Use of Specific Lysine Modifications to Identify the Site of Reaction between Cytochrome c and Ferricyanide*

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The site of the reaction between horse heart ferrocytochrome c and ferricyanide was investigated by measuring the reaction rate of cytochrome c derivatives specifically modified at single lysine residues to form trifluoroacetyl or trifluoromethylphenylcarbamyl amino groups. Cytochrome c derivatives singly modified at lysines 8, 13, 25, 27, 72, 79, and 87 surrounding the heme crevice had rate constants decreased from that of native cytochrome c by factors of 1.29, 2.03, 1.12, 1.35, 1.46, 1.29, and 1.19, respectively. Modification of a given lysine with the bulky trifluoromethylphenylcarbamyl group caused nearly the same decrease in reaction rate as modification with the trifluoroacetyl group, indicating that the effect was due to removal of an electrostatic interaction between the protonated lysine amino group and ferricyanide. Modification of lysines 22, 55, 99, and 100 at the right side, bottom, and back of cytochrome c had no effect on the reaction rate. These results indicate that the reaction site is located at the exposed edge of the heme and that the electrostatic interaction between ferricyanide and cytochrome c is dominated by the lysine amino groups surrounding the heme crevice, which include lysine 86, in addition to the ones listed above. We have used the specific lysine modification results to estimate the contribution of each lysine amino group to the electrostatic interaction and have developed a semiempirical relation for the total electrostatic interaction.

The mechanism by which cytochrome c transports electrons from cytochrome c to cytochrome oxidase in the mitochondrial membrane has been extensively studied, but remains poorly characterized because of the lack of detailed structural information or the complex membrane-bound oxidation-reduction partners of cytochrome c (1). For this reason, the reactions between cytochrome c and a number of small molecule oxidants and reductants have received considerable attention as well characterized model systems for the study of protein electron transfer reactions (2–5). The reaction between ferrocytochrome c and ferricyanide is one of the most rapid of these, with an overall rate constant of $k_w = 6$ to $16 \times 10^{10}$ M$^{-1}$ s$^{-1}$ as determined by stopped flow and T-jump measurements (6–8). Stellwagen and Shulman (9) have shown by NMR techniques that ferricyanide binds to cytochrome c in a rapid equilibrium before the rate-determining step and proposed the mechanism

$$k = k_w e^{-V/RT}$$

(1)

If $V$ is approximated by Debye’s formula (14),

$$V = 2.1175 \frac{e^{-a_s} + e^{-a_T}}{1 + \kappa a_s} \frac{Z_0 Z_B e^r}{r}$$

(2)

then the rate becomes

$$lnk = lnk_w - 3.576 \frac{e^{-a_s} + e^{-a_T}}{1 + \kappa a_s} \frac{Z_0 Z_B}{(a_s + a_T)}$$

(3)

where it is assumed that $r$, the distance between the centers of the two reactants, is $a_s + a_T$, $a_s$ and $a_T$ are the effective radii of the two reactants, $\kappa = 0.329 \sqrt{I} \text{ Å}^{-1}$, and the numerical constants are appropriate for aqueous solutions at 25°C. A somewhat different approach was taken by Broman (15), who proposed that the rate constant at ion strength $I$ could be related to the rate constant at zero ionic strength through the formula

$$k_I = k_w \gamma_A \gamma_B / (I + \kappa a_s)$$

(4)

where $\gamma_A$ and $\gamma_B$ are the activity coefficients of the reactants, and $\gamma_A b$ 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 relation (16),

$$lny = -1.17 Z_0^2 \frac{k}{I + \kappa a_s}$$

(5)

then the rate constant becomes

CII + Fe(CN)$_6^{3-}$ $\rightarrow$ CII-Fe(CN)$_6^{3-}$ $\rightarrow$ CIII-Fe(CN)$_6^{3-}$ $\rightarrow$

$k_1$ $k_2$ $k_3$

CIII + Fe(CN)$_6^{3-}$

A recent pulse radiolysis study by Broman and Shaffer (10) has now allowed the complete determination of all the kinetic parameters in Equation 1. The internal electron transfer rate constant $k_2$ was found to be $4.6 \times 10^4$ s$^{-1}$ and independent of ionic strength, while the complex formation and dissociation rate constants were strongly dependent on ionic strength. The reaction rates of cytochrome c with different transition metal complexes such as Co(phen)$_2^{3+}$, Ru(NH$_3$)$_6^{3+}$, Fe(EDTA)$_2^{2-}$, and Fe(CN)$_6^{3-}$ are correlated rather well with the relative Marcus theory (11, 12), suggesting that all the reactions occur by a common mechanism, probably involving outer sphere electron transfer through the exposed heme edge of cytochrome c.

Electrostatic interactions play an important role in all reactions of cytochrome c, and Wherland and Gray (13) 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, $k_w$.

$\gamma_A = \gamma_B$ and $\gamma_A b$ are the activity coefficients of the reactants, and $\gamma_A b$ 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 relation (16),

$$lnk = lnk_w - 3.576 \frac{e^{-a_s} + e^{-a_T}}{1 + \kappa a_s} \frac{Z_0 Z_B}{(a_s + a_T)}$$

(3)
Golldkorn and Schejter (17) have shown that Equation 6 is in good agreement with experimental data on the reaction between cytochrome c and ascorbate, provided that the ionic strength is less than 0.01 M. Equation 3 is numerically very similar to Equation 6 at low ionic strength, and Feinberg et al. (18) and Ilan et al. (19) have shown that it is in good agreement with the reactions between cytochrome c and various small molecule oxidation-reduction agents.

The above theoretical relations assume that each reactant is a sphere with a uniform charge distribution. This is clearly inadequate for cytochrome c, which has a highly asymmetric charge distribution. Koppenol (20) has corrected this by including a dipole moment term in the activity coefficient expression which he has shown to be very important over the ionic strength range 0.01 to 0.1 M. However, at ionic strengths above 0.1 M, quadrupolar and higher order terms will become increasingly important, and a model taking into account the positions of all the individual charges will be more appropriate. Stellwagen and Cass (21) and Ilan and Shafferman (10) have suggested from indirect evidence that lysines immediately surrounding the heme crevice of cytochrome c dominate the interaction with ferricyanide. In the present study, we have estimated the contribution of each lysine to the electrostatic interaction with ferricyanide by measuring the reaction rate of cytochrome c derivatives modified at single lysine residues.

**Materials and Methods**

Horse heart cytochrome c (Type VI), ascorbic acid, and potassium ferricyanide were obtained from Sigma. Ultrapure Tris base was purchased from Schwarz/Mann (Orangeburg, NY). All other chemicals used were of reagent grade.

Cytochrome c derivatives specifically trifluoroacetylated at single lysine residues were prepared by the procedures of Staudenmueller et al. (22, 23) and Smith et al. (24), while singly trifluoromethylphenyl-carbamylated cytochrome c derivatives were prepared by the method of Smith et al. (25). All of these singly modified derivatives were purified to greater than 95% and characterized by $^3$H NMR, peptide mapping, and visible spectrophotometry as discussed in the previous publications. They have the same oxidation-reduction potential and 695 nm absorption band as native cytochrome c, indicating that the heme environment is unaffected by the modification. They were never lyophilized during the purification procedure to avoid polymer formation.

The oxidation of the reduced cytochrome c was followed on a Durrum stopped flow spectrophotometer model D110 with a 2-cm light path cell and a dead time of ~2 ms. The spectrophotometer was connected to a C-1024 time averaging computer (Varian Associates, Palo Alto, CA) and a Sargent-Welch recorder model SRG. The electron transfer reactions were monitored by following the decrease in absorption of the reduced cytochrome c at 420 nm in 0.2 M ionic strength Tris-chloride buffer, pH 7.5, maintaining the temperature at 25°C (±0.5°C) with a circulating water bath. All measurements were made under second order kinetic conditions using 1 μM ferrocyanide and 2 μM ferricyanide. Native and derivatized cytochromes c were reduced with ascorbate and the excess reductant removed by passage through a Bio-Gel P-4 column equilibrated with 0.2 M Tris-chloride buffer, pH 7.5. Cytochrome c concentrations were determined by measuring the 550-nm absorbance, and the samples were purged with prepurified dry nitrogen before loading the flow system. Potassium ferricyanide supplied by Sigma was used without any further treatment. Ferricyanide solutions were made by weight, stoppered, and the mixture was shaken until it was clear. Ferricyanide solutions were also deoxygenated by flushing the aliquots with prepurified dry nitrogen. The second order rate constants were obtained from least square analysis of $(a_0 - b_0)^1 \ln b_0(a_0 - x)/a_0(a_0 - x)$ versus time plots, which were found to be linear. The tabulated error levels represent the standard deviation of at least 4 determinations.

**Results**

Holwerda et al. (26) have described the rate laws, activation parameters, and effect of ionic strength on the reaction of native and fully trifluoroacetylated cytochrome c with various inorganic oxidation-reduction reagents. We found that the reaction between native ferrocyanochrome c and ferricyanide followed second order kinetics with a rate constant of $1.07 \pm 0.05 \times 10^6$ M$^{-1}$ s$^{-1}$ in 0.2 M Tris-Cl, pH 7.5, 25°C, in good agreement with that found by other workers using stopped flow and T-jump techniques (6-8). Modification of the lysine 13 amino group with either CF$_3$CO— or CF$_3$Ph-NHCO— caused a 2-fold decrease in the rate constant, while modification of lysines 22, 27, 72, and 79 decreased the rate constant by ~1.5-fold (Table I, Fig. 1). The reaction rate was not affected by modification of lysines 22, 55, 99, and 100, which are well removed from the heme crevice region. These results strongly...
suggest that the reaction site for ferricyanide is at the exposed edge of the heme of cytochrome c, and that the lysines immediately surrounding the heme crevice are involved in electrostatic interactions with ferricyanide. The decrease in reaction rate due to modification of a given lysine is most likely due to elimination of the electrostatic interaction between the positively charged amino group and ferricyanide since the bulky CF$_3$PhNHCO$^-$ group caused nearly the same decrease as the CF$_3$CO$^-$ group. It is very significant that modification of lysines 22, 55, 99, and 100 removed from the heme crevice region had no effect on the reaction rate, even though the net charge on cytochrome c decreased from +7 to +6. Clearly, at an ionic strength of 0.2 M, the spherically symmetric charge model assumed in deriving Equations 3 and 6 is no longer valid, and short range interactions dominate the total electrostatic free energy.

A general equation for the electrostatic free energy of activation for the reaction between a protein and a small oxidation-reduction agent is

$$V = \sum_i V_i$$

(7)

where $V_i$ is the electrostatic free energy of interaction between charged group $i$ on the protein and the oxidation-reduction molecule. An approximation to $V_i$ can be obtained from the decrease in the reaction rate constant caused by modification of lysine $i$ to change the amino group charge from +1 to 0:

$$V_i \approx \Delta G^*_{\text{native}} - \Delta G^*_{\text{derivative}} = -RT \ln \left( \frac{k_{\text{active}}}{k_{\text{derivative}}} \right)$$

(8)

Equation 8 is a direct result of the Marcus theory formalism (11–13) which states that $\Delta G^*$ is the free energy of activation for the reaction involving cytochrome c or its singly modified derivative, and $k$ is the second order rate constant. This estimate of $V_i$ might also include favorable or unfavorable nonelectrostatic interactions between the modified lysine amino group and ferricyanide, but the near identity of reaction rate ratios for derivatives modified at a given lysine with the bulky CF$_3$PhNHCO$^-$ group and the CF$_3$CO$^-$ group suggests that these steric factors are not very important. In those cases where both CF$_3$CO$^-$ and CF$_3$PhNHCO$^-$ derivatives were available at a given lysine, only the smallest $-V_i$ value was tabulated in Table I.

It is also possible to estimate $V_i$ theoretically from Debye’s formula (Equation 2), which now takes the form

$$V_i = 2.1175 \left( \frac{e^{a_i} + e^{a_{i_B}}}{1 + \alpha a_i} \right) \left( \frac{Z_{\text{protein}}}{1 + \alpha a_i} \right) - \frac{e^{-r_i}}{R}$$

(9)

where $a_i$ is the effective radius of charge $i$, $a_B$ is the effective radius of the oxidation-reduction agent, and $r_i$ is the distance between charge $i$ and the oxidation-reduction molecule. Equation 9 was used to calculate $r_{i,\text{crystal}}$ and $r_{i,\text{deriv}}$ values from the experimental $V_i$ estimates, assuming $a_i = 3.0$ Å and $a_B = 4.5$ Å (Table I). The position of the charged groups in the activated complex was then optimized such that the $r_{i,\text{crystal}}$ values calculated from the x-ray diffraction structure of tuna ferrocytochrome c (27) were in the best possible agreement with the $r_{i,\text{deriv}}$ values (Table I). In this optimum configuration, ferricyanide is in van der Waals’ contact with the exposed edge of the heme in the vinyl group attached to Cys 17 as shown in Fig. 2. The agreement between the $r_{i,\text{crystal}}$ and $r_{i,\text{deriv}}$ values is quite good, especially when the uncertainty in the orientations of the lysine side chains is taken into account. Mandel et al. (27) found rather large differences in the orientations of the exterior side chains, including especially those of lysines 8, 25, 72, 73, 86, and 87, when comparing the crystal structures of two symmetry-independent forms of tuna ferrocytochrome c and tuna ferrocytochrome c. We found it was possible to adjust the orientations of the side chains of a model of tuna ferrocytochrome c to bring the $r_{i,\text{crystal}}$ values into coincidence with the $r_{i,\text{deriv}}$ values without assuming any unfavorable configurations. Specifically, the side chains of lysines 8 and 87 could be oriented more toward the front of cytochrome c to bring their $r_i$ values to 14 and 16 Å, respectively, and the side chain of lysine 27 could be moved slightly more toward the back of the molecule to bring its $r_i$ to 13 Å. The side chain of lysine 13 is somewhat more restrained than that of the other lysines since its amino group is in hydrogen-bonding contact with the carboxylate group of Glu 90 in all three of the above structures. It is, of course, possible that this interaction might be broken in the transition state and the side chain could be oriented closer to ferricyanide, bringing $r_{i,\text{crystal}}$ from 13 to 9 Å. It is apparent from the data in Table I that six or seven lysine amino groups within 15 Å of ferricyanide dominate the electrostatic inter-

![Fig. 2. Polyhedral surface charge map of the proposed transition state complex between ferrocytochrome c and ferricyanide. The ferricyanide ion is 11.98 Å from the heme iron on an axis passing through the nitrogen atom of the pyrrole ring bonded to Cys 17. All of the charged groups of horse heart ferrocytochrome c are drawn, but the positions were obtained from the structure of tuna ferrocytochrome c (27). In the case of the seven charged residues of the horse protein that are replaced by neutral residues in the tuna protein, the position of the most exposed atom of the tuna residue is plotted. The charged groups on the surface toward the viewer are connected to form irregular polyhedra. Top, standard view from the left side of cytochrome c (27); bottom, standard view from the front of cytochrome c (27).](image-url)
action. The only other charged group that is close enough to be involved is the carboxylate of Glu 90, which, with an $r_i^{\text{typical}}$ value of 16 Å, would make an unfavorable contribution to the electrostatic interaction.

**DISCUSSION**

The importance of electrostatic interactions to the reactions of cytochrome $c$ with transition metal oxidation-reduction agents is demonstrated most simply by the strong ionic strength dependence of the reaction rates (28). In the past, the electrostatic interaction has been calculated by assuming either that cytochrome $c$ has a spherically symmetric charge distribution of net charge $Z_A$ (17), or that a smaller site at the heme crevice with an effective site charge $Z_i$ is responsible for the interaction (26). A more precise way of expressing the total electrostatic interaction is as a sum of the electrostatic energies of interaction between each charged group on cytochrome $c$ and ferricyanide (Equation 7). This approach has also been used with considerable success by Friend and Gurd (29) to calculate the intramolecular electrostatic interactions of proteins such as myoglobin. In the present work, we have estimated the electrostatic interactions of individual lysine amino groups with ferricyanide by comparing the reaction rates of native and singly modified cytochrome $c$. It is apparent from these studies that a set of six or seven lysines surrounding the heme crevice of cytochrome $c$ dominates the total electrostatic interaction, and that the location of the reaction with ferricyanide is the exposed edge of the heme (Fig. 2). In principle, of course, specific modification of a lysine amino group could affect the reaction rate by mechanisms other than simple elimination of the electrostatic interaction. We have previously shown that the singly modified CF$_3$CO$-$/CF$_3$COH$-$ lysine cytochrome $c$ derivatives have the same oxidation-reduction potential, and visible absorption, estimated the electrostatic interactions of individual lysine groups with ferricyanide by comparing the reaction between totally trifluoroacetylated cytochrome $c$ and ferricyanide. This is consistent with an electron transfer mechanism involving vibronically coupled tunneling (39), recently supported by the discovery of a weak charge-transfer band in the ferrocyanide-cytochrome $c$ complex (40).

The chemical modification results show clearly that at 0.2 M ionic strength, the electrostatic interaction energy $V_i$ is a very rapidly decreasing function of $r_i$ and charged groups more than 17 Å away have a negligible interaction with ferricyanide. This is also demonstrated by the finding that the reaction between totally trifluoroacetylated cytochrome $c$ and ferricyanide was completely independent of ionic strength over the range 0.10 to 0.28 M, even though the net charge $Z_A$ was --12 (26). The closest charged groups on totally trifluoroacetylated cytochrome $c$ are Glu 90, and Glu 21 at 16 and 20 Å, respectively, from the proposed site of ferricyanide (27). At low ionic strength, however, the long range interactions become important, since the reaction between fully acetylated cytochrome $c$ and ferricyanide was found to be dependent on ionic strength below 0.05 M (19). It thus appears that at very low ionic strength, the electrostatic interaction can be adequately described by a spherically symmetric charge model, while at high ionic strength, the short range interactions become progressively more important. Although the use of Equation 9 in the present context can be criticized in a number of respects, the dependence of the theoretical $V_i$ values on $r_i$ is in good general agreement with the estimates obtained from the chemical modification results. Equation 9 assumes that all the charged groups that interact with ferricyanide are fully solvated, and the effective dielectric constant is that of water. This appears reasonable for the externally positioned lysine groups surrounding the heme crevice of cytochrome $c$. Another criticism to the use of Equation 9 is that the assumptions of the Debye-Hückel theory on which it is based are no longer valid at high ionic strength. However, Pitzer (30) has recently reviewed the application of rigorous Monte Carlo methods to electrolyte solutions and found that the correct radial distribution function is in excellent agreement with the exponential Debye-Hückel expression up to 0.42 M ionic strength. Even more pertinent to the present studies, Perlmutter-Haymann and Weissmann (31) studied the reaction between Co(NH$_3$)$_6$Br$^{2-}$ and OH$^-$ in the presence of nine different monovalent electrolytes at ionic strengths up to 1.5 M and found excellent agreement with the Debye-Hückel expression over the entire range.

Chemical modification studies have also been used to establish that the reaction sites on cytochrome $c$ for cytochromes $b_6$, $c_6$, and oxidase are all located at the heme crevice, suggesting a common electron transfer mechanism (22-25, 32-36). Our studies on the interaction between cytochrome $c$ and cytochrome $b_6$, support a theoretical proposal by Salemme (37) in which the complex is stabilized by complementary charge pair interactions between cytochrome $c$ lysines 13, 27, 72, and 79, and cytochrome $b_6$ carboxyl groups Glu 52, Glu 48, Asp 64, and the heme propionate, respectively. The dependence of the reaction rate between the two proteins on both specific lysine modifications and ionic strength demonstrated that these short range complementary charge pair interactions dominated the total electrostatic interaction (32, 38). It thus appears that the highly conserved lysines surrounding the heme crevice of cytochrome $c$ have evolved to direct the interaction of cytochrome $c$ with its oxidation-reduction partners. The location of the reaction site for ferricyanide at the heme crevice of cytochrome $c$ is consistent with an electron transfer mechanism involving vibronically coupled tunneling (39), recently supported by the discovery of a weak charge-transfer band in the ferrocyanide-cytochrome $c$ complex (40).

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