporphyrin IX) per mol of enzyme, which could be important for the regulation of the activity of the enzyme.[38] A protein with unknown function with 2 copper and 2 heme *b* per M_r of 400,000 was isolated from bovine erythrocytes.[39]

The binding of copper to several proteins has also been the object of many investigations and copper(II), not unlike cobalt(II), has been used as a probe for the binding site.[40]

Chapter 3 of Volume II and the last Chapter of Volume III deal with physiological aspects of copper and copper proteins and enzymes: the so debated role of superoxide dismutases and the metabolism of copper, respectively.

There remain the inevitable problems of nomenclature. The terms type-1, type-2, and type-3 copper seem to have lost their initial meaning. Several of the small blue proteins have been named prematurely by combining the name of a plant, a relative or a town, e.g., with "cyanin". When more is known of their structure and function a more general name with the indication of the species would be preferable (cf. "phytocyanin" in Chapter 6 of Volume I). A similar situation prevailed with erythrocuprein, hemocuprein, and hepatocuprein, which via cytocuprein were finally named Cu/Zn-superoxide dismutase. For semantic reasons terms like Semi-Met seem preferable to Half-Met for the hemocyanins (Chapter 6 of Volume II).

The astute reader will discover shades of opinion and outright contradictions, which illustrate the complexity of the subject. Many more amino-acid sequences and especially protein conformations are needed to understand the evolution of these fascinating proteins and the structure of their active sites.

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Chapter 2

ELECTRON PARAMAGNETIC RESONANCE OF COPPER PROTEINS

John F. Boas

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I. INTRODUCTION

Copper proteins play an important role in both plant and animal physiology, e.g., hemocyanin (Hc) is the oxygen-carrying protein in the hemolymph of many molluscs and arthropods; cytochrome *c* oxidase (which also contains iron) is the terminal oxidase in the respiratory chain; ascorbate oxidase catalyses the aerobic oxidation of L-ascorbate; and tyrosinase catalyses the ortho-hydroxylation of monophenols to diphenols and the subsequent dehydrogenation of *o*-diphenols to *o*-quinones. In some cases, such as ceruloplasmin, stellacyanin (St), and the azurins (Az), the physiological role of the copper protein may not be as clear-cut, as will be seen from the discussion in other chapters in these volumes and from earlier reviews.[1-5]

A characteristic of many copper proteins is their intense blue color, which is due to an optical absorption at around 600 nm with a molar absorption coefficient of between 1,000

and 10,000 M^{-1} cm^{-1}. This absorption coefficient is some 100 times larger than that found for simple copper amino-acid or peptide complexes.[1-3] A second characteristic of many copper proteins, and one usually associated with the intense blue color, is an unusual electron paramagnetic resonance (EPR) spectrum which has a hyperfine splitting when the magnetic field is parallel to the z or symmetry axis of around 0.0080 cm^{-1} or approximately one half of that of the simple copper complexes.[1-6] As shown by Malmström and co-workers in the early 1960s, both the blue color and the unusual EPR spectrum were associated with the activity of the multicopper enzymes ceruloplasmin and laccase.[6,7] Similar results were found for "blue" copper proteins containing only one copper atom.[1-4,8]

In general, copper proteins in their native state may contain either or both copper(I) or copper(II). Since copper(I) is not a paramagnetic ion, this form will only concern us in passing in this review. However, the copper(II) ion is paramagnetic and should give an EPR signal. The copper(II) ions in proteins have been broadly classified as type 1, type 2, and type 3 on the basis of their EPR signals, and this classification has been reviewed recently by Fee[3] and Boas et al.[4] Type 1 and type 2 are distinguished by their EPR spectral behavior, while type 3 does not give an EPR signal in the native state for reasons which we discuss below. Appendix 1 lists some of the naturally occurring copper proteins and gives representative values of the **g** and hyperfine parameters associated with their EPR spectra.

Type-1 copper(II) is associated with the intense blue color, and is found in the single copper proteins, such as the Azs and plastocyanin (Pc), and in the multicopper enzymes laccase, ceruloplasmin, and ascorbate oxidase.[3] As shown in Appendix 1, the EPR spectrum of type-1 copper is characterized by **g**-values similar to those of simple copper(II) chelates and by a value of the main hyperfine splitting approximately one half of that found for the simple complexes. The origin of this small hyperfine splitting is still one of the contentious issues in EPR studies of copper proteins. As pointed out by Malmström and Vänngård in 1960, it is difficult to explain this with the same model as that used for the simple complexes.[6] As we shall see, various theoretical models have been proposed for the blue copper site but, until recently, there has been little experimental evidence as to the nature and symmetry of the site and the surrounding ligands.

Type-2 copper gives EPR signals similar to those obtained for the simple copper(II) complexes. It is observed in association with the type-1 copper in ceruloplasmin, laccase, and ascorbate oxidase, and on its own in superoxide dismutase, the monoamine oxidases, galactose oxidase, and some other systems (e.g., see Boas et al.[4]). It has been argued that the label "type 2" should be reserved for those proteins where type-2 copper appears in association with type 1 (e.g., see Fee[3]). However, we will continue to refer to type-2 copper only on the basis of the EPR signals. The type-2 copper site appears to have an approximately square planar configuration, with nitrogen and oxygen being the coordinating ligands.[4,9]

Type-3 copper ions are characterized by an absorption band in the 300-nm region and the absence of an EPR spectrum.[2,4] They are found in ceruloplasmin, laccase, and ascorbate oxidase in association with type-1 and type-2 copper, and as the only form of copper in OxyHc and tyrosinase. The absence of an EPR spectrum led many workers to suggest that the copper was either copper(I) and hence diamagnetic, or was in a mixed valence complex with the copper(I) and copper(II) ions in close association (e.g., see the papers in Reference 1). An opposing school of thought held that the copper ions were copper(II) but were so close together that strong exchange coupling occurred, giving neither an observable paramagnetic susceptibility nor an EPR signal.[10] In the case of Hc, the recent evidence from resonance Raman spectroscopy,[11-13] extended X-ray absorption fine structure (EXAFS),[14-16] and from the magnetic measurements described in this chapter has tended to support the latter view. As in the case of the type-1 copper, the nature of the sites is controversial. There is still little direct evidence as to the type and symmetry of the surrounding ligands, although some indirect evidence is discussed in this chapter and in other chapters in these

volumes. In most cases, an X-ray structure determination is still required. Much of the speculation surrounding the type-1 and type-3 copper sites has arisen because it has proved very difficult to reproduce their magnetic and spectral properties in the laboratory.

In the clarification of the nature and function of the active sites of the copper proteins, EPR has had some successes, but has often not lived up to the expectations held for it. In principle, as pointed out by Brill and Venable,[17] one may obtain the following information from EPR studies:

1. The number of distinct magnetic centers
2. The orientation with respect to the crystal axes of the symmetry axes of the magnetic centers, when single crystals are studied
3. The symmetries of the protein environments of the magnetic centers, and the ground states of the transition metal ions
4. The delocalization of the electrons in the metal ion-protein bonds
5. Identification of the protein atoms bound to the metal ions if the former have nuclear magnetic moments
6. An estimate of the distance between paramagnetic metal ions

To these we may add:

7. An estimate of the proportion of copper ions in each type of magnetic center and of the number of metal ions not in an EPR-detectable center
8. Details of the chemical changes which take place either when the protein is modified or which occur during a chemical reaction

In practice such richness of detail is often not obtained due to difficulties such as those of obtaining large enough single crystals, of obtaining samples sufficiently free of extraneous copper or other impurities, of obtaining sufficient resolution in the EPR spectra, and of interpreting the data.

While conventional EPR alone has perhaps been of limited value in providing definitive answers on the nature of the copper sites in proteins, it has proved to be a more powerful technique when used in conjunction with magnetic susceptibility measurements and electron resonance techniques such as electron nuclear double resonance (ENDOR) and electron spin-echo spectrometry. When the results from these measurements are combined with those obtained from other techniques such as X-ray diffraction, resonance Raman spectroscopy, nuclear magnetic resonance (NMR), X-ray absorption spectroscopy, and photoelectron spectroscopy, a detailed picture of the copper sites is expected to emerge. Some of these techniques and the results obtained for various copper-protein systems are discussed in other chapters in these volumes.

This chapter will examine some of the general aspects of the application of EPR and its related techniques such as ENDOR and spin-echo spectroscopy to studies of copper proteins, and discuss their advantages and limitations. Since much of the available EPR data has been discussed in earlier reviews,[2-5] we will concentrate on what, to the author, are the more recent highlights, rather than attempt to give a totally complete and comprehensive review.

II. BASIC PRINCIPLES OF EPR

The copper(II) ion has the $3d^9$ electronic configuration with a single unpaired electron. The effective spin is therefore equal to the actual spin of the free ion, namely $S = 1/2$, so that a Kramers doublet is the lowest energy level. In a static magnetic field of flux density $\boldsymbol{B}$, the doublet is split into two levels which are labeled by the z component of the spin