Redox Protein Electron-Transfer Mechanisms: Electrostatic Interactions as a Determinant of Reaction Site in *c*-Type Cytochromes†

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ABSTRACT: The effect of ionic strength on the rate constant for electron transfer has been used to determine the magnitude and charge sign of the net electrostatic potential which exists in close proximity to the sites of electron transfer on various *c*-type cytochromes. The negatively charged ferricyanide ion preferentially reacts at the positively charged exposed heme edge region on the front side of horse cytochrome *c* and *Paracoccus* cytochrome c_2. In contrast, at low ionic strength, the positively charged cobalt phenanthroline ion interacts with the negatively charged back side of cytochrome c_2, and at high ionic strength at a positively charged site on the front side of the cytochrome. With horse cytochrome *c*, over the ionic strength range studied, cobalt phenanthroline reacts only at a positively charged site which is probably not at the heme edge. These inorganic oxidants do not react at the relatively uncharged exposed heme edge sites on *Azotobacter* cytochrome c_5 and *Pseudomonas* cytochrome *c*-551, but rather at a negatively charged site which is away from the heme edge. The results demonstrate that at least two electron-transferring sites on a single cytochrome can be functional, depending on the redox reactant used and the ionic strength. Electrostatic interactions between charge distributions on the cytochrome surface and the other reactant, or interactions involving uncharged regions on the protein(s), are critical in determining the preferred sites of electron transfer and reaction rate constants. When unfavorable electrostatic effects occur at a site near the redox center, less optimal sites at a greater distance can become kinetically important.

The amino acid sequence and tertiary folding of the polypeptide chain of a protein impart a uniqueness to its structure that can be seen, at one level, in the distribution of charged and uncharged amino acid side chains on the surface of the protein, which determines the electrostatic potential which surrounds the molecule. In previous studies, we have presented data which show that these electrostatic surface patterns are important components in determining reaction rate constants in vitro for some redox proteins, and therefore may be important factors in establishing biological specificity [cf. Tollin et al. (1986a) and Cusanovich et al. (1987)]. The electron-transfer rate constants can also be related to other properties, such as steric effects and redox potential differences, that theory predicts (Marcus, 1968; Marcus & Sutin, 1985) and experiments confirm (Tollin et al., 1984, 1986b; Przysiecki et al., 1985; Meyer et al., 1983, 1986) should influence reaction rate constants for these systems. In a recent investigation of the interaction between cytochrome *c* and cytochrome *c* peroxidase (Hazzard et al., 1988), we have shown that strong electrostatic interactions can actually decrease electron-transfer rate constants, as a consequence of the formation of nonoptimal complexes. Although this is not generally true for all protein–protein electron-transfer systems [cf. Tollin et al. (1987)], we felt that a detailed comparison of the electron-transfer reactions of *c*-type cytochromes with different surface charge distributions was of interest. Thus, in the present work, we have compared four cytochromes: horse cytochrome *c*, which has a generally positive electrostatic surface potential; *Paracoccus* cytochrome c_2, which has a positive potential in the vicinity of the exposed heme edge (the preferred site of electron transfer) and a much larger negative potential on the back side of the protein; *Pseudomonas* cytochrome *c*-551 and *Azotobacter* cytochrome c_5, which both have virtually no electrostatic potential close to the exposed heme edge, but are generally negatively charged elsewhere. As electron acceptors, we have used the following two oppositely charged inorganic oxidants: cobalt(III) phenanthroline, $[Co(phen)_3]^{3+}$, and ferricyanide, $[Fe(CN)_6]^{3-}$. The data demonstrate clearly that *c*-type cytochromes can utilize multiple electron-transferring sites, some of which can be substantially removed from the exposed heme edge, and that the interactions determined by the charged and uncharged regions on the protein surface can control the rate constant for electron transfer by specifying which of the multiple electron-transferring sites will be utilized by a potential reactant. These results are analogous to those obtained with plastocyanin, in which two sites, one acidic and one hydrophobic, have been implicated in electron-transfer reactions with inorganic complexes [cf. Sykes (1985) for a review], and for which electron transfer via the acidic patch is slower as a result of a greater separation between the redox centers. Although previous NMR studies of the interactions of inorganic complexes with cytochrome *c* have clearly indicated the existence of multiple binding sites, electron transfer has heretofore been assumed to occur only via the exposed heme edge [cf. Moore et al. (1984a)]. In this context, however, it should be noted that *covalent* attachment of an electron donor to sites which are more than 10 Å away from the heme has been shown to result in moderately fast electron transfer (30–50 s^{-1}), presumably occurring intramolecularly through the protein matrix (Winkler et al., 1982; Isied et al., 1984).

Materials and Methods

Horse heart cytochrome *c* (type VI) was purchased from Sigma Chemical Co. and used without further purification. Potassium ferricyanide was purchased from Mallinckrodt, and cobalt(III) phenanthroline was synthesized by using the pro-

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Table I: Electrostatic Analysis of Cytochrome Redox Kinetics

cytochrome	reactant	$E_{m,7}$ (mV)	V_{ii} (kcal/mol)	k_∞ (M^{-1} s^{-1})	Z_1
Pseudomonas c-551	cobalt phenanthroline[a]	270	−2.23	3.2×10^4	−1.7
	ferricyanide[b]		+3.73	3.1×10^5	−2.4
Azotobacter c_5	cobalt phenanthroline	315	−1.32	4.9×10^4	−1.0
	ferricyanide		+1.97	2.0×10^5	−1.5
Paracoccus c_2	cobalt phenanthroline (low μ/high μ)	250	−9.04/+3.63	$5.9 \times 10^1/3.5 \times 10^4$	−6.8/+3.6
	ferricyanide		−2.85	2.0×10^6	+2.1
horse c	cobalt phenanthroline	260	+2.53	7.0×10^3	+1.9
	ferricyanide		−5.35	1.2×10^6	+4.0

[a] $Z_2 = +3$; $E_{m,7} = 370$ mV. [b] $Z_2 = -3$; $E_{m,7} = 430$ mV; $E_{m,7}$ for ferricyanide varies by approximately 50 mV over the ionic strength range used in the present studies (O'Reilly, 1973).

cedure of Schilt and Taylor (1959). *Azotobacter vinelandii* cytochrome c_5 was purified as described in Swank and Burris (1969) and Campbell et al. (1973). *Paracoccus denitrificans* cytochrome c_2 was isolated according to Ambler et al. (1981a,b). *Pseudomonas aeruginosa* cytochrome c-551 was obtained as outlined by Ambler and Wynn (1973). Published extinction coefficients were used to calculate concentrations as follows: ferricyanide, $\epsilon_{420} = 1 \times 10^3$ M^{-1} cm^{-1}; cobalt phenanthroline, $\epsilon_{350} = 3.7 \times 10^2$ M^{-1} cm^{-1}; cytochromes c, c_2, c-551, and c_5 (ferrous form), $\epsilon_{550-555} = 3 \times 10^4$ M^{-1} cm^{-1}. All of the experiments were carried out in 5.0 mM potassium phosphate buffer containing 0.85 mM EDTA, pH 7.2 at 23.5 °C, at various ionic strengths (12–500 mM) obtained by the addition of appropriate amounts of NaCl. The reactions were studied by using stopped-flow spectrophotometry under pseudo-first-order conditions, and were monitored from 550 to 555 nm, depending upon which cytochrome was used as the electron donor. Some of the experiments were performed by using previously described instrumentation (Jung & Tollin, 1981), whereas others utilized a stopped-flow spectrometer obtained from Kinetic Instruments, Inc. The concentrations of the oxidants varied from 5 to 25 times greater than the concentration of the reductant. Pseudo-first-order rate constants (k_{obsd}) were obtained from linear semilog plots for at least three different oxidant concentrations at each ionic strength. An Olis Co. (Jefferson, GA) computer-fitting routine (SI-FIT) was also used to obtain k_{obsd} values. All of the reactions studied gave linear plots of k_{obsd} vs oxidant concentration. The apparent second-order rate constants were calculated from the slopes of the linear plots. The estimated error in these values is ±10%. Details of the procedures were as described previously [cf. Cheddar et al. (1986) and references cited therein]. The rate constant values obtained in the present study for horse cytochrome c and *Pseudomonas* cytochrome c-551 reacting with ferricyanide are consistent with those reported in the earlier study of Peterman and Morton (1977).

The X-ray crystal structures of the proteins were displayed and manipulated on an Evans and Sutherland PS390 graphic system. By evaluation of the kinetic results in terms of calculated electrostatic maps [using the methodology of Matthew (1985)], it was possible to identify probable reaction sites on the proteins and to evaluate the signs and magnitudes of the relative electrostatic potentials at these sites. In the calculation of electrostatic potential surfaces of the various cytochromes, a positive charge was placed on the heme iron. Furthermore, negative charges were placed on the heme propionates, although Moore et al. (1980) and Leitch et al. (1984) showed that, at least in *Pseudomonas* cytochrome c-551, the ionization constant for the propionate on pyrrole ring A (rear propionate) was close to neutrality (i.e., $pK_o = 6.3$ and $pK_r = 7.2$). It is known that the rear propionate in horse cytochrome c is ionized at pH 7 (Moore et al., 1984b) but the ionization state of the other propionates in the various cytochromes is unknown. Rather than show calculated maps for all possibilities, we have assumed the maximum charge in all cases. Smaller electrostatic potentials can be easily approximated by visually removing charge density in the immediate regions of the iron and propionates in the maps shown below.

The electrostatic map for *Paracoccus* cytochrome c_2 was calculated by using the existing low-resolution X-ray structure (Timkovich & Dickerson, 1973) and appropriate corrections to the amino acid sequence (Ambler et al., 1981a,b). Side chains were altered to correspond to the correct sequence, and the resulting structure was energy-minimized using Herman's routine in the FRODO software package.

The electrostatic model used to fit the rate constant vs ionic strength data (Watkins, 1986) in order to obtain an electrostatically corrected rate constant (k_∞) is described in Tollin et al. (1984). Although other theoretical treatments can be used, we have found that the relative values of k_∞ are model independent. This is a consequence of the fact that, at high salt concentrations (>500 mM), reactions generally become independent of ionic strength due to essentially complete masking of reactant charges by counterions. The analysis also provides an estimate of the electrostatic charge–charge interaction energy (V_{ii}) which permits, by assigning values for the charge on one of the reactants (Z_2) and for the intermolecular dielectric constant, an estimate of the active-site charge (Z_1) to be calculated. A dielectric constant of 50 and an active-site radius of 4.5 Å were used. These values have been previously shown (Meyer et al., 1984) to provide accurate estimates of the active-site charges for c-type cytochromes of known structure. To what extent the ionic strength effects on kinetics are influenced by uncharacterized structural changes [cf. Trewhella et al. (1988)] is difficult to ascertain. However, neither in the present work nor in our previous studies have we observed discontinuities in the ionic strength dependencies which might signal structural transitions.

Results and Discussion

In Figure 1 is shown the ionic strength dependency of the second-order rate constants for the transfer of an electron from ferrous horse cytochrome c to ferricyanide and to cobalt(III) phenanthroline. Table I presents the results of an electrostatic analysis of these data [cf. Tollin et al. (1984) for details of the methodology]. The sign of electrostatic interaction energies (V_{ii}) and the slopes of the ionic strength profiles indicate that the negatively charged ferricyanide ion is accepting an electron from horse cytochrome c in the vicinity of a positively charged region on the protein surface and that the positively charged cobalt phenanthroline ion is also reacting at a site on horse cytochrome c which is positively charged, although the magnitude of the charge in the latter case is significantly smaller (approximately half; cf. Table I). That both ionic strength dependencies show the same sign for the reactive site on horse cytochrome c is not unexpected, inasmuch as the electrostatic

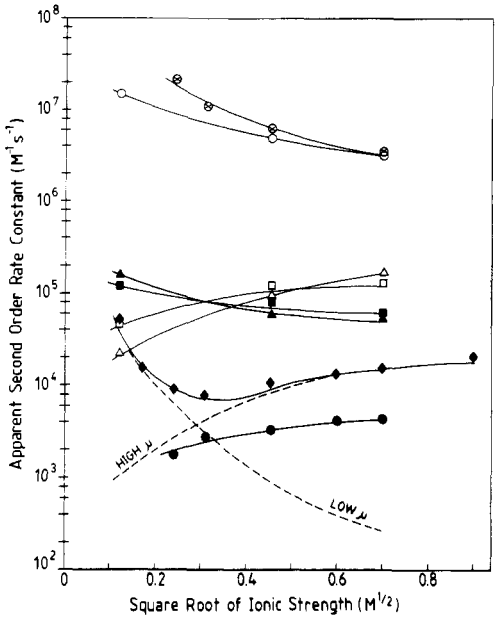

FIGURE 1: Semilog plots of ionic strength dependence of second-order rate constants for oxidation of reduced *c*-type cytochromes by ferricyanide and cobalt(III) phenanthroline. (⊗) Horse cytochrome *c* + ferricyanide; (●) horse cytochrome *c* + cobalt phenanthroline; (□) *Azotobacter* cytochrome c_5 + ferricyanide; (■) *Azotobacter* cytochrome c_5 + cobalt phenanthroline; (△) *Pseudomonas* cytochrome *c*-551 + ferricyanide; (▲) *Pseudomonas* cytochrome *c*-551 + cobalt phenanthroline; (○) *Paracoccus* cytochrome c_2 + ferricyanide; (♦) *Paracoccus* cytochrome c_2 + cobalt phenanthroline. Solid curves are theoretical fits (cf. Table I). In the case of the cytochrome c_2 + cobalt phenanthroline reaction, the dashed curves are the theoretical fits for the low and high ionic strength sites, and the solid curve is the sum of the two individual fits.

map of the closely related tuna cytochrome *c* shows that approximately three-fourths of the entire molecule (including the exposed heme edge) is enveloped in positive electrostatic potential, and only small scattered regions of negative charge exist [cf. Weber and Tollin (1985)]. The rate constant for ferricyanide oxidation, extrapolated to infinite ionic strength (k_∞) where electrostatic effects are no longer important, is approximately 200 times larger than the rate constant for cobalt phenanthroline,[1] even though the redox potentials for these two oxidants are similar (within 30–80 mV; cf. Table I). However, in striking contrast to the horse cytochrome *c* case, the k_∞ values for electron transfer from either cytochrome *c*-551 or cytochrome c_5 to ferricyanide are only 4–9 times larger than those for electron transfer from these cytochromes to cobalt phenanthroline. This large 20–50-fold difference between horse cytochrome *c* and cytochromes c_5 or *c*-551 suggests that factors in addition to redox potential and geometric constraints are involved in these reactions. We will return to this below.

In Figure 1 is shown the ionic strength profile of the rate constant for electron transfer from *Paracoccus* cytochrome c_2 to ferricyanide and to cobalt phenanthroline. It is immediately obvious that ferricyanide is reacting at a site on cytochrome c_2 which is dominated by a *positive* electrostatic potential and that at *low* ionic strengths cobalt phenanthroline is reacting at a site on cytochrome c_2 which has a net *negative* electrostatic potential; i.e., they are clearly reacting at different sites on the protein (we will consider the high ionic strength behavior below). The electrostatic map (Figure 2) of cytochrome c_2 [cf. also Weber and Tollin (1985)] shows a region of positive electrostatic potential close to the exposed heme edge, and a much larger region of negative electrostatic potential which is on the back side of the molecule and thus removed from the heme edge region. It must be the latter which is influencing the electron-transfer kinetics with cobalt phenanthroline at low ionic strength. Note also from Table I that the k_∞ value for electron transfer from cytochrome c_2 to ferricyanide is 60–30 000 times larger than the rate constant for electron transfer to cobalt phenanthroline (depending upon which extrapolated value is used). This result is analogous to that which was obtained for horse cytochrome *c*, but is much larger for the low ionic strength extrapolation than expected from the apparent intrinsic reactivity differences. This is a further indication that at low ionic strength cobalt phenanthroline is interacting at a site which is at a considerable distance, perhaps 10–15 Å on the basis of the crystal structure, from the heme edge. This is not an excessively long distance to allow effective electron transfer. For example, with ruthenium-labeled cytochrome *c* and azurin, rate constants of 25 and 1.9 s^{-1} have been measured for intramolecular electron transfer over approximately 12 Å (Kostic et al., 1983).

The redox potentials for cytochrome *c*-551, horse cytochrome *c*, and cytochrome c_2 are similar, whereas that for cytochrome c_5 is somewhat more positive (Table I). Furthermore, the steric factors involved in the reactivities of these cytochromes toward an uncharged small-molecule reductant (i.e., lumiflavin semiquinone) have been shown to be quite similar [cf. Meyer et al. (1983) for results with cytochromes *c*, c_2, and *c*-551; in unpublished work, we have obtained analogous results for cytochrome c_5]. In spite of these similarities, in terms of k_∞ values, ferricyanide reacts 4–10 times faster with horse cytochrome *c* and cytochrome c_2 than with cytochromes *c*-551 and c_5, and cobalt phenanthroline reacts about 5–7 times more slowly with horse cytochrome *c* and 500–800 times more slowly with cytochrome c_2 (low ionic strength) than with cytochromes c_5 and *c*-551. The simplest interpretation of these large differences in rate constants is that ferricyanide is reacting at the exposed heme edge with both horse cytochrome *c* and cytochrome c_2, because it is attracted there by the positive electrostatic potential. In contrast, cytochromes c_5 and *c*-551 both have a relatively uncharged, nonpolar, environment in the vicinity of the exposed heme edge (cf. Figure 2). Hence, the probability of close approach of ferricyanide (which has a high charge to size ratio and which is capable of forming hydrogen bonds) to this region is decreased, and the rate constants are significantly smaller than in the cytochrome *c* and c_2 cases. In contrast, cobalt phenanthroline appears to be more reactive with cytochromes *c*-551 and c_5 than with cytochromes *c* and c_2 (high ionic strength limit). This may be a consequence of the nonpolar character of the cobalt ligands, which could allow a closer approach to the exposed heme edge region in the former cases than in the latter. However, the cytochrome c_2 reaction with cobalt phenanthroline is clearly more complex. As noted above, at low ionic strength, this reagent apparently interacts at a negatively charged site, presumably somewhere in the vicinity of the back side of the cytochrome. As the ionic strength is increased, cobalt phenanthroline is influenced by a positive electrostatic potential during electron transfer, consistent with an interaction closer to the exposed heme edge. The k_∞ values for the low and high ionic strength extrapolations and the electrostatic map (Figure 2) support this point of view. Thus, the low ionic strength extrapolation yields a value which is considerably below that of horse cytochrome

[1] We have also found previously (Cheddar et al., 1986) that ferricyanide is approximately 300 times more reactive toward *C. pasteurianum* flavodoxin semiquinone than is cobalt phenanthroline.

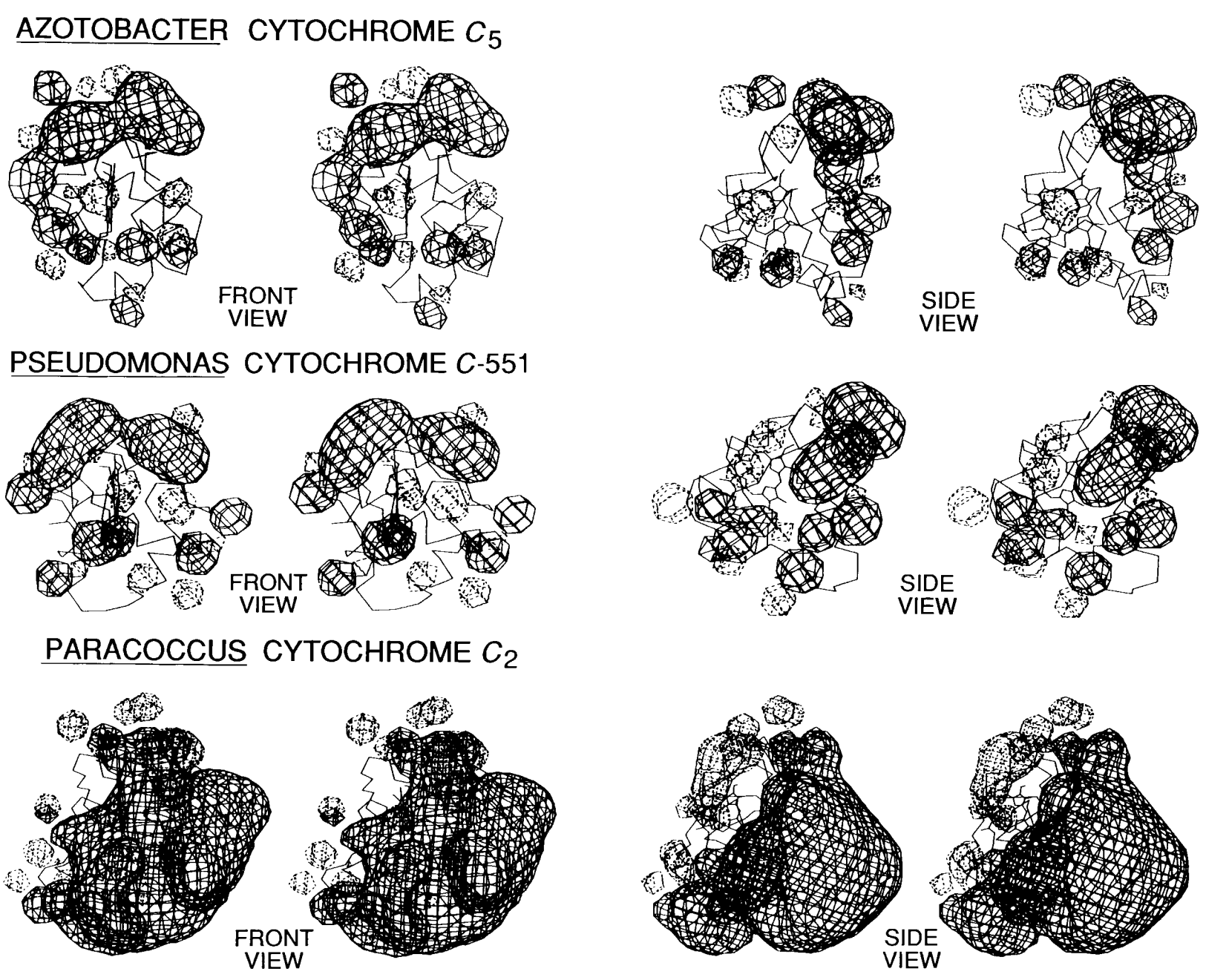

FIGURE 2: Stereoviews of electrostatic potential maps of *Azotobacter* cytochrome c_5, *Pseudomonas* cytochrome *c*-551, and *Paracoccus* cytochrome c_2. Calculations were made at pH 7, μ = 20 mM, and potentials are displayed at ±2 kT, except where otherwise noted. Solid lines are negative potential, and dashed lines are positive potential. Carbon atom backbones and the heme prosthetic groups are shown.

c (100-fold), whereas the high ionic strength limit is comparable to the values for cytochromes c_5 and *c*-551.

Ferricyanide is influenced by a negative electrostatic potential on both cytochromes c_5 and *c*-551, as shown by the shape of the ionic strength profiles (cf. Figure 1). Cobalt phenanthroline also is influenced by a negative potential on both cytochromes c_5 and *c*-551 (cf. Figure 1). The three-dimensional structures and the electrostatic maps (Figure 2) of cytochromes c_5 [cf. Carter et al. (1985)] and *c*-551 [cf. also Weber and Tollin (1985)] show that the exposed heme edge region is surrounded principally by uncharged and nonpolar amino acid side chains and that there is a negative potential at the periphery which extends to the back side of the proteins. It must be this negative potential which is affecting the reaction kinetics for these systems. It is striking that ferricyanide would apparently rather react at an electrostatically repulsive negative site on cytochromes c_5 and *c*-551 than at the hydrophobic site.

In summary, we have presented evidence which supports the contention that *c*-type cytochromes are able to use more than one site for electron transfer and that electrostatic interactions can provide a mechanism for controlling which site is operative under a given set of conditions.

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Registry No. $[Co(phen)_3]^{3+}$, 18581-79-8; $[Fe(CN)_6]^{3-}$, 13408-62-3; cytochrome *c*-551, 9048-77-5; cytochrome c_5, 51811-53-1; cytochrome c_2, 9035-43-2; cytochrome *c*, 9007-43-6.

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