Role of Specific Lysine Residues in Binding Cytochrome c_2 to the *Rhodobacter sphaeroides* Reaction Center in Optimal Orientation for Rapid Electron Transfer†

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ABSTRACT: The role of specific lysine residues in facilitating electron transfer from *Rhodobacter sphaeroides* cytochrome c_2 to the *Rb. sphaeroides* reaction center was studied by using six cytochrome c_2 derivatives each labeled at a single lysine residue with a carboxydinitrophenyl group. The reaction of native cytochrome c_2 at low ionic strength has a fast phase with a half-time of 0.6 μs that has been assigned to the reaction of bound cytochrome c_2 [Overfield, R. E., Wraight, C. A., & DeVault, D. (1979) *FEBS Lett. 105*, 137]. Modification of lysine-55 did not affect the half-time of this phase but decreased the apparent binding constant by a factor of 2. The derivatives modified at lysines-10, -88, -95, -97, -99, -105, and -106 surrounding the heme crevice did not show any detectable fast phase but only slow second-order phases due to the reaction of solution cytochrome c_2. These lysines thus appear to be involved in binding cytochrome c_2 to the reaction center in an optimal orientation for electron transfer. The involvement of lysines-95 and -97 is especially significant, since they are located in an extra loop comprising residues 89–98 that is not present in eukaryotic cytochrome *c*. The reactions of horse cytochrome *c* derivatives modified at single lysine amino groups with trifluoroacetyl or [(trifluoromethyl)phenyl]carbamoyl were also studied. The derivatives modified at lysines-22, -55, -88, and -99 far removed from the heme crevice had nearly the same half-times for the fast phase as native cytochrome *c*, 6 μs. In contrast, the derivatives modified at lysines surrounding the heme crevice all had slower half-times for the first-order phase, ranging from 24 to 50 μs. Modification of these lysines must change the orientation of bound cytochrome *c* in such a fashion that the rate of electron transfer is decreased.

The reaction between cytochrome c_2 and the photosynthetic reaction center from *Rhodobacter sphaeroides* is an attractive system for studying the relation between specific binding interactions and the rate of electron transfer. The recent X-ray crystallographic analysis of the reaction center from *Rb. sphaeroides* has provided detailed information on the conformation of the L, M, and H polypeptide chains and the location of the specialized bacteriochlorophyll dimer, $(BChl)_2$, within the protein (Allen et al., 1987a,b). The binding site for cytochrome c_2 is located on the periplasmic surface of the reaction center and contains negatively charged carboxylate groups that have been proposed to interact electrostatically with positively charged lysine residues surrounding the heme crevice of cytochrome c_2 (Okamura & Feher, 1983; Hall et al., 1987a). The reaction between ferrocytochrome c_2 and the reaction center displays multiphasic kinetics at low ionic strength (Overfield et al., 1979; Dutton et al., 1975). The fast phase with a half-time of 0.6 μs has been assigned to electron transfer from cytochrome c_2 bound at a "proximal" site on the reaction center to the photooxidized $(BChl)_2^+$. The slow first-order phase is due to the reaction of cytochrome c_2 bound at a "distal" site, while a second-order phase observed at low concentrations is due to the reaction of solution cytochrome c_2. Horse heart cytochrome *c* can substitute for *Rb. sphaeroides* cytochrome c_2 even though the two proteins have opposite net charges at pH 7. The positively charged horse cytochrome *c* binds about 10-fold more strongly to the proximal site than the negatively charged cytochrome c_2 (K_d = 0.3 vs 3.0 μM), but the rate for the fast phase of the reaction is slower ($\tau_{1/2}$ = 2 vs 0.6 μs) (Rosen et al., 1979; Overfield & Wraight, 1986).

In the present study, we have investigated the role of specific lysine amino groups in binding cytochrome c_2 to the reaction center and aligning the heme group for optimal rates of electron transfer. Detailed kinetic studies of both the fast and slow phases of the reaction were carried out on six different *Rb. sphaeroides* cytochrome c_2 derivatives each labeled at a single lysine residue with a 4-carboxy-2,6-dinitrophenyl group. Kinetic studies have also been carried out on horse cytochrome *c* derivatives to determine the effect of specific lysine modifications on the rate of intracomplex electron transfer. These studies provide an experimental test of models of the complex between cytochrome c_2 and the reaction center constructed by molecular modeling procedures (Allen et al., 1987b; Tiede et al., 1988).

Experimental Procedures

Materials. Horse heart cytochrome *c* (type VI) was obtained from Sigma Chemical Co. Cytochrome c_2 was isolated from *Rb. sphaeroides* by the method of Bartsch (1978). Reaction centers were prepared by using lauryldimethylamine oxide (LDAO)[1] detergent as described by Feher and Okamura (1978). The 4-carboxy-2,6-dinitrophenyl (CDNP) cytochrome c_2 derivatives were prepared as described by Hall et al. (1989). [(Trifluoromethyl)phenyl]carbamoyl (TFC) derivatives of horse cytochrome *c* were prepared by the procedure of Smith et al. (1977). Trifluoroacetyl (TFA) derivatives of horse

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[1] Abbreviations: CDNB, 4-chloro-3,5-dinitrobenzoic acid; CDNP, 4-carboxy-2,6-dinitrophenyl; TFA, trifluoroacetyl; TFC, [(trifluoromethyl)phenyl]carbamoyl; UQ_0, 2,3-dimethoxy-5-methyl-1,4-benzoquinone; LDAO, lauryldimethylamine oxide detergent; Tris, tris(hydroxymethyl)aminomethane.

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cytochrome *c* were prepared by the procedures of Smith et al. (1980). All other chemicals were purchased from Fisher Scientific Co. and were reagent grade.

Laser Rapid Kinetic Measurements. The rate of reduction of *Rb. sphaeroides* reaction centers by cytochromes *c* and c_2 was followed at 860 nm after excitation of the reaction centers with a laser pulse. The excitation source was a Nd:YAG laser (Quanta Ray DCR-2) which provided 15-ns pulses of 532-nm radiation as the second harmonic (200 mJ/pulse nominal power). The excitation pulse was focused 2 cm in front of the sample (250 μL in a 1-cm semi-microcuvette) by passing it through a fused silica lens (300 × 25 mm). The probe beam consisted of a 100-W tungsten halogen lamp which was passed through an 860-nm interference filter and focused on the sample by a fused silica lens. The probe beam was limited to a 1-s pulse by an electronically controlled shutter synchronized with the laser excitation. The angle between the probe and excitation beams was 9°. The analyzed probe beam area was restricted by an iris diaphragm placed behind the sample. The probe beam was passed through an additional 860-nm interference filter, a collecting lens, and a 600-nm long-pass filter and was detected with an R928 photomultiplier tube. Laser-induced transient signals were recorded on a 8100 Biomation waveform recorder and transferred to an IBM PC microcomputer for kinetic analysis. Single transients were used in the analyses without signal averaging. The power of the laser excitation pulse was adjusted to a value slightly larger than that needed to saturate the transient signal.

Reaction kinetics were measured under both low ionic strength and high ionic strength conditions. The high ionic strength reaction mixture contained 20 mM Tris-HCl, pH 8.0, 40 mM NaCl, 0.025% LDAO, 1 mM sodium ascorbate, 100 μM UQ_0, 0.5 μM reaction centers, and 2–10 μM cytochrome c_2. The reactions followed second-order kinetics under these conditions, and the second-order rate constant was calculated from the slope of a plot of the pseudo-first-order rate constants versus the cytochrome c_2 concentration. The low ionic strength reaction mixture consisted of 10 mM Tris-HCl, pH 8.0, 0.025% LDAO, 1 mM sodium ascorbate, 100 μM UQ_0, 1–10 μM reaction centers, and 2–20 μM cytochrome *c* or c_2. In most experiments, the cytochrome concentration was maintained at twice the reaction center concentration to ensure that the binding sites were saturated. For each reaction mixture, several transients were recorded at different time scale settings to follow the entire course of the reaction as described by Overfield et al. (1979). A Marquardt nonlinear least-squares program was used to fit the reaction transients to first-order, second-order, or multiphasic kinetic decay curves (Gagnon et al., 1988). For many of the derivatives, the two first-order phases dominated the reaction at high cytochrome concentrations, and the transient could be fitted by the function $f(t) = a_0 + a_1e^{-k_1t} + a_2e^{-k_2t}$.

Results

Reaction Kinetics of CDNP-Cytochrome c_2 Derivatives. The reactions of the CDNP-cytochrome c_2 derivatives were first studied at high ionic strength (50 mM) where the reaction of native cytochrome c_2 is dominated by a second-order phase with a rate constant of 2.1×10^8 M^{-1} s^{-1}. This phase has been assigned to the reaction of solution cytochrome c_2 with the photooxidized reaction center (Overfield et al., 1979). Modification of lysines-88, -97, -99, -105, and -106 at the left side of the heme crevice caused the most significant effects on the reaction, with decreases in the second-order rate constant ranging from 6- to 12-fold (Table I). In contrast, modification of lysine-55 to the lower right side of the heme crevice caused only a 2-fold decrease in the rate constant.

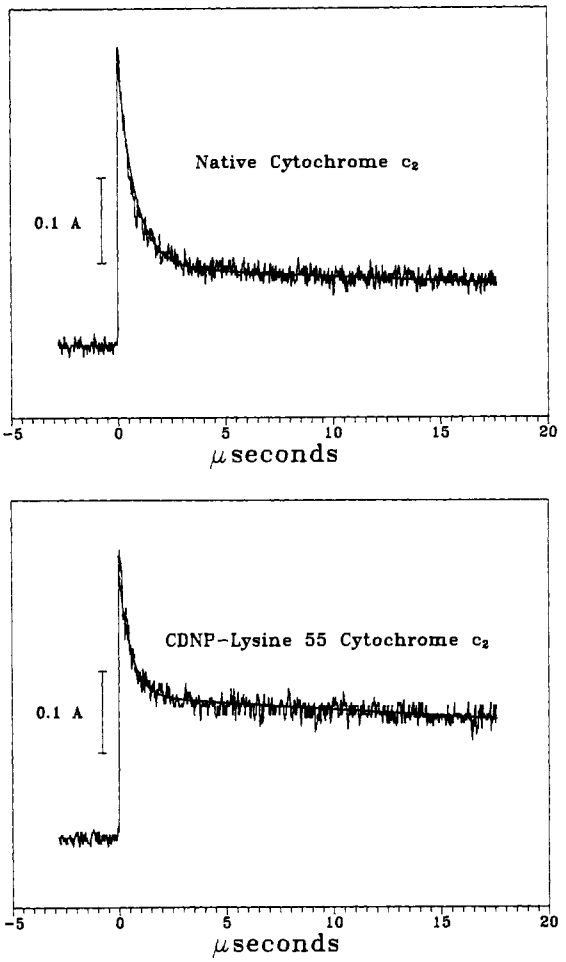

FIGURE 1: Reduction of photooxidized reaction centers by CDNP-cytochrome c_2 derivatives at low ionic strength. The reaction mixture consisted of 10 mM Tris-HCl, pH 8.0, 0.025% LDAO, 1 mM sodium ascorbate, 100 μM UQ_0, 10 μM reaction center, and 20 μM cytochrome c_2. The solid line gives the best fit of a biexponential equation to the data, as described under Experimental Procedures. (Top) Native cytochrome c_2; (bottom) CDNP-55 cytochrome c_2.

The reaction of native cytochrome c_2 at low ionic strength (5 mM) was found to involve three distinct kinetic phases, in agreement with the results of Overfield et al. (1979). The fast phase had a half-time of 0.6 ± 0.1 μs and accounted for 73% of the total reaction at a concentration of 10 μM reaction center and 20 μM cytochrome c_2 (Figure 1). The half-time of this phase was independent of reaction center concentration from 1 to 10 μM when the cytochrome c_2 concentration was maintained at twice that of the reaction center. An apparent binding constant of 1×10^5 M^{-1} was obtained from the concentration dependence of the amplitude of this phase. A second first-order phase with a half-time of 45 μs and an amplitidue of 26% was observed at high concentrations, and a slow second-order phase was present at low concentrations. The only derivative that exhibited a fast first-order phase was CDNP-lysine-55 cytochrome c_2, which had a half-time of 0.4 μs and a binding constant of 0.4×10^5 M^{-1} (Figure 1). The CDNP-cytochrome c_2 derivatives modified at lysines-10, -88, -95, -97, -99, -105, and -106 exhibited only slow second-order phases with rate constants ranging from 1.6×10^7 to 5.7×10^7 M^{-1} s^{-1} (Table I).

Reaction Kinetics of Horse Cytochrome c Derivatives. The half-time of the reaction of native horse cytochrome *c* at low ionic strength was found to be 15 μs, independent of concentration from 1 to 10 μM reaction center and from 2 to 20 μM

Table I: Second-Order Rate Constants for the Reaction of CDNP-Cytochrome c_2 Derivatives with *Rb. sphaeroides* Reaction Centers[a]

derivative	high ionic strength, $k \times 10^{-7}$ (M^{-1} s^{-1})	low ionic strength, $k \times 10^{-7}$ (M^{-1} s^{-1})
native *Rb. sph.*	21	80[b]
CDNP-10	6.6	3.6
CDNP-55	10.5	
CDNP-95	6.6	5.7
CDNP-97	1.8	4.2
CDNP-99	2.4	3.6
CDNP-105, -88[c]	2.2	4.4
CDNP-106	3.3	1.6
native *R. rubrum*	5.8	24

[a] The second-order rate constants were determined at high ionic strength by measuring the reduction of 0.5 μM photooxidized reaction centers by 2–10 μM cytochrome c_2 in a buffer containing 20 mM Tris-HCl, pH 8.0, 40 mM NaCl, 0.025% LDAO, 1 mM ascorbate, and 100 μM UQ_0. Second-order rate constants were measured at low ionic strength (5 mM) as described in Figure 1. [b] Overfield et al. (1979). [c] This fraction is a mixture of 55% CDNP-105 and 45% CDNP-88 derivatives.

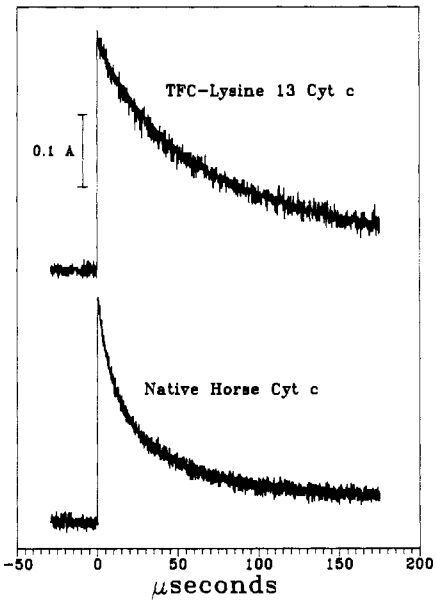

FIGURE 2: Reduction of photooxidized reaction centers by horse cytochrome *c* derivatives at low ionic strength. The reaction conditions were the same as those described in Figure 1. (Top) TFC-13 cytochrome *c*; (bottom) native horse cytochrome *c*.

cytochrome *c* (Figure 2). This is in good agreement with the results of Overfield and Wraight (1986), Rosen et al. (1979), and Ke et al. (1970). The use of a Marquardt nonlinear least-squares curve-fitting program revealed that the reaction consisted of two first-order phases, with half-times of 6 and 38 μs and amplitudes of 49% and 44%, respectively (Figure 2). The separation between the two phases became more apparent with the addition of 20 mM sodium chloride, as found by Overfield and Wraight (1986). The cytochrome *c* derivatives modified at lysines-7, -55, -88, and -100 had nearly the same half-times as native cytochrome *c* for each of the first-order phases (Table II). The half-times were slightly longer for the derivatives modified at lysines-22 and -25 on the right side of cytochrome *c*. In contrast, the derivatives modified at lysines-8, -13, -27, -72, -79, and -87 surrounding the heme crevice of cytochrome *c* all had significantly longer half-times ranging from 24 to 53 μs for the fast phase and from 140 to 530 μs for the slow phase (Figure 2, Table II). The half-times for the fast phase of the reactions were independent of concentration above 5 μM reaction center, indicating that they

Table II: Kinetic Constants for the First-Order Phases of the Reaction of Horse Cytochrome *c* Derivatives with the *Rb. sphaeroides* Reaction Center[a]

derivative	fast phase	slow phase
native horse	6 (49)	38 (44)
TFA-7	6 (35)	38 (60)
TFC-8	49 (68)	530 (23)
TFA-13	35 (35)	198 (36)
TFC-13	35 (60)	140 (26)
TFA-22	10 (53)	90 (25)
TFA-25	15 (46)	85 (38)
TFC-27	30 (69)	283 (22)
TFA-55	7 (38)	36 (46)
TFC-72	51 (71)	322 (25)
TFA-79	24 (38)	*b*
TFA-87	36 (66)	400 (22)
TFA-88	6 (35)	37 (44)
TFC-100	4 (50)	32 (49)

[a] The reactions were carried out in low ionic strength buffer with 10 μM reaction center and 20 μM cytochrome *c* as described in Figure 2, and the transients were fitted by a biexponential function as described under Experimental Procedures. The half-time of each phase is given in microseconds, and the amplitude of each phase (in parentheses) is given as a percent of the total reaction. [b] For this case, the slow first-order phase could not be resolved from the second-order phase.

were due to the reaction of bound cytochrome *c*. However, it was difficult to accurately resolve the two phases at concentrations below 5 μM because of the more limited signal to noise ratio. For this reason it was not possible to accurately measure the binding constants for the two phases.

Reaction Kinetics of R. rubrum Cytochrome c_2 and Yeast Cytochrome c. The reaction of native *Rhodospirillum rubrum* cytochrome c_2 with the *Rb. sphaeroides* reaction center did not show a detectable fast phase at low ionic strength but obeyed second-order kinetics with a rate constant of 2.4×10^8 M^{-1} s^{-1}. The second-order rate constant was found to be 5.9×10^7 M^{-1} s^{-1} in the high ionic strength buffer, which is 3.6-fold smaller than that of *Rb. sphaeroides* cytochrome c_2. The reaction of yeast cytochrome *c* had a fast phase with a half-time of 14 μs.

Discussion

Overfield et al. (1979) proposed a three-state model to account for the multiphasic kinetics of the reaction between *Rb. sphaeroides* cytochrome c_2 and the photosynthetic reaction center. The present studies support this model and provide information on the role of specific lysine residues in the interaction of cytochrome c_2 with the proximal and distal binding sites. The highly asymmetric distribution of lysine residues on the surface of *Rb. sphaeroides* cytochrome c_2 is shown in Figure 3, which was redrawn from the crystal structure of *R. rubrum* cytochrome c_2 by using the sequence alignment given in Table III. Although there is only a 39% sequence homology between the two proteins, 8 out of the 13 lysine residues in *Rb. sphaeroides* cytochrome c_2 are conserved in *R. rubrum* cytochrome c_2. Modification of specific lysine amino groups surrounding the heme crevice of cytochrome c_2 with the bulky, negatively charged CDNP group led to substantial decreases in the rate constants. These decreases are due to a combination of steric and electrostatic effects. The kinetic studies carried out at high ionic strength indicated that the lysine residues located in the sequence 95–106 on the left side of the heme crevice play an important role in the reaction of solution cytochrome c_2. This sequence contains five lysines conserved in *R. rubrum* cytochrome c_2 (95, 97, 99, 103, and 106) and three conserved in horse cytochrome *c* (99, 105, and 106). The very large decrease in rate for the fraction containing a 55–45% mixture of derivatives labeled at lysines-105 and -88 indicates

Table III: Optimal Alignment of the Amino Acid Sequences of *Rb. sphaeroides* Cytochrome c_2, *R. rubrum* Cytochrome c_2, and Horse Cytochrome *c* (Dickerson, 1980; Ambler et al., 1979)[a]

	10 20 30
Sp c_2	Q E G D P E A G A K A F N Q - C Q T C H V I V D D S G T T I A G
	10 20
Ru c_2	E G D A A A G E K V S K - K C L A C H T F D Q G G - - - - - -
	10 20
Hor c	G D V E K G K K I F V Q K C A Q C H T V E K G G - - - - - -
	40 50 60
Sp c_2	R N A K T G F N L Y G V V G R T A G T Q A D F K G Y G E G M K E
	30 40 50
Ru c_2	- A N K V G P N L F G V F E N T A A H K D N Y A - Y S E S Y T F
	30 40 50
Hor c	- K H K T G P N L H G L F G R K T G Q A P G F T - Y T D A N K N
	70 80 90
Sp c_2	A G A K G L A W D E E H F V Q Y V Q D P T K F L K E Y T G D A K
	60 70 80
Ru c_2	M K A K G L T W T E A N L A A Y V K D P K A F V L E K S G D P K
	60 70
Hor c	- - - K G I T W K E E T L M E Y L E N P K K Y I P - - - - - - -
	100 110 120
Sp c_2	A K G K M T F K - L K K E A D A H N I W A Y L Q Q V A V R P
	90 100 110
Ru c_2	A K S K M T F K - L T K D D E I E N V I A Y L K T L K
	80 90 100
Hor c	G - T K M I F A G I K K K T E R E D L I A Y L K K A T N E

[a] Residues that are conserved in two or three of the sequences are enclosed in boxes.

that both of these lysines are located at the binding domain. Modification of lysine-10 at the top of the heme crevice also decreased the reaction rate, but to a smaller extent. The reaction was even more sensitive to electrostatic interactions at low ionic strength, and modification of lysines-10, -88, -95, -97, -99, -105, and -106 surrounding the heme crevice completely eliminated both first-order phases of the reaction. This indicates that these lysines are involved in binding cytochrome c_2 to both the proximal and distal sites on the reaction center. Modification of lysine-55 to the lower right of the heme crevice decreased the binding constant by about 2 but did not affect the rate of the fast phase of the reaction, indicating that the optimal alignment of the heme group was maintained.

The involvement of lysines-95 and -97 is especially significant since they are located in the extra loop comprising residues 89–98 at the lower left of the heme crevice that is not present in eukaryotic cytochrome *c*. These residues might help orient cytochrome c_2 such that its heme group is optimally aligned for rapid electron transfer to $(BChl)_2^+$. This could account for the fact that the fast phase of the cytochrome c_2 reaction is about 10-fold faster than that of horse cytochrome *c* (Rosen et al., 1979; Overfield & Wraight, 1986). The involvement of these residues is also consistent with the recent studies of Tiede (1987), who used transient linear dichroism methods to measure the orientation of both cytochromes bound to reaction centers incorporated into phospholipid membranes. Cytochrome c_2 was found to bind in a more favorable orientation for rapid electron transfer than horse cytochrome *c*, with its heme group tilted 8° closer to the membrane normal and rotated by 32°.

Studies by Overfield and Wraight (1986) and Moser and Dutton (1988) have shown that the kinetics of the reaction of horse cytochrome *c* with the reaction center can be adequately described by the three-state model. Native horse cytochrome *c* binds much more strongly to the reaction center than native *Rb. sphaeroides* cytochrome c_2, and all of the horse cytochrome *c* derivatives bind strongly enough to exhibit a first-order phase in the reaction at low ionic strength. This is due in part to the fact that the TFA and TFC modifications only change the charge of the lysine from +1 to 0 while the CDNP modification changes the charge to −1. The half-time

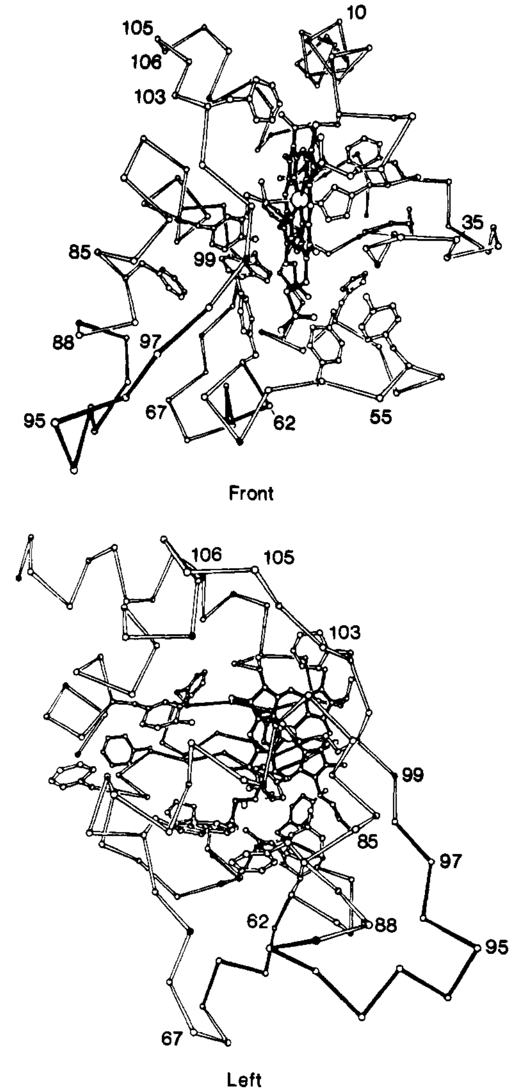

FIGURE 3: Crystal structure of *R. rubrum* cytochrome c_2 showing the α-carbons, aromatic residues, and heme ligands from the front and left side of the molecule [redrawn with permission from Figure 1 of Salemme et al. (1973)]. The predicted positions of the lysine residues of *Rb. sphaeroides* cytochrome c_2 are superimposed on the *R. rubrum* structure by using the sequence alignment shown in Table III. The heme group and the insertions into the eukaryotic cytochrome *c* sequence are shown in black.

of the fast phase of the reaction was increased 5–10-fold in the horse cytochrome *c* derivatives modified at heme crevice lysines (Table II). Modification of these lysines must change the orientation of bound cytochrome *c* in such a fashion that electron transfer from the heme group to $(BChl)_2^+$ is less favorable. The involvement of lysines-8, -27, -79, -86, and -87 in binding horse cytochrome *c* is especially significant since these residues are homologous to lysines-10, -35, -99, -105, and -106 surrounding the heme crevice of *Rb. sphaeroides* cytochrome c_2 (Table III). The distal cytochrome *c* binding site must also involve the heme crevice lysines, since the half-time for the slow phase was increased by modification of these lysines but not affected by modification of lysines-55, -88, and -100 on the bottom, left side, and back of cytochrome *c*, respectively. These results clearly indicate that specific lysine modification affects the rate of electron transfer within the bound complex as well as the binding strength. Previous studies of these derivatives at high ionic strength indicated that the heme crevice lysines were important to the second-order reaction but did not distinguish between effects on the binding strength and the electron-transfer rate (Hall et al., 1987b).

The kinetic studies on the cytochrome c_2 derivatives are consistent with molecular modeling studies which suggest that there are at least 11 complementary charge pair interactions between cytochrome c_2 and the reaction center (Tiede et al., 1988; Allen et al., 1987a). It is surprising, however, that *R. rubrum* cytochrome c_2 reacts so poorly with the *Rb. sphaeroides* reaction center. The reaction at low ionic strength displays no fast phase, and the second-order rate constant is over 10-fold slower than that of native *Rb. sphaeroides* cytochrome c_2, horse cytochrome *c*, or yeast cytochrome *c*. There is a 39% homology between the sequences of *R. rubrum* and *Rb. sphaeroides* cytochrome c_2, and most of the lysine residues on the left side of the heme crevice are conserved, including those in the extra loop at the lower left side. Molecular modeling studies have indicated that *R. rubrum* cytochrome c_2 should bind favorably to the *Rb. sphaeroides* reaction center (Allen et al., 1987b). There are, however, two extra lysines at residues 12 and 13 that are not present in *Rb. sphaeroides* cytochrome c_2 that might alter the orientation of the bound cytochrome and account for the slower rate of the reaction. We have previously shown that the reaction between *R. rubrum* cytochrome c_2 and *R. rubrum* reaction centers involves the lysines surrounding the heme crevice but were not able to isolate singly labeled derivatives (Hall et al., 1987b). In contrast, Rieder et al. (1985) have shown that the formation of a complex between the *R. rubrum* reaction center and *R. rubrum* ferricytochrome c_2 involves the back of the molecule on the opposite side from the heme crevice domain. This is probably a nonproductive product complex, but it is possible that this binding mode might have some functional significance.

Recent kinetic studies using the CDNP-cytochrome c_2 derivatives have shown that the binding domain for the *Rb. sphaeroides* cytochrome bc_1 complex is nearly identical with that for the reaction center, providing additional evidence that cytochrome c_2 functions as a diffusional carrier during electron transport in *Rb. sphaeroides* (Hall et al., 1989).

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