The Interaction between Cytochrome c_2 and the Cytochrome bc_1 Complex in the Photosynthetic Purple Bacteria *Rhodobacter capsulatus* and *Rhodopseudomonas viridis*†

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ABSTRACT: The rates of electron transfer from a ubiquinol analogue to cytochrome c_2 catalyzed by the cytochrome bc_1 complexes of *Rhodobacter capsulatus* and *Rhodopseudomonas viridis* were measured as a function of ionic strength. The effects of ionic strength on the kinetic parameters for the reactions are consistent with a role for electrostatic complex formation between cytochrome c_2 and the cytochrome bc_1 complex in the electron-transfer pathways in both photosynthetic purple non-sulfur bacteria. Additional support for a docking model in which positively charged lysines on cytochrome c_2 interact with negatively charged groups on the *Rb. capsulatus* cytochrome bc_1 complex was obtained from kinetic experiments using *Rb. capsulatus* cytochrome c_2 and equine cytochrome *c* in which specific lysine residues were altered by site-directed mutagenesis and chemical modification, respectively. Equine cytochrome *c*, which is a poor electron donor to the reaction center of *Rps. viridis*, is an effective electron acceptor for the *Rps. viridis* cytochrome bc_1 complex. Chemical modification of lysine residues on *Rps. viridis* cytochrome c_2 has a substantially greater effect on the reduction of the *Rps. viridis* reaction center by ferrocytochrome c_2 than on the oxidation of the *Rps. viridis* cytochrome bc_1 complex by ferricytochrome c_2. These data suggest that the docking site for *Rps. viridis* cytochrome c_2 on the *Rps. viridis* reaction center tetraheme subunit differs in structure from the docking site for the cytochrome on the *Rps. viridis* cytochrome bc_1 complex to a significant extent. In this respect, *Rps. viridis* differs from photosynthetic purple non-sulfur bacteria in which the reaction center does not contain a tetraheme subunit, where the binding sites for cytochrome c_2 on the reaction center and the cytochrome bc_1 complex appear to be quite similar.

The photosynthetic purple non-sulfur bacteria *Rhodobacter capsulatus* and *Rhodopseudomonas viridis* both contain a membrane-bound cytochrome bc_1 complex (Ljungdahl et al., 1987; Wynn et al., 1986; Cully et al., 1989; Robertson et al., 1993; Knaff, 1993). While the *Rb. capsulatus* complex has been extensively characterized, relatively few investigations of the *Rps. viridis* complex have been carried out (Knaff, 1993). As considerable sequence homologies exist between the subunits of the *Rps. viridis* complex and those of other photosynthetic bacteria (Hauska et al., 1988; Verbist et al., 1989), it is likely that the *Rps. viridis* complex has similar properties and plays a similar role to the complexes of other photosynthetic purple non-sulfur bacteria. In *Rb. capsulatus*, *Rhodobacter sphaeroides*, and *Rhodospirillum rubrum* the cytochrome bc_1 complexes play a key role in both photosynthetic and respiratory electron-transfer reactions coupled to the translocation of protons from the cytoplasm to the periplasmic space (Hauska et al., 1983; Crofts & Wraight, 1983; Rich, 1984; Dutton, 1986; Knaff, 1993). During photosynthetic electron flow, the cytochrome bc_1 complex serves as a ubiquinol:cytochrome-c_2 oxidoreductase, oxidizing ubiquinol reduced by the reaction center and reducing cytochrome c_2 oxidized by the reaction center. Cytochrome bc_1 complexes have been isolated and purified to apparent homogeneity from *Rb. sphaeroides*, *R. rubrum*, *Rb. capsulatus*, and *Rps. viridis* (Knaff, 1993). In both of the bacteria utilized in this study, *Rb. capsulatus* and *Rps. viridis*, the complexes have been shown to be composed of three peptide subunits containing four electron-carrying prosthetic groups: a *c*-type cytochrome containing covalently bound heme *c*, a *b*-type cytochrome containing two nonequivalent protohemes, and an iron–sulfur protein containing a single [2Fe–2S] center (Cully et al., 1989; Robertson et al., 1993). The genes coding for these subunits in *Rb. capsulatus* and *Rps. viridis* have been cloned, sequenced, analyzed, and found to be clustered on a single operon, *fbc* or *pet* (Gabellini & Sebald, 1988; Davidson & Daldal, 1987; Daldal et al., 1987; Verbist et al., 1989). Similar operon arrangements have been shown to exist in *Rb. sphaeroides* and *R. rubrum* (Majewski & Trebst, 1990; Yun et al., 1990; Shanker et al., 1992).

Although kinetics studies, biophysical measurements, and analysis by molecular genetics have provided a considerable amount of information concerning the structure and function of the *Rb. capsulatus* cytochrome bc_1 complex (Trumpower, 1990; Knaff, 1993), relatively little is known about its interaction with its electron-accepting partner, cytochrome c_2, a soluble *c*-type cytochrome that is located in the periplasmic

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space of this bacterium (Prince et al., 1975). Cytochrome c_2 functions in the transfer of electrons from the cytochrome bc_1 complex to the reaction center in photosynthetically grown *Rb. capsulatus* (Dutton & Jackson, 1972; Caffrey et al., 1992b) or to cytochrome oxidase in cells grown aerobically (Baccarini-Melandri et al., 1978). A 2.5-Å-resolution structure of cytochrome c_2 of *Rb. capsulatus* is available from crystallographic data (Benning et al., 1991), and the structural properties of the cytochrome have also been analyzed by NMR spectroscopy (Gooley et al., 1990). The amino acid sequence of *Rps. viridis* cytochrome c_2 is known from both direct protein sequencing (Ambler et al., 1976) and gene sequencing (Grisshammer et al., 1990). Although the protein has been crystallized (Miki et al., 1986), a tertiary structure is not yet available. Both *Rb. capsulatus* and *Rps. viridis* cytochrome c_2 show significant sequence homology to mitochondrial cytochrome *c* (Ambler et al. 1976; Ambler et al., 1979), and, in the case of *Rb. capsulatus* cytochrome c_2, similarities in tertiary structure have been shown to exist (Benning et al., 1991).

The mechanism of electron transfer catalyzed by the mitochondrial cytochrome bc_1 complex has been shown to involve the formation of a complex between cytochrome *c* and the cytochrome bc_1 complex that is stabilized by electrostatic interactions (Salemme, 1977; Margoliash & Bosshard, 1983). Cross-linking and chemical modification experiments have shown that the cytochrome c_1 subunit of the cytochrome bc_1 complex provides the negative charges for electrostatic binding to positively charged residues on mitochondrial *c* (Margoliash & Bosshard, 1983). Two acidic regions on mitochondrial cytochrome c_1 have been implicated in the formation of the electrostatically stabilized complex with mitochondrial cytochrome *c* (Capaldi et al., 1982; Broger et al., 1983; Stonehuerner et al., 1985). These acidic regions on cytochrome c_1 are conserved, to varying extents, in the four photosynthetic bacteria for which cytochrome c_1 sequences are available (Davidson & Daldal, 1987; Verbist et al., 1989; Majeswki & Trebst, 1990; Yun et al., 1990; Shanker et al., 1992). As cytochrome c_1 is known to be the immediate electron donor to cytochrome c_2 in photosynthetic bacteria (Meinhardt & Crofts, 1982; Snozzi & Crofts, 1985), it appears likely that some of these conserved glutamate and aspartate residues on the bacterial cytochrome c_1 are involved in binding cytochrome c_2. In fact, evidence is available indicating that electrostatic interactions do occur between the cytochrome bc_1 complexes and cytochrome c_2 in the photosynthetic bacteria *R. rubrum* and *Rb. sphaeroides* (Hall et al., 1987c,d, 1989; Bosshard et al., 1987). It has been reported that the rates of electron transfer from quinol to cytochrome c_2 catalyzed by the cytochrome bc_1 complexes of *R. rubrum* and *Rb. sphaeroides* are ionic strength dependent, with little effect on V_{max} and significant increases in K_m observed as the ionic strength increases (Hall et al., 1987c,d, 1989). Analysis of this data, using a semiempirical relationship first applied to mitochondrial electron-transfer reactions (Stonehuerner et al., 1979), has been utilized to estimate the number of electrostatically interacting pairs in the cytochrome c_1/cytochrome c_2 couple (Hall et al., 1987c,d, 1989). Experiments with lysine-modified derivatives of cytochromes *c* and c_2 support the conclusion that these electrostatic interactions involve lysine amino groups surrounding the exposed heme edge of cytochrome c_2 (Hall et al., 1987c,d, 1989). Recently, the role of conserved lysine residues at the exposed heme edge of *Rb. capsulatus* cytochrome c_2 in the interaction with the reaction centers of *Rb. sphaeroides* has been studied by site-directed mutagenesis (Caffrey et al., 1992b), but no information is available on the nature of the interaction of cytochrome c_2 with the cytochrome bc_1 complex in *Rb. capsulatus*.

In this work, complex formation between the cytochrome bc_1 complex and cytochrome c_2 of *Rb. capsulatus* has been studied at different ionic strengths by chromatography and ultrafiltration methods. The effects of ionic strength and of modification of lysine residues on cytochromes *c* and c_2 on the kinetic parameters associated with the ubiquinol:cytochrome-c_2/*c* oxidoreductase activity of the *Rb. capsulatus* cytochrome bc_1 complex have been determined in order to define the likely interaction domain on both *Rb. capsulatus* cytochrome c_2 and equine cytochrome *c* for the *Rb. capsulatus* cytochrome bc_1 complex. As the amino acid sequence of *Rps. viridis* cytochrome c_1 (Verbist et al., 1989) suggests that one of the putative binding site(s) for cytochrome c_2 may carry significantly less net negative charge than in *Rb. capsulatus*, similar kinetics studies have been carried out with *Rps. viridis* for comparative purposes. Experiments have also been carried out to determine whether equine cytochrome *c* can serve as an effective electron acceptor for the *Rps. viridis* cytochrome bc_1 complex. Although in *Rb. sphaeroides* and *R. rubrum* equine cytochrome *c* serves effectively both as an electron donor to the reaction center (van der Wal et al., 1987; Hall et al., 1987a,b) and as an electron acceptor from the cytochrome bc_1 complex (Hall et al., 1987c,d) and although equine cytochrome *c* is an effective donor to the *Rhodocyclus gelatinosus* reaction center (Matsuura et al., 1988), equine cytochrome *c* is a relatively ineffective electron donor to the *Rps. viridis* reaction center (Knaff et al., 1991; Meyer et al., 1991). It thus seemed of interest to investigate whether the ineffectiveness of equine cytochrome *c* as an electron-transfer partner for membrane-bound electron-transfer complexes isolated from photosynthetically grown *Rps. viridis* is limited to the reaction center or extends to the cytochrome bc_1 complex.

MATERIALS AND METHODS

The *Rb. capsulatus* cytochrome bc_1 complex was prepared as described previously (Robertson et al., 1993). The *Rps. viridis* cytochrome bc_1 complex was prepared essentially as described by Cully (1990). *Rps. viridis* reaction centers were prepared as described previously (Knaff et al., 1991). Native *Rb. capsulatus* cytochrome c_2 was purified according to the method of Bartsch (1978). The *Rb. capsulatus* cytochrome c_2 mutants were produced and purified as described previously (Caffrey et al., 1992a). *Rps. viridis* cytochrome c_2 was purified as described previously (Knaff et al., 1991). Equine cytochrome *c* (type III) was purchased from Sigma Chemical Co., and the (trifluoromethyl)phenylcarbamoyl (TFC) derivatives of equine cytochrome *c* were prepared as described by Smith et al. (1977). Carboxydinitrophenyl (CDNP) derivitization of *Rps. viridis* cytochrome c_2 was carried out by using the procedure described previously for *Rb. sphaeroides* cytochrome c_2 (Hall et al., 1989), and the average number of CDNP-labeled lysines per molecule was calculated by the method of Brautigan et al. (1978). 2,3-dimethoxy-5-decyl-6-methyl-1,4-hydrobenzoquinone (DBH) was kindly provided by Dr. David Birney (Texas Tech University).

Gel filtration cochromatography experiments were carried out in 20 mM Tris-HCl buffer (pH 8.00) containing 0.05% (w/v) dodecyl maltoside, using a Biogel P-100 (1 × 30 cm) column at a flow rate of 7.5 mL/h. Control experiments demonstrated that recovery of the individual components was ≥85% during chromatography on this column at both high and low ionic strengths. On this column, the *Rb. capsulatus*

cytochrome bc_1 complex eluted with the void volume, well separated from the elution positions of either cytochrome c_2 or equine cytochrome c alone, at both high and low ionic strength. Complex formation was also studied by using an Amicon stirred ultrafiltration cell with a YM-30 membrane that completely retained the *Rb. capsulatus* cytochrome bc_1 complex, but through which both equine cytochrome c and *Rb. capsulatus* cytochrome c_2 passed completely. The filtrates from each assay were collected, and the amount of cytochrome c_2 or equine cytochrome c was quantitated from ascorbate-reduced minus ferricyanide-oxidized spectra.

A Shimadzu Model UV-2100 spectrophotometer was used to monitor reduction of cytochromes catalyzed by the cytochrome bc_1 complexes from *Rb. capsulatus* and *Rps. viridis*. The ubiquinol:cytochrome-c_2/c oxidoreductase activities of the *Rb. capsulatus* and *Rps. viridis* cytochrome bc_1 complexes were assayed by following the rate of reduction of native, modified, or mutant cytochrome c_2 and native or modified equine cytochrome c at 550 nm after addition of DBH, as described by Berry and Trumpower (1985). Prior to the kinetic measurements, *Rb. capsulatus* and *Rps. viridis* cytochrome c_2 and equine cytochrome c were oxidized with a slight excess of potassium ferricyanide and chromatographed on a Dowex 1-X column equilibrated with potassium phosphate buffer (pH 7.40) containing 290 μM EDTA, to remove excess ferricyanide. Each assay mixture contained 2.90 nM *Rb. capsulatus* or *Rps. viridis* cytochrome bc_1 complex, 75 μM DBH, 290 μM EDTA, and cytochrome c_2 or equine cytochrome c at concentrations ranging from 0.5 to 50 μM in 50 mM potassium phosphate buffer, pH 7.40. Control experiments, in which no cytochrome bc_1 complex was added, were conducted at each cytochrome concentration, and the small rates of direct cytochrome reduction by DBH observed in these control experiments were subtracted from the rate observed in the presence of cytochrome bc_1 complex to give the catalyzed rate. The ionic strength of the buffer was varied by addition of NaCl. Eadie–Hofstee plots of the velocities, V, versus the V/[S] values were obtained by fitting the kinetic data with CricketGraph software on a Macintosh SE computer, and V_{max} and K_m values were extracted from the plots. The data obtained from the ionic strength dependence of the reduction rate of cytochrome c_2 by the cytochrome bc_1 complexes from *Rb. capsulatus* and *Rps. viridis* were fitted into an electrostatic pair model (Stonehuerner et al., 1979) for cytochrome c_2/cytochrome bc_1 complex interactions using a Quattro spreadsheet program. Kinetic measurements on the laser flash-induced oxidation of native and modified *Rps. viridis* cytochrome c_2 by *Rps. viridis* reaction centers were carried out as described previously (Knaff et al., 1991). The kinetics were measured using reaction mixtures that contained 1.1 μM reaction centers, 100 μM ubiquinone 0, 1 mM sodium ascorbate, and 20 μM native or modified cytochrome c_2 in 5 mM Tris-HCl buffer (pH 8.0). The data were fitted, and the pseudo-first-order rate constants were calculated, as described previously (Knaff et al., 1991).

RESULTS

Binding between the cytochrome bc_1 complex of *Rb.* capsulatus and cytochrome c_2 was measured by gel filtration chromatography on Biogel P-100 at either 10 or 310 mM ionic strength, as shown in Figure 1. Equimolar amounts of the cytochrome bc_1 complex and cytochrome c_2 were used in each run. At high ionic strength, cytochrome c_2 and the cytochrome bc_1 complex eluted separately, at positions expected from the differences in their molecular masses.

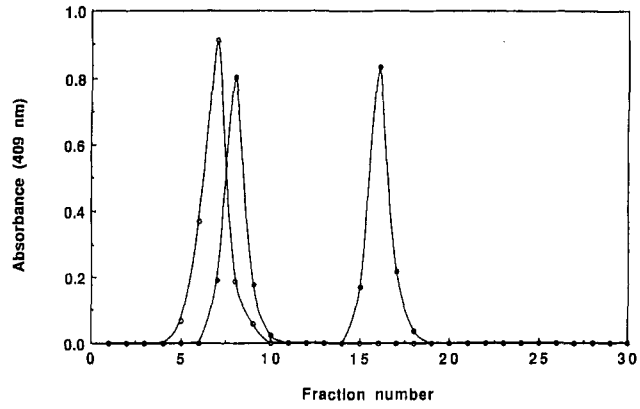

FIGURE 1: Complex formation between *Rb. capsulatus* cytochrome c_2 and the *Rb. capsulatus* cytochrome bc_1 complex. Equimolar amounts (10.0 nmol) of each protein in a total volume of 0.5 mL were chromatographed at low ionic strength (open circles) and high ionic strength (closed circles) on a Biogel P-100 column, as described in Materials and Methods.

However, at low ionic strength cytochrome c_2 comigrated with the cytochrome bc_1 complex, and no free cytochrome c_2 was detected. All of the cytochrome contained in the pooled cytochrome-containing fractions obtained from this gel filtration experiment conducted at low ionic strength was retained by a YM-30 ultrafiltration membrane (M_r = 30 kDa cutoff). When NaCl was added to the concentrated, pooled cytochrome-containing fractions to increase the ionic strength to 0.31 M, cytochrome was detected in the filtrate that passed through the YM-30 membrane. Analysis of the spectra of both the filtrate and the retentate indicated that >90% of the cytochrome c_2, but none of the cytochrome bc_1 complex, had passed through the membrane at high ionic strength. This demonstration that the only cytochrome-containing fraction obtained during gel filtration of a cytochrome bc_1 complex/cytochrome c_2 mixture at low ionic strength contained both cytochrome c_2 and the cytochrome bc_1 complex conclusively demonstrates that the two components associate at low ionic strength. Experiments in which equine cytochrome c replaced *Rb. capsulatus* cytochrome c_2 showed this same pattern of comigration with the cytochrome bc_1 complex at low ionic strength but not at high ionic strength, indicating that cytochrome c, like cytochrome c_2, forms an electrostatically stabilized complex with the *Rb. capsulatus* cytochrome bc_1 complex. Further evidence for complex formation was obtained from additional ultrafiltration experments. Although control experiments showed that the M_r = 12 kDa *Rb. capsulatus* cytochrome c_2 passes freely through the M_r = 30 kDa cutoff filter, an equimolar mixture of the cytochrome bc_1 complex and cytochrome c_2 at low ionic strength is completely retained by the ultrafiltration membrane, with no detectable cytochrome c_2 appearing in the filtrate. This is as expected if all of the *Rb. capsulatus* cytochrome c_2 is tightly bound to the *Rb. capsulatus* cytochrome bc_1 complex ($M_r \approx 100$ kDa). If the ionic strength was raised to 0.31 M, >85% of the cytochrome c_2, but none of the cytochrome bc_1 complex, was recovered in the filtrate. This result, like those of the gel filtration experiments, is the predicted outcome if the complex between cytochrome c_2 and the cytochrome bc_1 complex is stabilized by electrostatic forces that weaken at high ionic strength.

The kinetics of electron transfer from DBH to cytochrome c_2 catalyzed by the cytochrome bc_1 complexes from *Rb. capsulatus* and *Rps. viridis* obeyed Michaelis–Menten kinetics at all ionic strengths examined. Varying the ionic strength by addition of NaCl had little or no effect on V_{max}, but K_m

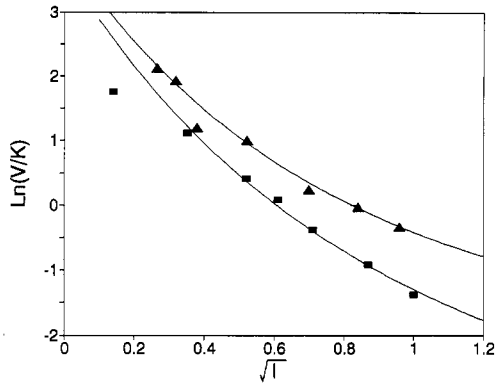

FIGURE 2: Ionic strength dependence of the kinetic parameters for the reduction of *Rb. capsulatus* cytochrome c_2 catalyzed by the *Rb. capsulatus* cytochrome bc_1 complex and the reduction of *Rps. viridis* cytochrome c_2 catalyzed by the *Rps. viridis* cytochrome bc_1 complex. V_{max}/K_m is reported in units of min^{-1}. The assays were carried out in 50 mM phosphate buffer, pH 7.40, with 0–1 M NaCl. The solid lines were obtained from eq 1 with $r = 4$, $n = 3.5$, and $\ln(V_{max}/K_m)_{infinity} = -2.3$ for *Rb. capsulatus* cytochrome c_2 (▲) and $r = 3.5$, $n = 3.8$, and $\ln(V_{max}/K_m)_{infinity} = -4.05$ for *Rps. viridis* cytochrome c_2 (■).

increased significantly with increasing ionic strength. Figure 2 summarizes the variation of V/K with ionic strength observed in these experiments. Similar dependencies on ionic strength have been observed previously for *R. rubrum* (Hall et al., 1987c) and *Rb. sphaeroides* (Hall et al., 1987d, 1989). The effects on the activity of the *Rb. capsulatus* complex appear to be entirely due to changes in ionic strength rather than to specific ion effects, as results similar to those observed with NaCl were obtained using KNO_3, $MgCl_2$, and Na_2SO_4 (Güner, 1992).

The ionic strength dependence of the reaction rates was used to analyze the electrostatic interactions in terms of the number, n, of charged pairs involved in the interaction between cytochrome c_2 and the cytochrome bc_1 complex. The data were fitted to a semiempirical equation (Stonehuerner et al., 1979) designed to analyze such interactions,

$$\ln(V_{max}/K_m) = \ln(V_{max}/K_m)_{infinity} + \frac{7.152ne^{\kappa(a-r)}}{(1+\kappa a)r} \quad (1)$$

where V_{max} is the maximal velocity, K_m is the Michaelis constant, $\kappa = 0.329(I)^{1/2}$ (I is the ionic strength), r is the distance between amino and carboxylate groups of the charged pair, a (1.7 Å) is the effective radius of these groups, and n is the number of charged pairs in the interaction of these two proteins. The best fits were obtained with $n = 3.5$ and $r = 4.0$ Å and with $n = 3.8$ and $r = 3.5$ Å for the interaction in *Rb. capsulatus* and *Rps. viridis*, respectively (Table I). The purely statistical uncertainties in the n and r values are ±20%. The n values derived from the empirical relationship shown in eq 1 should not be interpreted to mean that there are literally 4 charged pair interactions, but rather that the electrostatic interaction is equivalent to that arising from 4 isolated charged pairs. The theory is most useful for comparing different electrostatic interactions, and similar analyses of kinetic data for the reductions of cytochrome c_2 catalyzed by the cytochrome bc_1 complexes of *R. rubrum* (Hall et al., 1987c) and *Rb. sphaeroides* (Hall et al., 1987d, 1989) gave n values of 5 and 8, respectively.

The importance of electrostatic forces in the interaction of *Rb. capsulatus* cytochrome c_2 with the *Rb. capsulatus* cytochrome bc_1 complex having been established, experiments were designed to test the hypothesis (Ambler et al., 1979; Margoliash & Bosshard, 1983) that conserved lysine residues

Table I: Comparison of the Number of Interacting Pairs (n) in the Interaction of Cytochrome bc_1 Complexes and Cytochrome c_2 of Purple Non-Sulfur Photosynthetic Bacteria and of the Net Negative Charge on Regions I and II of Cytochrome c_1[a]

bacterium	n	net charge on c_1 region I	net charge on c_1 region II	reference
Rhodospirillum rubrum	5	−3	−4	Hall et al., 1987c
Rhodobacter sphaeroides	8	−5	−4	Hall et al., 1987d
Rhodobacter capsulatus	3.5	−4	−4	this work
Rhodopseudomonas viridis	3.8	0	−2	this work

[a] Region I runs from amino acid 88 through 107 in *Rb. capsulatus*, from amino acid 95 through 116 in *Rps. viridis*, from amino acid 90 through 108 in *Rb. sphaeroides*, and from amino acid 94 through 113 in *R. rubrum*. Region II runs from amino acid 209 through 218 in *Rb. capsulatus*, from amino acid 212 through 221 in *Rps. viridis*, from amino acid 211 through 220 in *Rb. sphaeroides*, and from amino acid 205 through 214 in *R. rubrum*. These sequences were taken from Davidson and Daldal (1987), Verbist et al. (1989), Yun et al. (1990), and Shander et al. (1992), respectively. The numbering starts with the initial methionine of the preprotein rather than with the N-terminal amino acid of the mature, processed cytochrome.

located near the exposed heme edge of cytochrome c_2 contribute the positive charges involved in complex formation. Three of these lysine residues, K12, K14, and K32 (Figure 3), were changed to either aspartate or glutamate by site-directed mutagenesis (Caffrey et al., 1992a). Figure 4 shows the effect of K12D, K14E, K32E, or K14E/K32E mutations in *Rb. capsulatus* cytochrome c_2 on the kinetic parameters associated with the activity of the *Rb. capsulatus* cytochrome bc_1 complex. The reduction of these cytochrome c_2 mutants catalyzed by the *Rb. capsulatus* cytochrome bc_1 complex, like that of the wild-type cytochrome, obeyed Michaelis–Menten kinetics. Replacing the positive charge normally contributed by these lysines with negative charges had virtually no effect on V_{max} but significantly increased K_m (Table II).

Equine cytochrome c shows significant sequence homology to *Rb. capsulatus* cytochrome c_2 (Ambler et al., 1979) and serves as an effective acceptor of electrons from the cytochrome bc_1 complexes of *R. rubrum* and *Rb. sphaeroides* (Hall et al., 1987c,d). Well-characterized derivatives of cytochrome c are available in which chemical modification of specific lysine residues eliminates the positive charge normally present and replaces it with an uncharged side-chain derivative. (Trifluoromethyl)phenylcarbamoylation of cytochrome c at lysines 13 and 72, both of which are located near the exposed heme edge of equine cytochrome c, had previously been found to have significant effects on the reduction of equine cytochrome c by the cytochrome bc_1 complexes of *R. rubrum* and *Rb. sphaeroides* (Hall et al., 1987c,d, 1989) and mitochondria (Ahmed et al., 1978). Figure 5 shows that eliminating the positive charge on either of these lysine residues has little effect on V_{max} but results in significant increases in K_m for the reduction of equine cytochrome c catalyzed by the *Rb. capsulatus* cytochrome bc_1 complex (Table III).

Although equine cytochrome c is an effective substitute for the cytochromes c_2 of both *R. rubrum* and *Rb. sphaeroides* in in vitro electron-transfer assays (Hall et al., 1987c,d, 1989; van der Wal et al., 1987) it has been demonstrated that the equine cytochrome is a relatively poor electron donor to the reaction center of *Rps. viridis* (Knaff et al., 1991; Meyer et al., 1991). It was thus of interest to determine whether equine cytochrome c could replace *Rps. viridis* cytochrome c_2 as an electron acceptor for the *Rps. viridis* cytochrome bc_1 complex. In contrast to the markedly different rates obtained for the oxidation of the two cytochromes by the *Rps. viridis* reaction center (Knaff et al., 1991), the K_m and V_{max} values obtained

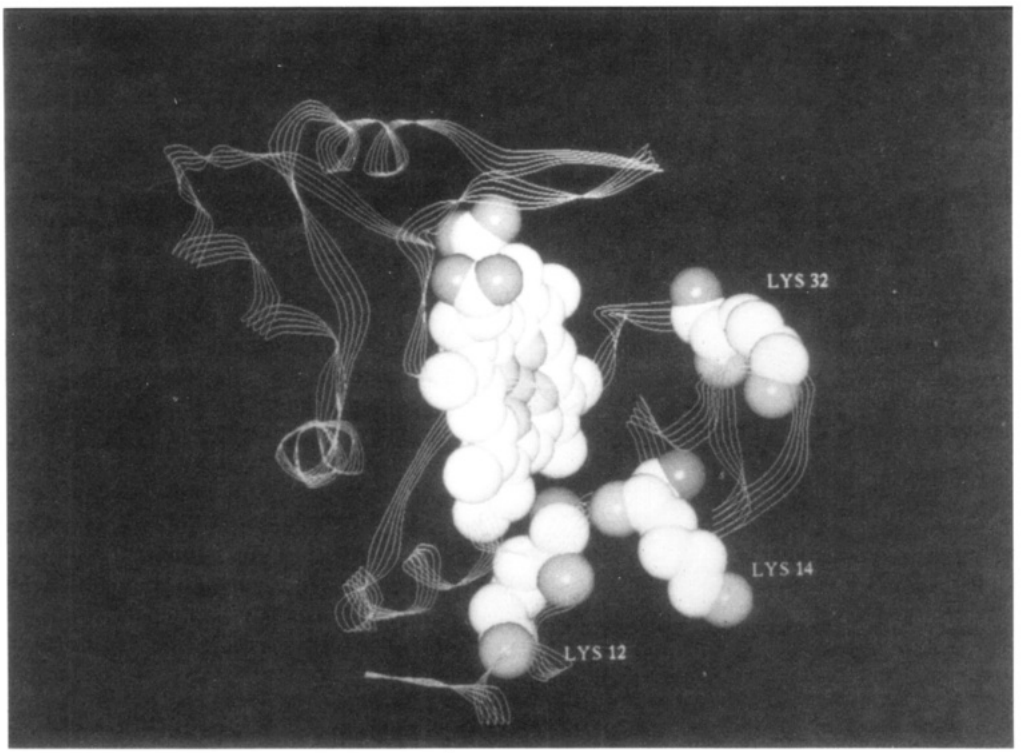

FIGURE 3: Ribbon structure of *Rb. capsulatus* cytochrome c_2. The heme group is shown edge-on. Lysine residues that have been altered by mutagenesis are indicated in white by amino acid sequence number (cytochrome c_2 coordinates were obtained from the Brookhaven Protein Data Bank and displayed on a Silicon Graphics Personal Iris computer).

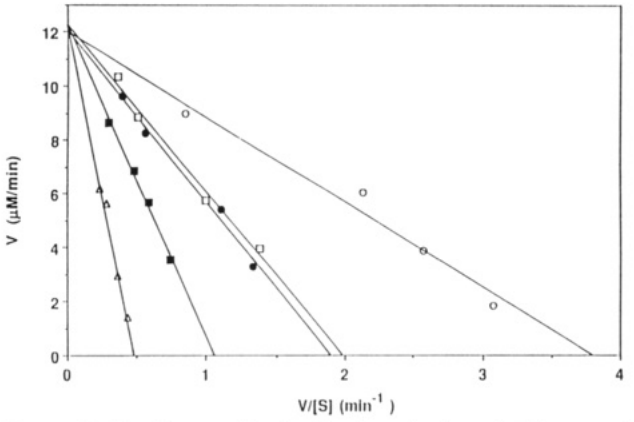

FIGURE 4: Steady-state kinetics for the reduction of wild-type and mutated *Rb. capsulatus* cytochrome c_2 catalyzed by the *Rb. capsulatus* cytochrome bc_1 complex; native cytochrome c_2 (○), K14E cytochrome c_2 (□), K12D cytochrome c_2 (●), K32E cytochrome c_2 (■), and K14E/K32E cytochrome c_2 (△). The velocity, V, is reported in μM/min, and $V/[S]$ is in min^{-1}. See Materials and Methods for details.

for the reduction of equine cytochrome *c* catalyzed by the *Rps. viridis* cytochrome bc_1 complex were similar to those measured for *Rps. viridis* cytochrome c_2 (Table IV).

The possible role of lysine residues on *Rps. viridis* cytochrome c_2 in the interactions between the cytochrome and either the reaction center or the cytochrome bc_1 complex was investigated by using CDNP-modified *Rps. viridis* cytochrome c_2 in which lysine residues on cytochrome c_2 have their normal charge of 1+ replaced by a charge of 1− (Brautigan et al., 1978). Modification of ca. 2.5 lysine residues on *Rps. viridis* cytochrome c_2 had relatively little effect on either the K_m or the V_{max} for the reduction of the cytochrome catalyzed by the *Rps. viridis* cytochrome bc_1 complex, producing only a 43% increase in K_m and actually resulting in a slight increase in V_{max} (Table IV). However, this lysine modification had a much greater effect on the oxidation of the cytochrome by the *Rps. viridis* reaction center, decreasing the pseudo-first-order constant for the oxidation of the cytochrome by the reaction center (measured at a cytochrome concentration of 20 μM and an ionic strength of 2.5 mM) 12.5-fold (data not shown).

Table II: Effect of Specific Lysine Mutations on the Kinetic Parameters for the Reduction of Cytochrome c_2 Catalyzed by the Cytochrome bc_1 Complex of *Rb. capsulatus*[a]

condition	k	K_m
native cytochrome c_2	68.9 ± 1.7	3.17 ± 0.36
K12D cytochrome c_2	69.5 ± 2.3	6.37 ± 0.77
K14E cytochrome c_2	70.7 ± 2.3	6.17 ± 0.81
K32E cytochrome c_2	70.7 ± 4.6	11.6 ± 1.6
K14E/K32E cytochrome c_2	70.1 ± 6.3	25.2 ± 2.7

[a] Kinetics were measured in 50 mM potassium phosphate buffer (pH 7.4), as described in Materials and Methods. The turnover number, k, is reported in s^{-1}, K_m is in μM, and experimental error is expressed as standard deviation from the mean.

DISCUSSION

Affinity chromatography of cytochrome bc_1 complexes from the photosynthetic bacteria *R. rubrum* and *Rps. viridis* on cytochrome *c*-coupled Sepharose provided early evidence for electrostatically stabilized complex formation between the cytochrome bc_1 complex and cytochrome c_2 of these bacteria (Wynn et al., 1986). In the case of *R. rubrum*, cochromatography and differential chemical modification experiments have provided additional evidence for complex formation (Bosshard et al., 1987). Analysis of kinetic data obtained from investigations of the reduction of cytochrome c_2 catalyzed by the cytochrome bc_1 complexes of *R. rubrum* and *Rb. sphaeroides* have provided substantial evidence for the existence of an electrostatically stabilized complex between these two reaction partners in these two photosynthetic bacteria. These studies have also provided considerable

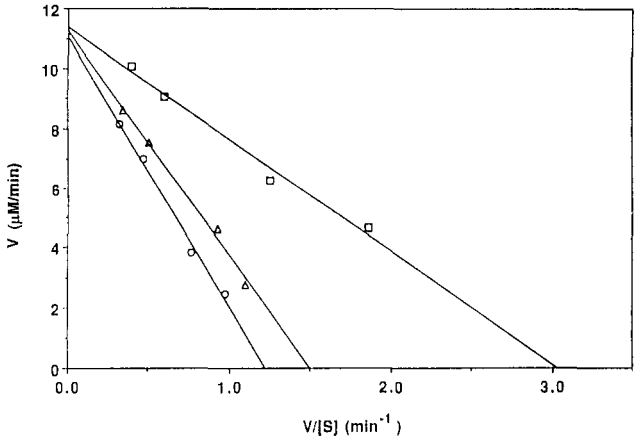

FIGURE 5: Steady-state kinetics for the reduction of native and lysine-modified derivatives of equine cytochrome *c* catalyzed by the *Rb. capsulatus* cytochrome bc_1 complex; native cytochrome *c* (○), TFC-K13 cytochrome *c* (△), TFC-K72 cytochrome *c* (□). The velocity, *V*, is reported in μM/min, and *V*/[S] is in min^{-1}. See Materials and Methods for details.

Table III: Effect of Specific Lysine Modifications on the Kinetic Parameters for the Reduction of Equine Cytochrome *c* Catalyzed by the *Rb. capsulatus* Cytochrome bc_1 Complex[a]

condition	*k*	K_m
native cytochrome *c*	65.5 ± 4.0	3.75 ± 0.23
TFC-K13 cytochrome *c*	64.9 ± 2.8	7.50 ± 0.74
TFC-K72 cytochrome *c*	63.8 ± 7.4	9.09 ± 0.62

[a] Experimental conditions and units are as in Table II.

Table IV: The Reduction of Equine Cytochrome *c* and *Rps. viridis* Cytochrome c_2 Catalyzed by the *Rps. viridis* Cytochrome bc_1 Complex[a]

condition	*k*	K_m
horse heart cytochrome *c*	43.4 ± 6.2	7.25 ± 0.35
native *Rps. viridis* cytochrome c_2	28.7 ± 1.4	4.62 ± 0.52
CDNP-modified *Rps. viridis* cytochrome c_2	31.4 ± 4.7	6.62 ± 0.36

[a] Experimental conditions and units are as in Table II.

evidence for the involvement of conserved lysine residues located near the exposed heme edge of cytochrome c_2 in complex formation with the cytochrome bc_1 complexes of these two bacteria (Bosshard et al., 1987; Hall et al., 1987c,d, 1989). It has been found that these conserved lysine residues also play a similar role in the docking of the cytochromes to the reaction centers of *Rb. sphaeroides* and *R. rubrum* (Hall et al., 1987a,b; van der Wal et al., 1987). We have now extended the earlier studies on the interaction between cytochrome bc_1 complexes and cytochrome c_2/c in *Rb. sphaeroides* and *R. rubrum* to *Rb. capsulatus* and *Rps. viridis*. Ultrafiltration and comigration chromatography experiments conducted with the *Rb. capsulatus* proteins (see above) strongly support the idea that electrostatically stabilized complex formation also occurs between cytochrome c_2 and the cytochrome bc_1 complex in this bacterium. The effect of ionic strength on the kinetics of the reactions catalyzed by the cytochrome bc_1 complexes of *Rb. capsulatus* and *Rps. viridis* provides additional support for the idea that electrostatic interactions between cytochrome c_2 and the cytochrome bc_1 complex are important in both of these photosynthetic bacteria.

It has been demonstrated that the cytochrome c_1 subunit of the cytochrome bc_1 complex is the direct reductant for cytochrome c_2 in photosynthetic bacteria (Meinhardt & Crofts, 1982; Snozzi & Crofts, 1985). A considerable amount of evidence suggests that cytochrome c_1 provides the negative charges necessary for electrostatic binding to positively charged lysine residues on mitochondrial cytochrome *c* (Margoliash & Bosshard, 1983), which is structurally related to cytochrome c_2 (Ambler et al., 1979; Dickerson, 1980; Meyer & Kamen, 1982; Benning et al., 1991). Cross-linking and differential chemical modification experiments have implicated two acidic regions on mitochondrial cytochrome c_1 in binding cytochrome *c* (Capaldi et al., 1982; Broger et al., 1983; Stonehuerner et al., 1985). One of these two regions (running from V209 through G218 in *Rb. capsulatus*) is well conserved in all four photosynthetic bacterial species for which cytochrome c_1 sequences are available, while the other region (running from G88 through D107 in *Rb. capsulatus*) is conserved in some but not all of these photosynthetic bacteria (Gabellini & Sebald, 1986; Davidson & Daldal, 1987; Verbist et al., 1989; Majewski & Trebst, 1990; Yun et al., 1990; Shanker et al., 1992).

Observations in several laboratories have demonstrated that as the ionic strength increased the affinity of the cytochrome bc_1 complex for cytochrome c_2 and the rate of electron flow from quinol to cytochrome c_2 catalyzed by the cytochrome bc_1 complex decreased substantially (Bosshard et al., 1987; Hall et al., 1987c,d, 1989). The ionic strength dependency of these reactions has been related to the number of charged pairs involved in electrostatic interactions between these two proteins, utilizing a semiempirical relationship developed by Stonehuerner et al. (1979) and Smith et al. (1981). The data obtained for complexes from *R. rubrum* and *Rb. sphaeroides* have been found to be consistent with this theoretical relationship (Hall et al., 1987c,d). Fitting the data obtained in the current study to this electrostatic theory gave essentially identical values of 3.5 and 3.8, respectively, for the number of charged pairs involved in the interaction between the cytochrome bc_1 complexes and cytochrome c_2 of *Rb. capsulatus* and *Rps. viridis* (Table I). An examination of the amino acid sequences for cytochromes c_1 (Davidson & Daldal, 1987; Verbist et al., 1989) reveals that one of the two putative cytochrome c_2-binding regions that is well conserved between the mitochondrial and the *Rb. capsulatus* cytochrome c_1 (region I, running from G88 through D107 in *Rb. capsulatus*) is poorly conserved in *Rps. viridis*. In fact, this region of *Rps. viridis* cytochrome c_1 has 0 net charge and contains only three acidic residues, while the same region of *Rb. capsulatus* cytochrome c_2 has a net charge of 4– and eight acidic residues (Table I). The second putative cytochrome c_2-binding region of cytochrome c_1 (region II, running from V209 through G218 in *Rb. capsulatus*) is better conserved, although this region also carries somewhat lower negative charge in *Rps. viridis* than in *Rb. capsulatus* (Table I). The fact that analysis of the ionic strength dependence of the kinetic parameters for cytochrome c_2 reduction in *Rb. capsulatus* and *Rps. viridis* gives values for the number of charged pairs involved in cytochrome c_1/cytochrome c_2 interaction that are so similar, despite the considerable differences in the negative charge present in region I of cytochrome c_1 in the two bacteria, raises the possibility that Region I may play a less important role than the better conserved Region II in cytochrome c_2 binding in photosynthetic bacteria.

To examine the interaction site on cytochrome c_2 and equine cytochrome *c* for the cytochrome bc_1 complex, we have used four mutants of *Rb. capsulatus* cytochrome c_2 in which specific lysine residues were altered by site-directed mutagenesis and two (fluoromethyl)phenylcarbamoylated equine cytochrome *c* derivatives in which specific lysine residues were chemically

modified. Mutation of *Rb. capsulatus* cytochrome c_2 lysines at positions 12, 14, or 32 (Figure 3) and modification of cytochrome *c* lysines at position 13 or 72, all of which are expected to participate in the electrostatic interaction between cytochrome c_2(*c*) and cytochrome c_1, markedly increased the K_m values for the reduction of these two cytochromes catalyzed by the *Rb. capsulatus* cytochrome bc_1 complex (Tables II and III). These results are consistent with the hypothesis that these lysine residues, located near the exposed heme edges of *Rb. capsulatus* cytochrome c_2 and of equine cytochrome *c*, contribute to the interaction of the cytochromes with the *Rb. capsulatus* cytochrome bc_1 complex, as is the case for the interaction between *Rb. capsulatus* cytochrome c_2 and the *Rb. sphaeroides* reaction center (Caffrey et al., 1992b). It should also be noted that the turnover numbers observed for the *Rb. capsulatus* cytochrome bc_1 complex are essentially identical, regardless of whether *Rb. capsulatus* cytochrome c_2 or equine cytochrome *c* is used as an electron acceptor (See Tables II and III). The fact that similar rates are observed, despite the significant differences in thermodynamic driving forces present for the two different acceptors (the E_m values of cytochrome c_2 and cytochrome *c* are +350 and +260 mV, respectively), suggests that the same rate-limiting step, probably a reaction within the cytochrome bc_1 complex, is operating in both cases.

Previous studies have demonstrated that, compared to *Rps. viridis* cytochrome c_2, equine cytochrome *c* is a very poor electron donor to the *Rps. viridis* reaction center (Knaff et al., 1991; Meyer et al., 1991). This observation was surprising, in the light of earlier observations that equine cytochrome *c* is an excellent electron donor to the reaction centers of *Rb. sphaerides*, *R. rubrum*, and *Rc. gelatinosus* (Overfield & Wraight, 1986; Hall et al., 1987a,b; van der Wal et al., 1987; Matsuura et al., 1988) and the fact that equine cytochrome *c* shows higher sequence homology to *Rps. viridis* cytochrome c_2 than to the cytochromes c_2 of these other photosynthetic bacteria (Ambler et al., 1976; Ambler et al., 1979; Dickerson, 1980). Our earlier studies have demonstrated that, in photosynthetic bacteria where equine cytochrome *c* is an effective surrogate for the native cytochrome c_2 as an electron donor to the reaction center, the equine cytochrome is an equally effective surrogate acceptor of electrons from the cytochrome bc_1 complex (Hall et al., 1987c,d). It thus appeared possible that equine cytochrome *c* might prove to be a poor substitute for *Rps. viridis* cytochrome c_2 as an acceptor of electrons from the *Rps. viridis* cytochrome bc_1 complex. However, as was shown above, very similar K_m and V_{max} values were obtained for the *Rps. viridis* cytochrome bc_1 complex-catalyzed reductions of equine cytochrome *c* and *Rps. viridis* cytochrome c_2 (Table IV). These findings suggest that the binding site for *Rps. viridis* cytochrome c_2 on its membrane-bound reductant, the cytochome bc_1 complex, differs in structure from that on its membrane-bound oxidant, the reaction center tetraheme subunit, as the former can accommodate equine cytochrome *c* well but the latter cannot. Further evidence for this suggestion comes from the observation that modification of a small number of lysine residues on *Rps. viridis* cytochrome c_2 has only a very modest effect on the ability of the cytochrome to accept electrons from the *Rps. viridis* cytochrome bc_1 complex (Table IV), but greatly impairs the ability of the cytochrome to donate electrons to the tetraheme subunit of the *Rps. viridis* reaction center (see above). Differences in relative reactivities for a number of cytochromes *c* during reduction by the mitochondrial cytochrome bc_1 complex, when compared to the reactivities observed during oxidation of these cytochromes by mitochondrial cytochrome oxidase, had been observed earlier by Errede and Kamen (1978).

The results obtained in this study with the CDNP-modified *Rps. viridis* cytochrome c_2 support our earlier suggestion (Knaff et al., 1991) that lysine residues are important in the docking of the cytochrome on the *Rps. viridis* reaction center. The observation that this modification of, on average, 2.5 lysine residues per *Rps. viridis* cytochrome c_2 had little effect on the kinetic parameters for reduction of the cytochrome catalyzed by the *Rps. viridis* cytochrome bc_1 complex cannot be taken as evidence that lysine residues are not involved in the interaction between *Rps. viridis* cytochrome c_2 and the *Rps. viridis* cytochrome bc_1 complex, but rather may indicate that a different set of cytochrome c_2 lysines are involved in the two binding processes. Experiments using *Rps. viridis* cytochrome c_2 modified at specific lysine residues will be necessary to explore this question further.

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