

METAL COMPLEXES OF PEPTIDES AND PROTEINS*

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Many of the functions of metal ions in biological processes presumably involve complex formation with proteins. The possible ligand groups in simple proteins are certain of the reactive groups of the amino-acid side chains, the terminal amino and carboxyl groups (if any), of the polypeptide chains and, in some cases, the amide groups in the peptide backbone. Our primary interest is the general reactivity of groups in proteins such as these ligand groups. The measurement of complex formation offers one way of obtaining a quantitative measure of such reactivity.

The study of metal-protein interactions involves two types of complications that an inorganic chemist generally would be unwilling to invite in the design of his experiments. In the first place, a protein contains a number of different kinds of potential ligand groups such as imidazole, amino, carboxyl, phenoxyl and often sulphhydryl, and these in various molar ratios to each other. In the second place, the structure of each protein places restrictions on the mutual approach and orientation of the potential ligand groups and thereby determines whether a given metal ion may combine with more than one ligand group at a particular point or site on the surface of the protein. Insofar as the ligand groups are held apart as discrete sites the behaviour of the protein will have something in common with an ion-exchange resin of rather low capacity, and the reaction at each ligand group on the protein will have much in common with the first step of complex formation between the given metal ion and the same ligand group in the form of a small molecule. In those cases where two or more ligand groups are held in a cluster a potential chelate site will exist. An exact model for this site may be difficult to construct in a small molecule. Conversely, the recognition and correct description of such sites in a protein may give a tremendous amount of information about the structure of the protein¹.

The problems and opportunities that confront one in studying a metal-protein system may be illustrated by the case which interests us most at the present, namely, sperm whale metmyoglobin equilibrated with cupric chloride². This protein is a particularly appropriate object for the study of reactivity, since the studies of Kendrew and his colleagues have established its structure in almost complete detail. The three-dimensional structure was

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determined by the technique of X-ray diffraction analysis of myoglobin crystals³⁻⁵.

A photograph of a model of the structure is shown in *Figure 15*. The thick white cord is not properly part of the model but traces the convolutions of the single polypeptide chain which winds around in such a way as to give a compact structure departing only moderately from the spherical shape. Where the white cord is straight it traces the axis of an α -helix formed by the regular arrangement of the peptide backbone in which every carbonyl oxygen atom is hydrogen-bonded to the amide nitrogen atom of the fourth residue beyond it in the polypeptide sequence. The regular helical regions are joined by irregular arrangements of the peptide backbone which are, nevertheless, comparably rigidly formed in the crystalline protein.

A feature of the structure is the presence of the haem group embedded in the molecule. The iron atom is bound in the chelate form of the large porphyrin ring, coordinated to four pyrrole nitrogen atoms. In its place in the structure of the protein the haem allows the iron to make a fifth coordination with a nitrogen atom of an imidazole ring forming part of a histidyl residue⁵. The sixth coordination position is occupied, in metmyoglobin which contains ferric iron, by a water molecule apparently bridging by way of a hydrogen bond to another imidazole group of a histidyl residue⁶. In the biologically functional form containing ferrous iron the sixth coordination position is available for reversible coordination of molecular oxygen. The rôle of the ligand occupying the fifth coordination position in moderating the absorption spectrum of proteins of this sort, as well as the interplay between the fifth and the sixth ligands, has been reviewed by Brill and Williams⁷.

The structure shown was determined by observations on the crystalline state. The most direct evidence as to the extent to which this structure persists in solution will be obtained from the detailed study of its reactivity. Several lines of evidence suggest that much of the crystalline structure is preserved in solution. Two separate studies of optical rotatory dispersion in solution have indicated a fractional content of the helical arrangement of the peptide backbone corresponding closely to the 75 per cent seen in the crystalline structure^{8, 9}. Viscosity studies indicate very slight asymmetry, again in keeping with the crystalline structure¹⁰. Again, the spectrum due to the haem group is much the same in the two states, which not only indicates that such primary interactions as those described for the histidyl residues are undisturbed but also that many more interactions involving the haem group in π -bonding, hydrogen-bonding and other relations to the protein are probably not drastically altered. When the native structure is disrupted by exposure to acid solution below pH 4.5 or to urea all these properties are changed drastically so that the similarity to the crystalline state disappears¹⁰⁻¹².

Although every side chain in the structure is not perfectly identified as yet, the X-ray observations are well supported by chemical determination of the sequence of amino-acids in the polypeptide chain by Edmundson working first in conjunction with Hirs and now with Kendrew¹³. A large number of interactions between side-chain groups, and some even between side-chain groups and the amide groups of the peptide

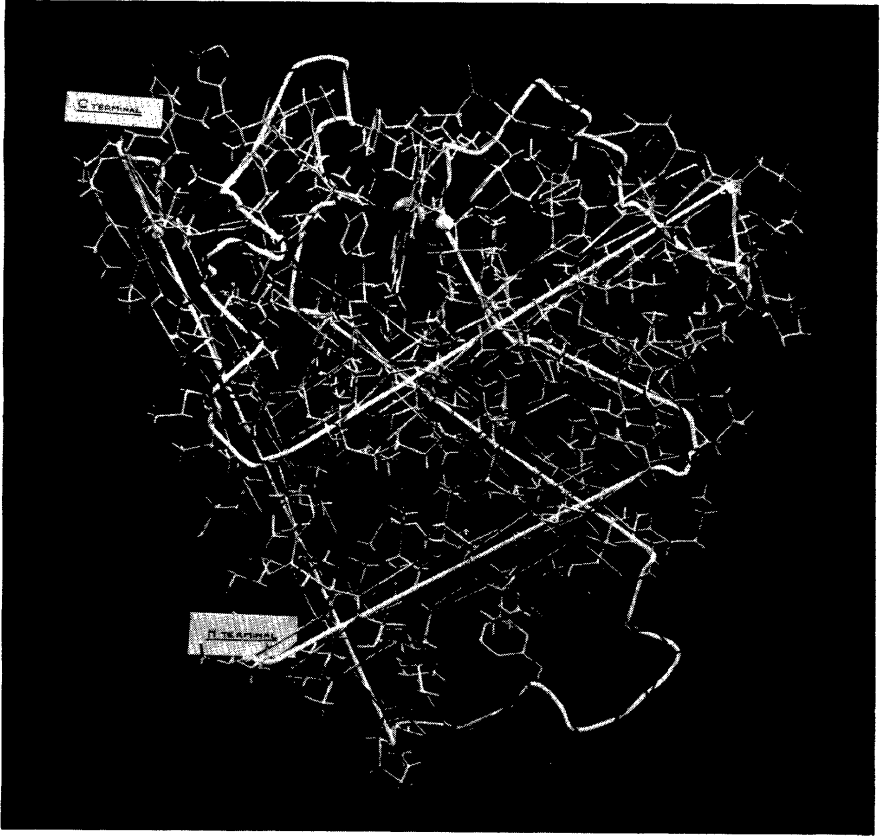


Figure 1. Photograph of model of myoglobin molecule⁵ (By courtesy of J. C. Kendrew)

backbone, have been observed and tabulated by Kendrew⁶. Although the X-ray diffraction technique does not observe hydrogen atoms directly it is possible to identify hydrogen bonds because two electronegative atoms joined by a hydrogen bond will lie closer together than van der Waals radii would allow but farther apart than the classical single covalent bond requires. Therefore, the structure indicates clearly not only which side-chain groups are buried and which exposed but which atoms in them are hydrogen-bonded and in what manner. The inside of the molecule is almost entirely non-polar; polar side chains either stick out into the environment (several extended lysyl side chains may be seen protruding in *Figure 1*, for example) or lie in what might be called an irregular interface between the non-polar interior and the external environment. These latter polar side chains are usually involved in one or more hydrogen bond interaction. The clustering of non-polar side chains on the inside and extensive hydrogen bond formation between polar atoms had been expected to some extent by classical protein chemists¹⁴⁻¹⁷, but the beautiful logic of the architecture revealed by the X-ray studies brings gratification far beyond that of confirmation of such hypotheses.

A comparably fine description of the arrangement and interactions of all parts of the myoglobin molecule in solution cannot be made at present. Wherever it is possible to evaluate the reactivity of side-chain groups it may be possible to infer whether certain groups are readily available for reaction or are buried, combined or in some way shielded from reaction. Because of the overlapping properties of different classes of groups, *e.g.*, amino, imidazole and phenoxyl, and the presence of more than one member of each class in most proteins, it has been thought wisest to employ concurrently several different methods of evaluating reactivity¹⁸.

Complex formation with metal ions is one of three approaches which we have been using to assess reactivity of groups or clusters of groups in myoglobin. In this application we would classify the method together with hydrogen ion binding as an equilibrium method which should be reversible in general.

A second main procedure makes use of the rate of reaction of a particular type of side-chain group with some appropriate compound. A compound which has proved most useful has been *p*-nitrophenyl acetate which is split catalytically by imidazole groups and can be studied with amino groups under pseudo first-order conditions^{18, 19}. Combination of either group with cupric or zinc ion prevents the reaction with *p*-nitrophenyl acetate. This catalytic procedure has the advantage that the protein undergoes only slight or temporary alteration and can be recovered essentially unchanged. This procedure has been used to probe imidazole groups in myoglobin¹¹.

A third main procedure involves measuring the rate of reaction with some reagent which yields stable derivatives. Two such reactions have been applied to myoglobin in our laboratory up to the present²⁰. The first is reaction with *O*-methyl isourea which combines with amino groups to yield the guanidino group and methanol. The effect of this reaction is to convert lysyl residues into homoarginyl residues without other apparent effects. The second reaction makes use of bromoacetate which alkylates the imidazole group of histidyl residues. Either or both nitrogen atoms in the ring may be

alkylated²¹. The reaction may be run at pH 7 in a pH-stat with only a slight degree of reaction with amino groups. In each case the modified protein is soluble, shows a spectrum very similar to that of the native material, appears to be of similar shape by the viscosity criterion, and shares with the native protein the ability to undergo a drastic change in acid solution or in urea. Both the homoarginine and the various carboxymethyl derivatives of histidine are measurable by routine methods after hydrolysis of the modified protein preparations.

We are now in a position to show a correlation which is beginning to develop between the three different approaches to the measurement of reactivity: equilibration with ions, catalysis by certain groups and formation of stable derivatives. All three methods indicate that certain of the imidazole groups are quite unreactive in the native protein. For example, prolonged reaction with bromoacetate at pH 7 or 8 yields a product in which only 8 of the histidyl residues are modified, leaving 4 unchanged. Since the haem absorption spectrum is unaffected it seems that one of the 4 imidazole groups which escapes alteration is that directly coordinated to the iron atom. If the structure of the protein is disrupted by urea the bromoacetate attacks essentially all the imidazole groups.

In parallel to the results of the bromoacetate reaction are earlier observations on the equilibria with hydrogen ions and reaction with *p*-nitrophenyl acetate¹¹. *Figure 2* shows the results of titration of myoglobin with hydrochloric acid and potassium hydroxide. The ordinate shows the number of

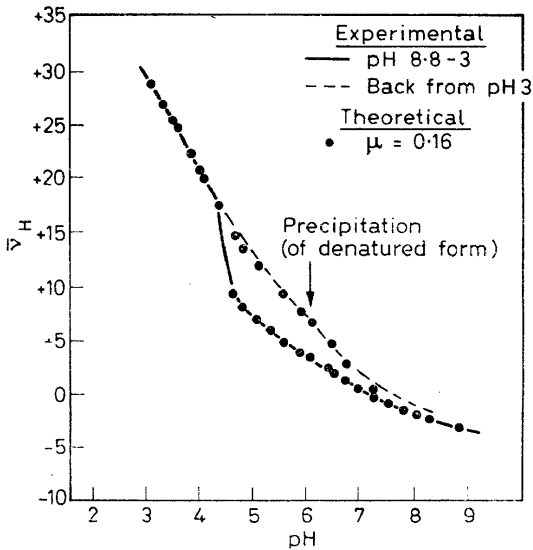


Figure 2. Experimental titration curves and computed points for myoglobin (By courtesy of *J. Biol. Chem.*¹¹)

moles of hydrogen ion bound per mole of protein referred to the isoionic condition near pH 7 as zero*. In acid solution below pH 4.5 the molecule

* See reference 11, footnote 4.

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appears to open up to accept hydrogen ions. Detailed analysis of the titration curve, including evaluation of heats of dissociation, indicates that the groups which become accessible abruptly below pH 4.5 are probably imidazole groups. On back titration, shown in the dashed curve, the total content of 12 imidazole groups is involved in the equilibrium. In the intact protein about 6 imidazole groups appear to be involved in the hydrogen ion equilibrium.

Similar conclusions may be drawn from measurements of the splitting of *p*-nitrophenyl acetate by myoglobin as a function of the average number of hydrogen ions bound, \bar{v}_H . In Figure 3(a) the ordinate shows the second-order rate constant, k_2 , plotted against \bar{v}_H under conditions described elsewhere¹¹. In Figure 3(b) the abscissa is shown transformed to represent $n_i - \bar{v}_{iH}$, which

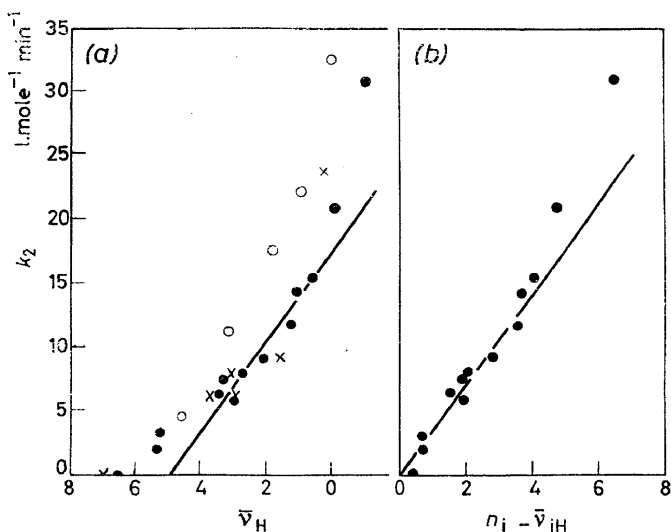


Figure 3. Dependence of k_2 on (a) \bar{v}_H , and (b) $n_i - \bar{v}_{iH}$; O: pH-stat method, $\mu = 0.06$; x: pH-stat method, $\mu = 0.16$; \circ : spectrophotometric method, $\mu = 0.09$ (By courtesy of *J. Biol. Chem.*¹¹)

in our nomenclature is equal to the average number of imidazole groups present in the basic, unprotonated form, assuming that a maximum of 6 imidazole groups enter into the equilibrium in the native protein. The linear relation holds over conditions corresponding to the range approximately from pH 5 to 7, above which the very reactive ϵ -amino groups begin to make a detectable contribution. By contrast, after disruption of the molecule by exposure to pH below 4.5, the rate of reaction with *p*-nitrophenyl acetate near pH 6 is increased, probably reflecting the release of previously unreactive imidazole groups. In summary, the results of equilibration with hydrogen ion and of reaction with *p*-nitrophenyl acetate and bromoacetate all indicate that 6 or 7 imidazole groups are far more reactive than 5 or 6 other such groups in the native molecule, and that essentially all

12 imidazole groups are reactive after disruption of the native structure.

Since beginning the hydrogen ion studies Dr Esther Breslow and the author have been making observations on the binding of cupric and zinc ions. These will be reported in detail elsewhere²² but can be summarized here. The principal conclusion from these observations is that the buried imidazole groups are probably involved in binding these metal ions. Such a conclusion suggests model peptide systems which should now be studied, a discussion of which will follow an outline of the evidence concerning the metal ion binding by myoglobin.

Measurement of the equilibria between myoglobin and cupric chloride by dialysis equilibrium or a precipitation method²³ showed that a small number of metal ions, 5 or 6 per mole of protein, were very strongly bound. The concentrations of unbound cupric ion were, therefore, held at sufficiently low levels to restrict binding to the first 6 or 7 ions per molecule, levels at which extensive binding to individual imidazole or amino groups would not be expected on the basis of association constants for appropriate model compounds. The reaction with *p*-nitrophenyl acetate was used to show that, for example, with an average of 4.3 cupric ions bound per mole there was no reduction in the catalytic activity of the protein at pH 6.40. This observation indicates that the combination with cupric ions did not affect the net number, or more precisely activity, of the freely reactive imidazole groups. Likewise, it was found that cupric ion binding to the guanidinated myoglobin derivative, in which all but one of the 19 lysyl residues were converted to homoarginyl residues, was essentially identical to that to the unmodified protein. This similarity of interaction extended from below pH 5 to pH 11. Thus the readily available amino groups, which appear to include almost the whole class²⁰, probably are not involved in the very tight binding of the first few cupric ions to combine.

The results in *Figure 4* show the sharp uptake of cupric ions, expressed in the upper ordinate as $\bar{\nu}$, the average number bound per mole of protein. The equilibrium pH values for the experiment beginning at pH 7.1 in the absence of metal ion are shown on the lower ordinate. It may be seen that the binding of the metal ions displaces the pH sharply to lower values. A similar but slightly less marked competition with hydrogen ions was found when zinc ions were substituted for cupric, and it was possible to show a high degree of competition between zinc and cupric ions when both were equilibrated with the protein at once.

The most direct evidence that cupric ions are taken up by those imidazole groups which are not in ready equilibrium with hydrogen ions in the native protein is obtained by titration of the protein in the presence of cupric chloride. The copper-protein complexes usually must be brought to a somewhat lower pH in order to take up a comparable quantity of hydrogen ions. In other words, in the presence of cupric ion the lower curve in *Figure 2* is displaced somewhat to the left. More significant is the fact that on back-titration with alkali the curve is almost exactly reversible without the hysteresis seen in *Figure 2*. These results are explained by assuming that the cupric ions compete preferentially for those sites which would normally take up hydrogen ions, or at least that some rearrangement brings about an equivalent stoichiometric behaviour.

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The stoichiometry of displacement of hydrogen ions accompanying the binding of cupric ion indicates that amide hydrogens of peptide bonds may be involved²⁴⁻²⁸. At pH 5 an average of slightly over one hydrogen ion is

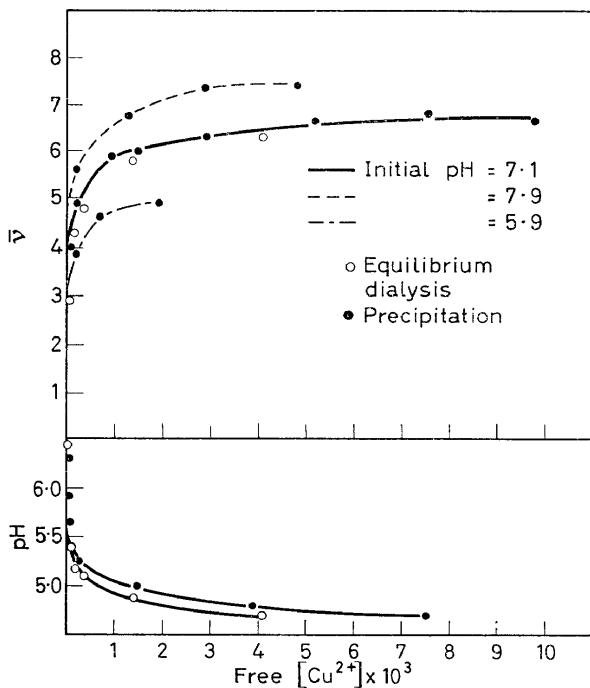


Figure 4. Binding of cupric ion by myoglobin

displaced per cupric ion bound, by pH 9 two are displaced and by pH 11 three are displaced. Since the experiments with guanidinated myoglobin gave similar results it is unlikely that amino groups of lysyl side chains contribute the displaced hydrogen ions. The behaviour is analogous to that shown in Figure 5 by 1:1 mixtures of Cu(II): glycyglycylglycine (curve 3) or Cu(II): glycyglycylglycylglycine (curve 4)²⁸.

The involvement of the cupric ions with normally unreactive imidazole groups and with neighbouring peptide bonds would be expected to disrupt the structure of the protein no less than the corresponding action of hydrogen ions. This effect is actually observed in a change in haem absorption spectrum, most marked at the Soret band near $410\text{ m}\mu$, which occurs when 2 or more cupric ions are bound. Solubility changes are also observed. Various observations which need not be described here suggest that the uptake of more than one cupric ion is a co-operative phenomenon, perhaps quite analogous to a most interesting all-or-none phenomenon observed a few years ago in the reversible interaction of phosphorylase with *p*-chloro-mercuribenzoate²⁹. The present interaction likewise is reversible: the technique that we favour for the isolation and purification of myoglobin involves steps in which zinc complexes are formed, isolated and dissociated¹⁰.

We hope shortly to be able to identify the histidyl residues in myoglobin, listing in one group the 6 or 7 that react readily with hydrogen ions, *p*-nitrophenyl acetate or bromoacetate, and in the other the 5 or 6 that accept hydrogen ions abruptly at low pH and seem to bind cupric or zinc ions preferentially. Since the amino-acid residues immediately preceding and following each histidyl residue in the polypeptide sequence are unique¹³, it

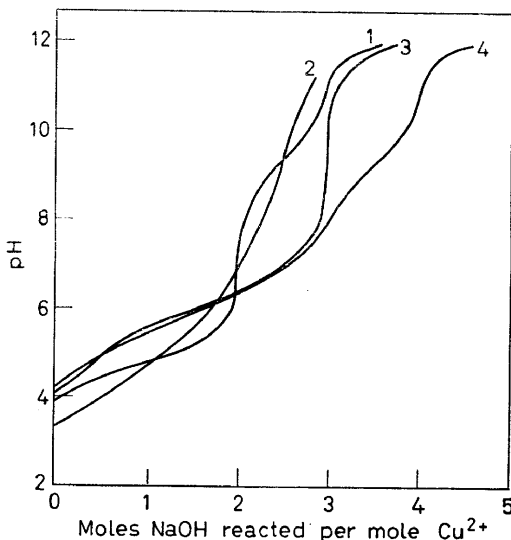


Figure 5. Titrations of 0.01M peptide and 0.01M CuCl_2 ; temperature 25° , $\mu = 0.16$; curve 1: glycylglycine; curve 2: glycylglycylsarcosine; curve 3: glycylglycylglycine; curve 4: glycylglycylglycylglycine (By courtesy of *J. Biol. Chem.*²⁸)

follows that a proper identification of the sequential neighbours of histidyl residues and carboxymethylhistidyl residues derived from bromoacetate-treated myoglobin will identify the members of each group positively. The most immediate application of such results would be to the interpretation of the structure summarized in *Figure 1*.

Two corollaries of interest for the interaction with metal ions concern us more here. One is that under suitable circumstances the sites of cupric ion binding might be more positively identified by making the bound cupric ions act to block the reaction with bromoacetate, just as cupric ion has been shown to block the reaction of *p*-nitrophenyl acetate with imidazole and amino groups^{18, 19}. The other is that one may study the peptide sequences involving histidyl residues in myoglobin to see whether those that appear to be involved in cupric ion binding are in any way distinctive. The express concern for the *sequence* of amino-acid residues rather than simply any neighbouring groups in space comes from the observation of the displacement of several hydrogen ions per bound cupric ion. The idea is based on analogy with tri- and tetrapeptides which have been studied extensively in solution^{24, 26, 28} and which have been worked out recently by X-ray diffraction studies of the crystalline state³⁰. In the protein, however, the imidazole groups of certain histidyl residues would be assumed to play the rôle of the

α -amino group in those parts of the chain far removed from the single real α -amino group. The amino-terminal region of the myoglobin molecule would qualify, of course, as one probable copper-binding locus^{2, 28, 31}.

The likelihood that a histidyl residue centrally placed in a peptide chain might enter into chelate formation with a cupric ion is difficult to assess at present. Our experience with carbobenzoxy-L-prolyl-L-histidylglycinamide³² and that of Martin and Edsall with acetylglycyl-L-histidine³³ pointed in each case to simple complex formation with cupric ion involving the imidazole group but not the amide nitrogen of the histidyl residue. On the other hand Ng and the author have found that cupric ion when it reacts with glycyl-L-histidine not only displaces a proton from a peptide bond, as was known before³³, but also essentially obliterates the ability of both the imidazole and the amino group to react with *p*-nitrophenyl acetate. The spectrum of the complex also is quite similar to that of the complex formed in equimolar proportions from cupric ion, glycylglycine and imidazole²⁷. These results indicate that a cupric ion combined with the imidazole group in a histidyl residue may at the same time combine with one true peptide nitrogen atom and with another nitrogen atom representing the location of a second peptide link up the sequence.

The fact that the imidazole groups in myoglobin seem to fall into at least two categories in their binding of cupric ion redirects attention to the nature of the amino-acid residues adjacent in the sequence to the histidyl residues. R. W. Roeske and the author are collaborating in the study of the properties of some model peptides related to histidine-containing sequences recognized by Edmundson and Hirs¹³. The sequence containing the histidyl residue directly bonded to the haem iron atom is of great interest in many ways. It has been shown to contain a seryl residue attached to the amino side of the histidyl residue. Spectral evidence indicates that this imidazole-iron bond is disrupted on treatment with acid below pH 4.5 or with cupric ion^{2, 11}. It is most likely that this histidyl residue is a special target for cupric ion, and we plan to study whether the adjacent seryl residue could be responsible for stabilizing the complex. One other serylhistidyl sequence has been identified in myoglobin. Very recently a histidylhistidyl sequence was also identified^{6, 34}, which should make a strong candidate for a copper-binding site in which a peptide nitrogen would probably take part. In a few other cases the residues adjacent to histidyl residues are not fully established; in others they do not contain appropriate ligand atoms in the side chains which might contribute to the stability of the complex.

Only by studying appropriate model peptides will it be possible to predict the behaviour of each individual potential metal-binding site in myoglobin. If a set of such sites can be predicted on the basis of model experiments to react with metal ions after a common pattern and with only minor variations in equilibrium constants then an attempt to describe appropriate average constants for the protein might be useful. The situation is clearly quite different from that which has been observed in the interaction of zinc ions with human serum albumin^{35, 36}. In that case a simple competition between zinc ions and hydrogen ions could be demonstrated, and it appeared that approximately 16 imidazole groups acted as a set of fairly homogeneous binding sites. The great simplicity of the zinc-albumin system may depend

on certain restrictions in the flexibility of the native structure, since at 37° a transition occurred which modified the interaction drastically³⁷.

The stress laid here on the importance of the nature of the amino-acid residues in a given length of peptide sequence should not draw attention away from the potential importance of clusters of ligand atoms in forming metal-binding sites. Just as the heme in myoglobin is constrained in an elaborate way involving interactions from all sides so we may expect that the most stably bound metal ions in metalloproteins will be held by clusters of side-chain groups. For example, phenoxyl groups of tyrosyl residues have been suggested as iron-binding ligands by Warner³⁸. It may be significant that one phenoxyl group in myoglobin appears to play a very important structural rôle both in the crystalline state⁶ and in solution³⁹. Some very remarkable interactions have also been attributed to sulphur-containing ligands^{40, 41}. The interpretation in general terms of the interaction of metal ions with elaborate three-dimensional clusters of ligands, nevertheless, can scarcely fail to be strengthened by a better knowledge of simpler metal-peptide interactions.

Any protein chemist will assure you that the protein with which he is best acquainted is unique and should not be looked upon as a typical protein. I should like to stress that myoglobin is a truly typical protein in that its structure and reactivity are highly specialized and characteristic only of it and possibly of a few related proteins⁴². Presumably it will be possible to show small but clear differences of this sort even between sperm whale myoglobin and the various myoglobins which have had such careful study in Professor Theorell's laboratory and elsewhere⁴³⁻⁴⁵. Quite different structures may prove to be responsible for the stability and function of metalloenzymes, for which the work of Malmstrom and Vallee should also be consulted^{46, 47}.

In studying every metal-protein system, however, the greatest care should be taken to correlate the measurements of metal interaction with other measures of reactivity such as the kinetic techniques which have been described here. The use of more than one metal ion may be helpful. For example, in the case of myoglobin the greater tendency of cupric ion than of zinc ion to displace hydrogen ions, for a given number of either metal ion bound, accords with the known differences between these metal ions in their interaction with the peptide bond^{19, 33}. In general, the presence or absence of competition between metal ions may be informative^{23, 48}. Techniques to detect association or dissociation of protein molecules, such as ultracentrifugation, and viscosity measurements to detect shape changes or swelling should be employed. Obviously, measurement of enzymic activity when present will offer a major tool. The close study of appropriate model peptides remains fundamental to the understanding of metal-protein interactions, and it is to be hoped that the continued development of knowledge of the crystal structure of metal-peptide complexes will parallel the growing knowledge of protein structure.

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