In summary, we conclude that at high μ, the rate-limiting step in the oxidation of CCP^{II} by C^{III} is a conformation change of the peroxidase to $*CCP^{II}$ (eq 3); this latter conformation possesses a more exposed heme and can rapidly transfer an electron to C^{III} on formation of a transient complex. Thus, the scheme in eq 3 represents conformational gating of electron transfer between the two proteins. At low μ, on the other hand, electron transfer between the *bound* proteins is at least partially rate-limiting and may be accompanied by rearrangement of the protein interface, which reduces the activation enthalpy. Further studies are under way to test the above conclusions and, particularly, to investigate the accessibility of the CCP^{II} heme in its acidic and alkaline forms.

Acknowledgments. This work was supported by grants from the NSERC (Canada) and the FCAR (Quebec). E.C. acknowledges a graduate fellowship from Concordia University. We wish to thank Professors Jack and Judith Kornblatt for interesting and helpful discussions.

Registry No. CCP, 9029-53-2; C, 9007-43-6; $Fe(CN)_6^{3-}$, 13408-62-3; $Fe(EDTA)^-$, 15275-07-7.

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Effect of Nonsynergistic Anions on Copper Transferrin

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Received March 27, 1987

The effect of perchlorate, thiocyanate, and chloride on dicopper transferrin has been studied through absorption and CD spectroscopy. It is shown that such anions displace copper(II) from its binding sites; then the metal binds to secondary sites. The release rate of copper, measured by using *o*-phenanthroline as the copper acceptor, is biphasic and is of dissociative type both in the presence and in the absence of perchlorate, though faster in the former case. Demetalation of copper protein by cyanide is also faster in the presence of perchlorate. Pyrophosphate is capable of demetalating the protein very efficiently. The mechanism appears to be more complex than in the previous case. The kinetics have been investigated and are discussed. The implications for iron transferrin are stressed.

Introduction

The mode of interaction with anions (both synergistic and nonsynergistic) represents a central topic in transferrin chemistry (Tf hereafter).[1,2] According to the recent X-ray results on lactoferrin, in each of the two binding sites the metal is bound to four protein residues (two Tyr, one His, and one Asp).[3] Furthermore it is probably bound to the synergistic anion (carbonate)[4] and to a solvent molecule.[5] The synergistic anion may bind both to the metal ion and to a positive side-chain residue (Arg and/or Lys) according to the interlocking sites model.[4] It is believed that important electrostatic interactions stabilize a closed conformation of the transferrin sites so that the bound metal is buried inside the protein and not exposed to the environment. Proton relaxation measurements are in agreement with such picture since they indicate that the metal bound water molecule is in slow exchange with the bulk solvent.[5-7] Very recent results from ^{35}Cl NMR[8] and difference UV spectroscopy[9] support the view of a closed conformation of the metal chromophore, not accessible to anions. Several positive residues (Arg and Lys) are present near both metal binding sites and have been demonstrated to determine metal site reactivity; they probably contribute to stabilize the native conformation of the protein and are the target of non-synergistic anions.[10-12]

The effect of nonsynergistic anions on the metal centers of transferrin was first reported by Price and Gibson,[13] who analyzed the changes induced by perchlorate on the EPR spectrum of iron transferrin. Subsequent studies demonstrated that relatively high concentrations of nonsynergistic anions with high affinity for positively charged groups of proteins, such as thiocyanate, perchlorate, pyrophosphate, and chloride, markedly affected the thermodynamic and kinetic properties of both metal binding sites.[14,15] This raised the interest of researchers toward this topic (the so called "salt effect") since these mechanisms of destabilization and labilization of the binding sites may represent the key of the process of metal release. In particular, Chasteen, through EPR difference spectroscopy, determined the general features of anion binding to iron transferrin; he estimated the affinity constants for the protein of a series of nonsynergistic anions, demonstrated that they cause similar spectral changes, and proposed that they bind to each protein domain, in a 2:1 ratio (per site), with strong positive cooperativity within each domain.[16] It was also shown that the spectral and kinetic effects induced by perchlorate parallel the effect induced by chemical modification of the Lys residues.[12]

Analogous effects induced by nonsynergistic anions were observed on other metal-substituted transferrins like the oxovanadium(IV) derivative[17] or the ytterbium(III) derivative.[18]

We have studied the effect of perchlorate, pyrophosphate, and other anions on the spectral properties of dicopper transferrin; we have also studied the influence of the two former anions on the rates of metal release. Copper(II) has been chosen because, as a bipositive ion, it has a relatively smaller affinity for the protein

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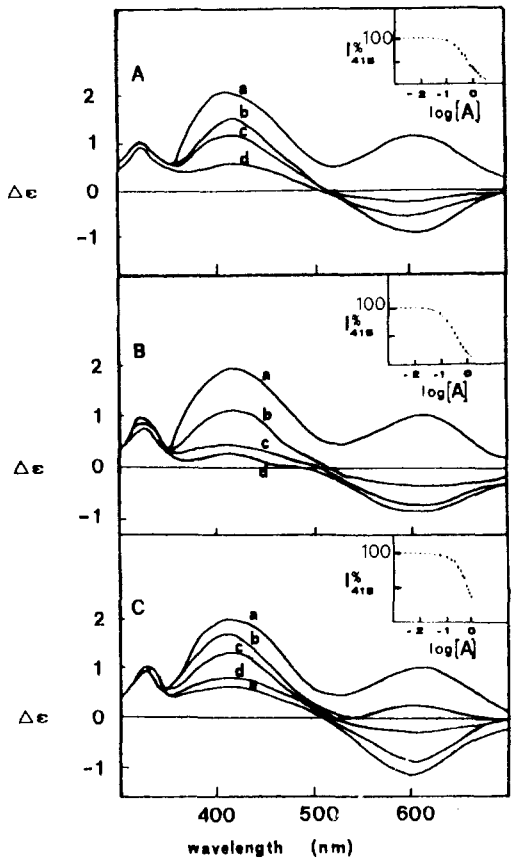

Figure 1. CD spectra of dicopper transferrin upon addition of increasing amounts of sodium perchlorate (A), sodium thiocyanate (B), and sodium chloride (C). The insets show the pattern of the variation in intensity of the band at 415 nm as a function of nonsynergistic anion concentration. The intensity is expressed as residual intensity in percent with respect to the calculated total variation $\Delta I = I_0 - I_\infty$. The best fitting curves are shown; best fitting parameters are reported in Table I. Conditions: 5×10^{-4} M dicopper transferrin, 5×10^{-3} M sodium bicarbonate, 50 mM Tris–HCl buffer, pH 8. Anion concentrations are as follows: (A) (sodium perchlorate) 0 (a), 0.23 (b), 0.45 (c), and 0.83 M (d); (B) (sodium thiocyanate) 0 (a), 0.24 (b), 0.54 (c), and 0.81 M (d); (C) (sodium chloride) 0 (a), 0.35 (b), 0.83 (c), 1.43 (d), and 2.06 M (e).

and a relatively small tendency to maintain a rigid ligand geometry arount the metal.

Experimental Section

Human serum transferrin in the apo form was purchased from Sigma Chemical Co. and further purified according to the standard procedure.[19] The concentration of the protein samples was determined through UV absorption spectroscopy. All the used reagents were of analytical grade.

Dicopper transferrin was prepared by adding two copper equivalents to millimolar solutions of apotransferrin in 0.1 M Tris buffer, pH 8, in the presence of 20 mM sodium bicarbonate, according to previously reported procedures.[20,21] Protein samples were then diluted to 5×10^{-4} M concentration for the spectral studies.

Absorption spectra were recorded on a Cary 17 D instrument with 1-cm path length cuvettes; CD spectra were recorded on a Jasco 500 D instrument at room temperature.

Kinetic measurements were performed on a Union-Giken RA 401 stopped-flow apparatus interfaced with a data collecting system for data acquisition and manipulation.[22] We used 1-cm path length cuvettes and followed the reaction at 430 nm; solution conditions were 50 mM Tris–HCl, pH 7.4, 25 °C, and 1×10^{-4} M final protein concentration.

Results

CD and Absorption Spectroscopy Studies. The CD spectra of dicopper transferrin consist of two charge-transfer bands at 315 and 415 nm, and a relatively broad positive band at 605 nm that corresponds to the d–d transition of bound metal ions (Figure 1).[23-26] The results of titrations of dicopper transferrin at pH 8 with increasing amounts of thiocyanate, perchlorate, and chloride as followed through CD spectroscopy are reported in parts A–C of Figure 1. The pattern of the spectral variations is rather similar in the three cases. Addition of these nonsynergistic anions causes a major variation of the d–d band at 605 nm (change in sign of the Cotton effect from positive to negative) and decreases the intensity of the charge transfer transition at 415 nm. The $\Delta\epsilon$ value of the latter band approaches zero for very high anion concentrations. On the other hand, the charge-transfer band at 315 nm undergoes only minor changes in intensity and shape. From a closer analysis of the spectral data it appears that the 420-nm band in absorption spectra and the 415-nm band in CD spectra consist of two transitions, separated by about 1000 cm^{-1}. From a plot of the variation in intensity of the CD band at 415 nm versus

Table I. Best-Fitting Parameters Obtained from the Analysis of the Variations of the CD Spectra of Dicopper Transferrin upon Addition of Nonsynergistic Anions[a]

anion	K_1	K_2	β_2
ClO_4^-	1.2	2.0	2.4
Cl^-	0.4	2.7	1.1
SCN^-	1.4	3.9	5.5

[a] Experimental data were analyzed by assuming an equilibrium of the type $CuTf + nL \rightleftarrows CuTf\text{-}L_n$ with $n = 2$ per each site.

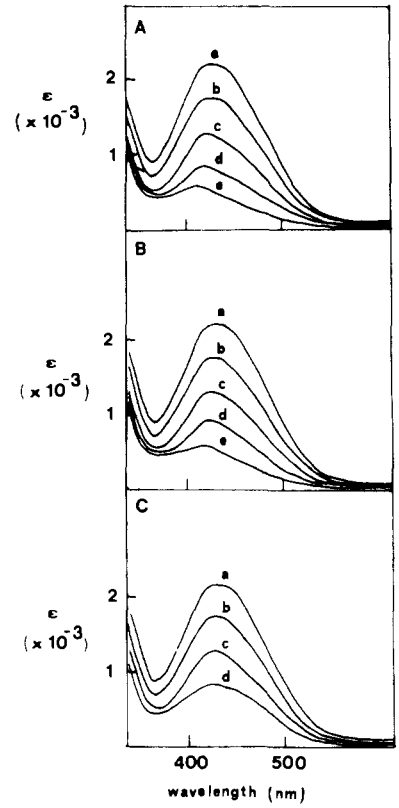

Figure 2. Effect of the addition of sodium perchlorate (A), sodium thiocyanate (B), and sodium chloride (C) on the visible absorption spectra of dicopper transferrin. Anion concentrations are as follows: (A) (sodium perchlorate) 0 (a), 0.10 (b), 0.42 (c), 0.72 (d), and 1.3 M (e); (B) (sodium thiocyanate) 0 (a), 0.10 (b), 0.22 (c), 0.35 (d), and 0.7 M (e); (C) (sodium chloride) 0 (a), 0.38 (b), 0.80 (c), and 1.4 M (d). Other conditions are as reported in Figure 1.

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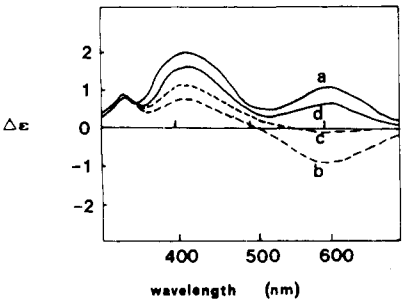

Figure 3. Reversal of the perchlorate effect by addition of increasing amounts of sodium bicarbonate as observed through CD spectroscopy: (a) dicopper transferrin; conditions as in Figure 1; (b) solution a plus 0.8 M sodium perchlorate; (c) solution b plus 20 mM sodium bicarbonate; (d) solution b plus 40 mM sodium bicarbonate.

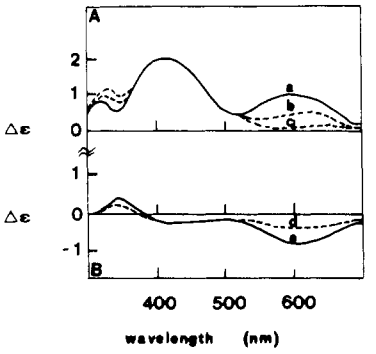

Figure 4. (A) CD spectra of apotransferrin in the visible region plus 2 (a), 3 (b), and 4 (c) equiv of copper(II) sulfate. (B) Relative difference spectra (d = b − a; e = c − a). Other conditions are as reported in Figure 1.

nonsynergistic anion concentration (Figure 1, insets), it appears that the slope of the curve is higher than expected for a simple equilibrium (MTf + L = LMTf); the experimental data are satisfactorily reproduced by assuming an equation of the type MTf + nL ⇄ MTfL$_n$ with n = 2 for each site as previously found for the iron derivative.[16] The apparent affinity constants obtained from the best fitting of the experimental data according to the above equation are reported in Table I; they clearly indicate the presence of important positive cooperativity in anion binding (the k_2 values are always higher than the k_1 values). The same conclusions are drawn by analyzing the disappearance of the charge-transfer bands at 420 nm in the absorption spectra (Figure 2).

Addition of sodium bicarbonate causes a reversal of the CD spectral effects of anions as shown in Figure 3.

CD spectra similar to those obtained in presence of thiocyanate, perchlorate, and chloride (Figure 1) could be obtained by computer subtracting the CD spectrum of a 2:1 Cu:Tf sample from the CD spectrum of a 4:1 Cu:Tf sample (Figure 4).

Kinetics of Copper Release. We used *o*-phenanthroline (*o*-phen) as the metal chelating agent and analyzed the patterns of absorbance decrease with time of the dicopper transferrin samples at 430 nm, respectively at zero and 50 mM perchlorate concentration.

At zero perchlorate concentration we observed that the metal removal reaction is biphasic with each phase accounting for about 50% of total absorbance; this is consistent with previous observations on copper ovotransferrin.[27] The overall process was treated as the sum of two exponential decays according to eq 1.[28] c_1 and

$$A_t = (A_0 - A_\infty)(c_1 e^{-k_{1\,obsd} t} + c_2 e^{-k_{2\,obsd} t}) + A_\infty \qquad (1)$$

c_2 were assumed to be equal and were fixed to 0.5 each (see later). The metal release rate in the presence of *o*-phen as copper acceptor

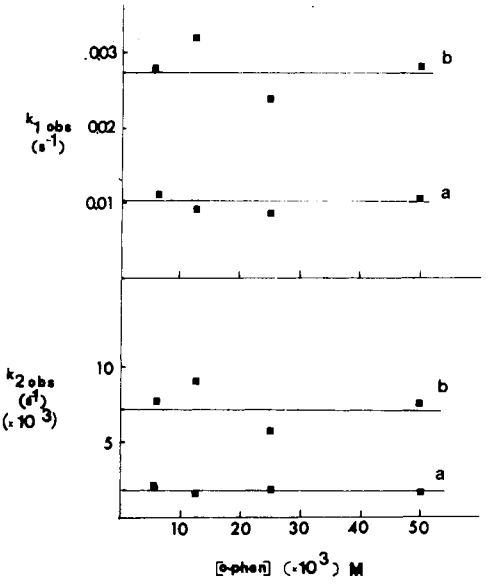

Figure 5. Dependence of $k_{1\,obsd}$ and $k_{2\,obsd}$ values on *o*-phen concentration in the absence (a) and in the presence (b) of 50 mM sodium perchlorate. Experimental conditions were 0.05 M Tris–HCl, pH 7.4, 25 °C, and 10^{-4} M protein concentration.

is ligand concentration independent in the *o*-phen concentration range 5×10^{-4} to 5×10^{-3} M, as shown in Figure 5. The estimated pseudo-first-order rate constants are $k_{1\,obsd} = 1.0 \times 10^{-2}$ s^{-1} and $k_{2\,obsd} = 1.9 \times 10^{-3}$ s^{-1}. These results strongly suggest that at low concentrations *o*-phen acts exclusively as external copper sink and does not participate to the molecular mechanism of copper removal; the latter probably consists of a dissociative mechanism governed exclusively by the protein.

Parallel experiments performed by using low concentrations of EDTA in the place of *o*-phen gave similar results, indicating that the dissociation rates, at least in the low concentration range, do not depend on the nature of the chelating agent.

Addition of 50 mM sodium perchlorate causes a marked increase in the rate of copper removal by *o*-phen. At this perchlorate concentration both $k_{1\,obsd}$ and $k_{2\,obsd}$ values are affected at comparable extents ($k_{1\,obsd} = 2.8 \times 10^{-2}$ s^{-1} to be compared with 1.0×10^{-2} s^{-1}; $k_{2\,obsd} = 7.5 \times 10^{-3}$ s^{-1} to be compared with 1.9×10^{-3} s^{-1}). Again, no dependence of k_{obsd} on the concentration of the chelating agent is observed (see Figure 5).

When dicopper transferrin is treated with pyrophosphate (PP) the metal dissociates from the protein and forms a copper(II)–pyrophosphate complex. In contrast to the previous cases, now the rate of metal release is dependent on pyrophosphate concentration. The experimental data were fitted to eq 1 to give a pair of $k_{1\,obsd}$ and $k_{2\,obsd}$ values for each value of pyrophosphate concentration. The experimental $k_{1\,obsd}$ and $k_{2\,obsd}$ values at increasing PP concentrations are shown in Figure 6; they exhibit a saturation behavior versus PP concentration. Stopped-flow analysis of the reaction of copper transferrin with pyrophosphate did not provide any evidence of the formation of short-life intermediate species.

Observations on the Interaction of Metallotransferrins with Cyanide. We observed a very marked difference in the reactivity of the copper centers toward cyanide depending on the presence of sodium perchlorate. In the absence of the latter anion 0.5 mM cyanide takes many hours to remove copper from 0.15 mM protein samples, whereas in its presence (at a concentration of 0.2 M) the process is complete in a few minutes.

At higher sodium perchlorate concentration (>1 M) addition of a twofold stoichiometric amount of sodium cyanide with respect to copper(II) concentration causes a fast disappearance of the negative CD band at 610 nm.

For comparison purposes we performed similar experiments on the iron derivative. We observed that 0.5 M perchlorate does not alter the CD spectrum of iron transferrin; however, its presence affects markedly the reactivity of iron transferrin towards cyanide.

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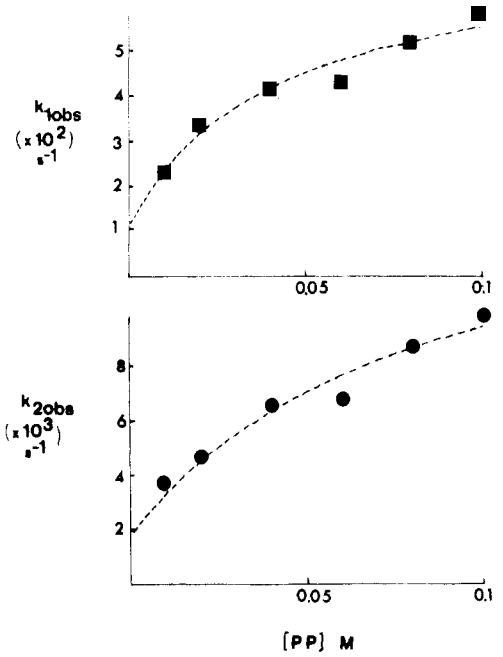

Figure 6. Dependence of $k_{1\,obsd}$ and $k_{2\,obsd}$ on pyrophosphate concentration in the reaction of copper removal from transferrin. Experimental conditions were 0.05 M Tris–HCl, pH 7.4, 25 °C, and 10^{-4} M protein concentration. Extrapolation to zero PP concentration gives respectively k_1 and k_2.

Cyanide at high concentrations (>1 M) binds iron in transferrin; the structural details of the process have been recently investigated by Chasteen.[29] Whereas in the absence of sodium perchlorate the process takes days to reach completion, in its presence (i.e. at a 0.5 M concentration) it is complete within a few hours. No further studies have been performed on this system.

Both of the above experiments further support the view that addition of lyotropic agents causes an unwrapping of the protein tertiary structure and a subsequent opening of the metal that facilitates the attack by metal chelators. CN^- has an easy access to the metal after perchlorate's interaction with the protein.

Discussion

Addition of nonsynergistic anions like perchlorate, chloride, or thiocyanate has profound effects on the CD and electronic spectra of dicopper transferrin. In particular the band at 420 nm in absorption spectra and 415 nm in CD spectra is completely abolished. Such a band had been previously assigned as a phenolate-to-copper charge transfer.[23] The present data indicate that it actually consists of two closely spaced bands that must arise from the two phenolate groups in each binding site. It appears therefore that nonsynergistic anions abolish tyrosinate coordination to copper; nevertheless, copper remains bound to the protein as indicated by the presence of a residual CD activity. A general interpretation of the experimental data could be afforded by taking into account early studies on the CD spectra of dicopper transferrin by Nagy and Tomimatsu, who demonstrated the presence of further binding sites for copper different from the primary sites.[30,31] In particular it was shown that copper bound to these sites gives rise to a positive band at 325 nm and a negative band at 610 nm; these transitions roughly correspond in sign, intensity, and position to those observed at the end of the CD titrations with nonsynergistic anions. Indeed, we could reproduce the CD spectrum of copper bound to the secondary sites of the protein by subtracting the spectrum of a 2:1 Cu:Tf sample from that of a 4:1 Cu:Tf sample. Such spectrum is quite similar to the final spectra of the titrations with nonsynergistic anions; the slight differences observed between the two sets of spectra are not surprising in view of the large difference in ionic strength in the two cases. So, it can be concluded that the effect of nonsynergistic anions, at high concentrations, consists in a continuous reduction of the thermodynamic affinity of copper for the primary binding sites to the point that coordination to the secondary sites, described by Nagy and Tomimatsu, is preferred. Such conclusion is strongly supported by the reversal of the effect obtained upon addition of sodium bicarbonate, which, as a synergistic anion, increases the apparent affinity constant of the metal for the primary sites.

Ancillary experiments were performed by adding stoichiometric amounts of iron(III) to dicopper transferrin samples both in the presence and in the absence of sodium perchlorate at pH 8; the typical iron transferrin spectral bands develop, indicating displacement of copper from the specific sites and formation of iron ternary complexes. However, under the strong iron transitions, it is still possible to detect some residual CD activity from copper bound to secondary sites (data not shown). This means that primary and secondary sites are not overlapping in agreement with previous observations.[30,31]

The order of efficiency of the different anions in causing migration of copper from primary to secondary sites at constant bicarbonate concentration parallels the Hofmeister lyotropic series (see the β_2 values reported in Table I), suggesting that site destabilization is achieved through binding of nonsynergistic anions to protein positive residues. Anyway, the present experiments do not allow us to establish whether there is direct competition at the positive residue(s) believed to be involved in the stabilization of the synergistic anion.

As a side result the assignment of the 315-nm band as a phenolate-to-copper charge transfer should probably be revised since this band is still present when tyrosinates are removed from coordination to copper. Imidazole-to-copper charge-transfer transitions are known to occur in this energy region[32] and may be the true origin of the above band. Indeed, at least one histidine is known to be present in the first coordination sphere of both primary sites,[3] and it is very likely that other imidazole residues are involved in coordination of copper at the secondary sites.

Thermodynamic labilization of copper bound to the primary sites may involve kinetic labilization and hence provide a clue to the mechanism of metal release from transferrin. Analysis of the kinetic data requires some theoretical considerations. Indeed, in order to relate the pseudo-first-order rate constants obtained from the analysis of the kinetic data to the microconstants for the dissociation of the metal from the C-terminal and N-terminal sites, one should consider the possibility that the two sites are interacting and analyze the system in terms of four microconstants as first described by Baldwin through the equation:[33]

$$A_t - A_\infty = \frac{A_0 - A_\infty}{2}\left[\left(2 - \frac{k_{1a}}{m_1 - m_2} - \frac{k_{1b}}{m_1 - m_3}\right)e^{-m_1 t} + \frac{k_{1a}}{m_1 - m_2}e^{-m_2 t} + \frac{k_{1b}}{m_1 - m_3}e^{-m_3 t}\right] \quad (2)$$

where $m_1 = k_{1a} + k_{1b}$, $m_2 = k_{2b}$, $m_3 = k_{2a}$. k_{1a} and k_{1b} refer to the dissociation of one copper ion from the a and b sites, respectively, in the fully metalated protein whereas k_{2a} and k_{2b} refer to the dissociation from the a and b sites when the other site is empty. In such analyses it has been often assumed that the two sites are similar but not identical. Under such assumption the following simplified equation holds:

$$A_t = \frac{1}{2}(A_0 - A_\infty)\left[\left(2 - \frac{m_1}{m_1 - m_2}\right)e^{-m_1 t} + \frac{m_1}{m_1 - m_2}e^{-m_2 t}\right] + A_\infty \quad (3)$$

Of course if no site–site interaction occurs, the Baldwin general equation reduces to eq 1. Since both eq 1 and 3 involve the same

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Table II. Kinetic Parameters Obtained from the Best Fitting of the Experimental Data Relative to Copper Removal by Pyrophosphate According to Eq 3 and 4[a]

k_1, s^{-1}	k_2, s^{-1}	K_1, M^{-1}	K_2, M^{-1}	k_3, s^{-1}	k_4, s^{-1}
1.0×10^{-2}	1.9×10^{-3}	25 ± 7	12 ± 4	7.3×10^{-2}	1.6×10^{-2}

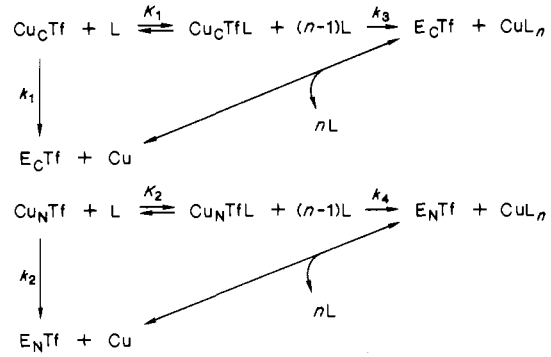
[a] k_1 and k_2 are the dissociation rate constants found in the experiment with *o*-phen alone.

number of parameters, namely A_0, $k_{1\,obsd}$, $k_{2\,obsd}$, A_∞ in one case and A_0, m_1, m_2, A_∞ in the other, the goodness of the fitting should be a valid indication of the presence of cooperativity. Our data could be reproduced reasonably well by eq 1 while no significant reduction of the mean square deviation was achieved by using eq 3. Moreover, the apparent percent of iron released from the fast phase, defined as $^1/_2[2 - m_1/(m_1 - m_2)]\gamma$ in terms of eq 3, was never far from 0.5, usually ranging between 0.4 and 0.6. In our opinion this means that the assumption that the two sites are similar and interacting is at least questionable. On the other hand, recent kinetic data on metallotransferrins suggest that very weak interactions, if any, occur between sites.[34,36] Therefore, we have chosen to analyze the experimental data by using eq 1; the obtained $k_{1\,obsd}$ and $k_{2\,obsd}$ values are in our opinion a reasonable approximation to the microscopic constants of the two sites.

Very recent results obtained on iron transferrin under the same experimental conditions (i.e. at pH 7.4, in the presence of 50 mM Tris–HCl buffer), indicate that the faster phase is associated to iron release from the C-terminal site;[37] this could reasonably hold also for copper transferrin.

The spontaneous dissociation rate of copper from transferrin, as measured in the presence of *o*-phen or EDTA, is sizable in comparison with that of iron, indicating that kinetic and thermodynamic labilities parallel each other. Addition of 50 mM sodium perchlorate further increases the rate of copper removal. Both pseudo-first-order kinetic constants increase by a factor of 3; it should be noted that at this perchlorate concentration no appreciable copper migration to secondary sites has occurred.

In order to analyze the kinetic data obtained in the presence of pyrophosphate (Figure 6) we have assumed that the overall mechanism of metal release from both the C-terminal and N-terminal sites could be described by the following schemes:



where "E" stands for empty site and K_1 and K_2 are the stability constants of the first equilibrium. The dependence of $k_{1\,obsd}$ and $k_{2\,obsd}$ on ligand concentration is thus given by eq 4 and 5, which

$$k_{1\,obsd} = \frac{[L]K_1k_3}{1 + [L]K_1} + \frac{k_1}{1 + [L]K_1} \quad (4)$$

$$k_{2\,obsd} = \frac{[L]K_2k_4}{1 + [L]K_2} + \frac{k_2}{1 + [L]K_2} \quad (5)$$

term 1 term 2

predict saturation kinetics versus ligand concentration. Term 1 refers to the "ligand-assisted" mechanism and term 2 to the dissociative mechanism. The $k_{1\,obsd}$ and $k_{2\,obsd}$ values were therefore fitted to the above equations by using for k_1 and k_2 the dissociation rate constants obtained from the experiments with *o*-phen. The k_3, k_4, K_1 ($=1/K_{M1}$) and K_2 ($=1/K_{M2}$) values obtained from a best-fitting analysis are reported in Table II.

By referring to Bates' mechanism,[38-40] we can describe the metal removal reaction from transferrin in the following way:

$$\mathrm{MTf} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \mathrm{MTf^*} \quad (\mathrm{I})$$

$$\mathrm{MTf^*} + \mathrm{L} \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} \mathrm{MTfL} \quad (\mathrm{II})$$

$$\mathrm{MTfL} \xrightarrow{k_3} \mathrm{ML} + \mathrm{Tf} \quad (\mathrm{III})$$

where step I is a conformational transition of the chromophore from a "closed" to an "open" state, step II describes the interaction of the ligand with the "open" conformation, and step III is the dissociation of the ternary complex. The rate-limiting step would be the transition from the "closed" to the "open" form. Saturation kinetics versus ligand concentration is expected.

On the basis of the above reported data, it appears that lyotropic anions have a marked effect on k_{obsd}. Since they bind to cationic sites of the protein in the native closed conformation, they probably affect the equilibrium position of the first step in Bates' mechanism; this can hold for pyrophosphate too. A refinement of the proposed kinetic mechanism is therefore needed to take into account such effects. Moreover, at least in the case of labile metals, another step should be usually introduced in the general kinetic scheme describing metal removal from the protein. This step, referring to the dissociative mechanism and tentatively expressed as

$$\mathrm{MTf} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \mathrm{MTf^*} \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} \mathrm{M} + \mathrm{Tf}$$

may however be neglected in the case of the native protein owing to the extremely low values of the spontaneous dissociation rate of iron.

Conclusions

Our spectral and kinetic data confirm the view that transferrins are extremely sensitive to nonsynergistic anions. The degree of sensitivity of the transferrin metal sites depends on the type of central metal; the tighter the metal binding the smaller the effect of anions. So, whereas the CD spectrum of the iron derivative is not affected by nonsynergistic anions, large CD spectral effects are seen for the copper(II) derivative. Presumably, the mechanism of action of nonsynergistic anions consists in the perturbation of the complex network of electrostatic and hydrogen bond interactions that stabilize the "closed" native conformation of the metal chromophore. The latter, originated by a protein folding that determines the formation of a metal binding pocket, is held firm by the cooperative binding of the synergistic anion.

Perchlorate, thiocyanate, and chloride probably bind to positively charged residues near the metal binding domain, perturb the electrostatic interactions that stabilize the metal chromophore, and cause loss of copper, which moves to secondary sites.

According to Bates' scheme the rate-limiting step for metal removal is a slow conformational transition involving the metal site. If this is true, from the present data it appears that the rate of this conformational change is heavily affected by both the type of metal ion and the presence of nonsynergistic anions with lyotropic properties. When the chelating molecule itself is a good lyotropic agent, as it is in the case of PP, a net increase in the efficiency of the metal removal process is observed. The kinetic

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data also indicate the presence of two clearly distinct metal release mechanisms for copper transferrin. The first, of dissociative type, is independent on both the nature and the concentration of the chelating agent whereas the second, "ligand-assisted", is strictly related to the type of chelating agent and exhibits saturation dependence on its concentration. The relative importance of the two coexistent mechanisms is also determined by the thermodynamic stability of the metal transferrin adduct; when the metal protein complex is weak, the dissociative mechanism is not negligible. Indeed, the thermodynamic stability of copper(II) transferrin can be estimated to be several orders of magnitude lower than that of iron(III) transferrin. Just to provide an estimate we can refer to a conditional stability constant for zinc(II) of 10^5 versus 10^{20} for iron(III).[41] Metal displacement studies performed on ovotransferrin indicate that copper and zinc should have similar affinities for the protein.[42]

Registry No. ClO_4^-, 14797-73-0; NCS^-, 302-04-5; Cl^-, 16887-00-6; CN^-, 57-12-5; $P_2O_7^{4-}$, 14000-31-8.

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Influence of Pentaamminechromium(III) on the Acidity of Coordinated Imidazoles and Pyrazole

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Received September 1, 1987

The pK_a's at 298 K, μ = 0.08, and the temperature dependence (273-338 K) for the deprotonation of the pyrrole NH of imidazole, 2-methylimidazole, and pyrazole coordinated to $Cr(NH_3)_5^{3+}$ are reported. Complexes were isolated as $[Cr(NH_3)_5LH](tfms)_3$ salts. Data summarized for various systems are as follows (ligand, pK_{298}, $\Delta H_a°$ in kcal/mol, $\Delta S_a°$ in eu): imidazole = imH, 9.35, 13.9 ± 0.3, 3.9 ± 1.2; 2-methylimidazole = 2-CH_3imH, 10.20, 17.0 ± 0.4, 10.4 ± 1.4; pyrazole = pyzH, 6.71, 10.6 ± 0.3, 4.7 ± 0.9. The $(NH_3)_5Cr^{III}LH^{3+}$ pK_a, $\Delta H_a°$, and $\Delta S_a°$ values are found to follow very closely the values previously determined for the $(NH_3)_5Co^{III}LH^{3+}$ analogues. A very weak additional band is found on the low-energy side of the $^4T_{2g} \leftarrow {}^4A_{2g}$ d-d transition of the Cr(III) center. This additional band is assigned to the $t_{2g}^3(d\pi) \leftarrow (\pi_1)_L$ LMCT transition from the imH and 2-CH_3imH rings at ca. 525 and 550 nm, respectively. These bands are analogous to the LMCT bands observed between low-spin Fe(III) and Ru(III) d^5 centers and imidazoles.

Introduction

The imidazole moiety of the amino acid histidine is an important functional group at the active site of a large number of metalloproteins.[1] The ability of this group to delocalize charge is important in its role to stabilize certain formal oxidation states such as Fe(III) and Fe(IV) in cytochromes or the oxygenated form of myoglobins and hemoglobins. Previous studies in these laboratories have probed the π interactions between the imidazole chromophore and various metal centers in octahedral coordination.[2-7] The pK_a of a coordinated imidazole pyrrole hydrogen is a useful measure of the extent that the charge of the central metal is transferred to the ligand by both σ induction and π donation from the ligand to the metal. Previous studies have been carried out on the low-spin $(CN)_5Fe^{2-}$ unit[2,3] and $(NH_3)_5M^{3+}$ units (M^{III} = Co(III), Rh(III), Ir(III), and Ru(III)).[2-5] These systems revealed that π-donation into a partially filled d^n set, d^5 in the case of Ru^{III}, is more important than the ionic potential of a metal center in raising the acidity of coordinated imidazoles. Most imidazoles are good σ donors and moderate π donors as ligands. Recently the 2-aldehyde-substituted imidazole, 2-CHOimH, was shown to be a strong π-acceptor group with a π-acceptor power comparable to that of pyrazine.[6,7] The 2-CHOimH ligand favors the lower oxidation states and forms stable complexes with low-spin $(NH_3)_5Ru^{2+}$ and $(CN)_5Fe^{3-}$ centers.[6,7] The π-acceptor power of 2-CHOimH stands apart from other substituted imidazoles that are only good π donors. We became interested in the influence of the metal center to serve as a better π-acceptor toward dπ-donating imidazole rings. If d^3 Cr(III) complexes of imidazoles and pyrazoles were prepared, one would have a good probe of the influence of imidazole π donation for comparison with other M(III) systems because ionic potential influences would be held nearly constant. The influence of Cr(III) on the pK_a of coordinated ligands can be rather large. For example, the pK_a of HCN is lowered from 9.21 to 1.27 upon coordination of the terminal nitrogen to $(H_2O)_5Cr^{3+}$.[11] $(NH_3)_5Cr^{3+}$ raises the acidity of water by 10 orders of magnitude, changing the pK_a from 15.0 to 5.0 upon coordination.[12] The synthetic routes to $(NH_3)_5CrL^{3+}$ species (L = imidazoles or pyrazole) have been accomplished by use of

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