

THE 'UNIQUE' METAL-BINDING PROPERTIES OF METALLOENZYMES

BO G. MALMSTRÖM

*Department of Biochemistry, University of Göteborg and
Chalmers Institute of Technology, Göteborg, Sweden*

ABSTRACT

(1) The theme of the survey is that metalloenzymes represent rather 'unique' metal complexes when compared to the simpler system generally studied by coordination chemists. This theme is introduced by a brief presentation of the historical development of the metalloenzyme field, particularly the contributions of O. Warburg and D. Keilin.

(2) Various types of metal ion-protein interactions and the distinction between non-specific and specific binding have been briefly reviewed.

(3) The 'unique' properties in the specific interactions with metalloenzymes are illustrated by a discussion of three different enzymes: carboxypeptidase, carbonic anhydrase and laccase (*p*-diphenol oxidase). In conclusion it is suggested that in these enzymes the 'unique' coordination imparts properties to the metal ion facilitating its role in the catalytic reaction.

METALLOENZYMES AND MODELS

The study of the role of metal ions in enzyme reactions provides a beautiful example of an area in which increased understanding has been intimately connected with the close interaction between traditionally distinct disciplines. Thus, the techniques and results of coordination chemistry, a field generally regarded as a branch of inorganic chemistry, have been utilized in investigations on one of the central problems of biochemistry, that of the relation between structure and function of enzymes. To my mind, a major impression from the progress made so far is that metalloenzymes represent rather 'unique' metal complexes in comparison with the simpler systems generally studied by coordination chemists. The nature of these 'unique' metal-binding properties and their relation to the catalytic function of the metal ion in a number of metalloenzymes will form the main theme of this survey.

The unusual coordination chemistry of metalloenzymes necessitates that arguments based on the properties of simpler 'model' complexes must be applied with caution and considerable judgement. This apparent antithesis between enzymes and models is well illustrated by the different attitudes taken by two investigators, D. Keilin and O. Warburg, who both played prominent roles in the early development of the field. Thus, it may be appropriate to introduce the subject by a brief account of some main points in the history of metalloenzymes.

G. Bertrand was the first investigator who suggested a metal as the active group in enzymes. This occurred in the 1890s in connection with his work on

laccase-catalysed oxidation of phenols¹. However, he was unable to provide a firm experimental proof of his idea, as indicated by his incorrect identification of the metal involved as manganese instead of copper². This was undoubtedly related to the low degree of purity of the enzyme preparations available at the time. Early claims for the involvement of metals in enzyme catalysis were, therefore, generally not based on identification but on the inhibitory effect of 'metal poisons', such as that of cyanide on cellular respiration. A new aspect was, however, introduced by Warburg in the 1920s, when he showed that charcoal-iron 'models' prepared from blood can catalyse certain oxidations. As cyanide was known to form strong complexes with iron, he suggested that iron-containing enzymes are catalysts in biological oxidations. On this basis, Warburg, in his book *Schwermetalle als Wirkungsgruppen von Fermenten*³, pictures himself as the discoverer of the key role played by iron in respiration. On the other hand, Keilin, in his review⁴ of Warburg's book, points out '... that charcoals as they are prepared from blood or from haemin have no more in common with haematin compounds than charcoal-iron prepared from Bismarck brown or safranin. The work of Prof. Warburg with Negelein and Brefeld between 1921 and 1924 on the properties of these charcoals, however interesting it was from a physiochemical point of view, has not been very helpful in the study of the mechanism of intracellular oxidation reactions'. Quite correctly, Keilin implies that his own pioneering work⁵ on the cytochromes supplied the first true demonstration of the involvement of iron in respiration. Keilin is also responsible for two subsequent major developments in the field of metallo-protein catalysis. Thus, together with Mann, he showed that copper is a constituent of laccase² and tyrosinase⁶ and that carbonic anhydrase is a zinc-containing protein⁷.

Keilin's attitude in the controversy just described, namely that there is an inherent danger in far-reaching, uncritical extensions of observations on models, has proved to be amply justified by recent extensive work on the structure and function of a small number of particularly well-characterized metalloenzymes, such as carboxypeptidase, carbonic anhydrase and laccase. It is quite fitting that two of these enzymes, laccase and carbonic anhydrase, were shown to be metalloenzymes by Keilin. As my own work in the last decade has centred around these two enzymes, it is also natural that they will dominate my present discussion.

While early investigators were led to an interest in the involvement of metals in enzyme catalysis through their work on important metabolic pathways, a different motivation has prompted many of the recent studies to be discussed here. Several authors⁸⁻¹¹ have stressed that metalloenzymes are particularly suitable objects for the elucidation of the relation between chemical structure and catalytic activity. One reason for this is the hope that the metal ion may provide a natural label of that portion of the protein which is directly involved in the reaction, the so-called active site. Another one is the fact that the metal ion often has physical properties, such as colour or paramagnetism, allowing a direct study of chemical events at this site. A third reason is the possibility of correlations with simple 'model' systems with regard to both coordination chemistry and catalytic properties. This point of view was first clearly enunciated in 1950 in a review by Lehninger⁸

who, however, did not follow up these thoughts with original work of his own on metalloenzymes. However, shortly afterwards two groups of biochemists initiated extensive series of investigations on metalloenzymes on the basis of similar considerations. One was B. L. Vallee⁹ and his associates who, in particular, have studied the state and function of zinc in carboxypeptidase. The other group was my own which, after an early interest in metal activation¹⁰, has concentrated its efforts on carbonic anhydrase and laccase. About the same time, an inorganic chemist, R. J. P. Williams¹¹, started publishing discussions on metalloenzymes, particularly those studied by the biochemical groups just mentioned, on the basis of model systems. Similar contributions, mainly concerning metal-containing oxidases, have later been made by another inorganic chemist, P. Hemmerich¹².

In the following I will first try to sketch the development of the concept of the 'unique' metal binding in metalloproteins by a consideration of two zinc-containing enzymes, carboxypeptidase and carbonic anhydrase. Hopefully, this choice is quite illustrative, as the research groups involved have taken quite different attitudes in their use of 'models', and only gradually has a common point of view emerged. This discussion will be followed by a somewhat fuller account of recent work on the state and function of three distinct forms of copper in 'blue' copper-containing oxidases¹³. These are perhaps even more unusual metal complexes than the zinc-containing enzymes. While because of the theme of my survey, stress will be laid on the state and chemical properties of the three forms of the metal, I will also include some comments on the relation of these characteristics to the function of the different ions in the catalytic reaction. In particular, such considerations have led to a major advance in our understanding of the reduction of oxygen to water, a most problematic reaction mechanistically^{13, 14}.

The 'unique' metal-binding properties of metalloenzymes can only be appreciated by a comparison with simpler systems. Thus, the two main sections of this exposition will be preceded by a brief account of metal ion-peptide complexes, particularly in relation to simple (non-specific) metal ion-protein interactions.

FUNDAMENTALS OF METAL ION-PROTEIN INTERACTIONS

As we are concerned here with the nature of metal-ion binding to proteins, those metalloenzymes, such as peroxidase, in which the metal ion is primarily bound to a prosthetic group will not be considered. The discussion will, in general, be limited to metalloenzymes and exclude metal-ion activated systems¹⁰. In the latter, the function of the metal ion may be to form a metallosubstrate, such as a Mg^{2+} -ATP complex participating in most kinase reactions¹⁵, but a metal ion-enzyme complex may also be involved, in which case the distinction from metalloenzymes is one of degree and not of kind¹⁶.

Possible ligand groups in metal-ion complexes with proteins are the polar side chains of certain amino acids, such as glutamic acid or histidine, terminal α -amino and α -carboxyl groups of the peptide chain and, in some cases, the amide groups of the peptide bonds. The interaction of these groups with metal ions has been studied extensively with amino acids and small peptides.

This area of investigation was reviewed more than ten years ago by Gurd and Wilcox¹⁷. Even if many studies on the stability of peptide complexes have appeared later, the major generalizations possible from this type of data were set forth already in their excellent survey. Recently an important review on the crystal structure of metal complexes with amino acids and peptides has been published¹⁸, and this provides much needed information on the structural background of the interactions. Unfortunately, with small complexes one may expect that the structures in solution and in the solid state may differ significantly. However, in one case in which this question has been studied in detail by spectroscopic methods, namely Cu^{2+} -triglycylglycine, it was found that the coordination was essentially the same in the two states, the main difference being the exclusion of water as axial ligands in the crystal¹⁹.

In proteins most of the potential ligand groups would be expected to be held apart and form discrete binding sites. Non-specific interactions with such sites are found with all proteins. They can be well described in terms of properties derived from simple model systems (amino acids and peptides), as shown, for example, in the pioneering investigation by Gurd and Goodman²⁰ on the binding of Zn^{2+} to serum albumin (cf. ref. 17).

One might predict that most proteins would, on purely statistical grounds, contain some polar groups in close enough proximity to allow chelate formation. This is borne out by the finding of preferred sites at low metal-ion concentrations with some proteins, such as myoglobin²¹ and serum albumin²². These sites are responsible for what I would like to call 'specific non-specific' binding; they are specific in the sense of being preferred sites in comparison to the type of sites discussed earlier, but non-specific in the sense that their complexes have no known biological functions. Also in these cases it has been possible to account for the stability and spectroscopic properties of the complexes by comparisons with models^{21, 22}.

As indicated in the introductory section of this paper, quite a different situation is found in the specific interactions of metalloenzymes. In the remainder of my discussion I will attempt to make this claim more concrete by a somewhat more detailed consideration of a few enzymes. I would like to state at the outset, however, that the information derived from models is indispensable as a background knowledge for viewing the 'unique' metal-binding properties of metalloenzymes. I would also like to stress that inorganic chemists, like Williams, have performed an extremely valuable service by continuously pointing out what complex-forming properties one would predict on the basis of results with simpler compounds. This has been a constant stimulus and guide, not least in the studies of my own research group. Thus, insofar as I will criticize certain claims based on model studies, this reflects mainly on the biochemist, who should be capable of producing data solid enough for him to trust them even when they deviate from predictions founded on basic coordination chemistry.

STATE AND FUNCTION OF METAL IONS IN CARBOXYPEPTIDASE AND CARBONIC ANHYDRASE

Detailed suggestions, based on model data, for coordination in a metallo-enzyme were first advanced for carboxypeptidase. Thus, Williams²³ in 1960

implied the presence of a $Zn^{2+}-S^{-}$ bond on the basis of the relative binding strength with different metal ions, particularly the higher affinity for Zn^{2+} compared to Co^{2+} , and the visible spectrum of the Co^{2+} enzyme. This idea was then extended, in collaboration with Vallee and Coleman²⁴, to comprise a nitrogen-sulphur chelate. Vallee and co-workers later tried to obtain more direct chemical support for this hypothesis, and as a result of an extensive series of investigations claimed that the ligands are the thiol group of a cysteine residue and the α -amino group of a *N*-terminal amino acid²⁵.

The relative stability constants with different metal ions and the visible spectrum of the Co^{2+} enzyme in the case of carbonic anhydrase²⁶ are very similar to the corresponding properties of carboxypeptidase. If the model arguments were correct, one would thus conclude that a thiol group is involved in the metal binding also in this enzyme. In fact, at a symposium in 1961, Williams²⁷ made such a proposal. However, at the same symposium, I²⁸ pointed out that the bovine enzyme lacks a sulphhydryl group. As all chemical data indicated an identical metal-ion coordination in all forms of the enzyme²⁹, this would seem to exclude a thiol group as a ligand even in the cases where there is one present, as in the human isoenzymes. This apparent difference compared to carboxypeptidase suggested that caution must be used in the application of arguments from models. This view was enforced by a critical evaluation of the experimental carboxypeptidase papers, since, as I expressed in another review³⁰, this shows that 'none of the experiments provides a definite demonstration of the nature of the binding site'. Fortunately it has been possible to settle the ensuing conflict definitively, as the three-dimensional structure is now available both for carbonic anhydrase³¹ and for carboxypeptidase³².

The beautiful crystallographic work of Lipscomb and co-workers³² on carboxypeptidase has furnished the structure of the largest molecule so far solved to high resolutions. It has also, together with sequence information,

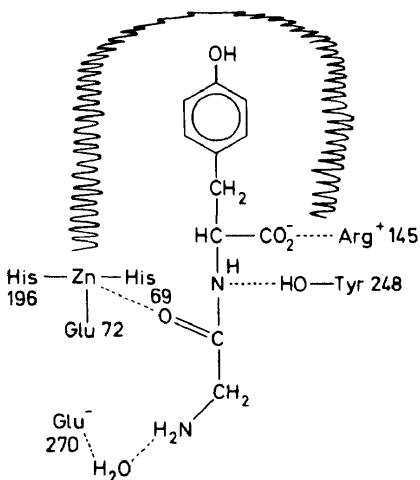


Figure 1. Schematic view of the active site of carboxypeptidase with glycytyrosine bound, as derived from the x-ray data³² and sequence information from the laboratory of H. Neurath (W. N. Lipscomb, personal communication).

for the first time provided firm evidence for the nature of the ligand groups in a metalloenzyme, as shown in *Figure 1*. It may be noted that the binding residues are not at all those previously suspected. A thiol group is, in fact, not even present in the enzyme, but there is a disulphide bridge. However, this, as well as the *N*-terminal residue, is more than 10 Å away from the metal ion. A similar distance had earlier been found, in a low-resolution structure³¹, for the thiol group in human carbonic anhydrase C.

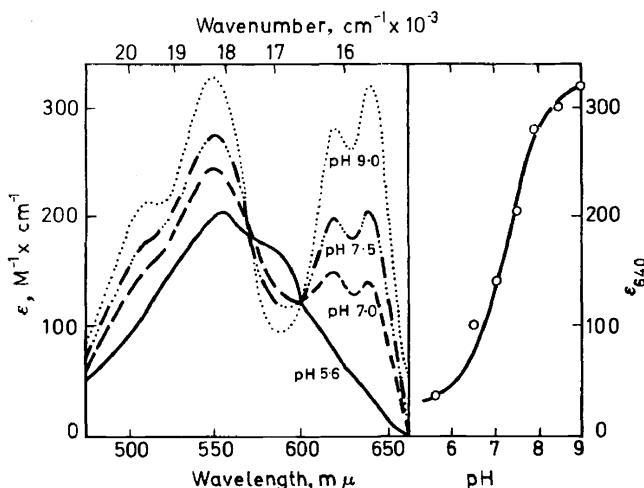


Figure 2. Absorption spectrum of human Co^{2+} -carbonic anhydrase B at different pH values²⁹.

The failure of certain model arguments in the case of carboxypeptidase should not mislead us to discredit the approach of utilizing the metal ion in metalloenzymes as a 'built-in reporter group' for events in the active site. The power of this technique, when judiciously applied, is nicely illustrated by the work of Lindskog on Co^{2+} -carbonic anhydrase³³. On the basis of spectroscopic and magnetometric data he concluded that the metal ion is in an irregular tetrahedral environment. Three ligands are here provided by the protein while the fourth one may be H_2O , OH^- or an inhibitor, such as a sulphonamide or an inorganic anion, as also confirmed by crystallographic data (B. Strandberg and A. Liljas, personal communication). In agreement with this, the bonding changes with pH or on the addition of an inhibitor, as illustrated by the example in *Figure 2*²⁹. Correlations with kinetic data³⁴ show that the high-pH form, involving an additional ligand (probably OH^-), is the catalytically active one. Furthermore, the additional ligand competes with inhibitors, as the inhibition is reversed by high pH^{33, 34}.

While the important work on carboxypeptidase and carbonic anhydrase deserves an extensive presentation, this has, in fact, already been made more competently by the original investigators at recent symposia^{32, 35}. Thus, I would just like to conclude this section by pointing to the power of combining the approaches sketched here with crystallographic results. For example, the structure in *Figure 1* shows the binding of a poor substrate, glycylytyrosine.

Comparisons with model building and biochemical data suggest that this is a poor substrate because it is bound in a way which interferes with the normal function of the glutamic acid residue at position 270. It is also suggested that the interaction between the carbonyl group of the substrate and the zinc ion is part of the normal catalytic mechanism. In fact, Lipscomb *et al.*³² have suggested a very plausible reaction scheme which is consistent with all available information, but for a discussion of this the reader is referred to the original paper. I would only like to stress that, while with both enzymes an essential role for the zinc ion in the catalytic mechanism is depicted, this function has no direct counterpart in the catalytic activity of simple zinc complexes. I will return to this question later.

THREE 'UNIQUE' FORMS OF COPPER IN 'BLUE' OXIDASES

The remainder of my presentation will be devoted to 'blue' copper-containing oxidases, a group of enzymes around which my own research interests have been entirely centred during the last few years. I hope to show that the metal coordination is perhaps even more unusual in these enzymes than in carboxypeptidase and carbonic anhydrase. On the other hand, there is so far very little structural information available about the copper-containing oxidases, so that the chemical basis for the unusual state cannot be viewed in any detail yet. Since, however, the purpose of a conference should be not only to present a finished picture of an important field but also to stimulate further research, it may be most apt that I will concentrate my discussion to this rapidly developing area of investigation.

A comparison with the properties of simple model compounds can easily lead to incorrect interpretations also with the copper proteins. For example, at a conference on metal binding in biology held in 1960, Williams³⁶ stated that models would suggest that tyrosinase is a Cu^{2+} protein while the 'blue' oxidases must contain a considerable fraction of their copper as Cu^+ . We now have evidence that the opposite situation holds¹³, namely that tyrosinase is a Cu^+ protein and that the blue colour of other copper proteins is given by an unusual form of Cu^{2+} . This comment is given here not as a criticism of Williams, whose statement is entirely correct in its explicit comparison with model compounds known at the time, but rather to emphasize the main point of this survey.

In this written presentation, my documentation will purposely be rather incomplete, as I have recently written a detailed review on the state and function of copper in biological systems¹³.

The forms of copper

The 'blue' oxidases have been found to contain three distinct forms of copper¹³. This is most extensively documented in the case of laccase (*p*-diphenol oxidase) from a fungus, *Polyporus versicolor*, and from a lacquer tree, *Rhus vernicifera*, so that my examples will mainly be taken from studies with this enzyme. However, most conclusions in regard to the state and function of the metal can probably be generalized and apply also to ceruloplasmin and ascorbic acid oxidase. In addition, the 'blue' Cu^{2+} found in certain electron-transport proteins, such as azurin, is probably closely related to the corresponding form in the oxidases.

The first demonstration of the heterogeneity of the copper in laccase came from quantitative e.p.r. (electron paramagnetic resonance)³⁷ and susceptibility measurements³⁸, which showed that two of the four copper ions present in each molecule are paramagnetic while the remaining two are diamagnetic. It was furthermore found that the paramagnetic ions are two Cu^{2+} in different chemical environments³⁹ and, therefore, designated Type 1 and Type 2 Cu^{2+} , respectively. This is shown by the experimental and simulated e.p.r. spectra given in *Figure 3*. The three forms of copper are thus: (1) Type 1 Cu^{2+} (the 'blue' Cu^{2+}), (2) Type 2 Cu^{2+} (the 'non-blue' Cu^{2+}), and (3) two diamagnetic copper ions (probably a spin-paired couple of Cu^{2+}); in parenthesis are given some further identifications to be discussed shortly.

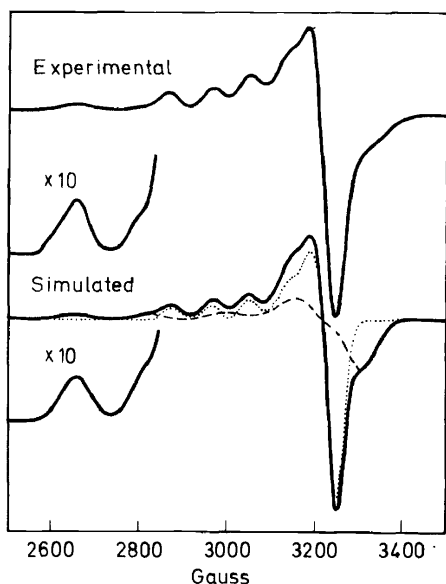


Figure 3. Experimental and simulated e.p.r. spectra at 9.2 GHz of *Polyporus laccase*³⁹. The simulation was formed on the assumption that two different forms of Cu^{2+} were present (dotted lines) in equal amounts. The full line in the simulated spectrum represents the sum of their contributions.

Each of these forms represents a 'unique' state of copper in comparison with model complexes. For the Type 1 Cu^{2+} this can be exemplified by its high extinction coefficient (*ca.* $5000 \text{ M}^{-1}\text{cm}^{-1}$) at 610 nm, its narrow hyperfine structure in the e.p.r. spectrum (0.009 cm^{-1} compared to the range 0.015 to 0.020 generally found in Cu^{2+} complexes; see *Figure 3* and ref. 40), and its unusually high redox potential ($> 500 \text{ mV}$). The association of the blue colour with Type 1 Cu^{2+} and the determination of the redox potential of this ion pose some problems since, as will be discussed later, it is generally reduced together with other electron acceptors in a cooperative manner⁴¹. However, the reduction achieved at high pH⁴² appears to be specific⁴³ for

Type 1 Cu^{2+} , and the cooperation between the sites can also be destroyed under certain conditions.

There have been several attempts to explain the unusual characteristics of the 'blue' Cu^{2+} (see ref. 13), but some of the early suggestions can now be eliminated. For example, the blue electron-transport proteins, azurin and stellacyanin, contain a single Cu^{2+} , so apparently it is not necessary to introduce an interaction between Cu^{2+} and Cu^+ to account for the spectral properties. The favoured interpretation is now that the symmetry of the ligand arrangement around the central ion deviates from the planar or tetragonal symmetry generally preferred by Cu^{2+} . This is supported by models⁴⁴ and theoretical calculations⁴⁵. It has also been directly demonstrated by e.p.r. spectra at 35 GHz³⁹ as well as by o.r.d. (optical rotatory dispersion) measurements⁴⁶. The Cotton effect in the o.r.d. curve in *Figure 4* shows that a blue chromophore is in a dissymmetric environment, and it has been possible to associate the main contribution with Type 1 Cu^{2+} .

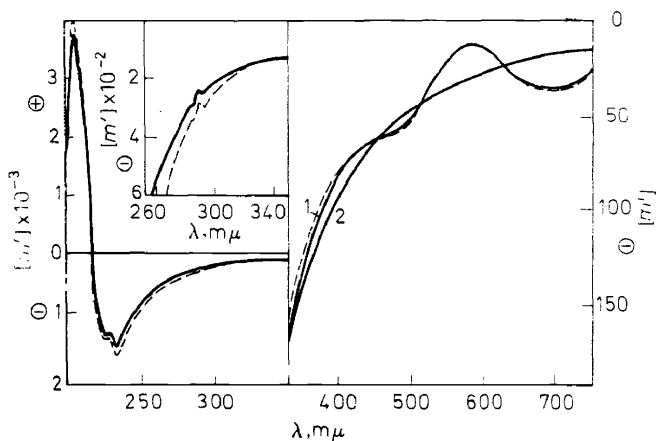


Figure 4 The o.r.d. curves for laccase A and B, and for the enzymes reduced with excess quinol⁴⁶.

The irregular coordination is probably responsible for the high redox potential, a property of some importance for the function of this ion in the catalytic reaction. This is also true regarding an early interpretation of the spectral data, suggesting that the electron-acceptor orbital is highly delocalized, which should facilitate rapid electron transfer. This idea, which was set forth in 1960 by Vännegård and myself in a paper⁴⁰ describing for the first time the unique state of copper in the 'blue' proteins, has been criticized on the ground that we failed to take into account effects of low symmetry⁴⁷. This criticism appears to be unjustified, as we explicitly stated that our treatment assumed axial symmetry but that this assumption may be incorrect. While we now know that the symmetry cannot be axial, the concept of a high degree of delocalization may still be valid¹³.

Type 2 Cu^{2+} appears to represent a more normal form of copper with regard to its spectral properties. It should be noted that it is termed 'non-blue'

only in relation to Type 1 Cu^{2+} but probably has an extinction coefficient around 650 nm which is somewhat higher than that found for tetragonal complexes¹³. It also appears to contribute slightly to the anomalous o.r.d. curve⁴⁶, in which case it also has a dissymmetric coordination. Its most distinctive 'unique' feature in laccase is an unusually high affinity for F^- ⁴⁸. The stability constant for the first F^- bound is so high that the reaction appears essentially irreversible. The binding of F^- can be observed by e.p.r. It leads to an inhibition of the oxidase activity, which indicates that the Type 2 Cu^{2+} is also an essential component of the enzyme. This has been confirmed by the reversible removal of this ion⁴⁹.

Three main possibilities must be considered for the diamagnetic copper ions: (1) Cu^+ , (2) low-spin Cu^{3+} , and (3) a spin-paired, binuclear Cu^{2+} — Cu^{2+} complex. A number of findings support the third possibility, but these can only be understood in terms of the redox titrations presented in the next section.

The electron-accepting sites in laccase

An unexpected finding was that three or four electrons per molecule are necessary to bleach the blue colour of laccase anaerobically⁴¹, despite the fact that the single 'blue' Cu^{2+} can accept one electron only. This is seen in *Figure 5*, which also shows that the degree of reduction is a linear function of the amount of reducing agent added. This result, in conjunction with e.p.r. titrations, demonstrates that there are two reducible sites in the protein other than the Type 1 and Type 2 Cu^{2+} . As all sites can be reduced by octacyanotungstate(IV), they must have a redox potential greater than 500 mV.

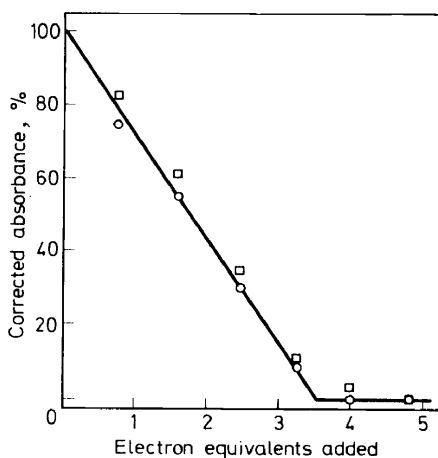


Figure 5. Anaerobic titration of *Polyporus* laccase with ascorbate⁵⁰. Absorbancy at 610 nm, ○; absorbancy at 330 nm, □.

Figure 5 also shows that an absorption band at 330 nm is reduced together with the blue colour, but it cannot be decided whether this absorption is also associated with the 'blue' Cu^{2+} or with the other sites. However, it has been

METAL-BINDING PROPERTIES OF METALLOENZYMES

found possible to differentiate the electron-accepting sites in a F^- -treated enzyme, as shown in *Figure 6*⁵⁰. This clearly demonstrates that the 330 nm band is associated with a two-electron-accepting site. As discussed in detail elsewhere⁴¹, there are three main reasons to think that this site consists of the two diamagnetic copper ions: (1) the stoichiometry, (2) the high redox potential, and (2) the 330-nm band. While spin-paired binuclear complexes of Cu^{2+} are known⁵¹, they are usually not completely diamagnetic at room temperature. Thus, again this pair represents a rather unique form of copper.

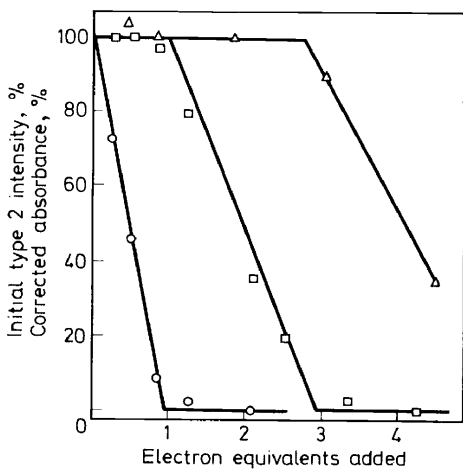


Figure 6. Anaerobic titration of F^- -treated *Polyporus laccase*⁵⁰. Absorbance at 610 nm, ○; absorbance at 330 nm, □; and percentage of initial Type 2 Cu^{2+} , Δ.

The linearity of the redox titration in *Figure 5* deserves some comments. Two situations could clearly lead to this result: (1) all sites have the same redox potential, or (2) there is a strong cooperativity, so that only fully oxidized or fully reduced molecules are present at equilibrium at each stage of the titration. The first possibility seems eliminated as one should then find molecules with only one of the Cu^{2+} ions in the diamagnetic pair reduced, but no new e.p.r. signals appear during the titration. However, a comparison with the lacquer-tree enzyme, in which Type 1 Cu^{2+} has a lower potential, suggests that a combination of the two explanations is the correct one. Thus, the diamagnetic pair is a cooperative, two-electron-accepting unit, while Type 1 Cu^{2+} happens to have the same high potential in the fungal but not in the tree enzyme¹³.

Functional considerations

While my primary concern here is with the unusual coordination properties of the copper ions in laccase, it may be in place to say a few words about their roles in the catalytic mechanism.

The function of the 'blue' Cu^{2+} is easy to visualize, as it probably acts as the primary electron acceptor in the one-electron oxidation of the organic substrate⁵². The main problem is instead how this one-electron site is

reoxidized by O_2 , a four-electron acceptor in view of the fact that H_2O is formed in this reaction. Reduction of O_2 in four consecutive one-electron steps does not seem likely. One reason is the high free-energy barrier for the first step¹⁴, which could perhaps be overcome by a strong stabilization of the O_2^- intermediate. Such a stabilization may occur in some enzymes⁵³. However, in view of the high redox potential, the barrier would seem too large with laccase. Furthermore, a one-electron mechanism must involve the highly reactive OH radical as an intermediate which, however, is not the case in enzymes having H_2O_2 as the final product of O_2 reduction.

An alternative mechanism would involve multi-electron steps. As has been discussed elsewhere¹³, two double-electron transfers involving the diamagnetic pair appears most likely. This pair in its reduced form would then also be the oxygen-binding site. Type 2 Cu^{2+} may also be associated with this site and contribute to the stabilization of the O_2^{2-} intermediate. Preliminary kinetic evidence^{50,54} supports a role in the reduction of oxygen for the sites other than the 'blue' Cu^{2+} .

CONCLUDING REMARKS

I hope that the preceding discussion has amply illustrated the thesis that the metal-ion coordination in the active sites of metalloenzymes shows many unusual features compared to the simple complexes generally studied by coordination chemists. My own experience with enzymes such as enolase, carbonic anhydrase and laccase led me to suggest this about ten years ago¹⁰, but the view seems only gradually to have won wider acceptance. However, recently some of the investigators earlier relying most heavily on model arguments have also expressed similar ideas⁵⁵.

The stress on the 'unique' metal-binding properties of metalloenzymes is, of course, no attempt at a return to vitalism. It just reflects the fact that a protein site may create an environment, for example, in regard to stereochemistry or local dielectric constant, which is quite hard to reproduce in a small molecule in aqueous solution. In this respect the effects are not limited to metals but also apply to other residues in active sites. As well-known examples may be mentioned that the active serine in chymotrypsin shows quite a different reactivity compared to most serine residues found in peptides or proteins, and that the histidine residues at positions 12 and 119 in ribonuclease display quite unique properties compared to other histidine residues. The basis of these special reactivities can now be discerned in those cases where detailed structural information is available⁵⁶. As metal ions even in a unique state still, of course, follow the laws of chemistry, models will continue to be useful when properly employed. It may just be well to remember that 'models' are not 'replicas'.

From the enzymologist's point of view, the most important feature of the 'unique' coordination is that it imparts properties to the metal ion facilitating its role in the catalytic reaction. Thus, it often also has catalytic functions generally not found in simple models. This is apparently what Vallee and Williams⁵⁵ imply with the term 'entatic' introduced by them, but it may be desirable to find more concrete expressions of such effects. I have already discussed how the low-symmetry coordination of a Cu^{2+} ion in laccase makes it a good oxidizing agent, both thermodynamically and

kinetically. The role of the zinc ion in carboxypeptidase is probably to polarize the carbonyl group attached to it (see *Figure 1*). It can be shown that the dielectric situation in the enzyme-substrate complex tends to intensify such an effect (W. N. Lipscomb, personal communication). It is thus apparent that by a judicious combination of structural information with the results from organic chemistry and reaction kinetics we should be able to give increasingly exact descriptions of mechanisms for metalloenzymes, as well as for enzymes in general.

REFERENCES

- ¹ G. Bertrand, *C.R. Acad. Sci., Paris*, **122**, 1132 (1896).
- ² D. Keilin and T. Mann, *Nature, London*, **143**, 23 (1939).
- ³ O. Warburg, *Schwermetalle als Wirkungsgruppen von Fermenten*, Editio Cantor: Freiburg (1949).
- ⁴ D. Keilin, *Nature, London*, **165**, 4 (1950).
- ⁵ D. Keilin, *Proc. Roy. Soc. B*, **98**, 312 (1925).
- ⁶ D. Keilin and T. Mann, *Proc. Roy. Soc. B*, **125**, 187 (1938).
- ⁷ D. Keilin and T. Mann, *Nature, London*, **144**, 442 (1939).
- ⁸ A. L. Lehninger, *Physiol. Rev.* **30**, 393 (1950).
- ⁹ B. L. Vallee, *Advanc. Protein Chem.* **10**, 317 (1955).
- ¹⁰ B. G. Malmström and A. Rosenberg, *Advanc. Enzymol.* **21**, 131 (1959).
- ¹¹ R. J. P. Williams, *Biol. Rev. Cambridge Phil. Soc.* **28**, 381 (1953).
- ¹² P. Hemmerich, *Z. naturwiss.-mediz. Grundlagenforsch.* **2**, 230 (1965).
- ¹³ R. Malkin and B. G. Malmström, *Advanc. Enzymol.* **33**, 177 (1970).
- ¹⁴ D. George, in *Oxidases and Related Redox Systems*, Vol. I, p 3, Ed. T. E. King, H. S. Mason and M. Morrison. Wiley: New York (1965).
- ¹⁵ M. Larsson-Raźnikiewicz, *Biochim. Biophys. Acta*, **85**, 60 (1964).
- ¹⁶ B. G. Malmström, *Federation Proc.* **20**, 60 (1961).
- ¹⁷ F. R. N. Gurd and P. E. Wilcox, *Advanc. Protein Chem.* **11**, 311 (1956).
- ¹⁸ H. C. Freeman, *Advanc. Protein Chem.* **22**, 257 (1967).
- ¹⁹ K. E. Falk, H. C. Freeman, T. Jansson, B. G. Malmström and T. Vännegård, *J. Am. Chem. Soc.* **89**, 6071 (1967).
- ²⁰ F. R. N. Gurd and D. S. Goodman, *J. Am. Chem. Soc.* **74**, 670 (1952).
- ²¹ F. R. N. Gurd, K. E. Falk, B. G. Malmström and T. Vännegård, *J. Biol. Chem.* **242**, 5724 (1967).
- ²² R. A. Bradshaw, W. T. Shearer and F. R. N. Gurd, *J. Biol. Chem.* **243**, 3817 (1968).
- ²³ R. J. P. Williams, *Nature, London*, **188**, 322 (1960).
- ²⁴ B. L. Vallee, R. J. P. Williams and J. E. Coleman, *Nature, London*, **190**, 633 (1961).
- ²⁵ T. L. Coombs, Y. Omote and B. L. Vallee, *Biochemistry*, **3**, 653 (1964).
- ²⁶ S. Lindskog and B. G. Malmström, *J. Biol. Chem.* **237**, 1129 (1962).
- ²⁷ R. J. P. Williams, in *Proceedings of the Fifth International Congress of Biochemistry*, Moscow 1961, Vol. IV, p 133, Ed. P. Desnuelle and A. E. Braunstein, Pergamon: Oxford (1963).
- ²⁸ B. G. Malmström, A. Rosenberg and S. Lindskog, in *Proceedings of the Fifth International Congress on Biochemistry*, Moscow 1961, Vol. IV, p 172, Ed. P. Desnuelle and A. E. Braunstein, Pergamon: Oxford (1963).
- ²⁹ S. Lindskog and P. O. Nyman, *Biochim. Biophys. Acta*, **85**, 462 (1964).
- ³⁰ B. G. Malmström and J. B. Neilands, *Ann. Rev. Biochem.* **33**, 331 (1964).
- ³¹ K. Fridborg, K. K. Kannan, A. Liljas, J. Lundin, B. Strandberg, R. Strandberg, B. Tilander and G. Wirén, *J. Molec. Biol.* **25**, 505 (1967).
- ³² W. Lipscomb, J. A. Hartsuck, G. N. Reeke, F. A. Quiocho, P. H. Bethge, M. L. Ludwig, T. A. Steitz, H. Muirhead and J. C. Coppola, *Brookhaven Symp. Biol.* **21**, 24 (1969).
- ³³ S. Lindskog, *Studies on the State and Function of Metal Ions in Carbonic Anhydrase*, Almqvist Wiksell: Stockholm (1968).
- ³⁴ S. Lindskog, *Biochemistry*, **5**, 2641 (1966).
- ³⁵ S. Lindskog, in *CO₂: Chemical Background and Physiological Aspects*, p 157. Ed. R. E. Forster, J. T. Edsall, A. B. Otis and F. J. W. Roughton, National Aeronautics and Space Administration: Washington, D.C. (1969).

BO G. MALMSTRÖM

- ³⁶ R. J. P. Williams, *Federation Proc.* **20**, 69 (1961).
- ³⁷ L. Broman, B. G. Malmström, R. Aasa and T. Vänngård, *J. Molec. Biol.* **5**, 301 (1962).
- ³⁸ A. Ehrenberg, B. G. Malmström, L. Broman and R. Mosbach, *J. Molec. Biol.* **5**, 450 (1962).
- ³⁹ B. G. Malmström, B. Reinhammar and T. Vänngård, *Biochim. Biophys. Acta*, **156**, 67 (1968).
- ⁴⁰ B. G. Malmström and T. Vänngård, *J. Molec. Biol.* **2**, 118 (1960).
- ⁴¹ J. A. Fee, R. Malkin, B. G. Malmström and T. Vänngård, *J. Biol. Chem.* **244**, 4200 (1969).
- ⁴² B. G. Malmström, R. Aasa and T. Vänngård, *Biochim. Biophys. Acta*, **110**, 431 (1965).
- ⁴³ J. A. Fee, B. G. Malmström and T. Vänngård, *Biochim. Biophys. Acta*, **197**, 136 (1970).
- ⁴⁴ D. C. Gould and A. Ehrenberg, *Europ. J. Biochem.* **5**, 451 (1968).
- ⁴⁵ W. E. Blumberg, In *The Biochemistry of Copper*, p 49, Ed. J. Peisach, P. Aisen and W. E. Blumberg, Academic Press: New York (1966).
- ⁴⁶ F. Bossa, G. Rotilio, P. Fasella and B. G. Malmström, *Europ. J. Biochem.* **10**, 395 (1969).
- ⁴⁷ G. Morpurgo and R. J. P. Williams, in *Physiology and Biochemistry of Haemocyanins*, p 113. Ed. F. Ghirelli, Academic Press: New York (1968).
- ⁴⁸ R. Malkin, B. G. Malmström and T. Vänngård, *FEBS Letters*, **1**, 50 (1968).
- ⁴⁹ R. Malkin, B. G. Malmström and T. Vänngård, *Europ. J. Biochem.* **7**, 253 (1969).
- ⁵⁰ R. Malkin, B. G. Malmström and T. Vänngård, *Europ. J. Biochem.* **10**, 324 (1969).
- ⁵¹ M. Kato, H. B. Jonassen and J. C. Fanning, *Chem. Rev.* **64**, 99 (1964).
- ⁵² L. Broman, B. G. Malmström, R. Aasa and T. Vänngård, *Biochim. Biophys. Acta*, **75**, 365 (1963).
- ⁵³ P. F. Knowles, J. F. Gibson, F. M. Pick and R. C. Bray, *Biochem. J.* **111**, 53 (1969).
- ⁵⁴ B. G. Malmström, A. Finazzi Agrò and E. Antonini, *Europ. J. Biochem.* **9**, 383 (1969).
- ⁵⁵ B. L. Vallee and R. J. P. Williams, *Proc. Nat. Acad. Sci. Wash.* **59**, 498 (1968).
- ⁵⁶ D. E. Koshland and K. E. Neet, *Ann. Rev. Biochem.* **37**, 359 (1968).