Reaction of Cytochromes c and c_2 with the *Rhodobacter sphaeroides* Reaction Center Involves the Heme Crevice Domain†

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Received December 30, 1986; Revised Manuscript Received March 12, 1987

ABSTRACT: In order to define the interaction domain on *Rhodobacter sphaeroides* cytochrome c_2 for the photosynthetic reaction center, positively charged lysine amino groups on cytochrome c_2 were modified to form negatively charged (carboxydinitrophenyl)- (CDNP-) lysines. The reaction mixture was separated into several different fractions by ion-exchange chromatography on (carboxymethyl)cellulose. Tryptic digests of these fractions were analyzed by reverse-phase peptide mapping to determine the lysines that had been modified. Fraction A was found to consist of a mixture of singly labeled derivatives modified at lysine-35, -88, -95, -97, and -105 and several other unidentified lysines comprising 32% of the total. 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 second-order rate constant for the reaction of native cytochrome c_2 with reaction centers was 2.0×10^8 M^{-1} s^{-1}, while that for fraction A was 20-fold less, 1.0×10^7 M^{-1} s^{-1}. This suggests that lysines surrounding the heme crevice of cytochrome c_2 are involved in electrostatic interactions with carboxylate groups at the binding site of the reaction center. The reaction rates of horse heart cytochrome c derivatives modified at single lysine amino groups with trifluoroacetyl or trifluoromethylphenylcarbamoyl were also measured. Modification of lysine-8, -13, -27, -72, -79, and -87 surrounding the heme crevice significantly lowered the rate of reaction, while modification of lysines in other regions had no effect. This indicates that the reaction of horse heart cytochrome c with the reaction center also involves the heme crevice domain.

The primary electron-transfer reaction in the photosynthetic bacterium *Rhodobacter sphaeroides* takes place in a membrane-bound reaction center consisting of three 30–35-kilodalton polypeptides and a number of prosthetic groups including bacteriochlorophyll, bacteriopheophytin, and ubiquinone (Feher & Okamura, 1978). Photooxidation of the specialized bacteriochlorophyll dimer results in formation of D^+ and electron transfer to bound ubiquinone. The oxidation of cytochrome c_2 by D^+ has been shown to be biphasic both in whole cells and for the purified reaction center in solution (Overfield & Wraight, 1980; Ke et al., 1970; Dutton et al., 1975). The fast phase was first order with a half-time of 3 μs and was assigned to electron transfer within a 1:1 complex of cytochrome c_2 and reaction center. The binding constant for this complex was found to decrease rapidly with increasing ionic strength, and no fast phase was observed at salt concentrations above 0.1 M (Overfield et al., 1979). The slow phase was second order with a half-time of 200 μs or longer and was attributed to the oxidation of cytochrome c_2 in solution. The rate constant for this reaction decreased rapidly with increasing ionic strength, both for *Rb. sphaeroides* cytochrome c_2 and for horse heart cytochrome c. Similar results have been obtained for the second-order reaction between *Rhodospirillum rubrum* reaction centers and both *R. rubrum* cytochrome c_2 and horse heart cytochrome c (Rickle & Cusanovich, 1979). Since the two bacterial cytochromes have a negative net change at pH 8, while horse heart cytochrome c has a positive net charge, it was suggested that the electrostatic interaction with the reaction center was controlled by the *local* charge distribution at the heme crevice. Salemme et al. (1973) have shown that 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. Since Okamura and Feher (1983) have shown that the binding domain on *Rb. sphaeroides* reaction centers consists of negatively charged carboxylate groups, it appears likely that the electrostatic interaction with the cytochromes would involve the highly conserved lysine amino groups surrounding the heme crevice. 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 the 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 eucaryotic 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 significant inhibition of the reaction with *Rb. sphaeroides* reaction centers. Kinetic studies were also carried out by using horse heart cytochrome c derivatives modified at specific lysine amino groups with CF_3CO- or $CF_3PhNHCO-$. We conclude that the reactions of both cytochromes occur at the heme crevice

† This work was supported in part by NIH Grants GM20488 and RR07101.

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[1] Abbreviations: CDNB, 4-chloro-3,5-dinitrobenzoic acid; CDNP, 4-carboxy-2,6-dinitrophenyl; UQ_0, 2,3-dimethoxy-5-methyl-1,4-benzoquinone; LDAO, lauryldimethylamine oxide detergent; MOPS, 3-(*N*-morpholino)propanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; TPCK, *N*-tosyl-L-phenylalanine chloromethyl ketone; DEAE, diethylaminoethyl; bicine, *N*,*N*-bis(2-hydroxyethyl)glycine; cyt c_2, cytochrome c_2; HPLC, high-performance liquid chromatography.

0006-2960/87/0426-4494$01.50/0

domain and involve electrostatic interactions between lysine amino groups surrounding the heme crevice and carboxylate groups at the binding site on the reaction center.

Experimental Procedures

Materials. Horse heart cytochrome *c* (type VI), 3-(*N*-morpholino)propanesulfonic acid (MOPS), Tris, TPCK-treated trypsin, and 2,3-dimethoxy-5-methyl-1,4-benzoquinone (UQ_0) were obtained from Sigma Chemical Co. Reaction centers were prepared with lauryldimethylamine oxide (LDAO) detergent as described previously (Feher & Okamura, 1978). Cytochrome c_2 was isolated from *Rb. sphaeroides* by the method of Bartsch (1978). 4-Chloro-3,5-dinitrobenzoic acid (CDNB) was purchased from Aldrich Chemical Co., recrystallized from benzene, dried over P_2O_5 in a vacuum, tightly sealed, and stored at −20 °C. (Trifluoromethylphenylcarbamoyl)- ($CF_3PhNHCO-$) lysine derivatives were prepared by the procedure of Smith et al. (1977). Trifluoroacetyl (CF_3CO-) derivatives were prepared by the procedure of Smith et al. (1980). All other chemicals were purchased from Fisher Scientific Co. and were of reagent grade.

Preparation of 4-Carboxy-2,6-dinitrophenyl (CDNP) Derivatives of Cytochrome c_2. Preparation of CDNP derivatives of cytochrome c_2 was based on modification of horse cytochrome *c* by CDNB as described by Brautigan et al. (1978). Cytochrome c_2 (37 mg) was oxidized by treatment with potassium ferricyanide and then chromatographed on a Bio-Gel P-2 column equilibrated with 0.2 M sodium bicarbonate, pH 9.0. A 160 mM CDNB solution was made from the recrystallized acid by using 0.2 M sodium bicarbonate, pH 9.0, and titrated to a pH of 9.0 by using 1 N sodium hydroxide. Cytochrome c_2 (750 μM) was mixed with 3.75 mM CDNB in 0.2 M sodium bicarbonate, pH 9, and allowed to react at 23 °C for 24 h. A second aliquot of 3.75 mM CDNB was then added, and after 42 h the excess CDNB reagent and hydrolysis products were removed by passing the sample through a Bio-Gel P-2 column equilibrated with 5 mM ammonium acetate, pH 5.4. The sample was applied to a (carboxymethyl)cellulose (Whatman CM32) 1.5 × 20 cm column equilibrated with 5 mM ammonium acetate, pH 5.4. The sample was eluted initially with 0.1 M ammonium acetate, pH 5.4. Native cytochrome c_2 was eluted with 0.2 M ammonium acetate, pH 5.4. The elution rate was 25 mL/h. Sample fractions that eluted in the void volume were combined, the pH was adjusted to 8.0 with Tris, and they were loaded onto a DEAE-agarose column equilibrated with 0.2 M bicine, pH 8.0. The sample was eluted from the DEAE column with an exponential gradient from 0.2 M bicine, pH 8.0, to 0.2 M bicine and 0.4 M sodium acetate, pH 8.0. Visible absorption spectra of the cytochrome c_2 derivatives were obtained on a Varian Cary 210 spectrophotometer. The average number of CDNP-labeled lysines per molecule was determined spectrally by the method of Brautigan et al. (1978). Redox potentials were measured in 0.1 M sodium phosphate, pH 7.0, by the method of Pettigrew et al. (1975).

Peptide Mapping. The cytochrome c_2 derivatives were dialyzed into 0.1 M bicine, pH 8.0, at a concentration of 1 μg/μL, and digested with TPCK-treated trypsin by using two 5% additions of the treated trypsin over a 20-h period at 37 °C. Tryptic digests were separated on a Brownlee RP-300 column with a linear gradient from 5 mM sodium phosphate, pH 7.0, to 100% methanol. The gradients were generated on a Spectra Physics SP 8700 solvent delivery system, and the eluent was monitored at 210 and 440 nm by using Spectraflow 757 and Tracor 970A variable-wavelength detectors in series. The amino acid composition of each purified peptide was determined by hydrolyzing the sample in 6 M HCl, containing 0.1% 2-mercaptoethanol, for 22 h at 110 °C in an evacuated, sealed tube. The hydrolysates were injected into a microbore amino acid analyzer equipped with ninhydrin detection (Durham & Geren, 1981).

Steady-State Kinetic Measurements. Horse cytochrome *c* and cytochrome c_2 derivatives were reduced with sodium ascorbate and chromatographed on a Bio-Gel P-2 column equilibrated with 10 mM MOPS, pH 6.5, and 0.025% LDAO, immediately prior to use. Assays were run in 10 mM MOPS, pH 6.5, 0.025% LDAO, and 40 mM NaCl. In addition, each assay medium contained 0.2–10 μM ferrocytochrome *c* or ferrocytochrome c_2, 40 nM UQ_0, and 15 nM reaction centers. Measurements of electron transfer between cytochrome *c* or c_2 and reaction centers were made spectrally by using an Aminco DW-2a Spectrophotometer illuminated from the side by a slide projector filtered to pass light above 640 nm. Activity was measured as a change in absorbance per minute at 550 nm as cytochrome *c* or c_2 underwent photooxidation.

Laser Rapid Kinetic Measurements. The oxidation of horse cytochrome *c* and cytochrome c_2 derivatives was followed at 550 nm with laser excitation of the reaction centers. The excitation source was a Nd:YAG laser (Quanta Ray DGR-2) which provided 15-ns pulses of 365-nm radiation as the third harmonic (100 mJ/pulse) or 532-nm radiation as the second harmonic (200 mJ/pulse). The excitation pulse was focused on the sample (150 μL in a 1-cm semimicrocuvette) by passing it through a fused silica lens (300 × 25 mm). The probe beam came from a 100-W tungsten halogen lamp which was passed through an interference filter and collimated 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 output. The angle between the probe and excitation beams was 9°. The analyzed probe beam area was restricted to that excited by the laser beam (3 mm) by using an iris diaphragm placed behind the sample. The probe beam passed through a monochromator (Kratos GM252) and was detected with a R928 photomultiplier tube. The signal was recorded on a 1010 Biomation waveform recorder, transferred to an IBM PC, and fitted to a first-order decay curve by least squares. The reaction mixture contained 20 mM Tris-HCl, pH 8.0, 40 mM NaCl, 0.025% LDAO, 1 mM sodium ascorbate, 100 μM UQ_0, 2–5 μM reaction centers, and 0–10 μM cytochrome. The photoexcitation of the reaction centers was found to be fully saturated by the laser pulse.

Results

Preparation of CDNP-cytochrome c_2 Derivatives. Rb. sphaeroides cytochrome c_2 (750 μM) was treated with 7.5 mM CDNB at pH 9.0 to modify positively charged lysine amino groups to negatively charged CDNP-lysines. The crude reaction mixture was separated by ion-exchange chromatography on Whatman CM 32 (carboxymethyl)cellulose as shown in Figure 1. Fraction A was found to have an average of 1 CDNP lysine per molecule, while fraction B, which eluted in the void volume, contained an average of 2.5 CDNP-lysines per molecule. The fraction marked native eluted in the same position as native cytochrome c_2 and contained no CDNP-lysines. Fraction B was further resolved into five fractions by chromatography on DEAE-agarose (data not shown). These fractions each contained an average of 2 or more CDNP-lysines per molecule.

Visible Absorption Spectra and Redox Potentials of CDNP-cytochrome c_2 Derivatives. There were no detectable differences between the visible absorption spectra of native

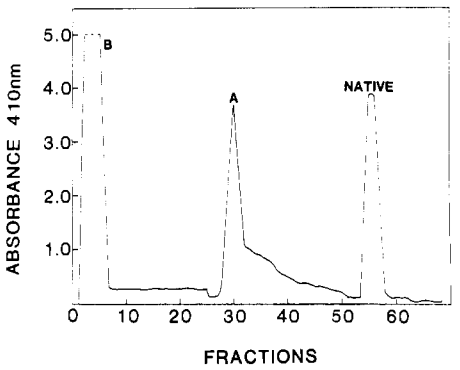

FIGURE 1: Chromatogram of *Rb. sphaeroides* CDNP-cytochrome c_2 derivatives. The reaction mixture (34 mg) was eluted on a 1.5 × 20 cm CM 32 column with 0.1 M ammonium acetate, pH 5.4, at a flow rate of 25 mL/h. The fraction size was 2 mL. At fraction 50, 0.2 M ammonium acetate was used to elute native cytochrome c_2.

cytochrome c_2 and fraction A in either redox state (data not shown). The presence of an unchanged 695-nm band in the oxidized state indicated that the bond between iron and Met-100 was intact. The redox potential of fraction A was found to be 342 ± 5 mV at pH 7.0, which was nearly the same as that measured for native cytochrome c_2 under these conditions, 352 ± 5 mV. The oxidized-state absorption spectra of the fraction B derivatives each contained a Soret band that was shifted to 400 nm or lower and a new band at 620 nm. These spectra were similar to that observed for ferricytochrome *c* in strongly acidic solutions (Theorell & Akesson, 1941) and indicate that the heme environment was distorted to such an extent that the Met-100 bond to iron was displaced. The fraction B derivatives could not be signficantly reduced by ferrocyanide, so their redox potentials were less than ~150 mV. These derivatives were not used for any further studies.

Identification of CDNP-Labeled Lysine Groups by HPLC Peptide Mapping. In order to identify the residues modified by CDNP, fraction A was hydrolyzed by trypsin and the resulting peptides separated by reverse-phase HPLC (Figure 2). CDNP-lysine absorbs at 440 nm, so it was possible to follow both protein and label simultaneously. The chromatogram of fraction A (Figure 2) shows five major CDNP-labeled peptides, which were identified by amino acid analysis with reference to the published amino acid sequence (Table I; Ambler et al., 1979). It was not possible to identify the remaining minor labeled peptides because they were not sufficiently pure. The assignments of both the labeled and unlabeled peptides were consistent with the known specificity of trypsin for lysine and arginine residues except for the atypical hydrolysis of the peptide bonds following Tyr-41 and Tyr-79. Integration of the 440-nm peaks of the chromatogram indicated that fraction A consisted of 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 minor CDNP-cyt c_2 derivatives.

Steady-State Kinetic Measurements. A steady-state assay was developed for studying the reaction between *Rb. sphaeroides* reaction centers and both horse heart cytochrome *c* and *Rb. sphaeroides* cytochrome c_2. Both reactions were found to obey Michaelis–Menten kinetics as shown in Figures 3 and 4. The V_{max}/K_m value for horse heart cytochrome *c* decreased with increasing ionic strength in the same fashion as the second-order rate constant measured by rapid kinetics methods (Figure 5). The steady-state kinetics of singly modified $CF_3PhNHCO$-cytochrome *c* derivatives are shown in Figure 3. Modification of lysine-13 caused the largest

Table I: Amino Acid Sequences of *Rb. sphaeroides* Cytochrome c_2, *R. rubrum* Cytochrome c_2, and Horse Cytochrome *c* (Dickerson, 1980)

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                    10                  20                  30
Sp c2   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 c2     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 c2   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 c2   - 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 c2   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 c2   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 F 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 c2   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 c2   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
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Table II: Effect of Specific Lysine Modifications on the Reaction between Horse Cytochrome *c* and *Rb. sphaeroides* Reaction Centers[a]

derivative	rapid kinetics, k(nat)/k(der)	steady state, (V/K)nat/(V/K)der
CF_3CO-Lys-7	1.8	
CF_3CO-Lys-13	3.2	
CF_3CO-Lys-22	1.1	
CF_3CO-Lys-25	1.7	
CF_3CO-Lys-79	2.9	
CF_3CO-Lys-87	2.7	1.9
CF_3CO-Lys-88	1.6	
$CF_3PhNHCO$-Lys-8	2.6	1.4
$CF_3PhNHCO$-Lys-13	7.7	2.5
$CF_3PhNHCO$-Lys-27	1.4	1.0
$CF_3PhNHCO$-Lys-72	4.0	1.6
$CF_3PhNHCO$-Lys-79	1.5	1.0
$CF_3PhNHCO$-Lys-100	1.1	1.0
CDNP-cyt c_2 fraction A	20.0	3.6

[a] Rapid kinetics measurements were carried out in 20 mM Tris-HCl, pH 8, 40 mM NaCl, 0.025% LDAO, 1 mM ascorbate, and 100 μM UQ_0. The second-order rate constant for native horse cytochrome *c*, k(nat), was 2.5 × 10^8 M^{-1} s^{-1}. The steady-state assays were carried out as described in Figure 4.

decrease in V_{max}/K_m, while modification of lysine-27, -79, and -100 had no effect (Table II). These results suggest that the interaction domain is located at the heme crevice of cytochrome *c* (Figure 6). The ionic strength used in these assays (40 mM) was found to give the largest difference between the V_{max}/K_m values of native and modified cytochromes *c*. Similar results were obtained with a pH 8 buffer containing 20 mM Tris-HCl, 40 mM NaCl, and 0.025% LDAO. The V_{max}/K_m value of CDNP-cytochrome c_2 fraction A was found to be decreased from that of native cytochrome c_2 by a factor of 3.6 (Figure 4). Although fraction A is a mixture of singly labeled derivatives, the modified lysines that have been assigned, 35, 88, 95, 97, and 105, are all located on the front surface of cytochrome c_2 (Figure 7).

Laser Rapid Kinetic Measurements. The transient oxidation of cytochrome *c* by laser-excited reaction centers was studied by using methods similar to those of Overfield and

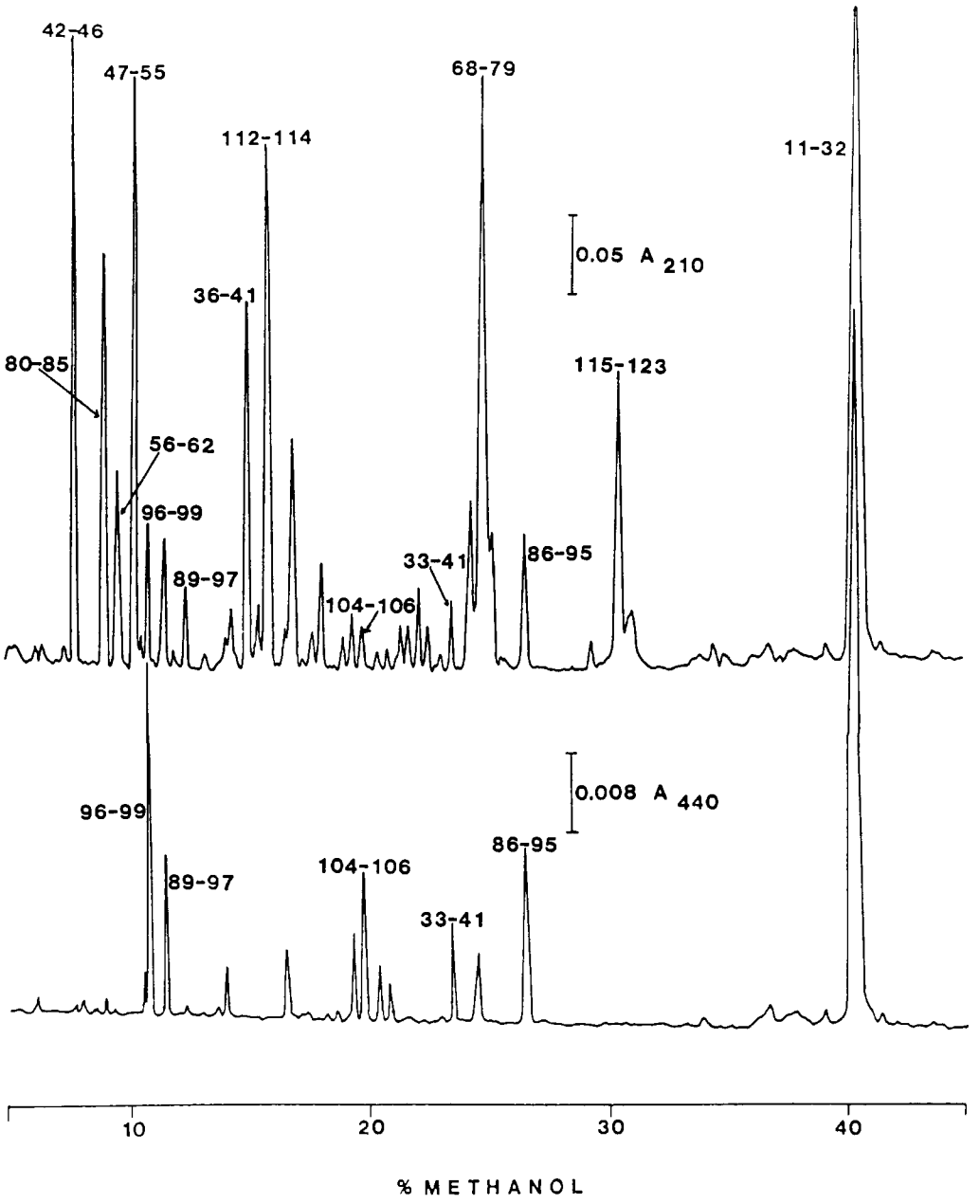

FIGURE 2: HPLC separation of trypsin-digested CDNP-cytochrome c_2 fraction A. The sample (500 μg) was dialyzed into 0.1 M bicine, pH 8.0, digested with TPCK-treated trypsin by using two 5% additions, and eluted on a Brownlee RP-300 column at 0.8 mL/min with a gradient from 5 mM sodium phosphate, pH 7.0, to 100% methanol.

Wraight (1980). The second-order rate constant was calculated from the slope of a plot of the inverse half-time of the reaction vs. the cytochrome *c* concentration. The second-order rate constant for native cytochrome *c* was 2.5×10^8 M^{-1} s^{-1}, in good agreement with the value reported by Overfield and Wraight (1980) at this ionic strength. The second-order rate constants of the CF_3CO- and $CF_3PhNHCO$-cytochrome *c* derivatives are given in Table II as the ratio k(native)/k(derivative). Modification of lysine-7, -8, -13, -25, -27, -72, -79, or -87 surrounding the heme crevice led to significant decreases in the rate constant, while modification of lysine-22 on the right side or lysine-100 on the back of cytochrome *c* had a negligible effect. van der Wal et al. (1987) have obtained similar results for the $CF_3PhNHCO$-lysine-8, -13, -72, and -100 derivatives measured at a lower ionic strength. The second order rate constant for native *Rb. sphaeroides* cytochrome c_2 was 2×10^8 M^{-1} s^{-1}, in good agreement with the value reported by Overfield and Wraight (1980). The rate constant for CDNP-cytochrome c_2 fraction A was 1.0×10^7 M^{-1} s^{-1}, 20-fold smaller than that of native cytochrome c_2. The transient oxidation curve of fraction A could be adequately fit by a single second-order reaction, with no evidence for a multiphasic reaction (Figure 8). However, it would have been difficult to deconvolute the transient curves of the different derivatives in fraction A if they did not have significantly different rate constants.

Electrostatic Interaction between Cytochrome c and Reaction Center. The hypothetical models for the interaction of cytochrome *c* with cytochrome b_5 (Salemme, 1976) and cytochrome *c* peroxidase (Poulos & Kraut, 1980) indicate that the electrostatic interaction is dominated by specific complementary charge-pair interactions between lysine amino groups on cytochrome *c* and carboxylate groups on the partner. Using this assumption, 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

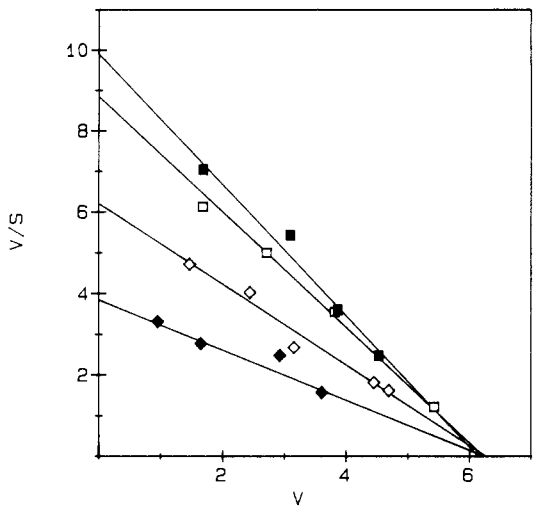

FIGURE 3: Steady-state reaction rates of singly labeled cytochrome *c* derivatives with *Rb. sphaeroides* reaction centers. The assay solution contained 10 mM MOPS, pH 6.5, 40 mM NaCl, 0.025 % LDAO, 40 nM UQ_0, and 15 nM reaction centers at 25 °C. The ferrocytochrome *c* concentration *S* ranged from 0.2 to 10 μM and the velocity *V* was measured as micromolar cytochrome *c* oxidized per minute. Symbols: native cytochrome *c* (■); $CF_3PhNHCO$-lysine-100 (□); $CF_3PhNHCO$-lysine-72 (◇); $CF_3PhNHCO$-lysine-13 (◆).

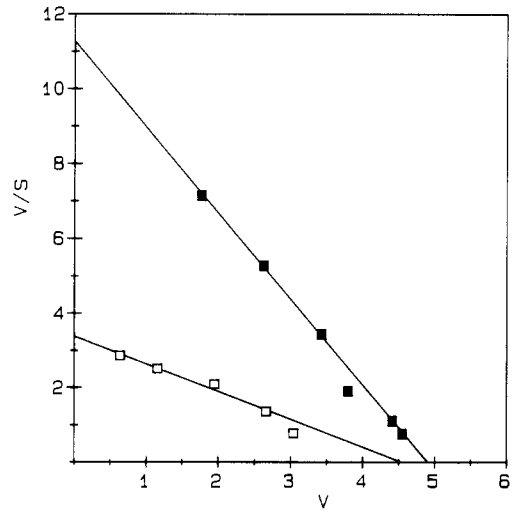

FIGURE 4: Steady-state reaction rates of native and CDNP-cytochrome c_2 fraction A with *Rb. sphaeroides* reaction centers. The assay solution contained 10 mM MOPS, pH 6.5, 40 mM NaCl, 0.025% LDAO, 40 nM UQ_0, and 15 nM reaction centers. The ferrocytochrome c_2 concentration ranged from 0.2 to 10 μM and the velocity, *V*, was measured as micromolar cytochrome c_2 oxidized per minute. Symbols: native cytochrome c_2 (■); CDNP-cytochrome c_2 fraction A (□).

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}. This equation provides a good fit to the ionic strength dependence of the reaction between horse cytochrome *c* and *Rb. sphaeroides* reaction center, assuming that there are nine charge pairs each having an r_i value of 4 Å (Figure 5). Each of these charge pairs would have a V_i value of −0.8 kcal/mol at an ionic strength of 50 mM, to give a total electrostatic interaction energy of −7.2 kcal/mol. This is in

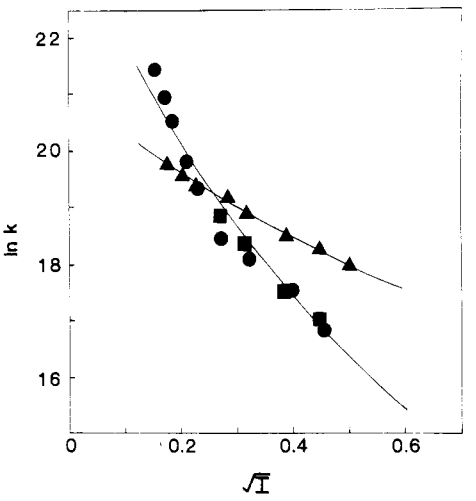

FIGURE 5: Effect of ionic strength on rate constant *k* for oxidation of cytochrome *c* or c_2 by *Rb. sphaeroides* reaction centers. The laser kinetic data for the reaction with horse cytochrome *c* (●) and *Rb. sphaeroides* cytochrome c_2 (▲) were taken from Overfield and Wraight (1980). The ln (V_{max}/K_m) values for horse cytochrome *c* obtained by steady-state kinetics (■) were shifted by a constant factor to bring them into alignment with the rapid kinetics data. The solid lines are theoretical curves obtained from eq 1 with $n = 4$, $r_i = 4$ Å for cytochrome c_2 and $n = 9$, $r_i = 4$ Å for horse cytochrome *c*.

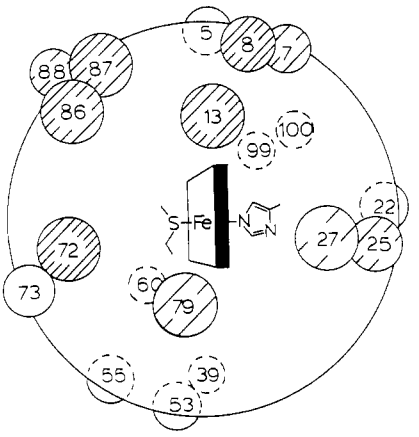

FIGURE 6: Schematic diagram of horse heart cytochrome *c* viewed from the front of the heme crevice. The approximate positions of the lysine residues are indicated by closed and dashed circles for residues located toward the front and back of cytochrome *c*, respectively. The estimated electrostatic 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). When two different derivatives were available for a given lysine, the smaller V_i value was used. The value for lysine-86 is not experimental but was assumed to be the same as that for lysine-87.

reasonable agreement with the chemical modification studies, which indiate the involvement of nine lysine groups surrounding the heme crevice at residues 7, 8, 13, 25, 27, 72, 79, 86, and 87. Lysine-73 and -88 at the edge of the heme crevice domain are not included since each has a nearby carboxylate group on the surface of cytochrome *c* that would cancel their contribution to the electrostatic interaction. There is also a carboxylate group located at Glu-90 at the top of cytochrome *c* that might partially cancel the contribution of one of the lysines. An estimate for V_i can be obtained from the change in reaction rate caused by modification of lysine-*i*: $V_i = -RT \ln [k(\text{nat})/k(\text{der } i)]$. These estimates range from 0.6 to 1.2 kcal/mol for most of the lysines surrounding the heme crevice of cytochrome *c* (Figure 6). They might, of course, also include contributions from favorable or unfavorable steric interactions. Equation 1 provides a good fit to the ionic strength

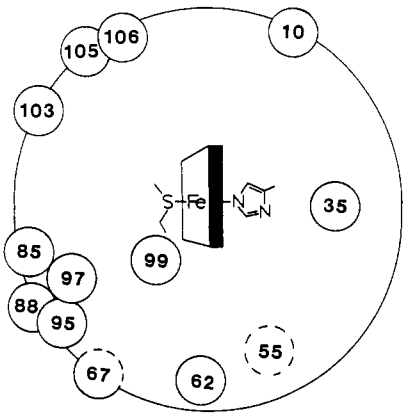

FIGURE 7: Schematic diagram of *Rb. sphaeroides* cytochrome c_2 viewed from the front of the heme crevice. The approximate positions of the lysine residues are indicated by closed and dashed circles for residues located toward the front and back of cytochrome c_2.

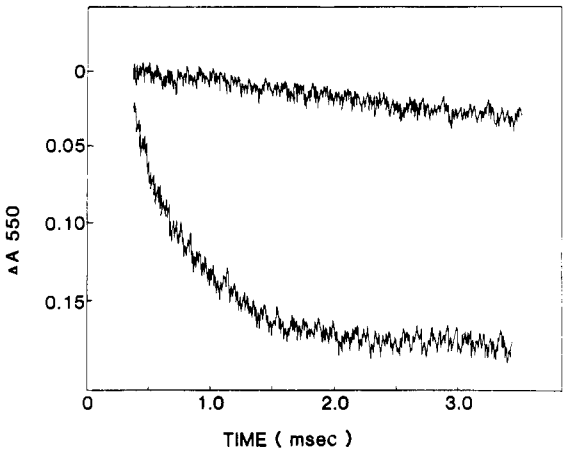

FIGURE 8: Laser rapid kinetic oxidation of native *Rb. sphaeroides* cytochrome c_2 (lower trace) and CDNP-cytochrome c_2 fraction A (upper trace) by *Rb. sphaeroides* reaction centers. The reaction mixture contained 5 μM reaction centers and 10 μM cytochrome c_2.

dependence of the reaction with *Rb. sphaeroides* cytochrome c_2, using $n = 4$ and $r_i = 4$ Å. The electrostatic interaction energy is thus about half as large for cytochrome c_2 as it is for horse cytochrome *c*.

Discussion

This study clearly demonstrates that the reaction between horse heart cytochrome *c* and *Rb. sphaeroides* reaction centers involves electrostatic interactions between lysine amino groups surrounding the heme crevice of the cytochrome and carboxylate groups on the reaction center. The binding domain closely resembles that for cytochrome oxidase (Smith et al., 1977, 1981; Ferguson-Miller et al., 1978; Rieder & Bosshard, 1980), cytochrome c_1 (Ahmed et al., 1978), cytochrome *c* peroxidase (Kang et al., 1978; Smith & Millett, 1980), and cytochrome b_5 (Stonehuerner et al., 1979). The CF_3CO– and $CF_3PhNHCO$– derivatives are well suited for evaluating the binding domain since the overall protein conformation and heme environment are not affected by the modification. We have previously shown that these derivatives have the same visible absorption, optical rotary dispersion, and proton NMR spectra as native cytochrome *c*. The redox potentials of the CF_3CO– derivatives were within 5 mV of that of native cytochrome *c*, while those of the $CF_3PhNHCO$– derivatives were within 10 mV (Smith et al., 1977). The steady-state assay was generally less sensitive to cytochrome *c* lysine modifications than the rapid kinetic assay, although the overall pattern of inhibition was similar. This is not unexpected, since the steady-state kinetic parameters are complicated functions that might not depend in a direct fashion on the reaction between cytochrome *c* and D^+.

Unfortunately, it was not possible to purify singly labeled CDNP-cytochrome c_2 derivatives. This was due in part to the much smaller amount of *Rb. sphaeroides* cytochrome c_2 available but also to the fact that this protein is anionic at pH 7, and ion-exchange chromatography on (carboxymethyl)-cellulose had to be carried out at pH 5.4. Even then, the conditions used in the purification of fraction A were found to be capable of resolving numerous different singly labeled horse heart CDNP-cytochrome *c* derivatives. 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. Although it is difficult to analyze the kinetics of such a mixture, certain limits can be placed on the rate constants of the individual derivatives. Since the second-order rate constant of fraction A was 20-fold smaller than that of native cytochrome c_2, at least 95% of the derivatives must have rate constants that are each decreased by a factor of at least 20. It should be noted that modification with the negatively charged CDNP group leads to much greater inhibition than with the CF_3CO– and $CF_3PhNHCO$– groups, and factors of 20 or more are common for other cytochrome *c* reactions (Ferguson-Miller et al., 1978). This 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. This figure was drawn with reference to the X-ray crystal structure of *R. rubrum* cytochrome c_2 (Salemme et al., 1973) by aligning the sequence of the two proteins (Table II; Dickerson, 1980). 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*.

We conclude that the reaction between *Rb. sphaeroides* cytochrome c_2 and the reaction center involves electrostatic interactions between lysine amino groups surrounding the heme crevice and carboxylate groups on the reaction center. The interaction domain appears to be similar to that used by horse heart cytochrome *c*, except that the magnitude of the electrostatic interaction is only half as large, judging from the ionic strength dependence of the reaction rate (Figure 5). Weber and Tollin (1985) have recently calculated the electrostatic interaction between flavodoxin and either horse cytochrome *c* or *R. rubrum* cytochrome c_2 using a discrete charge model. In both cases the electrostatic interaction is dominated by short-range interactions between lysines surrounding the heme crevice of the cytochrome and carboxylate groups of flavodoxin. However, long-range electrostatic repulsion by the large number of carboxylate groups on the backside of *R. rubrum* cytochrome c_2 decrease the binding energy at low ionic strength. As the ionic strength is increased above 0.1 M, the long-range interactions become less important and the binding energy of the cytochrome c_2 complex is less than 0.5 kcal/mol different from that of the horse cytochrome *c* complex. The localized charge model we have developed is only valid in the higher ionic strength region where the long-range electrostatic interactions can be neglected. The fact that modification of lysine-22 and -100 on the backside of horse cytochrome *c* did

not affect the reaction with reaction centers indicates that this assumption is reasonably valid at ionic strengths above 0.05 M. Long-range interactions may account for part of the decreased electrostatic interaction energy for the reaction with cytochrome c_2, but there also appear to be differences in the short-range interactions compared to the reaction involving horse cytochrome *c*.

The finding of Rieder et al. (1985) that the backside of *R. rubrum* cytochrome c_2 is involved in binding to the *R. rubrum* reaction center was unexpected because the heme crevice contains a number of lysines that are conserved in both bacterial and eucaryotic cytochromes. However, Rieder et al. pointed out that their technique measures a low ionic strength *binding* domain, which might be different from the domain involved in the electron-transfer reaction.

ACKNOWLEDGMENTS

We thank Dr. Robert Bartsch for a gift of *Rb. sphaeroides* cytochrome c_2 and Dr. David Knaff for helpful discussions and a gift of cytochrome c_2. We also thank Veronica Nehus for help setting up the steady-state assay.

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