Coordination Chemistry Reviews, 23 (1977) 119—129

Item of interest to coordination chemists

STRUCTURE AND FUNCTION OF COPPER PROTEINS

The third La Cura Workshop on this subject was held at Villa Giulia, Manziana (Rome), Italy, on 19—23 July, 1976

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(Received 31 January 1977)

ORGANISATION

It has almost become a tradition that Professors B.G. Malmström (Göteborg) and H.B. Gray (Pasadena) together with Professor B. Mondovi (Rome) call together, from laboratories in Europe and the US, colleagues who are working on Cu-proteins, for a few days of intensive discussions at a pleasant retreat in the old Etruscan Hill country north of Rome.

The present meeting was supported by EMBO, NSF and the Centro di Biologia Molecolare of CNR, Rome, and the Faculty of Pharmacy, University of Rome. There were no formal presentations scheduled, and it was mainly in the hands of the chairmen and discussion leaders to give the sessions the desired direction. Of great help in this undertaking was an outline of questions and challenges to be put forth on various topics, prepared by B.G. Malmström. Three days of the meeting were occupied by discussions on proteins with copper as the only metal, and almost two days by the copper—heme protein, cytochrome *c* oxidase.

DISCUSSIONS

It will not be possible in the present framework to do justice to all the contributions which made the meeting interesting and rewarding. Only a few observations, ideas or comments will be singled out, which seem particularly promising for future progress or which reflect a deviation from accepted views found in the literature or in the thinking of the majority of those assembled at Manziana.

Classification and stoichiometry

It was agreed that, although the subdivision of the copper species present in blue copper proteins into types 1—3 has served a useful purpose in the de-

scription of the properties of these proteins, it may be fallacious to adhere to their classification too rigorously in concluding that the structures around these various Cu-types in different proteins should be closely analogous. Such analogy is more likely to exist among the blue Cu-sites, as these stand out with rather unique properties, but it would be surprising to find close similarity between all sites that have been lumped together as type 2, e.g., between the Cu-site in such enzymes as diamine or galactose oxidases or the type 2 site of laccase. This designation of Cu-types should, therefore, be restricted to the blue proteins, for which they were originally introduced. This is particularly true for type 3 copper, which is the least explored among the Cu-types.

A discussion of the stoichiometry of Cu versus molecular weight and the ratio of the 3 Cu-types in the larger blue proteins such as ceruloplasmin and ascorbate oxidase, brought out the importance of the responsible reporting of analytical results in metal stoichiometries, which are often found and perpetuated in the literature in idealized, rounded-out numbers which may produce bias and thus inhibit progress toward a real solution. An example is the molar metal content of ceruloplasmin, which rests on metal analyses as well as molecular weight determinations which have both seen numerous revisions through the years. Indeed, for the proteins of high metal content there is almost no number or ratio of types of Cu per molecule that has not undergone revisions at some time, or that is entirely established even now. The question was raised whether this can realistically be expected. Not only do we face the possibilities of unavoidable error in biochemical analyses, but we must also consider that any preparative procedure has certain limits of reproducibility. Over and above these difficulties, however, looms the possibility — no longer to be considered entirely devious in these days — that the differences observed are indeed real and that there may be mixed populations of molecules with slightly varying metal content, as they have been observed, e.g., with the enzyme xanthine oxidase. We may be faced with deterioration, spurious metal binding to secondary sites, or with species resulting from natural turnover, breakdown and biosynthesis. For ceruloplasmin, e.g., there is both evidence supporting a 2 : 1 and a 3 : 1 ratio of type 1 to type 2 Cu (see ref. 1) and, obviously, these ratios cannot be considered independent of the total content of Cu per molecule, since it is generally agreed that type 3 copper occurs as a pair of Cu atoms per molecule. Evidence was mentioned for the simultaneous disappearance of type 1 and appearance of type 2 copper, pointing to the possibility of a transformation of one into the other. The question was even raised whether type 2 exists at all in the native state. There is, however, good evidence for the requirement of type 2 copper in catalysis as well as for its presence in ceruloplasmin in fresh serum, so that this extreme position is probably not justified. The discussion ended with the conclusion that no definitive answer can be given at this stage on the stoichiometry of the various copper types in the larger copper proteins.

Non-blue Cu-proteins

The state of research on galactose and diamine oxidases is such that definitive conclusions concerning the Cu site cannot be drawn at this time. With diamine oxidase the notion that pyridoxal phosphate is a constituent rather than an impurity in the enzyme has come into doubt. A good case was made (Hamilton, Penn. State), that in the enzymatically active form of galactose oxidase, Cu is oxidized by 1 e^- equivalent above the form detected by EPR, which provides support for the occurrence, in this case, of Cu^{3+}. The enzyme superoxide dismutase, which belongs in the class of non-blue Cu proteins, was not discussed at this meeting since a separate meeting concerned exclusively with this enzyme had just been held at Banyuls-sur-Mer (France).

Blue proteins, type 2 Cu

Evidence was presented (Wever, Amsterdam) from ligand superhyperfine structure in the $g_\perp$ region of ceruloplasmin, in which the type I Cu signal had been eliminated by reaction with NO, that 3 equivalent nitrogens are ligands to type 2 Cu. ^{14}NO and ^{15}NO gave identical results. Unfortunately, no splittings were observed at $g_\parallel$, which would have allowed firmer conclusions. By the use of water enriched in ^{17}O, a 12 G broadening of the low-field $g\parallel$ line of type 2 Cu in fungal laccase could be shown (Deinum, Göteborg). This effect was abolished when fluoride was added, an ion known to bind to type 2 Cu. From the strength of the effect it is concluded that water occupies an equatorial position on this Cu. An interesting approach to removing type 2 Cu from *Rhus* laccase by the combined use of EDTA and dimethylglyoxime (DMG) was reported (Graziani, Rome). It appears that DMG loosens the structure while EDTA removes the metal. During the discussion of the ligand structure around type 2 Cu the opinion was put forward (Gray, Cal. Tech.) that a protein is likely to hold the metal with the minimum number of ligands necessary to construct the proper site, with the remaining positions open to extraneous ligands.

Blue proteins, type 1 Cu

Since there are a number of blue Cu-proteins that have a single Cu atom of type 1 per molecule, much effort is of course directed toward learning about the properties of the blue Cu site from studies on these proteins. The amino acid sequences of a number of Azurins and plastocyanines are now known, and Malmström (Göteborg) reported the recent sequence determination of *Rhus* Stella-cyanin. Although much progress has been made toward understanding the structure of the metal site in blue Cu-proteins, the goal of synthesizing in vitro a well defined model for this site which incorporates all the salient characteristics, has thus far not been reached. This led Gray to the conclusion that tetrahedral Co-complexes involving a sulfur ligand are the

best models yet available. This theme was elaborated upon by Solomon (MIT), who presented mainly spectroscopic evidence [2] (near IR, CD, MCD) and ligand field arguments to support this notion. From such reasoning, the blue site is visualized as a distorted tetrahedral site, involving sulfur and 3 nitrogen ligands, although the presence of one oxygen ligand instead of a nitrogen is not excluded. Nuclear modulation, as detected by electron spin echo spectroscopy, strongly suggest (Peisach, New York) that *Rhus* Stellacyanin has one imidazole liganded to the copper, whereas in Azurin Cu is bound to two imidazoles. One or two of the nitrogen ligands may thus be furnished by imidazole while the third (nitrogen or oxygen) is assumed to be provided by a peptide group. With laccase, which in addition to a type 1 Cu has 3 other copper atoms per molecule, the near IR bands are very similar to those of the blue proteins containing one Cu atom only, but there are differences in the visible region. Thus, while the evidence for sulfur ligation of the Cu ion in the type 1 sites is growing, the single serious obstacle to accepting such a model has been removed by the finding (Deinum, Göteborg) that the single SH group of *Rhus* laccase may be available after all for ligation with Cu, a possibility which was previously thought to be excluded by the finding that 1 mole of mercurial could be bound per mole of laccase, and that the enzyme so treated suffered no loss of activity. More recent work indicates that the mercurial may be bound by another group or groups in the molecule. A new diagnostic of the sulfur ligand to the copper in the reduced state (in azurins from different bacteria and stellacyanin) was reported by Pecht (Rehovot). In measurements of difference spectra (reduced-oxidized form) a characteristic absorption band is found below 290 nm, with increasing intensity towards lower wavelengths [3]. Such an absorption spectrum is observed for the Cu^{I}—sulfur chromophore of Cu^{I}—metallothionein [4]. The band is absent in the apoprotein. Unfortunately, an absorption band in the 250 nm region cannot be expected to be useful with the multi-copper proteins. Pecht also drew attention to an absorption band of the oxidized proteins at 310—350 nm, which is attributed to a Cu^{2+}—peptide nitrogen link and which may also be present in copper oxidases.

Blue proteins, type 3 Cu

Indications had been available for some time that this copper, which is undetectable by EPR, occurs in pairs which are spin-coupled and act as a cooperative 2-electron acceptor during reduction. Evidence for this was presented by Solomon from a study of the temperature dependence of the magnetic susceptibility of *Rhus* laccase [5]. There is a small, though reproducible, deviation from Curie law behavior in the region of 100—170 K compatible with a Cu^{2+}—Cu^{2+} spin-coupling of $J = 170\ cm^{-1}$. As a convincing "control" to these experiments, there was presented a temperature curve of the susceptibility of mollusc hemocyanin, a protein in which Cu^{2+}—Cu^{2+} interaction may also be expected. No deviations were observed in this curve indicative of in-

teraction. Thus, if there is spin-coupling, $J > 600$ cm^{-1}. The alternative models of a copper(I)—disulfide system or a Cu^{I}—Cu^{III} pair have thus become less likely. It was agreed that for high spin Cu^{III} *d*—*d*, transitions in the near infrared should be detectable. No explanation can be given, however, for the origin of the absorption band at 330 nm, which is generally taken as a characteristic of the coupled copper pair in the blue Cu-containing oxidases. Analyses were reported (Lerch, Universität Zürich) showing the presence of 2 Cu per molecule of *Neurospora* tyrosinase (1.8—1.9 Cu per MW of 42,000) [6].

The copper in tyrosinase is EPR-nondetectable in the resting, reduced or oxygenated enzyme. However, upon addition of mercaptoethanol, a paramagnetic species, probably a mixed-valence Cu^{I}—Cu^{II} pair, is formed (Deinum and Lerch, Göteborg) [7]. A similar paramagnetic complex is formed with hemocyanin.

Electron-transfer properties and catalytic mechanism

What might be termed a heroic attempt to come to grips with the mechanism of electron transfer to and from blue proteins, *c*-type cytochromes and iron—sulfur proteins (HIPIP), was presented by Wherland (Cal. Tech.) [8]. On the basis of measurements of reaction rates of different proteins with a series of outer sphere donors and acceptors of varied properties, apparent rate constants were calculated for the self-exchange reaction (k_{11}) of protein redox-couples using Marcus theory. Some (tentative, because of the relatively small sample) generalizations were derived: donors and acceptors, with which the highest rates were achieved throughout, had hydrophobic π bonding ligands and showed a high entropy and high enthalpy of activation. For protein redox pairs, which are not physiological redox partners, the calculated rate constants agreed reasonably well with the observed ones, whereas with the pair *Pseudomonas* azurin—cytochrome *c* 551, which might naturally react with each other, the observed rate constants were between 2 and 3 orders of magnitude larger. It was also observed that few proteins gave consistent apparent k_{11} values with various donors or acceptors. It is thought that when there was little difference in k_{11}, the active site of the protein is relatively exposed, whereas it is least accessible in those proteins with which large k_{11} differences between various donors or acceptors were found.

It had been concluded previously, that in the reaction of *Pseudomonas* azurin with *Pseudomonas* cytochrome *c* 551, an equilibrium of a slowly and fast reacting form of azurin must be involved to explain the kinetics. The same conclusion was arrived at for the reaction of azurin with the *Pseudomonas* cytochrome oxidase—nitrate reductase (Brunori, Rome). Evidence was also presented for this system that a complex exists within which electron transfer occurs. Under certain conditions, this electron transfer rate becomes the limiting step. With azurin from *Alcaligenes faecalis* reacting with *Pseudomonas aeruginosa*, no evidence for the two conformers was found (Pecht and Rosen, Rehovot). Kinetic studies on the reduction of *Rhus* laccase by hydroquinone

were presented (Andréasson, Göteborg), which likewise could best be explained by the assumption of an equilibrium of two forms of the enzyme showing different reactivity towards substrate. This conclusion was based on spectrophotometric data collected at 615 and 340 nm at pH 6 and 7.4, and on computer simulation of the kinetic progress curves. The possibility was considered that one of the electrons needed for reduction of the type 3 site entered via the type 2 Cu. There was an interesting preliminary experiment reported (Fielden, Sutton, Surrey) in which native *Rhus* laccase and enzyme deprived of its type 2 Cu by the method of Graziani (see above) were exposed to a pulse of hydrated electrons (0.1 eq per mole of type 1 Cu). Only with the apoenzyme was there a diminution of the absorption at 615 nm, indicating that in the holoenzyme, electrons pass on fast to other sites on a path dependent on the presence of type 2 Cu. However, an extensive pulse-radiolysis study of this enzyme (Pecht and Goldberg [9]) has shown that in the holoenzyme, under similar conditions, a first order reduction of the 615 nm absorption, concomitant with the decay of a fast formed transient intermediate absorbing around 410 nm, is observed. Nevertheless, experiments of this kind, if conditions are properly worked out, ought to provide the sought-for answer on the participation of type 2 Cu in intramolecular electron transfer.

Concerning the reaction of blue oxidases with O_2, the most promising data available thus far are those on *Rhus* and fungal laccases obtained by the use of $^{17}O_2$ (Malmström and Vänngård, Göteborg). An EPR signal at $g = 1.91$ is observed on rapid mixing of fully reduced laccase with O_2 which is broadened when $^{17}O_2$ is used [10]. The characteristic optical (around 370 nm) and EPR absorptions correlate well. With fungal laccase the lifetime is ~1 s, with the *Rhus* enzyme ~10 s. Since only one line of the EPR spectrum can be observed because of overlap with the copper signals, the identification of the oxygen species involved is difficult. The species O^- is thought to be the most likely candidate at this time. Pecht reported on the observation of a species having an extinction of 850 M^{-1} cm^{-1} at 325 nm, produced specifically by the addition of an equimolar amount of H_2O_2 to *Rhus* laccase. In contrast to the transient intermediate observed by the Göteborg group for the reaction between the fungal laccase and H_2O_2, this species is stable for days and may be produced even at micromolar concentration of both enzyme and H_2O_2. Furthermore, it cannot be produced by any other oxidant except hydrogen peroxide. For the full reduction of this peroxy complex, six electron equivalents are required and reoxidation by O_2 yields the native enzyme.

In oxidative titrations of *Rhus* laccase by several oxidants (O_2, H_2O_2, $Mo(CN)_8^{3-}$ and Mn EDTA$^-$) the non-equivalence of the oxidation and reduction paths of this oxidase was observed. This is also expressed in different Nernst coefficients determined for each half of the redox cycle (2 for reduction and 1 for oxidation). This non-equivalence of titration pathways is an example of chemical hysteresis [11].

Cytochrome oxidase

The discussions on cytochrome *c* oxidase were not limited to the copper components, but also dealt extensively with the heme components, since there are more approaches available to deal with these. In fact, there is very little direct information on one of the copper atoms except that its presence can be ascertained by chemical analysis or EPR spectroscopy after denaturation. The difficulties encountered in research on this protein were summarized by the chairman of the first session: The protein is membrane-bound and has to be solubilized; different approaches to solubilization do not lead to identical products; differences in lipid content are most striking; the molecular weight of the active unit and the aggregation state of the protein under various conditions are not well defined; the substrate is itself a protein; the midpoint oxidation—reduction potential of the substrate is slightly higher than that of some components of the oxidase under certain conditions, so that the "reducing" substrate can be an oxidant for components of the oxidase; the protein has a very high affinity for O_2, placing high demands on anaerobic techniques in studies on oxidation—reduction properties; the optical absorption of the heme components dominates to the extent that very little information on the copper components can be derived from spectrophotometry, while the absorptions of the two hemes overlap; EPR spectroscopy detects at best two components (1 heme, 1 Cu) in the oxidized form of the enzyme, while at intermediate oxidation states more species of EPR signals for heme appear than there are components in the enzyme; there are slow spectral changes in the oxidized (resting) enzyme depending on a number of environmental conditions, and on oxidation—reduction rapid changes in optical and EPR spectra are followed, over hours, by slow changes.

There seems to be general agreement on the redox-active components of cytochrome *c* oxidase, namely two heme groups and two Cu atoms, and that these together make up the minimal functional unit, but the question was raised, whether the real active unit may not be comprised of a dimer of this minimal unit. It was reported (Van Gelder, Amsterdam) that at concentrations as they are generally used in spectrophotometric experiments a dimeric species, corresponding to 8 metal atoms, was predominating according to ultra-centrifuge studies. At concentrations of 0.1—1 mM as used in most EPR studies, higher polymeric forms may be expected to prevail, a situation which may well influence the observations made by this technique. It was pointed out that it may be necessary to adjust the concentration of the detergent that is always added to keep the enzyme in solution, to the amount of protein present rather than to the volume of the dissolving medium. There was also general concensus that the purified preparations mostly used in these days which have a heme content of >10 nmoles/mg protein do not differ in essential features. However, it was stressed by Wilson (Univ. of Penn.) that the membrane-bound enzyme does show behaviour different from that of the purified enzyme. The meeting accepted the idea that the two hemes and two copper

atoms are not equivalent in the oxidized form of the enzyme. This is supported by the quantities of heme or Cu represented in the EPR signals (viz. at most one of each) of the oxidized enzyme as well as by observations on the absorption band at 655 nm (Beinert, Univ. Wis.), quantitative evaluation of MCD spectra (Palmer, Rice Univ.) and Mössbauer spectroscopy [12]. There is no question that non-equivalence exists when electrons have entered the molecule or ligands have been bound. However, there still seems to be no safe ground on which the contributions of the two hemes to the absorption at ~600 nm can be assessed under all conditions. X-ray absorption spectroscopy indicates that two Cu^{II} atoms in different environments are present (Blumberg, Bell Labs.) and it is generally accepted that the prominent EPR signal of cytochrome *c* oxidase at $g \sim 2$ arises from one of the Cu atoms, despite its unusual EPR parameters. It was pointed out, however, that it was possible that the signal could arise from a sulfur free-radical in the neighborhood of a Cu^{I} ion, Peisach (Albert Einstein Coll. Med.). The information on the oxidized enzyme available from magnetic susceptibility data is conflicting, but there seems to be agreement that a non-interacting high-spin ferric species cannot be present and that the fully reduced enzyme contains a high-spin ferrous heme. Palmer, (Rice Univ.) presented evidence on the basis of a number of arguments including quantitative evaluation of MCD spectra, comparison of these to the MCD of other heme compounds and also including the effect of ligand interactions on MCD, for a model proposed earlier, a model which postulates the presence in the oxidized enzyme of two non-interacting species, a low-spin heme and a Cu atom, and a pair of antiferromagnetically coupled species, namely a high-spin heme and the second Cu atom. Palmer assumes that the high-spin as well as the low-spin heme retain their spin state throughout the reduction process. Such an assumption, of course, also would determine the interpretation of the EPR spectra, which show several ferric high-spin species during the process of reduction. Several workers in the field, however (Wilson (Univ. Penn.), Beinert (Univ. Wis.), Van Gelder (Univ. Amsterdam)), were not willing to accept this point of view. It is thought by Beinert and by Wilson that cytochrome *a*, which is low-spin initially, undergoes a spin-state change as cytochrome a_3 becomes reduced, although it was pointed out by Beinert that among the ~6 high-spin ferric heme species which can be observed under various conditions, some may well represent cytochrome a_3.

These considerations led to discussions on oxidation—reduction of cytochrome *c* oxidase and this was initiated by a consideration of the reduced form in the presence of CO, since one might expect some simplification with only 3 components to be reckoned with (cytochrome a_3^{2+}—CO being inert toward oxidation). However, disagreement was immediately apparent on this issue, since data obtained by Anderson et al. (introduced in absentia of the authors) [13] by coulometric titration combined with spectrophotometry, indicated release of 3 electrons from the reduced CO-blocked form, whereas Wilson (Univ. of Penn.) presented evidence from potentiometric titrations at varied pH and CO concentrations, that at high concentrations cytochrome

a_3—CO is titrated with an n value of 2 in unison with a second donor, which is thought to be the undetectable Cu [14]. There was otherwise little new information from redox titrations and it appeared that a scheme describing the events on oxidation—reduction, very similar to one proposed by Nicholls [15], found much appeal and acceptance. Since this also has a bearing on events seen in kinetic studies, it will be discussed in more detail later.

Among kinetic studies, only work on the reductive phase was considered after brief reference to the work on the O_2 reaction published by Chance et al. [16]. A significant set of observations was reported by Antonini (Univ. Rome), Brunori (Univ. Rome) and Greenwood (Univ. East Anglia) which leaves little doubt that the oxidized, resting form of the enzyme, as normally obtained on purification, is not the catalytically active one. The rate of reduction by cytochrome c of oxidase as prepared, is considerably slower than the reduction of enzyme that has undergone a cycle of reduction by cytochrome c and reoxidation by a pulse of oxygen (at a sufficiently low O_2 concentration, so that no excess O_2 remains) and is then exposed to an excess of cytochrome c. The difference in rates vanished at low enzyme concentrations. It is obvious that in ordinary catalytic assays at low concentrations, where any individual molecule is turning over many times, this effect is not observed, whereas in single-turnover type of experiments in the stopped-flow apparatus and at the high concentrations required for EPR spectroscopy, the effect is likely to be sizeable. Antonini suggested that a small fraction of an active species of the type

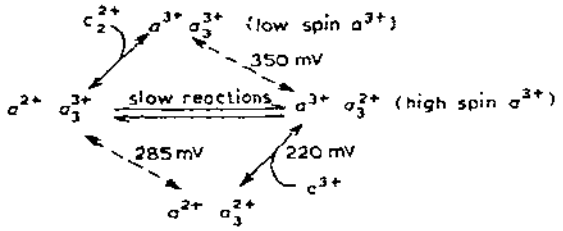
a^{3+} $\quad$ a_3^{2+}

Cu_d^{2+} $\quad$ Cu_u^{1+}

is formed. (The subscripts d and u stand for detectable or undetectable, respectively.) Similar observations were made when the same reaction sequences were carried out in the presence of CO. It had been shown previously by Gibson et al. [17] that the emergence of light sensitivity in the presence of CO is a useful measure of the rate of reduction of the a_3 component. With the resting oxidized enzyme this reduction and the emergence of light sensitivity had been found to be slow. However, with the O_2-"pulsed" oxidase, the combination of CO with a_3^{2+}, and heme a_3^{3+} reduction, were significantly accelerated. When the enzyme was allowed to autoreduce in the presence of CO (presumably to the CO-bound form of the species shown above as postulated by Antonini) and was then rapidly mixed with cyanide and O_2 and exposed to a flash of light, the rate of cyanide binding was 10 times that seen on exposure of the resting enzyme to cyanide under comparable conditions (Greenwoord, Univ. East Anglia). This is in line with previous observations that cyanide binding is more rapid when the enzyme is turning over than when it is in the oxidized state. Brunori (Univ. Rome) showed data from stopped-flow and temperature-jump experiments on the reaction with ferrocytochrome c of the "mixed valence" cytochrome c oxidase, again prepared by autoreduc-

tion in the presence of CO. Distinctly biphasic responses were observed which indicated a fast bimolecular (approximately 9×10^6 M^{-1} s^{-1} in both directions) and a slower unimolecular step (40–100 s^{-1}). Under all conditions the quantity of cytochrome c oxidized significantly exceeded the quantity of cytochrome *a* reduced. These results confirm previous findings that in the initial reductive phase, electrons are rapidly transferred to cytochrome *a* and partly also to an additional component, which, in the mixed valence enzyme, is most likely the EPR detectable Cu. This is also suggested by the observed absorbance decreases at 830 nm. These findings are not entirely in agreement with data published by Andréasson (Göteborg), who finds reduction of *a* only [18].

Van Gelder (Amsterdam) drew attention to the strong dependence on ionic strength of the reaction of reduced cytochrome *c* with the oxidase [19]. The rate of the on-reaction drops as the ionic strength increases. At pH 7.4 and 1°C the rate extrapolated to zero ionic strength is 2.3×10^8 M^{-1} s^{-1}. Petersen (Odense and Göteborg Univ.) presented detailed kinetic studies on the reoxidation of reduced oxidase by ferricytochrome *c* and the steady state kinetics with ascorbate, cytochrome *c* and O_2 in the presence and absence of inhibitors. The results were explained, as shown below, by a scheme referred to above and proposed by Nicholls (the Cu components are not considered in this scheme):



The most important aspect of this scheme is the equilibration of the half reduced forms. This scheme is indeed able to explain a number of independent observations obtained by titration and kinetic studies by means of optical and EPR spectroscopy. It remains for future work to show whether and how it could be expanded or modified so that the Cu components and also observations, which at this time do not seem compatible with it, can be accommodated.

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