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Ligand binding properties of cytochromes *c′*

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The cytochromes c′ bind CO, alkylisocyanides and CN^- with rate and equilibrium constants which are 10^2- to 10^6-fold smaller than other high-spin hemoproteins. The decreased affinity for exogenous ligands is largely associated with steric interactions at the heme coordination site. While CO and alkylisocyanides bind noncooperatively to the dimeric *Rhodospirillum molischianum* cytochrome *c′*, CO, alkylisocyanides and CN^- appear to bind cooperatively to the dimeric *Chromatium vinosum* cytochrome *c′* due to a ligand-linked dimer-monomer dissociation equilibrium. The differences between the cytochromes *c′* are thought to be due to differences in amino acid residues near the heme coordination site and subunit interface.

The cytochromes *c′* are mono- and dimeric heme proteins derived from photosynthetic, denitrifying and nitrogen fixing bacteria [1]. These cytochromes are thought to function in electron transport, although no specific role has been ascribed to them. The proteins have been found to exhibit properties similar to those of functionally different classes of hemoproteins. The cytochromes *c′* are autoxidizable and have a heme binding sequence pattern (-Cys-X-Y-Cys-His-) similar to that of the low-spin cytochromes *c*, but have optical absorption spectra and CO-binding properties resembling the O_2-binding high-spin hemoglobins and myoglobins.

Sequence studies suggest that the cytochromes *c′* may be related to other class II cytochromes, which include some low-spin cytochromes with a covalent heme attachment near the C-terminus [2]. Structural studies indicate that the cytochromes *c′* represent a unique family of *c*-type cytochromes whose subunit structure corresponds to a left-twisted array of four nearly parallel α-helices which resembles neither the mitochondrial cytochromes *c* nor the globins [3]. The cytochromes *c′* exhibit wide variations in their properties, typified by a 10^3-fold difference in CO equilibrium constants (Table I) [4] and widely divergent amino acid sequences. It is presumed that variations in the heme environment among these proteins provides the basis for these differences in properties. X-ray studies [3] have confirmed earlier observations that the heme iron in cytochrome *c′* is pentacoordinate, with a solvent-exposed histidine as the fifth ligand. The ligand binding properties of these proteins are anomalous relative to other high-spin hemoproteins.

In contrast to the hemoglobins and myoglobins that bind a variety of anionic and neutral ligands at the sixth coordination site in the ferric and ferrous states, the cytochromes *c′* have earlier been reported [6] to bind only two uncharged ligands, NO and CO at physiological pH. Equilibrium and kinetic studies [4] have indicated that the rates and affinities of CO binding to cytochromes *c′* are 10^2 to 10^5-fold smaller than those of globins and the peroxidases as observed in Table I.

Several factors have been considered [3] to account for the differences between the coordination properties of the cytochromes *c′* and those of other high-spin heme proteins. It has been suggested that the absence of a hydrogen bonding group capable of forming a stabilizing interaction with a bound ligand such as that observed in hemoglobin and myoglobin may militate against the formation of a hexacoordinate species. X-ray studies of *Rhodospirillum molischianum* cytochrome *c′* have suggested that the lowered affinity for CO, compared to the globins may reflect a steric hindrance at the sixth coordination site associated with close packing of residues about the distal heme surface such that some relocation of residues adjacent to the site must accompany ligand binding. It has further been proposed that the absence of a hydrogen bond from the unligated nitrogen of the bound imidazole to a backbone or side-chain carbonyl oxygen of the protein, as observed

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in other hemoproteins, may affect the coordination properties of the iron at the trans coordination site.

Further studies [17,18] of CO binding to the dimeric *Rs. molischianum* and *Chromatium vinosum* cytochromes *c′* by high-precision equilibrium methods have indicated striking differences in their properties. The *Rs. molischianum c′* binds CO in a noncooperative manner, while *C. vinosum c′* exhibits a cooperative CO-binding curve. Moreover, the overall CO affinity increases as the concentration of cytochrome *c′* decreases. Gel-filtration chromatography further showed that at micromolar cytochrome concentrations CO binding causes the reduced ligated dimer to dissociate into monomers. Carbon monoxide binding could thus be described by the following equilibrium involving a ligand linked dimer to monomer equilibrium;

$$D + CO \overset{K_d}{\rightleftarrows} D\text{-}CO + CO \overset{K_d}{\rightleftarrows} D(CO)_2 \overset{K_4}{\rightleftarrows} 2M\text{-}CO$$

where K_d is the intrinsic affinity constant and K_4 is the dimer-to-monomer dissociation constant. Thus, the cooperative binding curve results from the dissociation of the ligated dimer to ligated monomer.

Other studies [5,9] have indicated that the ligand-binding properties of the ferrous cytochromes *c′* are not limited to diatomic molecules. It was demonstratd that the *C. vinosum c′* binds ethylisocyanide, C_2H_5NC, as evidenced by spectroscopic changes similar to those observed for CO binding and the spectroscopic properties of the hemoglobin and myoglobin C_2H_5NC complexes which correspond to the conversion of a pentacoordinate high-spin complex to that of a hexacoordinate low-spin complex. Titration of the *C. vinosum c′* with C_2H_5NC yielded a binding constant which is only 5-fold smaller than that observed for Hb (Table I). By contrast, earlier studies [19] indicated that at 3 mM C_2H_5NC does not bind to *Rs. rubrum c′* at pH 7.0, consistent with the lower CO-binding affinity of this protein. These observations suggested that the binding site of *C. vinosum c′* may be significantly more accessible than that of the *Rs. rubrum c′*.

Subsequent studies [5,6] have demonstrated the binding of C_2H_5NC to two ferrocytochromes *c′* from *Rhodobacter sphaeroides* and *Rhodopseudomonas palustris* as well as that from *Rs. rubrum* at pH 7.0. As shown in Table I, the cytochromes exhibit more than a 10^3-fold difference in C_2H_5NC equilibrium constants, which are as little as 5-fold and as much as $4 \cdot 10^6$-fold smaller than the globins. Moreover, equilibrium constants for C_2H_5NC binding to the four cytochromes *c′* are separated into two widely different groups, with the value for *C. vinosum* being closely similar to the value for *Rb. sphaeroides* and the value for *Rh. palustris* similar to the value for *Rs. rubrum*. Table I indicates that there is no correlation between equilibrium constants for C_2H_5NC and CO binding. The most striking contrast is observed for *Rh. palustris*, which exhibits one of the lowest equilibrium constants for C_2H_5NC binding but the highest observed constant for CO binding.

The general lack of correlation between C_2H_5NC and CO binding constants suggested that electronic differences associated with differences in solvent or protein–histidine hydrogen bond strengths do not adequately account for the differences in C_2H_5NC binding constants. The C_2H_5NC binding constants are approx. 1–6 orders of mangitude lower than CO binding constants. In comparison, the C_2H_5NC binding constants are only 2–10-fold smaller than those of CO to pentacoordinate protoheme complexes [11]. Thus, while the ratios of binding constants $K^{CO}/K^{C_2H_5NC}$ for cytochromes *c′* from *C. vinosum* and *Rb. sphaeroides* are similar to ratios for the model systems, the ratios are much

TABLE I

Rate and equilibrium constants for ligand binding to cytochromes c′ and other hemoproteins

Hemoprotein	K^{CN} [a] (M^{-1})	k^{CN} [a] ($M^{-1}\,s^{-1}$)	K^{CO} [b] (M^{-1})	k^{CO} [b] ($M^{-1}\,s^{-1}$)	$K^{C_2H_5NC}$ [b] (M^{-1})	$k^{C_2H_5NC}$ [b] ($M^{-1}\,s^{-1}$)
Rb. sphaeroides c′[4,5]			10^4–10^5	$2.6 \cdot 10^3$ [c]	$3.86 \cdot 10^3$	
Rs. rubrum c′[4,5]			$1.3 \cdot 10^3$	8.4	1.2	
Rh. palustris c′[4,5]			$1.7 \cdot 10^6$	$3.3 \cdot 10^2$	2.2	
Rs. molischianum c′[4,5,6]			$4 \cdot 10^4$	$4.4 \cdot 10^2$	3.3	5
C. vinosum c′[4,7,8,9]	$1.3 \cdot 10^2$	$2.3 \cdot 10^{-3}$	$7.8 \cdot 10^4$	$1.4 \cdot 10^2$	$3.3 \cdot 10^3$	$3.2 \cdot 10^1$
G. dibranchiata[10,11]						
Hb(II)	$7.7 \cdot 10^4$	$4.9 \cdot 10^{-1}$	$6.4 \cdot 10^8$	$2.7 \cdot 10^7$	$4.6 \cdot 10^6$	$1.6 \cdot 10^5$
Hb[6,7,8,12]	$2.3 \cdot 10^6$	$1.1 \cdot 10^2$	$2 \cdot 10^7$	$2 \cdot 10^5$	$1.9 \cdot 10^4$	$2.7 \cdot 10^4$
Mb[7,8,11]	$1.4 \cdot 10^6$	$1.7 \cdot 10^2$	$3.3 \cdot 10^7$	$5.0 \cdot 10^5$	$1.7 \cdot 10^5$	$5.4 \cdot 10^4$
HRP[8,12,13,14]	$3.9 \cdot 10^5$	$1.1 \cdot 10^5$	$1 \cdot 10^6$	$4.9 \cdot 10^3$	$1.5 \cdot 10^2$	
CcP[8,11,15]	$5 \cdot 10^5$	$1.1 \cdot 10^5$	$2.2 \cdot 10^5$	$2.0 \cdot 10^4$	$1.2 \cdot 10^2$	2.0

[a] Equilibrium and rate constants correspond to total cyanide concentration at pH 7 and 25 °C.

[b] Equilibrium and rate constants correspond to pH 7.0 and 20 or 25 °C.

[c] Fast phase.

greater for *Rh. palustris* and *Rhodospirillum rubrum*, indicating that factors other than differences in trans ligand coordination contribute to the differences in C_2H_5NC binding between the cytochromes *c'*.

The differences in C_2H_5NC binding between the cytochromes *c'* were therefore proposed to be accounted for by steric restrictions associated with protein–ligand interactions at the sixth coordination site. The larger $K^{CO}/K^{C_2H_5NC}$ ratios observed for *Rh. palustris* and *Rs. rubrum* compared to *C. vinosum* and *Rb. sphaeroides* are consistent with smaller C_2H_5NC binding constants relative to those for CO due to steric effects involving C_2H_5NC, which has a structure analogous to CO but with the alkyl group providing additional steric bulk. The ratios, $K^{CO}/K^{C_2H_5NC}$, for cytochromes *c'* from *Rh. palustris* and *Rs. rubrum* indicate that the binding of C_2H_5NC to these proteins is more sterically hindered than CO. The $7 \cdot 10^5$ decrease in C_2H_5NC binding relative to CO binding for *Rh. palustris c'* in particular dramatically indicates that the packing of residues about the sixth coordination site permits relatively unhindered ligation of CO but severely hindered ligation of C_2H_5NC.

The proposed steric effects are correlated with and supported by the spectroscopic properties of the C_2H_5NC complexes. The Soret extinction coefficients and half-bandwidths exhibit more than a 2-fold difference, with the values of *C. vinosum* being most similar to those of *Rb. sphaeroides* and of *Rh. palustris* similar to those of *Rs. rubrum*. The extinction coefficients are much greater and correspondingly the half-band widths much smaller for *Rh. palustris* and *Rs. rubrum* compared to those of *C. vinosum* and *Rb. sphaeroides* cytochrome *c'*-C_2H_5NC complexes. The smaller Soret half-bandwidths and greater extinction coefficients for *Rh. palustris* and *Rs. rubrum* cytochrome *c'*-C_2H_5NC complexes has been associated with more restricted heme environments leading to reduced broadening in the absorption band [5]. Thus the observed correlation between the extinction coefficients, half-bandwidths and equilibrium constants for C_2H_5NC complex formation suggested that the differences in binding properties are associated with sterically hindered ligation to the heme.

Subsequent studies [6] of the binding of a homologous series of alkylisocyanides have been carried out for the ferrocytochrome *c'* from *Rs. molischianum*, for which the X-ray crystal structure has been determined. The Soret extinction coefficients and half-bandwidths of the alkylisocyanide complexes of *Rs. molischianum c'* are similar to those observed for *Rh. palustris* and *Rs. rubrum*.

The C_2H_5NC binding constant for *Rs. molischianum* cytochrome *c'* (Table II) is 10^7-fold less than the sterically unconstrained model, about 10^3–10^4-fold less than for Hb and Mb (Table I). The C_2H_5NC binding constant for *Rs. molischianum c'*, 3.3 M^{-1}, is similar to the value of 2.2 M^{-1} for *Rh. palustris c'* and 1.2 M^{-1} for *Rs. rubrum c'* (Table I). Thus, the equilibrium and spectroscopic properties of C_2H_5NC binding to *Rs. molischianum c'* are consistent with the proposal that a sterically hindered heme ligand environment in the *Rh. palustris* and *Rs. rubrum* cytochromes *c'* contributed to the lower C_2H_5NC binding constant and greater Soret extinction coefficients with narrower half-bandwidths.

TABLE II

Equilibrium constants and free energy parameters for alkylisocyanide and CO binding to Rs. molischianum cytochrome c' and a model heme complex

CO/alkyl-isocyanide	K (M^{-1})		ΔG_{PROT} (kcal/mol) [a]
	*Rs. moli schianum c** [a]	*model* [b]	
CO	$4 \cdot 10^4$	$3.90 \cdot 10^8$	5.35
Methyl	8.7	$0.11 \cdot 10^8$	8.2
Ethyl	3.3	$0.34 \cdot 10^8$	9.4
Propyl	13	$0.74 \cdot 10^8$	9.1
Butyl	59	$2.84 \cdot 10^8$	9.0
Amyl	95	$6.15 \cdot 10^8$	9.1
Hexyl	260	$15.1 \cdot 10^8$	9.1
t-Butyl	15	$0.78 \cdot 10^8$	9.0
n-Hexyl	100	$4.98 \cdot 10^8$	9.0

[a] Ref. 6.

[b] Protoheme mono-3-(1-imidazoyl)propylamide monomethyl ester in 2% myristyl trimethylammonium bromide [11].

The equilibrium constants for the binding of a series of alkyl isocyanides to the *c'* from *Rs. molischianum* provide a measure of the extent to which steric constraints indicated in the X-ray structure affect ligand binding. A decrease in the binding constant would be expected as the size of the alkyl group increases for a sterically hindered coordination site [12]. As shown in Table II, the equilibrium constants for *Rs. molischianum c'* follow the order methyl > ethyl < *n*-propyl < *tert*-butyl < *n*-butyl < amyl < cyclohexyl < *n*-hexyl. The results suggest that steric interactions contribute to the decrease in equilibrium constant from methyl to ethyl-isocyanide. The increase in equilibrium constant from ethyl to hexyl is associated with a hydrophobic heme coordination site in the protein, similar to that for the binding of alkylisocyanides to a sterically unconstrained protoheme model in aqueous detergent micelle [11]. The differences in equilibrium constant for alkylisocyanide binding to the model in detergent have been correlated with a difference in partition coefficients of the isocyanide between the micelle and the aqueous phase. The observed binding constants for the model increased as the size of the alkyl group increased. The results suggest then that both steric and hydrophobic effects are operative in alkylisocanide bindig to the *Rs. molischianum c'*.

The extent of steric interaction between the alkylisocyanide and protein in hemeproteins has been evaluated by considering the differences between the free energies of ligand binding to the heme model in the soap micelle and to the heme protein according to the expression:

$$\Delta G_{PROT} = RT \ln(K_{soap}/K_{obsd})$$

ΔG_{PROT} is a measure of both the steric interaction between the ligand and the protein at the sixth coordination site and the proximal effect at the fifth coordination site. This type of analysis is based on the assumption that the hydrophobic environment in the micelle is similar to that of the protein and that the homologous series of alkylisocyanides and CO will equally be affected by any proximal effect. Therefore, differences in ΔG_{PROT} between CO and alkylisocyanides are a measure of steric effects between the alkylisocyanides and the protein at the sixth coordination site. Table II also shows the ΔG_{PROT} values for the binding of CO and alkylisocyanides to *Rs. molischianum c'*. In the absence of any steric effect, ΔG_{PROT} would be constant for each of the alkylisocyanides. An increase in ΔG_{PROT} values as the size of the ligand increases is consistent with an increase in steric interaction.

The ΔG_{PROT} for CO binding to *Rs. molischianum c'* is 5.35 kcal/mol compared to 1.43 kcal/mol for Mb, about 0.0 kcal/mol for R-state Hb, and 3.4 kcal/mol for T-state Hb. The 3.4 kcal/mol increase in going from the R to the T state and the 1.43 kcal/mol for Mb has been interpreted in terms of an unfavorable proximal effect on the basis of the uniform dependence of ΔG_{PROT} for these proteins on ligand size and shape and the rate constants for complex formation. The rate constants for CO binding to cytochromes *c'* [4] are 10^4–10^6-fold slower than that for the model heme complex and 10^3–10^4-fold slower than those for Hb and Mb, which suggests that the very large ΔG_{PROT} of 5.35 kcal/mol observed for CO binding to the *Rs. molischianum c'* is more likely due to a steric effect than to a proximal effect. The ΔG_{PROT} for the binding of methylisocyanide to *Rs. molischianum c'* is about 3 kcal/mol greater than that for CO binding. Since electronic differences between model heme and heme protein would be expected to similarly affect CO and methylisocyanide, the large increase in the value of ΔG_{PROT} from CO to methylisocyanide is due to further steric interaction caused by addition of the third ligand atom. Addition of the fourth ligand atom, from methyl to ethylisocyanide, increases ΔG_{PROT} by about 1.2 kcal/mol and is also consistent with a further increase in steric interaction from methyl to ethylisocyanides, corresponding to a decrease in the affinity constant. Extending the alkyl chain from ethyl to hexyl or branching at the α-carbon causes no additional changes in ΔG_{PROT} which suggests that no additional steric interactions are present.

These results suggest that the extremely small equilibrium constants observed for the binding of methyl and other alkylisocyanides to *Rs. molischianum* ferrous cytochrome *c'* are in large part associated with a heme coordination site that is severely sterically hindered. The binding of a series of alkylisocyanides to the *C. vinosum c'* has also been studied (unpublished data) and indicates no further steric constraints to binding occur beyond that observed for CO, suggesting that the heme environment around the sixth coordination site is flexible. Likewise, the binding of alkylisocyanides is associated with a dimer-to-monomer equilibrium which appears to be much smaller than that for CO complexed to this cytochrome *c'*. In addition to the binding of other than diatomic ligands to the ferrocytochromes *c'*, the reversible binding of CN^- to the ferricytochrome *c'* from *C. vinosum* has also been demonstrated [7], in contrast to earlier studies. At pH 7.0 and 0.1 M KCN, the absorption spectrum of the *C. vinosum* cytochrome *c'* following several hours of equilibration is similar to those of the ferricytochrome *c* and metmyoglobin · CN^- complexes. Titration of the *C. vinosum c'* with CN^- yielded an observed equilibrium constant of $2.1 \cdot 10^4$ M^{-1} (Table I). The observed binding constant is 10^2–10^4-fold smaller than that observed for other high-spin hemoproteins. It was suggested that differences between these and earlier observations are due both to the greater CN^- concentration and longer equilibration times used. It was proposed that the lower binding constant for the cytochrome *c'* may result from steric interactions as well as perhaps weaker proximal imidazole bonding.

Subsequent kinetic studies [8] of CN^- binding to *C. vinosum* cytochrome *c'* indicate an extremely slow cyanide association rate constant in comparison to other high-spin hemoproteins (Table I). At neutral pH, the rate constant for CN^- binding is about 10^2-fold smaller than that for the monomeric *G. dibranchiata* Hb, 10^5-fold smaller than Hb and Mb, and as much as 10^7-fold smaller than the peroxidases. Thus, the much smaller affinity for cyanide binding to the *C. vinosum* cytochrome *c'* can be accounted for by the much smaller rate of association rather than a greater rate of dissociation due to much weaker binding which suggests that CN^- binding to this cytochrome *c'* is also characterized by severe steric hindrance. The proposed mechanism of CN^- binding is also unusual in that CN^- binding is characterized by a nonlinear cyanide concentration dependence of the observed rate constant at higher pH values, which is interpreted as involving an intermediate complex prior to coordination. The pH dependence indicates that cyanide anion is the major reactive form for binding to *C. vinosum c'*.

Since the earlier [7] measurement of the CN^- binding equilibrium, binding of CN^- to *C. vinosum* ferricytochrome *c'*, has been studied [21] to investigate possible allosteric interactions between the subunits of this

dimeric protein. Like CO, binding of CN^- to *C. vinosum* cytochrome *c′* appears to be cooperative. However, like CO, the affinity of CN^- binding increases as the concentration of cytochrome decreases. Moreover, gel chromatography shows that CN^- binding causes the dimer to dissociate to monomers, as shown by the change in elution volume relative to standard protein markers.

Analysis of the CN^- binding curve, resulting from spectrophotometric titration, by the previously described model for CO binding has yielded an intrinsic CN^- binding constant, K_d, of $3 \cdot 10^3$ corresponding to a total cyanide binding constant of $1.8 \cdot 10^1$. The dimer-to-monomer dissociation constant, K_4, for the CN^- complex is $7.4 \cdot 10^{-5}$ M, which is about 20-fold smaller than the value of K_4 for CO binding [18].

The smaller dimer-monomer dissociation constant for the cyanide-bound ferricytochrome *c′* may be due to structural differences between the proteins in the different oxidation states or differences in the nature of the CN^- and CO ligands. A preferential hydrogen bonding interaction between the bound CN^- (rather than CO) and Tyr-16 on the distal side of the heme iron may help to maintain the tertiary structure of the dimer upon ligation and thus contribute to a smaller dimer to monomer dissociation constant relative to CO.

While CN^- binding to the *Rs. molischianum* and other cytochromes *c′* has not been reported, it has been observed (unpublished data), that like CO, CN^- binding to *Rs. molischianum c′* does not appear to lead to the formation of CN^--bound monomer. The structural basis for the large differences in aggregation state of the cytochromes *c′* in the absence and presence of ligands is suggested by a comparison of the crystal structure of *Rs. molischianum c′* and the amino-acid sequences of several cytochromes *c′*.

The X-ray crystallographic structure of *Rs. molischianum* ferricytochrome *c′* [3] has indicated the nature of the amino acid interactions at the contact regions between the subunits of this protein. The subunit interface of *Rs. rubrum c′* is characterized by extensive hydrophobic packing interactions between the A and B helices of each monomer in addition to the salt link between the side-chain of Lys-10 of one subunit and one of the heme propionates of the other subunit and the potential intersubunit salt link formation between Lys-52 and Glu-45. The amino acid substitutions in *C. vinosum c′* at the AA′ and BB′ interfaces, based on sequence homology [22], result in similar favorable hydrophobic interactions while disallowing both salt linkages. Cytochrome *c′* from *C. vinosum* exhibits ligand-linked dissociation equilibria both in the reduced and oxidized states in contrast to the cytochrome *c′* from *Rs. molischianum* [17]. Thus, it appears that weaker subunit–subunit interactions in *C. vinosum c′* due to the absence of the two salt links, may contribut to the dissociation of the dimer into monomer in this protein.

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