Coordination Chemistry Reviews, 94 (1989) 17–45
Elsevier Science Publishers B.V., Amsterdam – Printed in The Netherlands

STRUCTURE–REACTIVITY STUDIES OF BLUE COPPER PROTEINS. AFFINITY LABELING OF ELECTRON TRANSFER PROTEINS BY TRANSITION METAL COORDINATION

OLE FARVER

The Royal Danish School of Pharmacy, Department of Chemistry, Copenhagen (Denmark)

ISRAEL PECHT

The Weizmann Institute of Science, Department of Chemical Immunology, Rehovot (Israel)

(Received 24 May 1988)

CONTENTS

ABBREVIATIONS

Az	azurin
Cyt	cytochrome
dipic	dipicolinic acid
HiPIP	high potential iron protein
Pc	plastocyanin
St	stellacyanin

A. INTRODUCTION

Electron transfer proteins contain prosthetic groups with transition metal ions constituting their redox centers. Resolution of the pathways through which electrons migrate to and from these centers is both an interesting and fundamental biophysical–chemical challenge. One obvious starting point for

0010-8545/89/$10.15

such an endeavour is the identification of reaction loci on the surface of redox proteins. Though detailed three-dimensional structures of redox proteins of different types have been available for more than a decade, resolution of their mechanisms of action lags behind. Thus, for example, the family of electron-mediating blue copper-containing proteins is characterized by the proteins having their metal centers relatively inaccessible to external ligands or solvent molecules. This raises the question as to where and how electrons proceed to and from the copper ion.

Different chemical methods have been devised for mechanistic investigations, and the designing of reagents with similarity in structure and reactivity has been quite successful. Specific covalent bond formation between a properly designed reagent and protein active sites is the foundation of "affinity labeling" methodology. The term "affinity labeling" was introduced in 1962 by Wofsy et al. [1]. The term was used by these authors to describe chemical modification of a protein in order to characterize binding sites in antibodies.

An affinity label, or active-site-directed covalent inhibitor, is a chemically reactive compound which is designed to resemble, for example, a substrate of an enzyme, so that it binds specifically to the active center and forms covalent bonds with neighboring amino acid residues. By so altering specific amino acid side-chains in enzymes, studies of the catalytic activity can provide useful insight into the role of the particular residues. If the modifying reagent has substrate-like structural and/or chemical features it is possible to achieve a covalent modification of the active center. A by now classic example is the identification of a histidine in the active center of chymotrypsin [2].

The present review considers in particular our own work on chemical modification, namely, coordination of substitution-inert transition metal ions, occurring as the result of a redox process, in electron transfer proteins in which the active site is constituted by a metal ion. This method has so far proven useful in studies of electron transfer to the metal centers in copper- and iron-containing proteins.

B. STRUCTURAL ASPECTS OF THE BLUE COPPER PROTEINS

(i) Structure and function

The biological role of the copper ion is to mediate or catalyze electron transfer reactions. In fact, with the exception of the oxygen-carrying hemocyanins, all other known copper proteins function in some sort of redox reaction. The increasing amount of structural information about the blue copper proteins available in recent years has allowed a more meaningful structure–function correlation for this important group of proteins [3–5].

In a type 1 copper site the Cu(II) state gives rise to the characteristic deep blue color with an exceptionally intense absorption band near 600 nm accompanied by a very small hyperfine splitting in the $g_{\parallel}$ region of the EPR spectrum. The absorption band is mainly due to S(π) to Cu($d_{x^2-y^2}$) charge transfer and the small hyperfine splitting is a consequence of the asymmetry of the copper environment [6]. Further, the redox potential of the type 1 center is usually rather high compared with that of many Cu(II)/Cu(I) complexes [3]. It should also be mentioned that the above properties have not so far been successfully reproduced in small copper complexes.

Three-dimensional structures are now available for three of these proteins in the cupric state. (For a recent detailed review we refer to Adman [3].) For plastocyanin, the structure of the Cu(I) state has also been determined [4]. Here we only give a brief account of the structural features which are of relevance to the affinity labeling work discussed in the following.

X-ray structure determination of plastocyanin from *Populus nigra* was performed originally at 2.7 Å resolution [7], and details are now available at 1.6 Å [8]. The folding topology can be described as a β barrel with the copper ion placed near one end ("north pole") of the molecule (Fig. 1). The four ligands are N (His-37), 2.04 Å; N (His-87), 2.10 Å; S (Cys-84), 2.13 Å;

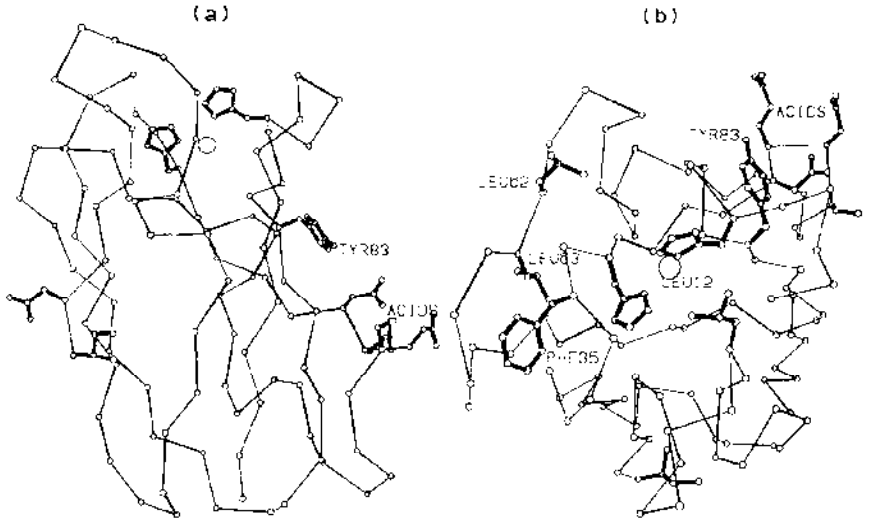


Fig. 1. Plastocyanin: polypeptide folding. (a) The drawing includes the Cu atom (large circle), the two copper-ligating histidines, tyrosine-83 and the acidic patch (42–45). (b) View looking down on the top of the molecule. Besides the residues shown in (a), this drawing also includes the hydrophobic side-chains near the copper site. The coordinates are taken from the Brookhaven Data Bank, file 1PCY [8].

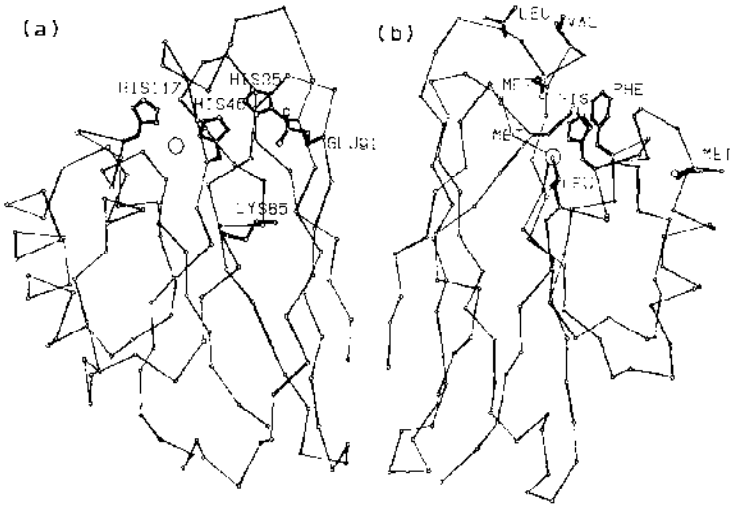


Fig. 2. Azurin: polypeptide folding. (a) Besides the Cu atom (large circle) and the two copper-ligating histidines (46 and 117) the drawing includes His-35, Lys-85 and Glu-91. (b) In this drawing the hydrophobic residues around His-117 are included. The ordinates are taken from the Brookhaven Data Bank, file 1AZU [10].

S (Met-92), 2.90 Å. The metal–ligand distances given here are those observed for the oxidized refined structure at 1.6 Å [8]. The copper ion is inaccessible to the solvent and embedded in a hydrophobic environment with only an edge of the imidazole ring of His-87 reaching the surface, making this a possible site for an outer-sphere-type electron transfer (Fig. 1(b)). Another important structural feature in plastocyanin is the presence of a negatively charged region made up of four adjacent carboxylates (residues 42–45). These are also shown in Fig. 1(a). Finally, the solvent-exposed Tyr-83 near the acidic patch should also be noticed (Fig. 1).

Three-dimensional structures have also been determined for two azurins, one from *Pseudomonas aeruginosa* [9,10] and the other from *Alcaligenes denitrificans* [11]. The *Pseudomonas* azurin structure was determined to a resolution of 2.7 Å [10]. Like plastocyanin, the polypeptide folding forms a β barrel and the copper ligands are homologous to those in plastocyanin: N (His-46), N (His-117), S (Cys-112), all at a distance of 2.0 Å, and S (Met-121) at 2.6 Å (see Fig. 2). The copper site here is also inaccessible to the external medium except for one edge of the imidazole ring of His-117. A number of hydrophobic residues lie in the region surrounding the exposed edge of the imidazole ring of His-117 (Fig. 2(b)). In contrast with plastocyanin, the distribution of charged residues on the surface has no remarka-

ble features and the invariant acidic and basic groups occur in pairs [10]. His-35, which has attracted interest as the result of kinetic work discussed below, is partially buried and lies distal to the copper-coordinating His-46 (see Fig. 2(a)).

The structure of azurin from *Alcaligenes denitrificans* exhibits only slight differences from that of *Pseudomonas* azurin [11]. The three copper ligands S, N and N are about 2.0–2.2 Å from the copper, while the methionine sulfur is further away. An important difference is in the structural arrangement of His-35, since hydrogen-bond formation seems to make its imidazole ring solvent-inaccessible. This feature has been substantiated for solution structures of the two azurins by NMR studies. His-35 in the *Pseudomonas* protein can exchange the N-δ hydrogen [12] while in *Alcaligenes faecalis* azurin, His-35 does not undergo protonation [13]. In the azurin from *Alcaligenes denitrificans*, however, this histidine appears to be protonated at pH < 4.5 [14].

(ii) Electron transfer in biological systems

Electron transfer reactions in solution have been the subject of extensive experimental and theoretical studies. As an authoritative treatment of the recent extensive development in the theory we refer to the excellent review by Marcus and Sutin [15]. We shall therefore give only a brief theoretical background here.

Biological electron transfer has several features in common with small-molecule electron transfer but also features which are different. The most important difference is that while in the latter case electron transfer is expected to occur with reactants at or near direct contact, with a reaction probability which depends on the reorganization energy λ and the standard free energy change ΔG°, in biological systems the redox centers are prevented from coming in direct contact. The rate is therefore expected also to vary exponentially with the separation distance r as $\exp(-\beta r)$ [15].

The active sites of electron transfer proteins represent only a small fraction of the total protein. The surrounding part, however, still plays important roles. One role is in recognition, i.e. in helping to optimize the relative orientations of the reaction partners, thereby controlling the probability of electron transfer. Another role is to provide a structure in which side-chain positions are properly adjusted to ensure effective electron transfer between the active sites.

Several polypeptide structures have been considered with respect to their electron transfer properties. A direct electron mediation along the peptide backbone is not very likely since electrons must cross gaps of sp^3 carbon atoms. Even if hydrogen bonding is taken into account the energy gap

between occupied and unoccupied π-electron levels is too large (greater than 3 eV) compared with kT to ensure electron mobility via unoccupied peptide orbitals [16].

A much more attractive mechanism for electron transfer seems to involve mediation through low-lying empty π^*-orbitals of aromatic amino acid side-chains and/or low-lying empty $3d$-orbitals of sulfur-containing side-chains. An illustration of facile adiabatic long distance electron transfer is seen in the outer-sphere $Ru(bpy)_3^{2+}$ -$Ru(bpy)_3^{3+}$ exchange. In this reaction the intermetal distance is 14 Å; therefore the reason why the reaction remains adiabatic is presumably the effective mixing of the metal t_{2g} orbitals with the π^* orbitals of the aromatic ligands [15]. Also "through space" coupling with direct interaction between π systems of intervening aromatic rings instead of "through bond" systems has been suggested by Taube for electron transfer in systems of mixed-valence ruthenium complexes [17]. In our studies of specific pathways for electron transfer to and from the active sites in the blue copper proteins, azurin, plastocyanin and stellacyanin, we have identified arrays of aromatic residues which may serve as effective π-electron conductors (see Section C). In this context, a very interesting study on interprotein electron transfer in a zinc-substituted cytochrome c peroxidase/cytochrome c complex has recently been reported [18]. Results were presented which demonstrate that with aliphatic residues in a certain position (82) in cytochrome c (produced by site-directed mutagenesis) the rate of intracomplex electron transfer is 10^4 times slower than with aromatic residues in this position. The rate enhancement by aromatic groups was interpreted to be the result of coupling of the heme π-electron systems by superexchange interaction through the intervening aromatic rings of residue 82 in cytochrome c and His-181 in cytochrome c peroxidase [18].

If electron transfer in proteins can be considered to proceed between spatially fixed and oriented sites, the first-order rate constant can be expressed as [15]

$$k = \kappa(r)\nu \exp(-\Delta G_r^*/RT) \tag{1}$$

$$\Delta G_r^* = \frac{\lambda}{4}\left(1 + \frac{\Delta G^\circ}{\lambda}\right)^2 \tag{2}$$

ΔG° and λ have the same meaning as above, ΔG_r^* is the free energy barrier of the reaction when the reactants are separated by a distance r. $\kappa(r)$ is the transition probability for electron transfer at the same separation distance r. and ν is a frequency. $\kappa(r)\nu$ can be expressed as [15]

$$\kappa(r)\nu = 10^{13} \exp[-\beta(r - r_0)] \qquad s^{-1} \tag{3}$$

If ν is interpreted as the frequency for nuclear motion along the reaction

coordinate (e.g. typically 10^{13} s^{-1}) then r_0 is the intersite distance for which the reaction is adiabatic ($\kappa = 1$). For redox proteins in which strong electronic coupling between the metal ions and their ligands exists, the distance of particular interest is the edge-to-edge distance between the redox sites, d. We can then write

$$\kappa\nu = 10^{13} \exp(-\beta d) \qquad s^{-1} \tag{4}$$

The value of β for aromatic systems is 12 nm^{-1} [15]. In case the driving force of a reaction is large enough to overcome the reorganization energy, the only barrier to the reaction is the electron transfer distance itself:

$$k = 10^{13} \exp(-\beta d) \qquad s^{-1} \tag{5}$$

Reduced plastocyanin Pc[Cu(I)] can be oxidized by electronically excited $^*Cr(phen)_3^{3+}$ in a precursor complex, and the intracomplex electron transfer rate for this highly exothermic reaction is 2.5×10^6 s^{-1} [19]. Inserting this rate constant and $\beta = 12$ nm^{-1} in eqn. (5) a value of $d = 1.27$ nm is calculated. This is in good agreement with the value estimated from the computer-generated model of the complex, where the edge-to-edge distance is 1.03 nm [19].

C. AFFINITY LABELING OF COPPER PROTEINS

The procedure for affinity labeling of redox proteins goes back to 1969. Kowalsky [20] reported that upon reducing iron(III) cytochrome c with Cr(II), the Cr(III) ion produced forms a substitution-inert complex with the protein. The choice of reductant, the chromous ion, offers several features advantageous to studies of electron transfer. Cr(II) complexes are generally strong reductants ($E = -51$ mV for the aqua complex) and their ligand exchange is exceptionally fast (for water exchange, $k = 10^8$ s^{-1} at 25°C [21]). In marked contrast, the product of oxidation, i.e. the respective Cr(III) complex, is substitution inert (for water exchange in $Cr(H_2O)_6^{3+}$, $k = 10^{-6}$ s^{-1}). Furthermore, because of the redox potentials the Cr(III) ion is not easily reduced or oxidized. This combination of chemical properties of the Cr(III)/Cr(II) couple was already exploited by Taube in the by now classic studies resolving inner-sphere electron transfer reactions [22]. As an illustration of the principle, the reduction of pentaamminechlorocobalt(III) with Cr(II) may be examined. The product of this reaction was found to be $(H_2O)_5CrCl^{2+}$. Hence a chloride-bridged activated complex has been formed during the reduction:

$$a_5CoCl^{2+} + Cr^{2+} \rightarrow \left[a_5CoClCr^{4+}\right]^* \rightarrow Co^{2+} + CrCl^{2+} + 5NH_3 \tag{6}$$

The essential feature of the procedure is that the coordination sphere of

Cr(II) in the activated complex, $[\ ]^{\ddagger}$, is assumed to be retained in the substitution-inert Cr(III) product. Therefore, after reducing a redox protein, the Cr(III) ion will remain in the very same coordination sphere from where the electron is donated to the redox center of the protein. Arguments doubting the validity of this conclusion have been raised. One point of criticism is the possible labilizing effect of the conjugate base form of the coordination sphere of Cr(III)(aq) at pH 6–7 where the labeling experiments were performed. This possibility, however, can be ruled out since the chromium label is only found coordinated to one specific site in each of the blue copper proteins examined so far (see below), in spite of the fact that a number of potentially competing chromium ligands are available in these proteins. Further, Cr(III) added to the native proteins at pH 7.0 can easily be removed again by dialysis. It is only via the $Cr(II) \rightarrow Cr(III) + e^-$ reaction that the inert Cr(III)–protein complex is formed [23–26].

Another criticism is that the Cr(III) ion initially coordinated to the copper protein may exchange an electron with Cr(II) in the solution and thereby become substitution labile and eventually find a thermodynamically more advantageous binding site. This exchange reaction is, however, negligible since $k_{ex} \approx 10^{-5}\ M^{-1}\ s^{-1}$, while the rate of reduction of, for example, St[Cu(II)] with Cr^{2+} is about $10^6\ M^{-1}\ s^{-1}$ [26].

Affinity labeling with chromium of plastocyanin from the photosynthetic system and of azurin from the bacterial respiratory system was initiated in 1981 [23,24]. Later, also the copper protein, stellacyanin, isolated from the sap of the *Rhus vernicifera* tree, was labeled with chromium [25,26].

With chromium labeling and identification of the binding sites in these three blue proteins the question that naturally arises is whether Cr(III) attached to a particular locus perturbs the normal function of the modified proteins. Therefore we initiated a study of the effect of the reactivity of these protein derivatives to check the functional significance of the labeled site with both physiological and non-physiological redox partners. These studies are discussed in Section D.

Ru(II)/Ru(III) has a pronounced tendency to form stable complexes with π-bonding ligands. Substitution-inert ruthenium–protein complexes may therefore be formed with proteins having solvent-exposed histidine or methionine side-chains. Such derivatives have provided important structural and kinetic information and will be discussed in Section E.

Pseudomonas azurin is an electron-carrying protein which shuttles between an oxidized (blue) Cu(II) state, (Az(II)) and a reduced (colorless) Cu(I) state, (Az(I)):

$$Az(II) + e^- = Az(I) \qquad E^\circ = 403\ mV \text{ at } 25\,^\circ C,\ pH\ 7.0 \qquad (7)$$

Being a strong reductant, Cr(II) will reduce Az(II) under anaerobic conditions [24] and the reduction can easily be monitored by following the disappearance of the blue color ($\epsilon_{625} = 5700\ M^{-1}\ cm^{-1}$; [27]). Oxidized azurin was found to be reduced stoichiometrically by Cr(II) and the Cr(III) ions produced remain bound to the reduced protein [24]. From the sequence determined by Ambler and Brown [28] it is known that proteolysis of azurin with trypsin and chymotrypsin produces well-characterized peptide fragments which can be separated chromatographically and identified. Thus trypsin cleaves the peptide bond on the carboxyl side of lysine or arginine, while chymotrypsin causes specific hydrolysis next to hydrophobic residues such as valine and leucine. Proteolytic cleavage of the modified azurin produced a series of peptides, and essentially all the chromium label was found attached to only one of these [24]. Amino acid analysis of the chromium-labeled peptides identified these as the stretches from Val-80 to Lys-97 (tryptic proteolysis) and from Thr-84 to Val-95 (chymotryptic fragment). The only two potential chromium ligands common to both of these peptide patches are Glu-91 and maybe Lys-85. Hence it was suggested that the Cr(III) ion is coordinated to the carboxylate of Glu-91 and also to the ϵ-amino group of Lys-85 [24]. As rationalized above, this is considered the site from where an electron is transferred from the reductant to the copper center of the protein. It is of interest to note that this location does not coincide with any of the heavy-atom sites in the UO_2^{2+} or $PtCl_4^{2-}$ derivative crystals [9]. This means that chromium coordination to the protein is not governed by a fortuitous high affinity metal-ion-binding side-chain. From the crystallographic data, the distance between the chromium and copper centers can be estimated as about 10 Å. A close inspection of the three-dimensional structure of azurin revealed that the Lys-85 to Glu-91 peptide patch defines an opening into the interior of the protein exposing N-δ of His-35 (Fig. 3). Calculations based on the atomic coordinates show that there is sufficient room to accomodate a water molecule in this opening. It is also noteworthy that the two imidazole rings of the above-mentioned His-35 and the copper ligand His-46 are virtually parallel and overlapping with an interplane distance of 3.8 Å. Thus the non-coordinating His-35 extends the effective π-system from the copper center towards the peptide loop, or in other words, the two imidazole rings provide a relay of π^* orbitals leading from the redox center of the protein to the binding site for Cr(III) (see Section B(ii)). This structural arrangement may then serve as a relay for resonance transfer of an electron from the surface of the protein to the Cu(II) ion and vice versa [24]. The following detailed mechanism for the chromium affinity labeling was suggested on the basis of the known chemistry of Cr(II)/Cr(III) reactions: the substitution-labile $Cr(H_2O)_6^{2+}$ ion coordinates to the carboxylate of Glu-91 and possibly also to the amino

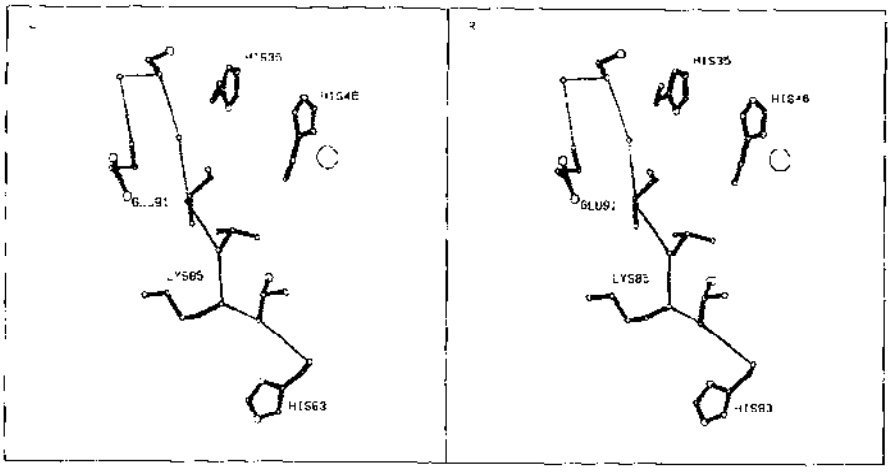
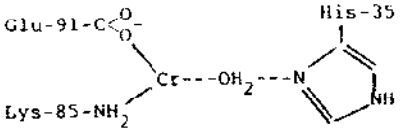


Fig. 3. Azurin. Stereodrawing of the His-83 to Glu-91 peptide strand together with the side-chains of His-35, the copper ligand His-46 and the copper atom (large circle). The data are taken from the same source as mentioned in the legend to Fig. 2.

group of Lys-85 as well as to a water molecule hydrogen-bonded to N-δ of His-35 [24]:



(8)

From this coordination sphere an electron can be rapidly transferred to the Cu(II) ion in the *Pseudomonas* azurin.

PMR studies have confirmed that the non-coordinated imidazole is titratable with a p$K \approx 7$, clearly demonstrating that His-35 is accessible to solvent [12,29,30]. Interestingly, this proton exchange is a relatively slow process which is most probably related to an equilibrium between an active and an inactive conformer of reduced *Pseudomonas* azurin (vide infra).

Proton NMR of *Alcaligenes faecalis* azurin clearly shows that His-35 does not undergo protonation in the pH range 4.0–9.5 [13], while in *Alcaligenes denitrificans* azurin a protonation is observed at pH < 4.5 [14]. Further, at pH 7.0 no evidence for a slowly attained equilibrium was found in the *Alcaligenes* Az–Cyt c_{551} electron transfer kinetics [31]. This may be taken as independent corroboration for the link between the slow conformational transition in *Pseudomonas* azurin and the protonation of His-35 in this

protein. Residues Ser-34 and Pro-36 in *Pseudomonas* azurin are substituted by Lys-34 and Thr-36 in the *Alcaligenes* protein. Also in *Alcaligenes* sp., residue 34 is a lysine and no slow isomerization step has been observed in this protein either [32]. This could be interpreted as a reflection of a different conformation of this region of the protein yielding another environment and/or accessibility to His-35. Alternatively, this could mean that the positively charged ε-amino group on Lys-34, being close to His-35, affects the behavior of the imidazole group of His-35 in the two *Alcaligenes* azurins.

The challenge of applying this labeling procedure to a multi-subunit enzyme, cytochrome oxidase (bovine), using Cr(II) is of interest in the present context [33]. Cytochrome oxidase contains four metal centers, two iron and two copper ions, all of which are thought to participate in electron transfer, leading to the catalytic reduction of dioxygen to water. It is commonly believed that the two large subunits termed subunit I and II carry the metal sites. Cytochrome oxidase was reduced with Cr^{2+} under anaerobic conditions, but in this complex system the stoichiometry was not well behaved, probably owing to oxidizing contaminants in the lipid and the solubilizing detergent. Also, a high degree of non-specific Cr(III) binding was observed. From gel chromatography of the resultant Cr(III)-enzyme complex, the conclusion was reached that the chromium label binds to subunit II of the oxidase, and it was proposed that it is this subunit which contains the electron-accepting metal centers. Especially worth noting is that subunit II of cytochrome c oxidase is one of the copper-binding entities having a polypeptide sequence with considerable homology to the well-characterized single-copper proteins azurin and plastocyanin [34].

Like the azurins the plastocyanins also undergo rapid reduction reactions between the blue Cu(II) state, Pc(II) and the colorless Cu(I), Pc(I) states:

$$Pc(II) + e^- = Pc(I) \qquad E^\circ = 370 \text{ mV at } 25\,^\circ C, \text{ pH } 7.0 \qquad (9)$$

Reducing Cu(II)–Pc with Cr(II), a substitution-inert Cr(III)–Pc(I) adduct is formed in a 1 : 1 ratio [23]. Applying the same line of argument as above, the chromium label is expected to remain in the very position from where the electron is transferred to the Cu(II) ion of plastocyanin. Enzymatic proteolysis of the Cr(III)-labeled protein with thermolysin, followed by separation and analysis of the fragments, led to the identification of the chromium-binding peptide. This peptide includes four side-chains which are potential ligands for the Cr(III) ion: Glu-42, Asp-43, Glu-44 and Asp-45. The electron is most probably transferred to the copper ion from this location in the protein structure (see Fig. 1). The carboxylate residues of this peptide are positioned in the intact protein next to Tyr-83, the solvent-exposed aromatic residue already mentioned. Independent support for this assignment is

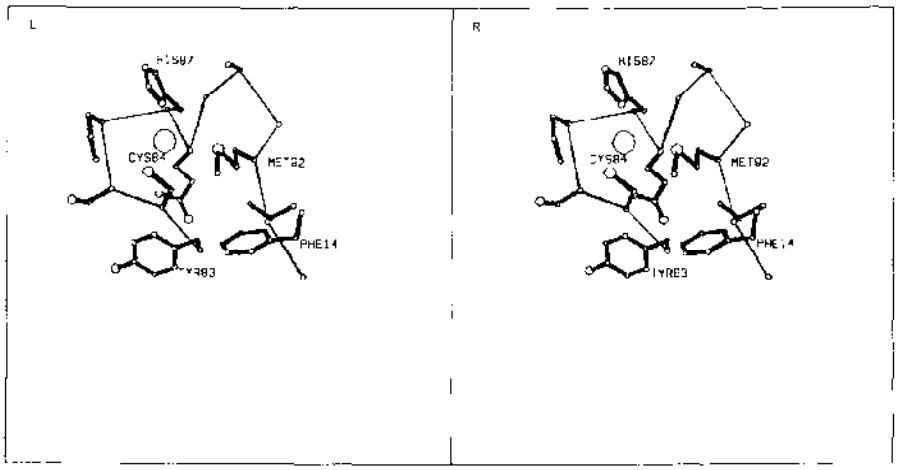


Fig. 4. Plastocyanin. Stereodrawing of details of the molecular structure viewed from Tyr-83 towards the copper center (large circle), including the highly conserved peptide patch Tyr-83 to Gly-94 and Phe-14. The data are taken from the same source as mentioned in the legend to Fig. 1.

provided by measurements of the fluorescence intensity of the tyrosyl residues of this protein [23]. French bean plastocyanin contains three tyrosines and no tryptophans. The crystallographic three-dimensional model shows that two tyrosines are buried inside the molecule while Tyr-83 protrudes into the solution. At neutral pH the quantum yield of chromium-labeled Pc(I) is 15% lower than that of the native reduced protein. Further, the pH dependence of the fluorescence intensity is also different for the labeled and the native protein. The distance between the copper ion and the assumed binding center for Cr(III) is 12 Å, and from the three-dimensional model [8] it is seen that the intervening region contains a number of highly conserved aromatic residues. On the basis of these observations, it was proposed that the electron transfer proceeds by electronic delocalization via a weakly coupled π^*-electron system from the solvent–protein interface to the copper center [23] (see Section B(ii)). The chromium binding site and the intervening aromatic residues, including the above-mentioned Tyr-83, are shown in Fig. 1 and details of the region from Tyr-83 to Cu are included in Fig. 4. The acidic patch, Glu-42 to Asp-45, is also the site where UO_2^{2+} is located in the heavy-atom derivative crystal, as calculated from the coordinates of the plastocyanin structure [8]. However, it should be emphasized that while Cr(III) ions added to plastocyanin are easily removed by dialysis,

only chromium(III) which is produced during the electron transfer process

$$Cr^{2+} + PcCu^{2+} \rightarrow Cr^{3+}\text{–}PcCu^{+} \quad (10)$$

is coordinated to the carboxylates in the substitution-inert Cr(III)–Pc complex.

One further single blue copper protein which has been reductively labeled by Cr(II) is *Rhus vernicifera* stellacyanin [25]. Towards inorganic redox agents this protein has an exceptionally high reactivity which has been interpreted as reflecting a relatively exposed redox center. The reduction potential of the Cu(II) ion in stellacyanin, being markedly lower than that of the blue proteins discussed above, together with the higher accessibility of the copper ion, cause the reduced form of the protein to be reoxidized with O_2 at a relatively fast rate [5]. When reduced with Cr(II) under anaerobic conditions, the labeled protein can be reoxidized with O_2 simply upon dialysis in aerated buffer. The reoxidized Cr(III)–St(II) adduct may then again be reduced under anaerobic conditions. In the experiments where

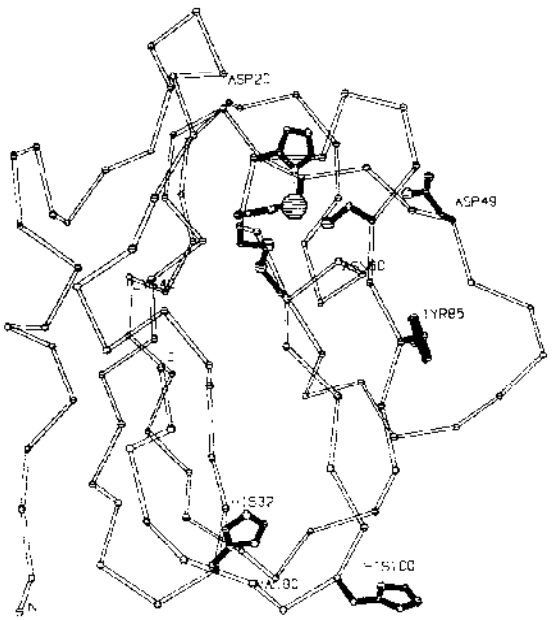


Fig. 5. Stellacyanin: polypeptide folding from energy minimization calculations [35]. The Cu atom (large shaded circle) and the four putative ligands His-46, Cys-87, His-92, Cys-93 (heavy lines) are shown. Some functionally important side-chains (see text) are also included in the drawing.

radioactive and non-radioactive chromium were used sequentially it has been found that only the original single chromium label, namely, the one equivalent attached in the first reduction step, remains coordinated to the protein [25]. This result indicates that Cr(II) reduces stellacyanin at a single specific site. The identity of this locus has recently been determined [26].

By thermolytic digestion of the ^{51}Cr-labeled protein, a single radioactive peptide was isolated which was identified as Val-48,Asp-49,Lys-50 in the stellacyanin sequence [26]. Thus Cr(III) is most likely to be chelated by the carboxylate and the amine side-chains of Asp-49 and Lys-50 respectively. Now, on comparing sequences the very interesting observation that Asp-49 is homologous to the invariant Asp-42 in all known plastocyanin sequences has emerged. As discussed above, this is one of the four potential ligands for the Cr(III) label in this protein. From sequence homology relationships between stellacyanin and plastocyanins, combined with the known three-dimensional structure of poplar plastocyanin, we have recently constructed a three-dimensional model for stellacyanin by energy minimization calculations [35]. This model shows that Tyr-85 in stellacyanin, like Tyr-83 in plastocyanin (vide supra), is solvent exposed and close to the binding site of Cr(III) (see Fig. 5). On this basis we have proposed an analogous electron transfer pathway over 12 Å from Cr(II) to Cu(II) via a weakly coupled π^*-system provided by the aromatic residues, leading to the sulfur atom of the copper-ligating Cys-87 [26] (see Section (B(ii)).

D. REACTIVITY OF Cr(III)-LABELED PROTEINS

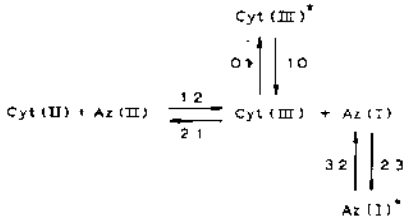
Identification of electron transfer sites by Cr(II)/Cr(III) reductive labeling naturally raises the question of the relevance of these loci to the biochemical reactions performed by these proteins. An important advantage of the affinity labeling procedure lies in the fact that specifically modified proteins are produced, namely, proteins carrying on their surface the labeling Cr(III) ion at the respectively defined labeling site. This modification is relatively minor compared with the commonly employed organic–chemical modifications. Still, its effect on the reactivity of the proteins with their respective physiological partners had to be determined in order to assess the functional role of the labeled site.

(i) Azurin

Azurins are found in bacteria from the *Pseudomonas* and *Alcaligenes* genera where they serve as electron mediators, most probably between cytochrome c_{551} and the bacterial cytochrome oxidase [36]:

$$\text{Cyt } c_{551} \overset{e^-}{\rightarrow} \text{Az} \xrightarrow{e^-} \text{Cyt oxidase} \xrightarrow{e^-} O_2 \qquad (11)$$

The electron exchange reaction between *Pseudomonas* azurin and Cyt c_{551} has been intensively studied and all the experimental results have recently also been reviewed [5]. As already found in the pioneering study of Antonini et al. [37], the reaction is not a simple bimolecular electron exchange process but a rather complex reaction involving not only electron transfer but also conformational transitions in both azurin and Cyt c_{551} [38,39]:



(12)

(The asterisk refers to an inactive conformer of the protein.)

The O_2 oxidation of Az(I) is catalyzed by Cyt c oxidase and exhibits a Michaelis–Menten-type mechanism:

$$V = \frac{V_{max}[\mathrm{Az(I)}]}{K_m + [\mathrm{Az(I)}]} \tag{13}$$

With the above reaction mechanisms and the identification of an electron transfer site on azurin by chromium labeling, the next step was to examine whether an effect of the Cr(III) binding on these electron transfer reactions of azurin can be resolved. Temperature-jump relaxation measurements of the electron exchange between Cyt c_{551} and chromium-labeled azurin revealed that the data could be fitted to the same reaction mechanism as that of native azurin and Cyt c_{551} [40].

However, a quantitative comparison of the data revealed that the chromium label attenuates the rates of both the electron exchange and of the conformational transition in Az(I) (see Table 1). This effect on the reactivity of azurin is interpreted as a result of the chromium ion being coordinated near His-35 in azurin. It probably perturbs an optimal docking of the two proteins by steric and/or electrostatic effects, hindering the maximal overlap of the π orbitals of the His-35 imidazole on azurin and the π orbitals on the Cyt c_{551} heme ring. This result means, in other words, that the electron exchange between the metal centers of the two proteins proceeds via these two conjugated ring systems (see Section B(ii)).

A conformational transition coupled to a proton transfer equilibrium has been demonstrated by chemical relaxation measurements on native Az(I)

TABLE 1

Kinetic and thermodynamic data for the azurin–cytochrome c_{551} reaction[a]

	Native azurin + cytochrome c_{551}	Cr(III)-labeled azurin cytochrome c_{551}
K_{01}	0.59	0.62
k_{12} ($M^{-1} s^{-1}$)	6.88×10^6	4.68×10^6
k_{21} ($M^{-1} s^{-1}$)	1.47×10^7	6.96×10^6
K_{12}	0.47	0.67
k_{23} (s^{-1})	1.67	0.58
k_{32} (s^{-1})	4.52	4.40
K_{23}	0.37	0.13
ΔH°_{01} (kJ mol^{-1})	44.0	38.5
ΔH°_{12} (kJ mol^{-1})	−14.6	−14.0
ΔH°_{23} (kJ mol^{-1})	−23.6	−31.8
K_{tot}	1.0	1.2
ΔH°_{tot} (kJ mol^{-1})	−4.7	−2.9

[a] Results of the numerical analysis of experimental data from ref. 40 using the reaction mechanism depicted in reaction scheme (12) which also defines all the symbols used. The measurements were performed at 25°C, 0.1 M Hepes, pH 7.0, 0.2 M KCl.

solutions [41–43]. The relaxation time of this process is similar to that observed for the slow Az(I) ⇌ Az(I)* equilibrium of reaction scheme (12). ΔH°_{23} for isomerization of Az(I) (reaction scheme (12) and Table 1) is negative. It has been demonstrated that protons are released from Az(I) upon increasing the temperature [41]; this means that His-35 is protonated [40] in the inactive conformer of the reduced azurin, Az(I)*. This also agrees with the conclusion reached by Mitra and Bersohn [13] from their NMR experiments on *Alcaligenes faecalis* azurin which show that electrons are efficiently transferred when His-35 is unprotonated. The time correlation between His-35 protonation and the slow conformational equilibrium between an active and an inactive conformer of reduced azurin can be described by a process in which a rapid proton exchange on His-35 in the active Az(I) conformer is coupled to a slow conformational transition between the two protonated isomers:

$$Az(I)^*H^+ \underset{k_f}{\overset{k_r}{\rightleftharpoons}} Az(I)H^+ \overset{K_a}{\rightleftharpoons} Az(I) + H^+ \qquad (14)$$

where the rate constants k_f and k_r are independent of pH. K_a is the acid dissociation constant. In Cr(III)-labeled azurin, the formation rate of the inactive form of Az(I) (k_f) is found to decrease by nearly two thirds compared with that of native azurin. In contrast, the rate of the reverse reaction (k_r) is unaffected by the modification. The proximity between the chromium label and His-35 suggests that the slow proton exchange of the

imidazole is affected by the presence of this cation or possibly by a change in the protein structure in this region, causing this decrease in rate.

The second half of the physiological cycle assigned to azurin involves electron transfer to the cytochrome c oxidase (reaction scheme (11)). Upon examination of the effect of chromium labeling on the catalyzed oxidation of Az(I) by O_2 [40], it was found that neither V_{max} nor K_m of this reaction is affected by Cr(III) modification of the azurin: $K_m = 4.2 \times 10^{-5}$ M, V_{max} = 1.6 mol oxidized azurin per mole Cyt ox. per second, $T = 25\,^{\circ}$C, pH 7.0, 0.1 M Hepes (see eqn. (13)). This segregation between the insensitivity of the cytochrome-oxidase-catalyzed oxidation of azurin and the attenuation, though small, of its electron transfer reactivity with Cyt c_{551} raised the possibility that there are two physiologically functional sites for electron transfer on azurin. One is engaged in electron exchange with Cyt c_{551} via His-35, and the other in electron transfer to cytochrome oxidase, most probably via His-117 in the hydrophobic "northern" end of the protein (see Fig. 2).

Highly interesting studies of the electron self-exchange rate of *Pseudomonas* azurin have appeared recently, all based upon ^{1}H NMR. Canters et al. [30,44] have analyzed line broadening of PMR lines of amino acid residues serving as copper ligands, His-46, His-117 and Met-121, and derived from them a bimolecular rate constant for electron exchange between Az(I) and Az(II) of 2×10^6 M^{-1} s^{-1} at 50 °C and neutral pH. A more recent extension of this study [30] reports a rate constant of 7×10^5 M^{-1} s^{-1} at 300 K, independent of pH in the range $5 < pH < 9$. The exchange rate has also been determined independently by Ugurbil and Mitra [45]. In this study the spin–lattice relaxation time of the $C_{(2)}$-H proton of His-35 was measured in mixtures of reduced and oxidized azurin at room temperature. Assuming that this proton is proximal to the Cu^{2+} ion, its T_1 value will be much smaller than the lifetime of the oxidized protein owing to electron transfer. From these data an electron exchange of 4.3×10^3 M^{-1} s^{-1} (pH 7.0) was calculated. This is 200-fold lower than the above-reported value [30]. The discrepancy between the results of the two groups is most likely due to the distance between Cu and His-35 $C_{(2)}$-H being 8.5 ± 0.5 Å rather than the 6 Å used by Ugurbil and Mitra [45]. Thus the close proximity model does not apply here. Interestingly, Ugurbil and Mitra also found that the self-exchange rate is independent of whether the imidazole is protonated or not. This aspect is further discussed below.

It is of interest to compare these experimentally determined self-exchange rates with values calculated by applying the Marcus cross-relation [15,46] using reported rates of outer-sphere electron transfer between azurin and different redox partners. The reversible electron exchange between *Pseudomonas* azurin and *Pseudomonas* Cyt c_{551} is a bimolecular reaction (see

above) and since the Cyt c_{551} self-exchange rate has been determined in a large number of experiments [32], the former reaction should be useful for calculations using Marcus theory. Indeed, the calculated self-exchange of azurin is 1.0×10^6 M^{-1} s^{-1} [5], in full agreement with the above-reported value of Canters et al. (7×10^5 M^{-1} s^{-1} [30]).

The other highly noteworthy aspect of the results emerging from the NMR studies of azurin is the observed independence of the self-exchange rate on His-35 imidazole protonation [30,45]. The previous discussion concerning the active and inactive conformers of reduced azurin, and the large body of kinetic evidence presented earlier, demonstrate a role for the protonation of His-35 in the electron transfer mechanism of the Az–Cyt c_{551} reaction. Since it was also demonstrated that this electron transfer occurs via His-35 [40], the inescapable conclusion is that the self-exchange between Az(I) and Az(II) proceeds via a different pathway. The hydrophobic "Northern" surface residues, including the partially accessible imidazole ring of His-117 (see Fig. 2) provide the most likely area for the efficient and pH-independent electron conduction.

(ii) Plastocyanin

Plastocyanins function as electron carriers in all higher plants and in many algae. It is widely accepted that plastocyanin mediates electrons between the membrane-bound cytochrome f/b_6 complex of photosystem II and the P-700 chlorophyll-containing center of photosystem I [5]:

$$\text{Cyt f} \overset{e^-}{\rightarrow} \text{Pc} \overset{e^-}{\rightarrow} P_{700} \tag{15}$$

The question now arises whether the negative patch on plastocyanin which can be labeled by chromium near Tyr-83 is involved in the above physiological electron transfer processes between photosystems I and II. Both photooxidation and photoreduction of native plastocyanin are series of complex reactions with rates that were found to decrease with increasing amounts of plastocyanin [47]. This observation was explained by a mechanism which involves dead-end complex formation between plastocyanin and both its reaction partners [47]. For photooxidation the following reaction scheme satisfies the observed kinetics:

$$\begin{array}{ccccccc}
[\text{Pc(I)}P^{+}_{700}] & \overset{K_{DE'}}{\rightleftharpoons} & \text{Pc(I)} + P^{+}_{700} & \xrightarrow{k_{ox}} & \text{Pc(II)} + P_{700} & \overset{K_{DE'}}{\rightleftharpoons} & [\text{Pc(II)}P_{700}] \\
\\
\text{Pc(I)} + [\text{Pc(I)}P^{+}_{700}] & & \xrightarrow{k'_{ox}} & & \text{Pc(II)} + [\text{Pc(I)}P_{700}] & & \\
\Big\uparrow\Big\downarrow K_{DE''} & & & & \Big\uparrow K_{DE''} & & \\
[\text{Pc(I)}_2P^{+}_{700}] & & & & [\text{Pc(II)Pc(I)}P_{700}] & &
\end{array} \tag{16}$$

where $K_{DE'}$ and $K_{DE''}$ are equilibrium constants for the formation of complexes between plastocyanin and P-700 in which the relative orientation of the proteins does not allow for electron exchange between the reductant centers (dead-end complex formation). Reduction of Pc(II) by cytochrome f proceeds by a similar mechanism.

The same mechanism could also be applied to the reactions of chromium-labeled plastocyanin. However, the modified plastocyanin is oxidized at a rate which is significantly slower than that of native plastocyanin ($k_{ox} = 1.8 \times 10^8$ M^{-1} s^{-1} for native plastocyanin and 6.9×10^7 M^{-1} s^{-1} for chromium-labeled plastocyanin) [47]. This suggests that, indeed, the region around the acidic patch and Tyr-83 carrying the chromium label is involved in electron transfer to P-700 (Fig. 1). As described above, the chromium-labeled region on plastocyanin contains several negatively charged carboxylates close to Tyr-83 [23]. This highly conserved negative patch could very well serve as the recognition site on plastocyanin, favoring interactions with an appropriate site on the reaction centers and optimizing the conditions for electron transfer. As discussed in Section C, a large number of aromatic residues are found next to this patch leading from the solvent–protein interface to the copper center [23] which could function as a π-conduction channel for electron transfer from Pc[Cu(I)] to P-700$^+$ reaction centers. This assignment of an anionic interaction site on plastocyanin is fully compatible with several recent reports. Haehnel et al. [48] have shown that Mg^{2+} ions affect flash-induced fast kinetics of P-700 reduction but have no effect on Cyt f oxidation. Davis et al. [49] have reported that Mg^{2+} has a pronounced effect on the affinity of photosystem I particles toward eukaryotic Pc(I).

The second potential electron transfer locus proposed on plastocyanin is the partially exposed imidazole ring of His-87, which constitutes one of the copper ligands [7,8] (cf. Fig. 1). This aromatic ring is imbedded in and largely masked by a number of invariant hydrophobic amino acid residues. Significantly, the photoreduction rates of oxidized plastocyanin by broken chloroplasts are found to be identical for the native and chromium-labeled plastocyanin. Since reduction of both native and chromium-labeled Pc(II) was found to be sensitive to the photosystem II electron transfer inhibitors (DCMU as well as DNP-INT [47]), and since Cyt f oxidation is stimulated by exogenous plastocyanin [50], it was concluded that the externally added Pc(II) reacts at the same site as the endogenous plastocyanin. On the basis of the kinetic results of the photoreduction of Pc(II) by broken chloroplasts, it was therefore suggested that electron transfer from the membrane-bound Cyt f to the copper center of plastocyanin proceeds via the His-87 imidazole. However, reduction of Pc(II) by photosystem I could not be entirely excluded [47]. Also, Hauska et al. [51] have found that added plastocyanin

failed to restore phosphorylation and suggested that it induces an artificial electron flow through P-700. A more recent study by Haehnel et al. [52] shows that exogenous plastocyanin, added to thylakoid vesicle fractions in the presence of DCMU and ascorbate, induces a rapid reduction of only 15% of total P-700$^+$ in right-side-out vesicles as compared with 70% in inside-out-vesicles. In contrast, Schmid et al. [53] found that antibodies raised against plastocyanin cause agglutination and further inhibit electron transport of the chloroplast. The same conclusion was reached by Böhme [54]. In view of the conflicting reports, caution should be maintained in drawing final mechanistic conclusions about the photoreduction of plastocyanin by chloroplasts.

Chemically modified plastocyanin has also been prepared by reaction with ethylenediamine, thus yielding an amide bond between protein carboxylates and one amino group of ethylenediamine [55–57]. This procedure is obviously not an affinity labeling in any sense of this term, yet it may provide mechanistic insights because negative surface charges of plastocyanin are converted into positive amino side-chains. Hence the reactivity of plastocyanin modified by this procedure may yield information about the site of interaction on plastocyanin in its reaction with photosystem I particles treated with Triton X-100. The most striking observation is that while in the absence of Mg^{2+} ions, native reduced plastocyanin does not serve as electron donor for P-700, after modifying an average of two of the five carboxylates of the amino acid residues 31–55 (i.e. including the 42–45 patch), the reaction rate becomes even larger than that of native plastocyanin + P-700 in the presence of 5 mM Mg^{2+} [56]. In the presence of magnesium ions, the K_m value for photooxidation of the above-mentioned modified plastocyanin is also smaller than that for the native plastocyanin. Upon more extensive modification of the carboxylates of plastocyanin, K_m decreases further as does the net negative surface charge of the protein. This is to be expected since the extent of electrostatic repulsion between plastocyanin and the negatively charged P-700 should diminish. All these observations provide further support for the notion that specific interactions prevail between a negative patch of carboxylates on plastocyanin and P-700.

In a thorough extension of the above study, Gross and coworkers have isolated and identified the different forms of ethylenediamine-modified plastocyanin and have examined the effect of this chemical modification on the rates of Cyt f oxidation and P-700 reduction [57]. They show convincingly that the site of interaction on plastocyanin for isolated Cyt f in the direct reaction between the two partners comprises the acidic residues 42–45 and 59–61. The effect of ethylenediamine modification of plastocyanin on the photoreduction of P-700 reaction centers by Pc(I) in the presence of 2.5–5 mM $MgCl_2$ is, however, less clear. While one form, modified at

residues 42–45 alone, showed the same reactivity as native Pc(I), modification of both the acidic patches, 42–45 and 59–61, showed twice as large a stimulation as when section 59–61 alone was modified [57]. The reason for the conflicting results from Cr(III) affinity labeling [47] and ethylenediamine modification of carboxylates on plastocyanin [55–57] is not clear at the moment. One possibility may be the different experimental approaches. Gross and coworkers [57] studied the bimolecular reduction of Pc(II) with isolated Cyt f by stopped-flow experiments, whereas we studied the rather complex photoreduction of Pc(II) by the Cyt f/b_6 complex bound to the membrane in intact chloroplasts (see reaction scheme (16) and ref. 47). Thus the positively charged residues around the heme edge may not be available to plastocyanin in this complex. In the photooxidation of Pc(I) by P-700, Gross and coworkers used Mg^{2+} ions to compensate for the charge effects [57] (both plastocyanin and P-700 reaction centers carry net negative charges) while we used photosystem I reaction centers in which the charge-compensating subunit (III) is still present [47].

The work of Gross and coworkers has been followed up by Takabe et al. [58] using essentially the same procedure for chemical modification of plastocyanin. They were able to separate plastocyanin having 1–4 mol of modified carboxylate per mole of plastocyanin; however, the position of these carboxyl residues on the protein is still unknown. Interestingly, their kinetic studies revealed that the rate of electron transfer from reduced Cyt f to oxidized singly and doubly modified plastocyanin decreased drastically as compared with the native plastocyanin [58]. On the other hand, this modification has only a minor effect upon photooxidation of reduced plastocyanin with P-700. With triply substituted plastocyanin, however, the effect is considerable and with four carboxylates modified, the maximum rate of Pc(I) photooxidation is obtained even in the absence of $MgCl_2$ [58]. Some carboxyl residues are evidently of importance in the Cyt f to plastocyanin electron transfer while others are decisive for Pc(I) to P-700 electron transfer. It would therefore be illuminating to find out how specific the modification is on the 16 carboxyl residues in spinach plastocyanin and to identify the modified residues.

An interesting derivative of plastocyanin has been prepared by covalently cross-linking it to Cyt c [59] or to Cyt f [60]. Cyt c was found to be still reducible by, for example, ascorbate in the Pc–Cyt c complex but inactive towards cytochrome oxidase, suggesting that the heme crevice is blocked to the enzyme. Unfortunately, no information was given concerning the reactivity of the plastocyanin. In the second study [60] the formation of a covalent Pc–Cyt f adduct was reported. In this adduct, both proteins could be reduced and oxidized by low molecular weight redox agents. The cross-linked amino acid residues have not been identified, but the 1 : 1 stoichiometry

was interpreted as a reflection of specific binding. However, since it has been demonstrated that the electron transfer from Cyt f in chloroplasts proceeds by a bimolecular reaction mechanism with no kinetic evidence for electron transfer in a precursor complex (reaction scheme (16) and ref. 47), it remains to be seen whether this chemical approach will provide further understanding of the molecular details of photosynthetic electron transfer mechanisms.

In a recent communication, Sykes and coworkers [61] have examined the reaction between oxidized plastocyanin modified by Cr(III) using the procedure reported earlier [23] and solubilized reduced Cyt f. At pH 8.0, the chromium labeling was found to cause a 40% decrease in the reduction rate of Pc(II). Further, upon attaching two Cr(III) ions to plastocyanin the rate decreases by 60%. However, it is not known at present where the second Cr(III) ion binds to the protein. The reduction of plastocyanin by isolated solubilized Cyt f is also inhibited by a +5-redox-inactive cationic cobalt(III) complex which provides support for electron transfer involving the negative patch [61]. No rate dependence on plastocyanin concentration was reported. In our earlier study [47], the photoinduced electron transfer, presumably from Cyt f present in broken chloroplasts, to plastocyanin was examined and it was found here that the chromium label had no effect on the reaction rate. To explain the inverse dependence of the rate on the total plastocyanin concentration, the formation of a dead-end complex between plastocyanin and the membrane-bound Cyt f/b_6 complex was assumed. A more detailed kinetic analysis of the isolated Cyt f/Pc system is necessary in order to decide whether the conflicting kinetic results can be simply due to a different mechanism in the simplified model system. Isolated Cyt f, which carries an overall negative charge at pH > 5.5, may use a positively charged binding site in its reaction with plastocyanin, which is not available in the intact Cyt f/b_6 complex.

Sykes and coworkers have also studied the effect of Cr(III) modification on the reactivity of plastocyanin with several inorganic complexes [62] and with non-physiological proteins as reductant partners [63]. Oxidation of Pc(I) labeled with one Cr(III) ion by $Co(phen)_3^{3-}$ showed reduced reactivity compared with that of the native protein. At pH 5.8 "biphasic" kinetics were observed. It was demonstrated earlier that the reaction between $Co(phen)_3^{3+}$ and native reduced plastocyanin involved a protonation equilibrium of the protein as well as the association of the Co(III) complex with Pc(I) [64]. At the present stage it is uncertain in what way the Cr(III) modification of plastocyanin affects this complicated behavior. Comparative studies, however, using $Ru(NH_3)_5py^{2+}$ as reductant and $Fe(CN)_6^{3-}$ and $Co(dipic)_2^-$ as oxidants, clearly showed that Cr(III) labeling of plastocyanin only affected the reaction between the protein and the positively charged redox partners [62].

Cr(III)-labeled Pc(II) reacts more slowly with horse heart Cyt c(II) than with native plastocyanin [63]. The reactions with native, singly and doubly labeled plastocyanin show simple bimolecular behavior in the concentration range 10–30 μM plastocyanin (pH 5.8, 25°C), and the average second-order rate constants are respectively 1.5×10^5 M^{-1} s^{-1}, 1.2×10^5 M^{-1} s^{-1} and 0.66×10^5 M^{-1} s^{-1}. The site of interaction between plastocyanin and Cyt c seems to be the same as that adopted by the positively charged inorganic reducing agents, since Cr(III) labeling exhibits similar effects. This implies electrostatic interaction between the carboxylate groups 42–45 on plastocyanin and the protonated ϵ-amino lysine groups on mammalian Cyt c.

In HiPIP reduction of the singly modified Pc(II), no change is observed in reactivity compared with native plastocyanin, whereas the two-fold modified Pc(II)–$[Cr(III)]_2$ reacts at a rate about 35% slower (25°C, pH 5.8) [63]. Since no effort was made in these studies to identify the site of binding of the second Cr(III) to plastocyanin, it is difficult to interpret the changes in reactivity of the modified plastocyanin.

E. REACTIVITY OF RUTHENIUM-MODIFIED PROTEINS

Recently, a rather attractive reagent for modification of proteins leading to derivatives with interesting reductant properties has been employed. This is the aquapentaammineruthenium(II) ion which, because of the pronounced tendency of ruthenium to π-bond formation, reacts quite selectively with certain amino acid residues of proteins. Thus a_5Ru^{2+} forms a stable complex with imidazole and thioether groups. Both the a_5RuL^{2+} and the oxidized a_5RuL^{3+} complexes are relatively substitution inert. Although the detailed mechanism of derivatization has not yet been elucidated, the procedure for labeling proteins with ruthenium is straightforward [65]. The method, however, cannot really be considered as an affinity labeling in the true sense of the term, yet it turns out to be of great value and interest, since it has been widely employed in designing systems where long-range intramolecular electron transfer can be studied in proteins [66]. Thus several pentaammineruthenium derivatives of sperm whale myoglobin [67,68], horse heart cytochrome c [70–74] and *Pseudomonas* azurin [65,69] have been prepared. A structural model of selected parts of ruthenium myoglobin is shown in Fig. 6. In azurin both the spectroscopic and electrochemical properties as well as peptide mapping studies identified the ruthenium binding site as His-83 on this protein [69]. Kinetic investigations have revealed that a relatively fast long-distance intramolecular Ru(II)-to-Cu(II) electron transfer can take place in the ruthenium modified protein [65]: flash photolysis can form an excited state $Ru(bpy)_3^{3+}$ which in the presence of Ru(III)-labeled azurin produces $a_5Ru(II)$–Az(II) rapidly. This step is then

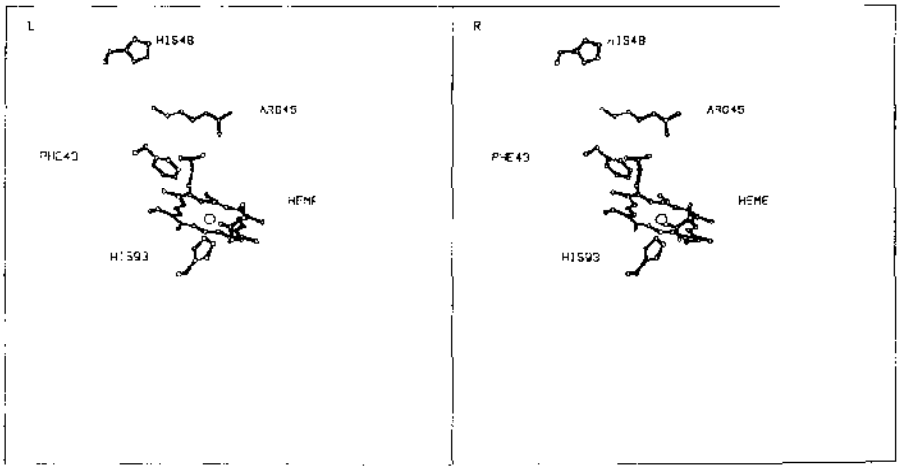


Fig. 6. Myoglobin. Stereodrawing of selected parts of sperm whale myoglobin. Details of the molecular structure of the region His-48 to the heme group. Ru is coordinated to His-48 N_ε [67] which is the atom next to the "H" of the His-48 label. The coordinates are taken from the Brookhaven Data Bank, file 1MBO [75].

followed by a first-order reduction of Cu(II), which is independent of protein concentration:

$$\text{Ru(II)–Az(II)} \xrightarrow{k_{et}} \text{Ru(III)–Az(I)} \tag{17}$$

The electron transfer rate constant k_{et} is 1.9 s^{-1} at pH 7.0 and interestingly it was found to be temperature independent over the wide range explored (−8 to +55°C) ($\Delta H^{\neq} < 4$ kJ mol^{-1}). This is in accord with results found previously by Gray and coworkers for ruthenium-modified Cyt c ($\Delta H^{\neq} < 6$ kJ mol^{-1}) [70–72]. The rate constant is much higher for intramolecular electron transfer from Ru(II) to Fe(III) in Cyt c ($k = 30$ s^{-1}) although the driving force for the azurin reaction is much larger, while the distance between the ruthenium-binding imidazoles (His-83 in azurin and His-33 in Cyt c) and the reductant centers of the proteins are almost the same. The different rates could be attributable to the different media, i.e. amino acid residues separating the metal ions in a through-space intramolecular mechanism. Alternatively, if a through-bond electron transfer occurs, then in this case the pathway in modified azurin involves twice as many peptide bonds as in modified Cyt c [65].

Isied and coworkers have also investigated the intramolecular electron transfer between the Ru(II) coordinated to His-33 and the Fe(III) center in Cyt c [73,74]. The Ru(II) Cyt c(III) intermediate was in this study generated

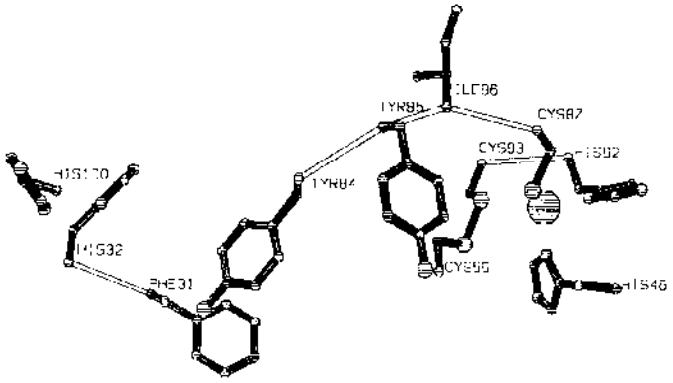


Fig. 7. Stellacyanin. View of a selected part of the protein in the region between the Ru(III/II)-binding histidines (32 and 100) and the copper center. The drawing is based on coordinates from energy minimization calculations on stellacyanin [35].

by pulse radiolysis and the intramolecular electron transfer within this complex was examined over a 40 °C temperature range. At 25 °C the first-order rate constant is 53 s^{-1}, and contrary to Gray and coworkers the rate was found to be temperature dependent ($\Delta H^{\neq} = 14.6$ kJ mol^{-1}). The reason for this discrepancy is not clear at the moment.

Our model-building studies of the three-dimensional structure of *Rhus* stellacyanin [35] suggested that the two free histidine residues (numbers 32 and 100) are solvent exposed (see Fig. 5). Moreover, they are probably close and form intermolecular hydrogen bonds. This makes stellacyanin an ideal system providing ligands for $Ru^{3+/2+}$. Indeed, a 2:1 [Ru(III)]$_2$–St[Cu(II)] complex is easily formed by reacting excess of [Ru(a)$_5$H$_2$O^{2+}] with St[Cu(II)]. followed by oxidation of Cu(I) with atmospheric O_2 [76]. The EPR spectrum of the oxidized complex shows no signal from the two Ru(III) d^5 ions, indicating a proximal binding which results in an electronic coupling between their spins. In the NMR spectrum of the [Ru(III)]$_2$–St[Cu(II)] complex the proton signals from C-2H and C-4H of His-32 and His-100 are absent, confirming Ru(III) complex formation with these residues. In the computed model of stellacyanin the distance from these two histidines to the copper center is 20 Å and the details of this region are shown in Fig. 7. Following the characterization of the Ru$_2$–protein complex, studies of long distance intramolecular electron transfer between the two metal centers were undertaken. The metal ions were reduced with CO_2^- radicals produced by pulse radiolysis [76]. Using a large excess of the Ru–protein complex, the

reducing radicals can react either with the Ru(III) ion or the Cu(II) ion:

$$CO_2^- + Ru(III)\text{–}St[Cu(II)] \rightarrow Ru(II)\text{–}St[Cu(II)] + CO_2 \quad (18a)$$

$$CO_2^- + Ru(III)\text{–}St[Cu(II)] \rightarrow Ru(III)\text{–}St[Cu(I)] + CO_2 \quad (18b)$$

Not surprisingly, considering the higher degree of accessibility of the His-coordinated Ru(III) ion compared with that of the buried Cu(II) ion, the hydrophilic reductant predominantly reduces the Ru(III) ion (reaction (18a)). The redox potential of $Ru(a)_5His^{3+/2+}$ is 50 mV while the potential of $Cu^{2+/+}$ in stellacyanin is 185 mV. The fate of the half-reduced Ru(II)–St[Cu(II)] complex is therefore one of the following: the Ru(II) ion can either transfer an electron to Cu(II) in another protein molecule in an intermolecular process (reaction (19a)) or reduce Cu(II) intramolecularly (reaction (19b)):

$$Ru(II)\text{–}St(II) + Ru(III)\text{–}St(II) \rightarrow Ru(III)\text{–}St(II) + Ru(III)\text{–}St(I) \quad (19a)$$

$$Ru(II)\text{–}St(II) \rightarrow Ru(III)\text{–}St(I) \quad (19b)$$

In all experiments a slow indirect reduction of Cu(II) was indeed observed, the rate of which is independent of either protein complex or CO_2^- concentration. The reaction was monitored both at 605 nm where Cu(II) has an intense absorption band and at 310 nm where $Ru(a)_5\text{–}His^{3+}$ has an extinction coefficient of 2000 M^{-1} cm^{-1}. The slow reduction of Cu(II) was concomitant with the reappearance of the 310 nm absorption. These observations clearly demonstrate that an electron is transferred from Ru(II) to Cu(II) in an intramolecular process, and the rate constant at 18°C is 0.05 s^{-1} [76]. The rate of the direct reduction of Cu(II) by CO_2^- was found to be the same for native and ruthenium-labeled stellacyanin. Thus it is possible to transfer electrons over a relatively long distance (20 Å) through the protein medium at a considerable rate. As shown in Fig. 7, in the intervening region between the two ruthenium-coordinating histidines and the closest copper ligand, the sulfur atom of Cys-93 contains three aromatic ring systems (one phenylalanine and two tyrosines). Therefore electron transfer through a weakly overlapping π^* system once more seems possible (see Section B(ii)).

F. CONCLUDING REMARKS

The progress made during the last years in the understanding of electron transfer in proteins has demonstrated that electron exchange can take place with considerable efficiency and at fast rates over long distances. It has also been shown by affinity labeling that specific residues are involved, which demonstrates that distance is not the only decisive factor governing the rate of transfer. Low lying empty π^*-orbitals of aromatic ring systems seem to

be one possible pathway. More experiments are needed in order to understand fully the factors that control the efficiency and specificity of long-range electron transfer in biological systems.

REFERENCES

1 L. Wofsy, H. Metzger and S.J. Singer, Biochemistry, 1 (1962) 1031.
2 E.B. Ong, E. Shaw and G. Schoellmann, J. Biol. Chem., 240 (1965) 694.
3 E.T. Adman, in P. Harrison (Ed.), Topics in Molecular and Structural Biology, Metalloproteins Part I, Macmillan, 1985, p. 1.
4 H.C. Freeman, in J.P. Laurent (Ed.), Coordination Chemistry, Vol. 41, Pergamon, Oxford, 1981, p. 29.
5 O. Farver and I. Pecht, in R. Lontie (Ed.), Copper Proteins and Copper Enzymes, CRC, 1984, p. 183.
6 H.B. Gray and E.I. Solomon, in T.G. Spiro (Ed.), Copper Proteins, Wiley, New York, 1981, p. 1.
7 P.M. Colman, H.C. Freeman, J.M. Guss, M. Murata, V.A. Norris, J.A.M. Ramshaw and M.P. Venkatappa, Nature (London), 272 (1978) 319.
8 J.M. Guss and H.C. Freeman, J. Mol. Biol., 169 (1983) 521.
9 E.T. Adman, R.E. Stenkamp, L.C. Sieker and L.H. Jensen, J. Mol. Biol., 123 (1978) 35.
10 E.T. Adman and L.H. Jensen, Isr. J. Chem., 21 (1981) 8.
11 G.E. Norris, B.F. Anderson and E.N. Baker, J. Mol. Biol., 154 (1983) 501.
12 K. Ugurbil and R. Bersohn, Biochemistry, 16 (1977) 3016.
13 S. Mitra and R. Bersohn, Proc. Natl. Acad. Sci. U.S.A., 79 (1982) 6807.
14 C.M. Groeneveld, M.C. Ouwerling, C. Erkelens and G.W. Canters, J. Mol. Biol., 200 (1988) 189.
15 R.A. Marcus and N. Sutin, Biochim. Biophys. Acta, 811 (1985) 265.
16 M.G. Evans and J. Gergely, Biochim. Biophys. Acta, 3 (1949) 188.
17 H. Taube, in B. Chance, D.C. DeVault, H. Frauenfelder, R.A. Marcus, J.R. Schrieffer and N. Sutin (Eds.), Tunneling in Biological Systems, Academic Press, New York, 1979, p. 173.
18 N. Liang, A.G. Mauk, G.J. Pielak, J.A. Johnson, M. Smith and B.M. Hoffman, Science, 240 (1988) 311.
19 B.S. Brunschwig, P.J. DeLaive, A.M. English, M. Goldberg, H.B. Gray, S.L. Mayo and N. Sutin, Inorg. Chem., 24 (1985) 3743.
20 A. Kowalsky, J. Biol. Chem., 244 (1969) 6619.
21 F. Basolo and R.G. Pearson, Mechanisms of Inorganic Reactions, 2nd edn., Wiley, New York, 1967.
22 H. Taube, Science, 226 (1984) 1028.
23 O. Farver and I. Pecht, Proc. Natl. Acad. Sci. U.S.A., 78 (1981) 4190.
24 O. Farver and I. Pecht, Isr. J. Chem., 21 (1981) 13.
25 G. Morpurgo and I. Pecht, Biochem. Biophys. Res. Commun., 104 (1982) 1592.
26 O. Farver, A. Licht and I. Pecht, Biochemistry, 26 (1987) 7317.
27 M. Goldberg and I. Pecht, Biochemistry, 15 (1976) 4197.
28 R.P. Ambler and L.H. Brown, Biochem. J., 104 (1967) 784.
29 H.A.O. Hill, J.C. Leer, B.E. Smith, C.B. Storm and R.P. Ambler, Biochem. Biophys. Res. Commun., 70 (1976) 331.
30 C.M. Groeneveld and G.W. Canters, J. Mol. Biol., 263 (1988) 167.
31 P. Rosen, M. Segal and I. Pecht, Eur. J. Biochem., 120 (1981) 339.

32 S. Wherland and I. Pecht, Biochemistry, 17 (1978) 2585.
33 G.D. Jones and M.T. Wilson, J. Inorg. Biochem., 21 (1984) 159.
34 G. Buse, in R. Lontie (Ed.), Copper Proteins and Copper Enzymes, Vol. III, CRC, 1984, p. 119.
35 S. Wherland, O. Farver and I. Pecht, J. Mol. Biol., 204 (1988).
36 T. Horio, J. Biochem. (Tokyo), 45 (1958) 195.
37 E. Antonini, A. Finazzi-Agro, L. Avigliano, P. Guerieri, G. Rotilio and B. Mondovi, J. Biol. Chem., 245 (1970) 4847.
38 P. Rosen and I. Pecht, Biochemistry, 15 (1976) 775.
39 M.T. Wilson, C. Greenwood, M. Brunori and E. Antonini, Biochem. J., 145 (1975) 449.
40 O. Farver, Y. Blatt and I. Pecht, Biochemistry, 21 (1982) 3556.
41 A.F. Corin, R. Bersohn and P. Cole, Biochemistry, 22 (1983) 2032.
42 S. Wherland and I. Pecht, unpublished results, 1979.
43 M.C. Silvestrini, M. Brunori, M.T. Wilson and V.M. Darley-Usmar, J. Inorg. Biochem., 14 (1981) 327.
44 G.W. Canters, H.A.O. Hill, N.A. Kitchen and E.T. Adman, J. Magn. Reson., 57 (1984) 1.
45 K. Ugurbil and S. Mitra, Proc. Natl. Acad. Sci. U.S.A., 82 (1985) 2039.
46 R.A. Marcus, Annu. Rev. Phys. Chem., 15 (1964) 155.
47 O. Farver, Y. Shahak and I. Pecht, Biochemistry, 21 (1982) 1885.
48 W. Haehnel, V. Hesse and A. Propper, FEBS Lett., 111 (1980) 79.
49 D.J. Davis, D.W. Krogman and A. San Pietro, Plant Physiol., 65 (1980) 697.
50 M. Avron and A. Schneyour, Biochim. Biophys. Acta, 226 (1971) 498.
51 G.A. Hauska, R.E. McCarty, R.J. Bergborn and E. Racker, J. Biol. Chem., 246 (1971) 3524.
52 W. Haehnel, R.J. Bergborn and B. Andersson, Biochim. Biophys. Acta, 637 (1981) 389.
53 G.H. Schmid, A. Radenz and W. Menke, Z. Naturforsch. Teil C, 30 (1975) 201.
54 H. Böhme, Eur. J. Biochem., 84 (1975) 87.
55 K.O. Burkey and E.L. Gross, Biochemistry, 20 (1981) 5495.
56 K.O. Burkey and E.L. Gross, Biochemistry, 21 (1982) 5886.
57 G.P. Anderson, D.G. Sanderson, C.H. Lee, S. Durell, L.B. Anderson and E.L. Gross, Biochim. Biophys. Acta, 894 (1987) 386.
58 T. Takabe, H. Ishikawa, S. Niwa and Y. Tanaka, J. Biochem., 96 (1984) 385.
59 L.M. Geren, J. Stonehuerner, D.J. Davis and F. Millett, Biochim. Biophys. Acta, 724 (1983) 62.
60 D.J. Davis and K. Hough, Biochem. Biophys. Res. Commun., 116 (1983) 1000.
61 D. Beoku-Betts, S.K. Chapman, C.V. Knox and A.G. Sykes, Inorg. Chem., 24 (1985) 1677.
62 S.K. Chapman, C.V. Knox and A.G. Sykes, J. Chem. Soc., Dalton Trans., (1984) 2769.
63 S.K. Chapman, C.V. Knox and A.G. Sykes, J. Chem. Soc., Dalton Trans., (1984) 2275.
64 A.G. Lappin, M.G. Segal, D.C. Weatherburn and A.G. Sykes, J. Am. Chem. Soc., 101 (1979) 2297.
65 N.M. Kostic, R. Margalit, C.-M. Che and H.B. Gray, J. Am. Chem. Soc., 105 (1983) 7765.
66 H.B. Gray, Chem. Soc. Rev., 15 (1986) 17.
67 R. Margalit, I. Pecht and H.B. Gray, J. Am. Chem. Soc., 105 (1983) 301.
68 R.J. Crutchley, W.R. Ellis and H.B. Gray, J. Am. Chem. Soc., 107 (1985) 5002.
69 R. Margalit, N.M. Kostic, C.-M. Che, D.F. Blair, H. Chiang, I. Pecht, J.B. Shelton, J.R. Shelton, W.A. Schroeder and H.B. Gray, Proc. Natl. Acad. Sci. U.S.A., 81 (1984) 6554.
70 J. Winkler, D. Nocera, K. Yocum, E. Bordignon and H.B. Gray, J. Am. Chem. Soc., 104 (1982) 5798.

71 K.M. Yocom, J.R. Winkler, D.G. Nocera, E. Bordignon and H.B. Gray, Chem. Scr., 21 (1983) 29.
72 D.G. Nocera, J.A. Winkler, K.M. Yocom, E. Bordignon and H.B. Gray, J. Am. Chem. Soc., 106 (1984) 5145.
73 S.S. Isied, G. Worosila and S.J. Atherton, J. Am. Chem. Soc., 104 (1982) 7659.
74 S.S. Isied, C. Kuehn and G. Worosila, J. Am. Chem. Soc., 106 (1984) 1722.
75 S.E.V. Phillips, J. Mol. Biol., 142 (1980) 531.
76 O. Farver and I. Pecht, 1988, submitted for publication.