Electrostatic Interaction of Cytochrome c with Cytochrome c₁ and Cytochrome Oxidase*

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The reactions of horse heart cytochrome c with succinate-cytochrome c reductase and cytochrome oxidase were studied as a function of ionic strength using both spectrophotometric and oxygen electrode assay techniques. The kinetic parameter Vₘₐₓ/Kₘ for both reactions decreased very rapidly as the ionic strength was increased, indicating that electrostatic interactions were important to the reactions. A new semiempirical relationship for the electrostatic energy of interaction between cytochrome c and its oxidation-reduction partners was developed, in which specific complementary charge-pair interactions between lysine amino groups on cytochrome c and negatively charged carboxylate groups on the other protein are assumed to dominate the interaction. The contribution of individual cytochrome c lysine amino groups to the electrostatic interaction was estimated from the decrease in reaction rate caused by specific modification of the lysine amino groups by reagents that change the charge to 0 or −1. These estimates range from −0.9 kcal/mol for lysines immediately surrounding the heme crevice of cytochrome c to 0 kcal/mol for lysines well removed from the heme crevice region. The semiempirical relationship for the total electrostatic energy of interaction was in quantitative agreement with the experimental ionic strength dependence of the reaction rates when the parameters were based on the specific lysine modification results. The electrostatic energies of interaction between cytochrome c and its reductase and oxidase were nearly the same, providing additional evidence that the two reactions take place at similar sites on cytochrome c.

A number of different experimental techniques have now been used to study the mechanism by which cytochrome c transports electrons from cytochrome c₁ to cytochrome oxidase in the mitochondrial membrane. In one approach, individual lysines on cytochrome c have been specifically modified to replace the positively charged amino group with a neutral CF₃CO—lysine (1–3), a neutral CF₃PhNHCO—lysine (4), or a negatively charged carboxyldinitrophenyl lysine (5). It was found that specific modification of those lysines immediately surrounding the heme crevice of cytochrome c decreased the rate of reaction with both cytochrome c₁ (6–8) and cytochrome oxidase (1–5), suggesting that these lysines facilitate both reactions. In the differential chemical modification approach used by Rieder and Bosshard (9), it was found that the lysines immediately surrounding the heme crevice of cytochrome c were protected from acetylation by complex formation with either cytochrome oxidase or cytochrome c₁ (9–11). It thus appears that the highly conserved lysines surrounding the heme crevice are involved in both the binding and reaction of cytochrome c with cytochromes c₁ and oxidase.

The great weight of chemical modification data available makes it attractive to estimate the contribution each lysine amino group on cytochrome c makes to the total electrostatic free energy of activation for the reactions with cytochrome c₁ and cytochrome oxidase. Although the decrease in reaction rate caused by modification of a lysine group could be due either to the elimination of the electrostatic interaction or to some type of steric effect, the steric effect should be much smaller with the CF₃CO— group than with the bulky CF₃PhNHCO— and carboxyldinitrophenyl groups. In the present paper, we have estimated the electrostatic free energy of interaction of each lysine by comparing the effects of the three different types of chemical modification on the reaction rate.

The total electrostatic interaction between charged reactants has also been estimated from the dependence of the reaction rate on ionic strength. In 1922, Bronsted (12) proposed that the rate constant at ionic strength, I, could be related to the rate constant at zero ionic strength through the formula:

\[ k_i = k_0 \frac{\gamma_A \gamma_B}{\gamma_{AB}} \]  

where \( \gamma_A \) and \( \gamma_B \) are the activity coefficients of the reactants, and \( \gamma_{AB} \) is the activity coefficient of the activated complex whose formation is assumed to be rate-limiting. If the activity coefficients are approximated by the Debye-Hückel relationship (13),

\[ \ln \gamma_i = -\frac{Z_i^2e^2}{2DKT} \left( 1 + \kappa a_i \right) \]

then the rate constant becomes:

\[ \ln k_i = \ln k_0 - \frac{e^2}{2DKT} \left( \frac{Z_A^2}{1 + \kappa a_A} + \frac{Z_B^2}{1 + \kappa a_B} - \frac{(Z_A + Z_B)^2}{1 + \kappa a_{AB}} \right) \]

where

\[ \kappa = \sqrt{\frac{8 Ne^2I}{1000DKT}} \]

\( Z_i \) is the charge on species \( i \), and \( a_i \) is the distance of closest approach between species \( i \) and buffer ions. Goldkorn and Schejter (14) have shown that equation 3 is in good agreement with experimental data for the reaction between horse heart cytochrome c and ascorbate, provided that the ionic strength...
Interaction of Cytochrome c with Cytochromes c and Oxidase

is less than 0.01 m. Koppenol (15) has recently extended this treatment by including in the activity coefficient expression a dipole moment term which can be very important over the ionic strength range 0.01 to 0.1 m. However, he cautions against applying the formula at higher ionic strengths. In a somewhat different approach, Wherland and Gray (16) have used the Marcus theory formalism to relate the rate constant to the electrostatic free energy of the activated complex, V, and the rate constant at infinite ionic strength, $k_e$:

$$k_1 = k_e e^{-V/RT}$$

(4)

If $V$ is approximated by Debye's formula (17):

$$V = 2.1175 \frac{e^{e^\alpha} + e^{e^\alpha}}{1 + e^{e^\alpha}} \frac{ZaZb e^{-\gamma}}{r}$$

(5)

then the rate constant becomes:

$$\ln k = \ln k_e - 3.576 \frac{e^{e^\alpha} + e^{e^\alpha}}{1 + e^{e^\alpha}} \frac{ZaZb}{(a + b)}$$

(6)

where it is assumed that $r$, the distance between the centers of the two reactants, is $a + b = 0.329 \sqrt{A}^{-1}$; numerical constants are appropriate for aqueous solutions at 25°C, and the distances are in angstroms. Equation 6 is numerically very similar to equation 3 at low ionic strength, and under these conditions, it is in good agreement with the measured reaction rates of a number of electron transport proteins with small oxidation-reduction agents (14, 16, 18).

Equations 3 and 6 are clearly inadequate for the reactions of cytochrome c with its oxidase and reductase because they predict that a change in the net charge $Z_a$ due to chemical modification of a lysine anywhere on cytochrome c would have the same effect on the reaction rates, whereas in fact only modification of those lysines immediately surrounding the heme crevice decreased the reaction rates (1-8). In the present paper, we have developed a semiempirical relationship that is in good agreement with both the experimental ionic strength dependence of the reaction rates and the chemical modification studies. The total electrostatic free energy of activation was calculated for both the oxidase and reductase reactions.

EXPERIMENTAL PROCEDURES

Materials—Horse heart cytochrome c (type VI). Mops, sodium cholate, and TMPD were obtained from Sigma Chemical Co. Tris was obtained from Schwarz/Mann. Native cytochrome c and CP,PHHCO—Lys 13 cytochrome c were prepared and purified as described by Smith et al. (4), while CP,CO—Lys 13 cytochrome c was prepared as described by Staudenmayer et al. (2). Beef heart succinate-cytochrome c reductase was purified by the method of Yu et al. (19).

Cytochrome c-depleted Keilin-Hartree particles were prepared by the method of Smith and Camerino (20) and stabilized with glycerol as described by Ferguson-Miller et al. (21). Protein was determined by the biuret method after solubilization of the Keilin-Hartree particles as described by Jacobs et al. (22).

Ascorbate-TMPD-Cytochrome Oxidase Activity—The cytochrome oxidase activity of native and CP,PHHCO—lysin 13 cytochromes c was measured polarographically with a Gilson model KM Clark electrode cell using the ascorbate-TMPD system (21). Assays were carried out at 25°C in 0.05 M Tris-Cl (pH 7.5) containing 0 to 350 mM NaCl, 250 mM sucrose, 7 mM ascorbate (from a fresh stock solution of 0.5 M Tris-ascorbate containing 1 mM EDTA and adjusted to pH 7.5), and 0.7 mM TMPD (from a fresh stock solution of 50 mM TMPD-2HCl). Keilin-Hartree particles were treated with 5% deoxycholate (1 mg/mg of protein) as described by Smith and Camerino (20) and added to the oxygen electrode cell to give a final concentration of 0.056 mg of protein/ml. The low base-line rate of oxygen consumption was subtracted from the rates of oxygen consumption measured after the addition of various concentrations of cytochrome c (0.01 to 54 mM, final concentration). Cytochrome c stock solutions were prepared in the same way as those used in the ascorbate-TMPD-cytochrome oxidase ionic strength dependence studies described above. The net velocities were calculated assuming 250 nmol of O2/ml of buffer.

Spectrophotometric Cytochrome Oxidase Activity—The cytochrome oxidase activity of native cytochrome c was measured spectrophotometrically using an Amino DW-2s spectrophotometer in the dual wavelength mode. Assays were carried out at 25°C in 50 mM Tris-Cl buffer (pH 7.5), the ionic strength of which was varied by addition of 0.5 M NaCl. Stock solutions of ferrocyanochrome c were prepared by reduction with ascorbate followed by chromatography on a small Bio-Gel P-60 column equilibrated with 50 mM Tris-Cl (pH 7.5) to remove the reducing agent and any polymeric material. After addition of various sized aliquots of the stock solutions to the reaction buffer, cytochrome c concentrations (0.1 to 5 mM) were determined from $A_{403-434}$ (nm) using $A_{403-434}$ nm = 83.4 mm$^{-1}$ cm$^{-1}$ (23). The rate of oxidation of cytochrome c was measured as $A_{403-434}$ (nm after addition of Keilin-Hartree particles (0.0008 mg of protein/ml, final concentration) prepared as above. Ionic strengths were calculated assuming $pK_a = 8.0$ for Tris and were corrected for dilution due to addition of aliquots of cytochrome c stock solutions and Keilin-Hartree particles.

Spectrophotometric Succinate-Cytochrome c Reductase Activity—The succinate-cytochrome c reductase preparation was treated with 5% cholate (1 mg/mg of protein) and diluted immediately prior to use with 50 mM Tris-Cl buffer, pH 7.5, containing 10 mM sodium succinate. The rate of reduction of cytochrome c following the addition of reductase was monitored at 420 nm on a Cary 14 spectrophotometer. The assay medium contained 0.1 to 12 mM ferrocyanochrome c, 10 mM succinate, 50 mM Tris-Cl, pH 7.5, and 0 to 0.8 M NaCl.

RESULTS

Ionic Strength Dependence of the Reactions between Cytochrome c and Cytochrome oxidase and Succinate-Cytochrome c Reductase—The effect of ionic strength on the activities of native and singly modified cytochromes c with beef heart cytochrome oxidase activity was measured in 50 mM Tris-Cl (pH 7.5) buffer containing 0 to 350 mM NaCl using the ascorbate-TMPD polargraphic assay (21). At the lowest salt concentration (50 mM Tris-Cl), low (0.01 to 0.25 mM) cytochrome c concentrations were used to ensure that the high affinity phase of the reaction was being measured (21), while at higher ionic strengths, the ascorbate-TMPD-cytochrome oxidase activity was determined at the lowest cytochrome c concentrations for which comparable rates of O2 uptake could be measured. Representative results for the native protein are presented as Eadie-Hofstee kinetic plots (Fig. 1) from which the parameter $V_{max}/K_m$ was obtained by extrapolating $V/S$ to $V = 0$ at each ionic strength. At higher ionic strengths, the reaction appeared to be monophasic with a maximum velocity about the same as the total (high affinity plus low affinity phases) maximum velocity at the lowest ionic strength. While $V_{max}$ was affected little or not at all by increasing ionic strength, Fig. 2 shows that $ln(V_{max}/K_m)$ decreased approximately linearly as $\sqrt{I}$ increased, suggesting the importance of electrostatic interactions in the cytochrome c-cytochrome oxidase reaction. The reaction of CF3PHHCO—lysin 13 cytochrome c was not lyophilized at any stage of their preparation or purification. Before use in assays, samples were chromatographed on small Bio-Gel P-60 columns equilibrated and eluted with 0.05 M Tris-Cl (pH 7.5) containing the appropriate NaCl concentration. The net velocities were calculated assuming 250 nmol of O2/ml of buffer.
of Keilin-Hartree particles to obtain comparable O2 uptake rates, suggesting that the reduction of cytochrome c might be rate limiting under these conditions. Alternatively, higher levels of Keilin-Hartree particles could be required to offset a loss of succinate dehydrogenase activity resulting from detergent pretreatment (24). Eadie-Hofstee plots were monophasic over the entire ionic strength range (not shown), and ln(Vmax/Km) decreased almost linearly as √I increased (Fig. 2).

The ionic strength dependence of the reaction between ferrocytochrome c and cytochrome oxidase was also studied spectrophotometrically using the same buffer system used for the polarographic assays. The reaction was carried out at the lowest possible cytochrome c concentrations to ensure that the high affinity phase was observed. As before, Vmax/Km values were obtained by extrapolating V/S to V = 0 on Eadie-Hofstee plots, and ln(Vmax/Km) decreased linearly as √I increased although with a slope slightly more positive than for the polarographic oxidase assay (Fig. 3). We also found that at ionic strength ≤0.05 M, Vmax/Km became nearly independent of ionic strength, and that the CF3PhNHCO—lysine 13 cytochrome c derivative had a Vmax/Km value approximately 20% larger than did the native cytochrome c. At ionic strengths above 0.1 M, however, the derivative had a Vmax/Km value about 6-fold less than did the native protein (data not shown) as was the case in the polarographic assay.

We have previously shown that the reduction of cytochrome c by succinate-cytochrome c reductase is rate-limited by the cytochrome c1-cytochrome c step only at low cytochrome c concentrations, and thus Vmax/Km is the only kinetic parameter that is a true measure of the cytochrome c1-cytochrome c reaction (6). At ionic strengths below 0.1 M Vmax/Km was independent of ionic strength, but at ionic strengths above 0.2 M, Vmax/Km decreased rapidly. Fig. 3 shows that again ln(Vmax/Km) decreased almost linearly as √I was increased over the range 0.4 to 1.0 M, reaffirming that electrostatic interactions are important in the reaction. The ionic strength dependence

**Fig. 1.** Polarographic cytochrome oxidase activity of native horse heart cytochrome c at 25 °C in 0.05 M Tris-Cl (pH 7.5), 250 mM sucrose, 7 mM ascorbate, and 0.7 mM TMPD. Keilin-Hartree particle concentration was 0.056 mg of protein/ml, and velocities are in micromolar concentrations of O2 reduced/min. The ionic strength was adjusted with NaCl to 0.038 M (○), 0.066 M (△), and 0.077 M (△).
and Ilan et al. (25) have shown that equations 3 and 6 are valid below 0.01 M ionic strength for the reactions of cytochrome c with small oxidation-reduction agents. Unfortunately, when the reactions between cytochrome c and its physiological oxidation-reduction partners cytochrome c1, cytochrome oxidase, and cytochrome c peroxidase are measured spectrophotometrically at ionic strengths below 0.1 M, the reaction rate is either not affected by specific lysine modifications and ionic strength changes or is affected in an opposite manner from that observed at higher ionic strengths (1, 5–7, present work). This is not because electrostatic interactions become unimportant at low ionic strength, but because the rate-limiting step in the reaction changes to one not involving them (1, 5–7). It is, therefore, desirable to develop a new relationship valid at ionic strengths above 0.1 M where all of the reactions are sensitive to electrostatic interactions.

It has been argued that a major reason equations 3 and 6 break down at higher ionic strength is that the Debye–Hückel (13) approximation is no longer valid. While it is true that the assumptions used to derive the Debye–Hückel formula are not valid at high ionic strength, the formula itself is not a bad empirical relationship for strong monovalent electrolytes. The experimental activity coefficients of monovalent electrolytes such as KCl, NaCl, and NH4Cl differ from equation 2 by less than 10% up to 0.5 M, provided that parameter α is optimized (26). While these errors are obviously important when considering fundamental theories of electrolytes, they are not serious for the type of semiempirical treatment considered here.

Pitzer (27) recently reviewed the application of rigorous Monte Carlo methods to monovalent electrolytes and found that the correct radial distribution function is in excellent agreement with the exponential Debye–Hückel expression upon which equation 5 is based up to 0.42 M ionic strength. Even more pertinent to the present investigation, Pimmert-Haymann and Weissmann studied the reaction between CO(NH2)2Br+ and OH− in the presence of nine different monovalent electrolytes at ionic strengths up to 1.5 M (28). All of the data were within 3% of equation 3 up to 1.5 M ionic strength using the parameters Z1 = +2, Z2 = −1, and α1 = α2 = aAB = 3 Å. The deviation was considerably larger for the divalent electrolyte Na2SO4.

The most serious deficiency in the use of equations 3 and 6 for reactions between proteins is the failure to consider individual charge interactions. A general expression for the electrostatic energy of interaction between two proteins is:

\[ V = \sum_j \sum_i V_{ij} \]

(7)

where the first summation is over all charged groups i on protein A, the second summation is over all changed groups j on protein B, and \( V_{ij} \) is the electrostatic energy of interaction between charges i and j. The electrostatic energies \( V_{ij} \) will be a complex function of the charges on i and j, the distance separating i and j, \( r_{ijn} \) the geometry of the two proteins, and the ionic strength. The problem is in some respects analogous to the intramolecular electrostatic stabilization of proteins, studied recently by Friend and Gurd (29). They found that for myoglobin at 0.01 M ionic strength, out of a total electrostatic stabilization of −9.7 kcal/mol, −7.8 kcal/mol was contributed by 12 specific charge-pair partners with \( r_{ij} \) values ranging from 1.8 to 4.6 Å. They found that \( V_{ij} \) decreased very rapidly as \( r_{ij} \) increased, with very few significant interactions with \( r_{ij} \) greater than 10 Å. Furthermore, they found that most of the charged groups on myoglobin had relatively large static solvent accessibility factors, indicating appreciable exposure to the solvent. At ionic strengths above 0.1 M, the charge interactions \( V_{ij} \) will decrease even more rapidly as \( r_{ij} \) increases, and it is expected that the overall electrostatic free energy of
interaction between different electron transport proteins will be dominated by short range charge-pair interactions. This concept is supported by the theoretical model for the activated complex between cytochrome c and cytochrome b5 proposed by Salemme (30), in which lysines 13, 27, 72, and 79 surrounding the heme crevice of cytochrome c form specific complementary charge interactions with the cytochrome b5 carboxylate groups of Glu 52, Glu 48, Asp 64, and the exposed heme propionate, respectively. A very significant feature of this model is that the distances between an amino group and a carboxylate group within a complementary charge pair interaction is about 3 Å, while the distance between this amino group and any other charged group is greater than 10 Å.

The following assumptions will be made to obtain a simplified expression for the electrostatic free energy of activation of cytochrome c reactions at ionic strengths above 0.1 M. (a) The total electrostatic interaction between the two proteins will be assumed to consist of n complementary charge pairs, each with \(Z_i = -Z'_j = 1\) and separation distance \(r_{ij}\). The different charge pairs will be assumed to be separated from each other by at least 10 Å, and all interaction energies \(V_{ij}\) with \(r_{ij}\) greater than 10 Å will be neglected. This reduces the double summation of equation 7 to a single summation. (c) All of the charged groups involved in the interaction are assumed to be fully solvated and equation 5 will be used to estimate \(V_{ij}\). The expression for the total electrostatic free energy of activation then becomes:

\[
V = -4.235 \sum \frac{e^{\alpha(r_{ij})}}{(1 + \kappa r_{ij})}
\]

(8)

Substitution of equation 8 into equation 4 yields:

\[
\ln(k/k_a) = 7.152 \sum \frac{e^{\alpha(r_{ij})}}{r_{ij}(1 + \kappa r_{ij})}
\]

(9)

**Electrostatic Free Energies of Interaction of Cytochrome c Lysines**—It is apparent from the chemical modification data shown in Figs. 4 and 5 that the different lysines surrounding the heme crevice of cytochrome c make different contributions to the overall electrostatic interaction. An approximation to the contribution a positively charged amino group on lysine \(i\) makes to the total electrostatic free energy of activation is given by

\[
V_i \approx \Delta G_{native}^* - \Delta G_{derivative}^* = -R T \ln Y_i
\]

(10)

where \(Y_i\) is the ratio of the rate constant of native cytochrome c to that of a derivative modified at lysine \(i\) with CF3CO— or
CF₃PhNHCO—(16). The corresponding formula for the carboxydinitrophenyl derivatives is

\[ V_i = -\frac{1}{2}RT \ln Y_i \]  

(11)
since the positively charged lysine is converted to a negatively charged group which would cause twice as large a change in the electrostatic free energy, neglecting any changes in \( r_i \). The agreement between the reaction rate ratios of the three different types of derivatives is quite good, indicating the major effect of modification was electrostatic. Any steric effect would be expected to be larger for the bulky CF₃PhNHCO— and carboxydinitrophenyl groups than for the CF₃CO— group.

The best estimate of the electrostatic free energy contribution of lysine \( i \) shown in Figs. 6 and 7 was assumed to be the smallest \( V_i \) value of the three types of derivatives. The \( V \) values of lysines 5, 39, and 53 were estimated from the differential acetylation rates (9) using a constant scaling factor to bring this technique into general correspondence with the chemical modification method. The final estimates shown in Figs. 6 and 7 indicate that the electrostatic free energies range from 0.94 kcal/mol for lysine 13 down to 0 kcal/mol for lysines at the back of the molecule. The total electrostatic free energy of activation is estimated to be the sum of all the individual electrostatic free energies shown in Fig. 6. There are no other charged groups surrounding the heme crevice of cytochrome \( c \) which would be closer than 10 Å to charged groups on cytochrome \( c_r \), with the possible exception of Glu 90 at the top of the heme crevice and the heme group itself. The total electrostatic free energy estimated in this way was \(-5.0 \) kcal/mol for the reductase reaction at 0.2 M ionic strength and \(-6.0 \) kcal/mol for the oxidase reaction at 0.05 M ionic strength.

To compare the chemical modification studies to the ionic strength studies, we have used the electrostatic free energy estimates, \( V_i \), and equation 8 to calculate the distance, \( r_i \), between lysine \( i \) and a carboxylate group presumed to be complementary to it on the other protein (Fig. 6). These estimates range from 3.4 Å for lysine 13 to 6.8 Å for some of the weaker complementary charge interactions involving lysines 8, 73, etc. In all cases, the effective radius of the individual amino and carboxylate groups, \( a \), was assumed to be 1.7 Å. It is possible that in some cases more than one carboxylate group contributed to the electrostatic free energy of a given lysine, but this is replaced in the calculation by one “equivalent” carboxylate at a distance which gives the correct energy. The distances calculated are in good general agreement with the distances found in the theoretical model of the cytochrome \( b_5 \)-cytochrome \( c \) complex (30). Equation 9 was then used to calculate the ionic strength dependence of the reaction rates, with good agreement between theory and experiment as shown in Figs. 2 and 3.

**DISCUSSION**

It should be emphasized that equation 8 is at best a first order approximation to the correct electrostatic energy of interaction between cytochrome \( c \) and its oxidation-reduction partners at ionic strengths above 0.1 M. It is only valid for proteins that have evolved a set of complementary charge interactions that dominate the total electrostatic interaction. The neglect of interactions between charges separated by more than 10 Å is supported by the chemical modification studies and by recent Monte-Carlo calculations of monovalent electrolytes which show the rapid decrease in electrostatic energy as \( r \) is increased (27). At ionic strengths above 0.05 M, \( V_i \) is less than one-tenth as large at \( r = 10 \) Å as at \( r = 3 \) Å. The most striking feature of the ionic strength dependence of the reaction rates is the very small deviation from linearity observed in the \( \ln(V_{max}/K_m) \) versus \( \sqrt{I} \) plots. This is the same pattern observed for the reactions between small molecules at high ionic strength (28) and supported the concept that the total electrostatic interaction is dominated by short range charge-pair interactions. Our use of Debye’s equation 5 as an empirical relationship for \( V_i \) is supported both by accurate Monte-Carlo calculations of the radial distribution function of monovalent electrolytes at high ionic strengths (27) and by the ionic strength dependence of the reactions between small molecules (28). Our studies of the ionic strength dependence of the cytochrome \( c \) oxidase reaction are in qualitative agreement with earlier studies by Nicholls (31, 32) and Davies et al. (33), using phosphate buffers, but polyvalent electrolytes have been shown to deviate much more significantly from the Debye-Hückel formulas than monovalent electrolytes (26, 28).

The slight difference in the ionic strength dependencies of the spectrophotometrically and polarographically measured oxidase reactions may be a reflection of the measurement of different reaction pathways under different and changing assay conditions. For example, Ferguson-Miller et al. (5, 21) have suggested that under the low ionic strength conditions normally used in the polarographic assay, oxidase-bound cytochrome \( c \) is reduced by TMPD at a rate which is significantly faster than the rate of dissociation of the complex. However, Hill and Nicholls (34) have reported that the mechanism of reduction of cytochrome \( c \) by TMPD appears to be affected by ionic strength.

Probably the best justification for the general approach taken here is the good agreement of equation 9, using parameters based on the chemical modification studies, with the experimental ionic strength dependence of the reaction rates. The electrostatic free energies of individual lysines, \( V_i \), shown in Fig. 7 are thought to be reasonably accurate because in most cases they are based on three different types of chemical modifications. It has been shown that these modifications do not lead to significant changes in the oxidation-reduction potential or the overall conformation of cytochrome \( c \) as determined by visible absorption spectrophotometry, optical rotatory dispersion, and proton magnetic resonance. The good correspondence between the ionic strength dependence of the oxidase and reductase reactions provides additional evidence that the two reactions occur at similar sites on cytochrome \( c \).

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Interaction of Cytochrome c with Cytochromes c and Oxidase

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Electrostatic interaction of cytochrome c with cytochrome c1 and cytochrome oxidase.
H T Smith, A J Ahmed and F Millett

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