Electrostatic and steric control of electron self-exchange in cytochromes c, c_{551}, and b_5

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ABSTRACT The ionic strength dependence of the electron self-exchange rate constants of cytochromes c, c_{551}, and b_5 has been analyzed in terms of a monopole–dipole formalism (van Leeuwen, J. W. 1983. *Biochim. Biophys. Acta.* 743:408–421). The dipole moments of the reduced and oxidized forms of *Ps. aeruginosa* cytochrome c_{551} are 190 and 210 D, respectively (calculated from the crystal structure). The projections of these on the vector from the center of mass through the exposed heme edge are 120 and 150 D. For cytochrome b_5, the dipole moments calculated from the crystal structure are 500 and 460 D for the reduced and oxidized protein; the projections of these dipole moments through the exposed heme edge are −330 and −280 D. A fit of the ionic strength dependence of the electron self-exchange rate constants gives −280 (reduced) and −250 (oxidized) D for the center of mass to heme edge vector. The self-exchange rate constants extrapolated to infinite ionic strength of cytochrome c, c_{551}, and b_5 are 5.1×10^5, 2×10^7, and 3.7×10^5 $M^{-1} s^{-1}$, respectively. The extension of the monopole–dipole approach to other cytochrome–cytochrome electron transfer reactions is discussed. The control of electron transfer by the size and shape of the protein is investigated using a model which accounts for the distance of the heme from each of the surface atoms of the protein. These calculations indicate that the difference between the electrostatically corrected self-exchange rate constants of cytochromes c and c_{551} is due only in part to the different sizes and heme exposures of the two proteins.

INTRODUCTION

Electrostatic interactions are recognized as one of the primary determinants of electron transfer to and from cytochromes (Feinberg and Ryan, 1981; Warshel and Russell, 1984; Matthew, 1985; Tam and Williams, 1985; Honig et al., 1986; Rogers, 1986; Tollin et al., 1986*b*; Gilson and Honig, 1988). Electrostatic interactions often control the rate of electron transfer between the cytochrome and its partner. In addition, the interactions are often important in determining the geometry of two proteins in a complex. Much of the work that has been done to probe the effect of electrostatics on bimolecular cytochrome electron transfer has been based on experiments in which the rate constants of these reactions are measured as a function of ionic strength (Wherland and Gray, 1978; Ferguson-Miller et al., 1979; Margoliash and Bosshard, 1983; Moore et al. 1984; Poulos and Finzel, 1984; Tam and Williams, 1985; Marcus and Sutin, 1985; Williams et al., 1985; Rogers, 1986; Dixon, 1988). Most early efforts to fit such data simply treated the protein as a monopole of adjustable charge; the best-fit charge was assumed to be that of the active site. Cytochrome c has also been extensively studied by specific functionalization of single residues; these experiments have mapped the residues which appear to be most important in cytochrome c–partner interactions for the reactions of cytochrome c with a variety of reaction partners (Margoliash and Bosshard, 1983; Concar et al., 1986; Bosshard et al., 1987; van der Wal et al., 1987; Bosshard and Ritzmann, 1988).

More recently, the full electrostatic surface maps of cytochrome c and one of its reaction partners, cytochrome c peroxidase, have been calculated (Northrup et al., 1987). Brownian dynamics has been used to calculate the approach of these two proteins in solution (Northrup et al., 1986*a* and *b*; 1987, 1988). In other work, molecular dynamics has been used to simulate the docking of cytochrome c with cytochrome b_5 (Wendoloski et al., 1987). These approaches give a clear picture of the approach and docking of two proteins, but demand extensive computer time.

It is therefore of interest to develop electrostatic models which are more sophisticated than treating the protein only as a monopole, but less demanding of computer time than full electrostatic surface–Brownian dynamic computations. A number of such formalisms have been developed including those by Wherland and Gray (1978), Stonehuerner et al. (1979), Koppenol (1980), van Leeuwen (1983), and Tollin et al. (1986*b*). Of these, that of van Leeuwen has few adjustable parameters and appears to be best at higher ionic strengths (> 0.1 M)(Rush et al., 1987; Dixon and Barbush, unpublished observations). Van Leeuwen's approach treats the protein

0006-3495/89/08/339/13 $2.00

as both a monopole and a dipole. Dipole moments can be either calculated from the crystal structures (Koppenol and Margoliash, 1982), or derived from a fit of the rate constant to the appropriate equations.

In this paper, we bring together data on cytochrome–cytochrome reactions as a function of ionic strength and calculations of cytochrome dipole moments to test the ability of the monopole–dipole model to fit experimental data of electron transfer between cytochromes. Three of the rate constants are those for self-exchange reactions, electron transfer between the oxidized and reduced forms of the same molecule:

$$\text{cyt}_a^{ox} + \text{cyt}_b^{red} \rightleftharpoons \text{cyt}_a^{red} + \text{cyt}_b^{ox}.$$

This reaction is useful because $\Delta G°$ is zero, i.e., the reactants and products are the same. Electron self-exchange rate constants electrostatically corrected to infinite ionic strength are presented for the self-exchange reactions of cytochromes *c*, c_{551}, and b_5. Cytochromes *c* and b_5 have much slower rate constants for electron self-exchange than does cytochrome c_{551}. Although the heme of cytochrome c_{551} is more exposed than that of horse heart cytochrome *c* (Stellwagen, 1978; Dixon et al., 1984), a quantitative analysis shows that the difference in rate constants is not due primarily to this difference in heme exposure.

EXPERIMENTAL

Sample preparation

Horse heart cytochrome *c* (type VI) was purchased from Sigma Chemical Co., St. Louis, MO, and used as received. Protein concentrations were calculated based on an extinction coefficent of 9,000 at 550 nm (Margoliash and Frohwirt, 1959). The data presented comes from two different samples. The trypsin-solubilized 82 amino acid form of cytochrome b_5 was used; details of the preparation will be reported elsewhere (Dixon et al., submitted for publication).[1]

Rate constant measurements

Proton NMR spectra were taken on a Varian VXR-400 spectrometer operating at magnetic field of 399.954 MHz with a 5-mm probe. The spectral width was 22.7 kHz. All spectra had more than 15,000 data points, and were the average of 64–128 scans. The NMR probe temperature was calibrated to ±0.4°C using a methanol thermometer (van Geet, 1968) and regulated to ±0.1°C in the range 5.0–60.0°C. Samples were run in μ = 0.1 M pH 7.0 Na_3PO_4 D20 (99.96% D) buffer, with added NaCl. The HOD in the NMR samples was <0.5%.

For calculation of the rate constants, a 180°-tau-90° sequence was used. The 180° selective pulse was applied through the decoupler. The strength of the pulse was 235 Hz, calibrated via the Block-Siegert shift (Abragam, 1961; Mersh and Sanders, 1982); pulse lengths were ~3 ms. A delay time (tau) of 0.002–1.5 s was used as the exchange period between the 180° pulse and the 90° detection pulse; each measurement had a series of 11 or 12 different tau (D2) values. Data was taken in blocks of 16 scans with two steady-state scans between each block. The magnetization was allowed to return to equilibrium for 1.5–2.5 s before each pulse sequence.

The data was analyzed by fitting the magnetization as a function of time to the equations for two exchanging species. Consider two exchanging species, a and b. Each one has its own chemical lifetime and magnetization lifetime T_1. During the mixing (D2) period, the inverted magnetization not only exchanges with the noninverted magnetization but also relaxes. The behavior of the magnetization of the two exchanging species is described by the following differential equation (Forsen and Hoffman, 1963) (a and b are the protons of heme methyl groups on oxidized and reduced cytochrome b_5, respectively):

$$dM_a(t)/dt + M_a(k_a + K_a) - M_b k_b = M_a^\circ K_a \quad (1a)$$

$$dM_b(t)/dt + M_b(k_b + K_b) - M_a k_a = M_b^\circ K_b, \quad (1b)$$

where M_a and M_b are the magnetization of the two exchanging species a and b at time *t*; M_a° and M_b° are the equilibrium magnetizations of a and b; k_a and k_b are the reciprocals of the lifetimes of species a and b; K_a and K_b are the reciprocals of the T_1s of a and b; and *t* is the value of D2. Eq. 1 has been solved for a number of experimental situations (Alger and Prestegard, 1977; Alger and Shulman, 1984), but not for the instance in which only one of the two peaks a and b is visible. In the current experiments, the heme methyl resonances of the oxidized protein are visible but the heme methyl resonances of the reduced protein are not because they are buried in the diamagnetic envelope. Therefore, Eq. 1 was solved to give two double exponential equations, and interest was focused on the equation pertaining to species a:

$$M_a(t) = C_1 \exp(\lambda_1 t) + C_2 \exp(\lambda_2 t) + M_a^\circ, \quad (2)$$

where

$$\lambda_1 = [-A + (A^2 - 4B)^{1/2}]/2.0 \quad (2a)$$

$$\lambda_2 = [-A - (A^2 - 4B)^{1/2}]/2.0 \quad (2b)$$

$$C_1 = M_{a(t=0)} - M_a^\circ - C_2 \quad (2c)$$

$$C_2 = -[(\lambda_1 + K_a + k_a) \cdot (M_a^\circ - M_{a(t=0)})]/(A^2 - 4B)^{1/2} \quad (2d)$$

$$A = k_a + k_b + K_a + K_b \quad (2e)$$

$$B = (k_a + K_a)(k_b + K_b) - k_a k_b. \quad (2f)$$

Electron self-exchange rate constants were obtained by fitting the intensities of heme methyl proton peaks recorded at a series of D2s to the above double exponential equation using the nonlinear least squares

[1]The form of bovine cytochrome b_5 studied is the 82 amino acid form and differs from the lipase-solubilized form that has been characterized crystallographically by removal of two residues from the amino terminus and nine residues from the carboxyl terminus. The sequence has been recently redetermined from the bovine liver cytochrome b_5 cDNA clone (Christiano, A. B., and A. W. Steggles. 1989. *Nucl. Acids Res.* 17:799) and from gas phase sequence analysis of tryptic peptides obtained from a tryptic hydrolysate of apocytochrome b_5 (Mauk, A. G., personal communication). The corrected sequence differs from that reported earlier (Ozols, J., and P. J. Strittmatter. 1969. *J. Biol. Chem.* 244:6617–6618) in that residue 57 is now known to be Asn and residues 11 and 13 are known to be Glu and Gln, respectively. Calculations were done on the reported crystal structure (Mathews et al., 1979; Mathews and Czerwinski, 1986) with these substitutions made.

ZXSSQ routine of the International Mathematical and Statistics Library, Houston, TX.[2]

T_1^{ox} values for the heme ring methyl groups of the fully oxidized cytochromes were measured on the same samples used for measurement of the electron self-exchange rate constants. A selective 180° pulse, inverting only the peak of interest, was used. As expected, this gives somewhat shorter T_1 values than the method in which all the peaks are inverted due to the contribution of the nuclear Overhauser effect to relaxation of the magnetization. For the cytochrome *c* experiments reported herein, the ratio of oxidized to reduced protein was obtained either by direct integration of the peak intensities or by fitting of the data to Eq. 2; the results were the same within experimental error. For cytochrome b_5, it was necessary to integrate the peak intensities; computer fitting gave more than one acceptable set of values ([oxidized]/[reduced] ratio and rate constant). It should be noted that one often observes more than one local minimum in the nonlinear least squares fits of slow exchange data to Eq. 2 when both the rate constant and [oxidized]/[reduced] ratio are allowed to vary. It is very important to know the [oxidized]/[reduced] ratio; it can be derived from NMR integration or optical spectra taken directly in the NMR tube (Dixon and Woehler, 1988).

A second method, 180°(nonselective)-180°(selective)-tau-90°, or inversion-inversion-recovery was used to visualize the exchange. In this experiment, the oxidized peak was first inverted by 180°, and then a hard pulse followed immediately, inverting all peaks by 180°. After these two pulses, all peaks were inverted with the exception of the peak irradiated by the first, selective, 180° pulse, which was along the $+Z$ axis (2 × 180° pulses). As the D2 period is increased in a series of experiments, the intensity of this peak decreases (due to exchange), and then increases back to its full size again (due to the relaxation of magnetization relaxation of all exchanging species). To fit the double inversion-recovery data only a minor change in the expression for C2 is necessary:

$$C_2 = -((\lambda_1 + K_a)(M_a^\circ - M_{a(t=0)} - k_a(M_a^\circ + M_{a(t=0)}))/(A^2 - 4B)^{1/2}. \quad (2g)$$

Dipole moment calculations

Dipole moments were calculated from the equations of Koppenol and Margoliash (1982) which are equivalent to the classical expression

$$D = \sum_i q_i r_i, \quad (3)$$

where D is the dipole moment vector of the protein, q is the partial charge of atom i, r is the vector from the center of the protein (center of mass) to atom i, and the sum is over all atoms. Charges were assigned with the charge tables from the AMBER force field (as implemented in MACROMODEL 2.0 by Dr. C. Still, Columbia University, New York, 1988) or from charges given by Karplus and co-workers (McCammon et al., 1979; Northrup et al., 1981). The charges in these two approaches are somewhat different, leading to somewhat different dipole moments.

The component of the dipole moment pointing along the vector from the center of mass to the exposed heme edge was obtained by calculating the dot product of the dipole moment vector and a vector from the center of mass to the exposed heme edge; these are given as D' values. Unless noted otherwise, all arginine and lysine residues, and the NH_2-terminus, were protonated; all carboxylic acids (including those of the heme and COOH-terminus) were deprotonated and histidine was in its neutral form.

The surface area of the protein was calculated using the algorithm of Connolly (1983*a* and *b*) as implemented in the Quantum Chemistry Program Exchange program (Indiana University, Bloomington, IN). This algorithm rolls a probe sphere over the protein and defines two types of surface: contact surface and reentrant surface. The calculations presented in this work refer only to the contact surfaces. Three probe radii were used: 1.5, 3.0, and 4.5 Å. Only the heavy atoms were used in the calculations; hydrogens were accounted for using the conventions of Blaney et al. (1982).

The distance from each of the surface atoms to the nearest atom on the heme was calculated by taking the distance from that surface atom to all atoms on the heme and then taking the minimum of this set of distances. Calculations were done on the structures of tuna cytochrome *c* (Takano and Dickerson, 1981*a* and *b*), *Ps. aeruginosa* cytochrome c_{551} (Matsuura et al. 1982), and cytochrome b_5 (Mathews et al., 1979; Mathews and Czerwinski, 1986) from the Brookhaven data bank (Bernstein et al., 1977).

RESULTS AND DISCUSSION

Measurement of self-exchange rate constants

The self-exchange reactions of cytochromes *c* and b_5 are both slow on the NMR time scale; i.e., one can see peaks arising from both the oxidized and reduced species in a mixture of the two. All rate constant measurements were made on the resonances of the methyl groups of the heme ring in the ferric oxidation state because these are three proton singlets well separated from the rest of the resonances in the spectrum. When one of these singlets is selectively inverted, it recovers with a rate that can be expressed in terms of not only the T_1 of the resonance in question, but also the T_1 of the resonance in the ferrous oxidation state as well as the rate for interconversion between the two oxidation states (Eqs. 1 and 2). The return to equilibrium of a given ferric resonance will be faster in a solution which has mixture of the two oxidation states than in a solution containing only the ferric protein because the inverted resonance is exchanging with a resonance which is uninverted in the former case.

Fig. 1 shows an experiment in which the 1-Me resonance of Fe(III) cytochrome b_5 was selectively inverted in a solution containing both oxidation states. Given the T_1 of the reduced species and the measured fraction of the two oxidation states (see Experimental), the only variable is the rate constant. A nonlinear least squares fit of the data gives a rate constant of $1.7 \times 10^4\ M^{-1}s^{-1}$.

After a selective 180° inversion of a given ferric resonance, the corresponding resonance in the ferrous protein will first decrease in intensity, as it exchanges with the inverted magnetization of the ferric proton, and then increase in intensity, as all the magnetization in the system returns to equilibrium. One cannot see this experimentally, however, because all of the methyl group reso-

[2]REFINE routine in FRODO (PS300 version 6.2); Quiochio, F. A., Department of Biochemistry, Rice University, Houston, TX.

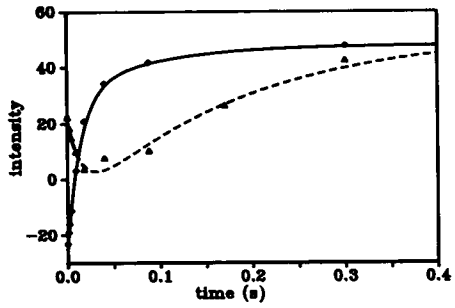

FIGURE 1 Intensity of the 1-Me peak (12 ppm) of cytochrome b_5 as a function of time after a 180° inversion. (♦) Selective 180° inversion; (▲) nonselective inversion of all resonances, followed by selective 180° inversion of the 1-Me peak. The lines are the theoretical fits to a rate constant of 1.7×10^4 M^{-1} s^{-1}. Conditions: μ = 0.60 M added salt, 25.0°C, and protein concentration = 4.0 mM with a $[cyt_{ox}]/[cyt_{red}]$ ratio of 1.

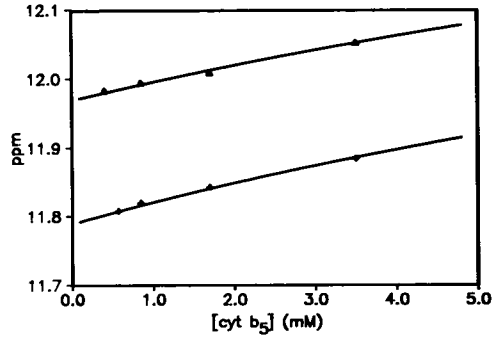

FIGURE 2 The chemical shift of the 1-Me resonance of cytochrome b_5 as a function of protein concentration. The data was fit to a chemical shift difference of 1.1 ppm between the monomer and dimer with K_{eq} = 25–30 M^{-1}. (+) at μ = 0.10 M added salt; (▲) at μ = 0.30 M added salt.

nances of the ferrous form of the protein are buried in the diamagnetic envelope. The self-exchange process can be visualized by an inversion-inversion-recovery pulse sequence in which a methyl resonance from the oxidized protein is selectively inverted by 180°. Immediate application of a hard pulse, which inverts all peaks by 180°, gives all peaks inverted except the peak of interest, which is again along the $+Z$ axis ($2 \times 180°$ pulses). As the D2 period is lengthened in a series of experiments, the intensity of this peak decreases (due to exchange), and then increases back to its full size again (due to the relaxation of magnetization relaxation of all exchanging species). Fig. 1 also illustrates the recovery of magnetization in this inversion-inversion-recovery experiment for cytochrome b_5. Although this 180°-180°-tau-90° sequence is useful to visualize the exchange, the rate constants calculated in this work were derived from fitting of data obtained from single inversion-recovery experiments as described in the experimental because the latter technique involves less spin manipulation and is therefore expected to be more accurate.

For accurate results, it is necessary to establish whether any protein–protein dimers form under the conditions of the experiment. The usual method involves measurement of the NMR spectrum as a function of concentration; protein–protein dimerization or aggregation manifests itself as changes in the chemical shifts or linewidths of the protein as a function of concentration (Hazzard and Tollin, 1985; Livingston et al., 1985; Timkovich, 1986; Satterlee, 1987; Satterlee et al., 1987; Tollin et al., 1987). For cytochrome b_5, small changes in the chemical shifts and linewidths of the hemin methyl resonances are seen; the chemical shift of the 1-Me resonance as a function of concentration is shown in Fig. 2. At this concentration the solution is 4% by volume protein, and line broadening might be due to factors other than specific protein–protein interactions at these high protein concentrations. If the line broadening and chemical shift changes are due to protein dimerization, the equilibrium constant calculated from the data in Fig. 2 is 25–30 M^{-1}, indicating a dimerization of only 10% at the highest protein concentration investigated (4 mM). Calculation of the association constant from the chemical shifts of the other ring protons or from the linewidths of these protons gave similar equilibrium constants. Experiments on cytochrome c revealed no change in the chemical shift or linewidth over the concentration range 1–10 mM, indicating no dimerization or aggregation in this concentration range.

Ionic strength dependence of the self-exchange rate constants of cytochromes c, c_{551}, and b_5

It has been known for many years that the electron self-exchange rate constants of some cytochromes c depend on the ionic strength of the solution. In the early 1970s, Gupta showed that the self-exchange rate constant for horse heart cytochrome c increased form approximately 10^3 M^{-1} s^{-1} at μ = 0.1 to 10^4 M^{-1} s^{-1} at μ = 1.0; the self-exchange rate constant for *Candida krusei* had a similar dependence on ionic strength, increasing from 10^2 to 10^3 M^{-1} s^{-1} over the same ionic strength range (Gupta, 1973). We have remeasured the self-exchange rate constant of cytochrome c as a function of ionic strength, extending the measurements to somewhat higher ionic strengths (Table 1). Our rate constants are slightly higher than those found by Gupta (Gupta et al., 1972; Gupta, 1973); this difference, which is at maximum a factor of 2, may reflect slight differences in experimental conditions.

TABLE 1 Electron self-exchange rate constant as a function of ionic strength

Protein	μ	k
	M	$M^{-1} s^{-1}$
Cytochrome c*	0.12	5.4×10^3
	0.13	6.7×10^3
	0.25	1.2×10^4
	0.30	1.6×10^4
	0.50	2.8×10^4
	0.80	4.2×10^4
	1.00	5.0×10^4
	1.50	5.9×10^4
Cytochrome b_5‡	0.12	2.6×10^3
	0.30	5.0×10^3
	0.60	1.7×10^4
	1.00	2.7×10^4
	1.50	4.4×10^4

*At 27°C. ‡At 25°C.

In addition, in NMR studies (solutions 0.5–5 mM in protein), the protein and its counterions contribute substantially to the ionic strength. All ionic strengths for NMR studies reported here are the sum of the added salt and the protein concentration multiplied by the total charge of the protein (Eley et al., 1984).

Not all cytochrome c self-exchange rate constants are dependent on ionic strength, however. The self-exchange rate constant of cytochrome c_{551} from *Pseudomonas aeruginosa* is almost independent of ionic strength, being $1.2 \pm 0.5 \times 10^7$ M^{-1} s^{-1} in 50 mM phosphate ($\mu = 0.17$ M) and $2.0 \pm 0.5 \times 10^7$ M^{-1} s^{-1} in a sample 0.5 M in NaCl ($\mu = 0.67$ M). The data for cytochrome c_{551} from *Pseudomonas stutzeri* is similar (Timkovich et al., 1988).

Cytochrome b_5 shows a dependence of the self-exchange rate constants on ionic strength that is similar to that of horse heart cytochrome c; rate constants (Dixon et al., submitted for publication) are given in Table 1.

Calculation of dipole moments

Protein dipole moments can be calculated from the crystal structure (see Experimental). The dipole moment is origin dependent (Jackson, 1975); dipole moments in this work are based on the origin at the center of mass. Use of the dipole moment depends on the model chosen for electron transfer. If electron transfer takes place only through the exposed heme edge, as is commonly supposed, then it is the component of the dipole moment through the exposed edge of the heme that is important. These are given herein as D' values.

The dipole moment of horse heart cytochrome c has been calculated previously by Koppenol and Margoliash (1982; Rush et al., 1987) as well as by Northrup et al. (1986*a*) (Table 2). The dipole moments of the oxidized and reduced forms of *Ps. aeruginosa* cytochrome c_{551} were calculated from the x-ray structure of the appropriate oxidation state of the protein (Matsuura et al. 1982). Using the charges as given by Karplus and co-workers (McCammon et al., 1979; Northrup et al., 1981), the dipole moments of the oxidized and reduced protein are 210 and 190 D, respectively; using the charge tables associated with the AMBER force field, the values are 190 and 170 D (both propionates deprotonated). The

TABLE 2 Parameters for cytochromes c (horse heart), c_{551} (*Ps. aeruginosa*), and b_5 (bovine)

Parameter	c (ox)	c (red)	c_{551} (ox)	c_{551} (red)	b_5 (ox)	b_5 (red)
No. of amino acids	103		82		82	
Approximate radius (Å)*	16.6		14.4		15.9	
Surface area, 1.5-Å probe (Å^2)	1673		1307		1561	
Percent of protein surface area that is heme	0.7		1.2		3.8	
Charge‡	7.5	6.5	−2	−3	−7.5	−8.5
Dipole moment (D)	325§	310§	210¶	190¶	−280¶	−330¶
Dipole projection through heme edge (D)	300¶	275¶	150¶	120¶	−250¶	−280¶
k_{self}, self-exchange ($M^{-1} s^{-1}$)	5.1×10^5		2×10^7		3.7×10^5	

*Wherland and Gray, 1979.
‡Both heme propionic acids deprotonated, other conventions in Experimental.
§Koppenol and Margoliash, 1982.
¶Calculated from the crystal structure.
¶From a fit of the self-exchange data.

difference in these values primarily serves as a reminder that dipole moments calculated for proteins are approximate values; all dipole moments below are based on charges from the tables of Karplus and co-workers. The projection of the dipole moment through the exposed heme edge (D') is 120 D for the reduced and 150 D for the oxidized protein.

The properties of *Ps. aeruginosa* cytochrome c_{551} are pH dependent. Near neutrality, this pH dependence has been interpreted in terms of titration of a single group with a pKa of 6.2 in the oxidized and 7.2 in the reduced form of the protein (Moore et al., 1980; Leitch et al., 1984). NMR studies indicate that the ionizing group is the propionic acid at the 7-position of the heme ring (Leitch et al., 1984). Deprotonation results in a decrease in redox potential of ~65 mV (Rogers et al., 1985; Rogers and Moore, 1988). Although this protonation affects many of the properties of cytochrome c_{551}, it has only a small influence on the dipole moment. The dipole moments of the oxidized and reduced proteins with the 7-propionic acid protonated were 230 and 210 D, respectively.

The dipole moment of the reduced protein is illustrated in Fig. 3, which shows the alpha carbon backbone of the protein, the heme, the center of mass (closest to the CHB, the δ-meso carbon of the heme) and the point at which the dipole moment vector extends through the surface of the protein (closest to the amino nitrogen of Lys-21). Small differences between the crystal structures of the oxidized and reduced forms of the protein (Matsuura et al., 1982) are not reflected in the dipole moment calculations. Calculation of the dipole moment of the oxidized protein using the reduced coordinates but with the charge on the iron increased by one gave essentially the same results as the calculation using the oxidized protein coordinates.

Crystal structures are available for both the oxidized and reduced states of the 93 residue form of cytochrome b_5 (Mathews et al., 1979; Mathews and Czerwinski, 1986). The structures of the two oxidation states are the same within experimental error. The structures are not quite complete, in that six residues are missing from the COOH-terminus and 2 from the NH_2-terminus due to disorder (see footnote 1). However, this is not a problem in the present calculations because the trypsin-solubilized form used in this work has only 82 amino acids (the 85 visible in the crystal structure minus the three COOH-terminal residues of that structure). The dipole moments of the reduced and oxidized protein are 500 and 460 D, respectively; the projections of these through the exposed heme edge are −330 and −280 D. The residue closest to

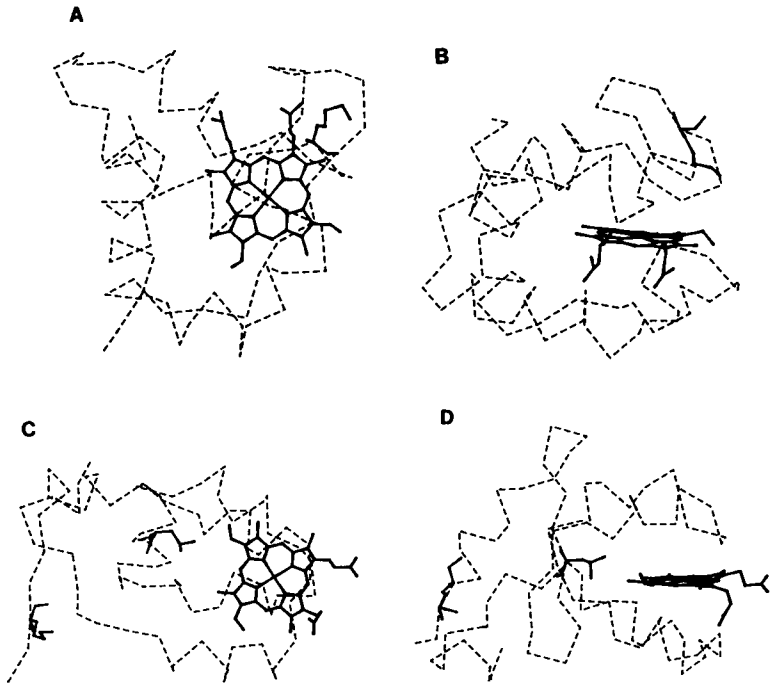

FIGURE 3 Illustrations of the dipole moments of cytochromes c_{551} and b_5. Each figure shows the alpha-carbon backbone as a dotted line. (*A* and *B*) Cytochrome c_{551}; the atom closest to the center of mass is CHB (δ-meso carbon of the heme); the residue closest to the point where the dipole moment vector crosses the protein surface is the Lys-21. (*C* and *D*) Cytochrome b_5; the residue closest to the center of mass is Leu-23; the residue closest to the point where the dipole moment vector crosses the protein surface is Lys-5.

the center of mass is Leu-23; the residue closest to the point where the dipole moment vector crosses the protein surface is Lys-5.

The dipole moment of the 93 residue form was approximated by adding the missing residues to the protein on a graphics screen, refining these residues only (see footnote 2) and then treating this structure as the crystal structure. A number of these residues are charged: the carboxyl group, lysine and glutamic acid at the carboxyl terminus, and lysine and the amino group at the amino terminus. Five different sets of geometries were tried; the dipole moment projections on the center of mass to heme edge vector ranged from -510 to -350 D for the reduced form and from -460 to -300 D for the oxidized form. This spread indicates how the dipole moment of a protein can depend on the conformations of only a few charged amino acids.

Self-exchange rate constants and dipole moments

A number of formalisms are available for treating the dependence of a reaction between two proteins on the ionic strength of the solution. That of van Leeuwen (1983) has proved to work quite well at the high ionic strength needed for NMR studies and to be relatively straightforward to implement. Van Leeuwen treats the electrostatics in terms of monopole–monopole, monopole–dipole, and dipole–dipole interactions. The formalism assumes electron transfer at the exposed heme edge and hence the component of the dipole through the heme edge is the value of the dipole moment used. The equations are:

$$\ln (k_1/k_{inf}) = -\{Z_{ox}Z_{red} + (ZD)(1 + \kappa R) + (DD)(1 + \kappa R)^2\}(q^2/4\pi\epsilon_0\epsilon kTR) f(\kappa) \quad (4)$$

$$ZD = (Z_{ox}D'_{red} + Z_{red}D'_{ox})/qR \quad (4a)$$

$$DD = D'_{ox}D'_{red}/(qR)^2 \quad (4b)$$

$$f(\kappa) = (1 - \exp(-2\kappa r))/2\kappa r(1 + \kappa r) \quad (4c)$$

$$\kappa = 0.329(\mu)^{1/2}, \quad (4d)$$

where Z_{ox} and Z_{red} are the net charges of the oxidized and reduced protein, D'_{ox} and D'_{red} are the components of the dipole moments through the exposed heme edge, r is the radius of the protein, $R = 2r$, k_1 is the rate constant measured at a given ionic strength and k_{inf} is the rate constant at infinite ionic strength. Given the charges and radii of the oxidized and reduced protein, one can fit the experimental data to give D'_{ox}, D'_{red} and the rate constant at infinite ionic strength, k_{inf}.

For horse heart cytochrome c, the fitted values of the dipole moments are $D'_{ox} = 300$ D, $D'_{red} = 275$ D, and $k_{inf} = 5.1 \times 10^5$ M^{-1} s^{-1} (Fig. 4). These fitted values are in very good agreement with the projection of the dipole moment on the heme plane calculated from the crystal structure coordinates, $D'_{ox} = 270$ and $D'_{red} = 255$ D (Rush et al., 1987; assumes charged propionates and amino acid residues). It should be noted that the fitted values of the parameters are sensitive to the value taken for the ionic strength of the solution. All ionic strengths in this work were calculated by adding the ionic strength due to the protein, as described above. If only the ionic strength of the added ions is considered for this reaction, the fitted values are $D'_{ox} = 250$, $D'_{red} = 220$ D, and $k_{inf} = 3.0 \times 10^5$ M^{-1} s^{-1}.

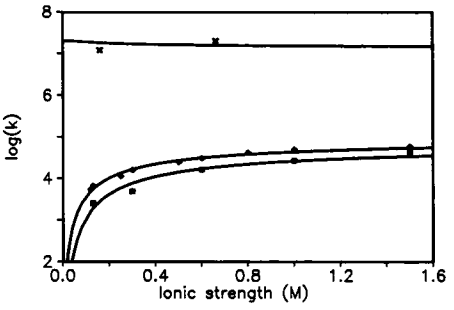

FIGURE 4 The electron self-exchange rate constants for cytochromes c (27°C), c_{551} (40°C), and b_5 (25°C) as a function of the ionic strength. (♦) Cytochrome c, $D'_{ox} = 300$, $D'_{red} = 275$, and $k_{inf} = 5.1 \times 10^5$ M^{-1} s^{-1}; (×) cytochrome c_{551}, $D'_{ox} = 150$ D, $D'_{red} = 120$ D, and $k_{inf} = 2 \times 10^7$ M^{-1} s^{-1}; (■) cytochrome b_5, $D'_{ox} = -250$ D, $D'_{red} = -280$ D, and $k_{inf} = 3.7 \times 10^5$ M^{-1} s^{-1}. Points are experimental: cytochrome c (this work), cytochrome c_{551} (Timkovich et al., 1988), cytochrome b_5 (Dixon et al., submitted for publication).

Experimentally, the self-exchange rate constant of cytochrome c_{551} is almost independent of ionic strength (Timkovich et al., 1988). Fig. 4 shows the dependence of the rate constant on ionic strength predicted using the dipole moments calculated from the x-ray structures. Indeed, there is only a very small predicted dependence of the rate constant on the ionic strength of the solution, largely because the proteins have only a small net charge (-2 in the oxidized and -3 in the reduced form). The lack of dependence of the rate constant on ionic strength precluded fitting of the data to give values for dipole moments; the rate constant at infinite ionic strength for cytochrome c_{551} was therefore taken to be that measured at moderate ionic strength, i.e., 2×10^7 M^{-1} s^{-1} (Timkovich et al., 1988; Keller et al., 1976). Fig. 4 also shows that the expected rate constant for cytochrome c_{551} increases as the ionic strength decreases. This is because the charge on the protein and the component of the dipole moment through the exposed heme edge are of opposite sign. It is not feasible to run NMR experiments at low ionic strength to observe the increase, however. As pointed out above, the ionic strength of solutions of

protein at NMR concentrations is made up from contributions of the protein as well as the added salt. For the ionic strength to be mainly from the added small ions, the small ions (buffer plus added salt) must be at least 0.1 M.

The dipole moments for cytochrome b_5 were obtained by fitting the experimental data to the equations above. This gives $D'_{ox} = -250$, $D'_{red} = -280$ D, and $k_{inf} = 3.7 \times 10^5$ M^{-1} s^{-1} (Fig. 4). Fig. 5 also shows a fit of k_{inf} to the dipole moments calculated from the structure shown in Fig. 3; when $D'_{ox} = -280$ D and $D'_{red} = -330$ D, k_{inf} is 7.3×10^5 M^{-1} s^{-1}. Both sets of values give reasonably good fits to the data. The larger dipole moments calculated from the crystal structures are compensated for by a larger k_{inf}. The two sets of D' values are similar but those calculated from the crystal structure are larger than those calculated from the ionic strength dependence of the self-exchange rate constant. The difference presumably arises from approximations made in the van Leeuwen approach although ion binding to specific protein sites cannot be eliminated as an explanation.

These calculations for cytochromes *c*, c_{551}, and b_5 show that cytochromes *c* and b_5 are very similar to one another, and that they are quite different from cytochrome c_{551}. For cytochromes *c* and b_5, the self-exchange data points lie almost on top of one another, and the fits of this data to values for D'_{ox}, D'_{red}, and k_{inf} gives very similar values for these parameters for the two proteins. This close match argues that similar factors control the rate constants for electron transfer in these two proteins. Cytochrome c_{551} is quite different, however, with slightly smaller dipole moments and a substantially larger k_{inf} of 2×10^7 M^{-1} s^{-1}. The origin of the difference between cytochrome *c* and cytochrome c_{551} is discussed in more detail below.

Protein–protein electron transfer and dipole moments

If the dipole moment is to be a useful protein parameter, it must predict the reactions of cytochromes with electron transfer partners. In addition to the self-exchange measurements reported above, two cross reactions of these three cytochromes have been studied as a function of ionic strength: the reaction of cytochrome *c* with cytochrome b_5 (Stonehuerner et al., 1979) and the reaction of cytochrome *c* with cytochrome c_{551} (Morton et al., 1970; Greenwood et al., 1971).

Stonehuerner et al. (1979) have measured the rate of electron transfer between reduced cytochrome b_5 and oxidized cytochrome *c* at moderate ionic strengths. At low ionic strength, these two proteins form a complex, which has been the subject of modeling (Salemme, 1976; Wendoloski et al., 1987), electron transfer (McLendon and Miller, 1985; McLendon et al., 1985; Mauk et al., 1986), optical (Mauk et al., 1982), and NMR (Eley and Moore, 1983; Hartshorn et al., 1987) studies. However, at the higher ionic strengths, electron transfer is bimolecular. Fig. 6 shows that the data for bimolecular electron transfer are fit well using the dipole moments given in Table 2 and a k_{inf} of 8.9×10^4 M^{-1} s^{-1}. There is some deviation of the calculated line from the experimental points; the experimental values depend more strongly on ionic strength than that predicted from the dipole moments obtained in the self-exchange experiments. The difference may be due in part to the effect of buffer, which was phosphate (with NaCl) in the self-exchange experiments and Tris-HCl in the cytochrome b_5–cytochrome *c* electron transfer experiment. The reaction is known to be sensitive to buffer composition. The ratio of the rate constants at 0.1 and 0.4 M ionic strength is 3.7 in phosphate and 22 in Tris-HCl buffer, respectively (Stone-

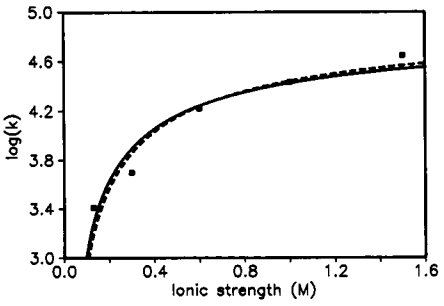

FIGURE 5 The electron self-exchange rate constant for cytochrome b_5 as a function of the ionic strength (25°C). The solid line is a best fit to the data for $Z_{ox} = -7.5$ and $Z_{red} = -8.5$; the fitted values are $D'_{ox} = -250$ D, $D'_{red} = -280$ D, and $k_{inf} = 3.7 \times 10^5$ M^{-1} s^{-1}. The dashed line is a fit of k_{inf} to the dipole moments calculated from the structure shown in Fig. 1: $D'_{ox} = -280$ D, $D'_{red} = -330$ D, and $k_{inf} = 7.3 \times 10^5$ M^{-1} s^{-1}.

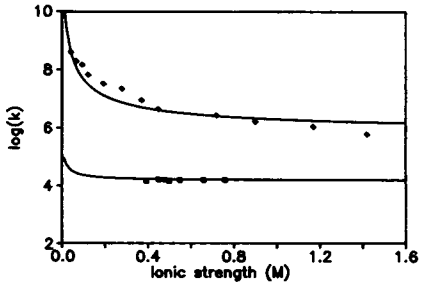

FIGURE 6 Electron transfer rate constants for cytochrome/cytochrome reactions as a function of ionic strength (dipole moments from Table 2): (■) cytochromes c/c_{551} (experimental data from Morton et al., 1970); (♦) cytochromes c/b_5 (experimental data from Stonehuerner et al., 1979).

huerner et al., 1979). Van Leeuwen (1983) has fit the cytochrome *c*–cytochrome b_5 electron transfer data in Tris-HCl buffer and obtained a value for the cytochrome b_5 D'_{red} of -570 D.

The rate constant for electron transfer between *Ps. aeruginosa* cytochrome c_{551} and cytochrome *c* has been studied by both Morton et al. (1970) and by Greenwood et al. (1971). For the bimolecular reaction measured directly (Morton et al., 1970), the rate constant did not depend on ionic strength, as shown in Fig. 6. Again, the data points are fit well using the dipole moments in Table 2 and a k_{inf} of 3.6×10^4 M^{-1} s^{-1}.

The reaction of cytochrome *c* with cytochrome *c* peroxidase has also been the focus of extensive study (Kang et al., 1977; Poulos and Kraut, 1980; Vitello and Erman, 1987; Liang et al., 1987; Moench et al., 1987; Poulos et al., 1987; Cheung and English, 1988; Hazzard et al., 1988). Using van Leeuwen's formalism, the data given by Kang et al. (1977) was not fit well using the charges and dipole moments given by Northrup et al. (1986*a*); this is presumably because cytochrome *c* peroxidase reacts with cytochrome *c* at two different sites, as found in the Brownian dynamic simulations of Northrup et al. (1986*b*, 1987, 1988).

Steric considerations

As discussed above, the electrostatically corrected rate constants for cytochromes *c* and c_{551} are quite different, with the latter being a factor of ~40 faster than the former. What is the origin of this difference? A straightforward idea is that the difference is due to the fact that cytochrome c_{551} has more of its heme edge exposed (Sutin, 1972; Stellwagen, 1978). In the simplest model, the rate constant for electron transfer between two hemes buried in proteins would be that of the two hemes with no steric constraint on electron transfer times the fraction of the surface area of each protein that is heme (θ). For self-exchange, this would give a rate constant $k_{protein} = k_{heme}\theta^2$ (Sutin, 1972). Because electron transfer is thought to fall off exponentially with distance, increased heme exposure should result in a faster electron transfer rate constant. Indeed, as we (Dixon et al., 1984; Shirazi et al., 1985), and others (Moore et al., 1984) have observed, small cytochromes *c* do have faster self-exchange rate constants than the larger proteins. However, for *c* and c_{551}, the heme exposure (i.e., the percent of the surface of the protein that is heme) is very similar. Using the Connolly algorithm and a probe sphere of 1.5 Å, we calculate that the former has 0.7% of its surface area as heme; the latter has 1.2%. This is not enough to account for the 40-fold difference in rate constants. The effect of size on electron transfer might be more subtle than only the extent to which the surface of the protein is exposed heme, however. Fig. 7 illustrates two proteins, one larger than the other. Consider an electron transfer partner approaching along the indicated vector. If the partner cannot penetrate the surface of the protein, the distance of closest approach to the heme is *r* for the small protein and *r'* for the large protein. Because $r' > r$ and because electron transfer is thought to fall off exponentially (Marcus and Sutin, 1985; Mayo et al., 1987; McLendon, 1988), the small protein should transfer electrons more quickly than the large protein. This size effect might be particularly important if atoms linked to the heme (e.g. the axial ligands or thioether bridges in cytochrome *c* [Tollin et al., 1986*a*]) are important in transferring electrons to the heme itself.

The hypothesis that size itself governs the electron transfer rate constant was tested by calculating the distance from every surface atom to the nearest heme atom, and then calculating a relative rate constant for electron transfer given this distance, the surface area of the atom in question, and the dependence of the rate constant on the distance to the heme. First, distances were calculated from each of the heavy atoms in the protein to the heme. The heme was defined in three ways: (*A*) as the ring atoms only, (*B*) as the ring atoms plus the first side chain atom off each of the ring positions and the sulfur atoms at the 2- and 4-positions, and (*C*) as model *B* plus the five heavy atoms of the axial histidine ring and the sulfur of the axial methionine.

Calculation of the surface area of each of the exposed atoms was performed using the Connolly algorithm (1983*a* and *b*; see Experimental). The distance from each of the surface atoms to the closest atom on the heme was measured by calculating the distance from the surface atom to each of the atoms of the heme, and then taking the minimum distance of this set. A histogram was then

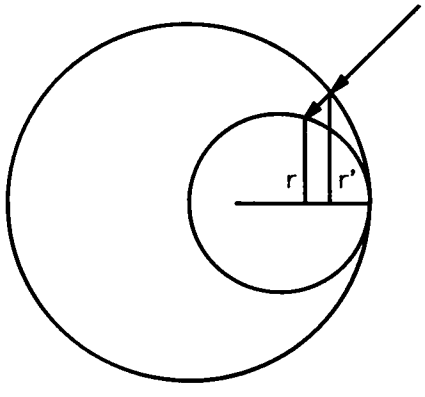

FIGURE 7 Schematic of electron transfer from a reagent to two different size proteins.

calculated in which all surface area lying between n and $n + 1$ angstroms from the heme was summed for each angstrom interval from $n = 0$ (heme edge) to the farthest atom from the heme (18 Å for cytochrome c and 17 Å for cytochrome c_{551}).

Fig. 8 shows this data for cytochrome c_{551} using models *A*, *B*, and *C*. As expected, models *B* and *C* have more exposure at the heme edge than does model *A*. Also, the more atoms in the heme set, the closer the surface area of the protein is to the heme, again as expected. Similar differences as a function of the heme model are seen for cytochrome c (data not shown).

Fig. 9 compares cytochromes c and c_{551} using a 1.5 Å probe sphere and model *C* for the heme. Three expected features are seen. First, there is no area at 1 Å, because this distance is less than a bond length. Second, the integrated area under the curve is smaller for cytochrome c_{551} than for cytochrome c, as expected because the former is a smaller protein (82 as opposed to 103 amino acids). Third, cytochrome c_{551} has more of its area closer to the heme, again as expected for the smaller protein.

These calculations were repeated using probe radii of 3.0 and 4.5 Å. As expected, the larger the probe radius the smaller the surface area. For example, the surface area of cytochrome c calculated using 1.5, 3.0, and 4.5 Å probes was 1,674, 952, and 564 Å^2, respectively. However, this difference is essentially only a scaling factor; no qualitative differences in the surface area as a function of distance from the heme were seen with the different probe sizes.

Electron transfer is thought to fall off exponentially with distance (Marcus and Sutin, 1985; Mayo et al., 1987; McLendon, 1988). For bimolecular reactions this becomes:

$$\text{rate} \propto \exp(-\alpha[\text{protein radius} + \text{partner radius}]), \quad (5)$$

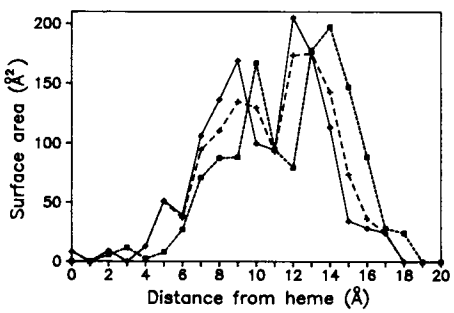

FIGURE 8 The surface area of the protein as a function of distance to the closest atom on the heme for cytochrome c_{551} and three models of the heme. Dimensions are in angstroms; probe radius was 1.5 Å. Data are in histogram form, i.e., the sum of the surface area of the protein lying between >1.0 and ≤2.0 Å from the heme is given at 2 Å. (■) Model *A*; (+) model *B*; (♦) model *C*.

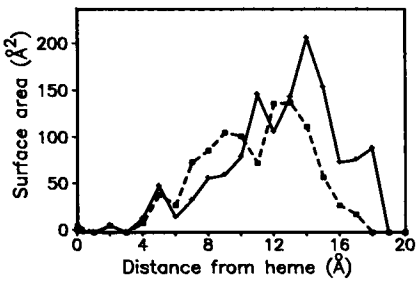

FIGURE 9 The surface area of the protein as a function of distance to the closest atom on the heme using a 1.5 Å probe for (■) cytochrome c_{551} and (+) cytochrome c.

where α is a constant which depends on the system and is expected to be close to 1.2 Å^{-1} for electron transfer between two cytochromes (McLendon, 1988). We have calculated relative rate constants by evaluating Eq. 5 at each atom of the protein surface, multiplying this value by surface area of the atom, and summing these contributions. This approach assumes that other contributions to the preexponential term are independent of the reaction being considered. For a given cytochrome, the value calculated depends somewhat on the model chosen for the heme. For example, for cytochrome c the values are 0.016 (model *A*), 0.117 (model *B*), and 0.117 (model *C*) (α = 1.2 Å^{-1}, r = 3 Å). For cytochrome c_{551} the values are 0.025 (model *A*), 0.260 (model *B*), and 0.261 (model *C*). This analysis indicates that electron transfer to atoms at the heme periphery would increase the electron transfer rate constant. Such electron transfer, through the bridge thioether linkages, has been proposed by Tollin et al. (1986*a*). A comparison of models *B* and *C* indicates that

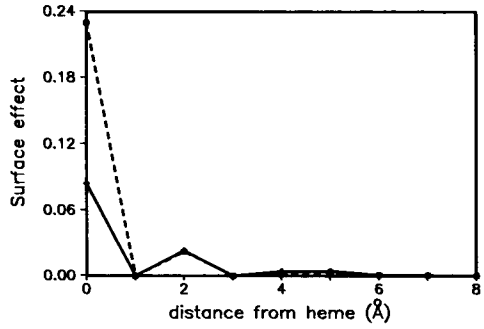

FIGURE 10 The relative amount of electron transfer at a given distance from the heme as a function of that distance. The model follows the formula $A \exp[-\alpha(r + 3)]$, where A is the surface area in square angstroms, α is a constant (1.2 Å^{-1}), r is the distance from the surface to the nearest point on the heme, and the radius of the electron transfer partner is taken as 3 Å. The model assumes no directional dependence of the rate constant as well as no dependence on the intervening residues. (■) Cytochrome c_{551}; (+) cytochrome c.

electron transfer through the axial ligands is unimportant in a steric sense.

Fig. 10 shows the relative value of the rate constant as a function of the distance from the heme for cytochromes *c* and c_{551} with an electron transfer partner with a 3 Å radius and a value of α of 1.2 $Å^{-1}$ (1.5 Å probe sphere and model *C* for the heme). The difference between cytochrome *c* and cytochrome c_{551} is small. The sum of these relative values is 0.117 for cytochrome *c* and 0.260 for cytochrome c_{551}. This implies that, although the rate constants for these two proteins differ by about a factor of 40 when extrapolated to infinite ionic strength, the origin of this difference is not due primarily to geometric constraints.

CONCLUSION

A fundamental question in this work is whether characterization of a protein as only a monopole and a dipole is adequate to predict the ionic strength dependence of the electron transfer rate constant. The van Leeuwen formalism has the advantage that there is only one adjustable parameter, the rate constant at infinite ionic strength, k_{inf}, when the dipole moment can be calculated from the *x*-ray structure. Our study shows that this model is adequate for cytochromes *c* and b_5. Rush et al. (1987) have recently investigated a series of reactions of horse heart cytochrome *c* with metal ion complexes of varying overall charge and have also found the van Leeuwen formalism to be a good predictor of rate as a function of ionic strength. Thus, for bimolecular electron transfer reactions occurring at a single site on each of the partners, a simple model for the dependence of the rate constant on ionic strength based on the protein charges and dipole moments is useful. The dipole moment is, as expected, somewhat dependent on the charge state of the iron and residues as well as on the partial charges assumed for each atom. However, differences in the positions of residues contribute substantially to differences in dipole moments. Thus, proteins may have dipole moments which fluctuate on a biological time scale.

Our work has shown that when the electron self-exchange rate constants of cytochrome *c* are extrapolated to infinite ionic strength, that of cytochrome c_{551} is ~40-fold larger than that of cytochrome *c*. A geometric analysis based on the static crystal structures of these proteins indicates that the faster self-exchange rate constant of the smaller cytochrome c_{551} is not due primarily to its greater heme exposure. The origin of the difference in these two self-exchange rate constants remains to be explained. Possibilities involve differences in reorganization energies or differences in the protein–protein complexes which arise from dynamic fitting of one structure to the other. The steric model does indicate, as has been proposed for many years, that electron transfer in these proteins occurs primarily at or near the exposed edge of the heme, even when the axial ligands are considered as possible pathways for electron transfer.

We thank A. G. Mauk for the cytochrome b_5. We thank S. Northrup for computer programs and useful discussions. Dr. Dixon thanks F. S. Mathews and R. Langridge for hospitality during the early stages of this work.

We thank the National Institutes of Health for support of this work (DK 38826) Dr. Dixon thanks the National Science Foundation for a Career Advancement Award (NSF CHE 8707447). The Varian VXR400 NMR spectrometer was purchased with funds from the National Science Foundation Instrumentation Program (CHEM-8049599). The molecular graphics facility was supported with funds from the United States Department of Education (2-4-01008).

Received for publication 16 December 1988 and in final form 14 April 1989.

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