Electrostatic Effects on the Kinetics of Oxidation-Reduction Reactions of c-type Cytochromes*

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The kinetics of the oxidation-reduction reactions between horse heart cytochrome c, Euglena gracilis cytochrome c552, and ions (ascorbate, ferricyanide, and ferrocyanide) was investigated as a function of ionic strength at pH 7, 25°C. The ionic strength was varied between 0.002 and 0.02 M. Data were analyzed according to four different functions of ionic strength. Results showed that the Kirkwood-Tanford smeared charge model holds well for the calculation of the activity coefficients and that the whole charges of these proteins are reflected in the rates of their reactions. Chemical modifications or changes in the pH that altered the charge of the proteins affected the primary salt effects as predicted by the smeared charge model.

In studies of reactions in which ions participate, an essential step is the investigation of ionic strength effects. This is important for two main reasons. First, the ionic strength dependence of the rate constants provides an appreciation of the effective electrostatic charges of the reactants; second, the extrapolations of the observed rate constants to zero ionic strength yield those values of the rate constants that must be used for the proper evaluation of activation parameters. The necessity of correct estimation of the enthalpies and entropies of activation is clearly enhanced in the elucidation of reaction mechanisms in which tunneling effects may be involved (1-3).

Previous investigations of the effect of ionic strength on the oxidation-reduction potentials of c-type cytochromes showed that the reduction of horse heart cytochrome c at neutral pH is accompanied by a net change of the charge of the protein from +8.3 to +7.5 (4), while for E. gracilis cytochrome c552 the charge is from -8 to -9 (5). These values were obtained by using the Kirkwood-Tanford (6-8) smeared charge model for globular proteins. The use of this model was justified by the estimated experimental values, which were very close to those expected from the chemical composition of the proteins and their prosthetic groups.

The same model should be expected to provide a correct description of the effects of ionic strength on the kinetics of oxidation-reduction reactions of c-type cytochromes. Thus, for example, an investigation of the rate of reduction of cytochrome c by the superoxide ion, O₂⁻, resulted in an estimated net charge of the protein of +6.3 (9). However, other results available in the literature are in conflict with this model. Thus, for the reduction of ferric horse cytochrome c by ferrous ethylenediaminetetraacetate, the apparent net charge of the protein was evaluated as +1.7 (10); for the oxidation of ferrous cytochrome c by cobaltic orthophenanthroline, the charge of the protein was estimated as +0.4 (11); while the charge of cytochrome c552 was found to vary between -0.4 and -1.8 in its reactions with several ionic oxidants and reductants (12). These values are much smaller than those expected from the chemical composition of these proteins and were interpreted as due to ionic strength effects on the rate constants involving only the charge at a specific site of electron transfer and not the whole charge of the protein (13, 14).

The unexpectedly low values estimated for the protein charges may be due to two different reasons. One possible reason is the range of ionic strength investigated, since deviations from simple Debye-Hückel behavior for small electrolytes are already apparent at I > 0.01 (15); another reason is the nature of the function employed to analyze the data. Most of the results previously described (10-12) were obtained in solutions of ionic strengths I > 0.04; furthermore, the data in several publications were analyzed in terms of functions of the ionic strength similar to those arising from the Kirkwood-Tanford model (6-8), while in others the Debye-Hückel limiting law was used.

The present investigation was undertaken in order to settle the apparent contradictions found in the literature. Cytochrome c, from horse heart, and cytochrome c552, from E. gracilis, were observed in their interactions with ionic oxidants and reductants. In all cases, when correct functions were used to express the activity coefficients of the proteins and the ionic strengths were kept in the range 0.002 to 0.01, the net charges estimated for the proteins were very close to those predicted from the chemical compositions of the latter. This behavior was maintained by the proteins when their charges were altered either by chemical modification or by manipulating the pH of the medium.

MATERIALS AND METHODS

Horse heart cytochrome c (Sigma Chemical Co., type VI) was percolated through Bio-Gel P-4 columns equilibrated with Tris-cacodylate buffer, 0.002 M, pH 7.2. When type III cytochrome c was used, it was further chromatographed on carboxymethyl cellulose columns (1 x 30 cm) using an eluant salt gradient of phosphate buffer, pH 7.0, from 0.01 to 0.1 M. The eluted protein was desalted on Bio-Gel P-4 columns, brought to isoelectric point, and percolated again through Bio-Gel P-4 column equilibrated with Tris-cacodylate buffer, 0.002 M, pH 7.2. Pyridoxal phosphate-cytochrome c was prepared as described elsewhere (16).

E. gracilis, strain Z, was grown at room temperatures in the light and its cytochrome c552 was purified according to Pettigrew (17). Just before use the protein was desalted on Bio-Gel P-4 columns and eluted with Tris-cacodylate buffer, 0.002 M, pH 7.2.

All of the reagents used were of analytical grade. The rates of reactions were studied by following the transmittance changes at 550 nm for horse cytochrome c and at 582 nm for Euglena cytochrome c552 on a Gibson-Durrum stopped flow spectrophotometer. Observed rate constants were obtained by least square analysis of at least four different runs.

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All of the kinetic measurements were performed at 25°C. The solutions were 10^-5 M to 10^-3 M in cytochrome c and were buffered at pH 7.2 with 0.002 M Tris-cacodylate; at pH 3.0, glycine-HCl, 0.002 M; at pH 4 to 5, sodium acetate-acetic acid, 0.004 M; and at pH 10.5, glycine-NaOH, 0.002 M. The ionic strength was provided by addition of appropriate amounts of various salts: NaCl, MgCl2, MnSO4, Na2SO4, and NaClO4, as well as by increasing concentrations of the buffer. No specific salt effects were observed, and the results presented here were those obtained with NaCl. The ionic strength of each solution was calculated as the sum of the contributions of the salt added; the buffer used (I = 0.001), the protein (I = 0.001) (4); and the contribution of the reductant or oxidant used. The range of ionic strength was from I = 0.002 to I = 0.02.

The reactions of cytochromes with oxidizing or reducing anions were studied at large excess of concentration of the latter. The observed pseudo-first order rate constants increased linearly with the concentration of the reactant in excess. From these linear plots, the second order rate constant, k, for the different reactions was estimated.

The reaction of horse heart ferricytochrome c with ascorbate is biphasic, and the rate constants for the last phase of the reduction increase linearly with ascorbate concentration (18). This study relates solely to the first phase of the reduction of horse cytochrome c by ascorbate.

In order to analyze the results, the following considerations were made. Assuming the Debye-Hückel treatment for the activity coefficients, and assuming further that the net charge of the transition state complex is the sum of the charges of the reactants, the general equation for the effect of ionic strength, I, on the second order rate constant, k, is (15):

$$\log k = \log k_0 + \frac{A(Z_p + Z_r)^2 - (Z_p^2 - Z_r^2)}{1 + B\alpha_1}$$  \hspace{1cm} (1)

where $A = 0.509$ and $R = 0.299$ in water at 25°C, $Z_p$ and $Z_r$ are the charges of protein and oxidation-reduction reactants, respectively, and $\alpha_p$, $\alpha_r$, and $\alpha$ are the distances of closest approach between the ions in the solution and the solvated protein, the oxidation-reduction reactant, and the transition state complex, respectively. For $\alpha_p$ and $\alpha_r$, values of 180 nm and 40 nm were used. Brgnsted's assumption (15) consists of replacing these distances of closest approach by a mean value, $\bar{\alpha}$, that was taken as 180 nm (4). This leads to:

$$\log k = \log k_0 + \frac{4A\bar{\alpha}_1 ((Z_p + Z_r)^2 - Z_p^2 - Z_r^2)}{1 + \bar{B}\alpha_1}$$  \hspace{1cm} (2)

which reduces to:

$$\log k = \log k_0 + 2A\bar{Z}_pZ_r\sqrt{\bar{\alpha}_1}$$  \hspace{1cm} (3)

the well known Brgnsted-Debye-Hückel expression.

For sufficiently small values of $\bar{\alpha}$, and I > $\bar{\alpha}$, Equation 3 reduces further to the limiting form:

$$\log k = \log k_0 + 2A\bar{Z}_pZ_r\sqrt{\bar{\alpha}_1}$$  \hspace{1cm} (4)

A somewhat different expression can be obtained by introducing into Equation 1 the assumption: $a_2 = a^* > a$. This leads to:

$$\log k = \log k_0 - \frac{AZ^2_1\sqrt{\bar{\alpha}_1}}{1 + Ba_1\sqrt{\bar{\alpha}_1}} + \frac{A(Z_p + Z_r)^2\sqrt{\bar{\alpha}_1}}{1 + Ba_2\sqrt{\bar{\alpha}_1}}$$  \hspace{1cm} (5)

Finally, an equation can be derived by introducing a "work term" for electrostatic interactions (19) into the expression given by Marcus (20) for the activated complex of outer sphere electron transfer reactions. The equation is (19):

$$\log k = \log k_0 - 1.6\frac{Z_pZ_r}{a_0 + \alpha_{\bar{\alpha}}} \left[ e^{-Ba_1\sqrt{\bar{\alpha}_1}} + e^{-Ba_2\sqrt{\bar{\alpha}_1}} \right]$$  \hspace{1cm} (6)

It should be noted that in Equation 6 the extrapolation is to infinite ionic strength, $k_0$. Equations 3, 4, 5, and 6 were used in the analysis of the data, as described below.

**RESULTS**

Fig. 1 shows the ionic strength dependence of the rate constants for the reduction of cytochromes c and c552, at pH 7.2 and 25°C, in terms of Equations 3 and 4. It is clear from this figure that the net charges estimated for the proteins depend very markedly on the nature of the function employed. Thus, the charges estimated according to Equation 1 are +8.3 and -7.8 for cytochromes c and c552, respectively. These values are very close to those expected from the chemical compositio-
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When Equation 4 is used, the calculated net charges are +3.3 for horse cytochrome c and -4.1 for cytochrome c552. It is important to notice that the absolute values of the charges estimated from Equation 4 are, although small in comparison to the full net charges, still definitely higher in the present experiments than in those reported by other authors. For example, the charge estimated for cytochrome c552 in its reaction with ascorbate was -0.5 (12). The only apparent reason for this discrepancy is the range of ionic strength investigated: 0.002 to 0.01 in our experiments, as compared to 0.04 < I < 0.2 (12).

While Equations 4 and 3 lead to clearly different estimates of the electrostatic charges of the proteins, Equations 5 and 6 give similar values to those obtained from Equation 3. This is shown in Fig. 2, where the effects of ionic strength on the oxidation of cytochrome c552 by ferricyanide are analyzed in terms of the three different functions.

The slopes that describe the ionic strength dependence of the rate of cytochrome c reduction by ascorbate were altered as expected by modification of the protein with pyridoxal phosphate. This is shown in Fig. 3.

Similarly, the slopes obtained for the reduction of cytochrome c552 by ferrocyanide were clearly dependent on the pH

![Figure 3](image-url)

**Fig. 3.** Plot of log k as a function of \((J/(1 + 6J))\) for the reduction of 5 x 10^{-4} M horse heart ferricytochrome c (A) and pyridoxal phosphate-1-ferricytochrome c (B) by ascorbate. Other conditions as in Fig. 1.

![Figure 4](image-url)

**Fig. 4.** The dependence on ionic strength at different pH values. Plot of log k/k0 as a function of \((J/(1 + 6J))\) for the reduction of 5 x 10^{-4} M Euglena ferricytochrome c552 by ferrocyanide at 25°C. Buffers used: pH 7.2: Tris-cacodylate, 0.002 M; pH values 5.4 and 4.5: sodium acetate-acetic acid, 0.004 M; and pH 3.0: glycine-HCl, 0.002 M.

### Table I

| Protein Reagent | pH | Theoretical charge of protein | Calculated charges of protein \(Z_p\) according to: | Rate constants extrapolated to \(I = 0\)  
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<td>Ascorbate</td>
<td>7.2</td>
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<td>+8.3</td>
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<tr>
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<td>6</td>
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Values obtained from different functions for the electrostatic charges of c-type cytochromes and for the rate constants of their reactions at zero ionic strength.

Theoretical charges of the proteins calculated from their chemical composition, assuming the following pK' values: aspartate, glutamate, and propionic side chains of heme: 4.5; histidine: 6.0; lysine and tyrosine: 10.5; arginine: 12.5; terminal amino group: 9.0; terminal carboxyl group: 2.0.

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of the medium (Fig. 4). The values estimated for the electrostatic charges of the proteins in their reaction with ferricyanide, ferrocyanide, and ascorbate under diverse conditions, using all of the functions described above, are summarized in Table I.

**DISCUSSION**

Two models can be used for the interpretation of salt effects on the rates of oxidation-reduction reactions of cytochromes. One model assumes that the salt effects depend only on the electrostatic charge of a site in the protein (13, 14); the other model involves the net charge of the whole protein. The “site” model predicts that a small electrostatic charge will be made evident by analysis of ionic strength effects; indeed, charges of +0.4 to +1.7 and −0.7 to −1.4 were reported for reactions of cytochromes c and ciso, respectively (10–12). The charges can be estimated by using different functions of the ionic strength. In this study we present a series of identical data analyzed in terms of four different functions.

In the first place, it is evident (Fig. 1) that the use of the Debye-Hückel limiting law leads to a markedly lower value of the estimated charge. This is obviously due to the fact that in the derivation of Equation 4, the approximation \( B a \sqrt{I} \ll 1 \) is made. This approximation can be justified only if \( a \mu I \) is very small, which, in turn, puts a rigid constraint on the experimental ionic strengths that may be used for studies of large polyelectrolytes, such as proteins. Our results show (Fig. 1 and Table I) that when \( I \) is kept below 0.01, the net charges estimated are distinctly larger than those obtained from the limiting law function in previous studies. This result emphasizes the fact discussed above that kinetic studies intended to determine electrostatic charges of proteins must be restricted to very low ionic strengths. Although this result in itself is not enough to disprove the “site” model, it certainly raises the question of the size that may be attributed to such a “site.”

Furthermore, the values of the rate constants extrapolated to \( I = 0 \) from two different regions of ionic strength are very different. Thus, for example, at 20°C the oxidation of cytochrome ciso by ferricyanide studied at 0.04 < \( I < 0.2 \) leads to \( k_0 = 5.0 \times 10^5 \) s\(^{-1}\) (12); while the same reaction studied at 25°C and 0.02 < \( I < 0.01 \) leads to \( k_0 = 2 \times 10^5 \) m\(^{-1}\) s\(^{-1}\) (Table I). These extrapolated values resulted from the use of Equation 4; if any of the other equations were used, the difference would be even larger. The use of functions that include the term \( B a \sqrt{I} \) was justified on theoretical and experimental grounds for cytochrome oxidation-reduction equilibria (4, 5). This justification implies a calculation of the activity coefficients of proteins that includes both their whole charge and their radii (4). A recent, more detailed treatment that takes into account the location of the charges on the protein surface leads to functions that are essentially identical with those obtained from the Kirkwood-Tanford smeared charge model at \( I < 0.05 \) (21). The use of functions with the term \( B a \sqrt{I} \) for the analysis of salt effects on kinetic studies is thus theoretically correct. On the other hand, the approximation implied in the use of the limiting law is unwarranted already at \( I = 0.01 \) for monovalent salts, the more so for reactions between large ions, such as iron hexacyanides or ascorbate, with proteins. Thus, the use of Equation 4 must be rejected a priori.

The model that involves the net charge of the whole protein leads to several predictions. First, the charges estimated from these equations, when proper values of the protein radii are used, should be those that can be evaluated from the amino acid and prosthetic group composition of each protein. This prediction is entirely corroborated for cytochromes c and ciso in reactions involving various ionic oxidants and reductants (Table I). It should be noticed that all of the functions that include terms of the \( B a \sqrt{I} \) form give essentially the same results (Fig. 2 and Table 1).

Moreover, the model predicts that if a cytochrome is chemically modified at random by a reagent that alters the charge at the modified sites, the ionic strength dependence of its reaction kinetics will be altered correspondingly: the charge estimated for the modified cytochrome will reflect the overall change in net charge of the whole molecule. Pyridoxal-phosphate-cytochrome c is a mixture of proteins with single modifications at lysine residues located at different positions in the molecular surface (16). The net charge estimated from the ionic strength dependence of the rate of pyridoxal-phosphate-cytochrome c reduction is 2 units less than that of the native molecule (Fig. 3 and Table I), as expected from the fact that 1 positively charged residue has been turned into a negatively charged modified residue. Since the modified groups are distributed in different areas of the protein surface, the result obtained could not have been predicted by the “site” model.

Finally, the net charge of the molecule may be changed by manipulating the pH of the medium. The smeared charge model predicts that the estimated net charge will vary according to the net charge of the protein at each different pH. Fig. 4 shows that in the case of cytochrome ciso, this result is indeed obtained (see Table I). The charges predicted from chemical composition, and assuming fixed values for the pK values of charged side chains, are in good agreement with the experimental values, except for the case of pH 5.4. This must probably indicate that the assumption of fixed values for the pK values is not justified at that particular pH. A more rigorous approach would take into account the actual pK values of the different groups; these can be estimated theoretically using the “solvent accessibility parameters” described by Gurd and co-workers (22, 23). However, the required crystallographic data are unavailable for cytochrome ciso. The lack of agreement for the pH 5.4, however, does not detract from the fact that the experimentally estimated charge is zero in the pH region corresponding to the isoelectric point of the protein (24). A similar study of cytochrome c is, unfortunately, precluded by the heme-linked ionization and conformation changes that this protein undergoes in the vicinity of its isoelectric point (25).

In conclusion, both equilibria and rates of oxidation-reduction reactions of cytochromes c and ciso in aqueous solutions are strongly affected by the ionic strength of the medium. For the investigation of primary salt effects, which represent an essential parameter in the study of these reactions, the Kirkwood-Tanford smeared charge model holds very well for these proteins within reasonable limits of the ionic strength.

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