Binding Site on *Rhodospirillum rubrum* Cytochrome c_2 for the *Rhodospirillum rubrum* Cytochrome bc_1 Complex†

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Received May 15, 1987; Revised Manuscript Received July 30, 1987

ABSTRACT: Cytochrome c_2 and the detergent-solubilized cytochrome bc_1 complex, both from *Rhodospirillum rubrum*, form a tight complex at a low ionic strength that can be isolated by gel permeation chromatography. The dissociation constant of the complex is estimated to be 10^{-6} M or less. The binding site for the cytochrome bc_1 complex on cytochrome c_2 was analyzed by differential acetylation of lysine residues in free and cytochrome bc_1 complex bound cytochrome c_2. In bound cytochrome c_2, three lysine residues at sequence positions 12, 13, and 97 were less reactive toward acetic anhydride. Lys^{13}, which is located above the exposed heme edge, was the least reactive, i.e., the most shielded by the cytochrome bc_1 complex. Correlating this information with the crystal structure of cytochrome c_2 indicates that the binding site for the cytochrome bc_1 complex on cytochrome c_2 involves a surface area above, and probably including, the exposed heme edge. This mode of binding is similar to that observed for horse cytochrome *c* interacting with the mitochondrial cytochrome bc_1 complex. A simplified version of the method of differential chemical modification is presented.

Rhodospirillum rubrum has been found to contain a membrane-bound cytochrome bc_1 complex (Wynn et al., 1986) that catalyzes the electron flow from quinol to soluble cytochrome c_2, which in turn reduces the photosynthetic reaction center (van der Wal & van Grondelle, 1983). The cytochrome bc_1 complex contains cytochrome c_1, two *b* cytochromes, and a Rieske iron–sulfur protein (Wynn et al., 1986). *R. rubrum* and other bacterial cytochrome bc_1 complexes are similar to the mitochondrial cytochrome bc_1 complex (Hauska et al., 1983), and horse cytochrome *c* can substitute for bacterial cytochrome c_2 in the reaction with both the cytochrome bc_1 complex (Wynn et al., 1986) and the photosynthetic reaction center (Rickle & Cusanovich, 1979). Since horse cytochrome *c* and bacterial cytochrome c_2 have closely related structures (Salemme et al., 1973), it is expected that the domain of interaction of cytochrome c_2 with the cytochrome bc_1 complex resembles the corresponding domain on horse cytochrome *c* reacting with mitochondrial cytochrome *c* oxidase and cytochrome bc_1 complex.

The electron-transfer domain of mitochondrial cytochrome *c* has been located at the molecular surface area surrounding the solvent-accessible heme edge, the domain being virtually the same for all the redox partners of the mitochondrial protein [for reviews, see Margoliash and Bosshard (1983) and Capaldi et al. (1982)]. Two independent methods have been applied to define the electron-transfer domain of mitochondrial cytochrome *c*: kinetic analysis of singly lysine-substituted cytochrome *c* derivatives and comparison of the rate of acetylation of lysines in the free and the redox partner bound protein. The latter approach is known as differential chemical modification (Bosshard, 1979). Both approaches are based on the observation that the stability of the electron-transfer complexes with cytochrome *c* is highly dependent on ionic strength and that lysines, of which many are sequence invariant, are involved in the formation of electron-transfer complexes and contribute to the overall molecular dipole moment of cytochrome *c* on which the reaction with redox partners strongly depends (Koppenol & Margoliash, 1982).

In the reaction of horse cytochrome *c* with bacterial reaction centers and bc_1 complexes, both kinetic and protein–chemical data indicate a "frontside" mode of binding (Bosshard et al., 1987; Hall et al., 1987a–c). Thus, the available experimental evidence overwhelmingly supports a single interaction domain for electron-transfer reactions on the frontside of horse cytochrome *c*, irrespective of whether the redox partner is of mitochondrial or bacterial origin. The situation is less clear for bacterial cytochrome c_2. Early conclusions from the comparison of cytochrome c_2 and cytochrome *c* structures predicted an interaction domain on the frontside of cytochrome c_2 (Salemme et al., 1973; Errede & Kamen, 1978). Kinetic data that were obtained with mixtures of lysine-modified derivatives of cytochrome c_2 from *R. rubrum* (Hall et al., 1987a) and *Rhodobacter sphaeroides* (Hall et al., 1987b) are best interpreted by a frontside domain for the reaction center and the cytochrome bc_1 complex from *Rhb. sphaeroides* (Hall et al., 1987b,c) and for the reaction center from *R. rubrum* (Hall et al., 1987a). However, the data with the *R. rubrum* reaction center are in direct contrast to those of Rieder et al. (1985), who have shown that the *R. rubrum* reaction center protects two lysine residues and the N-terminal amino group on the "backside" of cytochrome c_2 from reaction with acetic anhydride. Front- and backside are defined as in Salemme et al. (1973). Recent linear dichroism measurements have also suggested that mitochondrial cytochrome *c* binds to the *Rhb. sphaeroides* reaction center with a somewhat different orientation than cytochrome c_2 (Tiede, 1987).

In the present work it is shown that cytochrome c_2 and the cytochrome bc_1 complex, both from *R. rubrum*, form a tight complex. By employing a modified version of the method of differential chemical modification of lysine residues with acetic anhydride (Kaplan et al., 1971; Bosshard, 1979), the binding domain for the cytochrome bc_1 complex could be located above,

† This work was supported by the Swiss National Science Foundation (Grant 3.114-85 to H.R.B.) and by the National Science Foundation (PCM 84-08564 to D.B.K.).
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0006-2960/87/0426-7688$01.50/0

and probably including, the exposed heme edge of *R. rubrum* cytochrome c_2.

Materials and Methods

R. rubrum (strains G-9 and S-1) were grown (Snozzi & Bachofen, 1979) and cytochrome c_2 was isolated and purified from *R. rubrum* (strain G-9) (Sponholtz et al., 1976) as described. The R. *rubrum* cytochrome bc_1 complex was isolated from strain S-1 as before (Wynn et al., 1986), except that lauryl maltoside was substituted for octyl glucoside and the horse cytochrome *c* affinity chromatography step was omitted. Duroquinol:cytochrome c_2 oxidoreductase activity of the complex, assayed as before (Wynn et al., 1986), was 6 μmol (nmol of cytochrome c_1)$^{-1}$ h^{-1}. The ratio of bacteriochlorophyll *a* to cytochrome c_1 was 4.2, and that of cytochrome *b* to cytochrome c_1 was 1.8. The bc_1 complex contained no detectable P870 (cyt c_1:P870 = 10). Bacteriochlorophyll *a* was estimated from A_{773} (ϵ = 75 mM^{-1} cm^{-1}) after extraction into 7:2 (v/v) acetone/methanol (Clayton, 1963). Cytochrome c_1 was determined from $\Delta A^{red-ox}_{550-540}$ (ascorbate-reduced minus ferricyanide-oxidized, $\Delta\epsilon$ = 20 mM^{-1} cm^{-1}) or by pyridine hemochromogen analysis (Takaichi & Morita, 1981), cytochrome c_2 from $\Delta A^{red}_{550-540}$ (dithionite-reduced, $\Delta\epsilon$ = 20 mM^{-1} cm^{-1}), and cytochrome *b* by pyridine hemochromogen analysis (Takaichi & Morita, 1981).

[^{3}H]Acetic anhydride (100 Ci/mol) and [^{14}C]acetic anhydride (100 Ci/mol) were obtained from Amersham, α-chymotrypsin (3 times recrystallized, salt free) was from Fluka, protease from *Staphylococcus aureus* V8 was from Miles, and Sephadex G-75 (superfine) was from Pharmacia. Lauryl maltoside was prepared according to VanAken et al. (1986).

Binding studies and differential acetylation experiments were conducted in 10 mM triethanolamine hydrochloride containing 0.05% (w/v) lauryl maltoside, pH 8.0, unless stated otherwise. This buffer has an ionic strength of 4 mM. For peptide separation, the HPLC equipment described in Bechtold and Bosshard (1985) was used. Automated Edman degradation was performed with an Applied Biosystems protein sequencer, Model 470 A, and manual Edman degradation according to Chang (1981). ^{3}H/^{14}C ratios were determined by liquid scintillation counting in a Kontron Betamatic II instrument, with Kontrogel as a scintillant. Automatic correction for quenching and channel spillover was based on the external standard ratio method. The 2σ error of ^{3}H/^{14}C ratios due to statistical counting error was ±4% or less.

Binding of Cytochrome c_2 to Cytochrome bc_1 Complex. Binding was determined by chromatography of a solution containing the cytochrome bc_1 complex and cytochrome c_2 on a column (1 × 27 cm) of Sephadex G-75, eluted at 7.2 mL/h and 4 °C. On this column the cytochrome bc_1 complex eluted with the void volume (peak centered at 9.6 mL), well separated from the peak of cytochrome c_2 centered at 16.8 mL. To demonstrate the presence of cytochrome c_2 bound to the cytochrome bc_1 complex, the protein-containing fraction in the void volume of the column was made 0.25 M in NaCl and then passed through an ultrafiltration membrane (Diaflo UM/C-100, Amicon Corp.), which retained the cytochrome bc_1 complex. The pass-through was collected, and the amount of cytochrome c_2 present was quantitated from $\Delta A^{red-ox}_{550-540}$ in a reduced *minus* oxidized difference spectrum.

Differential Acetylation of Cytochrome c_2. Cytochrome bc_1 complex bound cytochrome c_2 and free cytochrome c_2 were reacted in parallel experiments (A and B) with trace amounts of radioactively labeled acetic anhydride. In experiment A, cytochrome c_2 (17 nmol) and cytochrome bc_1 complex (18.5 nmol) in a final volume of 960 μL were treated with [^{14}C]-

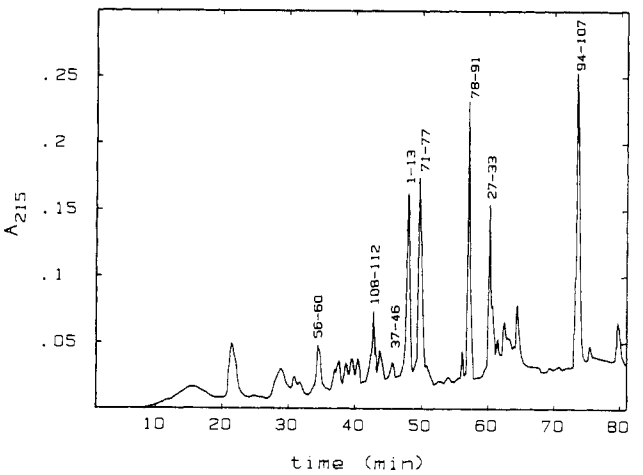

FIGURE 1: Separation of chymotryptic peptides of cytochrome c_2 on an Aquapore RP-300 reversed-phase column (Brownlee, Santa Barbara, CA). Separation was achieved by a 0.12% min^{-1} linear gradient of acetonitrile in 0.067% (v/v) aqueous trifluoroacetic acid. Numbers above peaks refer to sequence positions (see Table I).

acetic anhydride; in experiment B, the same amounts of cytochrome c_2 and cytochrome bc_1 complex were treated in the presence of 0.25 M NaCl with [^{3}H]acetic anhydride. NaCl was included in experiment B to dissociate the cytochrome c_2–cytochrome bc_1 complex. The reagents were of equal specific radioactivity and concentration in the trace-labeling steps. The radioactive acetic anhydride (1 μmol, 100 μCi) in toluene was added in four portions of 5 μL each at 5-min intervals. The reaction mixtures were placed in 5-mL cone-shaped Reacti-vials (Pierce Chemical Co.) kept on ice and stirred vigorously to disperse the toluene. In this way the acetic anhydride was diffused out of the toluene solution into the aqueous reaction mixture. Stirring was continued for 30 min. The degree of radioactive labeling was 0.63 mol of [^{3}H]acetyl and 0.56 mol of [^{14}C]acetyl per mole of cytochrome c_2.

Since the degree of trace labeling was less than 1 mol/mol, acetylation per se of one lysine could not alter the chemical reactivity of a neighboring lysine residue (Kaplan et al., 1971). Thus, the degree of isotopic labeling was a direct consequence of the binding event (Bosshard, 1979). By combining labeled cytochrome c_2 from experiments A and B and analyzing the ^{3}H/^{14}C ratio at each N^{ϵ}-acetyllysine, protection of surface-located lysines by the bc_1 complex could be monitored. ^{3}H/^{14}C = 1 indicated equal reactivity of a lysine in free and cytochrome bc_1 complex bound cytochrome c_2, whereas ^{3}H/^{14}C > 1 indicated lowered and ^{3}H/^{14}C < 1 indicated increased reactivity in bound cytochrome c_2. Before ^{3}H/^{14}C ratios were assigned to each lysine, carrier cytochrome c_2 (150 nmol) was added to the combined reaction mixtures, and cytochrome c_2 was separated from the cytochrome bc_1 complex by chromatography on Sephadex G-75 (conditions as in the binding studies, with 0.25 M NaCl added to the elution buffer). Thereafter, the ^{3}H,^{14}C-labeled cytochrome c_2 mixed with the carrier cytochrome c_2 was fully acetylated (Rieder & Bosshard, 1980), and phenolic *O*-acetyl groups were cleaved (Smyth, 1967) in order to achieve a chemically homogeneous derivative. The protein was digested with chymotrypsin [5% by weight, incubation at 37 °C for 6 h in 0.1 M *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonate, pH 8.0], and peptides were separated by HPLC (Figure 1). The peptide covering sequence positions 1–13 (Table I) was subdigested with Glu-specific protease from *S. aureus* V8 (conditions as for digestion with chymotrypsin) to yield peptides 1–8 and 9–13. Finally, ^{3}H/^{14}C ratios were determined at the level of the thiazolinone

Table I: Amino Acid Sequence of *R. rubrum* Cytochrome c_2 (Meyer & Kamen, 1982)[a]

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1          10         20         30         40         50         60
           * *                   •
EGDAAAGEKVSKKCLACHTFDQGGANKVGPNLFGVFENTAAHKDNYAYSESYTEMKAKGL

           70         80         90         100        110
                      *          *    *
TWTEANLAAYVKNPKAFVLEKSGDPKAKSKMTFKLTKDDEIENVIAYLKTLK
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[a] Peptides isolated by HPLC (Figure 1) and analyzed for $^3H/^{14}C$ ratios of N^{ϵ}-acetyllysines (Table IV) are underlined. Lysine residues marked by asterisk occupy sequence positions homologous to those of lysines of horse cytochrome *c* at the electron-transfer interaction site for the mitochondrial cytochrome bc_1 complex (Speck et al., 1979; Rieder & Bosshard, 1980; Smith et al., 1980).

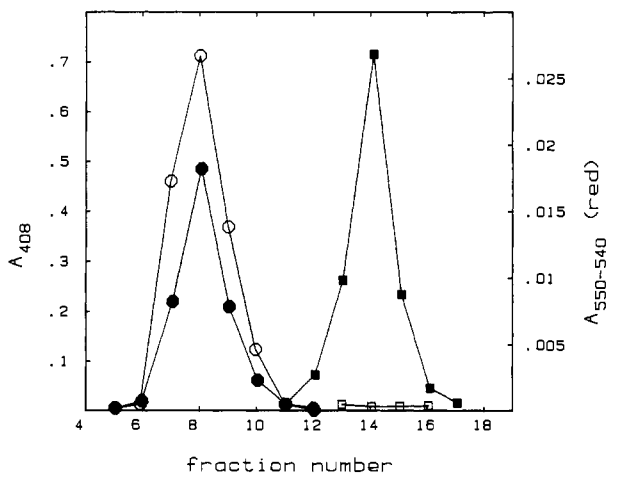

FIGURE 2: Binding of cytochrome c_2 to the cytochrome bc_1 complex. Cytochrome c_2 (6.4 nmol) and cytochrome bc_1 complex (5.1 nmol) in a total volume of 0.4 mL were chromatographed at low ionic strength (open symbols) on Sephadex G-75 (see Materials and Methods for details). Chromatography at high ionic strength was performed with 3.8 nmol of cytochrome c_2 and 4 nmol of cytochrome bc_1 complex in the sample applied (closed symbols). The eluate was analyzed for cytochrome bc_1 complex (A_{408}, circles) and for cytochrome c_2 ($A_{550-540}$, squares).

derivatives of N^{ϵ}-acetyllysine, the derivatives being obtained by automated (peptide 78–91) or manual Edman degradation (other peptides).

Results

Binding Studies. Binding was measured in a qualitative fashion by gel permeation chromatography. Samples containing approximately equimolar amounts of the cytochrome bc_1 complex and cytochrome c_2 were chromatographed on a column of Sephadex G-75 at 4 and 254 mM ionic strength (Figure 2). At low ionic strength, no trace of cytochrome c_2 could be detected in the fractions where cytochrome c_2 by itself was shown to elute in control experiments. This indicated that cytochrome c_2 was completely bound to the cytochrome bc_1 complex, the latter being eluted with the void volume. An attempt to measure the ratio of cytochrome c_2 to cytochrome bc_1 complex in the front peak spectrophotometrically was unsuccessful because the low amount of spectroscopically detectable heme precluded accurate quantification. Instead, cytochrome c_2 and cytochrome bc_1 complex from the front peak were separated by ultrafiltration at high ionic strength. Of the 6.4 nmol of cytochrome c_2 initially applied to the column, 2.9 nmol could be recovered from the peak represented by open symbols in Figure 2. Since no cytochrome c_2 was detected behind the front peak (open squares in Figure 2), the less than quantitative recovery might have been due to nonspecific adsorption of the small amount of cytochrome c_2 to the ultrafiltration membrane. In the second binding experiment conducted at high ionic strength, cytochrome c_2 was recovered quantitatively (i.e., >90%), as a single peak behind the peak of cytochrome bc_1 complex, at the same elution volume observed when cytochrome c_2 alone was chromatographed at high ionic strength.

Table II: Radioactivity in Thiazolinone Derivatives Obtained by Stepwise Edman Degradation of Peptides 71–77 and 78–91[a]

residue	^3H (dpm)	^{14}C (dpm)	residue	^3H (dpm)	^{14}C (dpm)
Val^{71}	163	89	Ser^{82}	81	96
Lys^{72}	563	470	Gly^{83}	81	93
Asn^{73}	181	156	Asp^{84}	75	102
Pro^{74}	105	98	Pro^{85}	78	101
Lys^{75}	934	1150	Lys^{86}	648	655
Ala^{76}	98	115	Ala^{87}	108	85
Phe^{77}	74	85	Lys^{88}	516	555
Val^{78}	84	65	Ser^{89}	105	122
Leu^{79}	92	88	Lys^{90}	743	596
Glu^{80}	74	65	Met^{91}	60	74
Lys^{81}	1212[b]	615[b]			

[a] Manual Edman degradation of peptide 71–77 and automated Edman degradation of peptide 78–91. [b] This $^3H/^{14}C$ ratio was corrected for higher labeling of Lys^{81} in 0.25 M NaCl ($^3H/^{14}C$ = 1.89, Table III), to give the corrected value of $^3H/^{14}C$ = 1.08 shown in Table IV.

It is not possible to accurately calculate a dissociation constant from the results of Figure 2. However, given the concentration of cytochrome c_2 and cytochrome bc_1 complex in the sample applied to the column and assuming that at least 80% of the cytochrome c_2 in the applied sample eluted together with the cytochrome bc_1 complex, it can be estimated according to the procedure of Dixon (1976) that the dissociation constant is between 10^{-7} and 10^{-6} M.

Differential Acetylation of Lysines. The rationale of the experiment is that binding of cytochrome c_2 to the cytochrome bc_1 complex will partially protect certain lysine residues from reaction with acetic anhydride. Identification of the shielded residues in the sequence of cytochrome c_2 may enable one to locate the binding site for the cytochrome bc_1 complex on the surface of cytochrome c_2. The essentials of the technique are that free cyctochrome c_2 and cytochrome bc_1 complex bound cytochrome c_2 were labeled with equal amounts of [^{3}H]acetic anhydride and [^{14}C]acetic anhydride, respectively, under reaction conditions that were as alike as possible during labeling of free and cytochrome bc_1 complex bound cytochrome c_2. Consequently, the $^3H/^{14}C$ ratio of N^{ϵ}-acetyllysines could be used as a measure of the reactivity of lysines in free and bound cytochrome c_2.

During reaction with [^{14}C]acetic anhydride, the cytochrome c_2–cytochrome bc_1 complex was in dynamic equilibrium with its free components, and the $^3H/^{14}C$ ratio is therefore a lower estimate of the true degree by which the rate of acetylation was affected in bound cytochrome c_2 (Bosshard, 1979). To get the $^3H/^{14}C$ ratios of individual lysines, the protein was digested, and the purified peptides were degraded by the stepwise Edman procedure. Edman degradation had to proceed at high yield in order to reveal the true $^3H/^{14}C$ ratio of closely spaced lysines; low yields would have smeared out the radioactivity between residues. Table II indicates that this was not the case. In addition, it is seen from Table II that the hydroxyl group of serine is much less reactive than the ϵ-amino group, as serines are not labeled above background.

The labeling pattern of free cytochrome c_2 was obtained in the presence of the cytochrome bc_1 complex and 0.25 M NaCl. At this high ionic strength, the gel filtration experiments described above and in Figure 2 indicate that none of the cytochrome c_2 is bound to the cytochrome bc_1 complex. In this way the concentration of the potentially acetylatable groups was held equal during the acetylation of free and bc_1-bound

Table III: Effect of Ionic Strength on Reactivity of Lysines toward Acetic Anhydride[a]

Lys no.	^3H (dpm)	^{14}C (dpm)	$^3H/^{14}C$
N-terminus	4142	3840	1.08
9	2826	3143	0.90
12	2113	2426	0.87
13	1014	1334	0.76
27	5447	5260	1.04
56	1361	955	1.43
58	2461	1570	1.57
72	1989	1895	1.05
75	2140	1910	1.12
81	4633	2445	1.89
86	3814	4125	0.92
88	1960	1588	1.23
90	2678	2155	1.24
94	2397	2020	1.19
97	1774	2052	0.86
109	861	905	0.95
112	1323	1036	1.28

[a] Cytochrome c_2 was labeled at 4 mM ionic strength with [^{14}C]acetic anhydride and at 254 mM ionic strength with [^3H]acetic anhydride. ^3H and ^{14}C radioactivity and $^3H/^{14}C$ ratios of thiazolinone derivatives of N^{ϵ}-acetyllysines are shown. Average $^3H/^{14}C$ ratio = 1.04 ± 0.16 (Lys^{56}, Lys^{58}, and Lys^{81} excluded).

Table IV: $^3H/^{14}C$ Ratios of N^{ϵ}-Acetyllysines and N-Terminal Glutamic Acid of Cytochrome c_2 Labeled in the Free Form with [^3H]Acetic Anhydride and Bound to the *R. rubrum* Cytochrome bc_1 Complex with [^{14}C]Acetic Anhydride

Lys no.	$^3H/^{14}C$	Lys no.	$^3H/^{14}C$
N-terminus	1.04	81	1.08[a]
9	0.80	86	0.99
12	1.59	88	0.93
13	3.77	90	1.25
27	1.06	94	1.12
56	1.16[a]	97	2.07
58	1.14[a]	109	1.17
72	1.20	112	1.13
75	0.81		

[a] Corrected for higher reactivity in the presence of 0.25 M NaCl (see text and Table III).

cytochrome c_2. The change of the ionic strength has some influence on the reactivity of the lysines (Rieder et al., 1985). To correct for this effect, a control experiment was conducted without cytochrome bc_1 complex at low (labeling with [^{14}C]acetic anhydride) and high ionic strength (labeling with [^3H]acetic anhydride). Equal reactivity, i.e., $^3H/^{14}C$ ratios around unity, was observed for most lysines, with the exception of Lys^{56}, Lys^{58}, and Lys^{81}, which were somewhat more reactive in 0.25 M NaCl, as had been observed previously (Rieder et al., 1985) (Table III). To obtain the results described below, the $^3H/^{14}C$ ratios obtained for these three residues were corrected by the appropriate value from Table III. The correction was $^3H/^{14}C$(corrected) = $^3H/^{14}C$(measured) − $^3H/^{14}C$(NaCl) + 1.

Table IV shows the $^3H/^{14}C$ ratios that reflect the differential reactivity of 16 out of a total of 17 lysines and of the N-terminal glutamic acid of free and cytochrome bc_1 complex bound cytochrome c_2. No reliable data could be obtained for Lys^{43} since the only peptide containing this residue (Table I) was highly contaminated and recovered in low amount (Figure 1). Clearly, all but three residues were equally reactive in free and cytochrome bc_1 complex bound cytochrome c_2. The mean $^3H/^{14}C$ ratio of the equally reactive residues was 1.06 ± 0.14. Lys^{12}, Lys^{13}, and Lys^{97} were significantly less reactive in cytochrome bc_1 complex bound cytochrome c_2, with Lys^{13} being the most shielded. If cytochrome c_2 is viewed with the surface-exposed heme edge facing the viewer, the three shielded residues are located above the heme cleft, on top of the molecule (Figure 3). Lys^{13} is closest to the exposed heme edge.

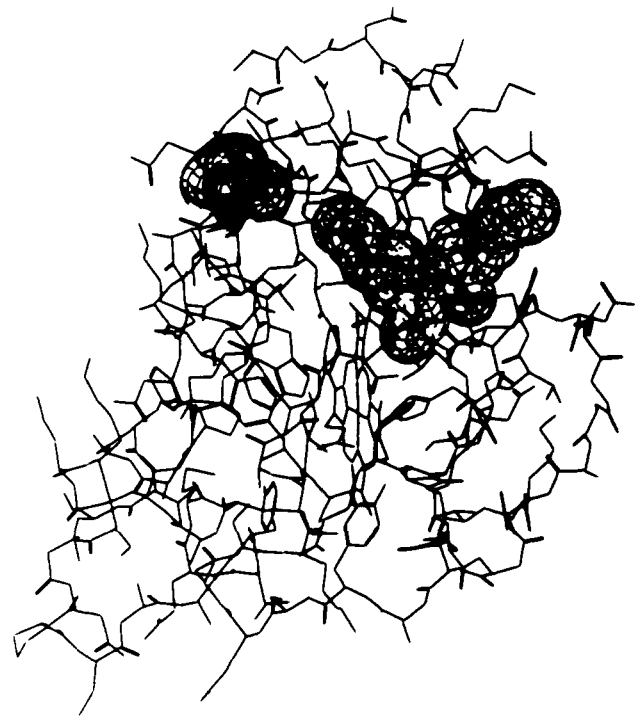

FIGURE 3: Model of cytochrome c_2 from *R. rubrum*. The molecule is drawn with the heme slightly tilted and the exposed edge facing the viewer. Van der Waals contours are shown for Lys^{12} and Lys^{13} forming the upper right cluster and Lys^{97} to the upper left of the heme. Lys^{13} is directly above the heme. These three residues are protected by the cytochrome bc_1 complex in the cytochrome bc_1 complex bound cytochrome c_2, as revealed by differential acetylation of the lysines of cytochrome c_2. Atomic coordinates were obtained from the protein Data Bank, Brookhaven National Laboratory.

DISCUSSION

Binding of Cytochrome c_2 to the Cytochrome bc_1 Complex. Detergent-solubilized cytochrome bc_1 complex from *R. rubrum* forms a noncovalent complex with cytochrome c_2 that can be isolated by gel filtration. Such a relatively high-affinity complex is not a general property of bacterial bc_1 complexes. For example, the cytochrome bc_1 complex isolated from *Rhb. sphaeroides* binds neither cytochrome c_2 nor cytochrome *c* (Yu et al., 1986). The stability of the present complex points to a specific protein–protein interaction between these two components of the *R. rubrum* electron-transfer chain. In analogy to the cytochrome bc_1 complex–cytochrome *c* complex isolated from mitochondria (Chang et al., 1976; Weiss & Juchs, 1978; Bosshard et al., 1979), we assume that the isolated bacterial cytochrome bc_1 complex–cytochrome c_2 complex corresponds to a virtual electron-transfer complex. On the basis of this assumption, a differential acetylation experiment was undertaken in search of the electron-transfer site on cytochrome c_2.

Differential Acetylation. Differential chemical modification of surface-located residues has been successfully applied in defining protein–protein interaction domains (Rieder & Bosshard, 1978, 1980; Pettigrew, 1978; Sinha & Brew, 1981; Hitchcock-de Gregori, 1982; Bechtold & Bosshard, 1985; Jackson & Harris, 1986; Burnens et al., 1987; Oomen & Kaplan, 1987). The method is based on a procedure originally devised by Kaplan et al. (1971). The usual experimental procedure has been the following. The protein under study was labeled, once free and once bound to its reaction partner,

with a trace amount of ^3H-labeled reagent, followed by complete modification with ^{14}C-labeled reagent in excess and under protein-denaturing conditions, to yield two batches of $^3H,^{14}C$-labeled protein. During the whole experiment the two batches of $^3H,^{14}C$-labeled protein had to be processed separately, and for each labeled lysine two $^3H/^{14}C$ ratios had to be compared in order to follow the chemical reactivity in free and bound protein.

The procedure adopted in the present study is simpler. Free cytochrome c_2 is labeled with one isotope, tritium in the present case, and bound cytochrome c_2 with the other. Any change of chemical reactivity can then be seen directly from a change in the $^3H/^{14}C$ ratio. Only a single derivative has to be analyzed, which speeds up analysis and eliminates errors arising from the comparison of peptides from parallel experiments. This simpler approach depends critically on identical reagent concentrations in acetylating free and bound cytochrome c_2 and on accurate knowledge of the specific radioactivity of the differently labeled reagents. A procedure similar to the one adopted here was described recently by Jackson and Harris (1986).

NaCl was added to dissociate the cytochrome bc_1 complex–cytochrome c_2 complex and thus produce the conditions for labeling of free cytochrome c_2. A control experiment revealed that the ionic strength did not change reactivity except in the case of three lysines (Table III). Those residues of equal reactivity in the control experiment had a mean $^3H/^{14}C$ ratio of 1.04 ± 0.16. This value and standard deviation serve as an indirect control of the reproducibility of the reaction conditions in the two experiments conducted with free and cytochrome bc_1 complex bound cytochrome c_2. We assume that ratios differing by more than two standard deviations from unity, i.e., $^3H/^{14}C$ ratios outside the range 1 ± 0.32, signal a change of reactivity in the cytochrome bc_1 complex–cytochrome c_2 complex.

Binding Site for Cytochrome bc_1 Complex on Cytochrome c_2. Three residues showed a $^3H/^{14}C$ ratio outside of 1 ± 0.32 (Table IV). Our hypothesis is that their lowered reactivity is directly caused by intermolecular contact with the cytochrome bc_1 complex. An alternative explanation would be that binding induces a conformational change in cytochrome c_2 which in turn decreases the chemical reactivity of residues outside of the protein–protein contact area. This possibility cannot be excluded. In our previous experiments, lowered reactivity of a lysine residue could always be correlated by independent kinetic experiments, which showed a higher K_m for the cytochrome c derivative with the same residue specifically modified. Such a correlation between differential acetylation and kinetic analysis of lysine-modified cytochrome c derivatives was observed with horse cytochrome c bound to mitochondrial cytochrome c oxidase, cytochrome bc_1 complex, cytochrome c_1, cytochrome c peroxidase, and bacterial flavocytochrome c-552 [Margoliash and Bosshard (1983) and references cited therein; Bosshard et al., 1986]. Also, localization of an epitope for a monoclonal antibody directed against horse cytochrome c by differential acetylation of lysines was confirmed by epitope mapping through conventional cross-reactivity measurements (Burnens et al., 1987). Thus, the approach of differential acetylation has now been tested by independent experiments in a large number of cases.

With the caveat that a conformational effect outside of the binding site for the cytochrome bc_1 complex cannot be ruled out, we interpret the present results in terms of a binding site for the cytochrome bc_1 complex that includes an area above the exposed heme edge (Figure 3). In mitochondrial cytochrome c the same area is part of the binding site for the mitochondrial cytochrome bc_1 complex. Lys^{12} and Lys^{97} of cytochrome c_2 are homologous to Lys^{13} and Lys^{87} of horse cytochrome c. Lys^{13} of *R. rubrum* cytochrome c_2 is an insertion with respect to the horse sequence, but Lys^{13} of cytochrome c_2 is in fact closer to the heme edge than invariant Lys^{12} (Salemme et al., 1973). The high $^3H/^{14}C$ ratio found for Lys^{13} may indicate that the heme edge constitutes a main feature of the binding site. A similar situation holds for equine mitochondrial cytochrome c, where the binding site for the cytochrome bc_1 complex extends to the frontside and includes Lys^{27}, Lys^{72}, and Lys^{79} (Margoliash & Bosshard, 1983). By analogy, we would have expected higher $^3H/^{14}C$ ratios for Lys^{27}, Lys^{75}, and Lys^{90} of *R. rubrum* cytochrome c_2 (homologous to Lys^{27}, Lys^{72}, and Lys^{79} in the horse cytochrome; see Table I). The fact that this was not observed does not necessarily rule out involvement of the frontside of cytochrome c_2 in binding, which was predicted from structure comparison of cytochrome c_2 and cytochrome c (Salemme et al., 1973) and from comparative kinetic data (Errede & Kamen, 1978). Equal rates of acetylation need not conclusively indicate that a residue is outside of the binding site.

Since the technique of differential acetylation does not involve electron-transfer measurements per se, conclusions reached from the differential acetylation experiment must be applied to the electron-transfer process with some caution. While we are confident that Lys^{12}, Lys^{13}, and Lys^{97} are at the intermolecular contact site, we cannot prove that this site is also the electron-transfer site for the reaction between cytochrome c_2 and the cytochrome bc_1 complex. Kinetic analysis of *R. rubrum* cytochrome c_2 derivatives with the *R. rubrum* cytochrome bc_1 complex will show if the present data are consistent with an electron-transfer site on the frontside of *R. rubrum* cytochrome c_2, as has been shown recently for the cytochrome c_2/cytochrome bc_1 complex pair from *Rhb. sphaeroides* (Hall et al., 1987c). Such experiments are currently under way.

ACKNOWLEDGMENTS

We thank Lotte Braun for expert technical assistance, Aidas Kriauciunas for assistance in the preparation of the cytochrome bc_1 complex, and Dr. Frank Millett for making his results available to us before publication and helpful discussions.

Registry No. Cytochrome c_2, 9035-43-2; L-Lys, 56-87-1.

REFERENCES

Bechtold, R., & Bosshard, H. R. (1985) *J. Biol. Chem. 260*, 5191–5200.

Bosshard, H. R. (1979) *Methods Biochem. Anal. 25*, 273–301.

Bosshard, H. R., Zürrer, M., Schägger, H., & von Jagow, G. (1979) *Biochem. Biophys. Res. Commun. 89*, 250–258.

Bosshard, H. R., Davidson, M. W., Knaff, D. B., & Millett, F. (1986) *J. Biol. Chem. 261*, 190–193.

Bosshard, H. R., Snozzi, M., & Bachofen, R. (1987) *J. Bioenerg. Biomembr. 19*, 375–382.

Burnens, A., Demotz, S., Corradin, G., Binz, H., & Bosshard, H. R. (1987) *Science (Washington, D.C.) 235*, 780–782.

Capaldi, R., Darley-Usmar, V., Fuller, S., & Millett, F. (1982) *FEBS Lett. 138*, 1–7.

Chang, J. Y. (1981) *Biochem. J. 199*, 557–564.

Chiang, Y. L., Kaminsky, L. S., & King, T. E. (1976) *J. Biol. Chem. 251*, 29–36.

Clayton, R. K. (1963) in *Bacterial Photosynthesis* (Gest, H., San Pietro, A., & Vernon, L. P., Eds.) p 448, Antioch, Yellow Springs, OH.

Dixon, H. B. F. (1976) *Biochem. J. 159*, 161–162.

Errede, B., & Kamen, M. D. (1978) *Biochemistry 17*, 1015–1027.
Hall, J., Ayres, M., Zha, X., O'Brien, P., Durham, B., Knaff, D., & Millett, F. (1987a) *J. Biol. Chem. 262*, 11046–11051.
Hall, J., Zha, X., Durham, B., O'Brien, P., Vieira, B., Davis, D., Okamura, M., & Millett, F. (1987b) *Biochemistry 26*, 4494–4500.
Hall, J., Zha, X., Yu, L., Yu, C.-A., & Millett, F. (1987c) *Biochemistry 26*, 4501–4504.
Hauska, G., Hurt, E., Gabellini, N., & Lockau, W. (1983) *Biochim. Biophys. Acta 726*, 97–133.
Hitchcock-de Gregori, S. E. (1982) *J. Biol. Chem. 257*, 7372–7380.
Jackson, P. J., & Harris, D. A. (1986) *Biochem. J. 235*, 577–583.
Kaplan, H., Stevenson, K. J., & Hartley, B. S. (1971) *Biochem. J. 124*, 289–299.
Koppenol, W. H., & Margoliash, E. (1982) *J. Biol. Chem. 257*, 4426–4437.
Margoliash, E., & Bosshard, H. R. (1983) *Trends Biochem. Sci. (Pers. Ed.) 8*, 316–320.
Meyer, T. E., & Kamen, M. D. (1982) *Adv. Protein Chem. 35*, 105–212.
Oomen, R. P., & Kaplan, H. (1987) *Biochemistry 26*, 303–308.
Pettigrew, G. (1978) *FEBS Lett. 86*, 14–16.
Rickle, G. K., & Cusanovich, M. A. (1979) *Arch. Biochem. Biophys. 197*, 589–598.
Rieder, R., & Bosshard, H. R. (1978) *J. Biol. Chem. 253*, 6045–6053.
Rieder, R., & Bosshard, H. R. (1980) *J. Biol. Chem. 255*, 4732–4739.
Rieder, R., Wiemken, V., Bachofen, R., & Bosshard, H. R. (1985) *Biochem. Biophys. Res. Commun. 128*, 120–126.
Salemme, F. R., Kraut, J., & Kamen, M. D. (1973) *J. Biol. Chem. 248*, 7701–7716.
Sinha, S. K., & Brew, K. (1981) *J. Biol. Chem. 256*, 4193–4204.
Smith, M. B., Stonehuerner, J., Ahmed, A. J., Staudenmeyer, N., & Millett, F. (1980) *Biochim. Biophys. Acta 592*, 303–313.
Smyth, D. G. (1967) *J. Biol. Chem. 242*, 1592–1598.
Snozzi, M., & Bachofen, R. (1979) *Biochim. Biophys. Acta 546*, 236–247.
Speck, S. H., Ferguson-Miller, S., Osheroff, N., & Margoliash, E. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 155–159.
Sponholtz, D. K., Brautigan, D. L., Loach, P. A., & Margoliash, E. (1976) *Anal. Biochem. 72*, 255–260.
Takaichi, S., & Morita, S. (1981) *J. Biochem. (Tokyo) 89*, 1513–1519.
Tiede, D. M. (1987) *Biochemistry 26*, 397–410.
VanAken, T., Foxall-VanAken, S., Castleman, S., & Ferguson-Miller, S. (1986) *Methods Enzymol. 125*, 27–35.
Van der Wal, H. N., & van Grondelle, R. (1983) *Biochim. Biophys. Acta 725*, 94–103.
Weiss, H., & Juchs, B. (1978) *Eur. J. Biochem. 88*, 17–28.
Wynn, R. M., Gaul, D. F., Choi, W. K., Shaw, R. W., & Knaff, D. B. (1986) *Photosynth. Res. 9*, 181–195.
Yu, L., Dong, J. H., & Yu, C. A. (1986) *Biochim. Biophys. Acta 852*, 203–211.

Labeling of Bovine Heart Cytochrome *c* Oxidase with Analogues of Phospholipids. Synthesis and Reactivity of a New Cardiolipin Benzaldehyde Probe†

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Received February 17, 1987; Revised Manuscript Received May 8, 1987

ABSTRACT: The syntheses of two new radioactive probes derived from cardiolipin and phosphatidylcholine are reported. These probes are derivatives of natural lipids and contain an amine-specific benzaldehyde in the head-group region. This functional group allows a choice of timing of the reaction (e.g., after equilibration and detergent removal) because an irreversible covalent bond is formed only upon the addition of reducing agent. These probes, as well as a benzaldehyde analogue of phosphatidic acid, and a water-soluble benzaldehyde reagent were covalently attached to bovine heart cytochrome *c* oxidase. After reconstitution into vesicles, the lipid–benzaldehyde probes selectively incorporated into the smaller polypeptides of the enzyme, while the remaining subunits (I–IV) exhibited little incorporation of label. The accessibility of amine groups labeled under the conditions used here was independent of the structural and charge differences between the benzaldehyde probes. This suggests that all three lipid probes react with polypeptides of the cytochrome *c* oxidase complex at general contact sites for membrane phospholipids. A water-soluble benzaldehyde reagent predominantly labeled subunits IV, Va, and Vb and polypeptides of VII–VIII. A comparison of these results facilitates a more refined view of the disposition of polypeptides of cytochrome *c* oxidase in respect to the lipid and aqueous phases.

Cytochrome *c* oxidase (ferrocytochrome *c*:oxygen oxidoreductase; EC 1.9.3.1) is the terminal enzyme of the electron transport chain in all eukaryotes and in some prokaryotes. It is a multimeric protein complex that accepts electrons from reduced cytochrome *c* and transfers them to molecular oxygen to form water. In eukaryotes, the three largest subunits are encoded by the mitochondrial genome and have been implicated in binding of the prosthetic groups (two heme irons and two copper ions) and proton pumping activity. The remaining subunits are coded for by the nuclear genome and may be involved in regulatory function of the enzyme complex. Some

† This work was supported by U.S. Public Health Service Research Grants GM 25698 and 34916.

0006-2960/87/0426-7693$01.50/0