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Temperature Dependence of the Unimolecular Reduction of Ferricytochrome *c* by Ferrocytochrome *c* Peroxidase at Low and High Ionic Strengths

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The oxidation of ferrocytochrome *c* peroxidase by horse ferricytochrome *c* has been investigated at 0.02 and 0.2 M ionic strengths (μ) in phosphate buffers, pH 7.0, over a range of temperatures from 0 to 26 °C. The absorbance–time curves for the reduction of cytochrome *c* are exponential at both μ's. At 3–10 μM peroxidase and 24 °C, the observed rate constants are 0.23 ± 0.02 s^{-1} (μ = 0.02 M) and 0.020 ± 0.002 s^{-1} (μ = 0.2 M), and the kinetics are independent of the cytochrome *c* concentration between 0.5 and 10 μM. The activation enthalpies and entropies are 4.6 kcal mol^{-1} and −46 eu at μ = 0.02 M and 17 kcal mol^{-1} and −7.8 eu at μ = 0.2 M, which indicates that the rate-limiting steps are different at low and high μ. The increased polarization of porphyrin cytochrome *c* fluorescence at μ = 0.02 M in the presence of the peroxidase clearly indicates that the proteins form a stable complex at low μ; at μ = 0.2 M, no polarization increase is observed. Therefore, the unimolecular rate-limiting step at high μ must occur prior to protein association. Oxidation of the peroxidase by both $Fe(CN)_6^{3-}$ and $Fe(EDTA)^-$ shows bimolecular kinetics at μ = 0.2 M with rate constants of (8.4 ± 0.4) × 10^4 and 70 ± 8 M^{-1} s^{-1}, respectively. Furthermore, these oxidations do not show limiting kinetics at reagent concentrations that yield half-lives 10-fold smaller than those for the cytochrome oxidation. Therefore, we speculate that at high μ the smaller reagents have better access to the peroxidase heme than cytochrome *c* and that the peroxidase must undergo a conformation change which increases its heme exposure (such as its acidic → alkaline transition) before it is oxidized by cytochrome *c*.

Introduction

The C/CCP[1] complex is currently being intensely investigated to provide insight into the structural factors that control biological redox rates.[2-4] Of particular interest is the role of the protein–protein interface in controlling interprotein electron transfer in noncovalent complexes formed by two proteins. The C/CCP complex is an obvious choice for such structure–function studies since the crystal structures of CCP[5] and C[6] are known to high resolution, and computer graphic modeling of the complex has been carried out by using the atomic coordinates of the proteins.[7,8]

Previously,[2] we reported electron-transfer rates between CCP^{II} and C^{III} ($\Delta E^\circ \sim 0.45$ V) using various eukaryotic C's at $\mu \leq 0.02$ M. Since the interaction of C with many of its biological partners is highly dependent on the ionic strength of the medium, we extended our investigation of the kinetics of reduction of *horse* C by the peroxidase over the range μ = 0.01–1 M.[9] Following rapid mixing of CCP^{II} (3–10 μM) and C^{III} (0.5–10 μM), exponential growth of C^{II} is observed at all μ's. The kinetics are *dependent* on μ but *independent* of both the CCP and C concentrations. Since first-order kinetics are observed under second-order conditions (i.e., [CCP] ~ [C]), a scheme involving rapid complex formation between the proteins followed by slow electron transfer within the complex is consistent with the observed kinetics:

$$C^{III} + CCP^{II} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} C^{III}/CCP^{II} \xrightarrow[\text{(slow)}]{k_3} C^{II}/CCP^{III} \quad (1)$$

(fast)

where the slant denotes noncovalent complexation[7,8] and the observed rate constant, k_{obsd}, is given by

$$k_{obsd} = \frac{k_3[C^{III}]}{K_d + [C^{III}]} \quad (2)$$

For the reaction to exhibit essentially first-order kinetics requires $[C^{III}] \gg K_d$ ($K_d = k_2/k_1$); thus, $K_d \sim 0.05$ μM if $[C^{III}] \geq 0.5$ μM. For the thermodynamically unstable C^{III}/CCP^{II} complex, K_d cannot be measured directly; however, for both the C^{III}/CCP^{III} and porC/CCP^{III} complexes, K_d's of ~0.1 μM and 1 mM were obtained at μ = 0.02 and 0.2 M, respectively at pH 6.0 by using absorbance difference[10] and fluorescence quenching[11] measurements. From these data, it is reasonable to assume that K_d for the C^{III}/CCP^{II} complex is ~0.05 μM at μ = 0.02 M but much larger at 0.2 M; thus, the unimolecular rate-limiting steps must *precede* protein association at high μ. One possibility is a slow conformation change in CCP^{II} prior to complexation as in the following scheme:

$$CCP^{II} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} {}^*CCP^{II} + C^{III} \underset{k_4}{\overset{k_3}{\rightleftharpoons}} C^{III}/{}^*CCP^{II} \xrightarrow{k_5} C^{II}/CCP^{III} \quad (3)$$

(slow) (fast)

where k_{obsd} for the reduction of C^{III} is now given by

$$k_{obsd} = \frac{k_1k_5k_3[C^{III}]}{(k_2 + k_3[C^{III}])(k_4 + k_5) - k_4k_3[C^{III}]} \quad (4)$$

Since the association of C and CCP is diffusion-controlled,[12] we assumed that $k_3[C^{III}] \gg k_2$; therefore, $k_{obsd} = k_1$.

The observed kinetics and K_d's are consistent with schemes outlined in eq 1 and 3. However, since the observed rates at low and high μ differ by only a factor of 10,[9] which corresponds to a 1.5 kcal mol^{-1} change in $\Delta G^\ddagger$, only minor changes in $\Delta H^\ddagger$ or $\Delta S^\ddagger$ need occur on increasing μ unless the mechanism is different at low and high μ. Furthermore, the activation parameters should

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(1) Abbreviations: μ = ionic strength; CCP^{II} = ferrocytochrome *c* peroxidase; CCP^{III} = ferricytochrome *c* peroxidase; C^{II} = ferrocytochrome *c*; C^{III} = ferricytochrome *c*; C/CCP is the *noncovalent* complex[7,8] formed between the proteins; porC = porphyrin cytochrome *c* (i.e., the iron-free derivative of cytochrome *c*).

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Table I. Observed Rate Constants for the Oxidation of Ferrocytochrome c Peroxidase by Horse Ferricytochrome c at 0.02, 0.1, and 0.2 M Ionic Strengths in Phosphate Buffers, pH 7.0[a]

μ = 0.02 M[b]		μ = 0.2 M[b]		μ = 0.1 M (KCl)[c]	
T, °C	k_{obsd}, s^{-1}	T, °C	k_{obsd}, s^{-1}	T, °C	k_{obsd}, s^{-1}
0	0.11	0	0.0015	4	0.0020
5	0.12	8	0.0032	9	0.0041
8	0.15	16	0.0086	13	0.0059
18	0.18	24	0.020	16	0.0090
24	0.23			21	0.015
				26	0.025

[a] The error in the observed rate constants is ≤10%. [b] At μ = 0.02 and 0.2 M, the phosphate concentrations are 0.01 and 0.1 M, respectively. [c] The ionic strength of 0.01 M phosphate buffer, pH 7.0, was adjusted to 0.1 M by using KCl (see text).

indicate whether electron transfer or protein rearrangement is rate-limiting at a given μ, which is important to understanding protein-mediated electron transfer.

Fluorescence polarization of porC as a function of the CCP to porC ratio is also presented at μ = 0.02 and 0.2 M to probe the presence of the C/CCP complex. Since more than one noncovalent complex may be formed between the proteins, the high salt form may have missed detection by fluorescence quenching[11] and absorbance difference measurements,[10] which are sensitive only to the separation and orientation of the hemes. Polarization, on the other hand, is also sensitive to the rotational relaxation time of the fluorophore, which would increase on complexation of the proteins.

Finally, since the CCP heme is ~10 Å below the protein surface,[5] it is of interest to determine if the rate-limiting steps at high salt are sensitive to the steric demands of the oxidant. Thus, we investigated the kinetics of the $Fe(CN)_6^{3-}$ and Fe(EDTA)$^-$ oxidation of CCP^{II} at μ = 0.2 M. These reagents were chosen because they are not likely to form ionically stabilized complexes with CCP since its ferric form carries a net negative charge of 9.5 at pH 7.0,[13] and because of its buried heme, the reactivity of CCP^{II} with reagents having hydrophobic [$Fe(CN)_6^{3-}$] vs hydrophilic [Fe(EDTA)$^-$] ligand surfaces may differ considerably, as observed previously for other metalloproteins.[14]

Experimental Section

Yeast CCP was isolated by the published procedure[15] and type VI (horse heart) C was obtained from Sigma and used without further purification. Aqueous solutions of ferric EDTA were prepared by mixing $Fe(NH_4)(SO_4)_2 \cdot 12H_2O$ with a slight excess of Na_2EDTA. All reactions were carried out in 0.01 and 0.10 M aqueous sodium phosphate buffers at pH 7.0, which have μ = 0.02 and 0.2 M, respectively. Because of the extreme O_2 sensitivity of CCP^{II}, the peroxidase was photochemically reduced in the cuvette and the oxidant was added by gastight syringe as described previously.[2] For the C^{III} oxidations, the kinetics were followed at 420 nm (CCP^{III} and CCP^{II} isosbestic point) by monitoring the growth of the C^{II} absorbance, and for the $Fe(CN)_6^{3-}$ and Fe(EDTA)$^-$ oxidations by monitoring the disappearance of CCP^{II} at 440 nm with a rapid-response spectrophotometer (HP Model 8451A; response time 0.1 s). A 400-nm UV cutoff filter was used to eliminate UV photoreduction by the polychromatic monitoring beam of the spectrophotometer. PorC was prepared from horse heart C and characterized by using published procedures,[11] and fluorescence polarization measurements were carried out on a Perkin-Elmer 44B spectrofluorimeter using HNP'B polarizers (Polaroid Corp.) as previously described.[16]

Results

The electron-transfer rate between horse C and CCP was measured between 0 and 26 °C at μ = 0.02 and 0.2 M. Rate data were limited to this temperature range since CCP^{II} undergoes a heme-linked transition around 30 °C at pH 7.0.[17] The observed rate constants are listed in Table I, and the results of the temperature analyses are given in Table II. To probe the existence of a phosphate effect in 0.1 M phosphate, the temperature dependence of the electron transfer was investigated in 0.01 M phosphate buffer adjusted to μ = 0.1 M with KCl, and the results are also summarized in Tables I and II. Since the data show no dependence on phosphate concentration, it seems unlikely that the unimolecular kinetics at high salt are due to a phosphate effect.

Table II. Analyses of the Temperature Dependence of Electron Transfer from Ferrocytochrome c Peroxidase to Horse Ferricytochrome c at 0.02, 0.1, and 0.2 M Ionic Strengths in Phosphate Buffers, pH 7.0[a]

	μ = 0.02 M[b]	μ = 0.2 M[b]	μ = 0.1 M (KCl)[c]
$\Delta G^{\ddagger}$,[d] kcal mol^{-1}	18	20	20
$\Delta H^{\ddagger}$, kcal mol^{-1}	4.6	17	17
$\Delta S^{\ddagger}$, eu	−46	−7.8	−7.9

[a] The activation parameters were determined from Eyring plots of ln (k/T) vs $1/T$ by using the data in Table I. The correlation coefficients were all >0.99. [b,c] See the corresponding footnotes to Table I. [d] Calculated at T = 295 K.

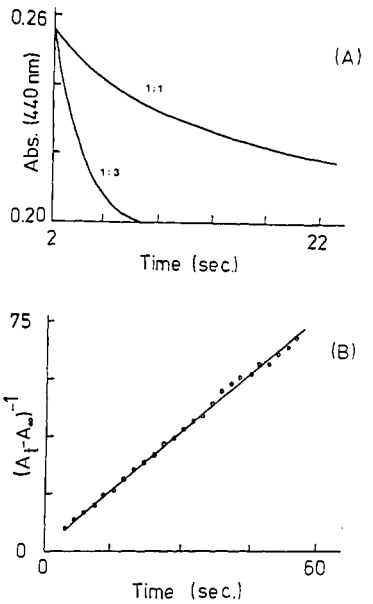

Figure 1. Oxidation of 3 μM CCP^{II} by $Fe(CN)_6^{3-}$ at μ = 0.2 M in phosphate buffer, pH 7.0, 25 ± 1 °C: (A) upper curve, absorbance change at 440 nm for equal concentration of reactants over ~5 half-lives ($t_{1/2}$ ~ 4 s); lower curve, absorbance change for a 3-fold excess of $Fe(CN)_6^{3-}$; (B) fit of the 1:1 reaction to second-order kinetics over ~15 half-lives. A_t and A_∞ are the absorbances at 440 nm at time t, and after the reaction was completed, respectively.

Figure 1a shows the spectrophotometric trace of the absorbance decrease at 440 nm when 1:1 and 1:3 ratios of CCP^{II}:$Fe(CN)_6^{3-}$ were mixed at μ = 0.2 M (0.1 M phosphate). Clearly, the reaction follows second-order kinetics under these conditions, and Figure 1b shows the second order fit over ~15 half-lives for the 1:1 reaction, which yields a rate constant of (8.4 ± 0.4) × 10^4 M^{-1} s^{-1}. Second-order kinetics were also observed at μ = 0.2 M for Fe(EDTA)$^-$ oxidation, and the rate constant obtained from measurements under pseudo-first-order conditions [10^2–10^3-fold excess of Fe(EDTA)$^-$ over peroxidase] is 70 ± 8 M^{-1} s^{-1}.

Figure 2 shows the results of fluorescence polarization measurements on porC as a function of the CCP to porC ratio. At μ = 0.2 M, the observed polarization is 0.132 ± 0.003 whether or not CCP^{III} is present in the solution. At μ = 0.02 M on the other hand, the observed polarization increases on the addition of CCP up to a 1:1 CCP to porC ratio and levels off at a value

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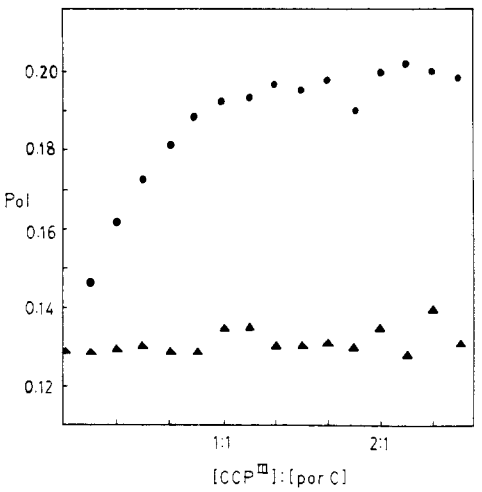

Figure 2. Titration of the fluorescence polarization (Pol) of 3 μM porphyrin cytochrome *c* with CCP^{III} at μ = 0.02 M (dots) and μ = 0.2 M (triangles). Measurements were carried out in 0.01 and 0.1 M phosphate buffers, pH 7.0, at 25 ± 1 °C, and the excitation and emission wavelengths were 515 and 620 nm, respectively.

of 0.198 ± 0.004. Thus, the polarization data corroborate the previous binding studies:[10,11] the proteins form a 1:1 complex at μ = 0.02 M but do not bind at μ = 0.2 M.

Discussion

From the activation parameters listed in Table II, it is apparent that at μ = 0.02 M the C^{III} oxidation is moderately temperature dependent ($\Delta H^{\ddagger}$ = 4.6 kcal mol^{-1}) whereas at μ = 0.2 M the reaction is strongly temperature dependent ($\Delta H^{\ddagger}$ = 17 kcal mol^{-1}). The observed rates differ by only 1 order of magnitude because of the large negative activation entropy at low μ (−46 eu) compared to that at higher μ (−7.8 eu). Such a large variation in the observed activation parameters supports a change in mechanism at high μ. It should be reemphasized at this point that first-order kinetics are observed under all experimental conditions, and the reaction rates are independent of protein concentrations. Therefore, bimolecular association is not partially rate-limiting at either μ.

C and CCP Electron Transfer at μ = 0.02 M. At μ = 0.02 M, and 3 μM reagent concentration, complex formation between C and CCP is extensive since the porC polarization levels off at a 1:1 ratio of the proteins (Figure 2). Thus, if there is no rearrangement of the C/CCP complex prior to electron transfer, then from Marcus' theory, the experimental activation enthalpy is approximated by[18]

$$\Delta H^{\ddagger} = \tfrac{1}{2}(\Delta H^{*}_{11} + \Delta H^{*}_{22} + \Delta H^{\circ}_{12}) \qquad (5)$$

where ΔH^{*}_{11} and ΔH^{*}_{22} are the enthalpies of reorganization for the electron exchange reactions of reagents 1 and 2, and ΔH°_{12} is the standard free enthalpy change for the electron-transfer reaction. Using ruthenated derivatives, Gray and co-workers determined values of 8 and 20 kcal mol^{-1} for ΔH^{*} for the Fe^{III}/Fe^{II} exchange reactions of C[19] and myoglobin,[20] respectively. A large ΔH^{*} is found for myoglobin because its heme is high spin in both redox states, and the ferric state possesses a heme-bound water molecule that dissociates on reduction.[20] EPR[21] and resonance Raman[22] studies indicate that the heme in CCP is also high spin but 5-coordinate in both redox states, so we adopt the ΔH^{*} value for myoglobin as an *upper* limit for CCP. A value of ~5 kcal mol^{-1} is obtained for ΔH°_{12} for C^{III}/CCP^{II} → C^{II}/CCP^{III} by adding eq 6 and 7. Insertion of the above values for ΔH^{*}_{11}, ΔH^{*}_{22} (1 = C; 2 = CCP), and ΔH°_{12} into eq 5, yields $\Delta H^{\ddagger}$ = 12 kcal mol^{-1}, which is significantly larger than the experimental value of 4.6 kcal mol^{-1}. This suggests that at low μ, electron transfer is probably partially rate-limiting and that it may be coupled to rearrangement at the protein interface, which reduces the activation enthalpy.

$$C^{III} + e^{-} \rightarrow C^{II} \quad \Delta H^{\circ} = -15.3 \text{ kcal mol}^{-1} \text{ (ref 19)} \qquad (6)$$

$$CCP^{II} \rightarrow CCP^{III} + e^{-} \quad \Delta H^{\circ} \sim 10 \text{ kcal mol}^{-1} \text{ (ref 23)} \qquad (7)$$

C and CCP Electron Transfer at μ = 0.2 M. In eq 3, a slow conformation change of free CCP is chosen as a likely rate-determining step to account for the observed first-order kinetics under conditions where the expected K_d (~1 mM[10,11]) is 3 orders of magnitude greater than the reagent concentration. The large observed $\Delta H^{\ddagger}$ (Table II) is consistent with a mechanism involving protein rearrangement rather than one involving electron transfer since the former can have activation enthalpies on the order of 10–20 kcal mol^{-1}.

Since phosphate is known to bind to C[24] and possibly also to CCP,[25] it was necessary to establish that the rate-limiting step in 0.1 M phosphate is not due to phosphate dissociation from the proteins. The kinetics in ~0.1 M KCl are identical with those observed in 0.1 M phosphate (Tables I and II); therefore, we conclude that ion-specific binding effects are not involved in the rate-limiting steps. Two other lines of evidence support this conclusion: (1) the enzyme kinetics of CCP using C as a substrate show similar trends in a variety of buffers including phosphate;[26] (2) since *both* the fast and slow steps observed here at μ = 0.02 and 0.2 M, respectively, are observed at intermediate phosphate concentrations,[9] the rate-determining steps cannot involve an equilibrium between phosphate-bound and phosphate-free forms of the proteins since this would lead to monophasic kinetics at all phosphate concentrations.

Oxidations of CCP^{II} by Fe(CN)$_6^{3-}$ or Fe(EDTA)$^{-}$ at μ = 0.2 M does not show a rate-limiting unimolecular step at reagent concentrations that yield half-lives of ≤4 s compared to 35 s for C^{III} oxidation. On the other hand, the oxidation of CCP^{II} at high salt by cytochrome *c*'s from different species (yeasts, tuna, bacterial c_{551}) is unimolecular in each case with k_{obsd} = 0.03 ±0.01 s^{-1}.[27] These observations support our interpretation of the slow unimolecular step in eq 3 as arising from a CCP conformation change, since it is conceivable that cytochrome oxidation at high salt requires transient broadening of the CCP heme channel,[5] whereas the smal molecules can directly access the buried heme (indeed, a K_d of ~2 mM has been observed for complexation of Fe(CN)$_6^{4-}$ to the CCP^{III} heme[13]). Therefore, a possible candidate for the slow CCP^{II} → *CCP^{II} step in eq 3 is the acidic → alkaline transition of CCP^{II}, which increases its heme exposure.[28] This transition has a pK_a = 7.7 at 25 °C[28] and ≤ 7.0 at 30 °C;[17] also, its first-order rate constant is close to k_{obsd} for cytochrome oxidation at high μ.[17]

Relevant to the present study is the postulation of Kang et al.[26] that CCP^{III} exists in low and high μ forms. This was proposed since the steady-state enzyme kinetics revealed large changes in V_{max}, with little change in K_m, on changing μ and pH. If different forms of CCP^{II} also exist in ionic strength equilibrium, then these forms must interconvert on a time scale that is slow relative to the electron-transfer rates since, at μ = 0.05 M, the absorbance–time curves for C^{III} + CCP^{II} → C^{II} + CCP^{III} are clearly biphasic[9] as mentioned above.

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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.

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Effect of Nonsynergistic Anions on Copper Transferrin

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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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