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# Interaction between Cytochrome *c* and Cytochrome $b_5$[†]

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ABSTRACT: The reduction of cytochrome *c* by cytochrome $b_5$ was studied over a wide range of ionic strengths in four different buffer systems. The reaction rate decreased linearly as the $I^{1/2}$ was increased, suggesting that electrostatic interactions are important in the interaction. The ionic strength dependence of the reaction rate was in quantitative agreement with the theory of Wherland & Gray [Wherland, S., & Gray, H. B. (1976) *Proc. Natl. Acad. Sci U.S.A. 73*, 2950] only if the effective radius of the interaction was 2 Å. This indicates that the interaction between the two proteins is best described as the sum of *n* complementary charge interactions, each involving a specific lysine on cytochrome *c* and a specific carboxyl group on cytochrome $b_5$. The number of complementary charge interactions, *n*, was calculated to be five to seven, in agreement with the results of our specific modification studies. Ultracentrifugation and gel permeation techniques were used to demonstrate that cytochrome $b_5$ and cytochrome *c* formed a stable complex at low ionic strength.

The development of a comprehensive theory of electron transport in biological systems has been hampered by the lack of detailed structural information on interacting components of these systems. The in vitro reaction between cytochrome $b_5$ and cytochrome *c* is a unique model system because the X-ray crystal structures of both proteins have recently been determined to high resolution (Argos & Mathews, 1975; Swanson et al., 1977), and the reaction rate is as rapid as the reactions of either protein with its physiological oxidants and reductants (Strittmatter, 1964). Although cytochrome $b_5$ was first discovered in the membrane of the endoplasmic reticulum, a very similar form has been found in other organelles, including the outer membrane of the mitochondrion (Borgese & Meldolesi, 1976). Matlib & O'Brien (1976) have suggested that the reaction between cytochrome $b_5$ and cytochrome *c* might have some limited physiological significance because under conditions of high intermembrane ionic strength cytochrome *c* is released from the inner membrane and can transport electrons from cytochrome $b_5$ located on the inner

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surface of the outer membrane to cytochrome oxidase on the inner membrane. Membrane-bound cytochrome $b_5$ from most sources is a single polypeptide chain consisting of two distinct regions, an amino terminus of ~95 amino acids which contains the heme prosthetic group and a carboxyl terminus of ~45 hydrophobic amino acids which "anchors" the protein to the membrane (Spatz & Strittmatter, 1971). Mild treatment with proteolytic enzymes cleaves the intact protein into the two fragments. Cytochrome *c* reacts as well with the water-soluble proteolytically cleaved protein fragment as it does with the intact membrane-bound protein, indicating that the active site of cytochrome $b_5$ is probably not affected by binding to a membrane (Strittmatter et al., 1972).

The mechanism of electron transport can probably be broken down into two separate steps, the highly specific binding interaction between redox proteins and the actual electron transfer within the bound complex. Recently, cytochrome *c* derivatives specifically modified at single lysine residues have been used to define the binding domain on cytochrome *c* for cytochrome $b_5$ (Ng et al., 1977), cytochrome oxidase (Smith et al., 1977; Ferguson-Miller et al., 1978; Staudenmayer et al., 1976, 1977), cytochrome $c_1$ (Ahmed et al., 1978; Speck et al., 1979), and cytochrome *c* peroxidase (Kang et al., 1978). In all cases, the binding site is located at the front of the cytochrome *c* molecule over the heme crevice and the positive charges on the five or six lysine groups immediately surrounding the heme crevice are involved in complementary charge interactions with negatively charged carboxyl groups on the other proteins. The cytochrome $b_5$ study is in agreement with the theoretical proposal of Salemme (1976) that cytochrome $b_5$ and cytochrome *c* can form a complex dominated by complementary charge interactions between cytochrome *c* Lys-13, -27, -72, and -79 and cytochrome $b_5$ carboxyl groups of Glu-52, Glu-48, Asp-64, and the most exposed heme propionate, respectively. In the proposed complex, the heme groups of the two proteins are nearly coplanar, with their edges separated by 8.4 Å. This separation distance is within the general range predicted for the thermally activated tunneling mechanism of electron transfer (Hopfield, 1974). Dailey & Strittmatter (1979) have recently used chemical modification techniques to show that Glu-47, -48, and -52 and the exposed heme propionate of cytochrome $b_5$ are involved in electrostatic interactions with cytochrome $b_5$ reductase.

In the present study we have further characterized the ionic interactions between cytochrome $b_5$ and cytochrome *c* by studying the effects of ionic strength on the reaction between the two proteins. The ionic strength dependence of the reaction rate was found to be in quantitative agreement with the theory of Wherland & Gray (1976) only if the effective radius of the interaction was 2.0 Å, suggesting that the interaction between the two proteins is best described as the sum of *n* complementary charge interactions, each involving a specific lysine on cytochrome *c* and a specific carboxyl group on cytochrome $b_5$. We also found that a stable complex was formed between cytochrome $b_5$ and cytochrome *c* at low ionic strength.

## Experimental Procedure

*Materials.* Horse heart cytochrome *c* (type VI), NADH,[1] and Mops were obtained from Sigma Chemical Co. EDTA and Tris were obtained from Fisher Scientific Co. $(F_3Me)$-PhNHCO-Lys-13 cytochrome *c* and native cytochrome *c* were prepared and purified as described by Smith et al. (1977). Calf liver cytochrome $b_5$, prepared as described by Strittmatter (1960), was a gift of Dr. F. S. Mathews.

*Cytochrome $b_5$ Assay.* The source of the cytochrome $b_5$ activity was a beef liver microsomal fraction prepared as described by Strittmatter et al. (1972). The microsomal preparation was treated with 5% deoxycholate (1 mg/mg of protein) and diluted with buffer immediately before use. The assay medium contained 0.5–20 μM cytochrome *c*, 50 μM NADH, and the indicated concentrations of buffer. The rate of reduction of cytochrome *c* following addition of microsomes was detected at 420 or 550 nm on a Cary 14 spectrophotometer using 1-cm cells. The cytochrome $b_5$ concentration of the microsomes was determined as described by Rogers & Strittmatter (1974).

*Complex Formation.* The procedure of Baggott & Langdon (1970) was used to study complex formation between horse heart cytochrome *c* and calf liver cytochrome $b_5$. Four milliliters of Sephadex G-75 equilibrated with 0.01 M Tris-HCl, pH 7.5, was added to a 15-mL conical centrifuge tube, most of the excess buffer was removed, and the tube was weighed. One milliliter of a solution containing a calibrated concentration of the protein(s) was added, and the tube was mixed for 30 min and then centrifuged in a clinical centrifuge to sediment the gel. One milliliter of supernatant was withdrawn and the concentration of the protein(s) determined by spectrophotometry. Then 1 mL of a calibrated solution of blue dextran was added, the tube mixed and centrifuged as before, and the supernatant assayed for blue dextran. The volume of the mobile phase ($V_m$) was determined from the blue dextran dilution factor, and the volume of the internal gel phase ($V_g$) was determined by $V_g = V_{total} - V_m$.

The dissociation constant for the equilibrium $c + b_5 \rightleftarrows cb_5$ was estimated from the equation $K = [c][b_5]/[cb_5] = [(\sigma_c' - \sigma_{cb_5})/(\sigma_c - \sigma_c')][b_5]$, where $\sigma_c$ is the gel partition coefficient for cytochrome *c*, $\sigma_c = [c_g]/[c]$, $\sigma_c'$ is the apparent gel partition coefficient for cytochrome *c* in the presence of cytochrome $b_5$, and $\sigma_{cb_5}$ is the gel partition coefficient of cytochrome *c* in the presence of a saturating concentration of cytochrome $b_5$.

Complex formation was also studied by ultracentrifugation in a Beckman Model E. The rotor was accelerated to 52 000 rpm, and photographs were taken at 280 nm every 20 min for 75 min. The sedimentation coefficient for uncomplexed cytochrome $b_5$ and cytochrome *c* was determined by using a solution containing 40 μM cytochrome and 5 mM Tris–Mops, pH 7.0, at 20 °C. Complexation was studied in a solution containing 20 μM each of cytochrome $b_5$ and cytochrome *c* in 5 mM Tris–Mops, pH 7.0.

## Results

The NADH-linked reduction of cytochrome *c* by liver microsomes results from the NADH–cytochrome $b_5$ reductase–cytochrome $b_5$ system (Strittmatter et al., 1972). We have previously established that the cytochrome $b_5$–cytochrome *c* step is rate limiting only at low cytochrome *c* concentrations and have found that the parameter $k_v = (v/S)_{v\to 0}$ obtained by extrapolating $v/S$ to $v = 0$ on an Eadie–Hofstee plot is a true measure of the cytochrome $b_5$ reduction of cytochrome *c* (Ng et al., 1977). $k_v/E_0$, where $E_0$ is the cytochrome $b_5$ concentration, was found to be $3.0 \times 10^7$ $M^{-1}$ $s^{-1}$ (0.1 M phosphate, pH 7.0, 25 °C), in reasonable agreement with the second-order rate constant for the reaction between the two purified proteins measured by stopped-flow techniques, $4.7 \times 10^7$ $M^{-1}$ $s^{-1}$ (Strittmatter, 1964).

The effect of ionic strength on the reduction of cytochrome *c* was studied in five different buffer systems: Tris-HCl, NaCl,

[1] Abbreviations used: $(F_3Me)$PhNHCO, (trifluoromethyl)phenylcarbamyl; $F_3Ac$, trifluoroacetyl; Tris, tris(hydroxymethyl)aminomethane; Mops, 4-morpholinepropanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; NADH, nicotinamide adenine dinucleotide (reduced form).

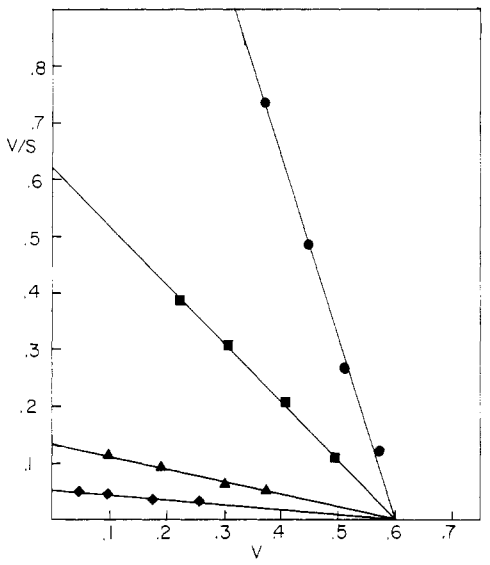


FIGURE 1: Ionic strength dependence of the reaction between cytochrome *c* and cytochrome $b_5$. All solutions contained 50 $\mu$M NADH and microsomes containing $1 \times 10^{-10}$ M cytochrome $b_5$. The initial velocities were measured in micromolar cytochrome *c* reduced per minute at 25 °C. *S* is in micromolar cytochrome *c*. Tris-HCl pH 7.5 buffers were 0.05 (●), 0.1 (■), 0.2 (▲), and 0.3 M (◆).

Na-Mops, Tris–Mops, and $Na_2HPO_4$. In each case higher ionic strength increased the apparent Michaelis constant $K_m$ of the reaction without affecting the apparent $V_{max}$ (Figure 1.) Ln $k_v$ was found to decrease linearly as $I^{1/2}$ increased up to ~0.6 M in all the buffer systmes, but the slope varied from about −12 for Tris-HCl and NaCl to about −7 for Na-Mops, Tris–Mops, and $Na_2HPO_4$ (Figure 2). When the reaction was studied at higher ionic strength in Tris-HCl buffer, the curvature in the ln $k_v$ vs. $I^{1/2}$ plot was not very large out to about $I^{1/2}$ = 1.3 M (Figure 3). The rate constant $k_v$ for ($F_3$Me)PhNHCO-Lys-13 cytochrome *c* was about one-third that of native cytochrome *c* at low ionic strength and became nearly equal to it at very high ionic strength (Figure 4). The slope in the ln $k_v$ vs. $I^{1/2}$ plot was slightly more positive than that of the native protein.

Two different techniques were used to demonstrate that a stable complex was formed between cytochrome $b_5$ and cytochrome *c*. The gel permeation technique of Baggott & Langdon (1970) was carried out using Sephadex G-75 equilibrated with 0.01 M Tris-HCl buffer. At this low ionic strength, cytochrome *c* has a relatively large gel partition coefficient, $\sigma_c$ = 2.4, indicating that weak ion-exchange effects increased the interaction with the gel. Addition of cytochrome $b_5$ displaced cytochrome *c* from the gel phase, and the dissociation constant of the cytochrome $b_5$–cytochrome *c* complex was estimated to be 20 $\mu$M, as described under Experimental Procedure. Cytochrome $b_5$ itself had a normal gel partition coefficient for a protein of its molecular weight.

Complex formation was also studied by sedimentation at 52000 rpm in the Beckman Model E ultracentrifuge. Cytochrome $b_5$ and cytochrome *c* had the same sedimentation coefficients, *s* = 1.70 S, in 5 mM Tris–Mops, pH 7.0, 20 °C, while a mixture containing 20 $\mu$M each of cytochrome $b_5$ and cytochrome *c* in 5 mM Tris–Mops had a sedimentation coefficient of 2.24 S. Addition of 0.1 M NaCl reduced the sedimentation coefficient back down to 1.70 S, the same as that of the uncomplexed proteins. The sedimentation coefficient expected for the cytochrome $b_5$–cytochrome *c* complex is 2.57 S, assuming a prolate ellipsoid shape with an axial ratio of 2. The sedimentation coefficient observed at low ionic strength

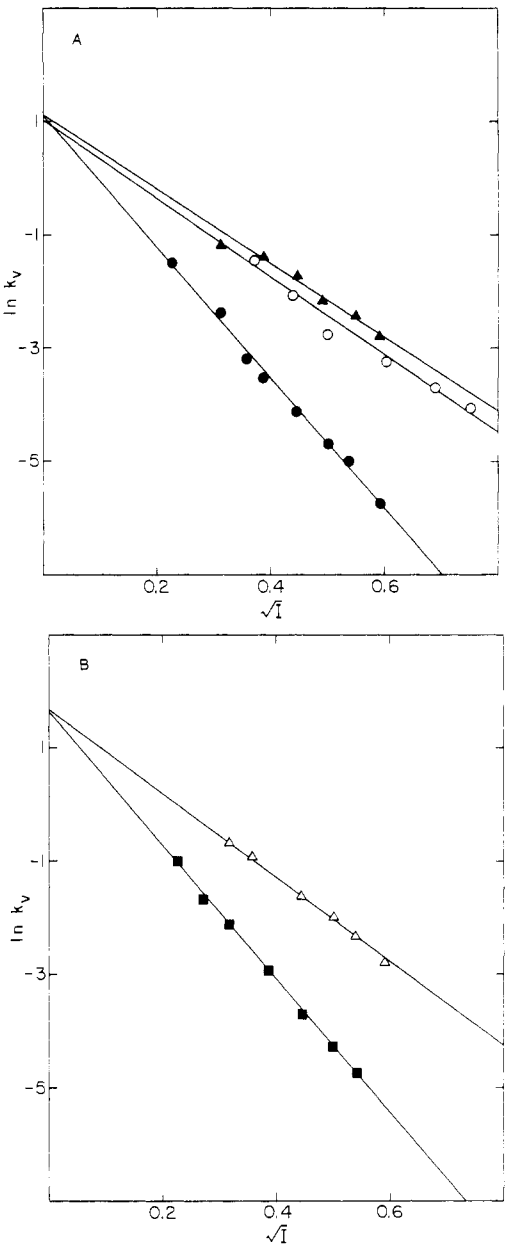


FIGURE 2: Effect of ionic strength on the rate constant $k_v$ for the reduction of cytochrome *c* by cytochrome $b_5$. $k_v$ was measured in reciprocal minutes at 25 °C in buffers containing 50 $\mu$M NADH and microsomes containing $1 \times 10^{-10}$ M cytochrome $b_5$. The following buffer systems were used. (A) 0.05 M Tris-HCl (pH 7.5) plus NaCl (●); $Na_2HPO_4$ (pH 8.0) (○); Na-Mops (pH 7.5) (▲). (B) Tris-HCl (pH 7.5) (■); Tris–Mops (pH 7.5) (△).

was not quite as large as this, probably because the solution contained both complexed and uncomplexed cytochromes in equilibrium.

## Discussion

A number of different theoretical approaches have been taken to estimate the importance of electrostatic interactions in electron-transfer reactions (Wherland & Gray, 1976; Koppenol et al., 1978). All of the equations derived reduce to the limiting Brønsted equation at low ionic strength:

$$\ln k = \ln k_0 + 2\alpha Z_1 Z_2 I^{1/2} \quad (1)$$

where $k_0$ is the rate constant at zero ionic strength, $Z_1$ and $Z_2$ are the total charges on the reactants, and $\alpha$ = 1.17 in aqueous solution at 25 °C. Deviations from this limiting law as $I^{1/2}$ is increased depend on $R_1$ and $R_2$, the "effective radii"

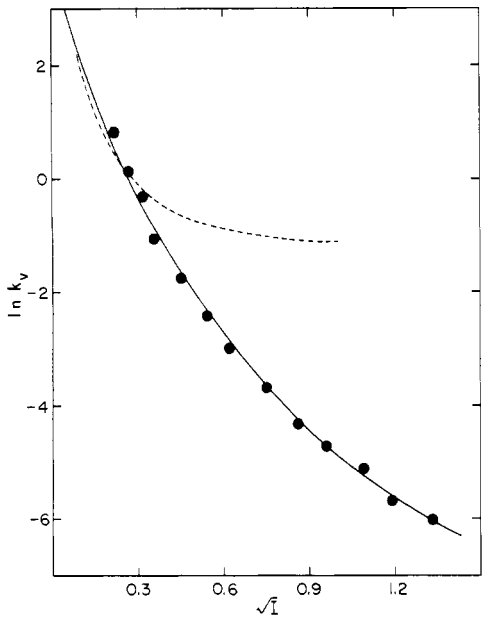


FIGURE 3: Effect of ionic strength on the rate constant $k_v$ for the reduction of cytochrome $c$ by cytochrome $b_5$ in Tris-HCl buffers (pH 7.5) containing 50 $\mu$M NADH and $1 \times 10^{-10}$ M cytochrome $b_5$ at 25 °C (●). The dashed line is a theoretical curve obtained from eq 2 with $R_1 = R_2 = 10$ Å and $Z_1 = -Z_2 = 4$, while the solid line was obtained from eq 2 with $R_1 = R_2 = 2.0$ Å and $Z_1Z_2 = -7$ or from eq 6 with $R = 2.0$ Å and $n = 7$.

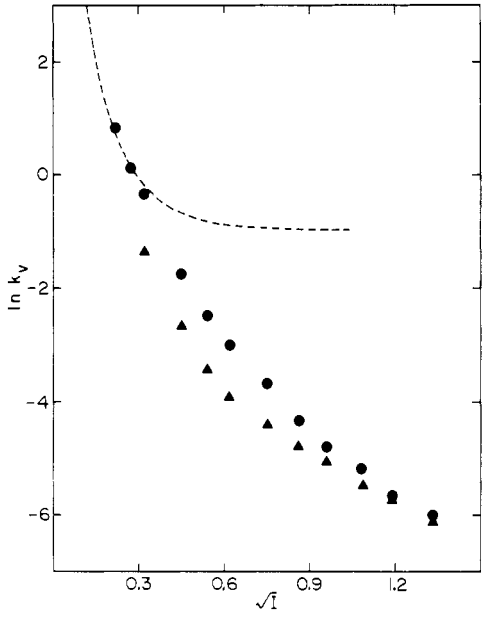


FIGURE 4: Ionic strength dependence for the reaction of cytochrome $b_5$ with native cytochrome $c$ (●) and ($F_3$Me)PhNHCO-Lys-13 cytochrome $c$ (▲) in Tris-HCl pH 7.5 buffer at 25 °C. The buffer contained 50 $\mu$M NADH and $1 \times 10^{-10}$ M cytochrome $b_5$. The dashed line is a theoretical curve obtained from eq 2 with $R_1 = R_2 = 16.5$ Å, $Z_1 = +9$, and $Z_2 = -6$.

of the reactants, the deviations being larger for larger $R$ values. The Wherland–Gray theory is based on the Marcus relation and gives

$$\ln k = \ln k_\infty - 3.576\left(\frac{e^{-\kappa R_1}}{1 + \kappa R_2} + \frac{e^{-\kappa R_2}}{1 + \kappa R_1}\right)\frac{Z_1Z_2}{R_1 + R_2} \quad (2)$$

where $k_\infty$ is the rate constant at infinite ionic strength and $\kappa = 0.329I^{1/2}$ Å$^{-1}$. The most surprising result of the present study was that the rate constant did not differ significantly from the simple Brønsted relation over the range $I^{1/2} = 0.2$–$0.6$ M, and deviations at higher ionic strength were relatively small. The best fit to the Wherland–Gray equation was obtained by using $R_1 = R_2 = 2$ Å and $Z_1Z_2 = -7$. These $R$ values are characteristic of the radii of individual amino and carboxyl groups but not of the cytochromes as a whole or even of "active sites". The theoretical curve for the reaction rate using parameters appropriate for the entire cytochrome $c$ and cytochrome $b_5$ molecules, $R_1 = R_2 = 16.5$ Å, $Z_1 = +9$, and $Z_2 = -6$, departed greatly from the experimental data (Figure 4). Use of parameters characteristic of the active sites of the two cytochromes, $R_1 = R_2 = 10$ Å and $Z_1 = -Z_2 = 4$, also gave poor agreement (Figure 3).

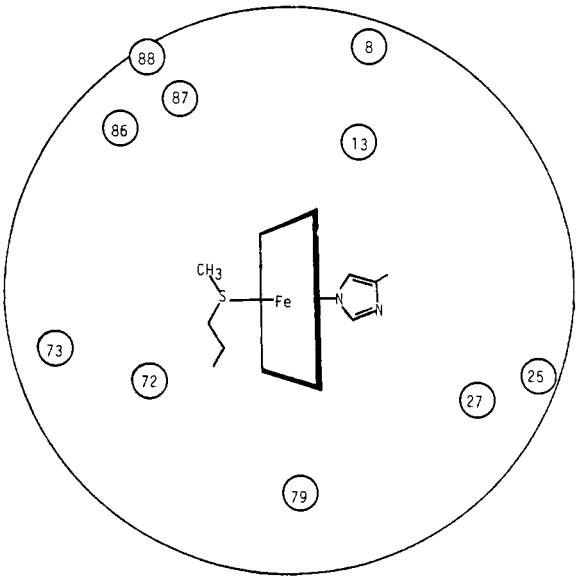


FIGURE 5: Schematic diagram of horse heart cytochrome $c$ showing the approximate positions of the lysine groups surrounding the heme crevice (Swanson et al., 1977).

Table I: Cytochrome $b_5$ Activity of Cytochrome $c$ Derivatives

| derivative | $k_v/E_0$ ($10^8$ M$^{-1}$ s$^{-1}$) | derivative | $k_v/E_0$ ($10^8$ M$^{-1}$ s$^{-1}$) |
|---|---|---|---|
| native | 3.0[a] | $F_3$Ac-Lys-88 | 3.0[b] |
| $F_3$Ac-Lys-13 | 1.1[a] | $F_3$Ac-Lys-99 | 3.1[a] |
| $F_3$Ac-Lys-22 | 2.9[a] | ($F_3$Me)PhNHCO-Lys-8 | 2.1[a] |
| $F_3$Ac-Lys-25 | 1.7[a] | ($F_3$Me)PhNHCO-Lys-13 | 1.1[a] |
| $F_3$Ac-Lys-55 | 2.9[a] | ($F_3$Me)PhNHCO-Lys-27 | 1.7[a] |
| $F_3$Ac-Lys-72 | 1.0[b] | ($F_3$Me)PhNHCO-Lys-72 | 1.2[a] |
| $F_3$Ac-Lys-79 | 1.7[b] | ($F_3$Me)PhNHCO-Lys-79 | 1.6[a] |
| $F_3$Ac-Lys-87 | 2.0[b] | ($F_3$Me)PhNHCO-Lys-100 | 3.0[a] |

[a] Ng et al. (1977). [b] M. B. Smith, J. Stonehuerner, and F. Millett (unpublished experiments).

A different approach toward development of a more realistic theory for ionic strength effects is suggested by the chemical modification studies of Ng et al. (1977) and M. B. Smith, J. Stonehuerner, and F. Millett (unpublished experiments). Fifteen different cytochrome $c$ derivatives were studied, each modified at a single lysine residue to leave an uncharged group. The derivatives modified at lysine-8, -13, -25, -27, -72, -79, and -87 surrounding the heme crevice each decreased the rate of reduction of cytochrome $c$ by cytochrome $b_5$ significantly (Table I). These decreases in reaction rate were most likely due to the removal of specific charge interactions rather than to steric effects because the bulky (trifluoromethyl)phenylcarbamyl group had nearly the same effect as the trifluoroacetyl group. It is very significant that modification of lysines only slightly removed from the heme crevice region, such as lysine-88, -55, and -22, had no effect on the reaction rate at

ionic strengths down to 0.05 M. If overall charge was the significant parameter in the reaction, then the change in the overall charge from +9 to +8 should have caused a change in the reaction rate by one-ninth of the total ionic strength effect, or $\Delta(\ln k) = 0.8$. This corresponds to a twofold reduction in $k_v$, which certainly could have been measured experimentally. At very low ionic strength, long-range interactions are probably important in the reactions between electron transport proteins. Koppenol et al. (1978) have calculated the electric potential field around cytochrome *c* and have suggested that at low ionic strength the overall charge and the dipole moment are both important in determining the rate of electron transfer. However, at higher ionic strength, the electric potential field around cytochrome *c* decreases drastically (from 1.5 kT/e at 0.0 M ionic strength to 0.03 kT/e at 0.1 M ionic strength), indicating that long-range effects become insignificant.

The above data support the concept that individual short-range complementary charge interactions determine the reaction rate at ionic strengths above 0.05 M. We shall assume for simplicity that the overall electrostatic interaction between cytochrome *c* and cytochrome $b_5$ consists of *n* specific complementary charge interactions. Each such charge interaction consists of a protonated amino group represented by a sphere of radius $R_1$ separated by a distance *r* from a deprotonated carboxyl group of radius $R_2$. It is assumed that both groups are fully solvated and do not interact electrostatically with any other groups. The electrostatic potential energy of one complementary charge interaction in the activated complex relative to infinite separation is given by

$$V = \tfrac{1}{2}\left(\frac{e^{\kappa R_1}}{1+\kappa R_1} + \frac{e^{\kappa R_2}}{1+\kappa R_2}\right)\left(\frac{Z_1 Z_2 e^2}{\epsilon}\right)\left(\frac{e^{-\kappa r}}{r}\right) \qquad (3)$$

where $\epsilon$ is the dielectric constant of water (Wherland & Gray, 1976). The total electrostatic potential energy between cytochrome *c* and cytochrome $b_5$ is thus

$$V = \sum_1^n -2.1175\frac{e^{-\kappa R}}{R(1+\kappa R)} \qquad (4)$$

where we have assumed that $Z_1 = -Z_2 = 1$, $R_1 = R_2 = R$, and $r = 2R$. Following the Marcus theory treatment of Wherland & Gray (1976)

$$\ln k = \ln k_\infty - 1.689V \qquad (5)$$

therefore

$$\ln k = \ln k_\infty + 3.576n\frac{e^{-\kappa R}}{R(1+\kappa R)} \qquad (6)$$

At lower ionic strength this reduces to a modified form of the Brønsted equation:

$$\ln k = \ln k_0 - 2.34nI^{1/2} \qquad (7)$$

which is identical with the original Brønsted equation except for the substitution of *n* for $-Z_1Z_2$. The best fit of eq 6 to the ionic strength data of Figure 3 gave $R = 2.0$ Å and $n = 7$, while eq 7, fitted to the data from $I^{1/2} = 0.2$–$0.6$ M (Figure 2), gave $n = 5$. The ionic strength dependence for the ($F_3$Me)PhNHCO-Lys-13 cytochrome *c* derivative was somewhat less than that of native cytochrome *c*, consistent with the removal of one out of five to seven complementary charge interactions.

There are several important deficiencies in the assumptions which led to the derivation of eq 6. First, eq 3 for the electrostatic potential energy is based on the Debye–Hückel theory, which is not rigorously valid at high ionic strengths. However, the range of validity of the Debye–Hückel theory is much greater for monovalent ions than for polyvalent ions. In fact, the Debye–Hückel formula fits the experimental activity coefficient data of monovalent ions such as $NH_4Cl$ reasonably well up to $I^{1/2} = 0.6$ M if *R*, treated as an empirical parameter, is adjusted to 2 Å (Lewis & Randall, 1961). The more theoretically rigorous theory of Mayer (1950) was found to be valid up to $I^{1/2} = 0.6$ M for monovalent electrolytes but did not differ numerically very much from the Debye–Hückel theory (Poirier, 1953). Equation 6 does not distinguish between the effects of different electrolytes, but the data indicated such effects were important. The smaller slope obtained in the ionic strength plots using Mops buffers was probably because the Mops anion is much larger and more diffuse than the $Cl^-$ anion and cannot interact as effectively with the amino groups on cytochrome *c*. The smaller slope obtained using phosphate buffers at pH 7.8 is most likely due to specific binding of $HPO_4^{2-}$ to cytochrome *c*. Both $Cl^-$ and the monovalent anion $HPO_4^-$ bind to cytochrome *c* with high affinity and a stoichiometry of 2:1 (Margalit & Schejter, 1973). Thus, the actual cytochrome *c* species tested in NaCl and Tris-HCl buffers contained two tightly bound $Cl^-$ anions. Although the binding sites for these anions are not known with certainty, at least one of them is probably at the heme crevice region where it would reduce the effective value of *n*. The specific binding of the phosphate dianion has not been studied, but it probably also binds tightly and further decreases the strength of one or more of the specific complementary charge interactions.

Another deficiency in eq 6 is that it was assumed that there were no electrostatic interactions between different complementary charge interactions, even though many of the groups involved are located on adjacent amino acid residues. These interactions would make a favorable contribution to the electrostatic potential energy of complex formation, and in fact if the *n* complementary charge interactions were brought infinitely close to one another the coefficient in eq 6 would change from *n* to $n^2$. However, since the distance between an amino group in one complementary charge interaction and a carboxyl group in an adjacent one is large (>10 Å) compared to the distance between an amino group and a carboxyl group in the same complementary charge interaction, this effect should decrease rapidly as ionic strength is increased and is probably not important above 0.1 M.

Finally, the assumption that all the complementary charge interactions are equally strong cannot be quite true because of structural restrictions on the positions of the amino and carboxyl groups. This is shown experimentally by the fact that modification of lysine-13, -25, -27, -72, and -79 each caused about a 2–3-fold reduction in reaction rate, while modification of lysine-8 and -87 caused only about a 1.5-fold reduction (Table I). In the original model-building studies of Salemme (1976), four complementary charge interactions were suggested to be important in the complex. An inspection of the cytochrome $b_5$ and cytochrome *c* models suggested that several additional interactions might be present if some minor structural changes were allowed, primarily of the lysine, aspartate, and glutamate side chains. Cytochrome $b_5$ contains a very dense surface cluster of seven carboxyl groups: Glu-47, Glu-48, Glu-52, Glu-60, Asp-61, Asp-64, and the heme propionate. It is suggested that they might interact with cytochrome *c* Lys-25, -27, -13, -86, -87, -72, and -79, respectively, although the additional interactions might not be as strong as the original four proposed by Salemme.

In view of the above deficiencies, eq 6 must be regarded as semiempirical and judged by its ability to fit the experimental

data. The reaction between cytochrome $b_5$ and cytochrome $c$ is uniquely suited to test this theory, and the excellent fit to the ionic strength dependence of the reaction rate, the reasonable value obtained for $R$, and the good agreement between the calculated number of complementary charge interactions and the results of the chemical modification studies all combine to justify the validity of the general approach. In extending this technique to reactions between other electron transport proteins, it will be important to use small monovalent electrolytes such as NaCl at ionic strengths above 0.1 M and where possible corroborate the results with chemical modification studies.

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# Proton and Carbon-13 Nuclear Magnetic Resonance Studies of Rhodopsin–Phospholipid Interactions†

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ABSTRACT: Proton and carbon-13 nuclear magnetic resonance ($^1$H and $^{13}$C NMR) spectra of rhodopsin–phospholipid membrane vesicles and sonicated disk membranes are presented and discussed. The presence of rhodopsin in egg phosphatidylcholine vesicles results in homogeneous broadening of the methylene and methyl resonances. This effect is enhanced with increasing rhodopsin content and decreased by increasing temperature. The proton NMR data indicate the phospholipid molecules exchange rapidly ($<10^{-3}$ s) between the bulk membrane lipid and the lipid in the immediate proximity of the rhodopsin. These interactions result in a reduction in either or both the frequency and amplitude of the tilting motion of the acyl chains. The $^{13}$C NMR spectra identify the acyl chains and the glycerol backbone as the major sites of protein lipid interaction. In the disk membranes the saturated *sn*-1 acyl chain is significantly more strongly immobilized than the polyunsaturated *sn*-2 acyl chain. This suggests a membrane model in which the lipid molecules preferentially solvate the protein with the *sn*-1 chain, which we term an edge-on orientation. The NMR data on rhodopsin–asolectin membrane vesicles demonstrate that the lipid composition is not altered during reconstitution of the membranes from purified rhodopsin and lipids in detergent.

The light-sensitive protein rhodopsin comprises at least 85% of the protein content of the outer segment membranes of visual receptor cells (Daeman, 1973). Rhodopsin is considered to be an integral membrane protein. Data from X-ray diffraction (Blaise, 1972; Blaurock & Wilkins, 1972; Chabre, 1975), neutron diffraction (Yaeger, 1975; Saibil et al., 1976), freeze-fracture electron microscopy (Chen & Hubbell, 1973), and electron paramagnetic resonance spectroscopy (Hong & Hubbell, 1972) indicate that at least part of the protein is in

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