The Binding Domain on Horse Cytochrome *c* and *Rhodobacter sphaeroides* Cytochrome c_2 for the *Rhodobacter sphaeroides* Cytochrome bc_1 Complex†

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ABSTRACT: The interaction of the *Rhodobacter sphaeroides* cytochrome bc_1 complex with *Rb. sphaeroides* cytochrome c_2 and horse cytochrome *c* was studied by using specific lysine modification and ionic strength dependence methods. The rate of the reactions with both cytochrome *c* and cytochrome c_2 decreased rapidly with increasing ionic strength above 0.2 M NaCl. The ionic strength dependence suggested that electrostatic interactions were equally important to the reactions of the two cytochromes, even though they have opposite net charges at pH 7.0. In order to define the interaction domain on horse cytochrome *c*, the reaction rates of derivatives modified at single lysine amino groups with trifluoroacetyl or trifluoromethylphenylcarbamoyl were measured. Modification of lysine-8, -13, -27, -72, -79, and -87 surrounding the heme crevice was found to significantly lower the rate of the reaction, while modification of lysines in other regions had no effect. This result indicates that lysines surrounding the heme crevice of horse cytochrome *c* are involved in electrostatic interactions with carboxylate groups at the binding site on the cytochrome bc_1 complex. In order to define the reaction domain on cytochrome c_2, a fraction consisting of a mixture of singly labeled 4-carboxy-2,6-dinitrophenylcytochrome c_2 derivatives modified at lysine-35, -88, -95, -97, and -105 and several unidentified lysines was prepared. Although it was not possible to resolve these derivatives, all of the identified lysines are located on the front surface of cytochrome c_2 near the heme crevice. The rate of reaction of this fraction was significantly smaller than that of native cytochrome c_2, suggesting that the binding domain on cytochrome c_2 is also located at the heme crevice. Since the same domain is involved in the reaction with the photosynthetic reaction center, cytochrome c_2 must undergo some type of rotational or translational diffusion during electron transport in *Rb. sphaeroides*.

The purple photosynthetic bacteria have been found to contain a cytochrome bc_1 complex that is similar in many respects to the mitochondrial cytochrome bc_1 complex (Crofts & Wraight, 1983; Prince & Dutton, 1978). The cyclic electron-transfer process in these bacteria is initiated when the photoexcited reaction center complex reduces ubiquinone to ubiquinol. The cytochrome bc_1 complex then transfers electrons from ubiquinol to cytochrome c_2, which in turn reduces the photoxidized bacteriochlorophyll dimer in the reaction center. Electron transfer through the cytochrome bc_1 complex is accompanied by vectorial proton translocation across the membrane, which is coupled to ATP synthesis. The cytochrome bc_1 complex has been purified from *Rhodobacter sphaeroides* by a number of different laboratories and shown to contain two *b* cytochromes, a Rieske iron–sulfur protein, and a cytochrome c_1 (Yu et al., 1984; Gabellini et al., 1982). It has only four polypeptide chains, in contrast to the mitochondrial cytochrome bc_1 complex that has eight to ten polypeptides (Capaldi, 1982). Horse heart cytochrome *c* can substitute for *Rb. sphaeroides* cytochrome c_2 in the reaction with both the cytochrome bc_1 complex and the reaction center, even though the bacterial cytochrome c_2 has a negative net charge at pH 7 while horse cytochrome *c* has a positive net charge. Therefore, it appears that the electrostatic interaction with the two complexes is controlled by the local charge distribution on the cytochrome. Although the crystal structure of *Rb. sphaeroides* cytochrome c_2 has not been determined, its sequence is quite homologous to that of *Rhodospirillum rubrum* cytochrome c_2, which has been studied by X-ray crystallography (Salemme et al., 1973). The distribution of lysine amino groups surrounding the heme crevice of *R. rubrum* cytochrome c_2 is remarkably similar to that of horse heart cytochrome *c*, suggesting that the interaction with both the cytochrome bc_1 complex and the reaction center would involve electrostatic interactions between the highly conserved lysine amino groups surrounding the heme crevice and carboxylate groups on the reaction partners. However, Rieder et al. (1985) have recently shown that the formation of a complex between *R. rubrum* reaction centers and cytochrome c_2 at low ionic strength protects only lysines on the "backside" of the cytochrome from modification with acetic anhydride. This observation suggests that there might be a fundamental difference between the site of electron transfer used by bacterial and eukaryotic cytochromes *c*.

We report here that modification of lysine amino groups on the front side of *Rb. sphaeroides* cytochrome c_2 with 4-chloro-3,5-dinitrobenzoic acid (CDNB)[1] results in inhibition of the reaction with the *Rb. sphaeroides* cytochrome bc_1 complex. Kinetic studies were also carried out by using horse heart cytochrome *c* derivatives modified at specific lysine amino groups with CF_3CO– or $CF_3PhNHCO$–. Our studies conclude that the reactions of both cytochromes occur at the heme crevice domain and involve electrostatic interactions between lysine amino groups surrounding the heme crevice and carboxylate groups at the binding site on the cytochrome

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[1] Abbreviations: CDNB, 4-chloro-3,5-dinitrobenzoic acid; CDNP, 4-carboxy-2,6-dinitrophenyl; $Q_0H_{10}Br(H_2)$, 2,3-dimethoxy-5-methyl-6-(10-bromodecyl)-1,4-benzoquinol; EDTA, ethylenediaminetetraacetic acid; cyt c_2, cytochrome c_2.

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bc_1 complex. Since the same domain on cytochrome c_2 is involved in the reaction with the reaction center (Hall et al., 1987), cytochrome c_2 must undergo some type of rotational or translational diffusion during electron transport from the cytochrome bc_1 complex to the reaction center.

Experimental Procedures

Materials. Horse heart cytochrome *c* (type VI) was obtained from Sigma Chemical Co. The cytochrome bc_1 complex from *Rb. sphaeroides* R-26 was prepared as described previously (Yu et al., 1984). Cytochrome c_2 was isolated from *Rb. sphaeroides* by the method of Bartsch (1978). The synthesis of reduced ubiquinol, $Q_0C_{10}Br(H_2)$, has been reported in the literature (Yu & Yu, 1982). The 4-carboxy-2,6-dinitrophenyl- (CDNP-) cytochrome c_2 derivative was prepared as described in the previous paper (Hall et al., 1987). Trifluoromethylphenylcarbamoyl ($CF_3PhNHCO$–) derivatives of horse cytochrome *c* were prepared by the procedure of Smith et al. (1977). Trifluoroacetyl (CF_3CO–) derivatives of horse cytochrome *c* were prepared by the procedures of Smith et al. (1980). All other chemicals were purchased from Fisher Scientific Co. and were of reagent grade.

Kinetics Measurements. An Aminco DW-2a spectrophotometer was used to measure the enzymatic activity of the *Rb. sphaeroides* cytochrome bc_1 complex. Activity was measured as the change in absorbance per minute at 550 nm as cytochrome *c* or c_2 underwent reduction by reduced ubiquinol via the cytochrome bc_1 complex. Prior to the kinetic measurements, horse cytochrome *c* and *Rb. sphaeroides* cytochrome c_2, native and derivatives, were oxidized with potassium ferricyanide and chromatographed on a Bio-Gel P-2 column equilibrated with 25 mM sodium phosphate, pH 7.0, and 300 μM EDTA. Each assay mixture used 1 mL containing 25 mM sodium phosphate buffer, pH 7.0, 300 μM EDTA, 7.4 μM $Q_0C_{10}Br(H_2)$, 2 nM *Rb. sphaeroides* cytochrome bc_1 complex, and 0–1 M NaCl. In addition each assay medium contained 0.2–8 μM ferricytochrome *c* or ferricytochrome c_2.

Results

Ionic Strength Dependence of Reduction of Horse Cytochrome c and Rb. sphaeroides Cytochrome c_2 by the Rb. sphaeroides Cytochrome bc_1 Complex. The effect of ionic strength on the reactions of native cytochromes *c* and c_2 with the cytochrome bc_1 complex was measured in 25 mM sodium phosphate buffer, pH 7.0, and 300 μM EDTA with 0–1 M NaCl. At ionic strengths below 0.2 M, the reaction with *Rb. sphaeroides* cytochrome c_2 had a zero-order time course at the lowest tested cytochrome c_2 concentration (0.5 μM) and the Michaelis constant K_m was too low to be measured. Figure 1 shows that the reaction rate measured at a constant cytochrome c_2 concentration of 1.4 μM was independent of ionic strength up to 0.2 M and then decreased rapidly with increasing ionic strength. The time course of the reaction became first order above 0.2 M ionic strength as the K_m for cytochrome c_2 increased rapidly. A similar pattern was observed for horse cytochrome *c*, except that the rate constant began decreasing at 0.1 M ionic strength. The rate constant was nearly the same as that of cytochrome c_2 below 0.1 M ionic strength but was up to 4-fold smaller at higher ionic strengths (Figure 1). Figure 2 shows that the reactions with native cytochromes *c* and c_2 obey Michaelis–Menten kinetics with K_m values in a conveniently measureable range at ionic strengths of 0.2 and 0.35 M, respectively.

Effect of Selective Lysine Modification on Reactions of Horse Cytochrome c and Rb. sphaeroides Cytochrome c_2 with the Cytochrome bc_1 Complex. The reaction of the *Rb. sphaeroides* cytochrome bc_1 complex with horse cytochrome *c* derivatives modified at single lysine amino groups was studied to determine the reaction domain. Figure 2 shows that the $CF_3PhNHCO$- and CF_3CO-cytochrome *c* derivatives obey Michaelis–Menten kinetics and have nearly the same V_{max} as native horse cytochrome *c*. The greatest effect on kinetic activity was observed for derivatives modified at lysine-7, -8 -13, -25, -27, -72, -79, and -87 surrounding the heme crevice

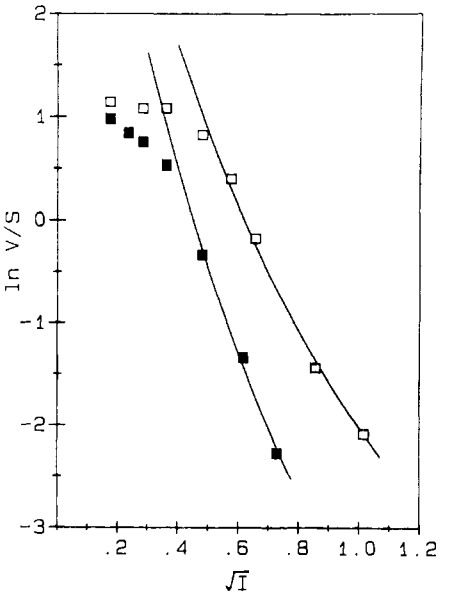

FIGURE 1: Ionic strength dependence of the reaction between the *Rb. sphaeroides* cytochrome bc_1 complex and horse cytochrome *c* (■) or *Rb. sphaeroides* cytochrome c_2 (□). The assays were carried out in a buffer containing 25 mM sodium phosphate, pH 7.0, 300 μM EDTA, 7.4 μM $Q_0H_{10}Br(H_2)$, 2 nM cytochrome bc_1 complex, and 0–1 M NaCl. The initial velocity *V* was measured from the change in absorbance at 550 nm, and the cytochrome *c* concentration *S* was held constant at 1 μM. The solid lines were both obtained from eq 1 with r_i = 4 Å and n = 8.

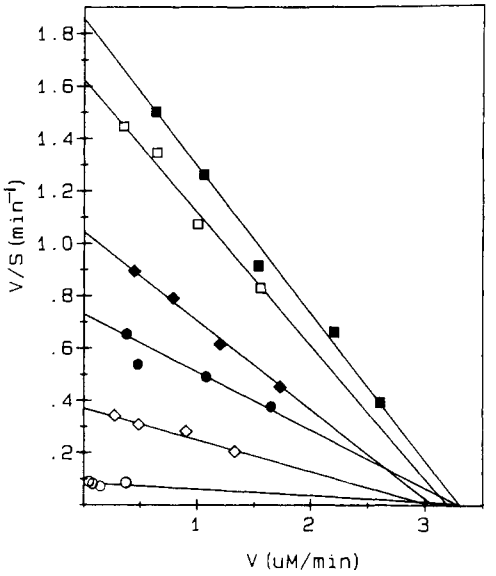

FIGURE 2: Steady-state kinetics for reaction of the *Rb. sphaeroides* cytochrome bc_1 complex with derivatives of horse cytochrome *c* and *Rb. sphaeroides* cytochrome c_2: native horse cyt *c* (■); $CF_3PhNHCO$-Lys-100 (□); $CF_3PhNHCO$-Lys-27 (◆); $CF_3PhNHCO$-Lys-13 (◇); native *Rh. sphaeroides* cyt c_2 (●); CDNP-cyt c_2 fraction A (○). The buffer was the same as that described in Figure 1, except that 150 mM NaCl was used for the horse cytochrome *c* studies and 300 mM NaCl was used for the *Rb. sphaeroides* cytochrome c_2 studies. The cytochrome *c* concentration ranged from 0.5 to 10 μM.

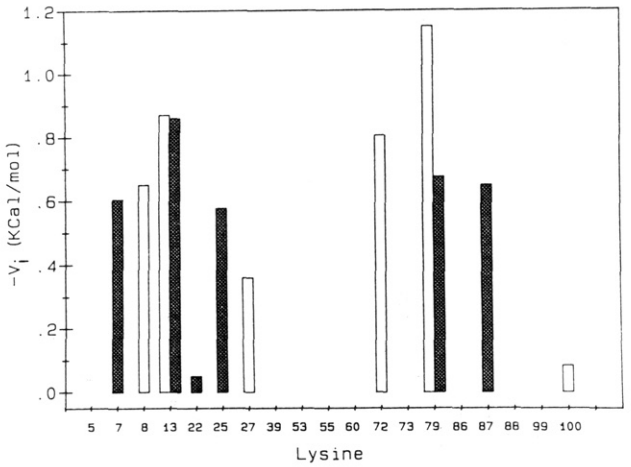

FIGURE 3: Effect of specific lysine modifications on reaction between horse cytochrome *c* and *Rb. sphaeroides* cytochrome bc_1 complex. V_i is defined as $-RT \ln [(V_{max}/K_m)\text{nat}/(V_{max}/K_m)\text{der } i]$. The open bars give V_i values for $CF_3PhNHCO$-cytochrome *c* derivatives, while the solid bars give V_i values for CF_3CO-cytochrome *c* derivatives. The steady-state assays were carried out as described in Figure 2.

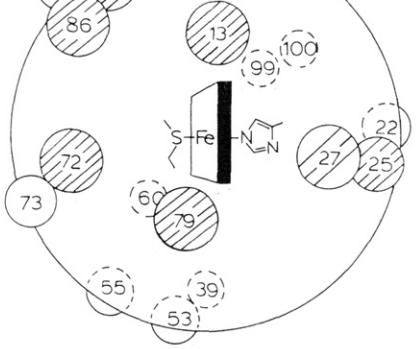

FIGURE 4: Schematic diagram of horse heart cytochrome *c* viewed from the front of the heme crevice. The approximate positions of the lysine residues are indicated by closed and dashed circles for residues located toward the front and back of cytochrome *c*, respectively. The estimated electrostatic free energy contribution of each lysine, V_i, is indicated by the number of diagonal hatch marks in the circle, with −0.10 kcal/(mol·hatch mark). The value given for lysine-86 is not experimental but was assumed to be the same as that for lysine-87.

that had V_{max}/K_m values 2–7-fold smaller than that of native cytochrome *c* (Figures 2–4). Modification of lysine-22 at the far right side of cytochrome *c* and lysine-100 at the back had no significant effect on the reaction with the cytochrome bc_1 complex. Figure 2 also shows kinetic measurements of the reaction of the cytochrome bc_1 complex with native *Rb. sphaeroides* cytochrome c_2 and CDNP-cytochrome c_2 fraction A. The V_{max}/K_m parameter of fraction A was 8-fold smaller than that of native cytochrome c_2. Fraction A is a mixture of singly labeled derivatives containing 6% CDNP-Lys-35-cyt c_2, 18% CDNP-Lys-88-cyt c_2, 9% CDNP-Lys-95-cyt c_2, 24% CDNP-Lys-97-cyt c_2, 11% CDNP-Lys-105-cyt c_2, and 32% other singly labeled derivatives (Hall et al., 1987). All of the assigned lysines are located on the front surface of cytochrome c_2.

Electrostatic Interaction between Cytochrome c and the Rb. sphaeroides Cytochrome bc_1 Complex. We have developed a semiempirical relationship for the electrostatic interaction of cytochrome *c* with its electron-transport partners that is in quantitative agreement with both the ionic strength dependence of the interaction and the effect of modifying specific lysine amino groups (Smith et al., 1981; Stonehuerner et al., 1979). The ionic strength dependence of the rate constant is related to the electrostatic interaction of *n* charge pairs by

$$\ln (k/k_\infty) = \sum_{i=1}^{n} -V_i/RT = \sum_{i=1}^{n} \frac{4.235e^{\kappa(a-r_i)}}{RT(1+\kappa a)r_i} \qquad (1)$$

where V_i is the electrostatic energy of the *i*th charge pair, r_i is the distance between the amino and carboxylate groups of that charge pair, *a* is the effective radius of these groups, and $\kappa = 0.329\sqrt{I}$ Å^{-1}. An estimate for V_i can be obtained from the change in reaction rate caused by modification of lysine-*i*: $V_i = -RT \ln [(V_{max}/K_m)\text{nat}/(V_{max}/K_m)\text{der } i]$. These estimates were in the range of −0.6 to −0.8 kcal/mol for most of the lysines surrounding the heme crevice (Figure 3). Although these estimates may include contributions from steric factors, the reasonably good correspondence between the effect of modifying a given lysine with the CF_3CO– group and the bulky $CF_3PhNHCO$– group indicates that this is not a major problem. For simplicity, it was assumed that each of the charge pairs involving lysines surrounding the heme crevice had a V_i value of about −0.65 kcal/mol, which corresponds to an r_i value of 4.0 Å, according to eq 1. The value of *n* was taken to be 8, which includes lysine-8, -13, -25, -27, -72, -79, -86, and -87. Lysine-7, -73, and -88 at the edge of the heme crevice domain were not included since they each have a nearby carboxylate group on the surface of cytochrome *c* that would cancel their contribution to the electrostatic interaction. The ionic strength dependence of the reacton rate calculated from eq 1 with $n = 8$ and $r_i = 4$ Å is shown in Figure 1. The agreement with the experimental data is quite reasonable at ionic strengths above 0.15 M, where the parameter V/S was equal to V_{max}/K_m. The best fit of eq 1 to the experimental data for the reaction with cytochrome c_2 at ionic strengths above 0.25 M was obtained with $n = 8$ and $r_i = 4$ Å (Figure 1).

Discussion

The results reported here demonstrate the importance of electrostatic interactions to the reaction of the *Rb. sphaeroides* cytochrome bc_1 complex with its natural oxidant cytochrome c_2 as well as with horse cytochrome *c*. The specific modification studies indicate that complex formation involves electrostatic interactions between lysine amino groups surrounding the heme crevice of cytochrome *c* and carboxylate groups on the cytochrome bc_1 complex. The binding domain on horse cytochrome *c* for the *Rb. sphaeroides* cytochrome bc_1 complex is nearly identical with the one for the bovine cytochrome bc_1 complex (Ahmed et al., 1978; Speck et al., 1979; Rieder & Bosshard, 1980). We have previously used a water-soluble carbodiimide to determine that carboxylate groups in the sequence 63–81 of bovine cytochrome cytochrome c_1 are involved in binding horse cytochrome *c* (Stonehuerner et al., 1985). Gabellini and Sebald (1986) have recently compared the sequences of cytochrome c_1 from bovine and yeast mitochondria with that from *Rb. sphaeroides* (although it appears that the latter might actually be a strain of *Rhodobacter capsulata*). They found that three carboxylate groups in the sequence 63–81 are conserved in all three proteins. These correspond to Glu-84, Asp-90, and Glu-94 of the *Rb. sphaeroides* sequence. Broger et al. (1983) have shown that (arylazido)-lysine-13 horse cytochrome *c* is cross-linked to bovine cytochrome c_1 somewhere in the highly acidic sequence 165–174. Two acidic residues in this sequence are conserved in the three species listed above, and correspond to Glu-17 and Asp-218 of the *Rb. sphaeroides* sequence. It therefore appears that the two acidic sequences 63–81 and 165–174 of bovine cyto-

chrome c_1 form the binding domain for cytochrome *c* and this domain is conserved in the proteins from *Rb. sphaeroides* and yeast.

The ionic strength dependence of the reaction rates indicates that the electrostatic interaction of *Rb. sphaeroides* cytochrome c_2 with the cytochrome bc_1 complex is nearly identical with that involving horse cytochrome *c* (Figure 1). Since the two proteins have opposite net charges, the local charge at the binding domain must dominate the electrostatic interaction. Unfortunately, it was not possible to purify singly labeled CDNP-cytochrome c_2 deriatives to characterize the interaction domain more precisely. CDNP-cytochrome c_2 fraction A consists of a mixture of singly labeled derivatives modified at lysine-35, -88, -95, -97, and -105, and other unidentified lysines comprising 32% of the total (Hall et al., 1987). Although it is difficult to analyze the kinetics of such a mixture, certain limits can be placed on the kinetic parameters of the individual derivatives. Since the V_{max}/K_m of fraction A was 8-fold smaller than that of native cytochrome c_2, at least 88% of the derivatives must have V_{max}/K_m values that are each decreased by a factor of at least 8. This result allows us to conclude that at least four of the lysine-35, -88, -95, -97, and -105 are involved in binding to the reaction center, regardless of the activity of the unidentified derivatives. All of these lysines appear to be located on the front surface of *Rb. sphaeroides* cytochrome c_2, as indicated in Figure 7 of the preceding paper (Hall et al., 1987). Lysine-88, -95, and -97 are located to the lower left of the heme crevice, while lysine-35 and -105 are at the right and top left of the heme crevice, respectively. Lysine-95 and -97 are conserved in *R. rubrum* cytochrome c_2, while lysine-35 is conserved in the two bacterial cytochromes as well as in horse heart cytochrome *c*. There are certainly enough lysine amino groups on the front surface of cytochrome c_2 to form eight complementary charge pairs with carboxylate groups on the cytochrome bc_1 complex as indicated by the ionic strength dependence data (Figure 1).

We have recently studied the reaction of horse cytochrome *c* and *Rb. sphaeroides* cytochrome c_2 with the photosynthetic reaction center from *Rb. sphaeroides* (Hall et al., 1987). The binding domains on horse heart cytochrome *c* for the reaction center and the cytochrome bc_1 complex are nearly identical as determined by the lysine modification method. Kinetic studies with CDNP-cytochrome c_2 fraction A indicated that the binding domain on cytochrome c_2 also involves lysine residues surrounding the heme crevice. Since the same domain is involved in binding to both the reaction center and the cytochrome bc_1 complex, it appears that cytochrome c_2 must undergo some type of rotational or translational diffusion during electron transport in *Rb. sphaeroides*.

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