Reactions of Isocytochrome c_2 in the Photosynthetic Electron Transfer Chain of *Rhodobacter sphaeroides*†

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ABSTRACT: *Rhodobacter sphaeroides* strains lacking cytochrome c_2 (cyt c_2), the normal electron donor to P_{870}^+ in light-oxidized reaction center (RC) complexes, are unable to grow photosynthetically. However, *spd* mutations that *s*uppress the *p*hotosynthetic *d*eficiency of cyt c_2 mutants elevate levels of the cyt c_2 isoform, isocyt c_2. We monitored photosynthetic electron transfer in whole cells, in chromatophores, and with purified components to ascertain if and how isocyt c_2 reduced light-oxidized RC complexes. These studies revealed that several fundamental aspects of photosynthetic electron transfer were similar in strains that use isocyt c_2 and wild-type cells. For example, P_{870}^+ reduction accompanied cytochrome *c* oxidation. In addition, photosynthetic electron transfer was blocked by the well-known cyt bc_1 complex inhibitors antimycin and myxothiazol. However, even at the increased isocyt c_2 levels present in these strains (∼40% that of cyt c_2 in wild-type cells), there was little, if any, of the rapid (<5 μs) electron transfer to P_{870}^+ that is characteristic of cytochromes bound to RC complexes at the time of the light flash. Thus, it appears that isocyt c_2 function limits the *in vivo* rate of P_{870}^+ reduction. Indeed, at low ionic strength *in vitro*, the apparent affinity of isocyt c_2 for RC complexes ($K_D \sim 40$ μM) is significantly lower than that of cyt c_2 ($K_D \sim 1.0$ μM). This reduced affinity does not appear to result from an altered mode of RC binding by isocyt c_2 since electrostatic interactions make similar overall contributions to the binding of both cyt c_2 and isocyt c_2 to this membrane-bound redox partner. Thus, sequence, structural, or local conformational differences between cyt c_2 and isocyt c_2 significantly alter their apparent affinities for this physiologically relevant redox partner.

The conversion of light into biological energy is a distinguishing feature of photosynthetic cells. In both procaryotes and eucaryotes, this function of the photosynthetic apparatus requires the concerted action of several pigment-binding proteins in addition to a number of soluble and membrane-bound electron transport proteins. Despite their crucial role in light energy conversion, detailed information is lacking about how the function of electron transport proteins is optimized to ensure efficient function of the photosynthetic apparatus. The following experiments seek to analyze the function of a recently discovered alternate electron donor to light-oxidized reaction center (RC)[1] complexes of the photosynthetic bacterium *Rhodobacter sphaeroides.*

In *R. sphaeroides*, light energy drives the oxidation of a bacteriochlorophyll dimer (P_{870}) at the external, or periplasmic, face of the RC complex, and the reduction of quinone on the inner, or cytoplasmic, side of the photosynthetic membrane (Shinkarev & Wraight, 1993; Lancaster et al., 1995). The semiquinone so formed is further reduced to the quinol state by a subsequent light-induced turnover of the RC. Quinol formation requires re-reduction of P_{870}^+ which, in wild-type *R. sphaeroides* cells, is carried out by the periplasmic electron transport protein, cytochrome c_2 (cyt c_2). Once quinol is formed, it leaves the RC, diffuses in the bilayer, and is oxidized by the ubiquinol−cyt c_2 oxidoreductase (cyt bc_1) complex. This cyclic flow of electrons, coupled to the formation of a proton gradient across the photosynthetic membrane, is completed by electron transfer from cyt c_1 to cyt c_2 (Gray & Daldal, 1995). Thus, photosynthetic energy transduction in wild type cells requires the productive interaction of cyt c_2 with two membrane-bound enzymes.

The central role that diffusible electron carriers like cyt c_2 play in photosynthetic energy transduction is underscored by the observation that phototrophs often contain more than one protein that is capable of reducing light-oxidized pigment−protein complexes [see Meyer and Donohue (1995) for a review]. Indeed, two related soluble *c*-type cytochromes (cyt c_2 and isocyt c_2) are potential electron donors to light-oxidized RC complexes in *R. sphaeroides* (Meyer

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[1] Abbreviations: cyt c_2, cytochrome c_2; isocyt c_2, isocytochrome c_2; RC, reaction center; RC, P_{870}, bacteriochlorophyll dimer; cyt bc_1, ubiquinol−cytochrome c_2 oxidoreductase.

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& Donohue, 1995). Despite an extensive analysis of photosynthetic membrane function in *R. sphaeroides* (Blankenship et al., 1995), the potential for isocyt c_2 to serve as an alternative electron donor to RC complexes was not uncovered until *spd* mutations, which *s*uppress the *p*hotosynthetic *d*eficiency of cyt c_2 mutants (Rott & Donohue, 1990), were shown to increase levels of this periplasmic redox carrier (Rott et al., 1992).

The periplasmic location (Rott & Donohue, 1990), properties (Fitch et al., 1989), and substantial amino acid identity between cyt c_2 and isocyt c_2 (Rott et al., 1993) are consistent with isocyt c_2 reducing light-oxidized RC complexes in Spd mutants. Since direct experimental support for P_{870}^+ reduction by isocyt c_2 is lacking, we sought to analyze if and how isocyt c_2 participated in photosynthetic energy transduction. While this photosynthetic electron transfer chain is in many respects similar to that present in wild-type cells, isocyt c_2 has a significantly lower apparent affinity for RC complexes than would have been predicted from its similarity to other members of the cyt c_2 family.

MATERIALS AND METHODS

Growth of Bacteria. These experiments used derivatives of *R. sphaeroides* CYCA65R7 [a cyt c_2-deficient mutant of Ga (Cohen-Bazire et al., 1956) that contains an *spd-7* mutation which increases isocyt c_2 levels (Rott & Donohue, 1990)]. When analyzing photosynthetic electron transport, the altered carotenoid composition of CYCA65R7 minimizes interference by light-induced pigment absorbance changes (Crofts et al., 1974).

Aerobic cultures of *R. sphaeroides* were grown at 32 °C in Sistrom's minimal medium A by vigorous agitation on a gyratory shaker or by sparging with 30% O_2, 69% N_2, and 1% CO_2. Photosynthetic cultures were maintained either by growth in completely filled culture vessels or by sparging with 95% N_2 and 5% CO_2. Where appropriate, 1 μg/mL tetracycline or 25 μg/mL kanamycin was added. Photosynthetic growth in the presence of tetracycline used Carolina Biological far-red 750 filters to minimize the formation of growth inhibitory products by antibiotic photooxidation (Donohue et al., 1988).

Escherichia coli strains were grown aerobically at 37 °C in LB media (Maniatis, 1982) with ampicillin (50 μg/mL) or tetracycline (10 μg/mL) as appropriate. S17-1 derivatives were used as donors for plasmid conjugation into *R. sphaeroides* (Donohue & Kaplan, 1991).

Isocyt c_2 Expression Plasmids. Plasmid pWF1R is a pRK415 derivative (Keen et al., 1989) that contains the isocyt c_2 structural gene (*cycI*; Rott et al., 1993) under control of the promoter for the *R. sphaeroides* B800−850 structural genes (*puc*; Lee & Kaplan, 1992). To generate this *puc*::*cycI* operon fusion, a *Sal*I−*Hin*cII restriction fragment, containing the entire *cycI* coding sequence, was made blunt with the Klenow fragment of DNA polymerase and ligated into the pUC19 *Sma*I site to generate pMAR11. A *Pst*I−*Xmn*I restriction fragment, containing 54 bp of *pucB* coding sequence and 716 bp of upstream DNA (Lee & Kaplan, 1992), was ligated into the *Pst*I−*Hin*cII sites of pMAR11. The *puc*::*cycI* operon fusion was then cloned as an *Eco*RI−*Hin*dIII restriction fragment into the analogous sites of pRK415.

For isocyt c_2 purification, strain CYCA65R7(pUI8474) was grown under photosynthetic conditions. Plasmid pUI8747 contains *cycI* under control of its native regulatory elements (Rott et al., 1993). From ∼50 g (wet weight) of late exponential phase cells, ∼2−5 mg of pure isocyt c_2 was routinely obtained (see below).

Analysis and Purification of c-Type Cytochromes. To analyze soluble cytochrome *c* content, exponential phase cells (∼5×10^8 cells/mL) were harvested and lysed in a French press. After removing unbroken cells and debris, ultracentrifugation was used to prepare soluble fractions (Rott & Donohue, 1990). Reduced (1 mM sodium ascorbate) minus oxidized (800 μM potassium ferricyanide) spectra were recorded at room temperature in an SLM Aminco DW2000 spectrophotometer. The cytochrome *c* content was estimated by subtracting the absorbance at 540 nm from that at the α-peak maximum assuming an E_{mM} of 20 (Hendry et al., 1981).

Periplasmic fractions were used for cytochrome purification with the following modifications to a previously described method (Rott et al., 1992). The 100% ammonium sulfate precipitate was brought to 50% ammonium sulfate prior to loading ∼40−80 mg of protein (∼0.5−1.0 mg of ascorbate reducible cytochrome *c*) on a 7.5 mm by 7.5 cm Tosohaas Phenyl 5PW column (Anspec Co., Ann Arbor, MI) connected to a System Gold HPLC (Beckman Instruments, Fullerton, CA). Proteins were eluted over a 30 min period with a linear 50−0% ammonium sulfate gradient. Simultaneous optical density readings at 280 and 410 nm were used to monitor cytochrome purification. Pooled fractions were dialyzed and concentrated prior to spectral analysis. To estimate cytochrome *c* recovery, published α-maxima of cyt c_2 (550 nm; Pettigrew et al., 1975) and isocyt c_2 (552 nm; Fitch et al., 1989) were used. To estimate purity, samples were solubilized (50 °C, no reductant) and separated on 15% sodium dodecyl sulfate−polyacrylamide gels. The isocyt c_2 midpoint potential was measured by oxidative and reductive titrations with a combination of sodium ascorbate, methyl-1,4-benzoquinone, potassium ferricyanide, and ferroceneacetic acid using a silver−silver chloride electrode.

Kinetics of Light-Induced Electron Transfer in Vivo. Chromatophores from exponential phase cultures (Crofts et al., 1985) were suspended in 100 mM KCl, 50 mM MOPS (pH 7.0). Assays for light-dependent redox changes used the indicated concentrations of RC complexes in the presence of valinomycin and nigericin (2 μM each). The redox potential was set (100 or 200 mV) by adding combinations of the following redox mediators: 1 μM each of 2-hydroxy-1,4-naphthoquinone and pyocyanin; 2 μM each of 2,3,5,6-tetramethyl-*p*-phenylenediamine, phenazine ethosulfate, and phenazine methosulfate; 10 μM each of *p*-benzoquinone, 1,2-naphthoquinone, 1,4-naphthoquinone, and duroquinone. As indicated, antimycin (10 μM) or myxothiazol (5 μM) was used to inhibit cyt bc_1 complex function (Meinhardt & Crofts, 1982; Gray & Daldal, 1995).

A computer-linked, single-beam, kinetic spectrophotometer (response time <2 μs) equipped with a xenon flash lamp (10 μs duration at half-maximal intensity) provided actinic light flashes (Bowyer et al., 1980). Light-induced redox changes were assayed as follows: absorbance increases at 542 nm reveal RC P_{870} photooxidation, while the subsequent decrease in absorbance at 542 nm signified P_{870}^+ reduction. Redox changes of total *c*-type cytochromes (the sum of cyt c_1 and either cyt c_2 or isocyt c_2) were obtained by subtracting absorbance changes at 542 nm from those at 551 nm. A

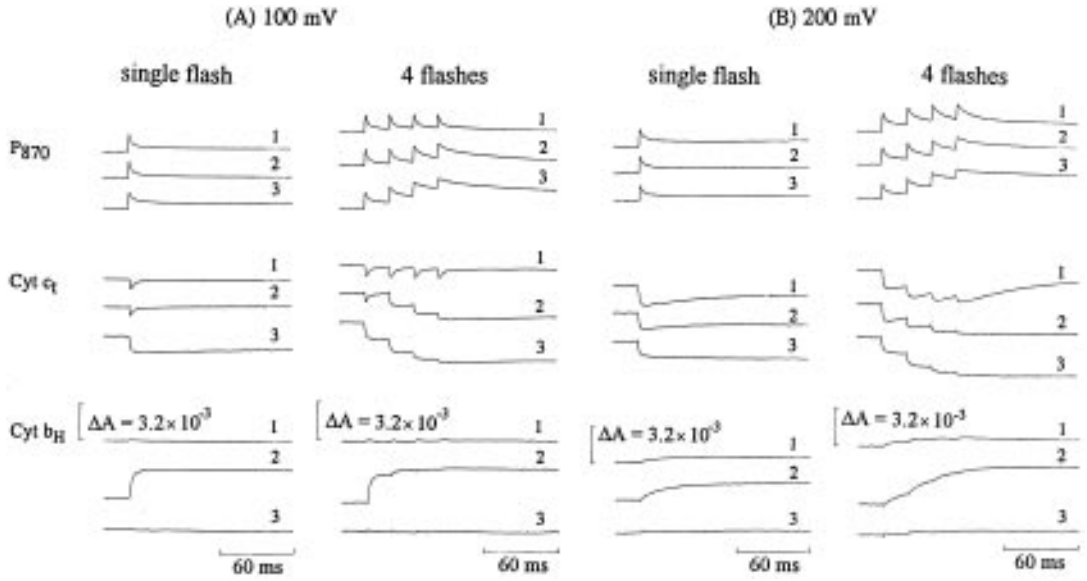

FIGURE 1: Kinetic traces of reaction center (542 nm), cytochrome b_H (561−569 nm), and *c*-type cytochromes (551−542 nm) of chromatophores of the Spd mutant, CYCA65R7, containing a a *cycI* expression plasmid (pVWF1R), in the absence of inhibitors (1) and in the presence of antimycin (2) or of myxothiazol (3). Chromatophores were suspended in 100 mM KCl, 50 mM MOPS (pH 7.0) to a concentration of 0.3 μM reaction center with the following redox mediators: 1 μM each of 2-OH-1,4-NQ and pyocyanin; 2 μM each of DAD, PES, and PMS; 10 mM each of r-BQ, 1,2-NQ, 1,4-NQ, and DQ. Also present were 2 μM each of valinomycin and nigericin. Traces shown are the average of 4, with 40 s (no inhibitors), 80 s (with antimycin), or 150 s (with myxothiazol) between measurements. The instrument response time was 200 μs. The redox potential was 100 mV (A) or 200 mV (B). The concentrations of antimycin and myxothiazol were 10 and 5 μM, respectively.

decrease in this parameter (cyt c_T) indicated oxidation of total *c*-type cytochromes while an increase reflected *c*-type cytochrome reduction. Similar light-induced absorbance changes at 561−569 nm reported on reduction and oxidation of the high-potential heme *b* of the cyt bc_1 complex (cyt b_H). Traces shown were taken from four averages, with 40 s (no inhibitors), 80 s (with antimycin), and 150 s (with myxothiazol) between measurements.

Extinction coefficients used to estimate the concentration of individual components from kinetic traces with whole cells or chromatophores were as follows: E_{mM}(cyt c_2)(550−542 nm) = 19; E_{mM}(P_{870}^+)(542 nm) = 10 (Dutton et al., 1975). For cyt b_H and cyt c_1, we used E_{mM}(cyt c_1)(551−542 nm) = 19 and E_{mM}(cyt b_H)(561−562 nm) = 20 (Bowyer et al., 1980; Meinhardt & Crofts, 1982). Finally, we assumed isocyt c_2 has an E_{mM} of 19 at 551−542 nm (Fitch et al., 1989).

Light-Induced Electron Transfer to Purified RC Complexes. RC complexes from *R. sphaeroides* strain R26 were purified as described previously (Maróti & Wraight, 1988). Samples were supplemented with ubiquinone-10 (Q-10, 20 μM) in Triton X-100 (0.03−0.04%), which served to maintain the RC complexes in suspension. Alterations to pH, ionic strength, and redox state are indicated in the figure legends.

Electron transfer from cyt c_2 or isocyt c_2 to RC complexes was assayed at 550 nm, following a single-turnover, xenon flash (half-width 10 μs) essentially as described previously (Overfield & Wraight, 1980a). An initial, rapid, and kinetically unresolved photooxidation of P_{870} (increase in absorbance) was followed by the simultaneous and coupled reduction of P_{870}^+ and oxidation of cytochrome (decrease in absorbance). Rate behavior was determined by exponential analysis of the flash-induced transients; the rates are expressed either as apparent first-order rate constants or half-times.

RESULTS

Increasing Isocyt c_2 Levels. To facilitate the study of PS electron transfer in *R. sphaeroides* cells which lack cyt c_2, we sought to develop an expression strain in which isocyt c_2 was present at levels comparable to cyt c_2 in wild-type cells. When a *cycI* expression plasmid (pWF1R) was placed in the Spd mutant CYCA65R7, the level of ascorbate-reducible soluble *c*-type cytochromes (∼322 pmol/mg of soluble protein) was roughly twice that of an isogenic strain lacking this plasmid (∼170 pmol/mg of soluble protein). However, the total soluble *c*-type cytochrome content of this isocyt c_2 expression strain, CYCA65R7(pWF1R), was lower than wild-type cells (∼700 pmol/mg of soluble protein). After correcting for the amount of ascorbate-reducible, soluble *c*-type cytochrome in photosynthetic cells that is not cyt c_2 (∼60 pmol/mg of soluble protein; Donohue et al., 1988), the isocyt c_2 level in this expression strain (∼260 pmol of isocyt c_2/mg of soluble protein) was ∼40% that of cyt c_2 in wild type cells (∼640 pmol of cyt c_2/mg of soluble protein).

Kinetics of Light-Induced Electron Transport in Vivo. When light-induced redox changes were monitored in the isocyt c_2 expression strain, the main features were similar to those of wild-type cells (Bowyer et al., 1980; Donohue et al., 1988). Each exciting flash that was applied to either whole cells (data not shown) or chromatophores (Figure 1) produced rapid oxidation and subsequent reduction of the RC bacteriochlorophyll dimer (P_{870}). The reduction of P_{870}^+ was accompanied by oxidation of *c*-type cytochromes; in turn, these proteins were reduced by electron transfer through the cyt bc_1 complex with kinetics that depended in a predictable fashion on the redox state and inhibitors present in the assay. The turnover of photosynthetic apparatus components observed after either single or multiple light flashes demonstrates the function of one or more *c*-type cytochromes in P_{870}^+ reduction.

If samples were initially poised at different redox potentials, the rates of reduction of cyt b_H and re-reduction of cyt c_T were more rapid when the quinone pool was ~30% reduced (100 mV; panel A of Figure 1) than when it was oxidized (200 mV; panel B of Figure 1). The kinetic traces obtained with whole cells were essentially the same as those shown here for chromatophores (data not shown), indicating that little, if any, isocyt c_2 was lost upon cell lysis. At the flash repetition sequence used, oxidized cyt c_T accumulated when the quinone pool was initially oxidized because a fraction of the cyt c_T remained oxidized after each flash. These effects are similar to those observed previously in wild-type cells and reflect the second-order reaction of quinol with the Q_O site of the cyt bc_1 complex.

Another important similarity between the photosynthetic electron transfer chain of this isocyt c_2 expression strain and that of wild-type cells is the inhibitory effects of myxothiazol and antimycin on cyt b_H and cyt c_T reduction after either single or multiple flashes (Figure 1). The effect of these well-characterized inhibitors of quinol oxidation in the Q_O site (Gray & Daldal, 1995) on both cyt b_H and cyt c_T reduction shows that cyt bc_1 complex function is required for photosynthetic electron transfer. Flash illumination gave rise to a transient reduction of cyt b_H (E_m ~ 50 mV; Meinhardt & Crofts, 1982), and the accumulation of this reduced form was minimal in the absence of inhibitor in either whole cells (data not shown) or chromatophores (Figure 1) with single or multiple light flashes. When similar experiments are performed in the presence of antimycin (to inhibit cyt b_H oxidation), cyt b_H reduction was not reversed on a rapid time scale.

Relative Levels of Individual PS Electron Transfer Chain Components. If similar experiments are performed with a train of eight light flashes to completely oxidize components of the high-potential chain, the amplitudes of light-dependent absorbance changes in the presence of inhibitors provide estimates of the relative concentration of RC complexes (P_{870}), cyt bc_1 (one cyt b_H per cyt bc_1 complex), and total cytochrome *c* (cyt c_T) (Crofts et al., 1985; Gray & Daldal, 1995). In wild-type cells, the RC:cyt bc_1 ratio is ~2:1 (Crofts et al., 1985; Table 1). In the isocyt c_2 expression strain, a RC:cyt bc_1 complex ratio comparable to wild-type cells (~2:1 with some variability depending on the E_h of the sample) is calculated (Table 1). While we provide estimates of these ratios from both whole cells and chromatophores, the later data are more reliable because it is easier to control their ambient redox potential.

When the cyt bc_1 and cyt c_T levels are compared in this isocyt c_2 expression strain, most of the cyt c_T change can be accounted for by cyt c_1 oxidation (Table 1). As expected, the RC:cyt c_T ratio calculated was similar in whole cells and chromatophores (Table 1), indicating that no significant loss of isocyt c_2 occurred during chromatophore preparation. In whole cells, the data suggest that isocyt c_2 is present at ~11−18% the level of RC complexes [i.e., RC:isocyt c_2 ratio of 2:(0.22−0.36)]. In chromatophores, the calculated RC:isocyt c_2 ratio was 2:(0.36−0.39). Thus, isocyt c_2 levels in this expression strain were roughly 40% that of cyt c_2 in wild-type cells (RC:cyt c_2 ratio of ~2:1).

P_{870}^+ Reduction by Isocyt c_2 in Vivo Lacks a Significant Rapid Component. Despite the above similarities, other aspects of the kinetics of light-dependent cytochrome c_T oxidation in chromatophores from this isocyt c_2 expression strain (Figure 1) are significantly different than those from wild-type cells (Bowyer et al., 1980). In wild-type cells, cyt c_T oxidation is well-known to be biphasic, with magnitudes of a fast ($t_{1/2}$ ~ 3 μs) and a slow phase ($t_{1/2}$ ~ 200 μs) that are variable with cyt c_2 content (Overfield et al., 1979; Bowyer et al., 1979). In chromatophores from this isocyt c_2 expression strain, there is only a small amount of cyt c_T oxidation at times shorter than 50 μs, with the large majority occurring with a slow kinetic ($t_{1/2}$ > 400 μs; Figure 2) that approximates the rate of cyt c_1 oxidation in wild-type cells. Time-resolved spectra (Figure 3) were similar at ~50 μs and 10 ms after a single flash, with α-band maxima at 551.5−552.5 nm. Unfortunately, the disproportionate ratio of isocyt c_2 and cyt c_1 in this strain (Table 1) and their overlapping optical properties precluded the resolution of time-dependent

Table 1: Levels of Photosynthetic Electron Transfer Chain Components

strain	relevant genotype	RC	cyt bc_1	cyt c_2	isocyt c_2
Ga	cycA$^+$	2[a]	1[a]	1[a]	~0.02[b]
CYCA65R7	Δ*cycA*, *spd-7*, pWF1R	2[c]	1.22[c]	—[d]	0.22;[c] 0.4[e]
CYCA65R7	Δ*cycA*, *spd-7*, pWF1R	2[f]	0.98[f]	—[d]	0.36;[f] 0.4[e]
CYCA65R7	Δ*cycA*, *spd-7*, pWF1R	2[g]	0.95[g]	—[d]	0.39;[g] 0.4[e]

[a] From Crofts et al. (1985). See text for details. [b] From Rott et al. (1992). [c] From whole cell analysis of the isocyt c_2 expression strain poised at an E_h of 200 mV (data not shown). RC and total *c*-type cytochrome levels estimated from the change in P_{870}^+ and cyt c_T after 8 flashes in the presence of myxothiazol; total cyt bc_1 complex from the cyt b_H levels after 8 flashes in the presence of antimycin; total isocyt c_2 from the difference between cyt c_T and cyt bc_1 complexes. [d] None present; the strain contains an inactive cyt c_2 gene (ΔcycA; Donohue et al., 1988). [e] From soluble cytochrome *c* assays on cells containing the *cycI* expression plasmid, pWF1R (data not shown). See text for details. [f] From kinetic analysis of chromatophores from the isocyt c_2 expression strain poised at an E_h of 200 mV (Figure 1). RC and total *c*-type cytochrome levels estimated from the change in P_{870}^+ and cyt c_T after 8 flashes in the presence of myxothiazol; total cyt bc_1 complex from the cyt b_H levels after 8 flashes in the presence of antimycin; total isocyt c_2 from the difference between cyt c_T and cyt bc_1 complexes. See text for details. [g] As for *f*, but with chromatophores poised at an E_h of 100 mV. Levels of the cyt bc_1 complex are underestimated at this E_h due to partial reduction prior to the first flash.

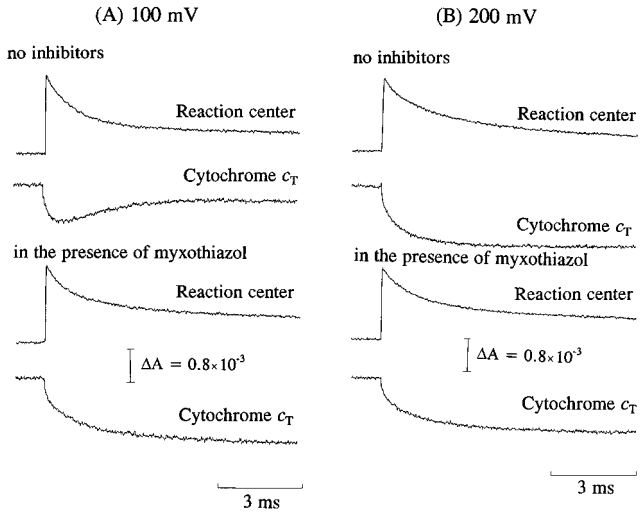

FIGURE 2: Flash-induced kinetics of reaction center (542 nm) and *c*-type cytochromes (551−542 nm) of CYCA65R7 (pVWF1R). Chromatophores were suspended in the same condition as described in Figure 1. Traces shown are the average of 15, with 40 s (no inhibitors) or 150 s (in the presence of myxothiazol) between measurements. The instrument response time was 10 μs. The redox potential was 100 mV (A) or 200 mV (B).

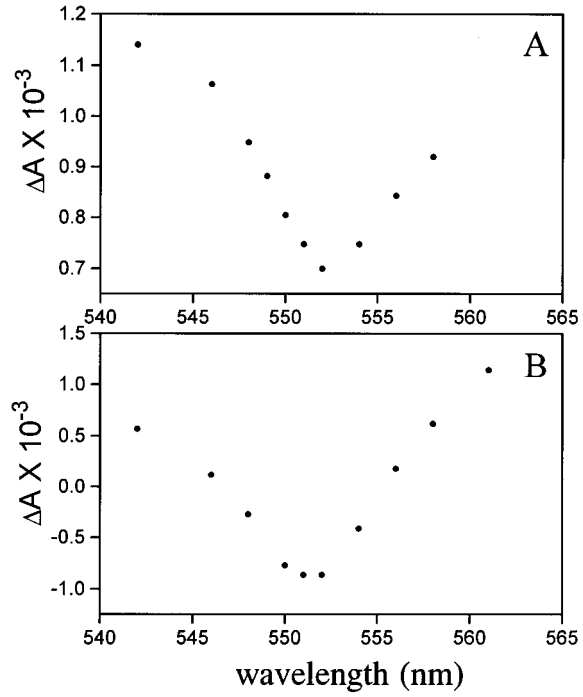

FIGURE 3: Time-resolved spectra of the flash-induced changes of chromatophore in the absence of inhibitors [The points show the change 50 μs (A) and 10 ms (B) after a single flash].

absorbance changes expected from the sequential oxidation of isocyt c_2 (552 nm; Fitch et al., 1989) and cyt c_1 (551 nm; Meinhardt & Crofts, 1982). Thus, the distinction between two kinetic phases and the nonexponential behavior expected of a second-order reaction between RC complexes and isocyt c_2 cannot be made with certainty using chromatophores from this isocyt c_2 expression strain.

Electron Transfer from Isocyt c_2 to Light-Oxidized RC Complexes. To directly monitor the rate of electron transfer from isocyt c_2 to light-oxidized RC complexes, P_{870}^+ reduction and cytochrome oxidation were monitored with purified components, and the behavior was compared to what is found when cyt c_2 is used as electron donor. The isocyt c_2 used for this analysis was purified from a strain (CYCA65R7[pUI8747]) that contains an inactivated cyt c_2 gene, yielding samples that were free of detectable contamination by other Coomassie or heme-staining polypeptides (data not shown). In addition, the apparent size of this isocyt c_2 sample (~15 kDa by SDS−PAGE) was consistent with that predicted by translation of the *cycI* gene (Rott et al., 1993), and its specific cytochrome *c* content (~65 nmol/mg of protein), α-maximum (~552 nm), and midpoint potential (~+289 mV) were indistinguishable (data not shown) from those reported previously (Fitch et al., 1989; Rott et al., 1992).

Because of the broad spectrum of P_{870}^+, flash-induced absorbance changes include contributions from P_{870}/P_{870}^+ at most wavelengths. At 550 nm, P_{870} oxidation to P_{870}^+ appears as an instantaneous absorbance increase. This is followed by a decrease which is due to both P_{870}^+ re-reduction by the cytochrome and cytochrome oxidation. These are coupled processes with identical kinetics, so the measurements at 550 nm can be described as either P_{870}^+ re-reduction or cytochrome oxidation.

At 4.4 μM cyt c_2, cytochrome oxidation was biphasic at low salt concentrations (Figure 4, top), with a fast phase

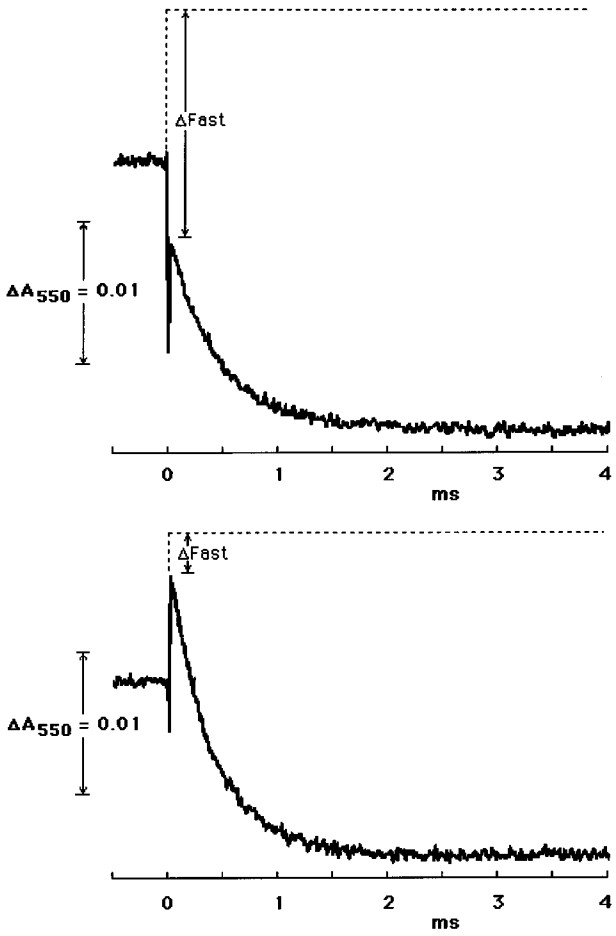

FIGURE 4: Kinetics of cyt c_2 (4.4 μM, top) and isocyt c_2 (6.8 μM, bottom) oxidation by isolated RC complexes. Shown are changes in absorbance at 550 nm after *R. sphaeroides* RC complexes (1 μM) are oxidized with a saturating flash of light. The dashed line shows the amplitude of the P_{870}^+ signal in the absence of cytochrome. The "deficit" between this and the first resolved time point of the kinetics is the fast phase (ΔFast) of cytochrome oxidation/P_{870}^+ re-reduction. Assays were performed in buffer containing 20 mM MOPS (pH 7.3), 20 μM Q-10, and 0.04% Triton X-100.

arising from cytochrome bound at the time of the flash, and a slow phase that reflects a subsequent collisional interaction (Overfield et al., 1979; Overfield & Wraight, 1980a,b; Moser & Dutton, 1988; Tiede et al., 1993; Wang et al., 1994). The fast phase ($t_{1/2} \sim 1$ μs) has been resolved in previous work (Overfield et al., 1979; Rosen et al., 1983; Tiede et al., 1993; Venturoli et al., 1993; Wang et al., 1994), but is not resolved here. However, because the fast phase is much faster than the resolution time, it appears as a deficit between the first time point after the flash and the initial amplitude of P_{870}^+ formation, which is determined in a sample with no cytochrome added (dashed lines in Figure 4). Clearly, at 4.4 μM cyt c_2, the fast phase accounted for more than half of the total reaction.

With isocyt c_2 (6.8 μM) under identical low salt conditions, cytochrome oxidation occurred in a predominant kinetic phase with $t_{1/2} \sim 250$ μs (Figure 4, bottom). Very little fast phase is evident, so the trace starts with an upward deflection due to P_{870}^+ formation. However, by comparing this response to the initial amplitude of P_{870}^+ in a sample without any electron donor added, there was a small (~12.5%) but reproducible deficit in the sample with isocyt c_2.

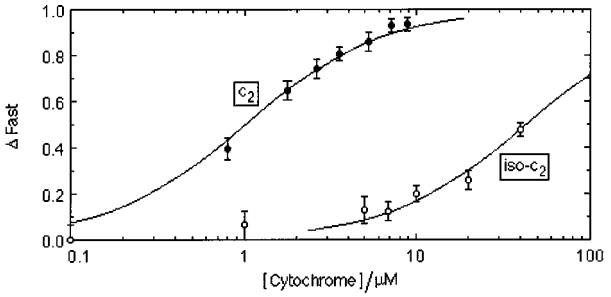

FIGURE 5: Relative amplitude of the fast phase of cytochrome oxidation (ΔFast) by isolated RC complexes. Assays were conducted on 0.5 μM RC complexes, 1 mM MOPS (pH 7.3), 20 μM Q-10, 0.03% Triton X-100, 10 mM KCl, 1 μM 1,4-naphthoquinone, 100 μM ascorbic acid, and either cyt c_2 (c_2, ●) or isocyt c_2 (iso-c_2, ○). The curves are drawn for apparent dissociation constants (K_D) of 1 μM (cyt c_2) and 42 μM (isocyt c_2).

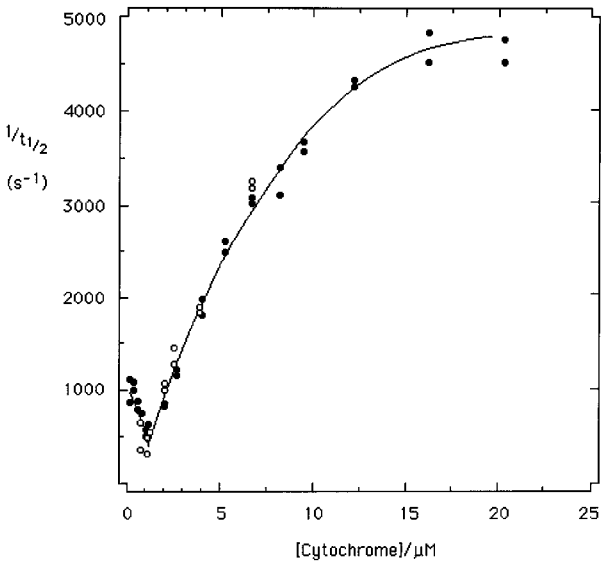

FIGURE 6: Concentration dependence of the slow phase of cytochrome *c* oxidation by isolated RC complexes. Assays were conducted as in Figure 5 except that cyt c_2 (●) or isocyt c_2 (○) concentrations were varied over the range indicated on the *x*-axis.

The possibility of a binding component for isocyt c_2 was further explored using higher concentrations of cytochrome (Figure 5) and of RC complexes (Figure 8). From the concentration dependence of the amplitude of the fast phase of cytochrome oxidation, an apparent dissociation constant, $K_D \sim 40$ μM, was estimated for isocyt c_2 (Figure 5). This is significantly weaker than that observed for cyt c_2 ($K_D \sim 1-3$ μM) under identical conditions in this (Figure 5) and other studies (Overfield et al., 1979; Rosen et al., 1980; Overfield & Wraight, 1980a,b; Tiede et al., 1993; Venturoli et al., 1993).

In contrast, the slow kinetic phases of cyt c_2 (top) and isocyt c_2 (bottom) oxidation after a light flash are virtually identical (Figure 4), and both proteins show typical second-order behavior at lower concentrations (Figure 6). The cusp-like feature at equimolar concentrations of cytochrome and RC complexes is a classic and diagnostic feature of a second-order reaction (Bashford et al., 1979). Because the kinetics of a second-order reaction are *not* exponential near the equimolar point, analysis by an exponential fit produces an apparent half-time which gets longer as this point is approached from either side. At molar ratios far from this point, the kinetics become pseudo-first-order and assume exponential character. From the slope in the region where

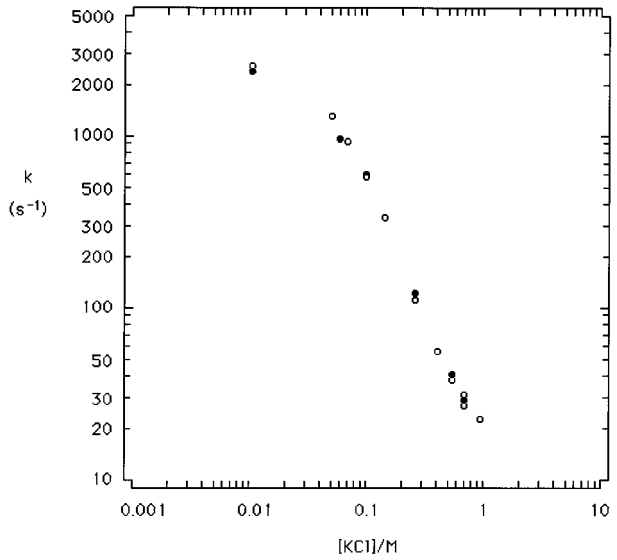

FIGURE 7: Salt dependence of the kinetics of the slow phase of cyt c_2 (●) and isocyt c_2 (○) oxidation by isolated RC complexes. Assays were conducted as in Figure 5 except that the KCl concentration was varied over the range indicated on the *x*-axis.

[cytochrome] > [RC], the second-order rate constant for isocyt c_2 (3×10^8 M^{-1} s^{-1}) is in excellent agreement with previous estimates for cyt c_2 (Overfield et al., 1979; Moser & Dutton, 1988; Tiede et al., 1993). At higher concentrations of cyt c_2, the rate appears to reach a limiting $t_{1/2}$ for this second-order reaction of ~200 μs, but this regime was not pursued with isocyt c_2. This behavior has been previously reported for cyt *c* and cyt c_2 (Overfield et al., 1979; Moser & Dutton, 1988; Tiede et al., 1993), and has been interpreted as indicating a rate-limiting process of binding or orientation of the cytochrome on the RC. However, for reasons which are, as yet, unknown, the value of the limiting rate is variable and is not seen at all in some preparations (Tiede et al., 1993; Venturoli et al., 1993).

Isocyt c_2 and Cyt c_2 Make Similar Electrostatic Contacts with RC Complexes. Electrostatic interactions contribute significantly to the formation of stable complexes between many members of the cyt c_2 family and their membrane-bound redox partners [Tiede et al., 1993; see Donohue and Meyer (1995) for a review]. To assess the electrostatic contribution to a productive interaction between isocyt c_2 and RC complexes, we tested the effects of salt and pH on the kinetics of electron transfer.

The effect of salt on the rate of the slow phase of P_{870}^+ reduction is virtually identical for both isocyt c_2 and cyt c_2, with a dramatic decrease as the salt concentration is raised above 10 mM (Figure 7). Very similar behavior was previously reported for the interaction of *R. sphaeroides* RC complexes with *R. sphaeroides* cyt c_2 and horse heart cyt *c* (Overfield & Wraight, 1980a,b; Moser & Dutton, 1988).

The observed pH dependence of the amplitude of the fast kinetic component of isocyt c_2 oxidation is also characteristic of a binding interaction that has a predominant electrostatic component. Using this assay, RC−isocyt c_2 binding is maximal at ~pH 7.8 and decreases rapidly at both lower and higher pH values, providing apparent pK_a values of ~6.3 and 9.5 (Figure 8). The higher pK_a value is very similar to that previously reported for cyt c_2 oxidation by RC complexes in phosphatidylserine vesicles (Overfield & Wraight, 1980a,b). Since a number of localized interactions contribute to tight

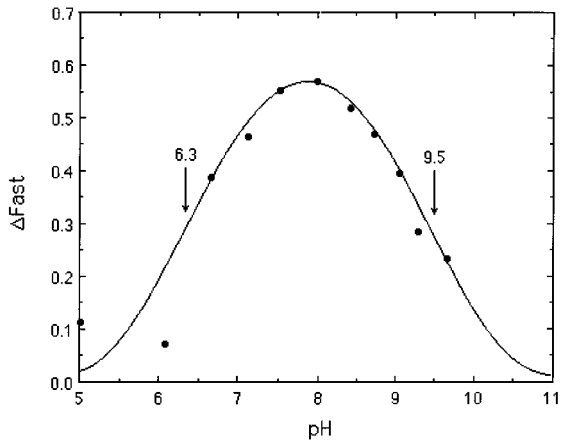

FIGURE 8: pH dependence of the fast phase of isocyt c_2 oxidation (ΔFast) by isolated RC complexes. Assays were conducted at 10 μM RC complexes, 40 μM isocyt c_2, 20 μM Q-10, 0.03% Triton X-100, 10 mM KCl, 1 μM 1,4-naphthoquinone, and 100 μM ascorbic acid. The curve is drawn to indicate titratable groups with apparent pK_a values of 6.3 and 9.5.

binding (Allen et al., 1987; Tiede et al., 1989, 1993; Caffrey et al., 1992), it is not possible *a priori* to assign the apparent pK_a values to titratable groups on the cytochrome or the RC from this analysis. Nevertheless, these results suggest that a similar series of electrostatic interactions contribute to the productive interaction of each electron donor with RC complexes. Thus, the reduced apparent affinity of isocyt c_2 for RC complexes does not appear to reflect the use of a significantly different mode of binding by this alternative electron donor.

DISCUSSION

These experiments sought to explain how *R. sphaeroides* Spd mutants accomplish photosynthetic energy transduction in the absence of cyt c_2, the normal electron donor to light-oxidized RC complexes. All Spd mutants contain elevated levels of isocyt c_2 (Rott & Donohue, 1990), and we know this protein is required for photosynthetic growth in the absence of cyt c_2 (Rott et al., 1993). Thus, the general features of photosynthetic electron transport described in the single strain we analyzed are likely to be applicable to all Spd mutants. Our results show that isocyt c_2 replaces cyt c_2 in reducing P_{870}^+ and oxidizing the cyt bc_1 complex. However, our experiments revealed some unexpected aspects of the isocyt c_2-dependent photosynthetic energy transduction in cells that use this alternate periplasmic electron donor.

Features of the Photosynthetic Electron Transfer Chain in Cells Using Isocyt c_2. P_{870}^+ reduction in whole cells or chromatophores coincides with the oxidation of one or more *c*-type cytochromes in both wild-type cells (Bowyer et al., 1980) and the Spd mutant we used that contains an isocyt c_2 expression plasmid. In wild type cells, it is known that the oxidation of cyt c_2 and cyt c_1 of the cyt bc_1 complex is coupled to P_{870}^+ reduction (Meinhardt & Crofts, 1982; Gray & Daldal, 1995). Electron transfer through the cyt bc_1 complex also appears to be required for photosynthetic electron transfer when cells use isocyt c_2 as an electron donor to RC complexes since two well-studied inhibitors of cyt bc_1 complex function (myxothiazol and antimycin) prevent electron transfer to P_{870}^+. While we have yet to monitor direct electron transfer from cyt bc_1 complexes to isocyt c_2, these functional assays and previous genetic tests (Rott et al., 1993) support the hypothesis that isocyt c_2 restores photosynthetic growth to all Spd mutants by mediating electron transfer from this membrane-bound donor to P_{870}^+.

An important difference between wild-type cells and those using isocyt c_2 as an electron donor to light-oxidized RC complexes was the failure to observe significant amounts of cytochrome *c* oxidation in the microsecond time range that is typical for whole cells or chromatophores containing normal levels of cyt c_2 (Bowyer et al., 1980; Donohue et al., 1988). In fact, the majority of the P_{870}^+ reduction by isocyt c_2 in whole cells or chromatophores occurred with a $t_{1/2} \sim 400$ μs. Because of the relatively low abundance of isocyt c_2 even in this expression strain and the similar α-maxima of this protein and cyt c_1, time-resolved spectra were not able to establish if the small amount of cytochrome *c* oxidation at times shorter than 50 μs reflected P_{870}^+ reduction by a minor fraction of the isocyt c_2 bound to RC complexes at the time of the flash. Despite the reduced apparent affinity of isocyt c_2 for RC complexes (see below) and its lower abundance, significant binding of this alternate electron donor could occur *in vivo* due to locally high effective concentrations in the small volume of the bacterial periplasm.

Most of the RC complexes in cells using isocyt c_2 as an electron donor are reduced ~100-fold slower than in wild-type cells. However, the slower rates of P_{870}^+ reduction do not limit photosynthetic growth since these cells have doubling times that approximate wild-type cells (Rott et al., 1990). In fact, maintenance of a light-driven protonmotive force is likely to be growth-limiting, and Spd mutants exhibit similar apparent rates of H^+ pumping through the cyt bc_1 complex (~7 ms; estimated from phase III of the carotenoid band shift) in Spd mutants as wild-type cells (~5 ms; Donohue et al., 1988).

In considering the lower rate of P_{870}^+ reduction in whole cells or chromatophores of this expression strain, it is important to note that isocyt c_2 levels were ~2-fold less than that of cyt c_2 in wild-type cells. Attempts to further increase isocyt c_2 abundance by placing *cycI* under control of other *R. sphaeroides* promoters have yet to be successful. Thus, we have been unable to test whether a larger periplasmic pool of isocyt c_2 would increase the fraction of protein bound to the RC and raise the amount of the total P_{870}^+ that is reduced rapidly after a light flash.

The apparent failure of the isocyt c_2 pool in this expression strain to saturate all the RC complexes reinforces why *spd* mutations are needed to restore photosynthetic growth of *R. sphaeroides* cyt c_2 mutants. For example, the ~20-fold increase in isocyt c_2 levels caused by the *spd-7* mutation produces a strain with approximately one electron donor for every five RC complexes under photosynthetic conditions (Rott et al., 1992). Thus, failure of *R. sphaeroides* cyt c_2 mutants to grow under photosynthetic conditions in the absence of an *spd* mutation reflects a limitation of the small isocyt c_2 pool (~1 for every 100 RC complexes) to support light-dependent electron transfer between the cyt bc_1 and RC complexes.

We have previously noted that the reaction domain for photosynthetic electron transfer with intact wild-type *R. sphaeroides* cells shows a stoichiometry of about 2 RC:cyt c_2:cyt bc_1 complex, and that carefully prepared chromatophores show a similar stoichiometry (Crofts & Wraight, 1983; Crofts et al., 1983; Crofts, 1986). We demonstrated

that the diffusion domain of cyt c_2 in chromatophores appears to about 10 cyt bc_1 complexes, but in cells it is more limited (~2 cyt bc_1 complexes; Fernandez-Velasco & Crofts, 1991). Joliot and colleagues have shown that the apparent equilibrium constant between cyt c_2 and RC is less than expected from the midpoint potentials, and have proposed a supercomplex structure for the photosynthetic apparatus to account for this and the limited diffusion domain (with the stoichiometry above). In this ingenious model, tightly bound cyt c_2 is restricted in its reaction domain to the partners in the supercomplex (Lavergne et al., 1989; Joliot et al., 1989; Lavergne & Joliot, 1991). It is clear from the results above that the photosynthetic chain in the isocyt c_2 expression strain we analyzed is not so organized. In both chromatophores and whole cells, the small fast phase of P_{870}^+ reduction indicates that a substantial fraction of isocyt c_2 does not bind to the reaction center in the configuration giving the fast phase seen at higher concentrations *in vitro*. In addition, the stoichiometry (2 RC:0.3 isocyt c_2) is incompatible with that expected for a supercomplex. The fact that the stoichiometry of the cyt bc_1 complex undergoing redox change is ~3-fold greater than the estimated concentration of isocyt c_2, and that the components undergo rapid electron transfer with a stoichiometry and kinetics similar to the wild-type, clearly indicates that rapid diffusion of isocyt c_2 must occur to connect cyt bc_1 complexes to RCs. In order to produce the kinetics observed, each isocyt c_2 molecule must interact with ~3 RCs and cyt bc_1 complexes during the rise kinetics ($t_{1/2}$ ~ 300 us), and ~6 of each during the reduction phase of the RC ($t_{1/2}$ ~ 1.5 ms in the absence of inhibitor).

Isocyt c_2 Has a Reduced Affinity for RC Complexes. Measurements of isocyt c_2 oxidation by isolated *R. sphaeroides* RC complexes *in vitro* revealed an apparent affinity for this protein that is ~40-fold lower than for cyt c_2. Several members of the cyt c_2 family from other organisms have similarly low affinities for *R. sphaeroides* RC complexes (Tiede et al., 1993), but the physiological relevance of this poor binding is minimal since these foreign cytochromes do not normally interact with this redox partner. In contrast, a productive isocyt c_2−RC interaction is essential for photosynthetic growth of *R. sphaeroides* Spd mutants. The relatively slow kinetics of *Rhodospirillum rubrum* RC reduction by *R. rubrum* cyt c_2 provide another example of physiological redox partners which are not optimized for high-affinity protein−protein interactions (Rickle & Cusanovich, 1979; Hall et al., 1987; Van der Wal et al., 1987).

In considering how members of the cyt c_2 family interact with RC complexes (Allen et al., 1987; Tiede et al., 1989, 1993; Caffrey, 1992), there are known to be opposite charges on the interaction surfaces on the RC (net negative charge on the periplasmic domain) and its potential electron donor (net positive charge on the cytochrome *c* heme face). This charge complementarity is reflected by the salt and pH dependence of the collisional process that controls the slow phase of cytochrome *c* oxidation by RC complexes. The virtually identical effects of salt on the second-order rate constants for P_{870}^+ reduction by both cyt c_2 and isocyt c_2 imply similar collisional interactions occur between reactants (RC complex and either cytochrome) with opposite effective charges at neutral pH values.

The reduction in the rate of the slow phase oxidation of *c*-type cytochromes by isolated RC complexes that occurs at high ionic strength (regardless of the value or sign of the net charge on the electron donor) has been taken as evidence that a determining feature in this interaction is the complementary nature of the local charges at the respective binding domains of these two redox partners (Overfield & Wraight, 1980a,b; Rosen et al., 1980; Moser & Dutton, 1988; Tiede et al., 1993). Thus, it is not so surprising to find strikingly similar effects of salt on the slow phase of cytochrome oxidation even though the primary amino acid sequence (Rott et al., 1993) and measured p*I* (Rott & Donohue, 1990) indicate that isocyt c_2 is more negatively charged than cyt c_2 at neutral pH values. These results and the presence of several conserved lysine residues in isocyt c_2 predict that the localized charge distribution at its heme cleft will be generally similar to that of other members in the cyt c_2 family. While the contribution of individual electrostatic contacts to forming a stable complex cannot be resolved from analyzing the salt or pH dependence of light-dependent cytochrome oxidation, our observations suggest that isocyt c_2 and cyt c_2 form comparable binary complexes with the RC.

Why Might Isocyt c_2 Bind RC Complexes with Lower Affinity than Cyt c_2? One potential cause for the lower affinity of isocyt c_2 for RC complexes is an acidic C-terminal domain (7/21 residues for a net charge of −6) that is absent in cyt c_2 and many other family members (Rott et al., 1993; Moore & Pettigrew, 1990). To explain the reduced apparent affinity of isocyt c_2 for RC complexes, it is possible that these additional negative charges in isocyt c_2 exert an inhibitory effect at the short protein−protein distances in the binary complex (Adir et al., 1996) that is not manifested at intermediate distances during an electrostatically guided docking event. Alternatively, structural perturbations, perhaps associated with the C-terminal extension, may increase the off rate of previously bound cytochromes by interfering with formation of a stable binary complex. The negative charge of this C-terminus could also have significant effects on the dipole moment at the isocyt c_2 docking surface.

Support for the potential significance of the C-terminal acidic domain in reducing the apparent affinity of isocyt c_2 for RC complexes can also be inferred from recent structural observations of *Rhodopseudomonas viridis* cyt c_2 (Sogabe & Miki, 1995). In this protein, the interaction of bulky, acidic, C-terminal side chains with other solvent-exposed residues significantly displaces in the polypeptide backbone around the docking surface. Thus, conformational changes at the docking surface that are brought about by their respective C-termini could explain the low affinity of *R. viridis* cyt c_2 (Tiede et al., 1993) and *R. sphaeroides* isocyt c_2 for *R. sphaeroides* RC complexes. In this regard, preliminary structural analysis of *R. sphaeroides* isocyt c_2 (Axelrod, personal communication) suggests that it is more closely related to *R. viridis* cyt c_2 (Sogabe & Miki, 1995) than to *R. sphaeroides* cyt c_2 (Axelrod et al., 1993). Additional structural information on isocyt c_2 should provide insight into whether this protein also makes altered contacts with RC residues (i.e., L162Y) that have been implicated in aiding formation of productive binary complexes by a nonelectrostatic mechanism (Farchaus et al., 1993; Wachtveitl et al., 1993; Wang et al., 1994).

In summary, we have analyzed the photosynthetic electron transfer chain allowing *R. sphaeroides* Spd mutants to transduce light into biological energy. From this analysis, we conclude that isocyt c_2 and the membrane-bound cyt bc_1

complex are essential components of the photosynthetic electron transfer chain of cells that use this alternate electron donor. While several features of this electron transfer chain are similar to those of wild-type cells, it is important to note that normal photosynthetic growth rates are achieved despite a significant difference in the apparent affinity of isocyt c_2 for RC complexes. In the future, it will be interesting to compare the properties of alternative photosynthetic electron transfer chains from well-studied bacteria like *R. sphaeroides* and *Rhodobacter capsulatus* (Jenney et al., 1994) to the schemes operating when oxygenic phototrophs use either plastocyanin, cyt c_6, or other electron donors to reduce light-oxidized photosystem I complexes (Donohue & Meyer, 1995; Zhang et al., 1994).

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