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MODELS FOR COPPER–PROTEIN INTERACTION BASED ON SOLUTION AND CRYSTAL STRUCTURE STUDIES

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A. INTRODUCTION

The biological action of copper usually takes place in association with certain specific proteins, referred to as copper proteins. These proteins generally behave as typical metalloproteins, and copper constitutes an integral part of their molecules. Extensive studies have been carried out on many copper proteins, but, as yet, there are no final data on the structures of the copper binding sites; see, for example, the reviews refs. 1–4. An important

approach to obtain more detailed information regarding the structures and reactions of the binding sites is to design and perform systematic model studies both in solution and in the crystalline state. The most recent data on such model studies in this field will be the main subject of this discussion.

(i) The function of copper proteins

Copper proteins are involved in many metabolic functions such as electron transfer reactions, the transport of oxygen molecules, and the transport and storage of copper ions. Table 1 lists some copper proteins with these functions and their copper contents.

The electron transfer reactions of copper proteins are greatly favoured by the low oxidation potential of copper(I); in fact, in its reactions with oxygen, the Cu^{I}–Cu^{II} couple is more favourable than that of any other "stable" redox couple of the transition series[6]. For instance, in the aqueous solutions of many low-molecular-weight copper(I) compounds, univalent copper is almost instantaneously oxidized into copper(II) by molecular oxygen[5]. Thus, it is not surprising that the last step in mitochondrial respiration, where oxygen is converted into water, is catalyzed by a copper protein, cytochrome oxidase[1]. In the case of iron(II), oxidation by oxygen is much less favoured; and in a slightly acid solution, say an aqueous solution of $FeCl_2$, very little or no oxidation occurs from the Fe^{II} to the Fe^{III} state[6]. Such a slow oxidation of iron(II) also occurs within the haemoglobins of mammals, who utilize haemoglobin for oxygen transport.

In the blood of certain invertebrates, on the other hand, the normal transporter of oxygen is a copper protein, haemocyanin[2,7] (Table 1). Each subunit of haemocyanin contains two copper atoms and the subunit combines with one single oxygen molecule. Haemocyanin combines and rapidly reaches its full capacity with oxygen molecules in water, even though the oxygen tension in water is relatively low. Then in the tissues the oxygen is almost compietely released. As a result, the efficiency of haemocyanin for the transport and utilization of oxygen is high[2].

The blood of mammals does not contain the same copper protein, haemocyanin, which combines and transports oxygen molecules in invertebrates. Instead, the main copper protein in the blood plasma of mammals is ceruloplasmin. Although it has been suggested that this protein is important for the storage of copper[1] and that it is responsible for the oxidation of ferrous ions[8], the true function of ceruloplasmin remains unknown. Of great importance for the transport of copper is the copper fraction bound to albumin[9], the most abundant protein in blood plasma. Whenever copper ions are absorbed from the intestine they are eventually bound to serum albumin; and at physiological concentrations, the 1 : 1 copper–albumin complex predominates[10] (cf. refs. 3, 11). In vivo the exchange rate of the Cu^{II} ions bound to albumin is relatively fast; this is important in order to facilitate the release of copper necessary for the biosynthesis of copper proteins[9].

Another important copper protein present in mammalian blood is the superoxide dismutase of the erythrocytes. This superoxide dismutase, which catalyzes the dismuta-

TABLE 1

The functions of copper proteins and their copper contents (cf. ref. 4)

Function	Example of protein	Copper content (g-atoms/mole)		
		Total	"Blue"	"EPR-nondetectable"
Electron transfer reactions	Azurin	1	1	
	Stellacyanin	1	1	
	Plastocyanin	1	1	
	Laccase	4[c]	1	2
	Ascorbate oxidase	8[c]	2	4
	Cytochrome *c* oxidase	2	[e]	1
	Tyrosinase	1		1
Transport of oxygen	Haemocyanin	2[d]		2
	Ceruloplasmin[a]	8[c]	2	4
	Superoxide dismutase[b]	2[c]		

[a] Weak oxidase activity, but unknown physiological function.
[b] Scavenger for superoxide anions but precise physiological role unknown.
[c] Includes an unlisted balance of "non-blue" Cu^{2+} copper.
[d] Per subunit.
[e] 50% "blue" and 50% "non-blue" Cu^{2+} copper.

tion of superoxide anion radicals to peroxide and oxygen[12], appears to be the copper protein most widely distributed among different cells. The dismutase activity has been discovered only recently[12]; and in the past a variety of names, such as erythrocuprein, cerebrocuprein and hepatocuprein, have been proposed for this copper protein depending upon the source from which it was prepared. This copper protein, superoxide dismutase, contains two copper and two zinc ions[13], and it is the copper ions that appear to be involved in the enzymatic function. EPR measurements indicate that the copper and zinc sites are in fact close to each other[14]. Just recently, the crystal structure of the bovine erythrocyte dismutase has been determined[15] to 5.5 Å.

Very little copper is required for the normal synthesis of copper proteins, and therefore any excess has to be eliminated before it causes toxic effects. For man, Wilson's disease, a congenital disorder with a familial incidence, seems to be the result of an abnormal copper metabolism due to the impairment of the normal excretion of copper through the bile and, as a result, copper starts to accumulate in the tissues (cf. Walshe in ref. 1). For many organs this leads to toxic effects, which in the case of the brain, liver and kidneys leads to progressive degeneration and characteristic deficiencies in function.

(ii) The state of copper in copper proteins

Malkin and Malmström have recently reviewed the state and function of copper in copper proteins[4]. They classify the protein copper into three main forms: two paramag-

netic forms and one EPR-nondetectable form*. One paramagnetic form of copper is present in those copper proteins that have an intense blue colour in the oxidized state. Many of these blue proteins contain several copper atoms per molecule, although only one single copper ion is necessary in order to produce the intense blue colour (Table 1). The other form of paramagnetic copper, called "non-blue" copper, yields optical density and EPR parameters that are not unusual; they are comparable to those found in many low-molecular-weight Cu^{2+} complexes[4].

Apart from the two paramagnetic forms there is also a kind of EPR-nondetectable copper present in copper proteins. This may indicate that univalent copper is involved. However, recent data, partly based on anaerobic redox titrations, seem to indicate that, at least in some blue oxidases, pairs of bivalent copper, Cu^{2+}–Cu^{2+}, rather than copper(I) ions are involved; the two single d^9 electrons are supposed paired, eliminating the EPR signal[4].

Thus extensive spectroscopic data, obtained mainly through the use of EPR, have furnished considerable amounts of detailed information regarding the nature of the copper involved in copper proteins. Also, using a pure copper isotope and combining EPR and magnetic susceptibility measurements, there are indications that, at least in a few copper proteins, nitrogen ligand atoms are involved[16, 17]. But, so far, in the absence of high-resolution X-ray crystallographic data, it has not been possible to draw any final conclusions regarding which specific kinds of ligand atom bind the copper in copper metalloproteins.

(iii) The importance of low-molecular-weight model complexes

It is generally believed that an enzyme provides not only the particular structure that constitutes the active site but that it also provides the environment necessary for catalysis. Thus within an aqueous solution, it seems that only a large molecule can supply the hydrophobic interior necessary for catalytic action, apart from the general requirements of a hydrophilic exterior contributing to the solubility. Because of this it may be assumed that the active site is not situated on the outer surface of the protein molecule. Also, it is unlikely that a small molecule, with a structure similar to that of the active site, would have any pronounced enzymic properties in dilute aqueous solution.

Even though a small molecule may have a low catalytic activity, it is in the study of the low-molecular-weight model system that it is possible to obtain highly accurate data rather than in the study of the protein itself. The interpretation of the data obtained

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* The main abbreviations used are: EPR, electron paramagnetic resonance; Gly, glycyl residue; His, histidyl residue; HMet, methionine in the form of a zwitterion; h, molar concentration of free H^+; β_n, cumulative equilibrium constant for the general reaction $Cu^{z+} + n\ A^{y-} \rightleftharpoons CuA_n^{(z-ny)+}$ ($\beta_0 = 1$); β_{1-n1}, equilibrium constant for the general reaction (cf. Section C), $Cu^{2+} + A^- \rightleftharpoons CuH_{-n}A + n\ H^+$; β_{1011}, equilibrium constant for the mixed amino acid complex (cf. Section C), $Cu^+ + A^- + B^- \rightleftharpoons Cu(A)B$; $Cu_pH_qA_r$, general formula for the complex formed by a reaction between copper ions and a polyprotic acid, H_QA; negative q means proton deficit obtained by displacement of protons from the ligand (for peptides, protons displaced from the peptide bonds).

from a small molecule is less ambiguous than those obtained from a large protein. This is especially true for metal complexes since, due to the presence of the metal ion, very accurate data can be recorded. Thus, a detailed investigation of a low-molecular-weight complex may yield important information regarding the geometry of the metal binding site for such metal ion–protein complexes, where the binding site is situated on the outer hydrophilic surface of the protein molecule. On the other hand, when the metal ion is bound very specifically to a site having a hydrophobic environment, then it might be possible, at least in a few cases, to describe the geometry via a solid model compound, or via a complex formed in a solvent of less polar properties than those of water. It appears, then, as if information regarding the geometry of the coordination structure in a copper protein can be obtained, as a first approximation, through studies on low-molecular-weight models both in solution and in the crystalline state. These models are structural models, and they quite generally indicate how proteins interact with copper ions; they should not be considered as dynamic models supposed to have an "enzymatic" function in an aqueous solution.

Many reviews have been devoted to copper proteins and the biological role of copper; see for instance refs. 1–4. There are also a few reviews dealing with certain aspects of low-molecular-weight "models" for copper proteins[1,2,18], including two extensive ones on the crystal structures of metal–peptide complexes[1,18] which survey the literature up to 1967. The need for additional accurate data on model systems involving the geometry of copper binding sites in proteins is still of great importance since, as yet, the complete nature of any single ligand atom that binds copper in a copper protein is not known.

The main emphasis within this article will be devoted to those model complexes that have been found to exist both in solution and in the crystalline state through the use of thermodynamic and X-ray diffraction methods. In addition, the copper(I)–copper(II) redox reactions will be discussed in relation to model studies on both copper(I) and copper(II) systems. Regarding spectroscopic properties, this discussion will be limited to only a few characteristic aspects of some copper proteins; spectroscopic data on low-molecular-weight copper complexes, some of them closely related to copper–protein interactions, have been previously reviewed[1–4,19,20]. Finally, the discussion will be confined to within a certain number of complexes assumed to have certain specific biological implications.

B. SOME ASPECTS OF THE COORDINATION CHEMISTRY OF COPPER

A classification of metal ions somewhat connected with electronegativity has been proposed by Ahrland et al.[21]. Metal ions termed type (a), or *hard*, form chemical bonds which are electrovalent; i.e. their coordination is governed mainly by electrostatic interaction existing between charges of opposite signs. In general, the higher the charge, and the smaller the radius of the metal ion, the stronger the complexes formed. Metal ions termed type (b), or *soft*, on the other hand, form chemical bonds of a covalent character.

The stability of their complexes does not increase regularly with charge and decreasing radius. Also, (b) -acceptors prefer large donor atoms rather than smaller ones[22].

An intermediate character, according to the above classification, prevails among those metal ions of the first transition series that have a large number of *d* electrons; for instance, the position of copper(II) in the order of increasing (b) character is as follows: $Zn^{II} < Co^{III} < Ni^{II} < Cu^{II}$. On the other hand, copper(I) is a true (b)-acceptor, a d^{10} system with a low oxidation number. According to these rules we find that the complexes of copper(I) follow the stability order[23]

$$RS^- > RNH_2 > OH^- \gg H_2O$$

Copper(II), which behaves as an intermediate ion, does not strictly follow the (a)-type affinity sequence

$$RCOO^- > RNH_2 > RS^- > H_2O$$

since it generally forms stabler complexes with nitrogen than with oxygen donor atoms.

In view of the above stability orders, it is not surprising that coordination through nitrogen and oxygen donors is important for copper(II) and that copper(I) forms very stable complexes with thiols and quite stable complexes with ammines. The copper(I) complexes of nitrogen ligands are generally more stable than the corresponding copper(II) complexes; the biligand Cu^I complex of ammonia is three orders of magnitude more stable than that of the harder Cu^{II} ion[24]. In addition, an increase in the (b) character in the series $Zn^{II} < Co^{III} < Ni^{II} < Cu^{II}$ has its counterpart in a tendency to form complexes with the amide nitrogen of the peptide bond. Thus, apart from Cu^{2+}, it is only Co^{3+} and Ni^{2+} of these intermediate ions that have the power to release a proton from the peptide bond; see for example ref. 25, and for a review see ref. 18.

The low affinity of the soft metal ions Cu^I, Ag^I, and Hg^{II} for water molecules may explain why they tend to accumulate in those tissues rich in lipids. Likewise, it is to be expected that the binding sites in copper proteins favouring univalent copper are sites having hydrophobic environments. This is based on the following potentials (versus the standard calomel electrode)[6]

$$Cu^{2+} + e^- \rightleftharpoons Cu^+ \qquad E = -89 \text{ mV} \tag{1}$$

$$Cu^+ + e^- \rightleftharpoons Cu \qquad E = +297 \text{ mV} \tag{2}$$

which indicate that the hydrated copper(I) ions are thermodynamically unstable in an aqueous medium (cf. ref. 26); copper(I) ions disproportionate into Cu^{II} ions and metallic copper. The equilibrium constant for the disproportionation reaction

$$2\,Cu^+ \rightleftharpoons Cu^{2+} + Cu^0(s) \tag{3}$$

has the value[27] of $1.6 \times 10^6\ M^{-1}$.

It is important to keep in mind, particularly as far as biological reactions are concerned, that this instability of the cuprous ion in aqueous solution is partly due to the compara-

TABLE 2
Equilibrium constants, K, for the reaction $2\,Cu^{+} \rightleftharpoons Cu^{2+} + Cu^{0}(s)$ in different solvents

Solvent	Anion	Temp. (°C)	log K
Water[126, 127]	Sulphate	25	6.2
	Perchlorate[27]	25	6.0
Methanol[28]	Perchlorate	25	4.55
Ethanol[28]	Perchlorate	25	1.55

tively strong solvation of cupric ions by water. Thus, the stability of Cu^{I} ions in solution can be considerably increased if solvents other than water, which do not enhance the solvation of cupric ions, are considered[28]. This is illustrated by Table 2, which shows that the equilibrium constant of reaction (3) decreases in the following order: water > methanol > ethanol.

(i) The nature of the ligands

Using the (a) and (b) type (or hard and soft) classification of metal ions, one observes that a metal ion with an intermediate character is best suited for interaction with proteins. This is particularly due to the fact that its main ligand atoms are oxygen and nitrogen. Of the oxygen ligands the carboxylate groups are the most important and of the nitrogen ligands the imidazole groups are the most important. For the coordination of Cu^{II} to the amide nitrogen of the peptide bond a chelate appears to be required, formed via an α-amino or an imidazole group[18]. The ϵ-amino group has a relatively high pK value, and therefore it apparently interacts in aqueous solution only with a strong complex-forming ion, such as the (b)-type Cu^{I} ion[24, 29, 30].

Complex formation via sulphur ligands may not be limited to the Cu^{I} ion. The considerably harder copper(II) ions may undergo redox reactions with the thiol groups of the proteins, as they do with the low-molecular-weight compounds, forming –S–S– bridges and copper(I) ions[31]. Whether or not copper(II) interacts with a single thiol group present in a protein is not known.

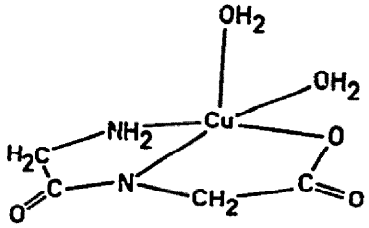
Apart from the metal ion affinity of the ligand atoms mentioned, most protein molecules contain one or a few special binding sites for metal ions, where stereochemical factors are also important. For instance, in albumin there appears to be a single specific copper(II) binding site, cf. refs. 3, 11; and in myoglobin there are two specific sites, one for a single Cu^{II} ion and one for a single Zn^{II} ion, even though these sites are fairly close to each other[32].

(ii) The coordination of the copper ions

When copper(II) is coordinated to oxygen and nitrogen ligand atoms, the geometry about the copper atom tends almost exclusively to be a distorted octahedron produced

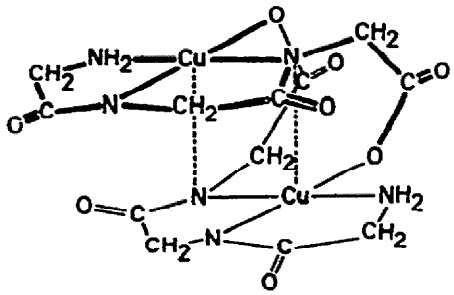
by four short and two long bonds. One or both of the long bonds may be removed completely, or be outside the distance considered as a bond, barely forming a van der Waals contact. The resulting square planar and square pyramidal geometry may be considered as a limiting case of a distorted octahedron. An empirical correlation between the stereochemistry of the copper(II) complexes and the ligand field effects produced by the four closest donor atoms has been noted by Freeman[18], and a discussion on this particular stereochemistry of cupric compounds has been published by Dunitz and Orgel[33].

Square pyramidal copper(II) coordination exists in the Cu^{II} structures of glycylglycine[34], I, and glycylglycylglycine[35], II,



I

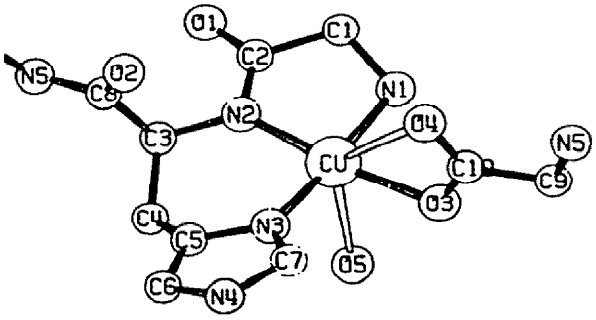
$Cu^{II}(GlyGly) \cdot 3H_2O$



II

$Na\ Cu^{II}(GlyGlyGly)H_2O$

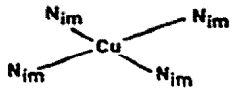
which is of particular interest with regard to models for copper–protein interaction. In these structures, I and II, the copper(II) ion is slightly lifted above the square plane in the direction of the fifth ligand atom. A six-coordinated copper(II) ion, usually in a tetragonal distorted octahedral geometry, is quite common among model structures for copper–protein interactions, for instance in the Cu^{II} structures of glycylhistidine[36] and glycylhistidylglycine[37], (III).



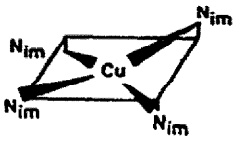
III

$Cu^{II}(Gly\text{-}L\text{-}HisGly)NaClO_4 \cdot H_2O$

Square planar geometry has been reported in a few cases, such as in the bis-imidazolato-copper(II)[38], IVa, and the dipotassium bis-biuretocuprate tetrahydrate[39] structures.



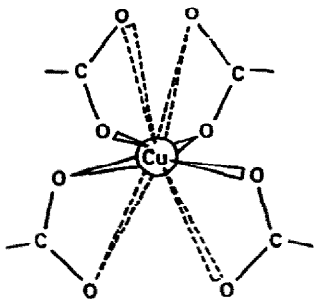
IVa



IVb

Bis-imidazolatocopper(II)

A coordination number of eight has been reported for two copper(II) structures, having the $CuO_4O'_4$ chromophore, with four short and four long bonds forming a distorted dodecahedron[30, 40]. One of these structures, tetra-(6-aminohexanoic acid)copper diperchlorate[30], having this extraordinary coordination, is important as a model for copper–protein interaction, V.



V

Cu^{II}(6-aminohexanoic acid)$_4 \cdot (ClO_4)_2$

Regular tetrahedral symmetry about copper(II) does not appear to have been reported to occur among low-molecular-weight models for copper proteins, but a flattened tetrahedral environment about one kind of copper exists in the bis-imidazolatocopper(II) structure, IVb, where four nitrogen atoms are the ligands[38] (cf. ref. 18).

Other kinds of Cu^{II} coordination may form during non-aqueous conditions. For instance, in pyridine, copper(II) and chloride form structural Cu_4Cl_6O units, where each copper has a trigonal bipyramidal coordination[41]. A trigonal arrangement is produced by three chloride ions about the copper, and an oxygen atom and a solvent molecule (pyridine) complete the bipyramid[41].

It follows from this discussion that the stereochemistry of Cu^{II} complexes varies considerably, and for copper–protein interactions an important implication seems to be that the ligand environment determines the coordination sphere about the copper to a much greater extent than the copper ion itself.

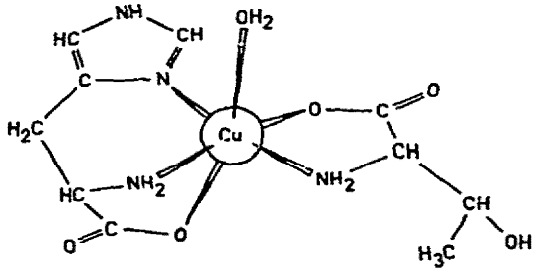
The stereochemistry of Cu^{I} complexes is usually quite different from that of Cu^{II} complexes[42]. The complete d^{10} electronic structure leads to a higher symmetrical en-

vironment for the central metal ion, and regular tetrahedral, trigonal, and linear symmetry have been reported. However, only a limited number of Cu^{I} structures are known, apparently due to their limited stability in the presence of oxygen. The few cases reported contain, in general, ligands such as acetonitrile, halides, or sulphur-containing ligands; they strongly favour the univalent oxidation state, and only in the sulphur case might there be any particular interest for copper(I)–protein interaction (see Section E). There are no complete structural data available on the $Cu^{(I)}$ interaction with such ligand atoms as ammine, amide and imidazole nitrogen atoms.

C. THE BIOLOGICAL SPECIFICITY OF COPPER IONS

Very little is known in detail regarding the pathways through which copper ions are transported in vivo in order to combine with the apoproteins within the cells and form the copper proteins. For mammals, one intermediary step involves plasma albumin, which transports labile copper in blood[9]. It has been suggested that copper is transferred from albumin to the apoproteins via the copper(II) complexes of amino acids[43–46], another labile copper(II) fraction present in blood plasma[43] (Sarkar and Kruck[1]). As a part of this mechanism there may be a ternary complex formed by albumin, copper(II) and an amino acid[43, 45, 46].

Amino acids are present both in blood plasma and most cells at a low but defined concentration; the total amount is about 2–30 m*M* (Table 3). This concentration is more than a thousand times higher than the quite low concentration of labile copper, which is approximately 1 μM in blood plasma. In these concentration ranges, and at a neutral pH, mixed amino acid complexes of the type Cu(A)B predominate rather than the 1 : 1 and 1 : 2 species of one single amino acid[47]; the most stable mixed complexes are formed by histidine (HA). One example is that of threonine, for which the structure has been determined[48], (VI).



VI

Cu^{II}(L-histidine)(L-threonine) · H_2O

This complex was first isolated and crystallized from human blood plasma (Sarkar and Kruck[1]); the stability constant[47] is $\beta_{101} = 10^{17.6}$.

The concentration of copper in blood plasma and the tissues is very low compared to the other complex forming metal ions (Table 3). This raises the question of how the

TABLE 3
Total concentrations of amino acids and of the main metal ions forming labile complexes in human tissues

Ion	Blood plasma (μM)	Liver[a] (μmole. kg^{-1})
Calcium	2,500	1,000
Magnesium	1,000	20,000
Zinc	20	1,000
Copper	1[b]	100
Amino acids[128]	2,500[c]	30,000[d]
Glutathione	0	3,000

[a] Listed data only correspond to the right order of magnitude.
[b] Labile copper.
[c] Histidine, 80; threonine, 130; cystine, 100 μM.
[d] Histidine, 1000; threonine, 10000; cysteine/cystine, 1600 μmole. kg^{-1}

copper ions compete with other metal ions for a certain binding site. One answer involves the very strong copper complexes of amino acids, which are much stronger than those of other metal ions[24]. Thus, protein binding sites which cannot compete with the amino acids for the copper(II) ions may instead react with the other metal ions. Therefore, it is suggested that it is the amino acids that are important for the regulation of the copper ion specificity. As an example, we may estimate the ratio between the concentration of a supposed protein complex, CuP, and that of the mixed histidine–amino acid complex.

$$[\mathrm{CuP}]/[\mathrm{Cu(A)B}] = [\mathrm{P}]\beta_{\mathrm{CuP}}(h^{-2}\,[\mathrm{HA}]\,[\mathrm{HB}]\beta_{1011}K_{\mathrm{HA}}K_{\mathrm{HB}})^{-1} \qquad (4)$$

It is assumed that several amino acids (HB) can form mixed copper complexes with histidine (HA) that are almost as stable as that formed by threonine. Also, the concentration of these amino acids in the form of their zwitterions, here denoted as [HB] and [HA], are approximated as being equal to the total concentrations of the amino acids. To begin with, we select three binding sites which do exist in metalloproteins and which consist of the following ligands: (1) three carboxylate groups (staphylococcal nuclease[49]), (2) three imidazole groups (cf. carbonic anhydrase[50]), and (3) two imidazole and one carboxylate group (cf. carboxypeptidase[51] and thermolysine[52]). As a first approximation, we estimate the stability constants for these sites from the data on the corresponding low-molecular-weight systems[24]: (1) $3\log\beta_1 = 5.1$, (2) $\log\beta_3 = 10.7$, and (3) $\log\beta_{2+1} = 9.6$. Then we introduce into eqn. (4) a value of 0.1 mM for the concentration of each protein site (P) and 0.1 mM for that of histidine (HA), 2 mM for those of the other amino acids (HB), and then the pK values ($pK_{\mathrm{HA}} = 8.9$ and $pK_{\mathrm{HB}} = 9.2$), and finally a pH of 7.5. This yields the following results: (1) $10^{-6.7}$, (2) $10^{-1.1}$, and (3) $10^{-2.2}$. It appears that from these data that none of these sites really form such a strong Cu^{II} complex as that of the mixed histidine complex.

On the other hand, with the application of these same calculations for zinc(II) ions, using the data listed in *Stability Constants*[24], we arrive at quite different results:

(1) $10^{-2.3}$, (2) $10^{1.9}$, and (3) $10^{1.0}$. In addition, considering the large difference in total concentrations between Zn^{II} and Cu^{II} (Table 3), these data indicate that, as far as sites number two and three are concerned, there should be more than 10,000 zinc ions bound per Cu^{II} ion. Thus, it is demonstrated that these sites really are specific for zinc ions, in spite of the fact that the copper(II) complexes have the larger stability constants. It should be noted that the true zinc(II) stability constants determined for carboxypeptidase and carbonic anhydrase are larger than those predicted here from low-molecular-weight complexes[3]. Thus, the zinc complexes will be even more predominant.

Neither Cu^{II} nor Zn^{II} ions, however, seem to interact with site number one, a pure carboxylate site. Instead this site may form complexes with iron(III), calcium(II) or magnesium(II) ions. For the latter two ions we estimate[24], assuming the 1:1 amino acid complexes and using an equation similar to eqn. (4), the values of $10^{0.6}$ and $10^{-1.5}$, respectively. Therefore, for calcium(II), site number one is more stable than the amino acid complexes. It is interesting to note that three calcium metalloproteins for which the structures are known, do bind the calcium ions via carboxylate groups[49,52,53].

Another example of protein binding sites for copper ions which seem to be regulated via the amino acid complexes are those sites which involve chelates formed by the terminal amino group and neighbouring peptide bond nitrogens. Among the metal ions listed in Table 3 only the copper ions have the capacity to react with such a site[18]. The effect of a histidyl residue within an N-terminal site is illustrated by three examples. We assume that these N-terminal sites contain the following amino acid residues: (4) Gly–Gly–Gly–Gly– . . . , (5) Gly–His–Gly– . . . , and (6) Asp–Ala–His– . . . . For the fourth and fifth site we estimate the value of the stability constants, $\beta_{1,-n1}$, from those determined on the triglycine[54], tetraglycine[24], and the glycylhistidylglycine[55] systems. For the sixth site, which is the same as albumin, we use the value determined for the human albumin complex[10,45] (n is the number of protons released by Cu^{II} from the peptide bonds): (4) $\log \beta_{1,-21} = -6.9$, (5) $\log \beta_{1,-11} = 6.5$, and (6) $\log \beta_{1,-21} = 3.0$. Then, using a pK value of 7.8 for the N-terminal amino group, we determine the ratio between the concentrations of the "protein-bound" copper and that bound to amino acids from the following equation.

$$[CuH_{-n}P]/[Cu(A)B] = [HP]\, h^{-n-1}\, \beta_{1-n1} K_{HP} (h^{-2}\, [HA]\, [HB]\, \beta_{1011} K_{HA} K_{HB})^{-1} \quad (5)$$

By using the same data as those of sites 1–3, the results are (4) $10^{-4.0}$, (5) $10^{1.9}$ and (6) $10^{5.9}$. It appears from these data that the N-terminal part of a peptide chain becomes a ligand for copper(II) only when it contains a histidine residue. The value of 0.0001, obtained for site number four, indicates that at physiological copper concentrations the amino acids *protect* most proteins from reacting with copper ions via their amino terminal ends. In our example, the sixth site does correspond to that found in human albumin; and the results, without a doubt, confirm that albumin contains the main fraction of labile copper in blood plasma[9].

The final results illustrate that during aerobic equilibrium conditions the low, but defined, concentration of amino acids seems to have an important function in regulating the specificity of the reactions for copper ions. Due to the strong complexes formed by amino acids, especially the mixed histidine complexes, the only Cu^{II} complexes able to form are those that are extremely stable. It should be kept in mind, however, that a protein site might have a stability constant that is much greater than that estimated from an aqueous model system. Also, the copper ions might be required to stabilize the conformation of the protein molecule, and then the situation may become much more complicated. But, at any rate, the relationship between the different ions is in principle correct; therefore, for most of these "weak" Cu^{II} sites, such as sites number two and three, copper(II) ions cannot be bound as long as zinc(II) ions are present.

In this discussion conditions similar to those occurring under aerobic equilibrium conditions have been assumed. This is approximately correct as far as blood plasma is concerned, but very little is known about the true conditions in the tissues. Under these aerobic equilibrium conditions very few or no free thiol groups can exist. A quite different situation may occur under anaerobic conditions. Then, there should be a defined concentration of thiol compounds which do form very stable copper(I) complexes[24] ($\beta_1 = 10^{19.2}$). It seems possible that glutathione or cysteine may play the same role as that of histidine for copper(II) although, as yet, this has not been experimentally verified. These copper(I) complexes may also form under aerobic non-equilibrium conditions after reduction of copper(II). This last condition seems to prevail within many cells, since it is here that cysteine predominates over cystine due to the presence of the glutathione reductase system[56]. However, oxidation of Cu^{I}–thiol compounds by molecular oxygen, with simultaneous formation of disulphides, is a relatively fast process and therefore the mixed Cu^{II}–histidine complex should still be the main low-molecular-weight copper complex[10] (cf. ref. 44). As a result, within aerobic cells, the formation of such a Cu^{I}–thiol complex is possible only as a quite short-lived step in the transfer of copper to the apoprotein. Nevertheless, such a copper(I) system makes it possible to understand the relatively fast transportation of copper ions from blood plasma into the cells[9,43,44] and furthermore, that some apoproteins react with copper(I) rather than copper(II) to form the mature copper proteins[57].

D. COPPER ION INTERACTION WITH NITROGEN AND OXYGEN DONOR ATOMS

Of the nitrogen donor atoms present in proteins those of the imidazole, α-amino, ε-amino, and peptide amide groups are the most important. The most important oxygen donor atoms are those of the carboxylate groups. It would then seem as if the α-amino acids, having both an α-amino group, a carboxylate group, and in a few cases also an additional binding group, would be suitable model compounds for studying copper–protein interaction; however, this is not so. The α-amino acids preferably form very stable five-membered chelate rings via the α-amino and carboxylate groups, involving ligand atoms which in a protein are present in the form of peptide bonds. Side-chains, which apparently

are the most important ligands in a protein, are very often not involved at all in copper(II) complexes of α-amino acids. This is well illustrated by the crystal structure of bis-(histidine)copper(II) dinitrate dihydrate, where the imidazole side-chain remains uncoordinated[58]. In a mixed chelate involving both threonine and histidine, however, copper(II) is bound not only to the α-amino group but also to the imidazole group[48], VI. As discussed in the previous section, this and similar complexes certainly have important biological functions in regulating the formation of highly specific copper–protein complexes, but they are not accurate models for copper–protein interaction.

(i) Copper(II) complexes of imidazole

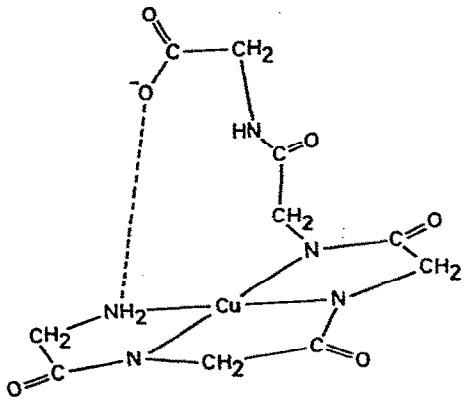
In the metalloproteins that have been analyzed by X-ray crystallography, it has been found that their metal ions are bound via side-chains of the proteins. For instance, in the zinc metalloproteins (carboxypeptidase[51], carbonic anhydrase[50], and thermolysine[52]) the zinc ions are bound via both imidazole and carboxylate groups. As far as the copper complexes of proteins are concerned, only one complex of myoglobin has been subjected to X-ray diffraction analysis[32]. In this complex an imidazole group binds just one single copper(II) ion on the protein surface. Additional binding of cupric ions leads to denaturation of the myoglobin molecule.

The zinc–imidazole systems, used as models for zinc–protein interaction, have furnished quite detailed information regarding tetrahedral[59] and octahedral zinc coordination[60]. The tetrahedral coordination has been found to exist in carbonic anhydrase[50], carboxypeptidase[51] and thermolysine[52]; the octahedral coordination has been found in insulin[61]. The copper(II)–imidazole structures, on the other hand, are different from those of zinc(II); they do not contain any discrete complexes. The copper(II)–imidazole systems usually form infinite chains in the crystalline state, where bridges are formed between the copper(II) ions[38,62]. One general rule appears to be that the four closest ligand atoms are nitrogen atoms from four different imidazole molecules, and that the anions, if present, occupy the fifth and sixth positions of a distorted octahedron[62].

In the bis-imidazolatocopper(II) structure[38], the geometry about one type of copper atom is nearly a square plane formed by the nitrogen atoms of four imidazole molecules (IVa). All of the imidazole molecules form bridges to the neighbouring copper atom, which coordinates the second imidazole nitrogen atom forming an infinite network. The coordination about the other type of copper in this structure[38] is considerably distorted from a square planar towards a tetrahedral symmetry (IVb). One interesting fact, as far as biological implications are concerned, is that, of the Cu^{II}–imidazole structures investigated so far[38,62], the lengths of the copper–nitrogen bonds vary considerably from 1.95 to 2.06 Å. As a result, a relatively wide range of changes in bond length can occur in a protein cavity. Also, the direction of the coordination varies among these structures[38,62]. Thus copper(II)–imidazole coordination is possible in a protein cavity, even though the stereochemistry does not permit all bonded nitrogen atoms to form a regular square plane.

(ii) Copper(II) complexes of the α-amino terminal end

The α-amino group is another nitrogen donor atom of great importance for copper–protein interaction, usually in combination with its neighbouring peptide bond amide groups. A prerequisite for such a binding site is, of course, that the α-amino group of the protein is available so that it can react with the copper ions. Also, the amino terminal end of the peptide chain has to be relatively flexible, in order to permit a copper(II) chelate to form without any major conformational change in the remaining part of the molecule. For low-molecular-weight peptides, which do not have a pronounced tertiary structure, as a general reaction, a stable chelate forms via the α-amino group and two or three peptide amide groups. This is well illustrated by the structure of Cu^{II}–pentaglycine[63], VII.



VII

$Na_2 Cu^{II}(GlyGlyGlyGlyGly) \cdot 4H_2O$

Also in solution, high precision *emf* data[54] measured over broad ranges of total copper(II) and ligand concentrations show that the corresponding triglycine species, $CuH_{-2}A^-$, predominates at neutral pH (Fig. 1). In this study[54] a detailed data treatment was used that does not introduce any a priori assumptions regarding the compositions of the complexes formed.

The presence of a histidine residue, in the second or third position of the amino terminal part of the peptide chain, enhances the stability of the corresponding complexes forming tridentate or tetradentate chelates via the α-amino, amide, and 1-imidazole nitrogen atoms. The calculations described in the previous section (Section C) do show that in mammalian tissues such a histidine residue is necessary in order to overcome the strong complexes formed by the amino acids. Also, at pH 7.5, the tetradenate complex is much stronger than the tridentate complex (Section C). These chelates are only formed from the animo-terminal end of the molecule, and not from the C-terminal end having a histidine residue in a similar position. This is illustrated by one of the structures of Cu^{II}–glycylhistidylglycine[37], III. Similar chelates also form with globular

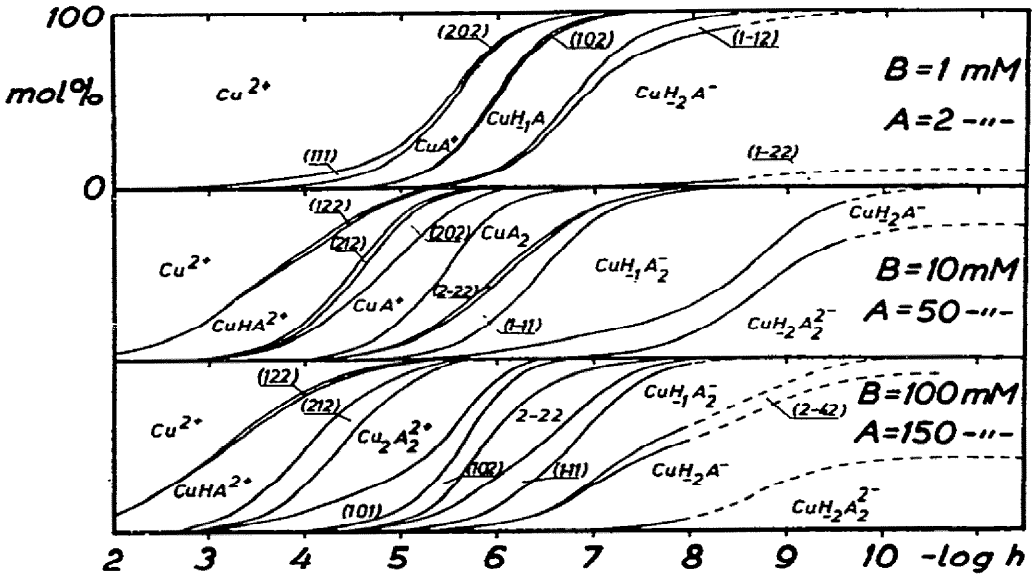
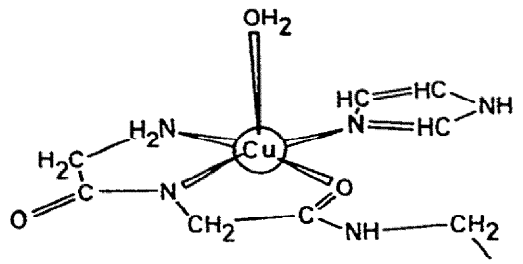


Fig. 1. Distribution of Cu^{2+} between different complexes in the copper(II)–triglycine system[54]. For a certain $-\log h$ (=pH) the number of moles per 100 moles of Cu^{2+} present as a given species is represented by the segment of the *vertical line* falling within the corresponding range. The minor species are indicated by their (*pqr*) triplets in the general formula $Cu_pH_qA_r$.

proteins, as indicated by the very strong first copper(II) site of serum albumin. Human albumin has the N-terminal amino acid sequence[64] Asp–Ala–His–Lys–... and bovine albumin has that of Asp–Thr–His–Lys–.... A great number of chemical data indicate that these proteins form tetradentate copper(II) chelates via the α-amino group, the two neighbouring peptide amide groups, and the histidine imidazole group[64] (for a review, see ref. 11), but, as yet, conclusive crystallographic data are lacking. Due to the very strong complex formed by such an N-terminal structure, at neutral pH, one may be inclined to suggest that similar complexes form among other proteins that have a histidine residue in the second or third position. However, the N-terminal amino acid sequences, as determined for two copper proteins, azurin and umecyanin, do not reveal any histidine residues[65, 66]. Whether or not such a particular N-terminal binding site is involved in other copper proteins is not known.

It should be understood that there are many degrees of rigidity occurring in different protein molecules, from the quite flexible amino-terminal end of albumin to the rigid peptide chain that cannot move at all. Accordingly, a peptide chain having a certain specific conformation may yield the binding site indicated by the structure, VIII, of the mixed copper(II) species formed by triglycine and imidazole[67].



VIII

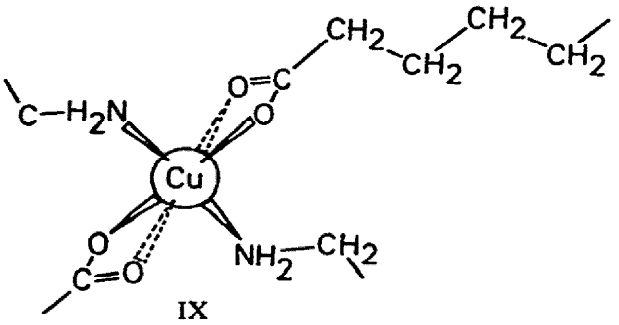
Cu^{II}(GlyGlyGly)(imidazole) · $2H_2O$

It follows from this model structure, VIII, that the ability for a protein to bind a copper(II) ion via the amino terminal end will be considerably increased if another ligand is also bound. Such a ligand may project from a different loop of the peptide chain, for instance an imidazole, or a carboxylate group, or it may come from an amino acid that forms a mixed complex (cf. ref. 45).

(iii) Copper(II) interaction with ε-amino groups

In a peptide chain there is only one single α-amino group, but within this chain there may be as many as ten or more lysine residues with ε-amino groups as their side-chains. Nevertheless, very little attention has been paid to this particular ligand in regard to copper–protein interaction, apparently due to its relatively high pK value[24] (~ 10.2). Clearly, high precision emf measurements, followed by detailed data treatment, show that in aqueous solution copper(II) ions do not form any complexes via the ε-amino group[68]. Thus, in the solution study of the Cu^{II}–6-aminohexanoic acid (HA) system, the data indicate that the coordination involves only the carboxylate groups[68]. This is confirmed by a structural analysis of the crystals, $Cu(HA)_4(ClO_4)_2$, grown in aqueous solution[30]. In this structure, V, copper is coordinated to a slightly tetrahedrally distorted square of oxygen atoms from four different ligands. The second oxygens of the carboxylate groups form an elongated tetrahedron at 2.88 Å from the copper atom; the coordination number is eight[30, 40].

In the crystalline state copper(II) also coordinates the nitrogen atoms of the ε-amino group, provided that the copper is first reduced and then slowly re-oxidized. This is illustrated in another structure of Cu^{II}–6-aminohexanoic acid, IX; four aminohexanoate molecules are coordinated to a central copper atom, two of them via a carboxylate oxygen atom and the other two via a nitrogen atom[30, 69].



IX

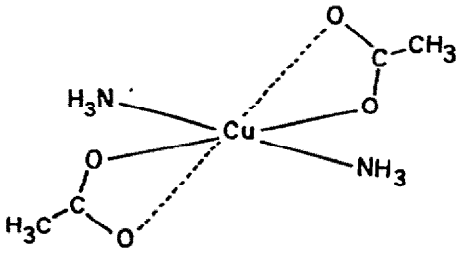
Cu^{II}(6-aminohexanoate)$_2 \cdot 2H_2O$

The centrosymmetric atoms that form an almost square plane are *trans* with respect to each other. We tentatively suggest that during the synthesis of this complex a linear copper(I) complex is first formed according to the following schematic reaction.

$$R \cdot H_2N{-}Cu^{I}{-}NH_2 \cdot R + 2\,R_1 \cdot COOH \rightleftharpoons$$

$$\rightleftharpoons R \cdot H_2N{-}[Cu^{II}(R_1 \cdot COO)_2]{-}NH_2 \cdot R + e^- + 2\,H^+ \qquad (6)$$

The formation of such a linear copper(I) complex, as a first step in this reaction, is in agreement with the fact that a similar complex is formed with ammonia[70]; it apparently also forms with imidazole molecules[55]. Furthermore, the linear copper(I)–ammonia complex has its counterpart in the copper(II)–diacetatodiamine structure[71], X.



X

$Cu^{II}(CH_3 \cdot COO)_2(NH_3)_2$

This structure[71], X, crystallizes in a similar space group, $P2_1/C$, to that of the 6-aminohexanoate structure IX. The environment about the copper is very similar in these two structures, IX and X, and so are the angles and bond lengths of the coordination polyhedra. From these data, it appears as if the complex involving ϵ-amino groups, IX, may be considered as a potential structural model for those copper binding sites that readily undergo reduction and oxidation. Such a binding site having "unidentate" ligands permits the ligands to move independently, and the coordination sphere can be rearranged or changed without any serious damage to the protein. For instance, a linear copper(I) complex, kept in position by two ϵ-amino groups and situated in a somewhat hydrophobic

environment, may be readily transformed into a copper(II) complex via oxidation and the simultaneous approach of two carboxylate groups (eqn. (6)).

(iv) Mononuclear copper(II) complexes of carboxylate ligands

In general, oxygen ligands present in proteins form less stable complexes than those of the nitrogen ligands (Table 4). This is also true at neutral pH, where, due to the relatively weak proton dissociation of the amino group, protons compete with Cu(II) ions. The pH dependence on the complex formation can be estimated by considering the following reaction

$$n\ HA + Cu^{2+} \rightleftharpoons CuA_n + n\ H^+ \tag{7}$$

The ratio of the concentrations between the complex, CuA_n, and the protonated ligand (HA) is determined from the equation

$$[CuA_n]/[HA]^n = bh^{-n}\beta_n \cdot K^n = bF_n \tag{8}$$

For the carboxylate group, the right hand side of eqn. (8) reduces to $\beta_n b$, since it may be considered completely dissociated at neutral pH. The free copper(II) ion concentration, b, will be considered constant in these calculations, and thus the stability order of the ligand will depend only on the quantity F_n. By using the constants (β_n) and the pK values of Table 4, the following stability order is valid at pH 7 for copper(II): glycine ($\log F_1 = 5.6$, $\log F_2 = 10.0$) > α-amino terminal site ($\log F_1 = 4.1$) ~ imidazole ($\log F_1 = 4.2$, $\log F_2 = 7.7$) > carboxylate ($\log F_1 = 1.6$, $\log F_2 = 2.6$) > ϵ-amino ($\log F_1 = 1.1$, $\log F_2 = 1.6$).

According to the above calculations, it is not to be expected that copper(II) carboxylate complexes will be formed on a protein surface as long as there are α-amino or imidazole groups available, or as long as the surrounding solution contains α-amino acids (cf. Section C). On a site somewhat removed from the protein surface, where the environment is less hydrophilic than the surrounding solvents, the forces between the positive (Cu^{2+}) and the negative (carboxylate group) charges become stronger. At such a half-hydrophobic site, a more stable (and inert) complex may form than that formed in aqueous solution[72]. In addition, carboxylic groups may also be available in a strictly hydrophobic cavity quite far from the outer surface. Studies[73] on acid dissociation constants in mixed solvents then indicate that carboxyl groups present in such a cavity may remain protonated at neutral "pH". But a low-molecular-weight mononuclear copper(II) complex usually involves four carboxylate ligands. And in hydrophobic cavity the formation of a complex of four carboxylates seems unlikely, since it would require a considerable amount of electrostatic energy. Thus, it follows from the reaction

$$Cu^{2+} + 4\ R \cdot COOH \rightarrow Cu(R \cdot COO^-)_4{}^{2-} + 2\ H^+ \tag{9}$$

that such a complex yields four charges originating from just two charges. If this complex

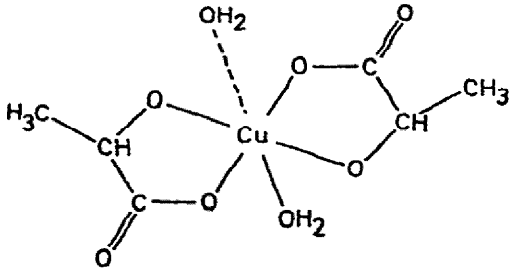
TABLE 4
Stability constants of mononuclear complexes and pK values of the ligands

Ligand	Cu^{II}		Cu^{I}		pK
	log β_1	log β_2	log β_1	log β_2	
Ammonia[24,70]	4.31	7.98	5.93	10.86	9.61 [b]
Imidazole[77,129]	4.33	7.87	5.78	10.98	7.11
Glycine[24]	8.2	15.2			9.6
Triglycine[54,78a]	5.66	10.17	6.2		8.55
Acetate[130]	1.62	2.60			4.61

[a] Involving the α-amino group, but no peptide bond amide groups.
[b] p$K(\epsilon - NH_3^+) = 10.2$.

does form it may be neutralized by a second metal ion forming a dimeric binding site, as occurs in thermolysine[52] (see Section F).

In low-molecular-weight systems, mononuclear complexes with only two carboxylate groups form if additional oxygen atoms are provided by substitution in position 2, thus yielding carboxylates of the type $R_1-C(OR_2)-COO^-$. Two five-membered chelate rings are formed, as illustrated by the structure of Cu^{II}–lactate[74], XI.



XI
Cu^{II}(D, L-lactate)$_2 \cdot H_2O$

However, in a hydrophobic protein cavity the formation of such a complex may require as much energy as that of the complex formed according to eqn. (9). It appears as if a third and a fourth oxygen atom can only approach the copper coordination sphere if there are neighbouring, positively charged groups present, such as lysine residues. Then, in principle, a chelate, similar to the lactate structure XI, can form via the oxygen atoms of the carbonyl and hydroxyl groups of a serine residue.

Our calculations show that on a protein surface carboxylate groups alone cannot compete with other ligands for the very limited amount of copper present in biological tissues. In a half-hydrophobic environment, slightly removed from the protein surface, the charges are enhanced and as a consequence, a mononuclear copper(II) complex might form. However, it seems as if such a cavity, as well as a strictly hydrophobic cavity, will favour binuclear and polymeric copper(II) complexes rather than mononuclear complexes, unless one or two positively charged side-chains are available. In addition, the calculations described in Section C indicate that the copper ions cannot compete with some of the

hard metal ions, Ca^{2+} or Mg^{2+}, for such a site. On the other hand, for copper ions, the mixed chromophore CuN_2O_{2-4} rather than a mononuclear copper(II) carboxylate site would be much more favoured as far as charge relations, stability constants, and low-molecular-weight model structures are concerned (cf. structures IX, X, Table 4, and the discussion in Section D-(*iii*)).

(v) Copper(I) complexes

It is generally understood that copper(I) is the true electron donor in redox reactions involving copper proteins. Nevertheless, very little detailed information is available regarding copper(I)–protein interaction.

One reason for the limited amount of information available is that copper(I) disproportionates in aqueous solution (eqn. (3)), which makes it difficult to study the labile and weak complexes of copper(I). One method to avoid this disproportionation is to introduce copper(I) in the form of a complex into the solution, using a ligand which forms much stronger complexes with copper(I) than it does with copper(II). Examples of such ligands are acetonitrile, some halides (chloride, bromide and iodide), and some sulphur-containing ligands. Most of the Cu^I crystal structures investigated so far contain one ligand of this kind, and consequently they yield only limited information regarding copper(I)–protein interaction. In solution studies involving an additional ligand the equilibria become much more difficult to interpret, since the additional ligand will compete strongly with the ligand under investigation. The system will then consist of four components: Cu^+, H^+, the model compound, and the additional ligand. Accurate data treatment for such a system would require five-dimensional measurements and apparently as many as ten thousand measuring points[75]. It should be noted that the few measuring points obtained from one single titration curve are not sufficient for an accurate quantitative data treatment on such a complicated system; such a limited amount of data will support many different assumptions regarding the compositions of the complexes.

Another method to introduce copper(I) into aqueous solution is to generate copper(I) ions into the solution containing the ligand, by using either a metallic copper[76, 77] or a copper amalgam electrode[78]. Through the use of a metallic copper electrode, Hawkins and Perrin[77] determined stability constants for a series of systems, including those of the Cu^I–imidazole system (Table 4). However, the composition of the assumed complexes was based on data that only involve a single value of the total concentrations of copper and ligand[77]. This data treatment does not exclude the existence of other species, such as polynuclear species.

Using copper amalgam, Bjerrum[70] produced univalent copper by reducing a copper(II) solution of ammonia. He reported that $\log \beta_1 = 5.93$ and $\log \beta_2 = 10.86$, at 18°C and 2 *M* ionic strength.

The method of generating copper(I) ions in the solution containing the ligand by using a two-phase copper amalgam electrode has been developed only recently[78]. One advantage of this method is that several measuring points may be recorded in a closed, anaerobic system, where the copper amalgam is also used to measure the copper ion activity. Two

systems have been studied by this method: Cu^{I}–Cu^{II}–triglycine[78] and Cu^{I}–6-amino-hexanoic acid[29]. In the first case[78], the main species was found to be a mixed copper(I)–copper(II) species, $Cu^{I}Cu^{II}H_{-3}A_2^{2-}$. Measurements over a range of copper and ligand concentrations indicate that this species predominates in the solution when the pH is higher than pH 7. The fact that three protons have dissociated from the peptide bonds in the species $Cu^{I}Cu^{II}H_{-3}A_2^{2-}$ indicates that at least one of these three protons was released by copper(I), and thus in this respect univalent copper has the same capacity as that of bivalent copper. The composition of this mixed species is very similar to that of the binuclear copper(II) species $Cu_2H_{-4}A_2^{2-}$, which exists both in solution[54] and in the crystalline state[35] II. For the species present in solution we may formulate the following redox reaction[78].

$$Cu_2^{II}H_{-4}A_2^{2-} + e^- + H^+ \rightleftharpoons Cu^{I}Cu^{II}H_{-3}A_2^{2-} \qquad (10)$$

It is interesting to note that the equilibrium data[54, 78] yield a standard redox potential of +340 mV for this reaction at pH 7.0. This is the same order of magnitude as the potentials reported for some copper proteins[79].

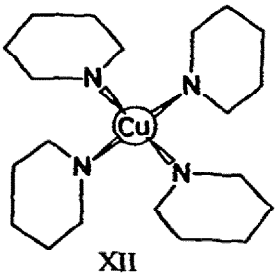
In the copper(I) system of 6-hexanoic acid, on the other hand, a completely different complex is formed, the tetranuclear $Cu_4^{I}A_4$ species[29]. This species differs from those proposed by previous authors on copper(I) systems of nitrogen donor atoms[24], but it has a composition very similar to several copper(I) complexes that exist in the crystalline state: the ammines, $(CuX \cdot RNH_2)_4$, the triethylarsine, $[CuI \cdot As(C_2H_5)_3]_4$, and the diethyldithiocarbamate, $[CuS_2CN(C_2H_5)_2]_4$, systems[42,80]. These structures contain a central metal group of Cu_4 tetrahedra with short copper–copper distances, 2.66–2.80 Å. A similar tetrahedral cluster of four copper(II) ions is found in the structure of the compound $Cu \cdot C_7H_{11}NO_2$, but the Cu–Cu distances are considerably longer and the copper atoms are oxygen-bridged[81]. (The ligand $C_7H_{11}NO_2$ is a tridentate Schiff base derived from acetylacetone and 2-aminoethanol.)

It has also been suggested that a four-nuclear cluster of copper ions may exist in copper proteins, such as in ceruloplasmin; see, for instance, Curzon and Cumings[1]. The numbers of copper atoms present in ceruloplasmin, laccase, and ascorbate oxidase yield integers when divided by four (Table 1); Frieden et al. [82] have pointed out that all the enzymes that reduce oxygen to water have a multicopper nature. In ceruloplasm in such a four-nuclear cluster of copper ions can explain many of the relations (Curzon and Cumings[1]), but some EPR data indicate that in the bivalent state the copper atoms are at least 6 Å apart (Blumberg[1]). If such a cluster exists, the valency change from Cu^{I} to Cu^{II} involves considerable rearranging, and the resulting Cu^{II}–Cu^{II} distances would be much longer than in the low-molecular-weight tetranuclear structure[81]. Such a large change in the copper environment must also involve a conformational change of the protein. Recent data[83] indicate that the models of four equivalent copper ions certainly have to be considerably modified, since there are neither four equivalent copper atoms in ceruloplasmin nor in the "blue" oxidases.

On the other hand, the copper ions in these oxidases are all functionally related, and they are involved in the same enzymatic reaction. Kinetic data do indicate that electrons

are transferred from one site to another[4]. One model structure[84] which indicates possible routes for the transfer of electrons within the protein is Cu^{II}(glycyl-L-alanine) · H_2O. In this structure[84] there are bridges formed both by carboxylate groups, Cu–O=C=O–Cu, and by peptide bonds, Cu–N=C=O–Cu, each of which connects two different copper atoms. It seems obvious that if such bridges ever occurred within a copper protein, they would facilitate the transfer of electrons between the copper sites.

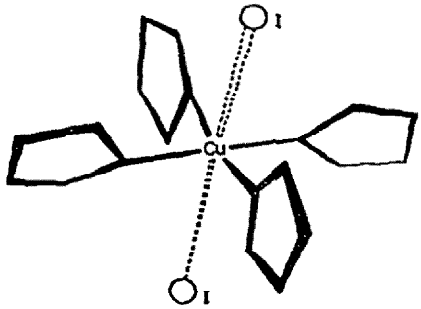
As far as the copper(I) coordinations to nitrogens are concerned, some additional contributions to copper(I)–protein interaction are found in two crystal structures: Cu^{I}–imidazole perchlorate[55] and tetrapyridine copper(I) perchlorate[85]. In the latter structure the coordination geometry is a regular tetrahedron, and copper(I) is bonded to four nitrogen atoms, each of which belongs to one pyridine molecule[85] giving structure XII.



XII

$Cu^{I}(pyridine)_4ClO_4$

Pyridine molecules are, of course, not usually present in proteins, and this structure is of direct interest only if we extrapolate to a similar imidazole structure. It is then interesting to note that four imidazole molecules appear to stabilize the copper(II) state. This is shown by the structure $Cu^{II}(imidazole)_4I_2$, XIII, where the environment about the copper atom is essentially square planar, with two quite distant iodine atoms (3.42 Å and 3.87 Å) completing a very distorted octahedron[86].

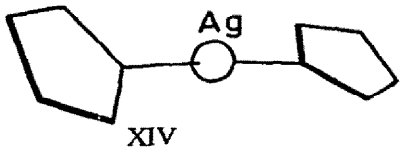


XIII

$Cu^{II}(imidazole)_4I_2$

Thus, not only in aqeuous solution but also, to a certain extent, in ethanol, the imidazole complex inhibits the formation of copper(I) from copper(II) and iodide. In more nonpolar solvents, however, copper(I) iodide precipitates. As expected from the data listed in Table 2, a hydrophobic environment favours the copper(I) state[86]. As a result, a copper(I) binding site of *four* imidazole groups, arranged in a tetrahedral geometry, may exist provided that the environment is hydrophobic.

Another possible copper(I) binding site, other than that of the four imidazole groups, is one having only two or three of these groups. A trigonal or a linear Cu^{I}–imidazole coordination is quite plausible if we extrapolate from those Cu^{I} structures that are available in the crystalline state[42,87]. Sites like these would permit the binding of additional ligands, a general feature for the so-called "non-blue" copper of copper proteins[4]. One promising model is copper(I)–imidazole perchlorate[55], but so far the copper(I)–imidazole crystals have only been available in a size suitable for powder diffraction analysis. The powder patterns indicate that these crystals are essentially isomorphous with those of bis-imidazole silver nitrate[88]. In this structure[88], XIV, silver is linearly coordinated to two imidazole molecules.



XIV

$Ag(imidazole)_2 \cdot NO_3$

In a protein cavity, a copper(I) binding site, having two or probably three imidazole side-chains, permits additional ligands to approach; for instance, carboxylate groups may project into the coordination sphere when copper(I) is oxidized to copper(II), a reaction of a kind similar to that illustrated by eqn. (6).

From this discussion it appears that as far as redox reactions of copper proteins are concerned, the most promising mononuclear models are those of the kind indicated by eqn. (6), since in the examples of ammines and imidazoles, experimental data indicate that both the Cu^{I} and Cu^{II} complexes exist. A linear copper(I) complex and a tetra-coordinated copper(II) complex permit the binding of additional ligands, forming a coordination number of three and four for copper(I) and a coordination number of five and six for copper(II)[4,18]. It is therefore proposed that $Cu^{I}N_2$ and $Cu^{II}N_2O_{2-4}$ chromophores are important models for those copper ions present in copper proteins, which undergo reversible reduction and oxidation reactions (cf. eqn. (6)). A study of the coordination of copper to four imidazole groups indicates that such a site may also be important as far as the redox reactions of copper proteins are concerned, provided that the environment is hydrophobic[86].

E. COPPER ION INTERACTION WITH SULPHUR LIGAND ATOMS

When various kinds of atoms are considered as ligands in copper proteins, it is important to be aware of the true valency state of the copper ions that enter the apoprotein during biosynthesis. During aerobic equilibrium conditions copper(II) rather than copper(I) is the predominating copper ion and as a result, nitrogen ligand atoms would be preferred to sulphur ligand atoms. On the other hand, within the cell most reactions are far from their equilibria and even during aerobic conditions, a certain number of free –SH groups are available. As has been previously suggested in Section C, some of the copper(II)

complexes that enter the cells might first be reduced and then transformed into a short-lived but very stable Cu^{I} complex of either glutathione or cysteine. Whether or not such a complex is important for the formation of copper proteins is not known.

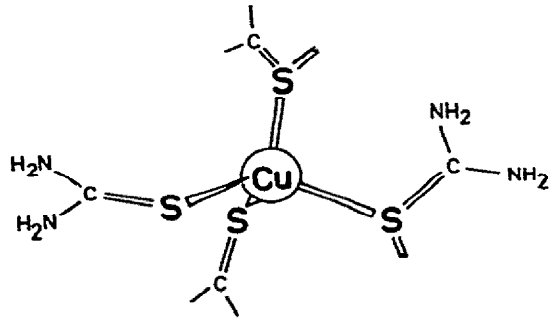
As far as the sulphur ligands present in proteins are concerned (such as thiols, thioethers, and disulphides), it is known that they interact with iron(II) and iron(III). This is indicated from X-ray diffraction studies on single crystals of iron-containing proteins and low-molecular-weight iron complexes[89–91]. In the corresponding copper systems, however, few data are available. As indicated from the (b)-type affinity sequence, the copper(I) ion rather than the two iron ions preferably interacts with sulphur donor atoms (cf. Section B(*i*)). The importance of copper-sulphur interaction in copper enzymes has been discussed in some detail by Hemmerich[1] and Beinert[1].

(i) Copper complexes of the thiol groups

Studies on non-haem iron proteins have demonstrated that thiol groups present in proteins are important ligands for the binding of iron ions[89]. With regard to the interaction of thiol groups with copper ions, it is not only the role of these groups in the protein binding of copper that is unknown, but also there are great difficulties in obtaining defined model complexes. For instance, it is not known whether or not a cysteinyl residue within a peptide chain can interact with copper(II) via both the thiol group and the neighbouring peptide bond amide group. Furthermore, when a mercaptan of the type $R \cdot CH_2SH$ is studied, it is oxidized[91] to $R{\cdot}CH_2{-}S{-}S{-}CH_2 \cdot R$ by copper(II), and it polymerizes[92] with copper(I). One method to overcome the last difficulty was used by Kolthoff and Stricks[91] in their study of the Cu^{I} complexes of cysteine. They used solutions that had high background concentrations of other ligands that form complexes with copper(I): 1 *M* NH_3 and 0.04 *M* SO_3^{2-}. They reported[91] a single 1 : 1 complex with $\log\beta_1 = 19.19$. However, both ammonia[24] ($\log\beta_1 = 5.9$) and sulphite[24] ($\log\beta_1 = 7.9$) from fairly stable Cu^{I} complexes and at these high concentrations, the formation of mixed complexes involving cysteine and one of these other ligands cannot be excluded. If it is possible for such a mixed species to form, the stability constants indicate that this mixed species will predominate in solution.

Thiol groups were also studied by Klotz et al.[93], who investigated the reaction of mercaptans with copper(II) chloride. A violet colour developed when the ratio of the total concentration of R–SH to Cu^{II} was larger than two. On this basis, as well as on the basis of other qualitative data, they concluded that the mixed-valent species $Cu^{I}_4Cu^{II}(RS)_4$ was formed[93].

As far as X-ray diffraction studies are concerned, some inferences regarding the copper–thiol interaction may be drawn from the data reported on the structures of thiourea and its derivative tetramethylthiourea. In one such structure[94], copper(I)–thiourea chloride, XV, the geometry about the copper atom is an almost regular tetrahedron with Cu–S distances from 2.28 to 2.43 Å.

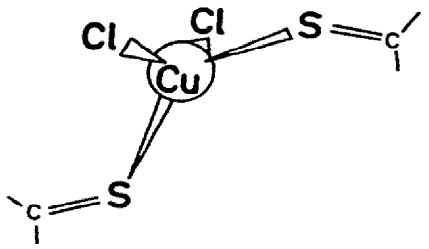


XV

$Cu^{I}(thiourea)_3Cl$

This geometry about the central metal ion is also found in the iron–sulphur systems of non-haem iron proteins, where each iron atom is tetrahedrally bound to four sulphur atoms[89].

Several other copper(I)–thiourea structures have been reported[95], but only a single copper(II) structure of this kind is known: the ruby-red copper(II)–tetramethyl thiourea chloride crystals[96], XVI.



XVI

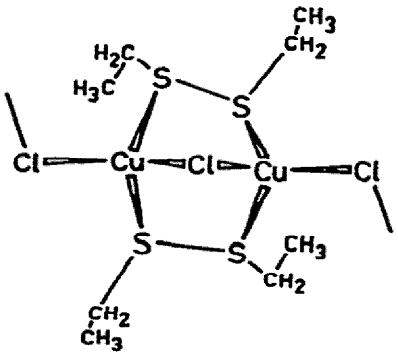
$Cu^{II}(tetramethylthiourea)_2Cl_2$

In this particular structure, XVI, the coordination geometry about the copper atom is best described as a considerably flattened tetrahedron with Cu–S distances from 2.297 to 2.339 Å.

Other structures that might simulate the copper ion interaction of thiol compounds are those of the thiocarbamates. In the copper(I)–diethyldithiocarbamate[80] and the copper(I)–dipropylthiocarbamate[97] structures there are clusters of four and six copper atoms, respectively, with the copper atoms located very close to each other (cf. Section D(*v*)). The copper(II) structure of a similar ligand, dipropyldithiocarbamate[98], differs from the copper(I) structures. In the copper(II) structure[98] there are two copper atoms located fairly close to each other, at 3.38Å. Each copper atom has a distorted square-pyramidal coordination to five sulphur atoms; the four closest sulphurs bind the copper via two four-membered chelate rings, each of which is formed by one ligand, and the fifth apical sulphur is shared by the neighbouring copper atom, thus forming a bimolecular unit[98] (cf. ref. 99).

(ii) Copper complexes of other sulphur ligands

The importance of the *disulphide* ligand, as regards copper–protein interactions, is substantiated by the presence of a disulphide bridge in almost every protein molecule. From the X-ray diffraction analysis of diethyldisulphide–copper(I) chloride, it is clearly indicated that copper(I) interacts with disulphide sulphur atoms[100]. In this structure, XVII, the copper atoms are tetrahedrally coordinated to two chlorine atoms and to two sulphur atoms, one from each of two molecules.

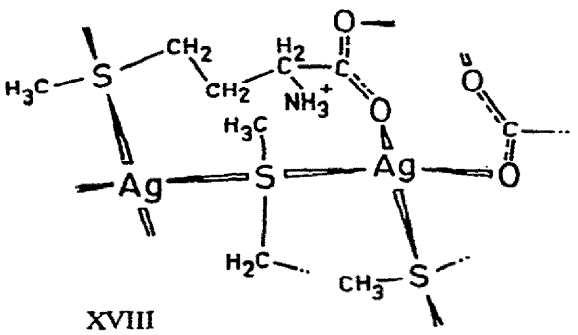


XVII

$Cu^{I}Cl \cdot (C_2H_5)_2S_2$

Here there is a fairly short Cu–Cu distance, 3.22 Å, between the pair of copper atoms which bind two disulphide molecules, XVII. Also, the copper–sulphur distances, 2.34 and 2.40 Å, are surprisingly short and of the same order of magnitude as the Cu–S distances in thiourea complexes[95], indicating a comparatively strong Cu–S interaction.

Thioether groups are usually more common in proteins than the disulphide groups. The thioether group is part of only one side-chain, that of methionine. The role of the thioether group in metal–protein interactions has recently been revealed by the X-ray crystallographic analysis of cytochrome *c*; in this structure a thioether sulphur atom occupies the sixth coordination position of the iron atom[89]. The copper(II) interaction of the thioether group, on the other hand, has mainly been studied in solution. Thioether carboxylates form both four- and five-membered chelate rings with copper(II), involving both oxygen and sulphur donor atoms[101, 102]. There are no similar studies reported for the corresponding copper(I) systems. But copper(I) does interact with the thioether group in the crystal structure[103] of $2(C_3H_6S_3) \cdot 3CuCl$. Some information of direct importance for copper(I)–protein interaction may be obtained by extrapolation from the data reported for two recent structures of silver(I)–methionine[104]. In these structures, Ag^{I} is coordinated in highly distorted tetrahedral and trigonal arrangements, XVIII.



XVIII

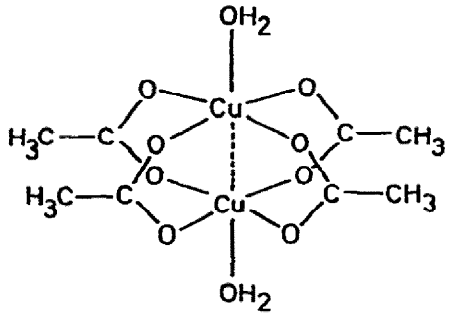
$Ag(D, L\text{-}HMet)NO_3$

Clearly in this environment, which is predominantly ionic, Ag^I is found to have a coordination geometry similar to that found in some copper(I) structures[42]. It is important to note that the Ag^I ion interacts more strongly with the thioether group than with the amino group (XVIII), and that Ag^I prefers carboxylate oxygen atoms to the amino nitrogen atoms. This methionine interaction of Ag^I, and probably that of copper(I), is very different from that of copper(II); in the Cu^{II}–methionine structure copper(II) does not interact with the thioether group[105]. There is little doubt that the Ag^I–methionine structures[104] are important models for metal–protein interaction; this particular environment of thioether and carboxylate groups may exist in many proteins.

F. MODELS FOR BINUCLEAR COPPER BINDING SITES

A series of studies by Malmström and collaborators has indicated that bivalent copper with a low magnetic moment exists in copper enzymes having oxidase activity, such as two laccases, human ceruloplasmin, and probably ascorbate oxidase (for a review, see ref. 4). For instance, redox titrations and EPR studies on *Polyporus* laccase have demonstrated that, apart from the "blue" and "non-blue" coppers, there is an additional site which accepts electrons only in pairs[106]. This site is characterized by the following properties[4]: (1) a redox potential greater than 0.5 V, (2) diamagnetic properties in the oxidized protein at room temperature, (3) no exhibition of any EPR signal in oxidized or reduced forms of the enzyme at 77°K, and (4) a near-ultraviolet absorption band of moderate intensity. On this basis, Malmström and coworkers[4,106] proposed that this additional electron-accepting site is associated with two copper ions present as a Cu^{2+}–Cu^{2+} unit. The exact nature of the ligands involved in this copper binding site is not known.

In low-molecular-weight systems interacting Cu^{2+} ions are not unusual, and binuclear complexes have long been recognized as providing special electronic and structural situations (for a review, see ref. 107). The well-known structure of $Cu_2(CH_3 \cdot COO)_4 \cdot 2H_2O$ illustrates that in binuclear carboxylates two copper ions are bridged in a pair via four carboxylate groups, with the water molecules occupying the terminal positions[108], giving structure XIX.



XIX

$Cu_2^{II}(CH_3COO)_4 \cdot 2H_2O$

The striking feature of this six-coordinate copper complex is the short distance between the two copper atoms (2.64 Å), which is only slightly greater than the interatomic distance in metallic copper (2.56 Å). In this and similar compounds, electron spin pairing occurs. At room temperature, binuclear copper(II) carboxylate compounds exhibit magnetic moments that are lower than the "spin-only" number for one unpaired electron per copper atom[107].

As far as aqueous solutions are concerned, there appears to be very little evidence for the existence of such a dimeric acetate species, although it forms in other less polar solvents[109]. In another carboxylate system, however, where the ligand also contains an amino group (the Cu^{II}–6-aminohexanoate system), equilibrium data indicate that binuclear species of the composition $Cu_2(RCOO^-)_3$ are formed[68] (Fig. 2).

In Section D(*iv*), it was previously mentioned that a protein may possibly bind copper ions via oxygen atoms in a mononuclear site. However, the formation of such a site via four carboxylate groups did not seem very likely in a hydrophobic cavity, since it would require a considerable amount of electrostatic energy. On the other hand, the formation of a binuclear complex, say a complex of the $Cu_2(CH_3COO)_4 \cdot 2H_2O$ type, XIX, may require less electrostatic energy. As illustrated by the following reaction

$$2\,Cu^{2+} + 4\,RCOOH \rightarrow Cu_2(R \cdot COO^-)_4 + 4\,H^+ \qquad (11)$$

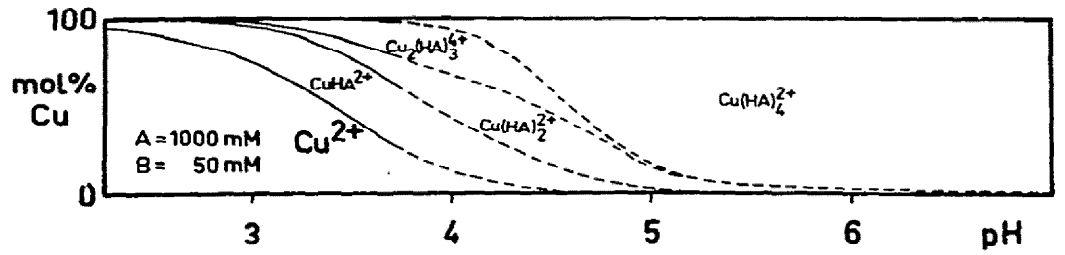
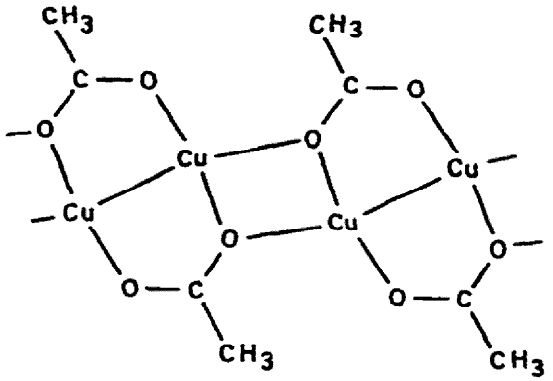


Fig. 2. Distribution of Cu^{2+} between different complexes in the copper(II)–6-aminohexanoic acid system[68]. For explanatory note, see legend to Fig. 1.

there is no net change in charges. Thus, in a hydrophobic protein cavity, the existence of a $Cu_2O_8O'_{0-2}$ chromophore seems more likely than that of a $CuO_4O'_{0-2}$ chromophore.

If such a binuclear carboxylate complex exists in a copper protein, one may ask if a pair of bivalent coppers can undergo a valency change into a pair of univalent coppers. A copper(I)–acetate structure, XX, recently determined by X-ray crystallography[110], provides an answer to this question.



XX

$Cu^I(CH_3COO)$

In this copper(I) structure, the Cu–Cu bond length is shorter, 2.544 Å, than that of the corresponding bond length in the copper(II)–acetate structure XIX, 2.64 Å. The environment about the copper(I) ion is unique in that it is square planar; the binuclear units are linked to other such units by longer Cu–O bonds, 2.31 Å, than those within the binuclear unit, 1.90 Å. Thus, this dimeric structure[110] is surprisingly similar to the corresponding copper(II) dimer[108], XIX. As a result, it is indicated from low-molecular-weight complexes that in a protein cavity a binuclear $Cu^I_2O_4O'_2$ chromophore may be transformed into a $Cu^{II}_2O_8O'_2$ chromophore by the simultaneous approach of two carboxylate groups projecting from a neighbouring peptide chain (cf. eqn. (6)).

As previously discussed in Section D (*iv*), it seems necessary for the existence of such a binuclear copper binding site, involving only oxygen ligand atoms, that it be situated in a hydrophobic cavity. In addition, when the ligands are supposed to be a series of oxygen atoms, it should be kept in mind that other metal ions, such as magnesium or calcium ions, may compete more successfully than copper ions for such a binding site (cf. Section C). The conditions may be different in a hydrophobic environment; the relatively strong complex-forming ions, Cu^I and Cu^{II}, may react according to eqn. (11), even though the carboxyl groups are protonated. On the other hand, the ions having the closed-shell electronic structure, in order to react properly, may require these ligands to be at least partly negatively charged.

Other binuclear copper complexes, apart from those having only oxygen ligand atoms, are those which also contain nitrogen ligand atoms. One example of such a complex, having a Cu–Cu distance of 2.64 Å, is the thiocyanate adduct of a copper(II)–carboxylate

dimer[111]. In addition, binuclear copper(II) complexes form by a reaction that can be regarded as a dimerization; examples are found in the copper(II) structures of salicylaldiminate derivatives[112], the Cu(II) structure of adenine[113], and the Cu^{II} structure of triglycine[35], compound II. Furthermore, pairs of copper atoms are also reported as existing in many structures having ligands other than those of oxygen and nitrogen atoms, for instance sulphur[98–100,114] and chlorine[115,116] ligand atoms.

Dimerization as well as oxobridging appear to have been reactions of importance during the formation of the binuclear copper(II) complex of glycylhistidylglycine[117] (Fig. 3). In this structure the α-amino, peptide, and imidazole nitrogen atoms of one molecule coordinate to each copper atom, and two such chelates form a dimer, held together by two oxygen atoms of two other peptide molecules; the coordination number is five (Fig. 3). Within the crystal these dimeric units are connected tetrahedrally and six such dimeric units form a ring[117]. These two structural units correspond to species of the composition $(Cu_2H_{-2}A_2)^0_n$, with values of n equal to 5 and 6, respectively. It is interesting to note that studies in solution independently indicate that very similar species also form at neutral pH, at relatively low concentrations[117,118]. In the solution studies, high-precision potentiostatic *emf* methods[11,119] and the small angle scattering technique were used[117]. Thus, the formation of such dimeric units is a favoured process, and it can be expected that a similar structure may exist in proteins. Whether or not this particular binuclear species (Fig. 3) has a subnormal magnetic moment is not known; but as previously suggested by Kato et al.[107], for structures having the same Cu–Cu distance, 3.5 Å, there may be a super-exchange mechanism that involves electron transfer through the bridging oxygen atoms.

A model for haemocyanin, which is similar to the structure of copper(II)–glycylhistidyglycine, Fig. 3, has been postulated by Gray from spectroscopic data[120]. Gray proposes that the oxidation of copper(I) to copper(II), by an oxygen molecule, may lead to the additional binding of two carboxylate groups projecting from the neighbouring peptide chain[120]. This is a reaction of the same kind as those previously discussed in some detail in Section D. For a closer juxtapositioning of Gray's model[120] with the structure of Cu^{II}–glycylhistidylglycine[117] (Fig. 3), it is necessary to replace one α-amino group by another ligand; it does not seem likely that a protein binding site contains two α-amino groups. Also, in the reduced state, the copper(I) ions of such a site may be bound only to two ligand atoms having a third coordination position available for an oxygen molecule. If such a site exists in a strictly hydrophobic environment, it seems possible that it can be used as a model for a copper binding site in a copper enzyme having oxidase activity. Then the oxidation of the copper pair will not only favour the two-electron step mechanism suggested by Malmström and coworkers[4,106], but it may also favour the release of protons (cf. eqns. (6) and (11)). The electrons and protons may react with the oxygen substrate in two redox cycles, first forming peroxide and then water (cf. ref. 4).

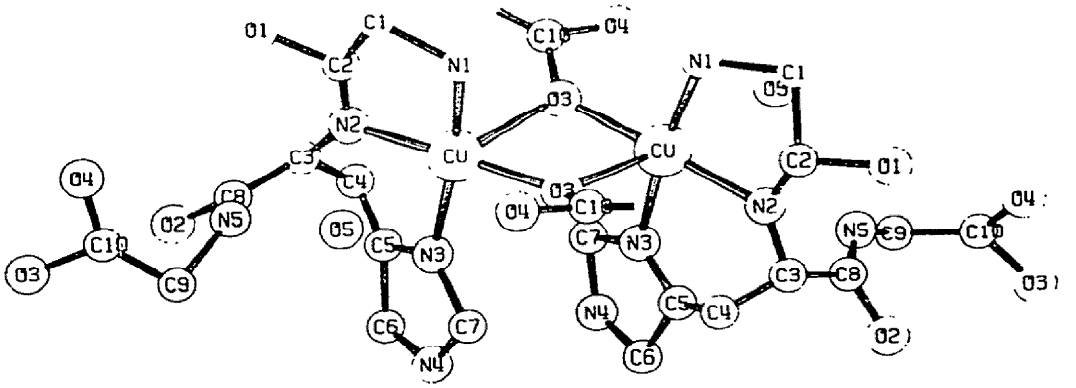


Fig. 3. Structure of Cu^{II}(glycyl-L-histidylglycine) · xH_2O (ref. 117).

G. MODELS FOR THE GEOMETRY OF "BLUE" COPPER BINDING SITES

A characteristic property of some copper proteins is their intense blue colour caused by EPR-detectable cupric ions. The extinction coefficients of the absorption band, at about 600 nm, vary from $3500\,M^{-1} \cdot cm^{-1}$ (azurin) to $5600\,M^{-1} \cdot cm^{-1}$ (ceruloplasmin) (cf. ref. 4). This absorption band is approximately 100 times as intense as the corresponding band of $Cu(NH_3)_4^{2+}$, which is $50\,M^{-1} \cdot cm^{-1}$ at 600 nm. It seems that this high extinction coefficient is due to a particular binding site only present in proteins since, as yet, it has not been found among low-molecular-weight model complexes. The presence of binuclear or polynuclear structures is ruled out as an explanation of the enhanced absorption band intensity, because some blue proteins contain only one copper ion.

Several theoretical interpretations have been made in order to describe the visible spectra and the EPR data of the "blue" copper binding sites. One of these, based on experimentally derived parameters, assumed that in ceruloplasmin d–d transitions and not charge-transfer bands were responsible for the visible spectra (Blumberg[1]). In this model the four closest ligands about the copper atom show relatively strong distortion from square planar geometry (Blumberg[1]). A similar result was obtained by Brill and Bryce[121] in their study on azurin. They were able to explain EPR parameters and ORD data on the basis of a mixture of the p and s orbitals into the $3d$-orbitals of the Cu(II) ion. Again, a distorted geometry which deviated from a square plane towards a tetrahedral configuration was indicated[121].

Consistent with these theoretical calculations[1,121] are studies on two model compounds[122,123]: γ-irradiated $Cu(CH_3CN)_4ClO_4$ and $(Cu_xZn)Hg(SCN)_4$. The EPR data of these model complexes are comparable to those found in the "blue" Cu^{2+} proteins. However, the ligands of these model complexes are not similar to those supposed to be present in the "blue" sites of the copper proteins. Although preliminary X-ray crystallographic data seem to indicate tetrahedral Cu^{2+} sites[122,123], the complete optical properties of the models have not yet been reported.

Some other examples of complexes that produce the blue colours are low-molecular-weight copper(II) complexes, which have either four ammonia (λ_{max} = 606 nm) or four imidazole molecules (λ_{max} = 590 nm) as ligands[79]; in solution the geometry about the copper in these complexes is not known, but the ammonia complex may correspond to a CuN_4O_{0-2} chromophore with square-planar, tetragonal-octahedral, or square-pyramidal geometry[124]. As far as a protein binding site of four imidazole groups is concerned, it seems important in order to enable reduction to occur that it should be situated in a hydrophobic environment[86] (cf. Section D (ν)). Many "blue" copper binding sites are characterized by a high redox potential, and thus they favour the copper(I) state[4]. However, as previously discussed, the copper(I) state is favoured in a non-aqueous environment (cf. Table 2 and the previous discussion in Section D). Therefore, in addition to the explanations offered by other authors for the redox potentials of "blue" copper binding sites (cf. ref. 4), we are inclined to propose that the "blue" copper sites are situated in hydrophobic cavities. It should also be noted that the geometry about one copper in bis-imidazolato-copper(II)[38], IVb, is a distorted tetrahedron, which is geometry of the same kind as those suggested by Blumberg[1] and by Brill and Bryce[121]. The corresponding copper(I)–imidazole structure is not known, but in a similar Cu^I structure, tetrapyridine copper(I) perchlorate[85], the environment about the copper is a regular tetrahedron, XII.

A Cu^{2+} site formed by four histidine side-chains, however, cannot be the single copper binding site of plastocyanin, since in this blue copper protein there are only two histidine residues[125]. Also, for azurin, another "blue" copper protein having a single copper binding site, the primary structure shows that there are just four histidine residues in the total protein molecule[66]. Studies on the binding of copper ions to azurin[66] indicate that the ligands which bind copper do not have pK values between 4 and 8. Thus, the binding site does not appear to consist of as many as four imidazole groups, which usually have a pK value of about 7.0. Other possibilities for the copper binding sites of plastocyanin and azurin might be that the ligands consist of either ϵ-amino groups, or both ϵ-amino and imidazole groups.

Another additional alternative regarding these binding sites of plastocyanin and azurin involves the peptide bond amide groups. Such sites might have a geometry very similar to that suggested by Gray[120]. He proposed, partly based on his experiences with Ni^{II} systems, that the blue proteins have copper(II) sites that are five-coordinated[120]. It is also indicated from the structures reviewed by Freeman that the blue Cu^{2+}–peptide complexes generally have copper atoms with a coordination number of five[1,18]. This is particularly correct for those complexes where not more than one peptide bond amide group is bound to copper(II). When there are two or more amide nitrogens bound to the copper atom then the colour changes to violet and λ_{max} decreases to about 550 nm (refs. 1, 18). Thus, if peptide bonds are involved, then in a "blue" copper site the chromophore may be of the $Cu^{II}N_2O_3$ type. The coordination of the amide nitrogen may require a chelate to be formed via an imidazole or an α-amino group; one or more of the oxygen atoms in this chromophore may be identified as carboxylate groups. Low-molecular-weight models for such a site are the Cu^{II}–glycylglycine[34] structure, I, and a part

of the Cu^{II}–glycylhistidylglycine[117] structure (Fig. 3). It is expected that such a chromophore, involving carboxylate group(s), should undergo a reaction similar to that indicated by eqn. (6); thus the carboxylate binding of copper is released when copper(II) is reduced to copper(I). At low pH (< pH 6) this would lead to an increase in the redox potential, as the pH decreases, with 0.06 V per pH unit and per carboxylate group released. Yet, such data have not been recorded for either azurin or plastocyanin. However, another small copper protein, stellacyanin, is characterized by this particular pH dependence of the redox potential (cf. ref. 4). Also, for stellacyanin, both the visible spectra and the EPR spectra change at alkaline pH in the same manner as they do when two or more amide nitrogens are bound to copper in low-molecular-weight biuret complexes[4]. In addition, analytical data[66] on azurin indicate that apart from the single copper ion and the peptide chain there are no other molecules, such as carbohydrate, present in the protein. There is only a single thiol group in the azurin molecule and when the copper ion has been removed from the protein this thiol group is not available for chemical reactions[66]. Thus, in azurin, the most plausible type of ligand atoms are nitrogen and oxygen atoms.

It follows from this discussion that a tetrahedrally distorted square plane, which is produced by four imidazole groups about a copper(II) atom and which is in a non-polar environment, can satisfactorily explain many of the properties characteristic of the "blue" copper binding sites. One or more of these ligands may, in principle, be replaced by amino groups but they will not allow, as the imidazoles do, relatively wide fluctuations in angles and distances of the copper–nitrogen bonds (cf. Section D(*ii*)). Another possibility for a "blue" copper binding site is one that has the coordination number of five[120]. If such a site involves the peptide bond amide group, then by extrapolation, the structure Cu^{II}–glycylglycine[34] and, in part, that of Cu^{II}–glycylhistidylglycine[117] can be considered as models for the geometry of such a site. Such a pentacoordinate copper binding site may require rearrangement of the geometry about the copper atom, when it is reduced. This would produce some conformational change in the protein, which may indicate how these two sites can be distinguished. However, it is important to emphasize that the models illustrated in this section do not actually reproduce the "blue" Cu^{2+} sites present in copper proteins; here they are discussed only as models, and as yet the correct nature of these sites remains unknown.

H. CONCLUDING REMARKS

Structural models for copper–protein interaction reside to a great extent on the coordination chemistry of low-molecular-weight systems, since no complete X-ray structure analysis of a copper protein has yet been achieved. Regarding these low-molecular-weight systems, high precision emf data and crystal structure studies indicate that there are three particular kinds of copper(II) complexes that may be considered as models for the geometry of the protein binding sites: the unique copper(II) chelates formed by the terminal amino group and neighbouring peptide bond amide groups; the complexes having $Cu^{II}N_2O_2O'_{0-2}$ chromophores, formed by the coordination of imidazole (or

ϵ-amino) and carboxylate groups; and the binuclear complexes having the pair of cupric ions bridged via carboxylate groups or oxygen atoms. It appears as if these three kinds of models may be identified in terms of particular binding sites present among proteins that bind copper ions specifically. An example of the amino terminal chelate, where the stability of the complex is considerably enhanced by the presence of a histidine residue, is found in serum albumin[64]. A $Cu^{II}N_2O_2O'_{0-1}$ chromophore and its corresponding univalent chromophore, $Cu^{I}N_2$, formed by the release of the carboxylate groups, may permit binding of additional ligand(s), say an anion, a property characteristic of the "non-blue" copper[4] in copper proteins. The binuclear complexes, which usually have low magnetic moments, may have their counterparts among the copper proteins containing "EPR-nondetectable" Cu^{2+} coppers[4] that accept electrons only in pairs.

In the preceding survey we have emphasized that there is a considerable difference between the copper complexes of proteins and those of small molecules in dilute aqueous solution. In an aqueous medium cuprous ions are unstable, partly due to the strong solvation of cupric ions. A protein molecule can provide the hydrophobic environment necessary to stabilize the copper(I) state; therefore, it is proposed that a hydrophobic environment is important for those protein-bound copper ions that undergo reduction and oxidation.

The existence of such a non-aqueous environment seems to be especially indicated regarding the copper binding sites responsible for the intense blue colour, since relaxation measurements show that these Cu^{2+} sites are probably not on the surface of the protein molecule[4]. It appears from some theoretical calculations that this blue colour is related to the symmetry of the site, and that the geometry about the "blue" copper should be one of a tetrahedrally distorted square plane[1,121]. As an alternative, it has been suggested that the "blue" copper may have a coordination number five[120]; an attractive but not conclusive suggestion for a "blue" site would then be that of a $Cu^{II}N_2O_3$ chromophore involving a peptide bond nitrogen atom. A five-coordinate copper(II) ion readily permits reversible reactions with a substrate via temporarily octahedral coordination[1,18]. But, as yet, the characteristic high extinction coefficient of the "blue" Cu^{2+} copper has only been found in proteins. The correct nature of the "blue" sites as well as the other sites remains unknown. Hopefully, X-ray structure analyses of blue copper proteins, now in progress, will soon be completed so that a more detailed picture of the copper binding sites will emerge.

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