calculated conformation of free unhydrated EnB resembles the experimental conformation of hydrated EnB better than that of the unhydrated one. A possible solution of this puzzle will be discussed elsewhere. We are also grateful to Dr. Benz for kindly calling our attention to EnB-mediated ion transport experiments (Benz, 1978), showing 1:1 complexing ratios where Ivanov et al. (1973) found 2:1 ratios. Our calculations indicate the possibility of both 2:1 and 1:1 ratios but are short of predicting the precise corresponding binding constants.

Registry No. EnB, 917-13-5; $(LacAla)_3$, 56760-72-6.

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Cytochromes *c′* in Their Reaction with Ethyl Isocyanide†

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ABSTRACT: The binding of ethyl isocyanide (EIC) to a representative number of cytochromes *c′* is demonstrated. Spectroscopic and equilibrium constants have been measured and compared for the binding of EIC to cytochromes *c′* from the photosynthetic bacteria *Chromatium vinosum*, *Rhodopseudomonas palustris*, *Rhodospirillum rubrum*, and *Rhodopseudomonas sphaeroides*. While the absorption spectra of the EIC complexes resemble those of EIC complexes of other high-spin hemoproteins, the Soret half band widths and extinction coefficients per heme exhibit more than a 2-fold difference with the values of *C. vinosum* being most similar to those of *Rh. sphaeroides* and of *Rh. palustris* similar to those of *Rs. rubrum*. The cytochromes exhibit binding equilibria consistent with the ligation of one molecule of EIC per heme in contrast to the reported binding of more than one molecule of CO per heme. The binding constants exhibit more than a 1000-fold difference with the values of *C. vinosum* being closely similar to those of *Rh. sphaeroides* and of *Rh. palustris* similar to those of *Rs. rubrum*. The lack of correlation between EIC and CO binding properties indicates that electronic factors do not determine the difference in EIC binding properties. The observed correlation between the extinction coefficients, half band widths, and equilibrium constants for EIC complex formation provides the first spectroscopic evidence that the differences in binding properties are associated with sterically hindered ligation to the heme. Although the differences in binding properties provide evidence of steric hindrance, the EIC binding constants for particular cytochromes *c′* indicate that the distal heme binding site is more accessible than previously indicated. The differences in spectroscopic and binding properties are discussed in terms of structural differences between amino acids thought to be associated with the distal heme binding site.

The cytochromes *c′* are mono- and diheme proteins derived from photosynthetic and denitrifying bacteria and are reported to comprise the largest and most widespread class of bacterial cytochromes known (Meyer & Kamen, 1982). These cytochromes are thought to function in electron transport although no specific role has been ascribed to them (Bartsch, 1978). The proteins have been found to exhibit properties similar to functionally different classes of hemoproteins. The cyto-

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chromes c' are autoxidizable and have a heme binding sequence pattern 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 (Bartsch, 1978). Sequence studies suggest that the cytochromes c' may be related to other class II cytochromes, which includes some low-spin cytochromes with a covalent heme attachment near the C terminus (Moore et al., 1982). Structural studies indicate that the cytochromes c' represent a unique family of c-type cytochromes whose overall tertiary structure resembles neither the mitochondrial cytochromes c or the globins (Weber et al., 1980, 1981).

The cytochromes c' have been studied by a variety of chemical and spectroscopic techniques, including optical (Horio & Kamen, 1961; Maltempo, 1976), near-infrared (Kamen et al., 1973), resonance Raman (Strekas & Spiro, 1974; Kitagawa et al., 1977; Teraoka & Kitagawa, 1980), magnetic circular dichroism (Rawlings et al., 1977), electron spin resonance (Ehrenberg & Kamen, 1965; Maltempo et al., 1974, 1980; Emptage et al., 1977), nuclear magnetic resonance (Emptage et al., 1981; La Mar et al., 1981; Jackson et al., 1983), Mössbauer (Moss et al., 1968; Maltempo & Moss, 1976), and X-ray (Weber et al., 1980, 1981). These proteins exhibit wide variations in their properties, typified by a 1000-fold difference in carbon monoxide equilibrium constants (Cusanovich & Gibson, 1973), a 113-mV difference in redox potentials (Kamen et al., 1971), which are highly pH dependent (Barakat & Strekas, 1982), widely divergent amino acid sequences (Ambler et al., 1981), and conflicting evidence as to the existence of a quantum mechanical admixture of intermediate- ($S = {}^3/_2$) and high- ($S = {}^5/_2$) spin states in the oxidized form (Scheidt & Reed, 1981; Moore et al., 1982). Variations in the heme environment among these proteins may provide the basis for these differences in properties. Recent X-ray studies have confirmed earlier observations that the heme iron in cytochrome c' is pentacoordinate with a solvent-exposed histidine as the fifth ligand (Weber et al., 1980, 1981). In contrast to the hemoglobins and myoglobins that bind a variety of anionic and neutral ligands to the ferric and ferrous states, the cytochromes c' have earlier been reported to bind only two ligands, nitric oxide and carbon monoxide, at physiological pH (Taniguchi & Kamen, 1963), and only carbon monoxide has been the subject of in-depth studies (Cusanovich & Gibson, 1973). Recently, we presented evidence for the binding of ethyl isocyanide (EIC) to *Chromatium vinosum* cytochrome c' (Kassner et al., 1983). In this paper, we demonstrate the binding of EIC to cytochromes c' from *Rhodopseudomonas palustris*, *Rhodopseudomonas sphaeroides*, and *Rhodospirillum rubrum*, compare the spectrscopic properties and equilibrium constants for the cytochromes from the four bacteria, and discuss the differences in terms of sterically hindered ligation.

Materials and Methods

Rhodopseudomonas palustris cytochrome c' was isolated according to the method of De Klerk et al. (1964). *Rhodospirillum rubrum* cytochrome c' was isolated according to the method of Bartsch et al. (1971). *Rhodopseudomonas sphaeroides* cytochrome c' was isolated according to the method of Sponholtz et al. (1976). Ethyl isocyanide was prepared according to the method of Jackson & McKusick (1955). Solutions of the reduced, unligated proteins contained 0.1M potassium phosphate buffer, pH 7.0, and a small amount of sodium dithionite to reduce and maintain the heme iron in the reduced state. A total of 2.5 mL of this solution was titrated with EIC in a standard rectangular cuvette fitted with a Teflon stopper. Additions of 0.1 M EIC were made to the solutions of *Rh. sphaeroides* cytochrome c' by using a 50-μL Hamilton syringe with a repeating dispenser attachment. Additions of pure EIC were made to the solutions of *Rh. palustris* and *Rs. rubrum* cytochrome c' in the manner just described. EIC was introduced by removing the cuvettes from the cell compartment, lifting the stopper, injecting the EIC solution, and then mixing thoroughly. All spectra were measured on a Cary 14R recording spectrophotometer. Following the addition of EIC, equilibrium was achieved within a few minutes as judged by the absence of further spectral changes. Absorption changes, measured at the Soret absorption maxima for the cytochrome c'–EIC complex (Table I), were used to determine the ligand stoichiometries and equilibrium constants. Ligand stoichiometry was determined from the slope of a plot of log $[(A - A_0)/(A_{100} - A)]$ vs. log [EIC], consistent with

$$\text{cyt-}c' + n\text{L} \rightleftharpoons \text{cyt-}c'\text{L}_n$$

$$K = [\text{cyt-}c'\text{L}_n]/([\text{cyt-}c'][\text{L}]^n)$$

$$\ln ([\text{cyt-}c'\text{L}_n]/[\text{cyt-}c']) = \ln K + n \ln [\text{L}]$$

$$[\text{cyt-}c'\text{L}_n]/[\text{cyt-}c'] = (A - A_0)/(A_{100} - A)$$

where A_0 and A_{100} are the limiting absorbances at 0 and 100% complex formation, respectively.

Each equilibrium constant was deterimined from the intercept of the linear least-squares line resulting from a plot of log $[(A - A_0)/(A_{100} - A)]$ vs. log [EIC]. Extinction coefficients for the cytochrome c'–EIC complexes were calculated from the spectra of the ligated proteins on the basis of reported extinction coefficients for the spectra of the reduced, unligated proteins (Bartsch, 1963).

Results

The addition of EIC to each of the cytochromes c' is accompanied by changes in absorption spectra, from a single band to two bands in the region from 500 to 600 nm, and a large increase in the extinction coefficient of the Soret band similar to that observed for CO binding to the cytochromes c'. Table I indicates observed absorption maxima and calculated extinction coefficients for EIC complexes of the cytochromes c' together with those of the corresponding CO complexes. The α and β bands of the EIC complexes have absorption maxima significantly blue shifted from those of the CO complexes. The visible absorption maxima of the EIC complexes show a small but perhaps significant blue shift in the α and β bands with *Rhodospirillum rubrum* < *Rhodopseudomonas palustris* < *Rhodopseudomonas sphaeroides* < *Chromatium vinosum*. The ratio of the extinction coefficients of the α and β bands is somewhat greater than 1 for the *C. vinosum* and *Rh. sphaeroides* cytochrome c'–EIC complexes and somewhat less than 1 for those of *Rh. palustris* and *Rs. rubrum*. As shown in Figure 1, the cytochromes c' exhibit profound differences in extinction coefficients, with that of *Rh. palustris* being 2-fold greater than that of *Rh. sphaeroides*. These extinction coefficients may be compared to values (mM^{-1} $heme^{-1}$ cm^{-1}) of 167 for protoheme mono[3-(1-imidazoyl)propylamide] monomethyl ester (Olson et al., 1983), 161 for horse myoglobin, and 193 for human hemoglobin (Antonini & Brunori, 1971). Table I also indicates a significant difference between the half band widths of the EIC complexes with that of *Rh. sphaeroides* being more than 2-fold greater than that of *Rh. palustris*. No such differences are observed for the CO complexes of these proteins.

Table I: Absorption Maxima and Extinction Coefficients of Some Ferrous Derivatives of Cytochromes *c'*

	absorption maximum (nm) (extinction coefficient [mM^{-1} $heme^{-1}$ cm^{-1}]) [half band width (nm)]					
C. vinosum						
c'[a]	569 (s)[b] (8.5)		547 (9.8)		437 (s)[b] (81.5)	426 (94)
c'–CO[a]		565 (11.5)		545 (13.4)		418 (282)
c'–EIC[c]		557 (18)		527 (17)		427.5 (189) [14.4]
Rh. sphaeroides						
c'[d]			546 (10.4)			425 (99)
c'–CO						
c'–EIC		556 (21.4)		527 (20.2)		426.5 (169) [16.6]
Rh. palustris						
c'[a]	570 (s)[b] (10)		552 (10.4)		435 (s)[b] (92)	426 (96)
c'–CO[a]		570 (11)		535 (12.6)		418 (262)
c'–EIC		555 (17)		526.5 (17.9)		427 (321) [7.6]
R. rubrum						
c'[a]	566 (s)[b] (11)		550 (11.5)		432 (s)[b] (96)	423 (98)
c'–CO[a]		564 (12)		534 (12.8)		416.5 (240)
c'–EIC		554.5 (19.2)		525.5 (19.5)		426 (245) [10.6]

[a] Bartsch, 1963. [b] Shoulder. [c] Kassner et al., 1983. [d] Bartsch, 1978.

Figure 2 illustrates a plot of log $[(A - A_0)/(A_{100} - A)]$ vs. log [EIC] for the titration of the cytochromes with EIC at pH 7.0, 25 °C. The plots for *C. vinosum* and *Rh. sphaeroides* yielded slopes 1.06 and 1.12, respectively, throughout the titration. The plots for *Rh. palustris* and *Rs. rubrum* yielded slopes of 1.03 and 0.955, respectively, for the indicated initial portion of the titration. At later portions of the titration, the slopes of the plots for *Rh. palustris* and *Rs. rubrum* exhibited a moderate increase attributed to the high concentration of EIC required for complex formation. The plots for the cytochromes *c'* are thus consistent with the binding of one ligand according to the equilibrium expression cyt-*c'* + L $\rightleftharpoons$ cyt-*c'*-L. The equilibrium constants for EIC binding to these cytochromes are shown in Table II together with equilibrium constants for CO binding and other properties of these proteins. The EIC binding constants for the cytochromes *c'* may be compared to values of 1.9×10^4 for hemoglobin (Reisberg & Olson, 1980), 3.2×10^5 for myoglobin (Stetzkowski et al., 1979), and 1.6×10^8 and 3.2×10^7 for a pentacoordinate protoheme complex in benzene and aqueous soap suspensions, respectively (Olson et al., 1983).

As indicated in Table II the cytochromes *c'* exhibit more than a 1000-fold difference in binding constants for EIC.

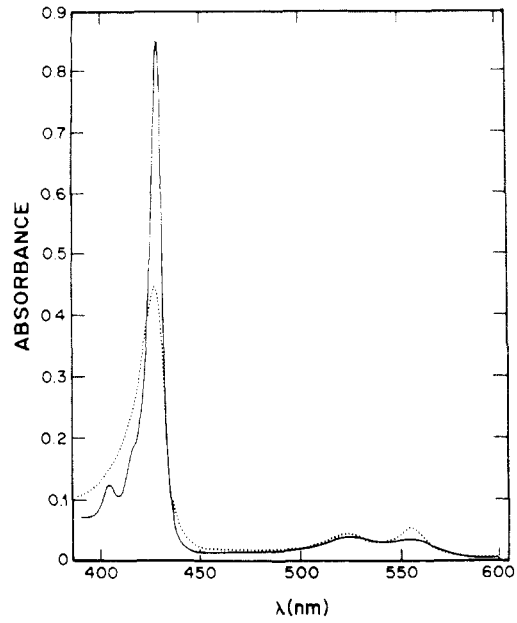

FIGURE 1: Absorption spectra of *Rh. sphaeroides* (···) and *Rh. palustris* cytochrome *c'*–EIC (—) complexes. The concentrations of both cytochromes are 2.86×10^{-6} M in 0.1 M potassium phosphate buffer, pH 7.0. The concentrations of EIC are 4.83×10^{-3} and 1.54 M, respectively.

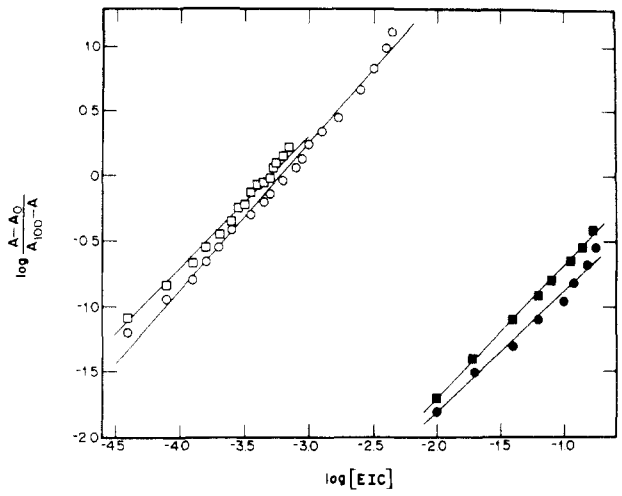

FIGURE 2: Effect of ethyl isocyanide concentration on the binding of *C. vinosum* (□) (Kassner et al., 1983), *Rh. sphaeroides* (○), *Rh. palustris* (■), and *Rs. rubrum* (●) reduced cytochromes *c'* in 0.1 M phosphate buffer at pH 7.0 and 25 °C.

Moreover, equilibrium constants for EIC 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 *Rh. sphaeroides* and the value for *Rh. palustris* similar to the value for *Rs. rubrum*. Table II indicates that there is no correlation between equilibrium constants for EIC and CO binding. The most striking contrast is observed for *Rh. palustris*, which exhibits one of the lowest equilibrium constants for EIC binding but the highest observed constant for CO binding.

Table II: EIC Equilibrium Constants and Other Properties of Some Cytochromes *c'*

	K^{EIC}	K^{CO}[b]	K^{CO}/K^{EIC}	$E_{m,7}$ (mV)[c]	M_r[c]	pI[c]
C. vinosum	3.29×10^3[a]	7.81×10^4	2.37×10^1	−5	29 000	5.0
Rh. sphaeroides	3.86×10^3	10^4–10^5	2.6–(2.6 × 10^1)	30	23 000	4.6
Rh. palustris	2.21	1.67×10^6	7.55×10^5	105	14 000	9.4
Rs. rubrum	1.22	1.26×10^3	1.03×10^3	−8	26 000	5.4

[a] Kassner et al., 1983. [b] Cusanovich & Gibson, 1973. [c] Kamen et al., 1971.

Discussion

The changes in absorption spectra associated with the binding of EIC are similar to those observed for the binding of EIC to hemoglobin and myoglobin and characteristic of the change from that of a pentacoordinate high-spin ferrous heme complex to that of a hexacoordinate low-spin ferrous heme complex. The positions of the α and β bands of the EIC complexes of the cytochromes c' relative to those of the CO complexes are consistent with observed differences between EIC and CO complexes of hemoglobin and myoglobin (Antonini & Brunori, 1971). Likewise, the binding of one EIC per heme is also characteristic of alkyl isocyanide binding equilibria of hemoglobin and myoglobin. This ligand binding behavior is however in sharp contrast to the more complex CO binding mechanism observed for these proteins, which indicated a stoichiometry of at least two CO molecules per heme (Cusanovich & Gibson, 1973). The binding constants are 8.3 $\times 10^3$ to 1.3 $\times 10^8$ fold smaller than those for a model heme complex but as little as 5-fold smaller than that for hemoglobin.

Several factors have been considered 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 myoglobin and hemoglobin (Antonini & Brunori, 1971) may mitigate against the formation of a hexacoordinate species (Weber et al., 1981). On the basis of early attempts (Taniguchi & Kamen, 1963) to demonstrate the binding of various anionic and neutral ligands of different size, it was concluded that the heme group is accessible only to small uncharged ligands. Subsequent studies of CO binding have also suggested that the heme group is situated in a protein-bounded cage with ligand binding controlled by hindered access of dissolved ligands to the heme binding site (Cusanovich & Gibson, 1973; Gibson & Kamen, 1966). These suggestions have been supported by X-ray studies of *Rhodospirillum molischianum* cytochrome c', which indicate that the distal heme surface is surrounded by aromatic or other hydrophobic amino acid residues that are oriented so as to restrict access of exogenous ligands to the sixth heme iron coordination site (Weber et al., 1980). It has also been suggested that the lowered affinity of the cytochromes c' for CO compared to the globins may reflect a steric hindrance at the sixth axial 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 (Weber et al., 1981). 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 in other heme proteins, may affect the delocalization of electronic charge through the imidazole ring (Peisach, 1975; Valentine et al., 1979) and the imidazole ligand field strength (Reed et al., 1979; Lundrum et al., 1980), which could affect the coordination properties of the iron at the trans coordination site (Weber et al., 1981). Correlations (Scheidt & Reed, 1981; Weber, 1982) between the structure and spectroscopic properties of the cytochromes c' together with model studies (Dolphin et al., 1977; Kastner et al., 1978; Reed et al., 1979; Ogoshi et al., 1980) support the concept of a weaker imidazole ligand field strength. Model studies have further indicated that CO binding to pentacoordinate heme complexes is dependent on the ligand at the fifth coordination position (Rougee & Brault, 1975; Scheidt et al., 1981).

The general lack of correlation between EIC and CO binding constants (Table II) suggests that electronic differences perhaps associated with differences in solvent or protein–histidine hydrogen bond strengths do not adequately account for the differences in EIC binding constants. The EIC binding constants are approximately 1–6 orders of magnitude lower than CO binding constants. In comparison, the EIC binding constants are only 2–10-fold smaller than those of CO to pentacoordinate protoheme complexes in benzene and aqueous soap suspensions (Olson et al., 1983). Thus, while the ratios of binding constants K^{CO}/K^{EIC} for cytochromes c' from *Chromatium vinosum* and *Rhodopseudomonas sphaeroides* are similar to ratios for the model systems, the ratios are much greater for *Rhodopseudomonas palustris* and *Rhodospirillum rubrum*, indicating that factors other than differences in trans ligand coordination contribute to the differences in EIC binding between the cytochromes c'.

The differences in EIC binding between the cytochromes c' are therefore more likely to be accounted for by steric restrictions associated with protein–ligand interactions at the sixth coordination site. The larger K^{CO}/K^{EIC} ratios observed for *Rh. palustris* and *Rs. rubrum* compared to *C. vinosum* and *Rh. sphaeroides* are consistent with smaller EIC binding constants relative to those for CO due to steric effects involving EIC, which has a structure analogous to CO but with the alkyl group providing additional steric bulk (Treidel, 1973). The ratios, K^{CO}/K^{EIC}, for cytochromes c' from *Rh. palustris* and *Rs. rubrum* are much greater than those observed for unrestricted binding in model systems, indicating that the binding of EIC to these proteins is more sterically hindered than CO. The 7×10^5 decrease in EIC binding relative to CO binding for *R. palustris* cytochrome 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 EIC due to the increase in size of the ligand associated with the two additional carbon atoms. The K^{CO}/K^{EIC} ratios may be compared to ratios of 11 and 4 $\times 10^3$ observed for the steric differentiation of *n*-butyl *tert*-butyl-isocyanides from CO in binding to a cyclophane heme model system (Traylor et al., 1980) designed to demonstrate distal-side steric effects in hemoprotein ligand binding. The reduction in ligand binding relative to unhindered model systems was accounted for in terms of a central steric effect caused by distal groups in close contact with the first or second atom of the bound ligand directly over the iron and a peripheral steric effect encountered by more distant groups in the ligand. Similar effects have been considered to account for steric differentiation of CO and O_2 in capped heme model complexes (Hashimoto et al., 1982). The present results indicate that both effects are operative in EIC binding to *Rh. palustris* and *Rs. rubrum* cytochromes c' and that the peripheral effect contributes to the steric differentiation between CO and EIC. These conclusions are supported by the spectroscopic properties of the EIC complexes in as much as the much smaller c'–EIC Soret half band widths for *Rh. palustris* and *Rs. rubrum* can be associated with more restricted heme environments, leading to reduce broadening in the absorption band (Rebane & Avarmaa, 1982) as observed for the spectra of porphyrins in restricted matrices at low temperature (Gurinovich et al., 1968). The fact that the ratios, K^{CO}/K^{EIC}, for the cytochromes c' from *C. vinosum* and *Rh. sphaeroides* are similar to those of the unrestricted models (Olson et al., 1983) indicates that the binding of EIC is not significantly more sterically hindered than CO. The K^{CO}/K^{EIC} ratios observed for *C. vinosum* and *Rh. sphaeroides* cytochromes c'

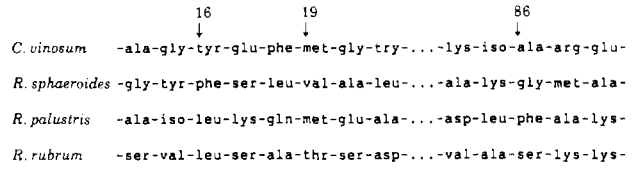

FIGURE 3: Partial amino acid sequences of some cytochromes c', indicating by number amino acid residues associated with the distal heme pocket of *Rs. molischianum* (Weber et al., 1981; Ambler et al., 1981).

suggest then that peripheral steric effects are not significantly encountered by EIC resulting in a small steric differentiation. The 10^4-fold smaller EIC binding constants observed for *C. vinosum* and *Rh. sphaeroides* relative to the unrestricted models may be associated with a central steric effect. Alternatively, these differences may suggest that electronic factors contribute to the lower binding constants for these cytochromes compared to those of other hemoproteins and model heme complexes. The weaker imidazole ligand field proposed to account for the magnetic properties of the ferric cytochromes c' could contribute to reduced binding of CO and EIC. However, the CO binding constants for relatively weak field axial ligands (Rougee & Brault, 1975) are larger than those observed for the *C. vinosum* and *Rh. sphaeroides* cytochromes c', suggesting that steric and, to a lesser degree, electronic factors account for the observed binding constants. Thus, a comparison of the EIC binding constants and the K^{CO}/K^{EIC} ratios for the four cytochromes c' with those observed for unrestricted ligation in model systems suggests that the differences between the EIC binding constants for the cytochromes as well as the differences between the proteins and the model systems are primarily due to steric factors, although the significantly lower CO binding constant for *Rs. rubrum* cytochrome c' suggests that this cytochrome would give a lower K^{EIC} in the absence of additional steric interactions.

It is appropriate to consider the structural basis for the proposed steric effects on EIC binding. X-ray diffraction studies of *Rs. molischianum* cytochrome c' indicate that residues Met-16, Leu-19, and Trp-86 pack about the sixth coordination site of the heme iron and that some relocation of the residues must accompany ligand binding (Weber et al., 1980, 1981). Figure 3 indicates segments of amino acid sequences in the four cytochromes c' that are thought to be homologous to the *Rs. molischianum* cytochrome c' (Ambler et al., 1981). It is significant to note that a comparison of the amino acids corresponding to residues 16, 19, and 86 in *Rs. molischianum* appears to provide little basis for the observed differences in EIC binding. Thus, it is observed that Met is found at residue 19 in *C. vinosum* and *Rh. palustris*, which exhibit widely different binding constants. By contrast, *C. vinosum* and *Rh. sphaeroides* cytochromes c', which exhibit the highest EIC affinity, have an aromatic residue at position 16 while *Rh. palustris* and *Rs. rubrum*, which have the lowest EIC affinity, have an aliphatic residue at this position. The relationship between the structural differences between the two types of amino acids and the suggested steric basis for differences in EIC binding is not, however, evident. While it is possible that the orientation of the benzene ring in the protein may lead to a reduced steric interaction with the bound EIC, the greatest dimension of the aromatic amino acid side chains is actually larger than that of Leu. Amino acids thought to be homologous to residue 86 in *Rs. molischianum* appear to be widely different among the four cytochromes c'. Although the steric bulk of the side chains of this residue is smaller in the cytochromes c' which give the greater EIC affinity, the differences in EIC binding constants cannot be readily correlated with differences in the size of the side chains. Thus, although it is possible that the detailed structural orientation of residues 16, 19, and 86 about the distal heme face in these cytochromes may provide a steric basis for the observed differences in EIC and CO binding constants, further crystallographic, sequence, and spectroscopic studies of these proteins may indicate that other amino acids in the four cytochromes c' occupy positions corresponding to residues 16, 19, and 86 in *Rs. molischianum*.

The large differences between the EIC binding constants and their correlation with the spectroscopic properties of the complexes provide further evidence that these and other alkyl isocyanides should provide important new probes of the heme environment in these proteins and thus contribute to our understanding of the functional and structural diversity between the cytochromes c' and other hemoproteins.

Registry No. EIC, 624-79-3; cytochrome c', 9035-41-0.

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ATP Synthesis Catalyzed by the Purified Erythrocyte Ca-ATPase in the Absence of Calcium Gradients†

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ABSTRACT: The Ca^{2+}-transporting ATPase of erythrocytes was isolated by calmodulin affinity chromatography. The backward reaction of the ATPase was investigated. The phosphorylation of the solubilized enzyme by P_i required Mg and was inhibited by Ca and vanadate in the micromolar concentration range. Significant amounts of phosphoenzyme could be obtained only in a medium containing high dimethyl sulfoxide concentrations (>25%) in order to diminish water activity at the phosphorylation site. The phosphoenzyme formed in this way could not phosphorylate ADP. However, upon addition of Ca^{2+} ions and dilution of dimethyl sulfoxide in the phosphorylated preparation (water activity jump), a highly reactive phosphoenzyme species was obtained which could transfer phosphate in nearly stoichiometric amounts to ADP to form ATP.

Most plasma membranes have been found to contain a Ca^{2+}-transporting ATPase activity which contributes to the maintenance of the low Ca^{2+} concentration in the cytosol. A very interesting property of this ATPase is its ability to interact in a Ca^{2+}-dependent way with the cytosolic regulator protein calmodulin. The interaction stimulates the ATPase activity (Jarret & Penniston, 1977; Gopinath & Vincenzi, 1977) and has made it possible to purify the enzyme from various plasma membrane sources by calmodulin affinity chromatography (Niggli et al., 1979; Caroni & Carafoli, 1981; Wuytack et al., 1980). Analysis of the reaction mechanism of the ATPase has

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