Roles of Dipolar Effects and Local Charge in the Ionic Strength Dependence of Redox Reactions between *c*-Type Cytochromes†

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ABSTRACT: Redox reactions between different *c*-type cytochromes were monitored by stopped-flow spectroscopy. Second-order rate constants were determined at different ionic strengths for the reactions of *Paracoccus denitrificans* cytochrome *c*-551i with its physiologic redox partner cytochrome *c*-550, and with the nonphysiologic partner horse heart cytochrome *c*. The latter two cytochromes are structurally quite similar and exhibit identical redox potentials but bear net charges of −7 and +7, respectively. Despite these opposite overall charges, the ionic strength dependencies for the reaction of each with the acidic cytochrome *c*-551i were very similar. The observed decrease in reaction rate with increasing ionic strength that was observed with cytochromes *c*-550 and *c*-551i, the latter of which bears a net charge of −20, cannot be explained simply on the basis of monopole−monopole interactions. These data were analyzed by two different methods: one which treats proteins as both monopoles and dipoles and considers the net charge; and another which neglects dipolar effects and considers only the local charge of the reactive site of the protein rather than net charge. The applicability of each method to the analysis of these data and to protein electrostatic interactions in general is discussed.

The analysis of the ionic strength dependence of the reaction between two proteins may potentially provide information about the protein orientations with respect to each other which are necessary for the reaction to proceed with optimum efficiency. When the structures of the reacting proteins are known, it is possible to analyze the observed ionic strength dependence with respect to monopole−monopole, monopole−dipole, and dipole−dipole interactions (Koppenol, 1980; Van Leeuwen, 1983; Eltis et al., 1991). The Van Leeuwen approach, which treats the protein as both a monopole and a dipole, has been used to characterize redox reactions between cytochrome *c* and plastocyanin (Zhou & Kostic, 1992, 1993; Brothers et al., 1993), and methanol dehydrogenase and cytochrome *c*-551i (Harris & Davidson, 1994), as well as self-exchange reactions between *c*- and *b*-type cytochromes (Dixon et al., 1989). Cytochrome *c* and plastocyanin possess opposite charges and exhibit a decreased reaction rate with increased ionic strength. Methanol dehydrogenase and cytochrome *c*-551i possess like charges and exhibit an increased rate of reaction with increased ionic strength. The self-exchange reactions also exhibit an increased reaction rate with increasing ionic strength. It has been noted, however, that not all proteins exhibit ionic strength behavior which is predicted by their overall net charge. The best example of this is given by *Paracoccus denitrificans* cytochrome *c*-550 (also referred to as c_2). Although it has a net charge of −7, it behaves like a cation with respect to the ionic strength dependence of its reactions with ferricyanide (Chedder et al., 1989), flavin (Meyer et al., 1984), and flavodoxin (Tollin et al., (1984). Based in part on these observations, it was suggested that the reaction of cytochrome *c*-550, and possibly other proteins, may be influenced more by local charge effects than by overall net charge (Meyer et al., 1984; Tollin et al., 1984; Cheddar et al., 1989). In this paper, we focus on the physiologic reaction between *P. denitrificans* cytochrome *c*-550 and its electron acceptor, cytochrome *c*-551i, both of which are acidic proteins and which exhibit a decreased rate of reaction with increasing ionic strength.

P. denitrificans has become a powerful and convenient system for studying the mechanisms of long-range intermolecular electron transfer reactions between soluble proteins. When this bacterium is grown with methanol or methylamine as a sole source of carbon and energy, each of these substrates is oxidized to formaldehyde by inducible periplasmic quinoproteins, methanol dehydrogenase and methylamine dehydrogenase (Davidson, 1993). The physiologic electron acceptor for methylamine dehydrogenase is an inducible periplasmic type I blue copper protein, amicyanin (Husain & Davidson, 1985). Also present in the periplasm of methylamine- and methanol-grown cells are the constitutive cytochrome *c*-550 and an inducible cytochrome *c*-551i (Husain & Davidson, 1986). These redox enzymes and proteins comprise soluble electron transfer chains which couple the oxidations of methanol and methylamine to respiration, with cytochrome *c*-550 ultimately donating to the membrane-bound respiratory chain those electrons which are generated by the dehydrogenase-catalyzed reactions (Kumar & Davidson, 1989). Two features of these soluble physiologic electron transfer chains are particularly noteworthy. (i) They include electron transfer reactions between soluble *c*-type cytochromes. (ii) Each of the redox proteins which comprise these electron transfer chains is relatively acidic. The latter observation raises questions as to the role of electrostatic interactions in stabilizing the protein−protein interactions which are necessary for the intermolecular electron transfer reactions.

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Cytochrome *c*-551i is the physiologic electron acceptor for methanol dehydrogenase (Long & Anthony, 1991) and also serves as an electron acceptor for methylamine dehydrogenase via amicyanin (Husain & Davidson, 1986). The complete amino acid sequence of the cytochrome is known (Van Spanning et al., 1991), and its three-dimensional structure has been determined from X-ray crystallographic analysis of a ternary complex of methylamine dehydrogenase, amicyanin, and cytochrome *c*-551i (Chen et al., 1994). Cytochrome *c*-551i is an unusual *c*-type cytochrome. It is relatively large (molecular weight = 22 000) and acidic (p*I* = 3.5). Its redox potential is +190 mV (Gray et al., 1986). Like other cytochromes *c*, its heme edge is exposed at the surface; however, the exposed heme is surrounded by primarily hydrophobic amino acid residues, and most of the negatively charged amino acid residues are located on the sides and back of the molecule away from the heme group (Chen et al., 1994). Cytochrome *c*-550 is the well-characterized cytochrome c_2 of *P. denitrificans*. Its crystal structure (Timkovich & Dickerson, 1976; Ambler et al., 1981) and amino acid sequence (Van Spanning et al., 1991) are also known. Like mammalian cytochrome *c*, it has an exposed heme edge which is surrounded by primarily positively charged amino acids, this despite its overall negative charge (p*I* = 4.5; Husain & Davidson, 1986).

The ionic strength dependence of the reaction between *P. denitrificans* cytochrome *c*-550 and cytochrome *c*-551i is characterized in this paper. Given the interesting similarities and differences with respect to the structure and charge distribution of cytochrome *c*-550 compared to the mammalian cytochrome *c*, the nonphysiologic reaction of cytochrome *c*-551i with horse heart cytochrome *c* was also examined. Comparison of the ionic strength dependencies of the reactions with cytochrome *c*-551i of the horse heart cytochrome *c* and *P. denitrificans* cytochrome *c*-550 is quite appropriate since the two possess the same redox potential of +250 mV and very similar three-dimensional structures, especially with regard to the position of the heme and the positive surface potential in the vicinity of the exposed heme edge. Therefore, any effects on reactivity with cytochrome *c*-551i that may be attributable to differences in overall charge and dipolar properties should be evident in a comparison of the ionic strength dependencies of the reactions of the two cytochromes.

EXPERIMENTAL PROCEDURES

Cytochromes *c*-550 and *c*-551i were isolated as previously described (Husain & Davidson, 1986) from *P. denitrificans* (ATCC 13543) which was grown aerobically on methylamine as a sole carbon source. Each cytochrome was further purified by chromatography over an AcA 54 size exclusion resin. Protein concentrations were calculated from previously published extinction coefficients (Husain & Davidson, 1986). Horse heart cytochrome *c* was purchased from Sigma and used without further purification. The fully oxidized and reduced forms of each cytochrome were generated by incubation with ferricyanide or ascorbate. Absorption spectra were recorded with a Milton Roy Spectronic 3000 Array Spectrophotometer.

Stopped-flow experiments were performed using an On-Line Instrument Systems (OLIS, Bogart, GA) stopped-flow sample handling unit coupled to Durrum optics. A 486 class computer controlled by OLIS software was used to collect data. All experiments were performed at 30 °C in 50 mM potassium phosphate, pH 7.5. Ionic strength was adjusted by the addition of NaCl, and in calculating the ionic strength, correction was made for the association between monohydrogen phosphate and sodium and potassium (Smith & Alberty, 1956). Reduced cytochrome *c*-551i was mixed with either oxidized cytochrome *c*-550 or horse heart cytochrome *c*. The former reaction was monitored at 419 nm where it exhibits a change in extinction coefficient of 7100 M^{-1} cm^{-1}. The latter reaction was also monitored at 419 nm where it exhibits a change in extinction coefficient of 9540 M^{-1} cm^{-1}. Typically, 3−4 data sets, each containing 500 data points, were averaged, and the data were fit using OLIS software to the equation for a single-exponential decay (eq 1), where

$$A_{419} = C[\exp(-kt)] + b \tag{1}$$

k is k_{obs}, C is a constant related to the initial absorbance, and b represents an offset value to account for a nonzero base line.

Dipoles were calculated from the crystal structures of horse heart cytochrome *c* (Bushnell et al., 1990), cytochrome *c*-550 (Benning et al., 1994), and cytochrome *c*-551i (Chen et al., 1994) using the programs QUANTA and CHARMM (Molecular Simulations, Burlington, MA). These programs calculate the dipole (P) using eq 2 where q_i is the partial

$$P = \sum q_i r_i \tag{2}$$

charge of the ith atom and r_i is the position of that atom relative to the center of mass of the molecule. The sum is over all atoms. The partial charges are assigned by the program according to Momany and Rone (1992).

The ionic strength dependencies of the observed reactions were analyzed by two different methods. Data were fit to eq 3 (Van Leeuwen, 1983) which includes contributions to

$$\ln k_\mu = \ln k_{inf} - [Z_1Z_2 + (\mathbf{ZP})(1 + \kappa R) + (\mathbf{PP})(1 + \kappa R)^2]\frac{e^2}{4\pi\epsilon_o\epsilon k_B TR} f(\kappa) \tag{3a}$$

$$f(\kappa) = \frac{1 - \exp(-2\kappa R_2)}{2\kappa R_2(1 + \kappa R_1)} \tag{3b}$$

the observed rate from monopole−monopole, monopole−dipole, and dipole−dipole interactions. In this equation, k_μ and k_{inf} are, respectively, the bimolecular rate constants at a given ionic strength and at infinite ionic strength; e is the elementary charge; Z_1 and Z_2 are net charges; R_1 and R_2 are the radii of each protein with $R = R_1 + R_2$; ϵ_o is the permittivity constant; ϵ is the dielectric constant of water; κ is the reciprocal thickness of the ionic atmosphere equal to $0.33I^{1/2}$; k_B is the Boltzmann constant; and T is temperature. The monopole−dipole interaction **ZP** and dipole−dipole interaction **PP** are defined in eqs 4 and 5

$$(\mathbf{ZP}) = \frac{Z_1P_2\cos\theta_2 + Z_2P_1\cos\theta_1}{eR} \tag{4}$$

$$(\mathbf{PP}) = \frac{P_1P_2\cos\theta_1\cos\theta_2}{(eR)^2} \tag{5}$$

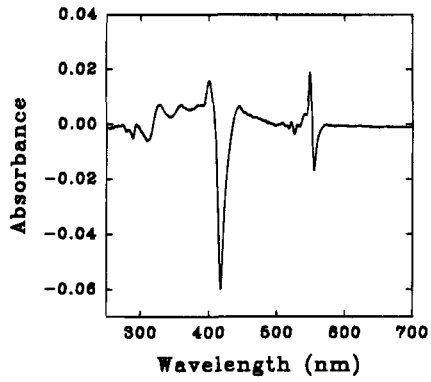

FIGURE 1: Difference spectrum for the reaction between cytochrome *c*-551i and cytochrome *c*-550. The spectrum was obtained after mixing reduced cytochrome *c*-551i (8.4 μM) with oxidize cytochrome *c*-550 (10.2 μM) in 50 mM potassium phosphate, pH 7.5.

where P_1 and P_2 are the dipole moments of the two reactants and θ_1 and θ_2 each define the angle between the dipole moment vector and vector from the center of mass to the reaction site on the surface for each protein. Data were also fit to eq 6 which neglects the contribution of dipolar effects

$$\ln k_\mu = \ln k_{inf} - V_{ii}X(I) \quad (6a)$$

$$V_{ii} = \alpha\varrho^{-2}D_e^{-1}Z_1Z_2r_{12} \quad (6b)$$

$$X(I) = (1 + \kappa\varrho)^{-1}\exp(-\kappa\varrho) \quad (6c)$$

and predicts the ionic strength dependence for a reaction which is influenced primarily by the charges of the interaction domains (Tollin et al., 1984; Meyer et al., 1984). V_{ii} is the electrostatic energy between reaction domains, Z is the charge on each domain, r_{12} is the distance between reactants, D is the effective dielectric constant at the interface, α is equal to 125 kcal mol^{-1} at 30 °C, and ϱ is the radius of the interaction domain which is set at 7 Å (Tollin et al., 1984).

Nonlinear curve fitting of data was performed with the Sigma Plot 5.0 (Jandel Scientific, San Raphael, CA) computer program.

RESULTS AND DISCUSSION

Although the cytochromes *c* used in these experiments have very similar absorption spectra, these spectra are not identical. Thus, on mixing reduced cytochrome *c*-551i with oxidized cytochrome *c*-550, the changes in the absorption spectra of the two cytochromes are not exactly complementary (Figure 1). A significant difference in absorption is observed at 419 nm, and, therefore, the redox reaction between the two cytochromes can be monitored spectrophotometrically at this wavelength. A very similar difference spectrum is obtained on mixing reduced cytochrome *c*-551i with horse heart cytochrome *c* (not shown), and the redox reaction between these two cytochromes can also be monitored at 419 nm.

The absorbance changes with time which were observed on mixing reduced cytochrome *c*-551i with oxidized cytochrome *c*-550 could be fit to a single exponential (Figure 2) under all conditions which were studied in these experiments. The absorbance changes with time which were observed on mixing reduced cytochrome *c*-551i with horse heart cytochrome *c* could also be fit to a single exponential (data not shown). For the reaction between cytochromes *c*-550 and

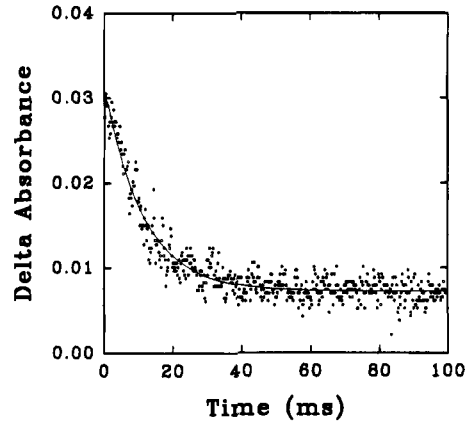

FIGURE 2: Reaction of reduced cytochrome *c*-551i and oxidized cytochrome *c*-550. Reduced cytochrome *c*-551i (1.7 μM) was mixed with oxidized cytochrome *c*-550 (3.5 μM), and absorbance was monitored at 419 nm. The solid line represents a fit of these data to eq 1 which yielded a k_{obs} of 87 s^{-1}.

c-551i, data were obtained at ionic strengths ranging from 0.16 to 1.1 M. At ionic strengths lower than 0.16 M, the reaction became too fast to reliably monitor by stopped-flow. At each ionic strength, the concentration of cytochrome *c*-550 was varied with a fixed concentration of cytochrome *c*-551i. Plots of k_{obs} against cytochrome *c*-550 concentration were linear in the concentration range studied, and if extrapolated, the fitted line passed through the origin. It was not possible to perform the reaction at higher concentrations because the background absorbance of the cytochrome mixture was too high to enable an accurate measurement of the relatively small change in absorbance. In parallel experiments at ionic strengths ranging from 0.11 to 0.38 M, the concentration of horse heart cytochrome *c* was varied with a fixed concentration of cytochrome *c*-551i. Plots of k_{obs} against cytochrome *c* concentration were also linear in the concentration range studied, and if extrapolated, the fitted line passed through the origin.

Although we were technically limited to the range of ionic strengths that could be studied, these data were collected in a physiologic range in which useful information could be obtained. For the Van Leeuwen approach (eq 3), at ionic strengths less than 0.1 M, monopole−monopole interactions dominate, and the effects of dipolar interactions will only be seen at ionic strengths greater than 0.1 M (Van Leeuwen, 1983). When applying this theory, investigators have routinely worked in the range of ionic strengths from 0.1 to 1.0 M (Dixon et al., 1989; Zhou & Kostic, 1992, 1993; Brothers et al., 1993; Harris & Davidson, 1994). The alternative formalism which neglects dipolar effects (eq 6) is theoretically valid at all ionic strengths, although it has been noted (Meyer et al., 1993) that for reactions between some proteins deviation of the predicted k_{obs} occurred at low ionic strength.

The simplest kinetic mechanism for the reaction of cytochrome *c*-551i with cytochrome *c*-550 is given in eq 7.

$$c\text{-}551i_{red} + c\text{-}550_{ox} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} c\text{-}551i_{red}{*}c\text{-}550_{ox} \underset{k_4}{\overset{k_3}{\rightleftharpoons}} c\text{-}551i_{ox}{*}c\text{-}550_{red} \quad (7)$$

Although the reactions are shown as reversible, the ΔE_m for the redox reaction is 60 mV which predicts that the equilibrium constant, k_3/k_4, will lie far to the right (K_{eq} =

Table 1: Ionic Strength Dependence of the Reactions of Cytochrome *c*-551i with Cytochrome *c*-550 and Horse Heart Cytochrome *c*

cytochrome	ionic strength (M)	k_1 (μM^{-1} s^{-1})[a]
P. denitrificans c-550	0.16	44
	0.21	34
	0.29	16
	0.37	11
	0.55	8.0
	0.75	5.0
	1.13	3.2
horse heart	0.11	36
	0.13	14
	0.16	12
	0.21	8.2
	0.29	3.9
	0.38	3.1

[a] Rate constants were obtained from plots of k_{obs} versus cytochrome *c*-551i concentration as discussed in the text.

10.4). Since the reaction step which is being monitored is k_3, the inability to observe saturation behavior for the reaction between the cytochromes indicates that under these conditions $k_3 \gg k_1[c\text{-}550] + k_2$. Thus, for this simple mechanism, $k_{obs} = k_1[c\text{-}550] + k_2k_4/(k_3 + k_4)$, and a value for k_1 may be obtained from the slope of the line that describes the relationship between k_{obs} and the concentration of the varied reactant (Hiromi, 1979). That these plots intersected the *y*-axis near the origin is consistent with k_4 being relatively insignificant. The same interpretation evidently applies for the reactions between cytochrome *c*-551i and horse heart cytochrome *c*. The values of k_1 obtained for each reaction at each ionic strength are given in Table 1. Despite the fact that cytochrome *c*-550 and horse heart cytochrome *c* have very different overall charges of −7 and +7, respectively, they exhibit similar ionic strength dependencies for their respective reactions with cytochrome *c*-551i. Reduced cytochrome *c*-551i possesses an overall net charge at a neutral pH of −20. Based solely on consideration of overall charge, the direction of the ionic strength dependence of the reaction with horse heart cytochrome *c* is as expected, but the observed ionic strength dependence of the reaction with cytochrome *c*-550 is not. The data obtained for the ionic strength dependencies of the second-order rate constants for the reactions of the cytochromes were analyzed by two different methods (eqs 3 and 6) in an effort to determine the relative importance of dipolar interactions, overall charge, and local charge effects at the reactive site.

The data shown in Table 1 were fit to eq 3 which includes contributions to the observed rate from monopole−monopole, monopole−dipole, and dipole−dipole interactions. The dipole moments used in these calculations were 330 D for oxidized horse heart cytochrome *c*, 717 D for oxidized cytochrome *c*-550, and 347 D for reduced cytochrome *c*-551i. It has been generally assumed that electron transfer between an external redox partner and horse heart cytochrome *c* occurs via the exposed methyl group of pyrrole ring C of the heme (Rush & Koppenol, 1987; Meyer et al., 1984). This position corresponds to an angle θ of approximately 30° which was used in this analysis. The analogous position on cytochrome *c*-550 corresponds to a θ of approximately 15°. The values of ln k_{inf} and θ for cytochrome *c*-551i were not defined and were obtained from the fits of each data set to eq 3. These fits are shown in Figure 3. With θ for horse heart cytochrome *c* set at 30°, fitted values were obtained

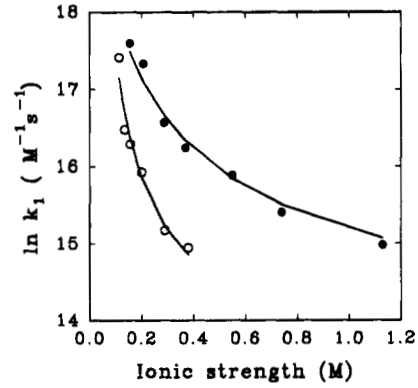

FIGURE 3: Use of eq 3 to analyze the ionic strength dependence of the reactions of cytochrome *c*-551i with (●) cytochrome *c*-550 and (○) horse heart cytochrome *c*.

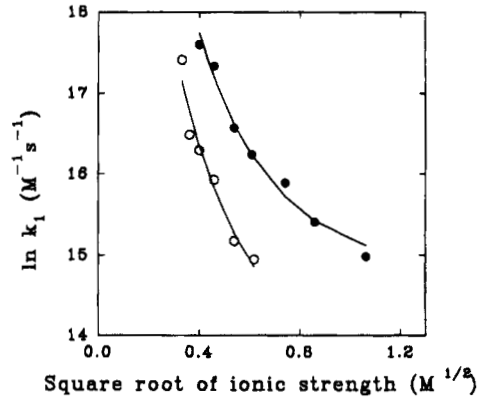

FIGURE 4: Use of eq 6 to analyze the ionic strength dependence of the reaction of cytochrome *c*-551i with (●) cytochrome *c*-550 and (○) horse heart cytochrome *c*.

of 31° for θ for cytochrome *c*-551i and 14.1 ± 1.1 for ln k_{inf}. With θ for cytochrome *c*-550 set at 15°, fitted values were obtained of 0° for θ for cytochrome *c*-551i and 15.8 ± 0.9 for ln k_{inf}. The errors in the fitted values of cos θ for cytochrome *c*-551i were rather large: 35% and 53%, respectively, for the reactions with cytochrome *c*-550 and horse heart cytochrome *c*.

An alternative to eq 3 which neglects the contribution of dipolar effects and predicts the ionic strength dependence for a reaction which is influenced primarily by the charges of the interaction domains of the reactants is eq 6. This equation has previously been used to analyze the interactions between cytochromes *c* and flavins (Meyer et al., 1984), cytochromes *c* and flavodoxin (Tollin et al., 1984), cytochromes *c* and inorganic oxidants (Cheddar et al., 1989), and cytochromes *f* and *c* with flavin semiquinones and plastocyanin (Qin & Kostic, 1992). The data shown in Table 1 were fit to eq 6. Rather than assuming values for the local charge, for each set of data the term V_{ii} was assigned as a single variable. As can be seen in eq 6, V_{ii} is directly proportional to the product of the charges. This variable and ln k_{inf} were not defined and were obtained from fits of the data to eq 6. These fits are shown in Figure 4. It can be seen that reasonably good fits were obtained for each set of data. For the reaction of horse heart cytochrome *c* with cytochrome *c*-551i, fitted values were obtained of ln k_{inf} = 13.5 ± 0.3 and V_{ii} = −13.9 ± 1.5. For the reaction of cytochrome *c*-550 with cytochrome *c*-551i, fitted values were obtained of ln k_{inf} = 14.8 ± 0.1 and V_{ii} = −14.4 ± 0.8.

If one attempts to explain the data in Table 1 solely in terms of monopole−monopole interactions, then cytochrome *c*-550 is behaving as though it were positively charged, in spite of its net charge of −7. Similar, atypical, ionic strength dependencies (decreasing rate constants with increasing ionic strength for like charged molecules) have been observed for the nonphysiologic reactions of *P. denitrificans* cytochrome *c*-550 with ferricyanide ion (Cheddar et al., 1989), flavin (Meyer et al., 1984), and ferrodoxin (Tollin et al., 1984). Those authors concluded that the negatively charged reactants were binding to cytochrome *c*-550 at a positively charged interaction domain. The data obtained here may also be interpreted as meaning that such a positively charged interaction domain is also the site of binding to a physiologic reactant, cytochrome *c*-551i. The only such cluster of positive charges on cytochrome *c*-550 is located surrounding the exposed heme group. This structural feature is shared with horse heart cytochrome *c*. The similar ionic strength dependence for the reaction with the mammalian cytochrome *c* may indicate that both it and cytochrome *c*-550 utilize this same domain for their reactions with the acidic cytochrome *c*-551i.

If one assumes that the local charges of the reaction domains of horse heart cytochrome *c* and cytochrome *c*-550 are equal (Tollin et al., 1984), then the statistically identical V_{ii} values for the reaction of each with cytochrome *c*-551i strongly suggest that the reactive site on cytochrome *c*-551i is the same for its reactions with each of the other two cytochromes. A difference of even +1 or −1 in the Z term for cytochrome *c*-551i would have a significant effect on the V_{ii} term (see eq 6) for the reaction with either cytochrome *c*-551i or horse heart cytochrome *c*. In apparent contradiction to this suggestion are the results of the analysis of these data by eq 3. When it is assumed that horse heart cytochrome *c* and cytochrome *c*-550 utilize analogous positions for their reactions with cytochrome *c*-551i, different θ values were obtained for the corresponding reaction sites on cytochrome *c*-551i. Taken alone, these results indicate that cytochrome *c*-551i utilizes different sites for its reactions with either of the other two cytochromes. It should be noted, however, that the angle θ defines a band of possible sites equatorial to the dipole vector of the protein, not a unique specific site on the surface. Furthermore, given the relatively large error associated with the fitted values for θ, it is not really possible to make definitive statements about the location of the reaction domain(s) on cytochrome *c*-551i. If cytochrome *c*-551i does, in fact, possess different reaction domains for each of the other two cytochromes, the possibility also remains that each site has the same local charge. If so, then the results of the two analyses would be compatible. Cytochrome *c*-551i is a relatively acidic protein. Unlike the other two cytochromes, cytochrome *c*-551i does not exhibit a positively charged region surrounding its exposed heme. That region is primarily hydrophobic, and negative charges are located on the sides and back of the protein away from this region (Chen et al., 1994). If the other two cytochromes are each reacting with cytochrome *c*-551i at a surface domain which is complementary in charge to their positively charged interaction domains, then these ionic strength data suggest that the interaction domain of cytochrome *c*-551i is not at the exposed heme site but at one of the distal regions of negative charge.

Of the formalisms which have been developed to describe the ionic strength dependence of reactions between proteins, there is as yet no consensus agreement on which if any is generally applicable. We have attempted to fit our data to two very different models, one which treats proteins as both monopoles and dipoles, considering overall net charge, and another which neglects dipolar effects and considers only local rather than net charge. Such a comparative treatment of data by alternative formalisms has not routinely been performed for redox reactions between proteins. Local charges cannot be primarily responsible for the ionic strength dependence of reactions between all proteins. We have previously demonstrated that the interaction between methanol dehydrogenase and cytochrome *c*-551i can be well described by Van Leeuwen theory (Harris & Davidson, 1994). In that case, it is believed that the sites of interaction on each protein are primarily hydrophobic. In other words, there is little if any local charge at either of the reacting sites. It has also been clearly demonstrated here that the ionic strength dependence of the physiologic reaction between cytochromes *c*-550 and *c*-551i cannot be explained in terms of simple monopole−monopole interactions which consider the overall net charges of the molecules. One must invoke either the importance of dipolar effects or the local charge effects or both to explain these data. Unfortunately, the analyses of the data by eqs 3 and 6 do not clearly distinguish either method as superior to the other. It is possible to fit the data to either model. The results of these analyses should also be interpreted with caution. In this study, relatively few data points are being fit to complex equations with multiple unknown variables. This is unavoidable given the constraints on this experimental system. For each data set, however, fitted parameters may be obtained by either method which describe the similar ionic strength dependencies of the reactions of the oppositely charged cytochromes *c* with cytochrome *c*-551i. The results of this study do not resolve the issue of which treatment is most appropriate, but they do provide a sound approach to systematically testing existing theories concerning the role of electrostatics in protein−protein interactions. If such a comparative analysis of data can be extended to other systems, particularly reactions between proteins of known structure, then it should be possible to accumulate a sufficiently large data base to intelligently evaluate the general applicability and perhaps limitations of these formalisms.

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