Soluble Variants of *Rhodobacter capsulatus* Membrane-anchored Cytochrome c<sub>y</sub> Are Efficient Photosynthetic Electron Carriers*  

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Photons (Ps) electron transport pathways often contain multiple electron carriers with overlapping functions. Here we focus on two c-type cytochromes (cyt) in facultative phototrophic bacteria of the *Rhodobacter* genus: the diffusible cyt c<sub>2</sub> and the membrane-anchored cyt c<sub>y</sub>. In species like *R. capsulatus*, cyt c<sub>y</sub> functions in both Ps and respiratory electron transport chains, whereas in other species like *R. sphaeroides*, it does so only in respiration. The molecular bases of this difference was investigated by producing a soluble variant of cyt c<sub>y</sub> (S-cyt c<sub>y</sub>), by fusing genetically the cyt c<sub>2</sub> signal sequence to the cyt c domain of cyt c<sub>y</sub>. This novel electron carrier was unable to support the Ps growth of *R. capsulatum*. However, strains harboring cyt S-cyt c<sub>y</sub> regained Ps growth ability by acquiring mutations in its cyt c domain. They produced cyt S-cyt<sub>y</sub> variants at amounts comparable with that of cyt c<sub>2</sub>, and conferred Ps growth. Chemical titration indicated that the redox midpoint potential of cyt S-cyt c<sub>y</sub> was about 340 mV, similar to that of cyt c<sub>2</sub> or cyt c<sub>y</sub>. Remarkably, electron transfer kinetics from the cyt bc<sub>1</sub> complex to the photochemical reaction center (RC) mediated by cyt S-cyt c<sub>y</sub> was distinct from those seen with the cyt c<sub>2</sub> or cyt c<sub>y</sub>. The kinetics exhibited a pronounced slow phase, suggesting that cyt S-cyt c<sub>y</sub> interacted with the RC less tightly than cyt c<sub>2</sub>. Comparison of structural models of cyt c<sub>2</sub> and S-cyt c<sub>y</sub> revealed that several of the amino acid residues implicated in long-range electrostatic interactions promoting binding of cyt c<sub>2</sub> to the RC are not conserved in cyt c<sub>y</sub>, whereas those supporting short-range hydrophobic interactions are conserved. These findings indicated that attaching electron carrier cytochromes to the membrane allowed them to weaken their interactions with their partners so that they could accommodate more rapid multiple turnovers.  

Biological energy transduction systems contain multiple electron carrier molecules connecting various chromophere-bearing membrane proteins. Often, these electron carriers are freely diffusible in inter-membrane spaces to link efficiently large multisubunit membrane-embedded complexes. For example, Photosystem I and Photosystem II are interconnected via the copper containing plastocyanin in chloroplasts. An iron bearing c-type cytochrome (cyt<sub>3</sub>) plays a similar role in photosynthetic (Ps) microbes under appropriate environmental conditions (2). In the case of the widely studied anoxic, purple non-sulfur, facultative phototrophs of the genus *Rhodobacter*, Ps growth relies on cyclic electron transfer (ET) between the photochemical reaction center (RC) and the cyt bc<sub>1</sub> complex (3, 4). In *Rhodobacter capsulatus*, either a soluble, freely diffusible cyt c<sub>2</sub> (5) or a membrane-anchored cyt c<sub>y</sub> (6, 7) reduces the photooxidized RC (8, 9). On the other hand, in *R. sphaeroides* only the cyt c<sub>y</sub> fulfills this role, as the cyt c<sub>y</sub> homologue of this species is unable to support Ps growth (10). The cyt c<sub>y</sub> cyts are structurally distinct from the cyt c<sub>2</sub> cyts as they have a linker domain of varying lengths in different species, attaching a highly conserved cyt c domain to a transmembrane anchor (7, 11). In *Rhodobacter* species, both cyts c<sub>2</sub> and c<sub>y</sub> can act as efficient electron carriers between the cyt bc<sub>1</sub> complex and the respiratory (Res) cyt c oxidases (C<sub>ox</sub>) (12, 13). Moreover, both *Rhodobacter* species also contain a hydroquinone oxidase (Q<sub>ox</sub>-dependent alternate Res pathway (14, 15) (Fig. 1). *R. capsulatum* mutants lacking cyts c<sub>2</sub> and c<sub>y</sub> are unable to grow by Ps. They can be complemented to Ps<sup>+</sup> by either *R. capsulatum* or *R. sphaeroides* cyt c<sub>y</sub>, but only by *R. capsulatum* (and not by *R. sphaeroides* cyt c<sub>y</sub>) (10, 16). On the other hand, *R. sphaeroides* mutants lacking only the cyt c<sub>2</sub> are Ps<sup>−</sup> (17), and they can be complemented to Ps<sup>+</sup> by cyt c<sub>2</sub> of either species, or by *R. capsulatum* (and not *R. sphaeroides*) cyt c<sub>y</sub> (10, 18).  

The soluble cyt c<sub>2</sub> and membrane-anchored cyt c<sub>y</sub> interact differently with their physiological redox partners, which are the RC, the cyt bc<sub>1</sub> complex, and the C<sub>ox</sub> (13, 19). During multiple turnovers, cyt c<sub>y</sub> transfers electrons from the cyt bc<sub>1</sub> complex to the RC more efficiently than cyt c<sub>2</sub> (19). Furthermore, whereas cyt c<sub>2</sub> interconnects a large number of RCs, cyt c<sub>y</sub> inter...
acts with a limited number of them (8, 19). In addition, in the absence of the cyt bc₁ complex, the steady-state amounts of cyt c₅₅ decrease, and the cyt c₂, kinetics change (9), suggesting a higher order of organization between the physiological partners (20, 21). Available data indicate that both the differing lengths of the linker portions of cyt c₂ (47), and the amino acid sequences of their cyt c domains are important for the ET properties (10). Although cyt c₂ can be fused to the linker portion of R. capsulatus cyt c₅₅ to yield a functional membrane-anchored cyt c₂ variant (cyt MA-c₂) (11), the cyt c domain of R. sphaeroides cyt c₅₅ does not substitute readily for its R. capsulatus counterpart (10).

To investigate the structural and functional features of cyt c₅₅ as an efficient electron carrier, and its interactions with its redox partners, genetically modified versions of membrane-anchored cyt c₅₅ were sought. Here, the production and properties of soluble, freely diffusible variants of cyt c₅₅ are described. Various cyt c₅₅ variants with differing linker lengths were fused to the cyt bc₁ complex to prohibit their diffusion and yielded novel cyt bc₁-c₅₅ fusion complexes (21). We found that cyt S-c₅₅ variants support native-like Ps growth provided that they are present at sufficient amounts in vivo. Remarkably, the RC-mediated oxidation kinetics of cyt S-c₅₅ are distinct from those exhibited by cyt c₅₅ and c₅₅. The findings suggest that, unlike the freely diffusing electron carriers (e.g. cyt c₂), which depend heavily on electrostatic contacts to recognize their physiological partners, membrane-anchored electron carriers (e.g. cyt c₅₅) are evolved to minimize binding interactions to maximize rapid multiple turnovers.

**EXPERIMENTAL PROCEDURES**

*Bacterial Strains and Growth Conditions*—The bacterial strains and plasmids used are described in Table 1. MPYE enriched medium (6) or Sistrom’s minimal medium A (22) supplemented with kanamycin or tetracycline as appropriate (10 or 2.5 µg/ml, respectively) were used for growth of R. capsulatus strains at 35 °C. Ps cultures were incubated under saturating light intensity in anaerobic jars containing H₂ and CO₂ generating gas packs (BBL 270304, BD Biosciences) (7). Escherichia coli strains were grown on Luria-Bertani medium containing ampicillin, kanamycin, or tetracycline (at 100, 50, or 12.5 µg/ml, respectively) as appropriate (6).

*Molecular Genetic Techniques*—R. capsulatus cyt S-c₅₅ was constructed by ligating the 3.4-kb EcoRI fragment of plasmid pHM5 containing the signal sequence of the cycA (structural gene of cyt c₂) (6) to the 0.8-kb EcoRI fragment of pRKE12 + containing the cyt c domain of cycY* (structural gene of cyt c₅₅ with a silent EcoRI site) to yield plasmid pYO12 (Table 1). The 1.2-kb KpnI-BamHI fragment of pYO12 carrying cyt S-c₅₅ was transferred to plasmid pRK415 using the same sites, yielding pYO100.

Spontaneous Ps⁷ revertants of cyt S-c₅₅ were isolated on tetraacycline containing MPYE plates under Ps growth conditions. Plasmids carrying cyt S-c₅₅ were transformed into E. coli strain HB101, and reintroduced into the R. capsulatus strain FJ2 (cyt c₂ - cyt c₅₅) using triparental matings (6) to identify chromosomal or plasmid-borne reversion events. The molecular nature of the plasmid-borne reversion mutations were determined by automated DNA sequencing of the 1.2-kb DNA fragment carrying cycY, as recommended by the manufacturer (Applied Biosystems Inc.). The M13 universal primer and the following cycY internal primers, FR1 (5’-CAGAGTGGCATGGCGG-3’), FR4 (5’-GGGCGCATCACCCTGT-3’), FF2 (5’-ATGGCAAGAAGCCGTCTG), YOR1 (5’-GGCTGACCAAGTATGATG-3’), YOF1 (5’-GGCAGCGGAAGCCGGGC-3’), and YOF2 (5’-TTGGCCGCGGCTCCAGA-3’) were used as sequencing primers as appropriate.

The double mutant cyt S-c₅₅-R35, which combined mutations R3 (H53Y) and R5 (K19R) isolated as described above, was constructed by site-directed mutagenesis of pYO26 carrying the cyt S-c₅₅-R5 allele using the QuiKChange mutagenesis kit (Stratagene Inc.) as recommended by the manufacturer (25 cycles at 95 °C for 30 s, 55 °C for 1 min, and 68 °C for 10 min) with mutagenic primers YO3 (5’-CCGCGATGAAAGCTCCAGAATGTTGATG-3’), YOR3 (5’-GGCTGAGAGAAGCCGGGC-3’), and YOF2 (5’-TTGGCCGCGGCTCCAGA-3’) as used for sequencing primers as appropriate.

**Biochemical and Biophysical Techniques**—Intra-cytoplasmic membrane vesicles (chromatophores), and chromatophore supernatant fractions (containing cytoplasmic and periplasmic proteins), were prepared from R. capsulatus cells grown under Res conditions in MPYE by using a French pressure cell at 18,000 p.s.i.) as described earlier (23). Chromatophore membranes used for SDS-PAGE/3,3’,5,5’-tetramethylbenzidine (TMBZ) or light-activated kinetic spectroscopy analyses were prepared in 50 mM MOPS buffer, pH 7.0, 1 mM phenylmethylsulfonyl fluoride dissolved in dimethyl sulfoxide and containing either 1 or 100 mM KCl, respectively (9). Protein concentrations were determined by the method of Lowry et al. (24), and 16.5% SDS-PAGE analyses were performed as described by Schägger and von Jagow (25). Samples were denatured in SDS-loading buffer for 5 min at 37 °C, and following electrophoresis, the c-type cytochromes were revealed by their peroxidase activities using TMBZ and H₂O₂ (26).

Optical spectra were recorded on a Perkin-Elmer UV-visible spectrophotometer (Lambda 30) fitted with an anaerobic redox cuvette, as needed. The difference spectra for c-type cytochromes were obtained with samples that were first oxidized by the addition of 20 µM potassium ferricyanide and then reduced by using either 1 mM sodium ascorbate, or a small amount of
solid, fresh dithionite. Chemical reduction-oxidation midpoint potential ($E_{m,7}$) determination of supernatant fractions of chromatophore membranes were performed according to Dutton (27) in 50 mM MOPS, 100 mM KCl, pH 7.0, in the presence of 15 to 30 μM of redox mediators (tetrachlorohydroquinone, 2,3,5,6-tetramethyl-1,4-phenylenediamine, 1,2-naphthoquinone-4-sulfonate, 1,2-naphthoquinone, phenazine ethosulfate, phenazine methosulfate, duroquinone, pyocyanine, 2-hydroxy-1,4-naphthoquinone, and anthraquinone-2-sulfonate) as described earlier (8). The optical changes that accompanied ambient redox potential changes were recorded between 500 and 600 nm, and the $E_{m,7}$ values determined by fitting normalized data to a single Nernst equation.

Light-activated, millisecond time scale kinetic spectroscopy was performed using chromatophore membranes reduced with sodium ascorbate, as described earlier (8, 9). Following a flash of actinic light, cyt $c_1$ re-reduction kinetics in the absence or presence of the cyt $bc_1$ complex inhibitor stigmatellin (2.5 μM), and as needed, in the presence of valinomycin (2.5 μM) were monitored at 550–540 nm. The carotenoid band shift was monitored at 490–475 nm to follow the generation of the transmembrane potential by cyclic ET (8, 9).

RESULTS

Soluble Variants of R. capsulatus Cyt S-$c_y$—A water-soluble, freely diffusible form of the membrane-anchored cyt $c_y$ from R. capsulatus (cyt S-$c_y$) was obtained by genetically fusing the signal sequence of cyt $c_2$ to the cyt $c$ domain of cyt $c_y$, as described under “Experimental Procedures” (Fig. 2). Initially, this construct did not complement the R. capsulatus strain FJ2 lacking both cyt $c_2$ and $c_y$ for Ps growth (Table 1), possibly because of the absence of adequate amounts of a soluble electron carrier. However, FJ2 derivatives harboring cyt S-$c_y$ reverted frequently to Ps (at a frequency of $10^{-6}$ to $10^{-7}$ on MPYE medium), and three such revertants (R3, R4, and R5) were retained for further studies. Molecular genetic analyses described under “Experimental Procedures” revealed that revertants R3 and R5 contained plasmid-borne copies of $cycY$, but contained a chromosomal mutation (X). Although the molecular nature of X was not determined, revertant R4 was cured of its plasmid by successive subcultures on MPYE medium without antibiotic selection to yield FJ2-R4 (cyt $c_2$, cyt S-$c_y$, X) (Table 1), used for some of the subsequent work due to its ability to enhance cyt $c$ production, as described below.
**R. capsulatus Soluble Cyt S-c\textsubscript{y} Variants**

**TABLE 1**

| Strain | Genotype | Phenotype | Ref. |
|--------|-----------|-----------|------|
| *E. coli* HB101 | F\textsuperscript{−} proA2 hisd20 (rb\textsuperscript{−} mB\textsuperscript{−}) recA13 ara-14 lacY1 galK2 rpsL20 supE44 rpsL20 supE44 proA2 xyl-5 mtl-1 | Wild type, Rif\textsuperscript{a} | 45 |
| XL1-Blue | recA1 endA1 gyrA96 thi-1 hsdR-17 supE44 relA1 lac [F proAB lacI q ΔM15 Tn10] | Tet\textsuperscript{b} | Stratagene |

\textsuperscript{a} *R. capsulatus* MT1131 is referred to as "wild type" as it is wild type with respect to its cyt c profile and growth properties. This strain was originally isolated as a "green" derivative of *R. capsulatus* SB1103 (46).

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**A** FJ2 (cyt c\textsubscript{y}, cyt c\textsubscript{yR3}, X\textsuperscript{−})

**B** FJ2-R4 (cyt c\textsubscript{y}, cyt c\textsubscript{y}, X\textsuperscript{−})

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**FIGURE 3. Growth properties of *R. capsulatus* strains carrying derivatives of cyt S-c\textsubscript{y}**. The cyts c\textsubscript{y}, c\textsubscript{yR3}, S-c\textsubscript{y}, R3, S-c\textsubscript{yR5}, and S-c\textsubscript{yR5} (panel A) and its isogenic derivative with the additional X4 mutation (FJ2-R4) (panel B) grown under Ps conditions on MPYE medium are shown. Note the Ps\textsuperscript{+} growth phenotype difference observed between FJ2 and FJ2-R4 both carrying cyt S-c\textsubscript{y}.

Ps\textsuperscript{+} growth abilities of cyt S-c\textsubscript{y} revertants were estimated by measuring their average colony sizes on MPYE medium in both FJ2 and FJ2-R4 backgrounds (Table 2). In the FJ2 background, cyt S-c\textsubscript{y}, cyt S-c\textsubscript{yR3} (H53Y), and S-c\textsubscript{yR5} (K19R) conferred Ps\textsuperscript{+}, Ps\textsuperscript{−/−} (slow Ps), and Ps\textsuperscript{+} phenotypes, respectively (Fig. 3A), whereas the double mutant cyt S-c\textsubscript{yR5} exhibited a Ps\textsuperscript{+} phenotype as vigorous as that of a wild type *R. capsulatus*. Moreover, the Ps growth phenotypes of all three cyt S-c\textsubscript{y} variants were further improved in the FJ2-R4 background to the point that even cyt S-c\textsubscript{y} that was Ps\textsuperscript{−} in FJ2 conferred a weak Ps\textsuperscript{−/−} growth in this strain (Fig. 3B).

**Amounts and Properties of Cyt S-c\textsubscript{y} Produced by Various Ps \textsuperscript{+} Revertants**—The c-type cyt profiles of Ps\textsuperscript{+} revertants, grown under Res\textsuperscript{−} conditions in MPYE medium, were examined by both SDS-PAGE/TMBZ analysis and reduced minus oxidized optical difference spectra. As expected, the membrane-bound cyt c\textsubscript{y} was absent in chromatophore membranes of FJ2 (cyt c\textsubscript{y}, cyt c\textsubscript{y−}) and FJ2-R4 (cyt c\textsubscript{y}−, cyt c\textsubscript{y−}, X\textsuperscript{−}), whereas the amounts of the cyts c\textsubscript{y}, c\textsubscript{yR3}, and c\textsubscript{yR5} were unchanged (data not shown). In chromatophore membrane supernatants prepared using FJ2 derivatives cyt S-c\textsubscript{y} was undetectable, whereas cyt S-c\textsubscript{yR5} (K19R), cyt S-c\textsubscript{yR3} (H53Y), and cyt S-c\textsubscript{yR5} (K19R and H53Y) were barely visible (Fig. 4A). In contrast, in the FJ2-R4 background the amounts of these c-type cytochromes, especially those of cyt S-c\textsubscript{yR5} and cyt S-c\textsubscript{yR5}, increased and became readily detectable. As expected, the cyts S-c\textsubscript{yR5} are slightly smaller (about 10 kDa) than cyt c\textsubscript{y} and cyt c\textsubscript{y}− (about 12 kDa) (Fig. 4B). Apparently, the effect(s) of the mutation(s) in FJ2-R4 is specific to cyt S-c\textsubscript{y} variants, because the amounts of native cyt c\textsubscript{yR3} or cyt c\textsubscript{y} were not affected significantly. The strain FJ2-R4 background was used for subsequent work instead of FJ2, as it enhanced production of the cyt S-c\textsubscript{y} variants (Table 2), and improved their Ps\textsuperscript{−} growth phenotypes (Fig. 3). We note that two additional TMBZ/H\textsubscript{2}O\textsubscript{2}-stained bands (about 19 and 34 kDa) were also detected in the supernatant fractions (Fig. 4). The higher molecular weight protein was identified by nano-LC-MS/MS analyses to correspond to *R. capsulatus* cyt c peroxidase (RRC03475).4

Semi-quantitative estimation of the amounts of cyt S-c\textsubscript{y} derivatives by using reduced minus oxidized optical difference spectra of chromatophore supernatants (Table 2) indicated that cyt S-c\textsubscript{yR5} and cyt S-c\textsubscript{yR5} were produced in FJ2-R4 at approximately one-third and equal amounts, respectively, to that of cyt c\textsubscript{y} found in a wild type *R. capsulatus* strain like MT1131 or its cyt c\textsubscript{y}− derivative FJ1 (Fig. 5A). Furthermore, the availability of a mutant producing high amounts of cyt S-c\textsubscript{y} allowed the determination of its redox midpoint potential (E\textsubscript{m}}) using chromatophore membrane supernatants without further purification, as it is the main c-type cytochrome that

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\textsuperscript{4} Onder, O., Turkarslan, S., Sun, D., and Daldal, F. (2008) *Mol. Cell. Proteomics* **7**, 875–890.
**R. capsulatus Soluble Cyt S-c\textsubscript{y} Variants**

TABLE 2
Properties of various soluble derivatives of *R. capsulatus* cyt c\textsubscript{y}.

| Plasmids          | Cyt derivative | Phenotype\textsuperscript{a} | Colony size\textsuperscript{b} | A\textsubscript{550–535} | Phenotype | Colony size | A\textsubscript{550–535} |
|-------------------|----------------|------------------------------|-------------------------------|--------------------------|-----------|--------------|--------------------------|
|                   |                |                              |                               | mm                       | mm        | mOD          | mm                       |
| pYO100            | Cyt S-c\textsubscript{y} | Ps\textsuperscript{+}        | Res\textsuperscript{+}       | 1.4                      | Ps\textsuperscript{+}   | 1.1          | 4.5          |
| pYO103            | Cyt S-c\textsubscript{R3} | Ps\textsuperscript{+}        | Res\textsuperscript{+}       | 12                      | Ps\textsuperscript{+}   | 1.5          | 5.2          |
| pYO105            | Cyt S-c\textsubscript{R5} | Ps\textsuperscript{+}        | Res\textsuperscript{+}       | 1.5                      | Ps\textsuperscript{+}   | 2.1          | 14.1         |
| pYO135            | Cyt S-c\textsubscript{R35} | Ps\textsuperscript{+}        | Res\textsuperscript{+}       | 2.4                      | Ps\textsuperscript{+}   | 2.4          | 49.2         |
| pHM14             | Cyt c\textsubscript{2} | Ps\textsuperscript{+}        | Res\textsuperscript{+}       | 360                      | Ps\textsuperscript{+}   | 2.3          | 163.2        |

\textsuperscript{a} Ps\textsuperscript{+} and Res\textsuperscript{+} indicate the ability to grow under photosynthetic and respiratory (Res) growth conditions, respectively. Ps\textsuperscript{+} and Ps\textsuperscript{−} refer to slow and no photosynthetic growth, respectively.

\textsuperscript{b} Amplitude of 550–535 nm absorbance of ascorbate reduced minus ferricyanide-oxidized spectra obtained using approximately 1.52 mg/ml of chromatophore supernatant proteins. Under similar conditions, values of 50.8 and 44.2 mOD were observed for the wild type strain MT1131 and the cyt c\textsubscript{y} mutant FJ1, respectively. All strains are described in Table 1.

A Cyt S-c\textsubscript{y} variant that absorbs around 550 nm in these fractions. Both oxidative and reductive chemical dark titrations of cyt S-c\textsubscript{y} variants yielded \( E_{m,7} \) values around +340 mV, which is similar (+365 mV) to that of the membrane-attached, native cyt c\textsubscript{y} (Fig. 5B). Thus, neither rendering cyt c\textsubscript{y} soluble, nor the K19R and H53Y amino acid substitutions, which apparently increased the steady-state amounts of cyt S-c\textsubscript{y} in the FJ2-R4 background, affected appreciably its \( E_{m,7} \) value.

Multiple Turnover ET Kinetics of Cyt S-c\textsubscript{y} Variants—ET kinetics exhibited by various cyt S-c\textsubscript{y} variants to the RC was examined by light-activated, time-resolved, optical difference spectroscopy (8). As expected, cyt c oxidation and re-reduction kinetics monitored at 550–540 nm wavelengths indicated no detectable ET activity to the RC either in FJ2 (not shown) (9) or FJ2-R4 backgrounds, although the previously established ET features of cyt c\textsubscript{y} were readily observed with control strains MT1131 (cyt c\textsubscript{y}, cyt c\textsubscript{y}, FJ1 (cyt c\textsubscript{y}), and pFJ631/FJ2 (cyt c\textsubscript{y}). In addition, pHM14/FJ2-R4 overproducing cyt c\textsubscript{2} exhibited kinetics similar to, but more amplified than, those seen with MT1131, in agreement with the increased cyt c\textsubscript{2} pool size. Strains pYO135/FJ2 or pYO135/FJ2-R4 harboring different amounts of cyt S-c\textsubscript{y} exhibited various levels of cyt c re-reduction activities consistent with their Ps\textsuperscript{+} phenotypes (Fig. 6, left column). Indeed, in all cases the full extent of cyt c oxidation was revealed by addition of the cyt bc\textsubscript{1} complex inhibitor stigmatellin (29, 30), which blocked cyt c re-reduction (Fig. 6, middle column). Of a train of eight flashes used in each case, complete cyt c oxidation required a different number of flashes in agreement with the different cyt c pool sizes in different strains (compare e.g. FJ1 containing wild type levels of cyt c\textsubscript{2} and pHM14/FJ2-R4 overproducing cyt c\textsubscript{2}). In addition, it was noted that the amounts of cyt c oxidation seen with cyt c\textsubscript{2} were larger than those seen with cyt S-c\textsubscript{y}, possibly indicating that the amounts of the latter electron carrier associated with RC were lower in vivo. In any case, the data estimated clearly that cyt S-c\textsubscript{y} was able to transfer electrons from the cyt bc\textsubscript{1} complex to the RC upon its light activation. Furthermore, carotenoid band shifts monitored at 475–490 nm wavelengths also confirmed the ET events between the physiological partners (Fig. 6, right column) (8). Indeed, the slower phases of the carotenoid band shifts were proportional to the extents of the ET activities in each case, demonstrating that transmembrane charge separation occurred upon establishment of the cyclic ET pathway between the cyt bc\textsubscript{1} complex and the RC via the cyt S-c\textsubscript{y} (Fig. 6, compare e.g. pYO135/FJ2-R4 to FJ2-R4). Remarkably, the modes of ET exhibited by the cyt S-c\textsubscript{y} was distinct from those seen with either cyt c\textsubscript{y} or cyt c\textsubscript{2} (8, 18, 19). However, the differences observed between the amounts of the carotenoid band shifts by different strains did not reflect any relationship between cyt c\textsubscript{y} and its efficiency in photosynthesis.

Comparison of Single Turnover Cytochrome c Oxidation Kinetics Exhibited by Different Electron Carrier Cytochromes—Kinetic differences between different electron carrier cytochromes were further examined by monitoring single turnover cyt c oxidations observed in the presence of stigmatellin.
In R. capsulatus, the presence of the fast oxidation phase in the cyt S-c₅ suggests that this cytochrome does not associate with the RC as tightly as does cyt c₂.

**DISCUSSION**

To shed further light to the molecular basis of the Ps ET competence of cyt c₅ from some species in comparison with those from others (7, 10), a soluble variant of this naturally membrane-associated protein was sought by fusing genetically its cyt c domain to the signal sequence of cyt c₂ (6). It was anticipated that a soluble, freely diffusible version of cyt c₅ would not be constrained by its membrane attachment, and reveal more readily the electron carrier properties of its cyt c domain. However, the cyt S-c₅ thus constructed was produced at very low amounts in vivo, and was unable to support the Ps growth of a R. capsulatus strain lacking both cyts c₂ and c₅. Whether these very low amounts reflected the poor biosynthesis or rapid degradation of cyt S-c₅ is unknown. Earlier, it was observed that in the absence of the cyt bc₁ complex, cyt c₅ is not detectable when cells are grown in enriched medium (9). Moreover, when the cyt c domains are exchanged between R. capsulatus and R. sphaeroides cyts c₂ (10), the latter chimeras were not stable in vivo. Fortunately, upon growth on enriched medium, strains carrying cyt S-c₅ yielded Ps⁺ revertants that produced more soluble cytochromes. Molecular analyses of these revertants indicated that they contained either single amino acid substitutions (e.g. K19R and H53Y) in the cyt c domain of cyt S-c₅ that increased their stability in vivo without changing their kinetic behaviors or had cyt S-c₅ unrelated chromosomal mutation(s) (e.g. FJ2-R4). Enhanced production of cyt S-c₅ derivatives in the latter revertants was reminiscent of protective associations between the cyt c₅ and its redox partners (9). However, the molecular basis of this chromosomal mutation(s) is unknown, and is beyond the scope of this work.

Dark, equilibrium titrations indicated that the E₅₅₇ of the cyt S-c₅, is very similar to that of cyt c₅ or cyt c₂, suggesting that rendering cyt c₅ soluble has not changed drastically its thermodynamic properties, even though its ET properties are modified. The three-dimensional structure of R. capsulatus cyt c₅ is not available, but its amino acid sequence is highly similar (63% identity and 75% similarity over 95 amino acids) to that of cyt c₅552 of Paracoccus denitrificans of known structure (32, 33). A structural model for the cyt c domain of R. capsulatus cyt c₅, built by homology modeling based on that of cyt c₅552 indicated that Lys-19 is on the surface of the protein and solvent exposed, whereas His-53 is slightly buried into the protein (Fig. 8). Thus, the larger side chain of Arg, substituting Lys at position 19, and more hydrophobic Tyr, substituting His at position 53, appear to have limited, if any, effects on the interactions of cyt S-c₅ with the RC (34), while increasing its stability and restricting its degradation.

A notable finding is the mode of ET kinetics exhibited by cyt S-c₅. Light-activated RC-coupled cyt c oxidation kinetics indicated clearly that electron donation by R. capsulatus cyt S-c₅ is much slower than that mediated by either cyts c₅ or c₂ (35–37). The rapid kinetics observed with cyt c₅ have been attributed to its close proximity to the RC, and to its inability to diffuse freely (19, 36). In the case of the cyt c₂, the biphasic kinetics observed...
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In each case, chromatophore membranes were prepared in 50 mM MOPS, 100 mM KCl buffer at pH 7, resuspended in the same buffer at the appropriate concentrations, and the ambient redox potential was poised at 100 mV, as described under “Experimental Procedures.” Light induced (a train of eight flashes) transient cyt c oxidation and re-reduction kinetics exhibited by various strains were monitored at 550–540 nm in the absence of inhibitor and in response to a single flash.

Docking of freely diffusible electron carriers (like cyt c2) to their redox partners (like the RC) is thought to be governed by protein–protein interactions mediated at least partly by complementary surface charges (34, 38, 41, 42). Different charge distributions on the docking surfaces of redox partners affect their mode of interactions, and mutations that affect the binding affinities of the partners also change the ET rates (e.g. the second-order rate constant, k2) between them. Upon docking, additional hydrophobic interactions are involved for precise orientation of the redox partners and subsequent optimal ET between them, as illustrated by extensive RC-cyt c studies (37, 38), or the cyt bc1-cyt c co-crystal structures (43). The kinetic data available for the cyt c2 that needs to diffuse to reach an oxidized RC (36, 38–40). In the case of cyt S-cy, kinetics, the quasi-absence of a fast phase, and the prominence of a slower phase together indicate that almost no cyt S-cy is associated with the RC prior to light activation. If this is the case, then comparison of appropriate strains with similar amounts of soluble electron carrier cytochromes suggests that the binding affinity of cyt S-cy to the RC is apparently lower than that of cyt c2 (Fig. 6) (35, 38, 40, 41). In agreement with this deduction, it was noted that cyt S-cy, unlike cyt c2, does not remain membrane-associated when chromatophores are prepared at low (e.g. 1 mM KCl) salt concentrations, and that increased steady-state concentrations of cyt S-cy sustain more vigorous Ps+ growth in various mutants.

FIGURE 6. Cyt c oxidation-reduction kinetics exhibited by various strains producing cyt S-cy derivatives. In each case, chromatophore membranes were prepared in 50 mM MOPS, 100 mM KCl buffer at pH 7, resuspended in the same buffer at the appropriate concentrations, and the ambient redox potential was poised at 100 mV, as described under “Experimental Procedures.” Light induced (a train of eight flashes) transient cyt c oxidation and re-reduction kinetics exhibited by various strains were monitored at 550–540 nm in the absence (left column) and presence (middle column) of the cyt bc1 complex inhibitor stigmatellin, which blocks re-reduction of cytochromes. Right column shows the 490–475 nm traces obtained using the same strains to monitor the carotenoid band shifts in response to the cyclic photosynthetic electron transport reactions in the absence of inhibitor and in response to a single flash.

FIGURE 7. Single turnover cytochrome c oxidation kinetics observed in various cyt S-cy producing strains. Samples were prepared, and traces (in the presence of stigmatellin) were recorded as described in the legend to Fig. 6, except that 2.5 μM valinomycin was present and data obtained by one hundred flashes were averaged to visualize the cyt c oxidation (550–540 nm) kinetics of strains harboring only the cyt c1 (pfJ631/FJ2, top row), cyt c2 (FJ1, middle row), or cyt S-cy-R35 (pYO135/FJ2-R4, bottom row). Note that the cyt c oxidation kinetics of the cyt S-cy are highly distinct from those seen with both the cyt c1 and cyt c2.
ing their redox partners (44). Thus, a lack of tight binding of the cyt c domain of cyt c, to the RC, along with its efficient interaction with the cyt bc1 complex, is consistent with both its rapid oxidation and re-reduction kinetics during multiple turnovers, and with the slower oxidation of its soluble derivative cyt S-cy (18, 19). It remains to be seen whether in the resting state, cyt c, remains associated mainly with the cyt c1 subunit of the cyt bc1 complex (21), rather than the RC, to ensure rapid and transient interactions upon light activation during multiple turnovers and Ps growth. If so, then limited numbers of RC-cyt c,-cyt bc1 complex can form “hardwired” photosynthetic units (21). These units then turn over efficiently to sustain vigorous Ps growth as long as the length of the linker portion of cyt c, is adequate (47).

In summary, this work demonstrates that the cyt c domain of the membrane-anchored electron carriers (e.g. cyt c,), apparently does not bind tightly to the RC due to the lack of strong protein-protein interactions between the partners. The findings suggest that attaching an electron carrier to the membrane, while restricting spatial diffusion, allows weaker binding to its partners to ensure rapid multiple turnovers. Unlike the freely diffusible electron carriers (e.g. cyt c2) that rely on complementary electrostatic interactions and large pool sizes, a small number of membrane-attached electron carrier cytochromes then suffices to support efficient Ps growth via enhanced multiple turnover rates, as observed with cyt c,y.

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