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Coordination compounds and life processes

Ivano Bertini, Luigi Messori and Maria Silvia Viezzoli
Department of Chemistry, University of Florence, Florence (Italy)

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CONTENTS

A. METAL IONS AND LIVING SYSTEMS

Life has developed in our planet on equilibrium with the earth crust and atmosphere. Living organisms have taken from the environment the substances that they needed for growth; at the same time they had to defend themselves against toxic compounds by developing appropriate strategies to neutralize them.

Life has changed the chemical composition of the planet, mainly through photosynthesis; we can state that, to a large extent, the evolution of living organisms has coincided with the evolution of chemicals available for being integrated into the biological processes. For example, a great change in the biological processes and in the structure of the involved biomolecules occurred on passing from a reducing to an oxidizing atmosphere. Oxygen became a convenient source of energy and life evolved to the present level of complexity. To defend themselves against the toxic effects of dioxygen and its derivatives and ensure their own survival, living organisms had to evolve complex enzymatic systems containing iron, copper and manganese,

to scavenge these new poisons (e.g. superoxide dismutases, catalases). At the same time, many biochemical structures had to be adapted to the new "oxygen world" and to the aerobic life: haemoglobins, myoglobins, haemerythrin, haemocyanin, and many other metalloproteins appeared on the scene.

The earth crust and the atmosphere are of interest to inorganic chemistry. This consideration implies that (i) the chemistry of biological processes needs the contribution of inorganic chemists; (ii) bioinorganic chemistry is central to science and has broad implications for many of its branches such as evolution of life, medicine, environmental sciences and others.

The above concepts are not yet widely accepted in the scientific culture, not even at the advanced research level. In middle Europe and in Italy, the biochemical culture is mainly in the hands of medical doctors who have poor contacts with chemists. As a result, the programs of university biochemical courses often do not even mention metal ions.

Pioneering work in the field was conducted in the period 1950–1970 by a few outstanding scientists. Perutz et al. solved the X-ray structure of haemoglobin [1,2]; Coleman [3], Lindskog [4,5], Lipscomb and co-workers [6] and Vallee et al. [7,8] gave fundamental contributions to the discovery and the characterization of zinc enzymes; Malkin and Malmstrom [9] and Beinert [10] clarified the bioinorganic chemistry of copper; Ballhausen and Gray [11] rationalized the electronic structures of the metal centres of metalloproteins; Williams et al. [12–14] worked on both chemical models of bioinorganic systems and metalloproteins, and was provocative with respect to the interpretation of the involvement of metal ions in living systems.

The appearance of the book edited by Eichhorn in 1973, [15] consecrated, in a certain sense, the birth of this new branch of science. The list of contributors included, among others, D.A. Buckingham, H.B. Gray, J.B. Neilands, E. Breslow, P.M. Harrison, P. Aisen, J.E. Coleman, T.G. Spiro, W.H. Orme Johnson, J.M. Rifkind, H.A.O. Hill, and R.H. Holm.

Presently, we do not have clear ideas about the role of all metal ions in living organisms. In certain roles, metal ions are present in such small amounts that they are hardly detected. In other cases, however, the chemistry of metal ions is known in detail. For example, the chemistry of zinc in several zinc enzymes is well characterized [16,17]. Analogously, a lot is known on the transport of dioxygen [18] and its utilization [19]. Electron transfer processes and the molecules involved have been extensively investigated [20] as have the chemistry of calcium [21], the role of metal ions in medicine [22], the interaction of metal ions with DNA [23], etc.

Other areas such as nitrogen fixation [24], hydrogen production [25] and photosynthesis [26] seem more complex; their systematic investigation has started only recently. A comprehensive book on biochemistry and metal ions has now appeared [17].

Whereas biophysical research, at a molecular level, characterizes the structure and the function of in vitro biomolecules, many more problems arise when dealing

with in vivo systems. Fundamental questions appear of the type: why is potassium inside the cell and sodium mainly outside?; which mechanisms regulate the concentration of metal ions inside a cell?; how is the biological response elicited by a toxic element?; and so on.

All these questions direct us towards the study of living organisms as a whole. This implies a kind of science somewhat different from what chemists are accustomed to, requiring the contribution of many specialists from different areas.

Let us take a bacterium and let it grow on a medium which contains certain substrates. We then proceed with the identification of the metabolites that it produces during the consumption of substrates. We can relate each metabolite to a net of enzymes which governs the degradation pattern and the enzymes to the genes encoding them. We can also introduce plasmids into the bacterium to improve the catabolic process. If the substrates on which the bacterium grows are pollutants, we have set up a research project of relevance to environmental sciences. Many steps in both aerobic and anaerobic microbial transformations involve redox reactions catalyzed by metalloenzymes.

The above considerations, though incomplete, imply that there is much to discover for the progress of science as a whole when studying the chemistry of metal ions.

B. FROM SACCONI'S COMPOUNDS TO METALLOPROTEINS THROUGH THE GRAY EFFECT

Only the availability of advanced biophysical investigation techniques has allowed the definite success of bioinorganic chemistry through the late 1960s and the 1970s.

In 1974, the senior author visited the laboratory of Harry Gray in Pasadena. Harry was investigating cobalt(II)-, nickel(II)- or copper(II)-substituted metalloenzymes as opposed to their natural analogues [27,28]. The visitor (I.B.) was surprised to see how seriously Harry was consulting the papers of Sacconi (and in part of the visitor) in order to compare the d–d spectra of metalloenzymes with those of the coordination compounds, characterized in Florence [29] and elsewhere.

The visitor realized soon that his background in theoretical and physical inorganic chemistry, which was the result of the teaching of Luigi Sacconi, would allow him to investigate such complicated systems as metalloenzymes. In Pasadena he had assisted Dave McMillin, now in Purdue, in preparing cobalt(II)-substituted zinc enzymes. As soon as he was back in Florence, he started studying cobalt(II)-carbonic anhydrase with acetate through electronic absorption spectroscopy and NMR. Andrea Scozzafava, junior assistant professor at that time, and the student Claudio Luchinat joined him in this venture. The first paper appeared in 1976 [30]. It reported that acetate binds to cobalt(II) in carbonic anhydrase, expanding its coordination number by one, from 4 to 5.

Transition metal ions are present in living organisms mainly bound to the

usual donor atoms: nitrogen, oxygen, sulphur. The donor atoms are generally provided by proteins through their amino acid residues. Histidine provides nitrogen, glutamate and aspartate provide oxygen, cysteine thiolate sulphur and methionine ethereal sulphur. Exogenous ligands, solvent often, provide further donor atoms. Alkaline and alkaline earth metal ions preferentially interact with cavities rich in oxygen atoms.

It follows that the approach to the investigation of metalloproteins is somewhat similar to the investigation of synthetic metal complexes. Indeed, quite often, coordination chemists have started synthesizing metal complexes as models of naturally occurring systems [31–33].

C. THE REACTIVITY OF METAL IONS IN ENZYMATIC ACTIVE CAVITIES

(i) Generalities

At variance with metal ions and coordination compounds in solution, metal ions in metalloproteins are located well inside a polypeptide chain which provides the ligands and tunes the hydrophobic or hydrophilic character of the region around them. It follows that the reactivity of metal ions in the active site of proteins may be completely changed with respect to conventional solution chemistry [34]. When a molecule such as a substrate or an inhibitor enters an enzymatic cavity, it liberates the solvent molecules trapped inside. This implies a large entropic increase that has no counterparts in bulk solution reactions. Furthermore, one has to take into account the enthalpic factors arising from hydrophobic and/or hydrophilic interactions, in addition, of course, to those arising from the binding of the molecule under investigation to the metal ion itself. Examples of this kind are many: we will discuss here some of them which have been contributed by the Florence group.

(ii) Zinc in carbonic anhydrase

Carbonic anhydrase is a zinc enzyme which catalyzes the simple reaction:

$$CO_2 + H_2O \rightleftharpoons HCO_3^- + H^+$$

This reaction, fundamental for the life of higher organisms, is so slow at neutrality that life would not be permitted ($k = 3.5 \times 10^{-2}\ s^{-1}$) in the absence of the enzyme [34]. On the other hand, the reaction:

$$CO_2 + OH^- \rightleftharpoons HCO_3^-$$

is very fast ($k = 8.5 \times 10^3\ s^{-1}\ M^{-1}$) [35].

At pH 10, where $[OH^-] = 10^{-4}$, the two reactions have nearly the same rate. It follows that the peculiar role of the enzyme is that of providing a ZnOH group

at pH 7 in such a way that the reaction proceeds through the following steps [36]:

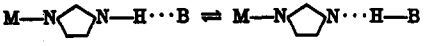
$$Zn{-}OH + CO_2 \rightleftharpoons Zn{-}(HCO_3^-)$$

$$Zn{-}(HCO_3^-) + H_2O \rightleftharpoons Zn(OH_2) + HCO_3^-$$

$$Zn(OH_2) \rightleftharpoons Zn{-}OH + H^+$$

The low pK_a of the $Zn(OH_2)$ moiety is reached through three factors: (a) a tetrahedral complex with neutral ligands; (b) the interaction with other acidic groups; and (c) the hydrophobicity of the cavity.

Three neutral histidines are the zinc ligands [37,38]. It should be noted that bound histidines are often hydrogen bonded to other residues (backbone CO, COO^-, Asn, etc.) (Fig. 1). In this way, the enzyme has full control of the orientation of the metal ligands [39]. Furthermore, if the hydrogen bonds are strong, two chemical species may coexist, schematically represented by the following scheme:



The relative population of the two species depends on many factors, such as the charge of the metal M, the charge density of the base B, and so on. Thus, the protein environment tunes the basicity of the metal ligands [39].

In human carbonic anhydrase, the NH groups of the three coordinated histidines are hydrogen bonded to Glu-117, to Gln-92 and to Asn-244. Other acid/base groups, in addition to $ZnOH_2$, are present in the cavity, e.g. histidines and, in some

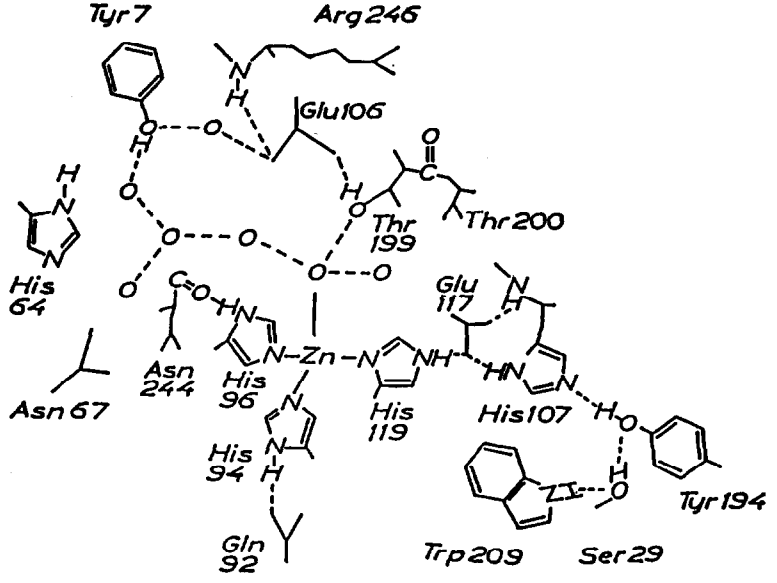


Fig. 1. Schematic drawing of the active site of human carbonic anhydrase II.

isoenzymes, arginines. Part of the cavity is formed by hydrophobic groups such as Trp, Val, Lys, etc. whereas part is hydrophilic with threonines.

Ab initio calculations are available [40], which analyze the above factors with respect to the pK_a of $ZnOH_2$ and to the nucleophilicity of the ZnOH moiety. The hydrophobic part of the cavity is thought to be responsible for attracting CO_2 [41]. A reasonable intermediate step could be with CO_2 interacting with the NH of residue Thr-200 and pointing, with the other end, towards the metal. In this way the OH nucleophile, which is oriented by zinc and by the hydrogen bond with Thr-199, would be in a position perfectly suitable for nucleophilic attack.

Anions are capable of binding zinc and are therefore competitive inhibitors of bicarbonate dehydration [42]. From analysis of the electronic spectra of the cobalt(II)-substituted derivative, we proposed that sulphonamide binds in a tetrahedral way by replacing the coordinated water; indeed, the absorption spectra of the inhibited enzyme in the visible region are intense, indicating tetracoordination (Fig. 2) [43]. The X-ray structure eventually confirmed this hypothesis (Fig. 3) [37]. We also suggested that thiocyanate and acetate add to the coordination sphere without substituting any of the other four ligands [43]. Both the low intensity of the absorption spectra and the presence of a band at 14 000 cm^{-1} (Fig. 2) were taken as evidence of five coordination. Again, the crystallographic structure confirmed this suggestion for the thiocyanate derivative [37].

(iii) Zinc in carboxypeptidase

Carboxypeptidase A is a zinc enzyme which catalyzes the hydrolysis of the carboxy terminal amino acid of a peptidic chain [44–46]. The enzyme shows marked preference for substrates with aromatic amino acids. Extensive crystal data on bovine carboxypeptidase A and a number of adducts with pseudo-substrates and inhibitors are available. They allow deep insight into the structural features of the enzyme (Fig. 4) [47–51]. It can be observed that, near the metal centre, there is a hydrophobic pocket capable of accommodating the aromatic ring of the substrate. Two nearby residues are strategic for the stabilization of the substrate: an arginine (Arg-145), at the bottom of the cavity, forms a hydrogen bond with the carboxylate group of the incoming peptide; a tyrosine (Tyr-248) forms a hydrogen bond with the peptidic NH. Until 1990, it was believed that the carbonyl group could bind zinc in such a way that the carbon atom is activated for nucleophilic attack. This mechanism looked very similar to that proposed by inorganic chemists for catalysis by metal ions and coordination compounds. Then all the debate in the literature focused on the groups carrying out the nucleophilic attack. The following groups were the probable candidates for the attack:

(a) Glu-270, leading to an anhydride intermediate [52];
(b) water, activated by the nearby Glu-270 (general base mechanism) [53]; and
(c) a possible ZnOH group (metal hydroxide mechanism) [16].

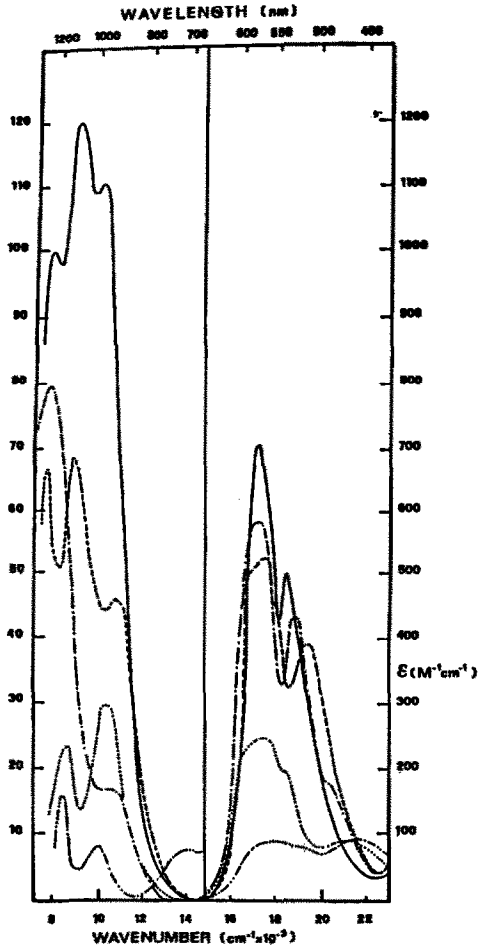


Fig. 2. Electronic spectra of cyanide (——), cyanate (–·–), acetazolamide (– – –), azide (···), and thiocyanate (–···–) adducts of cobalt(II)-substituted bovine carbonic anhydrase II [43].

Nowadays, it is more and more believed that the peptidic carbonyl interacts with Arg-127 and is thus activated for the nucleophilic attack (Fig. 5). The reaction proceeds by the attack of the zinc-bound water on the scissile carbonyl according to the following sequence of events.

Zinc is coordinated by two histidines (His-69 and His-196) and one glutamate (Glu-72). The zinc-coordinated water molecule is hydrogen bonded with Glu-270. This hydrogen bond plays a strategic role in the whole catalytic process. As a proof of its importance, we remember here that, in the absence of Glu-270, the genetically engineered enzyme becomes nearly inactive [54]. We may think that the water-Glu

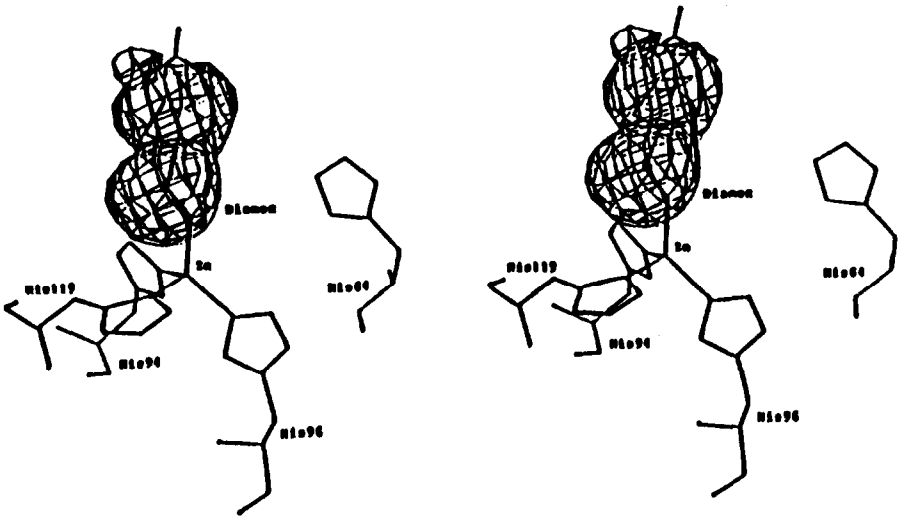


Fig. 3. Stereo view of the acetazolamide (diamox) adduct of HCAII [37].

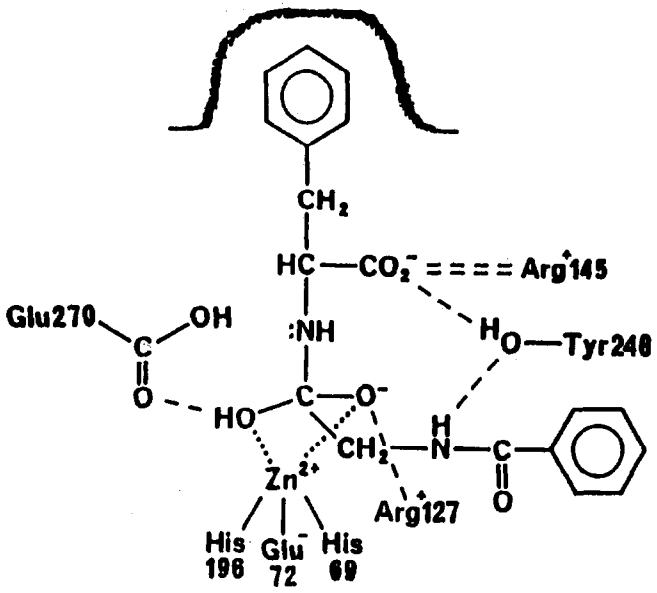
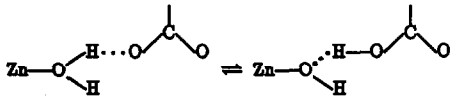


Fig. 4. Schematic drawing of the active site of carboxypeptidase A.

270 hydrogen bond is shared by the two counterparts in a tautomeric equilibrium:



The zinc-hydroxo species is perfectly oriented to perform the nucleophilic attack on

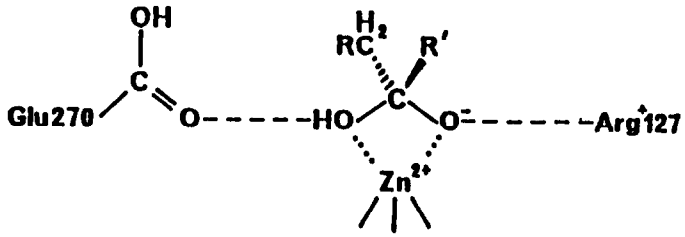


Fig. 5. The nucleophilic attack to the scissile carbonyl of the substrate in carboxypeptidase.

the carbonyl carbon of an amino acid residue oriented in the cavity as previously described. Once the attack is performed, one proton is provided by Tyr-248, which takes it back from the indissociated form of Glu-270. This situation roughly corresponds to having the amino acidic residue bound to Arg-145 with COO^- and to Glu-270 with NH_3^+. Finally, water substitutes the carboxylate group. The products leave and the $Zn{-}OH_2$/Glu-270–COO^- hydrogen bond forms again.

$$Zn{-}O\diagdown_{H} + R'{-}NH{-}CO{-}R + H^+ \rightarrow Zn{-}O{-}C\lessdot^{OH}_{R} + R'NH_2$$

If the hydrogen bond net around the coordinated water accommodates a further proton (acidic form), the enzyme becomes inactive [55,56].

Amino acids and even carboxylates interact with Arg-145. Their binding affects the electric potential inside the cavity in such a way that the Glu-270/water interaction is weakened. It follows that anions such as thiocyanate, cyanate and azide can now bind zinc. Many complexes such as CPA-AA, CPA-$(AA)_2$ and CPA-AAX (X = pseudo-halide) have been spectroscopically described for cobalt(II) carboxypeptidase [57–61] and, in some cases, their structure confirmed by X-ray analysis [62].

(iv) Copper in copper zinc superoxide dismutase

Superoxide dismutases (SOD) from higher organisms [63,64] contain two identical subunits each with a copper–zinc dimeric unit. The molecular mass of the dimer is 32 000. Copper(II) sits at the bottom of a cavity; above it there is an arginine residue (Arg-143) (Fig. 6). The enzyme in vitro catalyzes the dismutation of the ion superoxide [65,66]. When the positive Arg-143 residue is substituted with a residue bearing a negative charge, such as a glutamate, the enzyme looses activity [67]. The substitution of Arg-143 with an isoleucine reduces the activity to 11% [67]. The measurement of the activity is performed at low O_2^- concentration and, probably, it is just a measure of k_{on}. The activity of the mutants on residue 143 parallels the affinity constant for azide [67]. When the negative Glu-133 is substituted by the neutral Gln, the enzyme exhibits faster reaction rates and also the affinity for N_3^- is

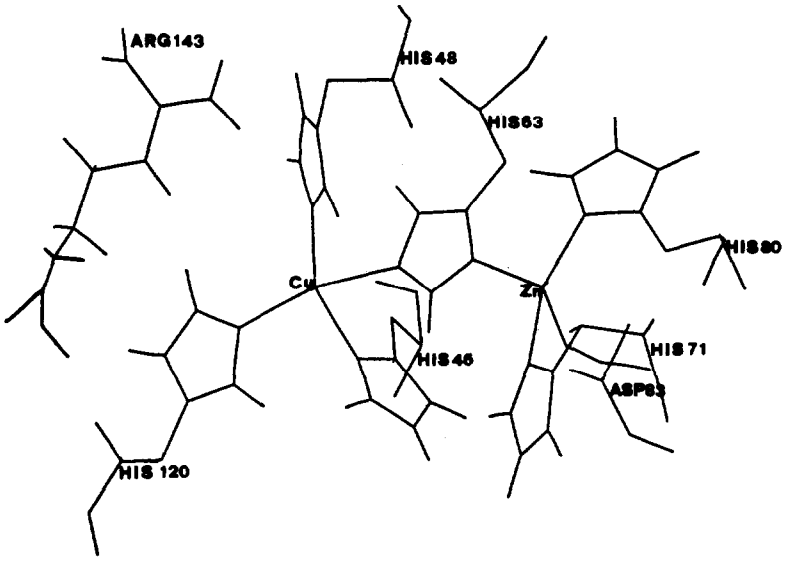


Fig. 6. Computer graphics representation of the active cavity of copper zinc superoxide dismutase.

increased [68]. It seems that electrostatic charges determine both the affinity of azide and the enzymatic activity [67,68]. The change of arginine with lysine also shows that the position of the positive charge is important. This is an indication that the ion superoxide enters the cavity; it is probably accommodated between the copper ion and Arg-143.

Despite the fact that the reaction:

$$Cu^{2+} + O_2^- \rightarrow Cu^+ + O_2$$

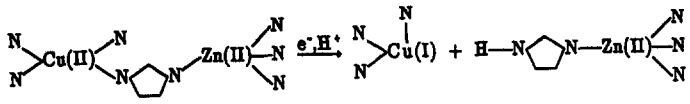
is thermodynamically favoured, the presence of a hydrogen bond with O_2^- prevents the electron flowing from oxygen to copper [69–71]. This indicates that the further step of the reaction is a bimolecular reaction of the type:

$$Cu^{2+}{-}O_2^- \cdots H^+ + O_2^- \rightarrow Cu^+ \cdots O_2^- \cdots H^+ + O_2$$

The hydrogen bond with Arg-143 would then be responsible for attracting one electron from the copper(I) ion to reform the catalyst and the peroxide anion. Studies of the mutants which cannot form the hydrogen bond (typically Ile-143) indicate that the hydrogen bond can break, say, at the picosecond level; then the oxidation of O_2^- occurs. Copper has been found to interact with a semicoordinated water molecule at 2.5 Å [72,73]. Its presence or absence in the various investigated mutants is not relevant for the enzymatic activity [74]. It follows that its substitution by O_2^- is not a rate-limiting step.

The X-ray structure of the oxidized enzyme shows that a histidinato bridge (Fig. 7) connects the two metal ions. Other residues complete the coordination

polyhedron. The enzyme can be reduced chemically with dithionite. Upon reduction, the bridge breaks down and the bridging ligand is protonated on the side of copper.



This is nicely demonstrated by ^{1}H NMR spectroscopy [75] and by the electronic spectra of the derivative with cobalt(II) in the place of zinc(II) [76]. Apart from the breaking of the histidine bridge, the structure of the enzyme remains essentially the same. This reasoning suggests that the first step of the oxidation of superoxide requires a proton. Since the concentration of protons at neutral or higher pH values is small, this requirement is a severe limiting step of the reaction. From unique measurements on O_2^- saturated solutions, Fee has suggested that the protons in the reaction are provided by H_2O [77]. Even the profile of the reduction potential of the enzyme with pH, measured electrochemically (Fig. 8), indicates that the reduction reaction is of the type [78]:

$$Cu^{2+} + e^- + H^+ + E \rightarrow Cu^+ + EH$$

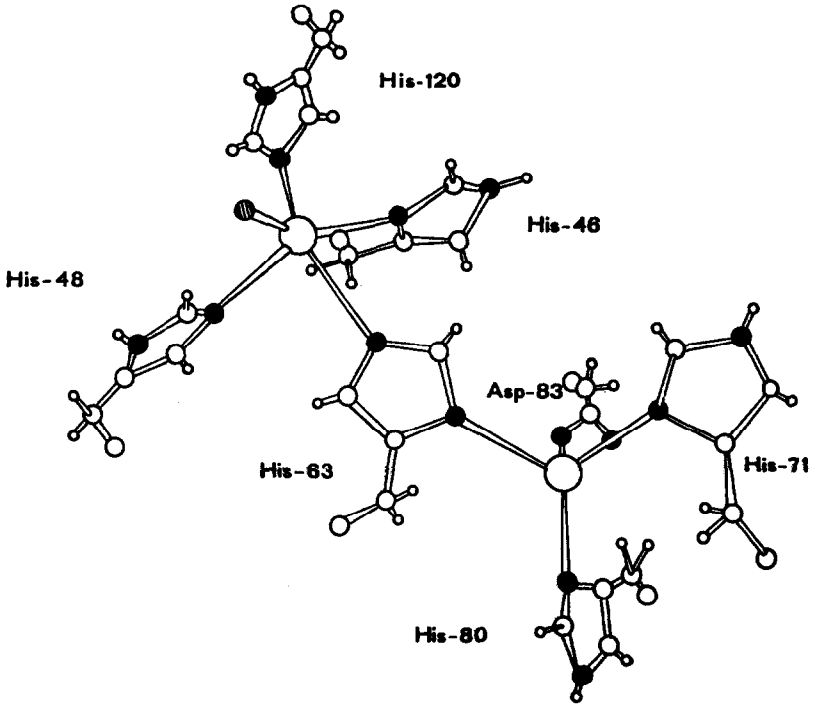
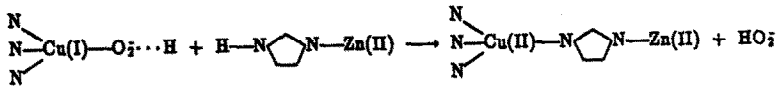


Fig. 7. Schematic drawing of the metal sites in superoxide dismutase.

A further O_2^- binds copper(I) and the oxidation of copper(I) occurs with the assistance of the formerly bridged histidine.



Anions such as cyanide and azide are competitive inhibitors of the dismutative reaction [79,80]. They bind copper(II) and partially remove one His ligand [81,82].

(v) Iron in peroxidases

Peroxidases are an important class of metalloenzymes which contain one iron(III) haeme moiety, with a coordinated apical histidine, at the active site (Fig. 9) [83–85]. The iron(III) ion is high-spin; the reduction potential of the couple Fe^{3+}/Fe^{2+} is negative. Probably, the latter feature is due to a partial anionic character of the coordinated histidine as only this class of haeme proteins shows the occurrence of strong hydrogen bond interactions between the apical histidine and a nearby carboxylate residue [85]. Several peroxidases, from different sources, exhibit molecular weights in the range 30 000–50 000, all having a single haeme group per molecule

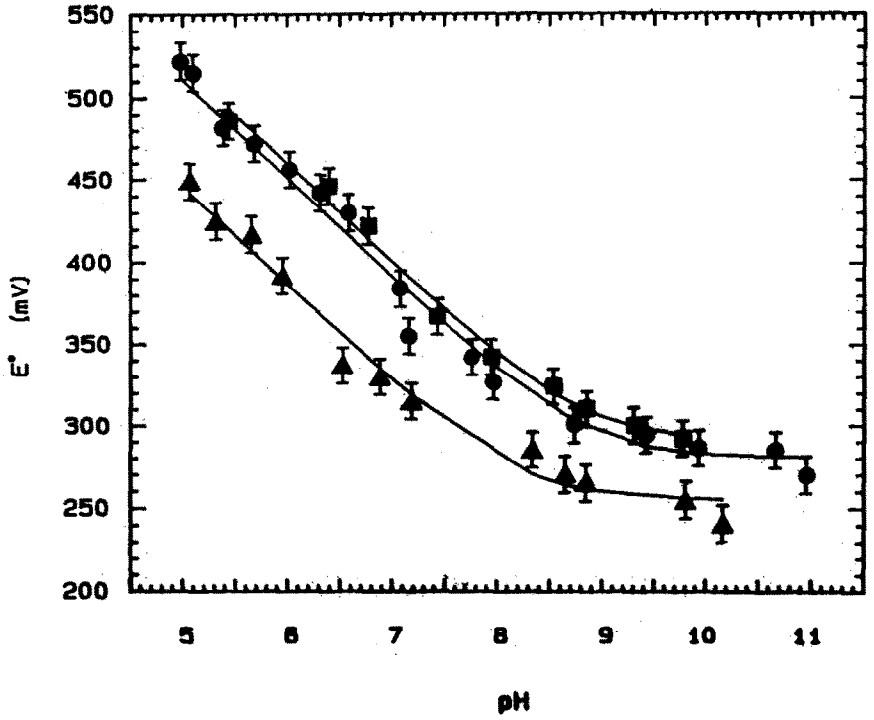


Fig. 8. pH dependence of the redox potential for HSOD (●), HSOD-AS (■), and BESOD (▲) [78].

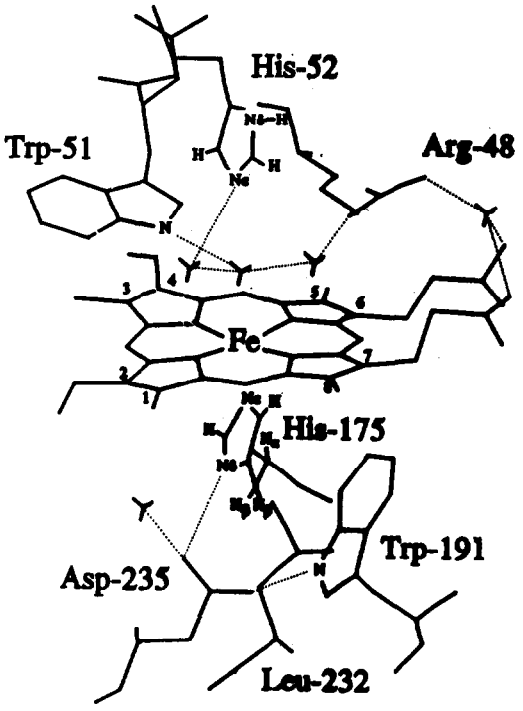


Fig. 9. Schematic drawing of the active site of cytochrome *c* peroxidase.

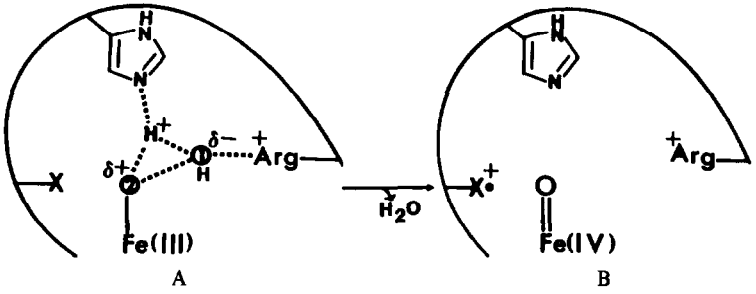


Fig. 10. Schematic representation of the peroxidase transition state (A) and Compound I (B). X represents the site of free radical formation [85].

[83,84]. On the distal side of the haeme, a histidine and an arginine are usually found, in addition to other hydrophobic groups [85]. The enzyme reacts with hydrogen peroxide to provide a two-electron oxidized derivative. It has been shown that the resulting derivative contains a ferryl group plus a radical, located on the porphyrin ring or on a close aromatic residue [86,87]. This species is usually called Compound I (Fig. 10). The arginine and the distal histidine residues assist the incoming hydrogen peroxide molecule in the course of the heterolytic cleavage of the O—O bond [85]. Compound I behaves as a powerful oxidizing agent, mainly through radical reactions.

Different peroxidases act on different substrates. One member of the peroxidase family is capable of oxidizing the naturally occurring lignin polimer [88,89]; it is called lignin peroxidase. Cyt *c* peroxidase oxidizes cytochrome *c*; horseradish peroxidase oxidizes various aromatic substrates [83,84].

The cyanide derivatives of these enzymes are invariably low-spin, and thus particularly suitable for ^{1}H NMR investigations in spite of their being paramagnetic [90–96]. Apart from structural information, ^{1}H NMR studies permitted the shift pattern of the apical His to be related to the Fe^{3+}/Fe^{2+} reduction potential of the native enzyme; correlations with the imidazolate character of the apical histidine were also attempted [96].

Mutants of cytochrome *c* peroxidase in which the aspartate residue hydrogen bonded to the apical histidine is removed display a lower oxidation potential and a consistent ^{1}H NMR pattern [97].

In horseradish peroxidase and, possibly, in lignin peroxidase, the radical in Compound I is a porphyrin ring [98,99]. In Cyt *c* peroxidase, the radical is a tryptophan [86,87]. Substitution of tryptophan with phenylalanine provides a derivative whose Compound I again exhibits the porphyrin ring in a radical form [86,87,100].

D. THE ELECTRONIC STRUCTURE OF POLYMETALLIC SYSTEMS IN PROTEINS

Just as polymetallic systems have attracted the interest of inorganic chemists for their challenging electronic structure, bioinorganic chemists have been attracted by the role of polymetallic systems in proteins. We will mention here the copper–cobalt, copper–copper and copper–nickel heterodimetallic centres in superoxide dismutase, the Co_4 cluster in Co_7 metallothioneins, and the Fe_2S_2 and Fe_4S_4 clusters in iron–sulphur proteins.

(i) Metallo-substituted superoxide dismutases

Zinc in copper–zinc superoxide dismutase can be removed and substituted by other divalent metal ions, usually without breaking the histidine bridge [64]. When zinc is substituted by paramagnetic metal ions, the presence of the aromatic bridge allows the establishment of magnetic coupling [101]. In the case of cobalt(II) in the zinc site, we have a $S = 1$ ground state separated from the excited $S = 2$ state by 33 cm^{-1} (Fig. 11). This corresponds to a J value of ~ 17 cm^{-1} if the antiferromagnetic coupling is described by the Hamiltonian $J\hat{S}_1 \cdot \hat{S}_2$, $\hat{S}_1$ operating on $S_1 = 1/2$ and $\hat{S}_2$ on $S_2 = 3/2$. This is the energetic description.

Another effect is that the electron of copper(II) relaxes at a rate similar to that of the electrons of cobalt(II) [102]. Indeed, the magnetic coupling provides an efficient relaxation mechanism for both spins. As a result, hyperfine shifted ^{1}H NMR spectra of the Cu_2Co_2SOD species can be obtained (Fig. 12). The spectra could be assigned

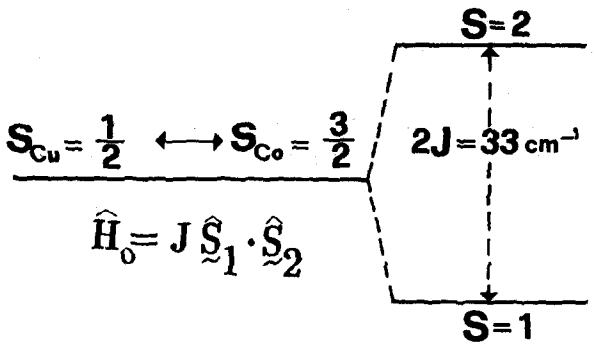


Fig. 11. Scheme of the energy levels in Cu_2Co_2SOD.

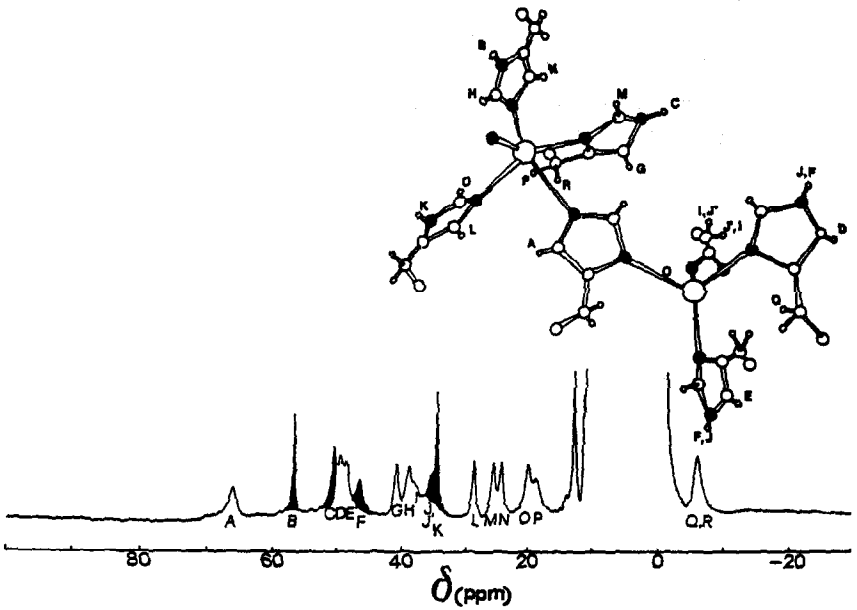


Fig. 12. 300 MHz ^1H NMR spectrum of Cu_2Co_2SOD. The filled signals disappear in D_2O. The spectrum is recorded in acetate buffer at pH 5.5 [103].

with the help of ^1H NOE experiments [103,104]. Cu_2Co_2SOD has proved to be an important spectroscopic probe (a) to demonstrate that the structure of the Cu_2Co_2-SOD is identical to that of the native enzyme [104]; (b) to follow the structural variations which occur in a series of mutants [68,105,106]; and (c) to monitor the interactions with anions [82]. All these topics have been reviewed recently [81].

In Cu_2Cu_2SOD, the magnetic coupling povides a J value of 26 cm^{-1} with the $S=0$ ground state separated by 52 cm^{-1} from the $S=1$ state [107]. The electronic relaxation rates of the two copper ions are almost identical to those of the copper ion in native SOD [108].

In Cu_2Ni_2SOD, there is probably antiferromagnetic coupling with an $S = 1/2$ ground state [109]. The tetrahedral nickel(II) ion has electronic relaxation rates larger than those of tetrahedral cobalt(II) [110]. The ^1H NMR spectra are sharp (Fig. 13); even 2D NMR spectra could be obtained [111]. From analysis of the spectra, it emerges that the environment of the copper(II) centre is virtually identical to that found in Cu_2Co_2SOD.

(ii) Cobalt(II) metallothioneins

Metallothioneins are small proteins (MW $\simeq$ 6000) with a high content of cysteines [112,113]. They probably function as storage proteins for divalent metal ions. Metallothioneins can bind up to seven zinc(II) or cadmium(II) ions per molecule [114]. The X-ray structure of the Cd_5Zn_2 protein has shown that the seven metal ions are arranged in two clusters of four and three, as reported in Fig. 14 [115]. The solution structure of the Cd_7 derivative determined by 2D NMR spectroscopy had already shown the overall arrangement of the two clusters [116,117].

The paramagnetic cobalt(II) derivative of metallothionein provides a good

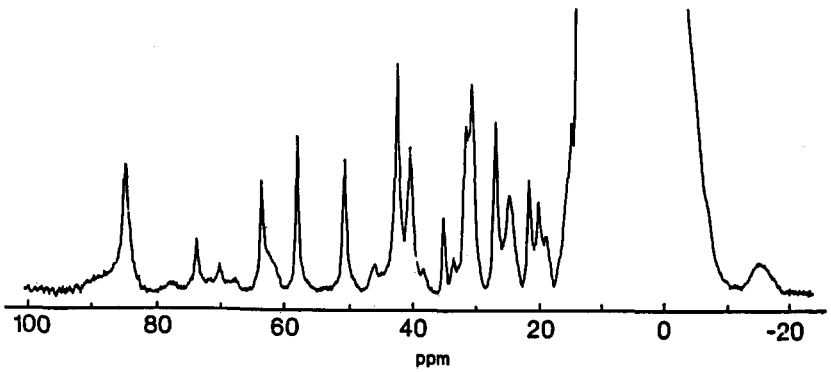


Fig. 13. 200 MHz ^1H NMR spectrum of Cu_2Ni_2SOD. The spectrum is recorded in phosphate buffer at pH 6.5.

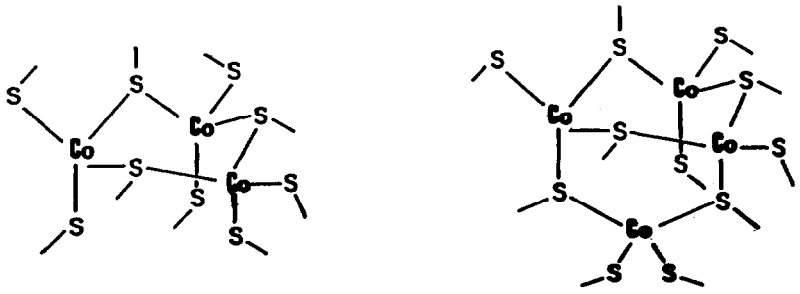


Fig. 14. Scheme of the two clusters (Co_3 and Co_4) in cobalt(II)-substituted metallothionein [118].

chance to follow the progressive formation of the two metal clusters. We have titrated the demetallized protein with cobalt(II) using magnetic susceptibility and ^{1}H NMR (Fig. 15) [118]. From these studies, it appears that, at low cobalt(II):protein stoichiometries (cobalt(II)/protein < 3), cobalt(II) binds the protein and exchanges among various binding sites. At a cobalt(II)/protein ratio of 3:1, a trimetallic cluster is obtained with strong antiferromagnetic coupling. Above a 4:1 ratio, the cluster transforms into a Co_4 cluster. Then the second Co_3 cluster is formed. Owing to the different dynamic properties, only the Co_4 cluster generates sharp ^{1}H NMR spectra (Fig. 15).

The temperature dependence of the ^{1}H NMR spectra has been theoretically interpreted as consistent with five similar J values among the four cobalt ions, the

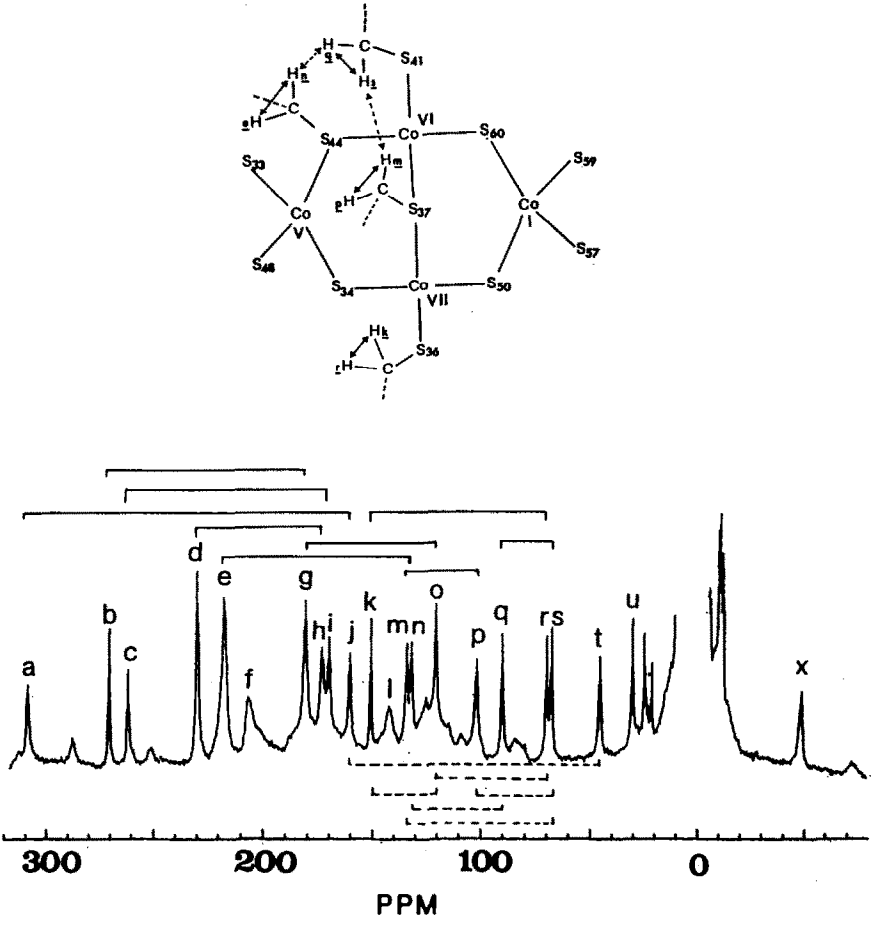


Fig. 15. 200 MHz ^{1}H NMR spectrum of the Co_4 cluster in cobalt(II)-substituted metallothionein. The broken and the continuous lines indicate the connectives, detected through NOE experiments, between some protons of the cluster [118].

sixth J value being close to zero [119]. The J values are about 50 cm^{-1} [119]. Preliminary ^1H NOE studies of the Co_4 cluster suggests that it is roughly similar to the Zn_4 cluster [120].

(iii) Iron sulphur proteins with an Fe_2S_2 cluster

The Fe_2S_2 iron sulphur proteins [121–124] are electron carrier proteins of low molecular weight (about 6000) (Fig. 16) [121]. In the oxidized form, both iron ions are in the 3+ oxidation state whereas in the reduced state they are 3+ and 2+, respectively [125]. The two ions are antiferromagnetically coupled with $J = 400$ cm^{-1} in the oxidized state and 200 cm^{-1} in the reduced state [126–129]. Much interest has been raised by the latter situation because the ground state $S = 1/2$ can be investigated by EPR [128]; in addition, the magnetic coupling between $S = 5/2$ and $S = 2$ increases the electronic relaxation rates and allows investigation by ^1H NMR (Fig. 17) [130]. ^1H NMR studies have nicely shown that there is no equivalence between the two iron ions and that the iron centre in the 3+ oxidation state is always the same [131]. This behaviour could be unambiguously ascertained based on the fact that the four signals of the four β-CH_2 protons of the cysteines bound to iron(III) were identified as well as those of the β-CH_2 protons of the cysteines bound to iron(II) [129–131]. Nuclear Overhauser effect studies established that the cysteines bound to iron(II) have two protons of two different CH_2 moieties very close to each other [131]. Then, inspection of the X-ray structure [132] allowed the assignment of the iron(II) as the one on the surface of the protein; this is the reducible iron ion. The sulphur donors of the latter iron ion form three hydrogen bonds with peptidic NH groups, whereas the other iron ion forms only one hydrogen bond

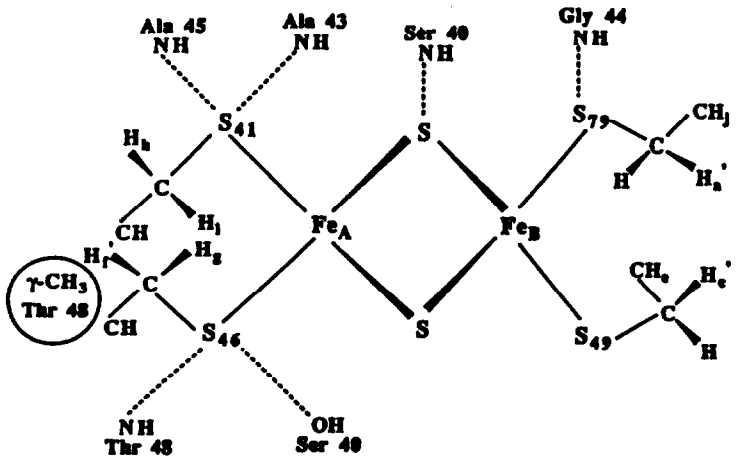


Fig. 16. Schematic drawing of the active site of *Spirulina platensis* Fe_2S_2 ferredoxin [131].

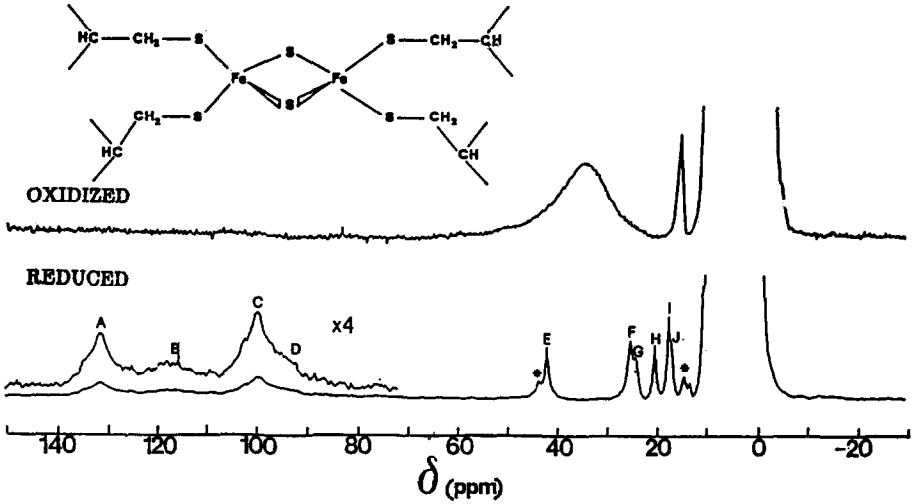


Fig. 17. Typical ^1H NMR spectra of oxidised and reduced Fe_2S_2 ferredoxins from spinach [130].

[132]. Hydrogen bonds give rise to a positive electrostatic field around the metal ion which increases the redox potential.

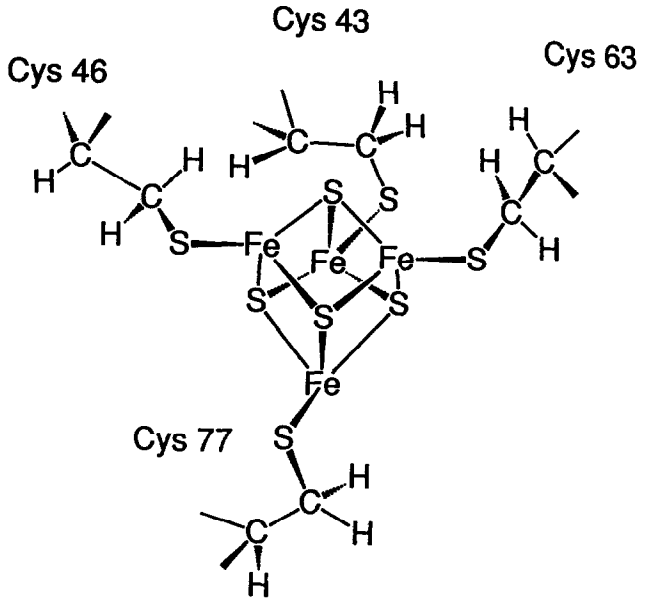


Fig. 18. Schematic drawing of the Fe_4S_4 cluster of HiPIP from *Chromatium vinosum*.

(iv) Iron sulphur proteins with an Fe_4S_4 cluster

The Fe_4S_4 clusters are classified according to the proteins to which they belong [121–124]. We have studied the clusters belonging to high-potential iron sulphur proteins (HiPIPs) and those belonging to ferredoxins. The general structure of Fe_4S_4 cluster is shown in Fig. 18. The role of the cluster in HiPIPs is not yet clear [121–124]; on the other hand, it has been established that ferredoxins serve as electron transfer proteins [132,133]. The formal oxidation states of the iron ions in the Fe_4S_4 cluster of HiPIP correspond to three Fe^{3+} and one Fe^{2+} in the oxidized form and to two

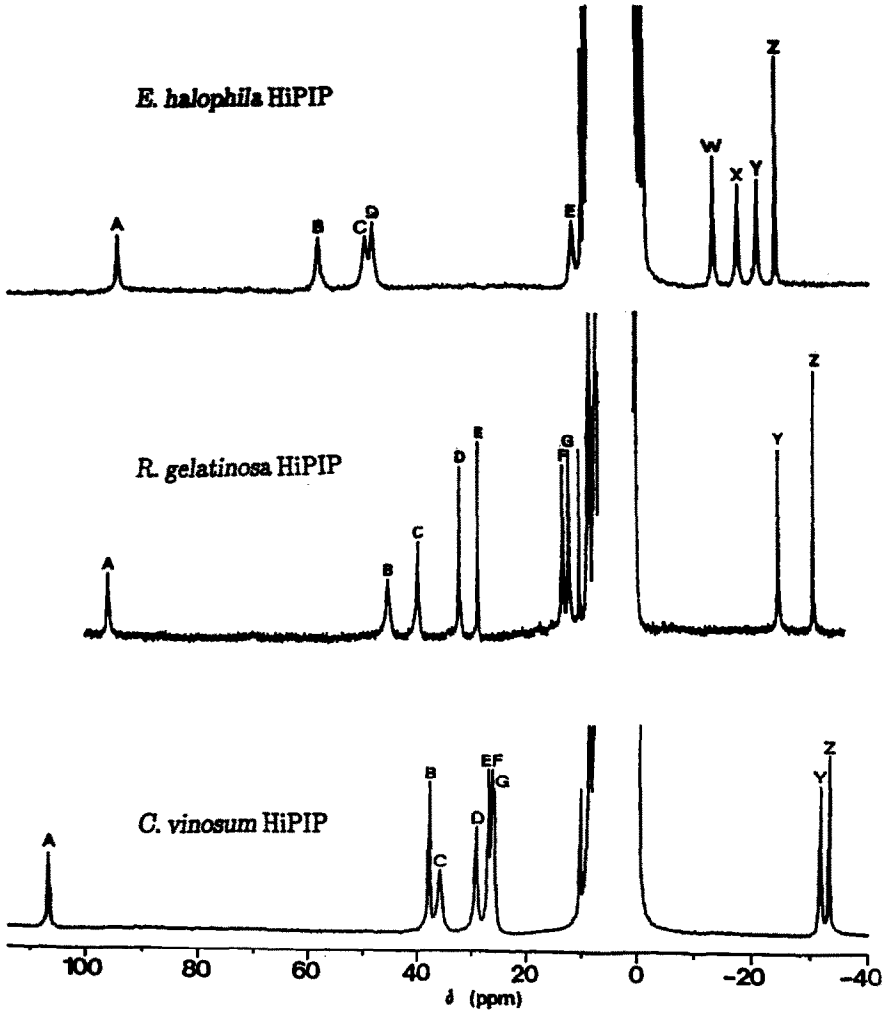


Fig. 19. ^1H NMR spectra of a series of oxidised HiPIPs form different sources containing the Fe_4S_4 cluster [137].

Fe^{3+} and two Fe^{2+} in the reduced form [134]. The reduction potentials range from 450 to 200 mV [135]. On the other hand, in ferredoxins, formally we find two Fe^{3+} and two Fe^{2+} in the oxidized form and three Fe^{2+} and one Fe^{3+} in the reduced form [121–124].

The ^1H NMR spectra (Fig. 19), recorded and interpreted in Florence [136–139] together with Mössbauer results [140,141], permitted a deeper insight into the electronic structure of these fascinating clusters.

Using an appropriate theoretical model for antiferromagnetic coupling, a satisfactory description of the electronic structure of the clusters could be achieved. In oxidized HiPIP there is a mixed valence pair with oxidation state 2.5+ whereas the other two iron ions are in the oxidation state 3+. This result is obtained with a smaller antiferromagnetic coupling in the Fe(III)–Fe(II) pair and a larger one in the Fe(III)–Fe(III) pair. The smaller antiferromagnetic coupling can be determined by the occurrence of an electron delocalization contribution.

From inspection of the X-ray structure [142,143], it appears that the cluster is at the surface of a hydrophobic region, the mixed valence iron pair and one iron(III) ion are partially exposed to the solvent, and the other iron(III) ion is completely buried. On reduction, a cluster corresponding to that of oxidized ferredoxin is obtained. All the iron ions are in the 2.5 oxidation state. It appears that there is electronic delocalization at least pairwise. The ground state is $S = 0$.

To date, reduced ferredoxins have been studied at a lesser extent. Nevertheless, from preliminary investigations it appears that the electronic situation can be described in terms of a pair with 2.5 oxidation state plus two isolated iron(II) ions [144].

E. METAL TRANSPORT AND STORAGE

Another fascinating field of bioinorganic chemistry is represented by the metal transport processes [145]. Transferrins are proteins designed to bind trivalent metal ions though some affinity for divalent metal ions has been demonstrated [146–149]. These proteins are important to regulate free iron levels in the biological fluids in which they are found.

They are all monomeric proteins, of molecular mass 80 000, with the capacity to bind together two iron(III) ions and two carbonate anions tightly but reversibly. The relationship between the metal and the anion is synergistic in the sense that neither is bound strongly in the absence of the other [150–154].

Crystallographic analyses of diferric human lactoferrin [155–157] and rabbit serum transferrin [158] have defined the location and the nature of the iron sites in transferrins. Transferrins have essentially the same bilobal structure with one iron site in each lobe located in a cleft between two domains (Fig. 20). The iron ligands (two tyrosines, one histidine, one aspartate and the bidentate carbonate) are the same in every case, giving rise to a distorted octahedral geometry (Fig. 21).

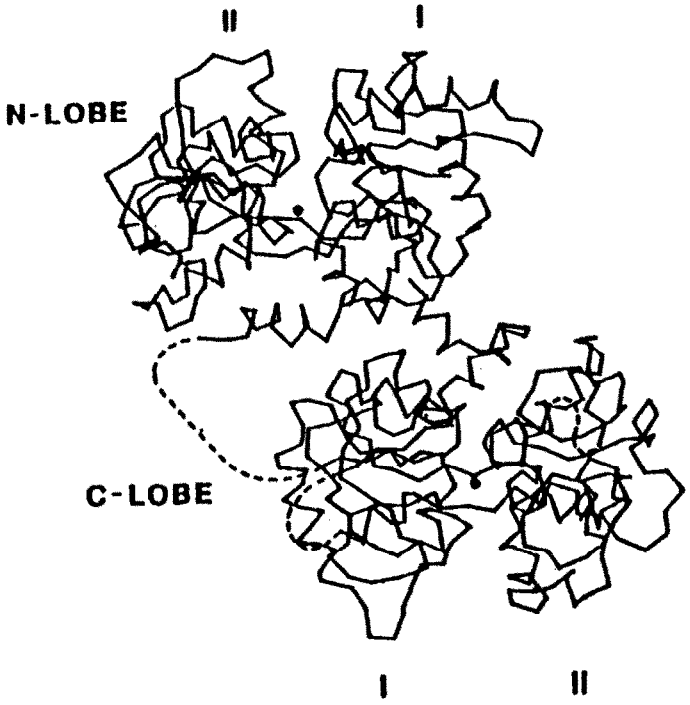


Fig. 20. General structure of rabbit serum transferrin as deduced from X-ray data [158].

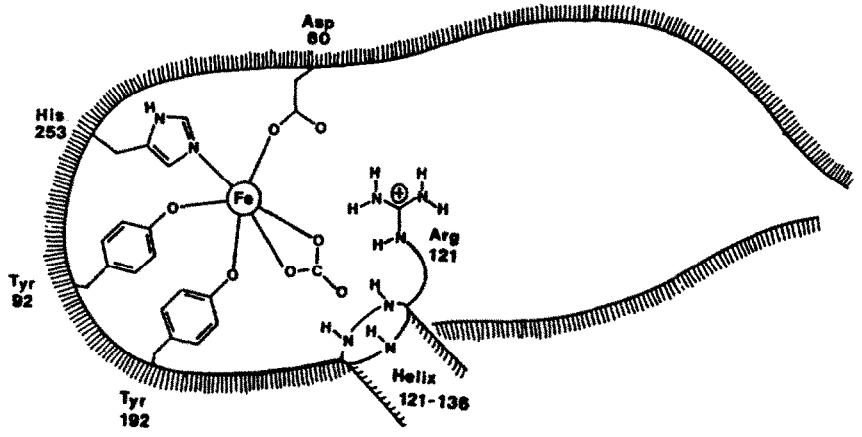


Fig. 21. Schematic drawing of the metal in transferrin [156].

A number of spectroscopic studies have been performed on native transferrins and a series of metallosubstituted derivatives with the aim of characterizing the solution chemistry of these metalloproteins, mainly based on EPR [159–161], resonance Raman [162], visible [163] and NMR spectroscopies [164–165].

The use of sensitive investigation tools resulted in the detection of small but significant structural differences between the C-terminal and the N-terminal site, which are reflected in important functional differences. Indeed, the C-terminal site exhibits a larger resistance to acids whereas the N-terminal site is kinetically more labile.

Yet, the molecular mechanism of metal uptake and release from transferrins has not been elucidated in detail. Physiologically, metal release occurs when the complex between transferrin and its membrane receptor is internalized in the cytoplasm; in the cytoplasm, transferrin releases iron through a still unknown mechanism [166] (Fig. 22). There have been different proposals according to which release may occur either by acidification or by a reductive mechanism. The first hypothesis seems to be the favoured one.

Interestingly, we can reproduce in vitro a system to simulate metal release from transferrin. Analysis of the rate of release under different solution conditions provides interesting hints concerning the molecular mechanism of release. A mechanism of the following type has been proposed [167]:

$$Fe(III)Tf{-}CO_3^{2-} \rightarrow Fe(III){-}Tf{-}CO_3^{2-}* \quad (1)$$

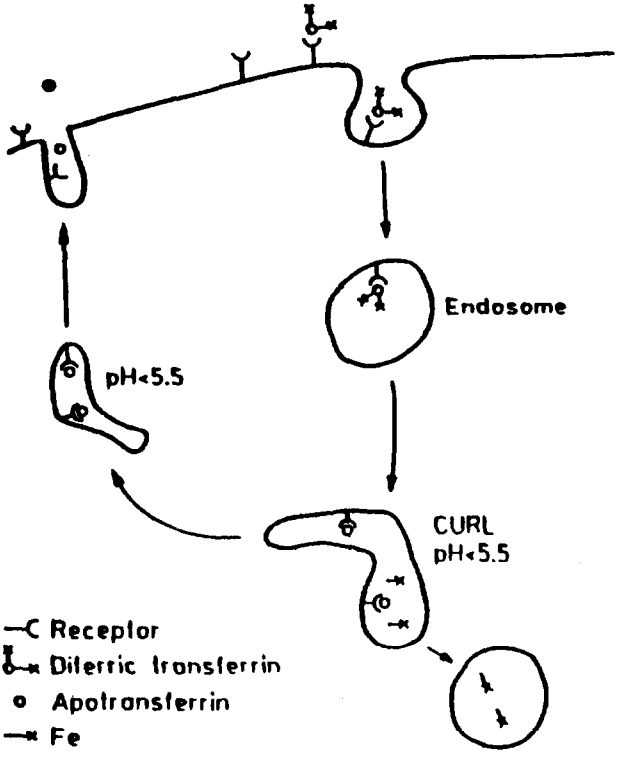


Fig. 22. Scheme of the physiological mechanism of iron release for serum transferrins [145].

$$Fe(III)–Tf–CO_3^{2-} * + Chel \rightarrow Chel–Fe(III)–Tf–CO_3^{2-} \quad (2)$$

$$Chel–Fe(III)–Tf–CO_3^{2-} \rightarrow Fe(III)–Chel + Tf–CO_3^{2-} \quad (3)$$

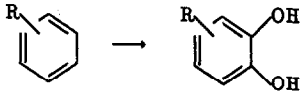
$$Tf–CO_3^{2-} + H^+ \rightarrow Tf + HCO_3^- \quad (4)$$

in which the rate-limiting step (1) is represented by a conformational change of the protein from a "close" to an "open" state. This proposal has been supported by recent crystallographic data on apolactoferrin as compared with iron lactoferrin [168]. According to these results, apolactoferrin exists in an "open conformation" which should roughly correspond to the activated conformation of the holoprotein prior to metal release.

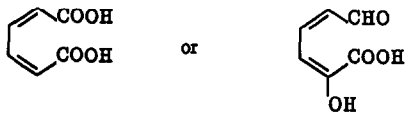
F. PERSPECTIVES

The understanding of the structure and function at a deep level of metalloproteins opens new frontiers on the importance of such systems at the metabolic level. The possibility of encoding a gene and expressing the corresponding protein makes this field of research quite fascinating. The gene for a specific protein can be isolated or synthesized and introduced into a simple living organism such as the bacterium *E. Coli* or a yeast strain. Sometimes, gene technology allows one to obtain very high level expression of the protein. Under these conditions, the protein can be produced as ^{15}N-enriched or ^{13}C-enriched or, alternatively, some amino acid can be isotopically labelled in a selective way. This result is of fundamental importance for NMR investigation since detection of heteronuclear correlations in 2D or 3D experiments enormously simplifies the assignment problem, which is the first obligatory step to determine the structure in solution. Site-directed mutagenesis provides proteins with amino acids selectively changed. This allows the investigation of the structure function relationship. When the protein is a catalyst, the possibility of changing the active site allows us to monitor the thermodynamic reaction profile and the kinetic parameters. In principle, computer systems make it possible to predict the changes needed to achieve a given purpose. For example, cuprozinc superoxide dismutase is a dimer. At the interface between the two subunits there are hydrophobic residues. By changing some of them with hydrophilic residues, a half-weight monomeric protein has been obtained. So, the combined use of computer-based techniques (from simple computer graphics to molecular mechanics and molecular dynamics) and site-directed mutagenesis provide a very powerful strategy for the investigation of the biological function of a protein. In order to determine the structure of the new derivative, proper techniques should be used. If the X-ray structure of the native protein is available, X-ray diffraction methods represent a rapid technique to obtain the structure of the mutants. Alternatively, spectroscopic tools should be used. Among them, NMR is the most powerful.

The investigation of proteins is very relevant for environmental studies. It seems that nature uses general pathways to degrade xenobiotics. For example, aromatic systems are metabolized by bacteria to produce *o*-catechols.



Then dioxygenases open the ring to produce:



Degradation proceeds until Krebs cycle compounds are obtained.

The various bacteria differ from the enzymes which carry out these reactions. Either the enzymes perform the same type of reactions but differ for the size of the cavity, redox properties, etc. or different enzymes provide different metabolic pathways. It is a current technique to transfer plasmids which encode a certain group of enzymes from a microrganism to another. This is a way of controlling chemical reactions through living organisms!

REFERENCES

1 M.F. Perutz, M.G. Rossmann, A.F. Cullis, H. Muirhead, G. Will and A.C.T. North, Nature, 185 (1960) 416.
2 M.F. Perutz, H. Muirhead, J.M. Cox and L.C.G. Goaman, Nature, 219 (1968) 131.
3 J.E. Coleman, Nature, 214 (1967) 193.
4 S. Lindskog, J. Biol. Chem., 238 (1962) 945.
5 S. Lindskog, Biochemistry, 5 (1966) 2641.
6 G.N. Reeke, J.A. Hartsuck, M.L. Ludwig, F.A. Quiocho, T.A. Steitz and W.N. Lipscomb, Proc. Natl. Acad. Sci. U.S.A., 58 (1967) 2220.
7 B.L. Vallee and H. Neurath, J. Am. Chem. Soc., 76 (1954) 5006.
8 B.L. Vallee and R.J.P. Williams, Proc. Natl. Acad. Sci. U.S.A., 59 (1968) 498.
9 R. Malkin and B.G. Malmstroem, Adv. Enzymol., 33 (1970) 177.
10 H. Beinert, in J. Peisach, P. Aisen and W.E. Blumberg (Eds.), The Biochemistry of Copper, Academic Press, New York, 1963, p. 285.
11 C.J. Ballhausen and H.B. Gray, in A.E. Martell (Ed.), Coordination Chemistry, Vol. 1, Van Nostrand Reinhold, New York, 1971, pp. 3–83.
12 R.J.P. Williams, Biol. Rev., 28 (1953) 381; Q. Rev., 24 (1970) 331.
13 J.J.R. Frausto da Silva and R.J.P. Williams, The Biological Chemistry of the Elements, Clarendon Press, Oxford, 1991.
14 A.E. Dennard and R.J.P. Williams, Transition Metal Chem., 2 (1967) 115.
15 G.L. Eichhorn (Ed.), Inorganic Biochemistry, Elsevier, Amsterdam, 1973.
16 I. Bertini, C. Luchinat, W. Maret and M. Zeppezauer (Eds.), Zinc Enzymes, Birkhäuser, Boston, 1986.
17 I. Bertini and C. Luchinat, in H.B. Gray (Ed.), Bioinorganic Chemistry, Mary Miller University Books, Los Angeles, 1992.

18 J.M. Rifkind, Adv. Inorg. Biochem., 7 (1988) 155.
G.B. Jameson and J.A. Ibers, in H.B. Gray (Ed.), Bioinorganic Chemistry, Mary Miller University Books, Los Angeles 1992.
19 T.G. Spiro (Ed.), Metal Ions in Biology, Vol. 2, Wiley, New York, 1980 and references cited therein.
J.S. Valentine, J.N. Burstyn and L.D. Margerum, in A.E. Martell and D.T. Sawyer (Eds.), Oxygen Complexes and Oxygen Activation by Transition Metals, Plenum Press, New York, 1988, pp. 175–187.
J.S. Valentine, in H.B. Gray (Ed.), Bioinorganic Chemistry, Mary Miller University Books, Los Angeles 1992.
20 H.B. Gray, B.G. Malmstrom, Biochemistry, 28 (1989) 7499.
H.B. Gray and W.R. Ellis, Jr., in H.B. Gray (Ed.), Bioinorganic Chemistry, Mary Miller University Books, Los Angeles 1992.
21 S. Forsen, in H.B. Gray (Ed.), Bioinorganic Chemistry, Mary Miller University Books, Los Angeles 1992.
22 S.J. Lippard, (Ed.), Platinum, Gold and Other Metal Chemotherapeutic Agents, ACS Symp. Ser. 209, American Chemical Society, Washington, DC, 1983.
23 J.K. Barton and S.J. Lippard, in T.G. Spiro (Ed.), Metal Ions in Biology, Vol. 1, Wiley, New York, 1980, p. 31.
T.V. O'Halloran, in H. Sigel (Ed.), Metal Ions in Biological Systems, Vol. 25, Dekker, New York, 1989, p. 105.
J.K. Barton, in H.B. Gray (Ed.), Bioinorganic Chemistry, Mary Miller University Books, Los Angeles 1992.
S.J. Lippard, in H.B. Gray (Ed.), Bioinorganic Chemistry, Mary Miller University Books, Los Angeles 1992.
24 E.I. Stiefel and S.P. Cramer, in T.G. Spiro (Ed.), Metal Ions in Biology, Vol. 7, Wiley, New York, 1985, p. 89.
E.I. Stiefel, in H.B. Gray (Ed.), Bioinorganic Chemistry, Mary Miller University Books, Los Angeles 1992.
R.N.F. Thorneley and D.J. Lowey, in T.G. Spiro (Ed.), Metal Ions in Biology, Vol. 7, Wiley, New York, 1987, p. 221.
25 J.J.G. Moura, I. Moura, M. Teixeira, A. Xavier, G.D. Fauque and J. Le Gall, in H. Sigel (Ed.), Metal Ions in Biological Systems, Vol. 23, Dekker, New York, 1988, p. 285.
26 J. Deisenhofer, H. Michel and R. Huber, Trends Biochem. Sci., 10 (1985) 243.
27 R.C. Rosenberg, C.A. Root and H.B. Gray, J. Am. Chem. Soc., 97 (1975) 21.
28 R.C. Rosenberg, C.A. Root, P.K. Bernstein and H.B. Gray, J. Am. Chem. Soc., 97 (1975) 2092.
29 R. Morassi, I. Bertini and L. Sacconi, Coord. Chem. Rev., 11 (1973) 343.
30 I. Bertini, C. Luchinat and A. Scozzafava, Biochim. Biophys. Acta, 452 (1976) 239.
31 J.T. Groves and R. Neumann, J. Am. Chem. Soc., 111 (1989) 2900.
32 J.M. Berg and R.H. Holm, in T.G. Spiro (Ed.), Iron Sulfur Proteins, Vol. 4, Wiley, New York, 1982, p. 1.
33 J.T. Groves and J.R. Olson, Inorg. Chem, 24 (1985) 2715.
34 C. Faurholt, J. Chem. Phys., 21 (1924) 400.
35 J.T. Edsall and J. Wyman, Biophys. Chem., 1 (1958) 550.
36 S. Lindskog, Adv. Inorg. Biochem., 4 (1982) 115.
37 E.A. Eriksson, T.A. Jones and A. Liljas, in I. Bertini, C. Luchinat, W. Maret and M. Zeppezauer (Eds.), Zinc Enzymes, Birkhäuser, Boston, 1986, p. 317.
38 K.K. Kannan, M. Ramanadham and T.A. Jones, Ann. N.Y. Acad. Sci., 429 (1984) 49.

39 D.W. Christianson, Adv. Protein Chem., in press.
40 I. Bertini, C. Luchinat, M. Rosi and A. Sgamellotti, Inorg. Chem., 29 (1990) 1460.
41 I. Bertini, E. Borghi and C. Luchinat, J. Am. Chem. Soc., 101 (1979) 7069.
42 Y. Pocker and S. Sarkanen, Adv. Enzymol., 47 (1978) 149.
43 I. Bertini, G. Canti, C. Luchinat and A. Scozzafava, J. Am. Chem. Soc., 100 (1978) 4873.
44 B.L. Vallee, A. Galdes, D.S. Auld and J.F. Riordan, in T.G. Spiro (Ed.), Zinc Enzymes, Wiley Interscience, New York, 1983, p. 26.
45 D.S. Auld, J.F. Riordan and B.L. Vallee, in H. Sigel (Ed.), Metal Ions in Biological Systems, Vol. 25, Dekker, New York, 1989, p. 359.
46 D. Christianson and W.N. Lipscomb, Acc. Chem. Res., 22 (1989) 62.
47 D.C. Rees, M. Lewis and W.N. Lipscomb, J. Mol. Biol., 168 (1983) 369.
48 D.C. Rees and W.N. Lipscomb, Proc. Natl. Acad. Sci. U.S.A., 80 (1983) 7151.
49 D. Christianson and W.N. Lipscomb, J. Am. Chem. Soc., 108 (1986) 545.
50 D. Christianson and W.N. Lipscomb, in I. Bertini, C. Luchinat, W. Maret and M. Zeppezauer (Eds.), Zinc Enzymes, Birkhäuser, Boston, 1986, p. 121.
51 D.W. Christianson and W.N. Lipscomb, J. Am. Chem. Soc., 110 (1988) 5560.
52 M.E. Sander and H. Witzel, in I. Bertini, C. Luchinat, W. Maret and M. Zeppezauer (Eds.), Zinc Enzymes, Birkhäuser, Boston, 1986, p. 207.
53 D.A. Buckingham, D.M. Foster and A.M. Sargeson, J. Am. Chem. Soc., 92 (1970) 6151.
54 W.J. Rutter, personal communication, 1991.
55 D.S. Auld and B.L. Vallee, Biochemistry, 9 (1970) 4352.
56 D.S. Auld, I. Bertini, A. Donaire, L. Messori and J. Moratal, Biochemistry, 31 (1992) 3840.
57 C. Luchinat, R. Monnanni, S. Roelens, B.L. Vallee and D.S. Auld, J. Inorg. Biochem., 32 (1988) 1.
58 I. Bertini, R. Monnanni, G.C. Pellacani, M. Sola, B.L. Vallee and D.S. Auld, J. Inorg. Biochem, 32 (1988) 13.
59 I. Bertini, C. Luchinat, L. Messori, R. Monnanni, D.S. Auld and J.F. Riordan, Biochemistry, 27 (1988) 8318.
60 R. Bicknell, A. Schaeffer, I. Bertini, C. Luchinat, B.L. Vallee and D.S. Auld, Biochemistry, 27 (1988) 1050.
61 I. Bertini, C. Luchinat, R. Monnanni, J.M. Moratal, A. Donaire and D.S. Auld, J. Inorg. Biochem., 39 (1990) 9.
62 D.W. Christianson, S. Mangani, G. Shoham and W.N. Lipscomb, J. Biol. Chem., 264 (1989) 12849.
63 J. Fee, in H. Sigel (Ed.), Metal Ions in Biological Systems, Vol. 13, Dekker, New York, 1981, p. 259.
64 J.S. Valentine and M.W. Pantoliano, in T.G. Spiro (Ed.), Metal Ions in Biology, Vol. 3, Wiley, New York, 1981, p. 291.
65 J.M. McCord and I. Fridovich, J. Biol. Chem., 244 (1969) 6049.
66 I. Fridovich, Adv. Enzymol., 41 (1974) 35.
67 L. Banci, I. Bertini, C. Luchinat and R.A. Hallewell, J. Am. Chem. Soc., 110 (1988) 3629.
68 E.D. Getzoff, D.E. Cabelli, C.L. Fisher, H.E. Parge, M.S. Viezzoli, L. Banci and R.A. Hallewell, Nature, in press.
69 R. Osman and H. Basch, J. Am. Chem. Soc., 106 (1984) 5710.
70 M. Rosi, A. Sgamellotti, F. Tarantelli, I. Bertini and C. Luchinat, Inorg. Chim. Acta, 107 (1985) L21.
71 M. Rosi, A. Sgamellotti, F. Tarantelli, I. Bertini and C. Luchinat, Inorg. Chem., 25 (1986) 1005.
72 N.J. Blackburn, S.S. Hasnain, N. Binsted, G.P. Diakun, C.D. Garner and P.F. Knowles, Biochem. J., 219 (1984) 985.

73 I. Bertini, F. Briganti, C. Luchinat, M. Mancini and G. Spina, J. Magn. Reson., 63 (1985) 41.
74 L. Banci, I. Bertini, R.A. Hallewell, C. Luchinat and M.S. Viezzoli, Eur. J. Biochem., 184 (1989) 125.
75 I. Bertini, C. Luchinat and R. Monnanni, J. Am. Chem. Soc., 107 (1985) 2178.
76 L. Banci, I. Bertini, C. Luchinat, R. Monnanni, A. Scozzafava and B. Salvato, Gazz. Chim. Ital., 116 (1986) 51.
77 J.A. Fee and C. Bull, J. Biol. Chem., 261 (1986) 13000.
78 H.A. Azab, L. Banci, M. Borsari, C. Luchinat, M. Sola and M.S. Viezzoli, submitted for publication.
79 A. Rigo, R. Stevanato and P. Viglino, Biochem. Biophys. Res. Commun., 79 (1977) 776.
80 A. Rigo, R. Stevanato and G. Rotilio, Biochem. Biophys. Res. Commun., 63 (1975) 1013.
81 I. Bertini, L. Banci, C. Luchinat and M. Piccioli, Coord. Chem. Rev., 100 (1990) 67.
82 L. Banci, A. Bencini, I. Bertini, C. Luchinat and M. Piccioli, Inorg. Chem., 29 (1990) 4867.
83 J. Everse, K.E. Everse and M.B. Grisham (Eds.), Peroxidases in Chemistry and Biology, Vols. 1 and 2, CRC Press, Boca Raton, 1990.
84 K.E. Hammel and M.A. Moen, Enzym. Microbiol. Technol., 13 (1991) 15.
85 T.L. Poulos, in G.L. Eichhorn and L.G. Marzilli (Eds.), Heme Proteins, Elsevier, Amsterdam, London, New York, 1988.
86 B.M. Hoffmann, J.E. Roberts, T.G. Brown, C.H. Kang and E. Margoliash, Proc. Natl. Acad. Sci. U.S.A., 76 (1979) 6132.
87 B.M. Hoffmann, J.E. Roberts, C.H. Kang and E. Margoliash, J. Biol. Chem., 256 (1981) 6556.
88 M. Tien, Crit. Rev. Microbiol., 15 (1987) 141.
89 M. Tien and T.K. Kirk, Proc. Natl. Acad. Sci. U.S.A., 81 (1984) 2280.
90 J.D. Satterlee and J.E. Erman, Biochemistry, 30 (1991) 4398.
91 V. Thanabal, J.S. de Ropp and G.N. La Mar, J. Am. Chem. Soc., 109 (1987) 7516.
92 V. Thanabal, J.S. de Ropp and G.N. La Mar, J. Am. Chem. Soc., 110 (1988) 3027.
93 J.S. de Ropp, G.N. La Mar, H. Wariishi and M.H. Gold, J. Biol. Chem., 266 (1991) 15001.
94 J.S. de Ropp and G.N. La Mar, J. Am. Chem. Soc., 113 (1991) 4348.
95 L. Banci, I. Bertini, P. Turano, J.C. Ferrer and A.G. Mauk, Inorg. Chem., 30 (1991) 4510.
96 L. Banci, I. Bertini, P. Turano, M. Tien and T.K. Kirk, Proc. Natl. Acad. Sci. U.S.A., 88 (1991) 6956.
97 J.D. Satterlee, J.E. Erman, J.M. Mauro and J. Kraut, Biochemistry, 29 (1990) 8797.
98 J.T. Groves and G.A. McClusky, Biochemical and Clinical Aspects of Oxygen, Academic Press, New York, 1979, p. 277.
99 J.T. Groves, W.J. Kruper and R.C. Haushalter, J. Am. Chem. Soc., 102 (1980) 6375.
100 L.A. Fishel, J.E. Villafranca, J.M. Mauro and J. Kraut, Biochemistry, 26 (1987) 351.
101 I. Morgenstern-Badarau, D. Cocco, A. Desideri, G. Rotilio, J. Jordanov and N. Dupre', J. Am. Chem. Soc., 108 (1986) 300.
102 L. Banci, I. Bertini, C. Luchinat and A. Scozzafava, J. Am. Chem. Soc., 109 (1987) 2328.
103 I. Bertini, G. Lanini, C. Luchinat, L. Messori, R. Monnanni and A. Scozzafava, J. Am. Chem. Soc., 107 (1985) 4391.
104 L. Banci, I. Bertini, C. Luchinat, M. Piccioli, A. Scozzafava and P. Turano, Inorg. Chem., 28 (1989) 4650.
105 I. Bertini, L. Banci and C. Luchinat, in L. Ave (Ed.), Metal Clusters in Proteins, ACS Symp. Ser. 372, American Chemical Society, Washington, DC, 1988, p. 70.
106 L. Banci, I. Bertini, D.E. Cabelli, R.A. Hallewell, C. Luchinat and M.S. Viezzoli, Inorg. Chem., 29 (1990) 2398.

107 J.A. Fee and R.G. Briggs, Biochim. Biophys. Acta, 400 (1975) 439.
108 I. Bertini, L. Banci, R.D. Brown III, S.H. Koenig and C. Luchinat, Inorg. Chem., 27 (1988) 951.
109 L.-J. Ming, L. Banci, C. Luchinat, I. Bertini and J.S. Valentine, Inorg. Chem., 27 (1988) 4458.
110 L. Banci, I. Bertini and C. Luchinat, Nuclear and Electron Relaxation, VCH, Weinheim, 1991.
111 I. Bertini, C. Luchinat, L.-J. Ming, M. Piccioli, M. Sola and J.S. Valentine, Inorg. Chem., in press.
112 J.H.R. Kaegi and A. Schaeffer, Biochemistry, 27 (1988) 8509.
113 M. Vasak and J.H.R. Kaegi, in H. Sigel (Ed.), Metal Ions in Biological Systems, Vol. 15, Dekker, New York, 1983, p. 213.
114 J.H.R. Kaegi and Y. Kojima, Experientia Suppl., 52 (1987) 25.
115 W.F. Furey, A.H. Robbins, L.L. Clancy, D.R. Winge, B.C. Wang and C.D. Stout, Science, 23 (1986) 704.
116 A. Arseniev, P. Schultze, E. Worgotter, W. Braun, G. Wagner, M. Vasak, J.H.R. Kaegi and K. Wuethrich, J. Mol. Biol., 201 (1988) 637.
117 P. Schultze, E. Worgotter, W. Braun, G. Wagner, M. Vasak, J.H.R. Kaegi and K. Wuethrich, J. Mol. Biol., 203 (1988) 251.
118 I. Bertini, C. Luchinat, L. Messori and M. Vasak, J. Am. Chem. Soc., 111 (1989) 7296.
119 I. Bertini, C. Luchinat, L. Messori and M. Vasak, J. Am. Chem. Soc., 111 (1989) 7300.
120 I. Bertini, C. Luchinat, L. Messori and M. Vasak, in preparation.
121 T.G. Spiro (Ed.), Iron–Sulfur Proteins, Vol. 4, Wiley, New York, 1982.
122 L.H. Jensen, in H. Matsubara, Y. Katsube and K. Wada (Eds.), Iron Sulfur Protein Research, Japan Scientific Societies Press/Springer Verlag, Berlin, 1987, p. 3.
123 A.J. Thompson, in P. Harrison (Ed.), Metalloproteins, Part 1, Verlag Chemie, Weinheim, 1985, p. 79.
124 R. Cammack, in H. Matsubara, Y. Katsube, K. Wada (Eds.), Iron Sulfur Protein Research, Japan Scientific Societies Press/ Springer Verlag, Berlin, 1987, p. 40.
125 J.F. Gibson, in I. Bertini and R.S. Drago (Eds.), ESR and NMR of Paramagnetic Species in Biological and Related Systems, Reidel, Dordrecht, 1980.
126 J.P. Gayda, J.F. Gibson, R. Cammack, D.O. Hall and R. Mullinger, Biochim. Biophys. Acta, 434 (1976) 154.
127 J.C. Salerno, T. Ohnishi, H. Blum and J.S. Leigh, Biochim. Biophys. Acta, 494 (1977) 191.
128 J.F. Gibson, D.O. Hall, J.H.M. Thornley and F.R. Whatley, Proc. Natl. Acad. Sci. U.S.A., 56 (1966) 987.
129 W.R. Dunham, G. Palmer, R.H. Sands and A.J. Bearden, Biochim. Biophys. Acta, 253 (1971) 373.
130 I. Bertini, G. Lanini and C. Luchinat, Inorg. Chem., 23 (1984) 2729.
131 L.B. Dugad, G.N. La Mar, L. Banci and I. Bertini, Biochemistry, 29 (1990) 2263.
132 T. Tsukihara, K. Fukuyama, M. Nakamura, Y. Katsube, N. Tanaka, M. Kakudo, K. Wada, T. Hase and H. Matsubara, J. Biochem. (Tokyo), 90 (1981) 1763.
133 J. Rabinowitz, Methods Enzymol., 24 (1972) 431.
134 I. Bertini, F. Briganti, C. Luchinat, A. Scozzafava and M. Sola, J. Am. Chem. Soc., 113 (1991) 1237.
135 A.V. Xavier, J.J.G. Moura and I. Moura, Struct. Bonding (Berlin), 43 (1981) 188.
136 I. Bertini, F. Briganti, C. Luchinat and A. Scozzafava, Inorg. Chem., 29 (1990) 1990.
137 L. Banci, I. Bertini, F. Briganti, C. Luchinat, A. Scozzafava and M. Vicens Oliver, Inorg. Chem., 30 (1991) 4517.

138 I. Bertini, F. Briganti, C. Luchinat, L. Messori, R. Monnanni, A. Scozzafava and G. Vallini, FEBS, 289 (1991) 253.
139 I. Bertini, F. Capozzi, S. Ciurli, C. Luchinat, L. Messori and M. Piccioli, J. Am. Chem. Soc., 114 (1992) 3332.
140 C.L.Thompson, C.E. Johnson, D.P.E. Dickson, R. Cammack, D.O. Hall, U. Weser and K.K. Rao, Biochem. J., 139 (1974) 97.
141 E. Munck and T.A. Kent, Hyperfine Interactions, 27 (1986) 161.
142 C.W. Carter, Jr., J. Kraut, S.T. Freer, Ng. H. Xuong, R.A. Alden and R.G. Bartsch, J. Biol. Chem., 249 (1974) 4212.
143 C.W. Carter, Jr., in W. Lovemberg (Ed.), Iron–Sulfur Proteins, Vol. III, Academic Press, New York, 1977, p. 157.
144 I. Bertini, F. Briganti, C. Luchinat, L. Messori, R. Monnanni, A. Scozzafava and G. Vallini, Eur. J. Biochem., 204 (1992) 831.
145 R.R. Crichton and M. Charloteaux-Wauters, Eur. J. Biochem., 164 (1987) 485.
146 N.D. Chasteen, Adv. Inorg. Biochem., 5 (1983) 201.
147 J.H. Brock, in P. Harrison (Ed.), Metalloproteins, Part 2, Macmillan, London, 1985, p. 183.
148 I. Bertini, S. Mangani, L. Messori, S. Mobilio and P. Orioli, Biophys. Synchrotron Radiat., 2 (1987) 176.
149 D.C. Harris and P. Aisen, in T.M. Loehr (Ed.), Iron Carriers and Iron Proteins, VCH Weinheim, 1989, p. 239.
150 M.R. Schlabach and G.W. Bates, J. Biol. Chem., 250 (1975) 2177.
151 G.W. Bates and M.R. Schlabach, J. Biol. Chem., 250 (1975) 2182.
152 J.L. Zweier, J.B. Wooten and J.S. Cohen, Biochemistry, 20 (1981) 3505.
153 I. Bertini, C. Luchinat, L. Messori, A. Scozzafava, G. Pellacani and M. Sola, Inorg. Chem., 25 (1986) 1782.
154 I. Bertini, L. Messori, G.C. Pellacani and M. Sola, Inorg. Chem., 27 (1988) 761.
155 B.F. Anderson, H.M. Baker, E.J. Dodson, G.E. Norris, S.V. Rumball, J.M. Waters and E.N. Baker, Proc. Natl. Acad. Sci. U.S.A., 84 (1987) 1769.
156 E.N. Baker, B.F. Anderson, H.M. Baker, M. Haridas, G.E. Norris, S.V. Rumball and C.A. Smith, Pure Appl. Chem., 62 (1990) 1067.
157 C.A. Smith, H.M. Baker and E.N. Baker, J. Mol. Biol., 219 (1991) 155.
158 S. Bailey, R.W. Evans, R.C. Garratt, B. Gorinsky, S. Hasnain, C. Horsburgh, H. Jhoti, P.F. Lindley, A. Mydin, R. Sarra and J.L. Watson, Biochemistry, 27 (1988) 5804.
159 R.A. Pinkowitz and P. Aisen, J. Biol. Chem., 247 (1972) 7830.
160 D.A. Folajtar and N.D. Chasteen, J. Am. Chem. Soc., 104 (1982) 5775.
161 J.L. Zweier, P. Aisen, J. Peisach and W.B. Mims, J. Biol. Chem., 254 (1979) 3512.
162 Y. Tomimatsu, S. Kint and J.R. Scherer, Biochemistry, 15 (1976) 4918.
163 P. Aisen, A. Aasa and A.G. Redfield, J. Biol. Chem., 244 (1969) 4628.
164 I. Bertini, C. Luchinat and L. Messori, J. Am. Chem. Soc., 105 (1983) 1347.
165 I. Bertini, C. Luchinat, L. Messori, R. Monnanni and A. Scozzafava, J. Biol. Chem., 261 (1986) 1139.
166 E. Morgan and E. Baker, Fed. Proc. Fed Am. Soc. Exp. Biol., 45 (1986) 2810.
167 R.E. Cowart, N. Kojima and G.W. Bates, J. Biol. Chem., 257 (1982) 7560.
168 B.F. Anderson, H.M. Baker, G.E. Norris, S.V. Rumball and E.N. Baker, Nature, 344 (1990) 784.