Role of Specific Lysine Residues in the Reaction of *Rhodobacter sphaeroides* Cytochrome c_2 with the Cytochrome bc_1 Complex†

Joan Hall,‡ Xiaohui Zha,‡ Linda Yu,§ Chang-An Yu,§ and Francis Millett*,‡

Department of Chemistry and Biochemistry, University of Arkansas, Fayetteville, Arkansas 72701, and Department of Biochemistry, Oklahoma State University, Stillwater, Oklahoma 74078

Received August 10, 1988; Revised Manuscript Received October 7, 1988

ABSTRACT: The reaction of *Rhodobacter sphaeroides* cytochrome c_2 with the *Rb. sphaeroides* cytochrome bc_1 complex was studied by using singly labeled cytochrome c_2 derivatives. Cytochrome c_2 was treated with chlorodinitrobenzoic acid to modify lysine amino groups to negatively charged carboxydinitrophenyllysines and separated into eight different fractions by ion-exchange chromatography on a Whatman SE 53 (sulfoxyethyl)cellulose column. Peptide mapping studies indicated that six of these fractions were modified at single lysine amino groups. Each of the derivatives had the same V_{max} value as native cytochrome c_2 in the steady-state reaction with the *Rb. sphaeroides* cytochrome bc_1 complex. However, the K_m values of the cytochrome c_2 derivatives modified at lysines 10, 55, 95, 97, 99, and 106 were found to be larger than that of native cytochrome c_2 by factors of 6, 2, 3, 32, 13, and 8, respectively. These results indicate that lysines located in the sequence 97–106 on the left side of the heme crevice have the greatest involvement in binding the cytochrome bc_1 complex. The involvement of lysine 97 is especially significant because it is located in an extra loop comprising residues 89–98 that is not present in eukaryotic cytochrome *c*.

The purple bacterium *Rhodobacter sphaeroides* contains a cytochrome bc_1 complex that transfers electrons from ubiquinol to cytochrome c_2 during photosynthetic electron transport. Its functional properties are remarkably similar to those of the mammalian mitochondrial cytochrome bc_1 complex (Crofts & Wraight, 1983; Prince & Dutton, 1978), but its subunit composition is much simpler, consisting of only four polypeptide chains (Yu et al., 1984; Gabellini et al., 1982). Horse heart cytochrome *c*, which has a positive net charge, can substitute for the negatively charged *Rb. sphaeroides* cytochrome c_2 in the reaction with the *Rb. sphaeroides* cytochrome bc_1 complex, but it has a significantly larger K_m value (Hall et al., 1987a). We have previously shown that the reaction of horse cytochrome *c* with the cytochrome bc_1 complex involves six lysine residues that surround the heme crevice (Hall et al., 1987a). In the present study we have purified six different *Rb. sphaeroides* cytochrome c_2 derivatives, each labeled at a single lysine residue with 4-carboxy-2,6-dinitrophenyl. Steady-state kinetic studies with these derivatives indicate that the lysines located in the sequence 97–106 on the left side of the heme crevice have the greatest involvement in binding the cytochrome bc_1 complex.

Experimental Procedures

Materials. Cytochrome c_2 was isolated from *Rb. sphaeroides* by the method of Bartsch (1978). The cytochrome bc_1 complex was isolated from *Rb. sphaeroides* R-26 chromatophores by using Triton X-100 solubilization and calcium phosphate–cellulose chromatography as described previously (Yu et al., 1984). The preparation contained 8.3 nmol/mg cytochrome c_1 and had a ubiquinone–cytochrome *c* reductase activity of 12.6 μmol of cytochrome *c* min^{-1} (mg of protein)$^{-1}$. The synthesis of reduced ubiquinol, $Q_oC_{10}Br(H_2)$,[1] was carrried out as reported by Yu and Yu (1982). Tris and TPCK-treated trypsin were obtained from Sigma Chemical Co. 4-Chloro-3,5-dinitrobenzoic acid (CDNB) was purchased from Aldrich Chemical Co. and purified as described by Brautigan et al. (1978).

Preparation of 4-Carboxy-2,6-dinitrophenyl (CDNP) Derivatives of Cytochrome c_2. Oxidized cytochrome c_2 (28 mg, 750 μM) was mixed with CDNB (4.88 mM) in 0.2 M sodium bicarbonate, pH 9.0, and allowed to react at room temperature for 24 h. The reaction mixture was oxidized with potassium ferricyanide and passed through a Bio-Gel P-2 column equilibrated with 2.5 mM ammonium acetate, pH 5.4. The sample was applied to a 1.5 × 30 cm (sulfoxyethyl)cellulose (Whatman SE 53) column and eluted with an exponential gradient from 2.5 mM to 0.4 M ammonium acetate, pH 5.4, at a rate of 25 mL/h. The visible absorption spectra of the derivatives were recorded on a Hewlett-Packard spectrophotometer, and the average number of CDNP-labeled lysines per molecule was calculated by the method of Brautigan et al. (1978).

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 0.1 μg/μL TPCK-treated trypsin for 20 h 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 SP8700 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 as described by Durham and Geren (1981).

Steady-State Kinetic Measurements. The rate of reduction of ferricytochrome c_2 by the cytochrome bc_1 complex was

† This work was supported by NIH Grants GM20488 and RR07101 to F.M., USDA Grant 87-CRCR-2433, and OAGS Grant 5417 to C.-A.Y.

‡ University of Arkansas.
§ Oklahoma State University.

[1] Abbreviations: CDNB, 4-chloro-3,5-dinitrobenzoic acid; CDNP, 4-carboxy-2,6-dinitrophenyl; $Q_oH_{10}Br(H_2)$, 2,3-dimethoxy-5-methyl-6-(10-bromodecyl)-1,4-benzoquinol; Tris, tris(hydroxymethyl)aminomethane; TPCK, *N*-tosyl-L-phenylalanine chloromethyl ketone; Bicine, *N*,*N*-bis(2-hydroxyethyl)glycine; cyt c_2, cytochrome c_2; HPLC, high-performance liquid chromatography; EDTA, ethylenediaminetetraacetic acid.

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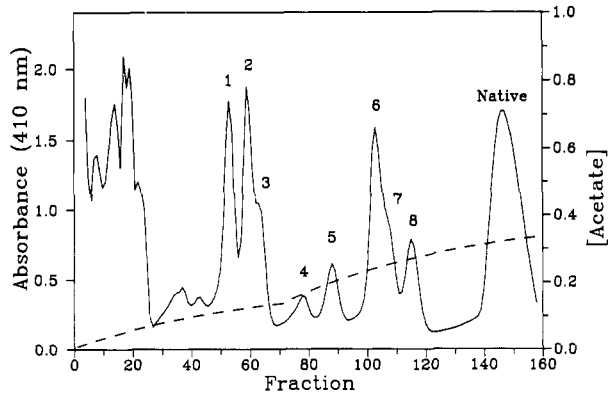

FIGURE 1: Separation of *Rb. sphaeroides* CDNP-cytochrome c_2 derivatives on a SE 53 (sulfoxyethyl)cellulose column. The reaction mixture of CDNP-cytochrome c_2 derivatives (28.5 mg) was loaded on a 1.5 × 30 cm SE 53 column and eluted with a gradient from 2.5 mM to 0.4 M ammonium acetate, pH 5.4, at a flow rate of 25 mL/h. The fraction size was 1.6 mL.

measured on a Hewlett-Packard 8452A diode array spectrophotometer using the dual-wavelength mode at 416–434 nm. The assay solution contained 25 mM sodium phosphate, pH 7.0, 300 mM NaCl, 300 μM EDTA, 10 μM $Q_0C_{10}Br(H_2)$, 5 nM *Rb. sphaeroides* cytochrome bc_1 complex, and 0.5–16 μM ferricytochrome c_2.

Results

Preparation of CDNP-Cytochrome c_2 Derivatives. *Rb. sphaeroides* cytochrome c_2 was treated with CDNB to modify positively charged lysine amino groups to negatively charged CDNP-lysines and then chromatographed on a Whatman SE 53 (sulfoxyethyl)cellulose column as shown in Figure 1. Fractions 1–8 were found to contain an average of 1 CDNP-lysine/molecule, while the fractions eluting before fraction 1 contained an average of 2 or more CDNP-lysines/molecule. The separation obtained on the SE 53 column was significantly better than that obtained previously by using Whatman CM32 (carboxymethyl)cellulose or Pharmacia SP Sepharose (Hall et al., 1987b,c). The visible absorption spectra of all the CDNP-cytochrome c_2 fractions were identical with that of native cytochrome c_2 once the contribution of the CDNP-lysine was subtracted. The presence of an unchanged 695-nm band in the oxidized state indicated that the bond between iron and methionine 100 was intact. The redox potentials of the singly labeled derivatives were all within the range 345–357 mV at pH 7.0 except for fraction 3, which was 337 ± 5 mV. The redox potential of native cytochrome c_2 was found to be 352 ± 5 mV under these conditions, in agreement with the value reported by Pettigrew et al. (1975).

Identification of CDNP-Labeled Lysine Groups by HPLC Peptide Mapping. Tryptic digests of the derivatives were separated by reverse-phase HPLC to identify the residues modified by CDNP (Figure 2). CDNP-lysine absorbs at 440 nm, so it was possible to follow both protein and label simultaneously. The chromatogram of fraction 1 was the same as that of native cytochrome c_2 except for the presence of one CDNP-labeled peptide, which was identified by amino acid analysis to be CDNP-96–99. Since trypsin does not cleave the peptide bond following a CDNP-labeled lysine, derivative 1 is labeled at lysine 97. The other fractions were analyzed in the same way, and the assignments are given in Tables I and II.

Reaction between the Cytochrome bc_1 Complex and the CDNP-Cytochrome c_2 Derivatives. Hall et al. have previously found that the reaction between the *Rb. sphaeroides* cytochrome bc_1 complex and native *Rb. sphaeroides* cytochrome c_2 obeys Michaelis–Menton kinetics and that the K_m value is extremely small at low ionic strengths and then increases rapidly as the ionic strength is increased above 0.25 M. The present studies were carried out at an ionic strength of 0.35 M so that the K_m value of native cytochrome c_2 was in a measurable range (0.65 μM). Figure 3 shows that the

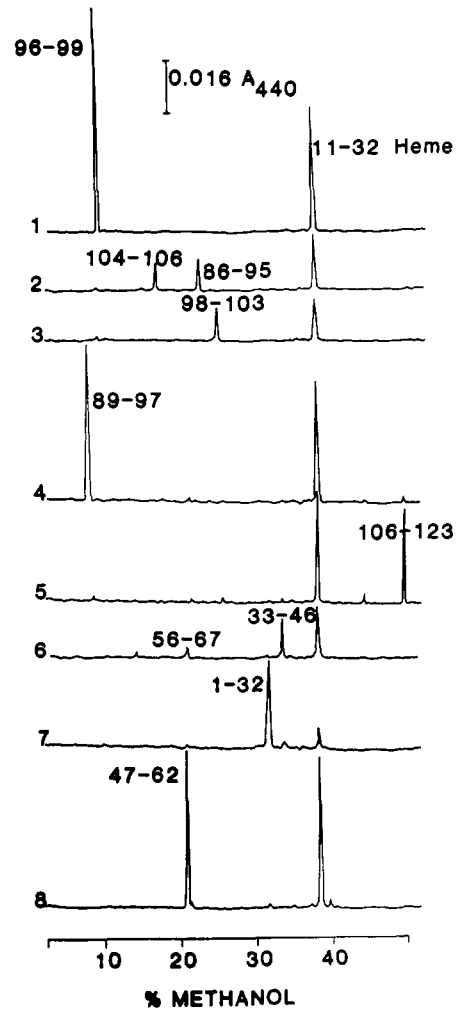

FIGURE 2: HPLC separation of the trypsin digests of CDNP-cytochrome c_2 fractions 1–8. The samples (60 μg) were eluted on a Brownlee RP-300 column at 0.8 mL/min with a gradient from 5 mM sodium phosphate, pH 7.0, to 100% methanol. Only the 440-nm traces are shown.

Table I: Steady-State Kinetic Parameters for the Reaction of CDNP-Cytochrome c_2 Derivatives with the *Rb. sphaeroides* Cytochrome bc_1 Complex[a]

fraction	lysine modified	R
1	97	31.7
2	88, 105 (45%, 55%)	13.3
3	99	13.3
4	95	2.6
5	106	7.8
6	35, 62 (75%, 25%)	5.2
7	10	5.9
8	55	2.2
R. rubrum cytochrome c_2		8.3
horse cytochrome *c*		4.2

[a] The V_{max}/K_m values for the reactions of the cytochrome c_2 derivatives were measured by using the conditions described in Figure 4 and are displayed as the ratio $R = (V_{max}/K_m)_{native}/(V_{max}/K_m)_{derivative}$, where native refers to native *Rb. sphaeroides* cytochrome c_2. The R values of *R. rubrum* cytochrome c_2 and horse cytochrome *c* relative to native *Rb. sphaeroides* cytochrome c_2 were also measured under the same conditions.

Table II: 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]

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
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 - - - - - -
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 - - - - - -
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
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
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
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
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
Hor c	- - - K G I T W K E E T L M E Y L E N P K K Y I P - - - - - - - -
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
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
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 conserved in two or three of the sequences are enclosed in boxes.

CDNP-cytochrome c_2 derivatives have nearly the same V_{max} as native cytochrome c_2. However, the V_{max}/K_m values were decreased substantially for derivatives modified at lysines 10, 88, 97, 99, 105, and 106, which are all located on the front of cytochrome c_2 (Figures 3 and 4; Table I). The 5-fold decrease in V_{max}/K_m observed for fraction 6 is probably due to modification of lysine 35, which is located on the front of cytochrome c_2, rather than to lysine 62, located on the back.

Discussion

The kinetic studies of the CDNP-cytochrome c_2 derivatives indicate that the lysine residues located in the sequence 95–106 on the left side of the heme crevice play the most important role in the interaction with the cytochrome bc_1 complex (Figure 4). This sequence contains five lysines conserved in *Rhodospirillum rubrum* cytochrome c_2 (95, 97, 99, 103, and 106) and three conserved in horse cytochrome *c* (99, 105, and 106) (Table II). The involvement of lysines 95 and 97 in the interaction is especially significant, since these residues are located in the extra loop comprising residues 89–98 that is not present in eukaryotic cytochrome *c*. This might account for the fact that the binding of *Rb. sphaeroides* cytochrome c_2 to the cytochrome bc_1 complex is considerably stronger than that of horse cytochrome *c*, since most of the other lysines surrounding the heme crevice are conserved in the two proteins. The relatively small effect of modifying lysines 55 and 95 indicates that they are located at the extreme edge of the binding domain. The present work suggests that a total of seven or eight lysine amino groups are involved in binding the cytochrome bc_1 complex, in agreement with previous ionic strength dependence studies (Hall et al., 1987a).

It is surprising that *R. rubrum* cytochrome c_2 interacts even more poorly than horse cytochrome *c* with the *Rb. sphaeroides* cytochrome bc_1 complex (Table I). There is a 39% homology between the sequences of *R. rubrum* and *Rb. sphaeroides* cytochromes c_2, and most of the lysine residues on the left side of the heme crevice are conserved, including those in the extra

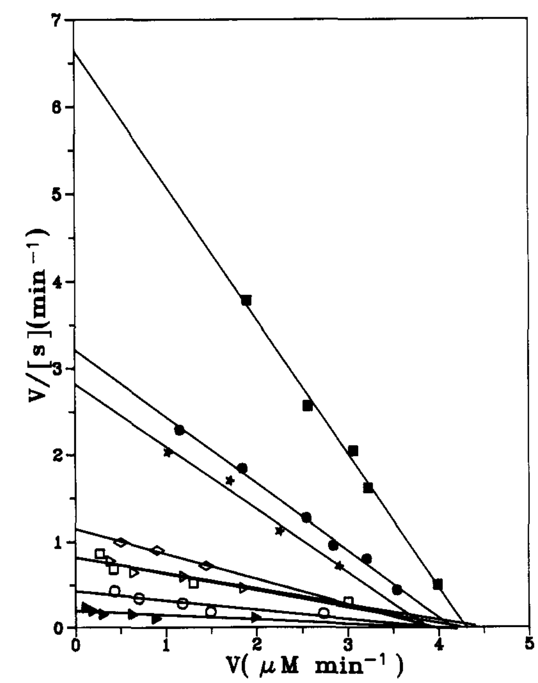

FIGURE 3: Steady-state kinetics for the reaction of the *Rb. sphaeroides* cytochrome bc_1 complex with CDNP-cytochrome c_2 derivatives: Native *Rb. sphaeroides* cytochrome c_2 (■); CDNP-cytochrome c_2 fractions 1 (▲), 3 (○), 4 (★), 5 (△), 7 (◇), 8 (●); native *R. rubrum* cytochrome c_2 (□). The velocity, *V*, is reported in μM/min, and *V*/[S] is in min^{-1}. The assay buffer contained 25 mM sodium phosphate, pH 7.0, 300 mM NaCl, 300 μM EDTA, 10 μM $Q_0C_{10}Br(H_2)$, 5 nM cytochrome bc_1 complex, and 0.5–16 μM cytochrome c_2.

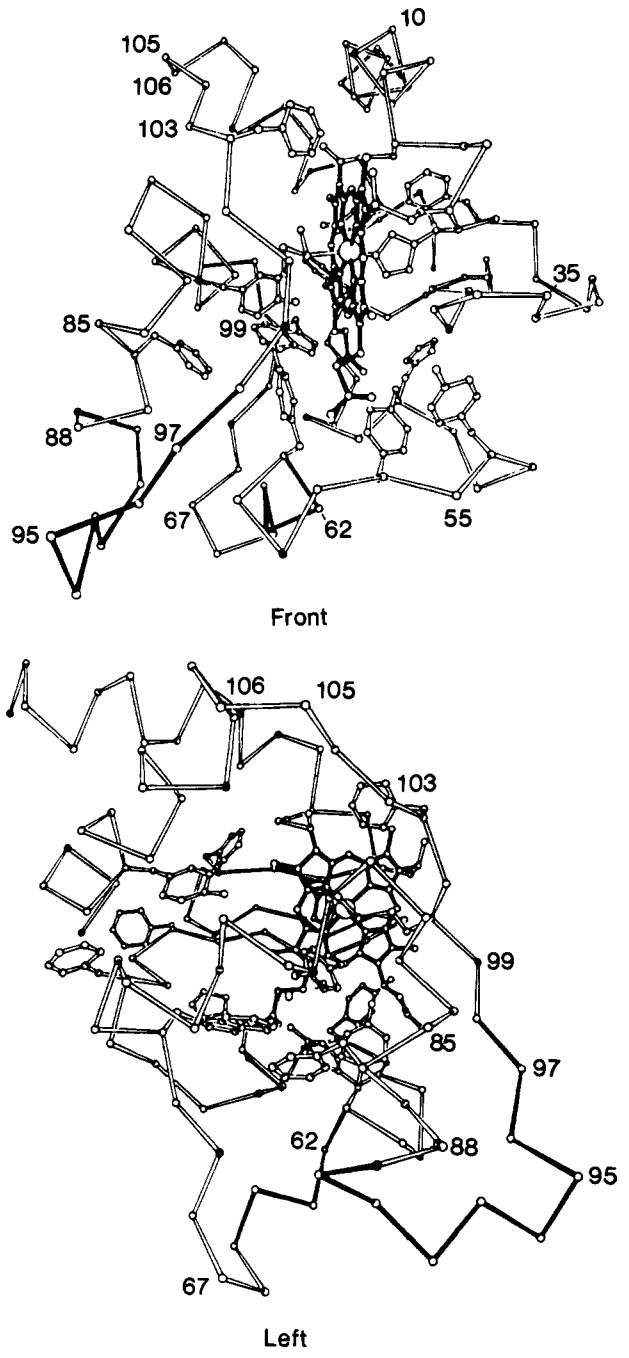

FIGURE 4: Crystal structure of *R. rubrum* cytochrome c_2 showing the α-carbons, aromatic residues, and heme ligands from the front and left sides of the molecule [redrawn with permission from Figure 1 of Salemme et al. (1973)]. The predicted positions of the lysine residues of *Rb. sphaeroides* cytochrome c_2 are superimposed on the *R. rubrum* structure by using the sequence alignment shown in Table II. The heme group and the insertions into the eukaryotic cytochrome *c* sequence are shown in black.

loop at the lower left side (Table II). There are, however, two extra lysines at residues 12 and 13 that are not present in *Rb. sphaeroides* cytochrome c_2. Bosshard et al. (1987) have recently found that formation of a complex between *R. rubrum* cytochrome bc_1 and *R. rubrum* cytochrome c_2 protected only lysines 12, 13, and 97 at the top of the heme crevice from modification by acetic anhydride. This suggests a significantly different binding domain than we have found in the present work for the *Rb. sphaeroides* proteins or previously for *R. rubrum* (Hall et al., 1987d). However, the method used by Bosshard et al. (1987) identifies the binding domain involved in the stable product complex, which might be different from the domain involved in the actual electron-transfer reaction between the two proteins.

The CDNP-cytochrome c_2 derivatives described here are also being used for a detailed study of the reaction between cytochrome c_2 and the photosynthetic reaction center from *Rb. sphareoides*. Preliminary results indicate that the binding domain for the reaction center is nearly identical with that for the cytochrome bc_1 complex, providing additional evidence that cytochrome c_2 functions as a diffusional carrier during electron transport in *Rb. sphaeroides*.

ACKNOWLEDGMENTS

We thank Patricia O'Brien for help with the amino acid analyzer, Professor David Knaff for a gift of *R. rubrum* cytochrome c_2, and Dr. Melvin Okamura for a gift of *Rb. sphaeroides* cytochrome c_2.

Registry No. Lys, 56-87-1; cytochrome c_2, 9035-43-2; ubiquinol–cytochrome *c* reductase, 9027-03-6.

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