Structural and Functional Characterization of the Unusual Triheme Cytochrome Bound to the Reaction Center of Rhodovulum sulfidophilum*

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The cytochrome bound to the photosynthetic reaction center of Rhodovulum sulfidophilum presents two unusual characteristics with respect to the well-characterized tetraheme cytochromes. This cytochrome contains only three hemes because it lacks the peptide motif CXCH, which binds the most distal fourth heme. In addition, we show that the sixth axial ligand of the third heme is a cysteine (Cys-148) instead of the usual methionine ligand. This ligand exchange results in a very low midpoint potential (~160 ± 10 mV). The influence of the unusual cysteine ligand on the redox potential of this distal heme was further investigated by site-directed mutagenesis. The midpoint potential of this heme is upshifted to +310 mV when cysteine 148 is replaced by methionine, in agreement with the typical redox properties of a His/Met coordinated heme. Because of the large increase in the redox potential of the distal heme in the mutant, both the native and modified high-potential hemes are photooxidized at a redox potential where only the former is photooxidizable in the wild type. The relative orientation of the three hemes, determined by EPR measurements, is shown different from tetraheme cytochromes. The evolutionary basis of the concomitant loss of the fourth heme and the down-conversion of the third heme is discussed in light of phylogenetic relationships of the Rhodovulum species triheme cytochromes to other reaction-center-associated tetraheme cytochromes.

In photosynthetic organisms, the first step in the conversion of light energy into chemical energy occurs in the reaction center (RC). Upon absorption of a photon by the RC, an electron is transferred from a primary electron donor, located on the periplasmic side of the membrane, to an acceptor on the cytoplasmic side. In purple photosynthetic bacteria the primary electron donor is a bacteriochlorophyll dimer (P), and the electron acceptor a quinone molecule (QA). This light-induced charge separation at the level of the RC initiates a cyclic electron transfer, which leads to the formation of a transmembrane proton-motive force ultimately used for ATP synthesis.

In most photosynthetic bacteria, a tetraheme cytochrome c operates as secondary electron donor to the photosoxidized primary donor (for a review, see Ref. 1). Three-dimensional high-resolution structures of the RC and its associated cytochrome subunit have been determined for Blastochloris (formerly Rhodopseudomonas) viridis (2) and Thermochromatium tepidum (3). In the case of B. viridis, the four hemes are arranged linearly and are labeled, starting from P, heme 1(III), heme 2(IV), heme 3(II), and heme 4(I).2 The spatial arrangement of the four hemes presents a high-low-high-low sequence in terms of redox potential (4–7). This does not impede rapid electron transfer from the distal hemes to P*. Nevertheless, function of the alternating arrangement of the midpoint potentials remains elusive. The fast kinetics of electron transfer, from the tetraheme cytochrome to P* were extensively studied in B. viridis (8). The highest potential heme 1(III), which is closest to P, equilibrates with P* within 100 to 200 ns. The complete re-reduction of P* is then limited by the interheme electron transfer between heme 3(III) and heme 1(III) (2–μs half-time). All of these works contributed to give reliable outlines of tetraheme cytochromes bound to the RC.

Recently, Masuda and co-workers (9) discovered in Rhodovulum (R.) sulfidophilum a new type of RC-bound cytochrome. Its amino acid sequence, deduced from the puC gene, contains only three possible Cys-X-X-Cys-His heme-binding motifs as the binding motif of heme 4(I) is absent. In addition, the sequence analysis suggested that the distal residue responsible for the axial coordination of heme 3(II) (usually methionine) was possibly replaced by a cysteine. In the present work we have coupled site-directed mutagenesis to biophysical measurements to characterize the unusual triheme RC-bound cytochrome of R. sulfidophilum. Relative orientations, redox mid-
point potentials, and fast interheme electron transfer have been determined for the WT and a mutant of the axial ligand of heme 3III. The redox arrangement of the hemes was found to be high-low-ultra-low in R. sulfidophilum, at variance with the accepted high-low-high-low sequence in the tetraheme cytochromes. These features provide a new case for the understanding of long range electron transfer and the evolution of multicenter redox proteins.

EXPERIMENTAL PROCEDURES

Bacterial Growth, Membrane Preparation, and Isolation of RC-LH1 Complexes—R. sulfidophilum cells were grown for 24 h, in Hutner medium supplemented with 0.3 mM NaCl under continuous illumination in anaerobic conditions. The growth medium was supplemented with 50 μg ml⁻¹ streptomycin in the case of the C148M mutant. For membrane preparations, cells were harvested by centrifugation at 4000 × g for 10 min, re-suspended in 20 mM Tris-HCl (pH 7), and disrupted by a French press at 50 megapascal. The unbroken cells were separated from the membrane supernatant by centrifugation at 10,000 × g for 10 min. The membrane fragments were spun down at 250,000 × g for 90 min and re-suspended in 20 mM Tris-HCl, 100 mM KCl (pH 7). For RC-LH1 complex purification, the membranes were solubilized by addition of 0.5% Triton X-100. This suspension was loaded on a continuous sucrose gradient and centrifuged at 200,000 × g overnight. RC-LH1 complexes were collected at the bottom of the gradient (0.3–0.9 M), concentrated using Microcon centrifugal filters (30 kDa), and re-suspended in 20 mM Tris-HCl, 100 mM KCl (pH 7), 0.05% Triton X-100 for redox titrations, or in 20 mM MOPS (pH 7), 0.05% Triton X-100 for EPR experiments.

Construction of Plasmids and Site-specific Mutagenesis—The whole puf operon, containing genes coding for the L, M, and C (cytchrome) subunits of the RC complex and the α and β subunits of the light-harvesting complex of R. sulfidophilum was cloned (9). The Nael-SphI fragment containing the 3′-region of pufM, the pufC gene, and the 5′-region of open reading frame 641 was cut out and inserted into plasmid pUC118 at the SmaI-SphI sites, yielding pRSS113. Site-directed mutagenesis was performed based on the two-stage PCR method (10), using the LAPCR in vitro mutagenesis kit (Takara). To replace the 148th cysteine residue of the cytocomplex subunit with a methionine residue, the following oligonucleotide was used: 5’-GATCACCAAGAA-CATQATCGAGGGCCACC-3’ (the corresponding methionine codon is underlined). The plasmid containing mutations on the pufC gene was named pSCC148M. This plasmid and pRSS113 containing the wild type pufC gene were ligated with Km²-suicide vector pJP5603 (11) at the EcoRI site and transferred into the cells of strain PUF1 of R. sulfidophilum, in which the 0.4-kb region flanked by SalI restriction sites in the pufC gene was replaced by the 2.0-kb SmI-SphI cassette (12). The mobilizing plasmid S17-1 (13) was used for this conjugal transfer of the plasmid. Km² SmI² cells of R. sulfidophilum were selected as single crossover candidates. The insertion of the plasmid into the genomic DNA by a single crossover recombination was confirmed by DNA sequence analysis of the pufC gene amplified by PCR from the genomic DNA of the mutant strains. The mutant, in which the 148th cysteine of the triheme cytochrome subunit was replaced by a methionine, was designated C148M. The strain in which the wild pufC gene was incorporated into the genomic DNA by a single crossover recombination was denoted cis-WT.

EPR Spectroscopy—Membrane fragments, resuspended in 20 mM MOPS (pH 7), were oxidized by addition of 2 mM potassium ferricyanide. The membranes were then spun down and re-suspended in 20 mM EDTA (pH 7) to remove manganese and ferricyanide. After renewed pelleting, the membrane fragments were re-ruspended in 20 mM MOPS (pH 7). EPR spectra were taken on a Bruker ER 300 X-band spectrometer fitted with an Oxford Instruments helium cryostat and temperature control system. The instrument settings were: temperature, 15 K; microwave power, 6.7 mW; microwave frequency, 9.43 GHz; modulation amplitude, 2.5 millitels. For the study of angular dependence of EPR signals, oriented multilayer membranes were obtained by drying the membrane fragments on Mylar sheets in a 90% humidity atmosphere for ~24 h in the dark at 4 °C (14).

Redox Titrations—The redox titrations of the isolated RC-LH1 complexes, re-suspended in 20 mM Tris-HCl (pH 7), 100 mM KCl, 0.05% Triton X-100, were performed with an anaerobic spectrophotocatalytic cell (100 μm thick) adapted to a high sensitivity xenon flash-lamp spectrophotometer previously described in Ref. 15. To eliminate the broad-band spectral contribution of the mediators (methyl viologen and benzyl viologen), the cytochrome changes were estimated with respect to a baseline drawn between 548 and 562 nm. The working electrode (gold grid, InterNet Inc.) was modified by 2-pyridinecarboxaldehyde thiocarbazone (Sigma). The following redox mediators were used at a concentration of 100 μM each (E_m (midpoint redox potential) indicated): potassium ferricyanide (III) (+430 mV), 1,1′-dimethylferrocene (+340 mV), tetrachloro-1,2-benzoquinone (+280 mV), hexaamineruthenium(II) chloride (+195 mV), 1,2-naphthoquinone (+145 mV), trimethylphenyldihydroquinone (+100 mV), 2-methyl-1,4-naphthoquinone (menadione) (0 mV), 2-hydroxy-1,4-naphthoquinone (-145 mV), anthraquinone-2-sulfonic acid (-225 mV), benzyl viologen dichloride (-350 mV), and methyl viologen dichloride hydrate (-440 mV).

Fast Optical Absorption Spectroscopy—Electron transfer rates between the cytochrome subunit and the primary electron donor of the R. sulfidophilum RC were investigated by fast optical time resolved spectroscopy. A laboratory built laser-based spectrophotometer (16) was used to monitor absorbance changes in the nanosecond to second time range.

RESULTS

Amino Acid Sequence of the Trihemes and Site-directed Mutagenesis—The R. sulfidophilum puf operon encompasses pufA, pufB, pufL, pufM, and pufC in this order from the 5′-upstream region. Site-directed mutation of the pufC gene, C148M, was introduced through a single crossover recombination between the genomic DNA of strain PUF1 (pufC deletion mutant (9)) and the pUC118-based pSCC148M plasmid (see “Experimental Procedures” and Fig. 1). The single crossover recombination was expected to occur between the Nael site of pufM and the Sall site of pufC. The gene recombination on the genomic DNA of the mutant was confirmed by PCR using primers annealing to the 3′-region of pufL (L530F) and the middle region of pufC.
Identification of Paramagnetic Species Arising from the RC-associated Cytochrome in the Wild Type and C148M Mutant—

The EPR spectrum in Fig. 2, top, was recorded on oxidized RC-LH1 complexes purified from the wild type strain of *R. sulfidophilum*. EPR signals because of low spin hemes with His/His or His/Met ligation. The $g_{\perp}$ peak at 2.47 was completely absent in the spectrum of oxidized membrane fragments (Fig. 2) and represented only a very minor component in the spectra recorded on dehydrated, partially ordered membranes (Fig. 3). This signal is probably because of a denatured form of one of the other hemes, most probably the g = 2.63 species (see “Discussion”).

In addition to the EPR species detected in the isolated RC-LH1 complexes, the spectra obtained on membrane fragments featured additional $g_{\perp}$ peaks at higher $g$ values (Fig. 2, spectra labeled WT mbs). The comparison of spectra taken on purified cytochrome $bc_1$ complex (17) indicated that the large majority of these additional heme species can be attributed to this enzyme (Fig. 2, bottom spectra). From the oxidized-minus-ascorbate-reduced difference spectrum (Fig. 2), only the cytochrome $c_1$ and $g = 2.95$ hemes are reducible by ascorbate.

Replacement of cysteine 148 by methionine in the C148M mutant resulted in conspicuous spectral changes (Fig. 2, spectra labeled C148M mbs). (i) The $g_{\perp}$ = 2.63, $g_{\parallel}$ = 2.26, and $g_{\perp}$ = 1.79 signals were absent in the mutant, demonstrating that these spectral parameters in the wild type corresponded to the heme ligated by Cys-148. (ii) Additional signal intensity was present in the region of $g_{\perp}$ = 3.1 to 3.2 in the C148M mutant. (iii) The redox difference spectrum demonstrates that the modified heme is reducible by ascorbate.

Orientation of the Three Hemes in the Wild Type and C148M Mutant—Fig. 3A shows representative spectra from partially oriented membrane multilayers (14) of the WT and C148M mutant, as well as difference spectra (mutant minus wild type) highlighting the low spin heme species resulting from the cysteine to methionine ligand change. In line with the results on unoriented membranes shown above, additional signal amplitude in the $g = 3.1$ to 2.3 region was observed in the mutant. The difference spectra show that the heme modified by the mutation has its $g_{\perp}$ peak at 2.15 (Fig. 3A, bottom spectrum). Polar plots of signal amplitudes for the three heme species attributed to the RC-associated cytochrome in wild type membranes are shown in Fig. 3B. For the $g = 2.63$ heme only, all three principal tensor axes could be observed. Because the $g_{\perp}$ line, and 1.79 ($g_{\perp}$ trough). Both the 2.63 and 2.47 $g_{\parallel}$ peaks are far outside the range of field positions typically found for low spin hemes with His/His or His/Met ligation.
direction is close to the heme normal (18), knowledge of the inclination of \( g_z \) to the membrane plane is sufficient for deducing at which angle a given heme lies with respect to the membrane. The heme species having \( g_z \) at 3.12, 2.95, and 2.63 were thereby found to lie at angles of 90°, 60°, and 35° with respect to the plane of the membrane. The polar plot depicted in Fig. 3C represents the peak of the mutation-modified heme at \( g_z = 3.16 \). The orientations of the \( g_z \) directions in the \( g_z = 3.16 \) heme and in the corresponding heme in the wild type membranes are similar within the precision of the experiment (cf. Fig. 3B, right polar plot and C), indicating that no major conformational changes has occurred.

**Redox Titrations in Isolated RC-LH1 Complexes**—In a first set of experiments, the redox midpoint potentials of both the primary electron donor P and the photooxidizable hemes were determined. The light-induced absorbance changes of P+/P were measured at 610 nm, 50 \( \mu \)s after the actinic flash, as a function of the imposed redox potential in isolated RC-LH1 complexes (Fig. 4A). When one heme of the bound cytochrome is reduced prior to the flash, electron transfer to P+ is faster than 50 \( \mu \)s (see below). The absorbance change at 50 \( \mu \)s will then display two waves in the redox titration: the high potential one corresponds to the titration of P+/P proper, and the low-potential one is indicative of the cytochrome/cytochrome redox potential. For both the WT and the C148M mutant, the photooxidation of P titrates at an apparent midpoint potential (\( E_m \)) of +450 mV. At lower ambient potential (\( E_a \)) the signal disappears because of the fast re-reduction of P+ by the bound cytochrome. The \( E_m \) of the photooxidizable high potential heme(s) of the triheme cytochrome are thus found equal to +245 and +310 mV for the WT and C148M mutant, respectively. Similar information was obtained by monitoring directly the absorbance changes indicative of the flash-induced cytochrome oxidation (not shown). At potentials lower than ~200 mV no heme photooxidation is observed because of the reduction of the primary electron acceptor, with \( E_m \) of ~150 mV (not shown).

To determine the \( E_m \) of other hemes of the RC-bound triheme of *R. sulfidophilum*, dark redox titrations were performed in the \( \alpha \)-band of cytochromes in purified RC-LH1 complexes.\( ^3 \) For the WT (Fig. 4B, circles) the data are best fitted with three one-electron Nernst curves of nearly equal amplitude. The \( E_m \) of these components are +265, +30, and ~160 mV. The +265 mV value determined for the highest potential heme is in good agreement with the \( E_m \) derived from the photooxidation experiments (+245 mV, see Fig. 4A). Difference absorption spectra for each of the three different WT hemes peak between 552 and 554 nm in the \( \alpha \)-band (Fig. 4C). When the ligand Cys is replaced by a Met (C148M mutant), the best fit is obtained with two Nernst curves with \( E_m \) equal to +85 and +310 mV (Fig. 4B, squares). This high potential is in agreement with the value determined for the photooxidizable heme in the C148M mutant (Fig. 4A). The 2-fold increase of the amplitude of the high potential wave suggests that two hemes of similar midpoint potentials contribute to this component (Fig. 4B, squares). The shift by about 50 mV of \( E_m \) at 30 mV and \( E_m \) at 265 mV in the WT to \( E_m \) at 85 mV and \( E_m \) at 310 mV may tentatively be ascribed to the second mutation (Asp-285 into Gly), which was revealed by sequencing.

**Photooxidation of Heme(s) in Isolated RC-LH1 Complexes**—According to the above midpoint potential determination and assignment, one expects that, at moderate ambient redox potential, only one heme would be photooxidizable in the WT,

\( ^3 \) Accurate redox titrations of the triheme cytochrome cannot be performed on membranes, because of the presence of a large amount of cytochrome bc\(_{1}\) complex (19).
measured 10 ms after the first actinic flash (responds to the light-induced difference spectra of absorption changes $B_{sulfidophilum\ \text{WT}}$). The heme(s) and the primary electron donor were measured in high potential heme as shown by the small 610-nm change at 1.5 s apart (Fig. 5). For the WT (panel A), a large amount of the photooxidized $P^*$ is rapidly re-reduced after the first flash by a high potential heme as shown by the small 610-nm change at 50 $\mu$s and the negative signal observed at 424 nm because of cytochrome photooxidation. After the second and subsequent flashes, the formation of $P^*$ is observed, whereas no further cytochrome photooxidation occurs. This result is in line with the redox data. At variance, in the C148M mutant (panel B), two hemes can be photooxidized by a series of saturating flashes as the system reaches a steady state regime from the second flash onwards. This result is again in accordance with the redox titrations described above, showing a recovery of the usual behavior of tetraheme RC-bound cytochromes, which contain, like C148M, two His/Met-coordinated high potential hemes. UV-visible absorption changes linked to photooxidation of the WT high potential heme are presented in Fig. 5C (upper trace). The $\alpha$-band is centered at 554 nm, in accordance with redox titration (Fig. 4C). In the case of the C148M mutant (Fig. 5C, lower traces), the absorption changes induced by the first and second excitation flash are very similar, however, peaking at a lower wavelength, lying between 552 and 554 nm, revealing the participation of the additional mutated heme.

Fast Kinetics of Inter-heme Electron Transfer—Flash-induced absorbance changes were measured with nanosecond time resolution in the $Q_x$ band of the primary electron donor $P$ (610 nm, see Fig. 6) or in the $\alpha$-band of cytochromes (Fig. 7) to determine the intramolecular electron transfer properties of both the WT and C148M mutant under ascorbate-reduced conditions. In the case of the WT, the reduction of the photooxidized primary donor is essentially monophasic, with 90% of $P^*$ being reduced with a half-time of 1.6 $\mu$s. 10% of $P^*$ remains oxidized in the >10-μs range in agreement with the experiment shown in Fig. 5A. In contrast, for the C148M mutant, the re-reduction of $P^*$ is complete and biphasic: the 1.8-μs half-time fast phase accounts for 60% of the amplitude, whereas 40% of the $P^*$ decays with $t_{1/2} = 11$ μs.

Fig. 6 describes the light-induced absorbance changes in the $\alpha$-band of cytochromes detected at various times after flash excitation. For both the WT (upper panel) and C148M mutant (lower panel), the signal detected at 100 ns is due to $P^*$, and to the carotenoid band shift caused by the photo-induced membrane potential. In the case of the WT, a trough centered at 555 nm develops from 500 ns to 5 μs with a half-time of 1.5 μs in agreement with the kinetics observed for $P^*$ decay. In the C148M mutant, in the 100-ns to 5-μs time range, the trough wavelength is centered at 555 nm, as for the WT, while at longer times, a gradual shift toward shorter wavelengths is observed. A global fit analysis of the data with a biexponential decay revealed two phases of cytochrome oxidation: a fast component of $t_{1/2} = 2.1$ μs (dot-dashed line) and a slower phase of $t_{1/2} = 8.7$ μs (solid line), in fair agreement with that measured for the $P^*$ reduction (Fig. 6, squares). This shows that the oxidation is shared between two hemes absorbing at 553 and 555 nm, respectively, in line with the presence of two hemes of midpoint potentials around 310 mV obtained in the dark titration.
DISCUSSION

Correlation of Redox/Spectral Species to Hemes Bound by the Three Binding Motifs in the Amino Acid Sequence—Three distinct redox and EPR species could be attributed to the hemes of the R. sulfidophilum RC-associated triheme cytochrome. The wiping of the $g_s = 2.68$ spectrum and the concomitant appearance of the $g_s = 3.16$ spectral species induced by the Cys-148 to Met mutation unambiguously identified these paramagnetic centers as arising from heme 3(II). The loss of the $-160 \text{ mV}$ titration wave in the mutant identifies this redox species as belonging to the heme 3(II) in agreement with the EPR observation that this heme is not reducible by ascorbate and in line with redox properties of His/Cys-ligated hemes (see below).

A second low potential species ($E_m = +30 \text{ mV}, g_s = 3.12$) was detected in the WT. In typical purple bacterial tetraheme cytochromes, heme 2(IV) has a His/His ligation pattern and consequently displays a low redox potential. The conservation of the axial ligands in the sequence of the R. sulfidophilum triheme cytochrome (9) suggests that this heme has a similarly low $E_m$ value also in the triheme cytochrome. The $+30 \text{ mV}$, $g = 3.12$ heme is therefore attributed to heme 2(IV).

Both in the optical redox titrations and in the EPR spectra, only one species (the $+265 \text{ mV}, g_s = 2.95$ heme) was found to possess a relatively high redox potential, allowing reduction by ascorbate. Because only the ligation pattern (His/Met) of heme 1(III) is compatible with a high potential heme, we ascribe the $E_m = +265 \text{ mV}, g_s = 2.95$ species to heme 1(III).

Global Structure of the R. sulfidophilum Triheme Cytochrome—As summarized in Fig. 8, the three hemes of the triheme cytochrome, corresponding to hemes 3(II), 2(IV), and 1(III) seen in the x-ray structures of the B. viridis and T. tepidum reaction centers, lie at angles of 35°, 90°, and 60°, respectively, with respect to the membrane. These angles deviate from the roughly 90°, 45°, and 90° seen in the three-dimensional structures of the above mentioned RCs. Similar deviations have also been observed by EPR in the tetraheme cytochromes of Allochromatium vinosum and Rubrivivax gelatinosus (20, 21) and were rationalized by a slightly differing docking conformation of the multiheme cytochromes to the RC cores (21). However, the crystal structure of the photosynthetic reaction center from T. tepidum published recently (3) challenges this interpretation. The geometric arrangement of the four hemes in the tetraheme cytochrome of T. tepidum with respect to the RC core is virtually indistinguishable from that seen in the structure of B. viridis. The amino acid sequence of the T. tepidum tetraheme subunit, however, is highly homologous to that of its A. vinosum counterpart, for which heme orientations different from those seen in the structures have been determined by EPR (21, 22). This discrepancy does not appear to reflect a systematic error of the EPR technique because the orientations of hemes found by EPR on the B. viridis tetraheme subunit agree well with those determined by crystallography. A key to understanding these discrepancies may lie in the peculiar structure of the multiheme-containing RCs. The cytochrome subunit roughly resembles a cylinder protruding straight from the membrane plane into the periplasmic space. Preparation of the stacked membrane multilayers may induce local deviations forcing the cytochrome subunit (and the whole RC) away from the sterically unfavorable native position in which the multiheme subunit would act as a spacer between two adjacent membranes. On the other hand, the quasi-crystalline arrangement of RCs in photosynthetic membranes from B. viridis (23) may stabilize the “upright” position of the tetraheme cytochrome permitting the true angles to be measured by the spectroscopic techniques in this special case.

Although the absolute values of heme orientation determined by EPR deviate from the published crystal structures for several organisms examined so far, the general pattern of the two correlated structural pairs, i.e. hemes 1(III)/4(I) and hemes 2(IV)/3(II) having roughly similar orientations, is conserved in all purple bacterial tetraheme cytochromes studied by EPR on partially ordered membranes. This, however, is not the case for the R. sulfidophilum triheme protein. Whereas a slight inclination/rotation of the crystal structure template is sufficient to reproduce the angles obtained for hemes 1(III) and 2(IV) in R. sulfidophilum, the orientation of heme 3(II) (35°), i.e. the cysteine ligated heme, significantly differs from that of its canonical partner heme 2(IV) (90°). This modified orientation is not because of the presence of the atypical 6th ligand, because the orientation remains the same when the cysteine is replaced by the “normal” methionine ligand. We therefore suppose that the structural collapse of the distal tip of the cytochrome subunit induced by the absence of heme 4(I) has structural reper-
cussions on its neighborhood thereby modifying the tilt angle of heme 3(II).

Heme 3(II), the Second Physiological Case of Cys/His-ligated Heme—$g_s$ peaks of low spin hemes with His/Met, His/His, or His/Lys axial ligation generally lie above $g = 2.8$ (24). The suspiciously low rhombicity of the EPR spectrum of heme 3(II) with its $g_s = 2.63$ indicated the presence of a different 6th ligand, possibly a cysteine residue as indicated by the spectral similarities to cysteine coordinated model proteins (25). The present mutagenesis study confirms this hypothesis. A case of Cys/His ligation in the native state of a heme-containing enzyme was recently reported for the SoxAX system involved in thiosulfate oxidation (26). Strikingly, SoxAX was also purified from R. sulfidophilum. The EPR parameters of one of the two spectral species attributed to Cys/His-ligated hemes in SoxAX resemble those obtained for heme 3(II). The second spectral species in SoxAX is characterized by an even lower rhombicity with $g_s = 2.42$, i.e. close to that additionally detected in isolated RC-LH1 from R. sulfidophilum and attributed to a partially denatured form of heme 3(II) (see above). It seems likely to us that the $g_a = 2.63$ to $g_s = 2.47$ transition corresponds to a relief of steric constraints on the conformation of the two axial heme ligands, similar to what has been described for His/His- and His/Met-ligated hemes (27, 28). In SoxAX, the cysteine ligand of one of the two Cys/His hemes is probably chemically modified (26). The mass spectroscopic analysis excludes this possibility for the triheme cytochrome in the R. sulfidophilum RC (data not shown). It is of note that the presence of a cysteine as axial ligand to the heme iron is sufficient to confer a very low potential to this heme. Raphael and Gray (29) already evidenced a similar downshift on the potential of soluble cytochrome c whose usual methionine ligand was replaced by cysteine.

Electron Transfer Characteristics of the Triheme Cytochrome—The model of the R. sulfidophilum triheme cytochrome, described above in terms of arrangement of midpoint potentials and orientations of the hemes relative to the membrane plane, suggests that the unusual WT cytochrome contains only one high potential photooxidizable heme (heme 1(III)) under moderate redox potential conditions. This is clearly demonstrated by both spectroscopic measurements with a series of saturating flashes (Fig. 5) and the fast kinetics of electron transfer between the cytochrome subunit and the primary electron donor of the RC (Figs. 6 and 7). Indeed only one heme is photooxidized during a series of saturating flashes for the WT. In agreement with this, the re-reduction of $P^+$ is found monophasic with a half-time of 1.6 $\mu$s. This corresponds to the rate of electron transfer between $P^+$ and the closest heme 1(III). In the case of C148M, the large increase in redox midpoint potential of heme 3(II) linked to the Cys to Met replacement renders this heme photooxidizable. This is demonstrated by the observation that two hemes are photooxidized during a series of flashes. The reduction of $P^+$ in the C148M mutant is clearly biphasic. The fast phase ($t_{1/2} \sim 2 \mu$s) corresponds to the electron transfer between $P^+$ and heme 1(III). In addition, $P^+$ is fully re-reduced only after the electron transfer between heme 1(III) and heme 3(II) is completed. This explains the occurrence of the slow phase ($t_{1/2} \sim 10 \mu$s) of $P^+$ re-reduction and the shift to a shorter wavelength of the light-induced absorbance changes associated with this phase.

It should be noted that a systematic discrepancy is observed concerning the equilibrium constant between P and the closest heme that is expected to affect the titration data; and the effect of the membrane potential (in chromatophores or cells), which would also decrease the equilibrium constant between P and the closest heme in kinetic experiments. This phenomenon probably occurs also on R. sulfidophilum chromatophores under light-induced electron transfer. Moreover, the slowing down of the electron transfer between $P^+$ and heme 1(III) (from 1.6 to 2.1 $\mu$s) and the decrease of the relative amplitude of the fast phase (from 90 down to 60%) observed in the case of the C148M mutant is also explained by such electrostatic effects in addition to those induced by the mutation of Asp-285 into Gly.

The Triheme Systems Derive from a Common Ancestor—All RC-associated triheme cytochromes detected so far are monophyletic, deriving from a single ancestor closely related to the tetraheme cytochrome of Roseobacter denitrificans (31). Structurally and functionally, the triheme cytochromes distinguish themselves from the tetraheme counterparts by three distinct features, i.e. (a) the conversion of the high potential heme 3(II) to a low potential induced by the methionine to cysteine ligand change; (b) the absence of the CXXCH binding site of heme 4(I) and its associated methionine 6th axial ligand; and (c) the loss of three (Glu-79, Glu-85, and Val-67 (see Refs. 32–34)) of the four main amino acids involved in docking of soluble electron donors. Three different scenarios for the mutational event(s) entailing these alterations are possible. (i) The triggering event was the deletion of a sequence stretch containing the motif that binds heme 4(I) and docks the soluble electron carriers. To explain the additional change in ligand of heme 3(II), a role for the $E_c$ change of heme 3(II) in suppressing the deleterious effects of the loss of heme 4(I) must be invoked. (ii) Alternatively, the methionine to cysteine ligand change may have been the initial event entailing loss of heme 4(I) as a suppressor mutation as in (i). (iii) Excision of the heme 4(I) binding site and heme 3(II) ligand change may have occurred simultaneously in an indel (insertion-deletion) event, exchanging a sequence stretch comprising both sequence features in question by a new (foreign) stretch derived from a distal location in the genome.

In both hypotheses i and ii, the concomitance of the loss of heme 4(I) together with the docking site and the $E_c$ change of heme 3(II) is rationalized by functional constraints. First, another docking site for soluble electron donors that connects the cytochrome $bc_1$ complex to the RC has to be present on the triheme cytochrome. The presence of this new docking site may explain the large difference of orientation observed for heme 3(II) in triheme cytochrome compared with the usual tetrahemes. Owing to the low accessibility of heme 1(III), we propose that electron transfer between the soluble cytochrome $c_{549}$ (35) occurs via an uphill step involving heme 2(IV) or heme 3(II) or both. In addition, the very low midpoint potential of heme 3(II) may be linked to the involvement of this heme in an alternative electron transfer not involving the cytochrome $bc_1$ complex. So far, the respective experiments have been carried out under “standard” conditions only, i.e. growth in Hutner (non-reducing) medium and electron transfer assays at mildly reducing ambient potentials. Rhodovulum sulfidophilum, however, is able to grow under a variety of rather “non-standard” environmental conditions, e.g. by collecting reducing equivalents from oxidation of thiosulfate or dimethylsulfide. Dimethylsulfide dehydrogenase, for example, has been shown to deliver electrons into the photosynthetic electron transfer chain in R. sulfidophilum (36). Comparison of growth properties between wild type and the C148M mutant on media containing low potential (mostly sulfur-derived) electron
donating substrates and determination of the docking site of the soluble electron donors \( c_{549} \) might therefore help to elucidate the grounds of the peculiar double-site alteration in the RC associated triheme cytochromes.

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