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Oxidation of Heme Proteins by Copper(II) Complexes. Rates and Mechanism of the Copper Catalysed Autoxidation of Cytochrome c, Myoglobin and Hemoglobin

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The mechanism of the autoxidation of ferrocytochrome c catalysed by some copper(II) complexes has been established. In most cases the rate determining step was found to be the one electron oxidation of the cytochrome by the copper(II) species, which was followed by the rapid reoxidation of the resulting Cu(I) complexes by molecular oxygen. The rate constants for the electron transfer reactions of several copper(II) complexes with ferrocytochrome c, myoglobin and hemoglobin have been measured at 25 °C. It was shown that these rates for the copper complexes, as well as those for several other oxidants, are consistent with simple outer sphere electron transfer through the heme edge for all the heme proteins. The redox potential of the reacting species was found to be the dominant factor in determining the relative rate of the electron transfer reactions. This enables the catalytic activity of different copper complexes to be predicted.

Introduction

Catalysis of autoxidation by copper(II) complexes has been frequently observed in diverse areas of chemistry, but is nevertheless only poorly understood. Recently the copper(II) aquo ion catalysed autoxidation of hemoglobin has been intensively investigated [1, 2], in part because of its possible relevance to *in vivo* oxidation. Copper aquo ion was used in these studies even though only very low concentrations of free copper will be present *in vivo* relative to the concentration of copper bound to the abundant complexing species such as peptides, phosphate and citrate, as well as high molecular weight species such as albumin. We have investigated the catalytic effect of a range of copper complexes on heme autoxidation. This work forces conclusions of significance to hemoglobin autoxidation, but also of importance to the general problem of copper catalysis. The studies were directed at two main questions: (i) What are the major factors affecting the relative catalytic power of various copper complexes? Why, for example, is CuEDTA catalytically inactive? and (ii) What are the important differences between hemeproteins that affect their susceptibility to catalytic autoxidation?

The mechanism of the catalysed autoxidation of hemoglobin is difficult to establish because of complications arising from the presence of multiple hemes and the binding of oxygen to the hemes. Therefore, we have predominately studied the simpler heme proteins cytochrome c and myoglobin, but we consider that our main conclusions are applicable to many other substrates including hemoglobin. A mechanism has been established for the catalysed autoxidation of these proteins. This supports the less well founded mechanism proposed for hemoglobin.

Copper histidine and copper citrate complexes have previously been shown [3] to catalyse the autoxidation of ferrocytochrome c, but the complicated kinetic behavior did not permit the assignment of a detailed molecular mechanism. Their observations are however consistent with the general pattern of behavior we have found.

Experimental

Copper complexes were prepared *in situ* by mixing aqueous solutions of appropriate concentration of copper nitrate and the ligands. Solutions were buffered at a pH of 6.15 with 10^{-2} *M* 2-(n-morpholino)-ethanesulphonic acid (MES), and maintained at an ionic strength of 0.1 *M* with sodium nitrate. Concentrations of the reactant complexes were calculated from published stability constants where they differed from stoichiometry significantly. These differences were always small (less than 10%). Reduced cytochrome c was prepared by reduction of degassed, buffered, solutions of oxidized horse heart cytochrome c (Sigma Type (III) or Type (VI)) with sodium dithionite, followed by gel filtration on a 1 × 20 cm column of Sephadex G 25.

Sperm whale myoglobin (Sigma Type (II)) and human hemoglobin (Sigma Type (IV)) were reduced by the same procedure as cyt c. Concentrations of cyt c and myoglobin were calculated from published extinction coefficients [4, 5].

Equilibrium Constants

Equilibrium constants for reactions such as that described by equation (1) were determined from visible spectral determination of the cyt c(II) and cyt c(III) concentrations from published extinction coefficients [4]. In detail, 3 ml of a copper(II) solution of accurately known concentration was purged of oxygen by bubbling with argon or nitrogen in a 1 cm spectrophotometer cell fitted with a suba seal. After bringing to 25 °C in a temperature controlled cell block of a Varian 635 UV–visible spectrophotometer, approximately 10 microlitre of deoxygenated ferrocytochrome c was injected through the suba seal, and the oxidation of cytochrome followed by the change in absorbance at 549 nm. When reaction was complete, the absorbance was recorded at 549 nm and at three isobestic points (504, 541, 525 nm). The ratio of oxidized to reduced cyt c at equilibrium could then be calculated. Approximately 100 microlitre of a 0.25 *M* deoxygenated solution of EDTA was then injected into the cell. This leads to a reversal of the reaction, that is the Cu(I) in solution is oxidized by cyt c(III), since the EDTA displaces the equilibrium to the left. The Cu(I) concentration at equilibrium was then determined from the difference in concentrations of oxidized cytochrome before and after the addition of EDTA.

Kinetics

Pseudo first order rate constants, k_{obs}, were determined from first order plots of the changes in absorbance at appropriate wavelengths, as monitored on the Varian 635 or a stopped flow spectrophotometer. All kinetic and equilibrium measurements were carried out at 25 °C, at pH 6.15 and at an ionic strength of 0.1, unless otherwise specified.

Results and Discussion

Oxidation of Cytochrome c

Solutions of reduced cyt c at pH 6 and above autoxidize in air only very slowly in the absence of catalysts such as copper ions (see below). Addition of a range of copper complexes results, in the presence of sufficient oxygen, in the relatively rapid and complete oxidation of cyt c(II) as judged by the visible absorption spectrum of cyt c. The rate observed for this catalytic autoxidation depends on the nature of the metal complex.

In the absence of oxygen, partial oxidation of cyt c still occurs on the addition of solution of $Cu(aq)^{2+}$, bis (2,2′-bipyridyl)copper(II) ion ($Cu(bipy)_2^{2+}$) or bis-(1,10-phenanthroline)copper(II) ion ($Cu(phen)_2^{2+}$), with initial rates comparable to the rates in the presence of air. Complete oxidation does not occur; the reaction reaches an equilibrium position which depends on both the concentration and type of copper complex used. This reaction in the absence of oxygen is, we believe, the result of a simple electron transfer reaction, for example that given by equation (1)

$$Cu(bipy)_2^{2+} + \text{cyt c(II)} \rightleftharpoons Cu(bipy)_2^{+} + \text{cyt c(III)} \quad (1)$$

Visible absorption spectra of the products are consistent with this equation. More significantly, the equilibrium constants (Table I) for the reaction with $Cu(bipy)_2^{2+}$ and $Cu(phen)_2^{2+}$ were found to be in good agreement with the equilibrium constants calculated from the independently measured redox potentials for the copper complexes [6] and cyt c [7]. $Cu(bipy)_2^{2+}$; Found $(2.9 \pm 0.5)10^{-3}$, Calculated $(4.3 \pm 2)10^{-3}$. $Cu(phen)_2^{2+}$; Found $(5.2 \pm 0.6)10^{-2}$, Calculated $(3.5 \pm 2)10^{-2}$. This observation not only supports the assignment of the spectral changes to reaction (1), but also means that binding of the copper complexes to cyt c is negligible (or at least there is a negligible difference between the binding of the copper complex in its two oxidation states). The measured equilibrium constant for $Cu(aq)^{2+}$ depends on the copper and cytochrome concentrations, as anticipated as it was known (unpublished observations) that the copper ion binds to cyt c under these conditions.

In the presence of oxygen, the Cu(I) complexes are rapidly reoxidized to Cu(II), thus completing the

TABLE I. Equilibrium Constants (K) for the Oxidation of Ferrocytochrome c by $Cu(phen)_2^{2+}$ and $Cu(bipy)_2^{2+}$ Ions[a].

$Cu(phen)_2^{2+}$		$Cu(bipy)_2^{2+}$	
10^3 (Initial $[Cu(phen)_2^{2+}]$) *M*	10^2 K	10^3 (Initial $[Cu(bipy)_2^{2+}]$) *M*	10^3 K
0.21	6.2 (1)	0.52	2.7 (1)
0.42	5.0 ± 1.9 (2)	1.04	3.5 (1)
0.52	6.0 ± 0.8 (6)	2.08	2.9 ± 0.5 (4)
1.04	3.4 ± 0.2 (2)	4.16	2.5 (1)
2.08	5.1 ± 0.8 (8)		

[a]All data were obtained at 25 °C and ionic strength 0.1. $Cu(phen)_2^{2+}$ and $Cu(bipy)_2^{2+}$ results were measured on solutions buffered, at pH 6.1 and 5.0 respectively, with 10 m*M* MES.

catalytic cycle. If the electron transfer step is rate determining then the autoxidation rate is expected to follow the equation:

$$d\,[\text{cyt c(III)}]/dt = k_{12}\,[\text{cyt c(II)}]\,[\text{Cu(phen)}_2^{2+}] \quad (2)$$

This behavior is exhibited by the copper phenanthroline catalyzed reaction. Linear, pseudo first order plots were obtained with excess $Cu(phen)_2^{2+}$. Figure 1

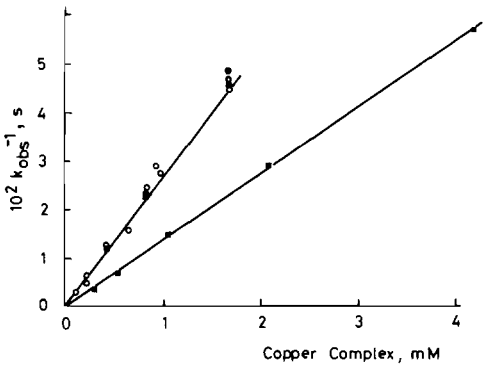

Fig. 1. Rate of oxidation of ferrocytochrome c by $Cu(phen)_2^{2+}$, ○, and $Cu(bipy)_2^{2+}$, ■. Dependence of the pseudo first order rate constant, k_{obs}, on the copper concentration in the presence, ○, and absence, ●, of oxygen.

demonstrates that the first order rate constants, k_{obs}, derived from these plots are consistent with equation (2). The second order rate constant $k_{12} = (2.72 \pm 0.3)$ 10 M^{-1} s^{-1} was obtained from the slope of Fig. 1. Moreover, the initial rate of reaction in the absence of oxygen was found to be in good agreement (Fig. 1) with the data obtained in air.

With the $Cu(bipy)_2^{2+}$ and the copper aquo ions at pH 6.1 the first order plots were not found to be linear, the extent of non-linearity being dependent on the experimental conditions. Saturating the solutions with pure oxygen rather than air increased the rates of these reactions, and improved the linearity of the first order plots. These observations are consistent with our proposed mechanism if the rate of reoxidation of the Cu(I) complexes by molecular oxygen is similar to the rate of the reaction of Cu(I) with ferricytochrome c, the reverse of reaction (I). No direct experimental determination of the rate of such oxidations under our experimental conditions, in particular at low Cu(I) concentrations have been reported, but the rate of autoxidation at higher concentrations is sufficiently slow [8] that it is reasonable to suppose that it does not effectively dominate over the reoxidation of Cu(I) by oxidized cytochrome. This conclusion is also supported by other observations. At lower pH, 3–5, when the rate of autoxidation of Cu(I) is more rapid, good linear first order plots were obtained. Data obtained at pH 5 for $Cu(bipy)_2^{2+}$ catalyzed autoxidation are also presented in the Fig. 1. These results are consistent with the second order rate equation (equation 2) yielding a rate constant $k_{12} = (1.39 \pm 0.1)\,10\,M^{-1}\,s^{-1}$.

With Cu(II) aquo ion the rate of oxidation was not found to be strictly first order in the cyt c(II) concentration, at least partly because of the relatively slow rate of reoxidation of the Cu(I). However, since the uncomplexed copper(II) binds to cyt c, bound copper may also be kinetically active resulting in complicated kinetic behavior. The data in Fig. 7, which is a plot of the initial rate of oxidation as a function of the total copper(II) concentration, is also evidently more complicated than that found with the other copper complexes studied. We have so far been unable to satisfactorily interpret these and similar results.

From the mechanistic scheme proposed, in which the first and essential step is a simple reversible electron transfer reaction, it follows that copper complexes with high redox potentials will completely and rapidly oxidize cyt c without reoxidation of the Cu(I) by molecular oxygen. This prediction was confirmed by experiment. Both bis(2,9-dimethyl-1,10-phenanthroline)copper(II) ion (E^0 = 0.59 volt) [6] and (2,5-dithiahexane-1,6-dicarboxylato)copper(II), Cu(DTA) (E^0 = 0.48 volt) [9] rapidly and completely oxidize cyt c(II). The rates of both reactions were found to be first order in both the cyt c(II) and the copper complex concentration. Second order rate constants may be found in Table III. Complete kinetic data for these systems will be published elsewhere.

The proposed mechanism also leads directly to the prediction that copper complexes with low redox

TABLE II. Rate Constants for the Autoxidation of Ferrocytochrome c in the Presence of Some Copper Complexes that do not Catalyze the Reaction[a].

$10^3\,[Cu^{2+}]$, M	10^3 [ligand], M[a]	Half life, hr	k_{obs}, s^{-1}
1.04	1.25	30	6.4×10^{-6}
1.04	10.40	29	6.6×10^{-6}
10.40	12.48	25	7.7×10^{-6}
1.04	2.08	30	6.4×10^{-6}

[a]EDTA was used in all experiments except for those recorded in the last line for which *trans*-[14]-diene was used.

TABLE III. Comparison of Calculated and Observed Rate Constants for the Oxidation of Ferrocytochrome c and Deoxymyoglobin[a].

Oxidant	Deoxymyoglobin k_{12}(My), M^{-1} s^{-1}	Ferrocytochrome c k_{12}(cyt), M^{-1} s^{-1}	Ratio k_{12}(My)/k_{12}(cyt)		
			Observed	Calculated	
				uncorr.	corr.
$Cu(phen)_2^{2+}$	4.3×10^4	27	1600	50	73
CuDTA	1.8×10^5	5.1×10^3	35	36	32
$Cu(dmphen)_2^{2+}$	2.8×10^6	1.0×10^6	2.8	23	34
$FeCDTA^-$/$FeEDTA^-$	1.45×10^2	1.1×10^2	1.3	35	21
$Fe(CN)_6^{3-}$	1.6×10^7	6.7×10^6	2.4	28	10
Ferricytochrome c	2.2×10^3	300	7.3	40	66

[a]Conditions for the copper oxidations may be found in the captions to the relevant figures. Rate constants for $FeEDTA^-$-cyt c oxidation were taken from ref. 27; $FeCDTA^-$–My data from Cassatt *et al.* (1975); $Fe(CN)_6^{3-}$–cyt data from ref. 28; $Fe(CN)_6^{3-}$–My data from ref. 16; ferricytochrome–ferrocytochrome exchange data from ref. 29; ferricytochrome–My data from ref. 22.

potentials will be much less effective catalysts since the rate of the electron transfer step is expected [10] to decrease with decreasing redox potential (the measured rates of electron transfer do follow this pattern [11]). This prediction was also experimentally verified. In the presence of EDTA and copper, cyt c(II) is oxidized at a rate which is independent of the concentrations of these reagents (Table II). The same rate of oxidation was found [12] in the presence of (*trans* [14] diene)copper(II) ion (Table II). This slow rate is ascribed to the rate of the uncatalyzed autoxidation at this pH. The rate constants found agree well with those reported by Sawada *et al.* [13]. Very slow rates of autoxidation are also found in the presence of bathocuproine disulfonate (following an initial rapid reduction in which the impurity Cu(II) is reduced to Cu(I)). This Cu(I) specific reagent breaks the catalytic cycle by preventing reoxidation of the Cu(I) formed in the initial electron transfer step.

Some further copper complexes with redox potentials above 0.1 volt, including complexes of imidazole, citrate ions and dipyridylammine all catalyze the autoxidation of cyt c(II) with rates in the same range as the phenanthroline and bipyridyl complexes. Davison and Hamilton [3] have previously found that mixtures of copper and citrate ions catalyze oxidation, but with more complex kinetics than we have found with other copper complexes. The complex behavior of these solutions is probably a consequence of the presence of a complicated mixture of copper complexes, including dimeric species, and possibly of binding to cyt c of the negatively charged complexes. More surprisingly, Davison [3] also reported that bis(histidine)copper(II) ion, $Cu(his)_2$, was an effective catalyst despite the low value of the redox potential for reduction of this complex. However, the observation that excess histidine slowed down the reaction, and that the reaction was highly pH dependent, suggests that the active species in solution is not $Cu(his)_2$ but either $Cu(his)^+$ or $Cu(aq)^{2+}$. We have confirmed and extended his observations. Pseudo first order behavior was also found in our work, yielding the rate constants shown in Fig. 2. These results are not quantitatively consistent with the simple hypothesis that only one of the species $Cu(aq)^{2+}$, $Cu(his)^+$, or $Cu(his)_2$ is the kinetically active species in solution, based on the concentrations of these species calculated from the published equilibrium constants [14].

Some conclusions are nevertheless apparent. Firstly the slow rate found at high histidine concentrations requires that the rate constant for $Cu(his)_2$ catalysis be less than 3×10^{-2} M^{-1} s^{-1}. Secondly the absence of any pronounced inflection around a ligand to metal ratio of 1:1, together with other observations on $Cu(aq)^{2+}$ catalysis, suggests that the $Cu(aq)^{2+}$ ion is the kinetically dominant species in solution. Any of the multitude of other species in solution may however also contribute to the observed rates.

Thus we have not found any genuine exceptions to the observation that the catalytic activity of the copper complexes is qualitatively related to redox potential. Only copper complexes of intermediate redox potential show strong catalytic activity because

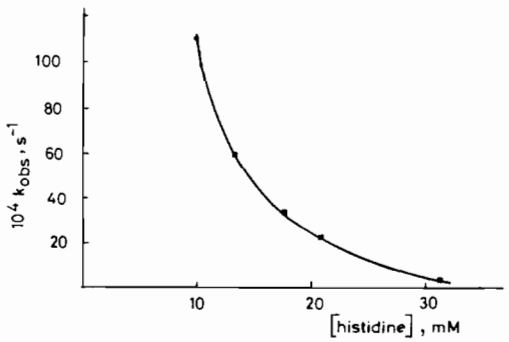

Fig. 2. Rate of oxidation of ferrocytochrome c by copper(II) in the presence of histidine. Dependence on the histidine concentration.

electron transfer becomes too slow at low redox potentials, and at high redox potentials although electron transfer is rapid the rate of reoxidation of the Cu(I) complex becomes too slow for effective catalysis. It is evident however that there are circumstances where the simple relationship between catalysis and redox potential may not hold. Probably the most important of these is the variation in the degree of association of the reactants. For example, negatively charged copper complexes such as the copper-citrate ions will associate more strongly with the positively charged cytochrome which will usually result in more rapid oxidation. If, however, the interaction is strong and specific to particular sites on the protein, large increases or decreases in rate may be observed.

It is also apparent that our predictions need to be modified if the redox potential of the substrate is very different from that of cytochrome. Copper complexes with low redox potentials may act as catalysts for substrates of similar redox potential since in such cases electron transfer will be thermodynamically more favourable and the rate of electron transfer will probably be appreciable. It will be difficult to find a suitable catalyst for substrates of much higher redox potential than cyt c. Our assumption that the rate of the electron transfer step is rate determining and not the rate of oxidation of the copper(I) by molecular oxygen also may not always be valid. Indeed, with a few complexes we found that the rates of the two reactions were comparable.

Electron transfer to the heme from the Cu(II) complexes probably occurs through the edge of the heme that is in contact with the bulk solvent, as is thought to commonly occur in the electron transfer reactions of cyt c [15].

Oxidation of Myoglobin and Hemoglobin

We have also examined the reactions of copper complexes, other than the aquo ion, with myoglobin and hemoglobin. Both proteins are oxidized by $Cu(phen)_2^{2+}$ in a similar way to cyt c but at higher rates. With myoglobin, in the presence of more than a tenfold excess of the copper complexes, absorbance changes at 425 or 435 nm yielded good linear first order rate plots. Rate constants, k_{obs}, derived from these results are plotted against $Cu(phen)_2^{2+}$ concentration in Fig. 3. The data in Fig. 3 are consistent with simple second order behavior, as in equation (2), with an apparent rate constant $k = 148 \pm 5\ M^{-1}\ s^{-1}$. We have also shown that the rate of reaction with myoglobin decreases with increasing oxygen concentration, as expected if deoxymyoglobin is the active reductant rather than oxymyoglobin (Fig. 4). Such behavior is commonly found in oxidation reactions of myoglobin [16, 17].

It is consistent with the mechanism:

$$MyO_2 \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} My + O_2$$

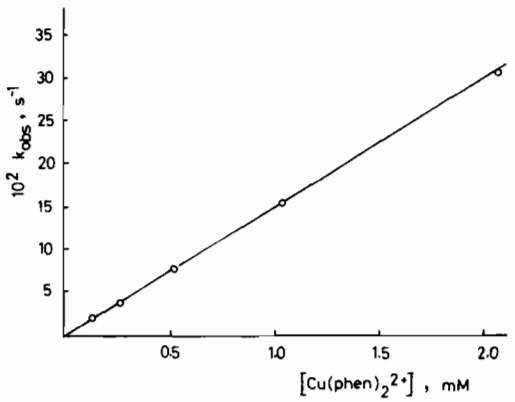

Fig. 3. Rate of oxidation of myoglobin by $Cu(phen)_2^{2+}$. Dependence of the pseudo first order rate constant, k_{obs}, on the concentration of $Cu(phen)_2^{2+}$.

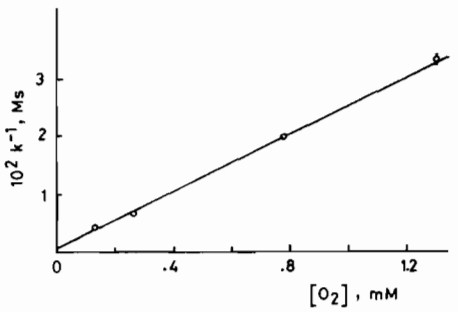

Fig. 4. Rate of oxidation of myoglobin by $Cu(phen)_2^{2+}$. Dependence of the reciprocal of the second order rate constant, k, on the concentration of oxygen.

$$My + Cu(phen)_2^{2+} \xrightarrow{k_{12}} My^+ + Cu(phen)_2^+$$

$$(Cu(phen)_2^+ \xrightarrow{O_2} Cu(phen)_2^{2+})$$

where My, MyO_2 and My^+ represent deoxymyoglobin, oxymyoglobin and metmyoglobin respectively. The rate constant, k, for oxidation of deoxymyoglobin by the copper complex was calculated from the equation $k_{12} = kK[O_2] = kk_{-1}[O_2]/k_1$, where K, the association constant for the binding of oxygen to deoxymyoglobin, was taken to be 1.1×10^6 [5]. Similar kinetic behavior was found in the oxidation of myoglobin by the copper complexes of DTA and dmphen, although in these systems k_1 is close enough to $k_{12}[CuL]$ to require the use of a double reciprocal plot [17]. – see Figs. 5 and 6. The intercepts of these plots should be equal to the reciprocal of k_1, which has previously been shown [5, 17] to be $10\ s^{-1}$. The slopes of the plots yielded the rate constants, k_{12}, given in Table III.

The kinetics of oxidation of hemoglobin by $Cu(phen)_2^{2+}$ are similar to those of myoglobin, except that good first order behavior was not observed. Initial rates were however proportional to the copper concentration, and inversely proportional to the

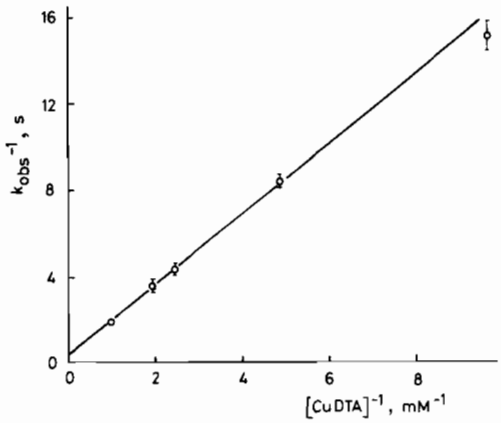

Fig. 5. Rate of oxidation of myoglobin by CuDTA. Dependence of the pseudo first order rate constant, k_{obs}, on the reciprocal of the CuDTA concentration.

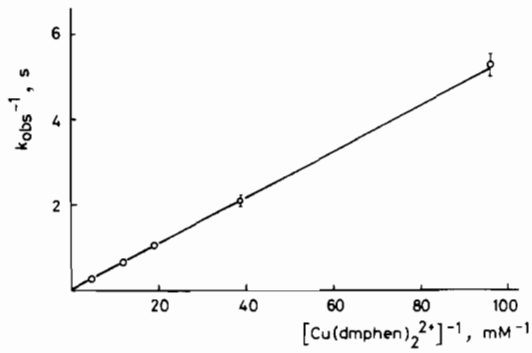

Fig. 6. Rate of oxidation of myoglobin by $Cu(dmphen)_2^{2+}$. Dependence of the reciprocal of the pseudo first order rate constant, k_{obs}, on the reciprocal of the $Cu(dmphen)_2^{2+}$ concentration.

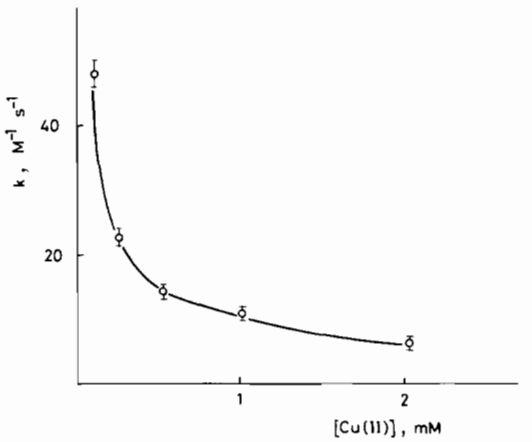

Fig. 7. Rate of oxidation of ferrocytochrome c by Cu(II) aqua ion. Dependence of the initial apparent second order rate constant, k, on the copper concentration.

oxygen concentration (k approx. 80 M^{-1} s^{-1}). Again, as with myoglobin, this behavior indicates that deoxygenated forms of hemoglobin are the most reactive species in solution. A rate constant that is applicable to such forms has therefore been calculated from the fraction of hemoglobin that is not fully saturated with oxygen. This fraction was estimated from the Adair constants given by Imai and Yonetani [18]. The rate constant of approximately 10^4 M^{-1} s^{-1} derived in this way is less than the corresponding value for myoglobin.

Electron transfer from copper to the heme thus occurs readily with both myoglobin and hemoglobin as well as with cyt c. By analogy to cyt c and to the reaction of myoglobin with other small redox reagents [17] electron transfer most probably occurs through the edge of the heme that protrudes to the outside of the protein.

Our observations on copper aquo ion catalysis of cytochrome parallel those recently described by Rifkind *et al.* [1], and Winterbourn and Carrell [2] on the copper catalyzed autoxidation of hemoglobin. In both systems the mechanism basically involves an electron transfer step leading to Cu(I) and Fe(III), followed by reoxidation of the Cu(I) by molecular oxygen. In both systems copper binding to the protein also plays an important role. Our work on the simpler cytochrome system thus supports the general mechanistic conclusions of these authors. It appears likely however that *reversible* electron transfer occurs both through the heme edge as well as through β-cysteine-93 bound copper within, of course, the constraints of the equilibrium constants for the electron transfer reactions. Reaction through the β-cysteine-93 will be favoured at low copper concentrations because of the preferential location of copper at this site, and because the redox potential of the cysteine bound copper is probably higher than free copper aquo ion. At higher concentrations electron transfer via the heme edge is likely to also occur, and probably accounts for the observation [2] that the rate of oxidation of hemoglobin continues to increase with increasing copper concentration, even when the binding sites are saturated.

Comparison of the Rates of Oxidation of Ferrocytochrome c and Deoxymyoglobin

Table III lists the experimentally determined rate constants for the oxidation of cytochrome c(II) and deoxymyoglobin by several oxidants. It can be seen from these results that (i) deoxymyoglobin is more reactive than cytochrome c(II) and that (ii) the order of reactivity of the oxidants is the same for both proteins, and in particular for the copper complexes that we have investigated. This latter order correlates with the redox potentials of the copper species.

The relative magnitudes of the rate constants in Table III may be largely rationalised by the Marcus expression [10, 19], $k_{12} = (k_{11}k_{22}K_{12}f)^{1/2}$ which relates the rate constant, k_{12}, of an electron transfer reaction to the electron exchange rate constants, k_{11}

and k_{22}, for the redox couples in the reaction and to the equilibrium constant, K_{12}, for the reaction (f is related to the other constants in the expression. It usually falls in the range 0.1 to 1.0). Now if it is assumed that the redox potential is the only kinetically significant difference between the heme proteins then the ratio of the rate constants for deoxymyoglobin and cytochrome c oxidation may be calculated from the Marcus equation. The calculated values listed in Table III are within two orders of magnitude of the experimental ratio. At least part of the discrepancy between the calculated and the observed ratio resides in differences in the size and charge of the two proteins, which will affect the rate of collision between the reactants in the electron transfer and exchange reactions (alternatively, the charge and size of the proteins will affect the degree of association of the reactants prior to electron transfer). Qualitatively, because cytochrome c has a higher positive charge (at pH 6) and smaller diameter than myoglobin, positively charged oxidants will react relatively more slowly with cytochrome and negatively charged species relatively more rapidly. Thus $k_{12}(My)/k_{12}(cyt)$ is higher than expected for $Cu(phen)_2^{2+}$ oxidation and lower than expected for $Fe(CN)_6^{3-}$ oxidation. Wherland and Gray [15] have described a quantitative treatment for the estimation of this effect. Corrections based on their method gave the predicted ratios given in the last column of Table I.

Considering the simplifying assumptions that are necessary to do these calculations the agreement between the observed and calculated ratios is remarkably good. We therefore conclude that our approach is basically sound and that our initial assumption – that the electron transfer behavior of the two heme proteins is intrinsically identical – is a good assumption. This conclusion suggests that the protein is at most only playing a minor role (apart from charge and size effects) in the reactivity of the heme to oxidation. Our supposition that the electron transfer occurs with both proteins through the edge of the heme exposed to the solvent is consistent with both these conclusions. It is not necessary therefore to invoke special protein properties, such as protein binding sites or protein mediated electron transfer, to understand their rates of electron transfer. However, it should be emphasised that the protein will play an important role in determining the extent of association between the oxidant and reductant and therefore in the rate of electron transfer especially in reactions between two proteins. For example, the overall rate of electron transfer between cytochrome c and cytochrome oxidase is greatly enhanced by the strong association between these proteins.

It is also interesting that the spin state of the protein does not appear to play an important role in the electron transfer rates, since cyt c(II) is in the low spin state whereas deoxymyoglobin has a high spin ground state.

Similar, but more limited, comparisons can be made for the oxidation of hemoglobin. The rate constants found for $Cu(phen)_2^{2+}$ (10^4 M^{-1} s^{-1}), $Fe(CN)_6^{3-}$ (7×10^4 M^{-1} s^{-1}) [16] and FeCDTA (2 M^{-1} s^{-1}) [20] are less than those for myoglobin as was anticipated simply on the basis of the redox potential of hemoglobin (0.17 volt) [21]. No quantitative treatment of the results has been attempted (because of the difficulties resulting from the presence of four interdependent hemes) but the basic data suggest that the conclusions drawn about the electron transfer properties of myoglobin also apply to hemoglobin. At first sight it is perhaps surprising that the heme in myoglobin and hemoglobin is not protected from oxidation to the non-functional iron(III) form. This might be done by, for example, burying the heme totally in the protein. On the other hand the accessibility of the heme to redox reagents is probably advantageous in the reduction of the oxidised species in the enzyme systems designed for this purpose.

With our copper complexes as well as with a number of other oxidants (Table III), oxidation of myoglobin apparently occurs entirely through deoxymyoglobin rather than by direct oxidation of oxymyoglobin, despite the much higher concentration of oxymyoglobin in air-saturated solutions (direct oxidation of oxymyoglobin by cyt c(III) might occur to a limited extent, although the predominant pathway still involves deoxymyoglobin [22, 23]). Why is oxymyoglobin reluctant to oxidise? It could be argued that the presence of bound oxygen prevents access of the oxidants to the iron atom [16]. We do not like this proposition because it appears unlikely that large ions such as $Cu(dmphen)_2^{2+}$ or cyt c would be able to attack the iron atom any more readily in oxymyoglobin than in the deoxy form. Such speculation is unnecessary anyway, because the low reactivity of oxymyoglobin is neatly explained by its relatively high redox potential; that is the redox potential for the MyO_2/MyO_2^+ couple. This redox potential is higher than that for the My/My^+ couple because preferential binding of oxygen to the reduced state will stabilize this state. The association constant for oxygen binding to metmyoglobin must be low both for experimental and theoretical reasons. If it is less than 0.1 M^{-1}, then E^0 for the oxygenated couple must be higher by more than 0.35 volt. This in turn implies, through the Marcus equation discussed above, that the deoxymyoglobin will be more than a thousand times more reactive than oxymyoglobin.

The final line in Table III compares the rates of the uncatalysed autoxidation of cytochrome and myoglobin. The ratio observed is greater than that for the other oxidants, which, we suggest, is a conse-

quence of different mechanisms for the two heme proteins. Cytochrome c appears to be oxidised by simple outer sphere electron transfer forming superoxide ion [13, 24], whereas myoglobin autoxidation probably involves the bound oxygen as well as deoxymyoglobin.

In general, we conclude from this work that cytochrome, myoglobin and hemoglobin are oxidised by copper complexes at a rate that depends largely on the difference in redox potential between the heme and the copper complex. The only other important factor appears to be the extent of association of the reactant species prior to electron transfer. We also feel that this conclusion will also apply to the oxidation of other substrates. For example the copper(II) complex catalysed oxidative polymerisation of phenols [25] has been found to correlate with the redox potential of the copper complexes investigated.

Provided the redox potential of the copper containing species is known or can be estimated, its catalytic effectiveness can be predicted. What then can be deduced about the possibility of *in vivo* copper catalysis? Some simple rules based on E^0 values determined for metal complexes in general [26] and copper complexes in particular [6] leads to the following predictions: (i) Complexes with ligands of saturated ligands with oxygen or nitrogen donor atoms, examples EDTA, glycine, and ATP, will have low redox potentials and therefore will be poor catalysts. (ii) Complexes of saturated ligands with sulphur donor atoms will have higher redox potentials and therefore will be good oxidants for the hemes, but may be poor catalysts because the Cu(I) state may not be readily oxidised by molecular oxygen. Thus, for example, copper bound to cysteine in hemoglobin is a relatively strong oxidant. (iii) Complexes of unsaturated ligands with oxygen or nitrogen donor atoms tend to have intermediate redox potentials and therefore make good catalysts.

We would therefore expect that copper catechol complexes would make good catalysts as well as complexes of the nitrogen heterocycles. Examples of this type of complex include copper bound to cytochrome, and copper complexes of tyrosine and histidine. The latter complex is apparently, however, less effective than the aquo ion. Though exceptions to these rules may be readily found, it is likely that they will still have some useful predictive value.

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