Biochimica et Biophysica Acta 894 (1987) 468–476
Elsevier

BBA 42670

Properties of the reaction center of the thermophilic purple photosynthetic bacterium *Chromatium tepidum*

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(Received 15 June 1987)

Key words: Photosynthesis; Reaction center; Cytochrome *c*; Purple bacterium; Thermophilic chromatiaceae; (*C. tepidum*)

Reaction centers were purified from the thermophilic purple sulfur photosynthetic bacterium *Chromatium tepidum*. The reaction center consists of four polypeptides L, M, H and C, whose apparent molecular masses were determined to be 25, 30, 34 and 44 kDa, respectively, by polyacrylamide gel electrophoresis. The heaviest peptide corresponds to tightly bound cytochrome. The tightly bound cytochrome *c* contains two types of heme, high-potential *c*-556 and low-potential *c*-553. The low-potential heme is able to be photooxidized at 77 K. The reaction center exhibits laser-flash-induced absorption changes and circular dichroism spectra similar to those observed in other purple photosynthetic bacteria. Whole cells contain both ubiquinone and menaquinone. Reaction centers contain only a single active quinone; chemical analysis showed this to be menaquinone. Reaction center complexes without the tightly bound cytochrome were also prepared. The near-infrared pigment absorption bands are red-shifted in reaction centers with cytochrome compared to those without cytochrome.

Introduction

Chromatium tepidum is a newly discovered thermophilic purple sulfur bacterium [1,2]. Its growth temperature optimum of 48–50 °C is the highest known for a purple photosynthetic bacterium. The intracytoplasmic membrane obtained from *C. tepidum* exhibits considerable thermostability compared with related mesophilic species [3]. Two kinds of antenna complex have been purified from this organism [3,4]. One of these antenna complexes, B-917, has its absorption maximum at the longest wavelength for any BChl-*a*-containing organism.

Reaction centers have been isolated from a large number of species of the purple nonsulfur bacteria (reviewed in Ref. 5), also known as the α and β branches of the purple bacteria [6]. In contrast, reaction centers have been purified from only a few purple sulfur bacteria (the γ branch), including *Chromatium vinosum* [7–10], *Thiocapsa pfennigii* [11] and some species of *Ectothiorhodospira* [12,13].

The reaction centers of purple sulfur bacteria have previously been found to contain a tightly bound *c*-type cytochrome with both low and high-potential hemes (reviewed in Refs. 5, 14). A

Abbreviations: LDAO, lauryl-*N*,*N*-dimethylamine-*N*-oxide; BChl, bacteriochlorophyll, BPh, bacteriopheophytin; SDS, sodium dodecyl sulfate; m°, millidegree; PAGE, polyacrylamide gel electrophoresis.

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similar cytochrome is also found in some purple nonsulfur bacteria, e.g., *Rhodopseudomonas viridis* [15,16]. The function of this cytochrome, especially the low-potential hemes, is not well understood. The low-potential hemes of the tightly bound cytochromes are capable of being photooxidized at cryogenic temperatures [17]. This cytochrome oxidation was the first and remains one of the clearest and most intensively studied examples of quantum mechanical tunneling processes in biochemical systems (reviewed in Ref. 18).

The multiheme membrane-bound *c*-type cytochrome from *C. vinosum* was first isolated and characterized in 1971 by Kennel and Kamen [19], and has since been studied by several research groups (reviewed in Refs. 5, 14).

In *C. vinosum*, there has been some uncertainty about whether the low-potential hemes (*c*-553) and the high-potential hemes (*c*-556) were part of the same or different peptides [20,21]. In other organisms that contain the tightly bound cytochrome, e.g., *Rhodopseudomonas viridis*, it is clear that all four hemes are part of a single polypeptide [15,16].

We have purified and partially characterized reaction centers from *C. tepidum*. A preliminary report of this work has been presented [22].

Materials and Methods

C. tepidum was cultured anaerobically under continuous illumination with a 60 W incandescent lamp at 10 cm distance for 3 or 4 days at 50 °C in the medium reported by Madigan [1,2]. Chromatophores were prepared as previously reported [3]. Reaction centers were isolated from the whole chromatophore membrane by an lauryl-*N*,*N*-dimethylamine *N*-oxide (LDAO) treatment followed by DEAE-Sephacel column chromatography. A chromatophore suspension with an absorbance of 50 at 855 nm was incubated in the dark at 40 °C in the presence of 0.25% LDAO for 1 h, followed by centrifugation at 200000 × *g*. The supernatant liquid containing crude reaction centers was dialyzed against 20 mM Tris with 0.05% LDAO at pH 8.5, and then chromatographed on a column of DEAE-Sephacel previously equilibrated in 20 mM Tris/0.05% LDAO (pH 8.5), 4 °C. The reaction center was eluted by the same buffer with a 0–250 mm NaCl gradient. The fractions containing reaction centers (determined by the absorption spectra) were rechromatographed under the same conditions. Final purification was obtained using a 4.5 × 250 mm DEAE-analog HPLC column (Sota chromatography) with a 0–300 mM NaCl gradient over 30 min at 2 ml/min in 20 mM Tris (pH 8.5)/0.05% LDAO. Two resolved reaction center peaks eluted from the HPLC column, one with and one without attached cytochrome (see Results).

Absorption spectra were recorded on Shimadzu UV-160, UV-360 or Cary 219 recording spectrophotometers. CD spectra were obtained on a recording circular dichrometer, Jasco J-500 C, with a Jasco DP-500 data processor. The temperature was regulated by a thermostatted recirculating water-bath. For the observation of the near-infrared region an S-1 type photomultiplier (R1767, Hamamatsu) was used, 10 mm and 2 mm quartz cells were used in combination with appropriate spacers. Laser-flash-induced absorption changes were measured by the method described earlier [23].

Low-temperature cytochrome photooxidation (77 K) was measured with a Cary 219 spectrophotometer and a 0.3 mm path cuvette equipped with a cold finger and a clear glass dewar. Purified chromatophores were brought to 50% glycerol and frozen in liquid N_2 in the cell. A dark spectrum was recorded and stored in an Apple computer. The sample was then illuminated for 2 min with white light from a 100 W tungsten halogen lamp and then left in the dark for 1 min before the light spectrum was recorded and stored. The difference spectrum was generated by computer subtraction of the dark spectrum from the light spectrum.

Room-temperature cytochrome photooxidation was measured with a Cary 219 spectrophotometer. Red actinic illumination was provided by a 100 W tungsten halogen lamp with a Ditric 650 long-pass filter and a fiber-optic light pipe. The photomultiplier tube was protected by a Corning 4-97 and a Ditric 620 short-pass filter. Spectra were stored on an Apple computer before, during and after illumination, and the light-minus-dark difference spectra were obtained by subtraction.

Discontinuous SDS-polyacrylamide gel electrophoresis was carried out using a 12.5% acrylamide

gel and a Hoefer Mighty Small electrophoresis unit. Samples for electrophoresis were treated with 2% SDS, 10 mM dithiothreitol and 6 M urea and then heated for 1 min at 100 °C. Sucrose or glycerol was not used in the dissociation buffer as their addition caused gelation of the sample after heating. Heme staining was carried out as described by Thomas et al. [24].

Treatment with sodium borohydride was as described by Ditson et al. [26].

Quinone analysis generally followed the procedure given by Collins [26]. Cells or purified reaction centers were lyophilized, weighed, and then extracted in 20 ml of 2 : 1 chloroform/methanol by stirring for 2 h. The extract was filtered through Whatman No. 1 filter paper and the filtered extract dried under vacuum using a rotary evaporator. The extract was dissolved using two 1-ml portions of 2 : 1 chloroform/methanol and applied to a Merck Soft-Plus silica gel 60 TLC plate. The plate was developed with petroleum ether (boiling range 30–60 °C)/diethyl ether, 85 : 15. Quinone bands were determined by observation under long- and short-wave ultraviolet light and also by the use of a reduced Nile blue stain [27]. The bands were scraped off the plate, dissolved in 2 : 1 chloroform/methanol, filtered to remove the silica, dried under vacuum using a rotary evaporator and redissolved in absolute ethanol. Identification of the type of quinone was obtained from the reduced-minus-oxidized ultraviolet difference spectrum [28]. The number of isoprene side-chain units was determined by HPLC on an analytical Whatman Partisil 10 ODS3 column by monitoring absorbance at 269 nm using an isocratic solvent system of 9 : 1 methanol/isopropanol at a flow rate of 2.5 ml/min. Synthetic ubiquinone-10 was obtained from US Biochemical and contained a sufficient quantity of shorter side-chain length ubiquinones to determine their retention times. Menaquinones-1 to 10 were a gift of Drs. U. Manz and S. Weber of F. Hoffman-La Roche & Co., Basel, Switzerland.

Results

Three near-infrared absorption peaks are observed in the purified reaction center complex from *C. tepidum* (Fig. 1, solid line). These peaks at 885, 800 and 756 nm are due to absorbance from the special pair, BChl *a*, mostly accessory BChl *a* and BPh *a*, respectively. While the 885 nm absorption band is about 15 nm to longer wavelength than that typically found in BChl-*a*-containing purple bacteria, it is similar to that found in *C. vinosum* [7,8]. In the visible portion of the spectrum the 596 nm band is due to the Q_x transition of BChl *a* and the 530 nm band has contributions from cytochrome *c* (see below), carotenoid and the Q_x transition of BPh *a*. The peak at 410 nm is due to cytochrome, the 366 nm band can be attributed to the Soret bands for BChl *a* and BPh *a*, and the 280 nm peak is due to aromatic amino acids.

From 33 g of cell paste (12000 $A_{850} \times$ volume in ml of chromatophores), 100 $A_{800} \times$ volume in ml of reaction center with a ratio of $A_{280} \div A_{800} = 1.50$ could be isolated. The best ratio of $A_{280} \div A_{800}$ obtained was 1.45 for reaction center with the bound cytochrome. The reaction center can be isolated both with and without the tightly bound cytochrome as shown by the lack of a 410 nm peak in the absorption spectrum of Fig. 1 (dashed line). The amount of reaction center isolated without the attached cytochrome varied from 10–50% of the total reaction center isolated as determined by comparing the integrated peak areas from the HPLC detector (monitoring at 800 nm). Rechromatography of each of the two reaction center peaks resulted in a single peak with retention times identical with that of the material eluted from the initial separation (not shown).

The difference spectrum of the cytochrome-containing minus the cytochrome-free reaction centers is shown in Fig. 2, normalized at the 800 nm BChl peak. The cytochrome-free reaction centers have a very similar absorption spectrum to the cytochrome-containing ones, with the principal difference being the absence in the former of the characteristic cytochrome absorption bands. In addition, small shifts in the wavelengths of the near infrared absorption bands are observed in the two samples. The peak of the reaction center photoactive BChl is 885 nm in the cytochrome-containing samples and 875 nm in the cytochrome-free reaction centers. The accessory BChl absorption band is found at 800 nm in the cytochrome-containing samples and 799 nm in the

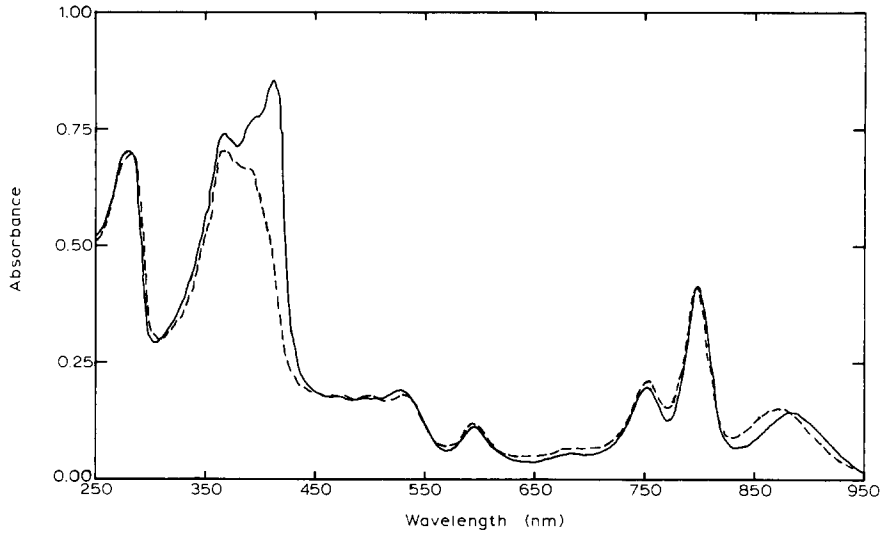

Fig. 1. Ultraviolet/visible absorption spectrum of *C. tepidum* reaction centers in 20 mM Tris/0.05% LDAO (pH 8.0). Solid line, with bound cytochrome; dashed line, without bound cytochrome.

cytochrome-free samples. No effect is observed on the position of the 755 nm BPh absorption band. The near-infrared region of the difference spectrum is expanded in the inset to Fig. 2.

Both reaction center complexes were found to be fully photoactive. The laser-flash-induced difference spectrum (Fig. 3) is similar to that found for other purple bacterial reaction centers [29]. The recombination time ($1/e$) for reaction centers without added quinone is 35 ms (Fig. 3 inset and Fig. 4A). When ubiquinone is added to the sample, the decay is extended to 1.6–1.8 s (Fig. 4B). Addition of *o*-phenanthroline, an inhibitor of the $Q_A^- \rightarrow Q_B$ reaction, restores the more rapid decay kinetics (Fig. 4C), establishing that the 35 ms decay is due to the $P\text{-}870^+Q_A^- \rightarrow$ P-870 Q_A reaction, and that the reaction centers as isolated contain only a single active quinone.

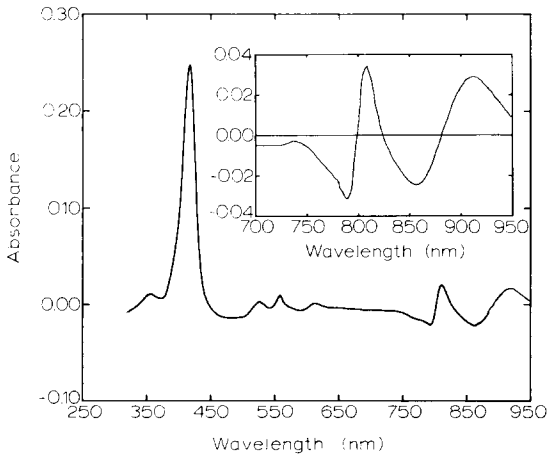

Fig. 2. Difference spectrum of the cytochrome-containing minus the cytochrome-free reaction centers of *C. tepidum* from Fig. 1. The spectra were normalized at the 800 nm absorption. The inset shows an expanded view of the near-infrared region.

When ascorbate is added to the reaction centers with cytochrome, the apparent absorption change at 885 nm due to P-870 photooxidation is largely eliminated, due to extremely rapid rereduction of oxidized P-870 by reduced cytochrome (Fig. 4D). The absorbance at 556 nm (α peak of high-potential cytochrome) increases due to P-870 photooxidation in the absence of reductant (Fig. 4E). In the presence of reductant, the cytochrome oxidation is submillisecond (Fig. 4F). In reaction centers without the cytochrome, addition of ubiquinone also increased the P-870 rereduction time from 35 ms to approx. 1.8 s (Fig. 4G). Upon addition of 2 mM sodium ascorbate to this sample the time constant was reduced to 76 ms, although the apparent amplitude was reduced by about 15% (Fig. 4H). This is in contrast to the behavior observed in the cytochrome-containing sample (Fig. 4D; note the different time scales). Adding 2 mM *o*-phenanthroline to the sample further reduced

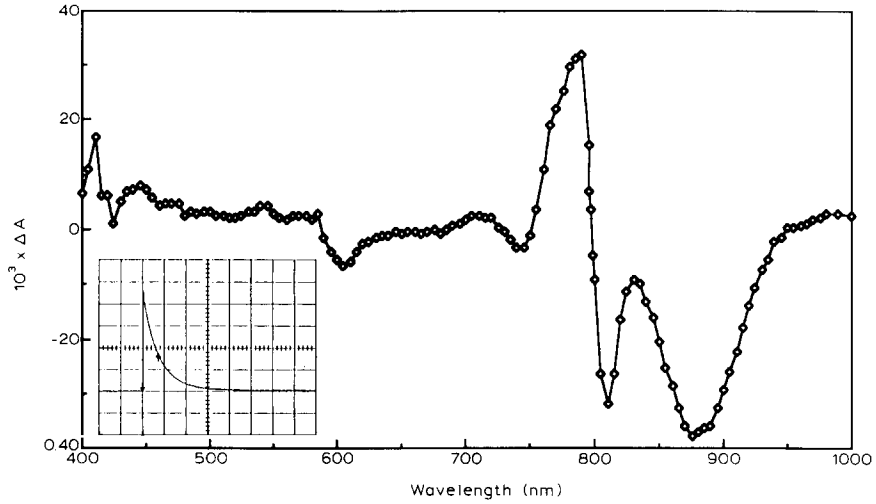

Fig. 3. The saturating laser flash-induced difference spectrum of purified *C. tepidum* reaction centers (with bound cytochrome). The inset shows the laser flash-induced photobleaching of reaction centers measured at 880 nm (50 ms/div, $\Delta A = 3.4 \cdot 10^{-3}$ per div).

the time constant to 36 ms, which was the same as before any additions (not shown). The acceleration of decay of P-870$^+$ by ascorbate presumably reflected a direct rereduction of P-870$^+$ by ascorbate. This decay time appeared to be faster in samples with higher ascorbate concentration, although this was not investigated in detail.

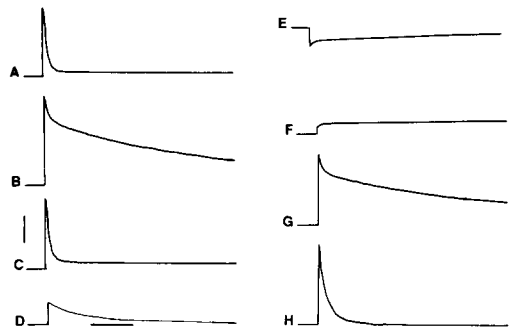

Fig. 4. Laser flash-induced absorption changes in *C. tepidum* reaction centers. An upward deflection indicates an absorption decrease. Traces A–D were monitored at 885 nm; trace A, reaction center, with cytochrome, no additions; trace B, same as A except 100 $\mu g \cdot ml^{-1}$ ubiquinone-10 added; trace C, same as B except additionally 1 mM *o*-phenanthroline added; trace D, same as A except 1 mM sodium ascorbate added. Traces E and F, same conditions as traces A and D, but monitored at 556 nm. Traces G and H, reaction centers without cytochrome monitored at 875 nm. Trace G, 300 $\mu g \cdot ml^{-1}$ ubiquinone-10; Trace H, 2 mM sodium ascorbate plus 100 $\mu g \cdot ml^{-1}$ ubiquinone-10. $A_{800} = 0.10$ for all traces. The horizonal bar is 200 ms in all traces except D, where it is 20 ms. The vertical bar is $\Delta A = 7.0 \cdot 10^{-3}$ for trace A, $5.7 \cdot 10^{-3}$ for B, $7.1 \cdot 10^{-3}$ for C, $5.9 \cdot 10^{-3}$ for D, $2.9 \cdot 10^{-3}$ for E, $3.4 \cdot 10^{-3}$ for F, $6.9 \cdot 10^{-3}$ for G and $7.2 \cdot 10^{-3}$ for H. Other conditions as in Fig. 1.

Quinone analysis of whole cell extracts of *C. tepidum* showed both menaquinone (1.3 μmol per g dry cells) and ubiquinone (5.6 μmol per g dry cells) to be present (not shown). These amounts are comparable to those reported in *C. vinosum* by Takamiya et al. [30]. The menaquinone consisted of a mixture of isoprenoid side-chains of lengths 6, 7 and 8 units in the approximate ratio of 11 : 4 : 85, respectively. In isolated reaction centers only menaquinone 8 was detected. This result is consistent with the finding that in *C. vinosum* Q_A is menaquinone [10,31].

Chemically induced reduced-minus-oxidized absorption difference spectra in the cytochrome α-band region (Fig. 5) reveal the presence of both high-potential cytochrome *c*-556 (hydroquinone reducible) and low-potential cytochrome *c*-553 (dithionite reducible) in the purified cytochrome-containing reaction centers. The absorption coefficients for the cytochromes can be estimated by comparison to the BChl absorption. Assuming that $\epsilon_{801} = 288\ mM^{-1}\ cm^{-1}$, as found for *Rhodobacter sphaeroides* [32] and that there are two copies of both *c*-556 and *c*-553, the difference absorption coefficients at the α peak for each heme of *c*-553 and *c*-556 are 17.3 and 12.8

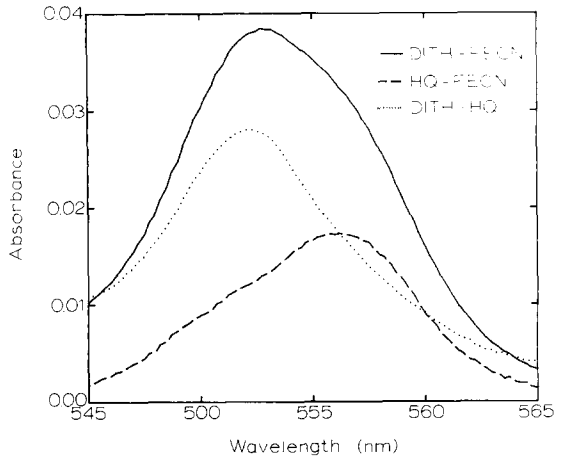

Fig. 5. Chemically induced oxidized-minus-reduced absorption difference spectra of purified cytochrome-containing *C. tepidum* reaction center in the visible portion of the spectrum. Solid line is dithionite-minus-ferricyanide; dashed line is hydroquinone-minus-ferricyanide; dotted line is dithionite-minus-hydroquinone. $A_{800} = 0.188$.

$mM^{-1} \cdot cm^{-1}$, respectively. These values are likely to be underestimates, because some reaction centers (10–25%) with no cytochrome were present as contaminants.

The cytochrome in the isolated reaction center complexes can be photooxidized at room temperature (Fig. 6). The difference absorption spectrum clearly shows that it is the high-potential cytochrome that is oxidized under these conditions, with maximum photobleaching observed at 557 nm. This experiment was carried out with 1 mM ascorbate and 100 μg ubiquinone/ml; under these conditions only the high-potential cytochrome is expected to be substantially in the reduced state. The low-potential cytochrome is capable of photooxidation at 77 K (Fig. 7). This behavior is typical of that observed in other photosynthetic bacteria that contain reaction centers with tightly bound cytochrome.

SDS-polyacrylamide gel electrophoresis of the cytochrome-containing sample revealed four polypeptides of apparent molecular masses 25, 30, 34 and 44 kDa. The heaviest peptide stains positively for heme (Fig. 8). The cytochrome-free reaction centers exhibit only the three lower-molecular-weight bands, none of which stains for heme.

Fig. 9 shows the absorption and circular dichroism spectra for the cytochrome-containing reaction center from *C. tepidum* in the visible and near-infrared. Except for the long wavelength of the special pair absorbance and a small but distinct shoulder on the long-wavelength side of the 810 nm negative CD, the spectral features in the CD spectra are very similar to those of other

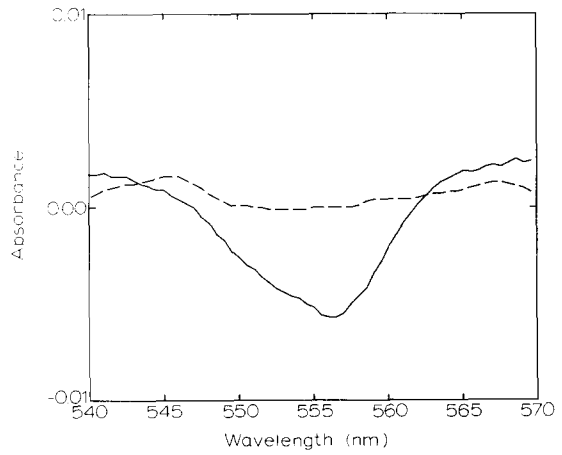

Fig. 6. Room-temperature light-induced cytochrome photooxidation in *C. tepidum* reaction centers, measured as described in Materials and Methods. Solid line, cytochrome-containing reaction centers; dashed line, cytochrome-free reaction centers. Each sample had $A_{800} = 0.153$. Other conditions as in Fig. 1. Both samples contained 1 mM sodium ascorbate and 100 $\mu g \cdot ml^{-1}$ ubiquinone.

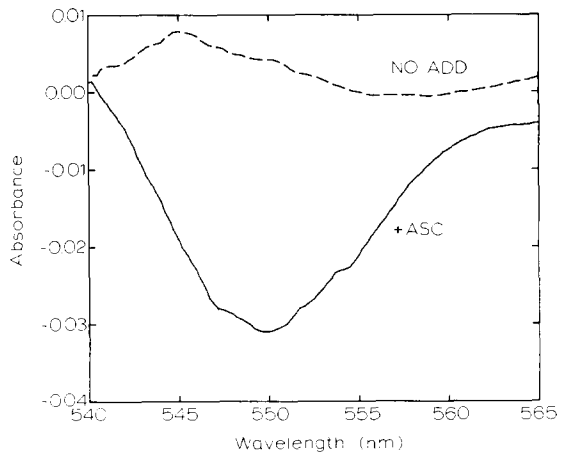

Fig. 7. Cytochrome *c*-553 photooxidation in *C. tepidum* chromatophores at 77 K. The solid line is a sample with 10 mM ascorbate added and the dashed line is a sample with no additions. Additional details are given in Materials and Methods.

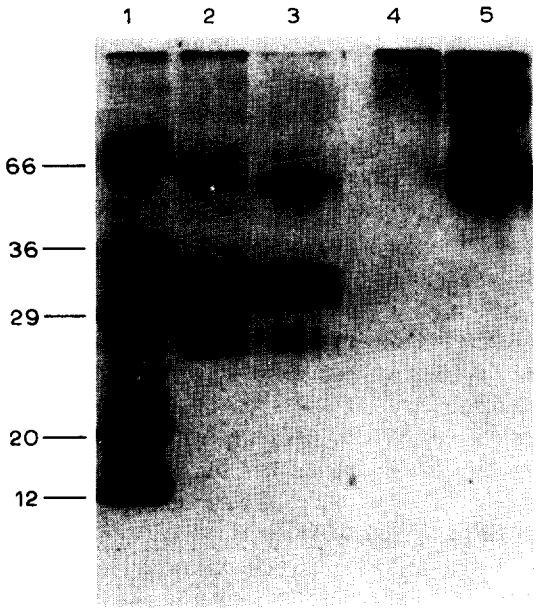

Fig. 8. Discontinuous SDS-PAGE of *C. tepidum* reaction center in 12.5% acrylamide. Lane 1 is molecular weight standards, lanes 2 and 4 are reaction center without bound cytochrome, and lanes 3 and 5 are reaction center with bound cytochrome. Lanes 1–3 are stained with Coomassie blue, while lanes 4 and 5 are stained for heme.

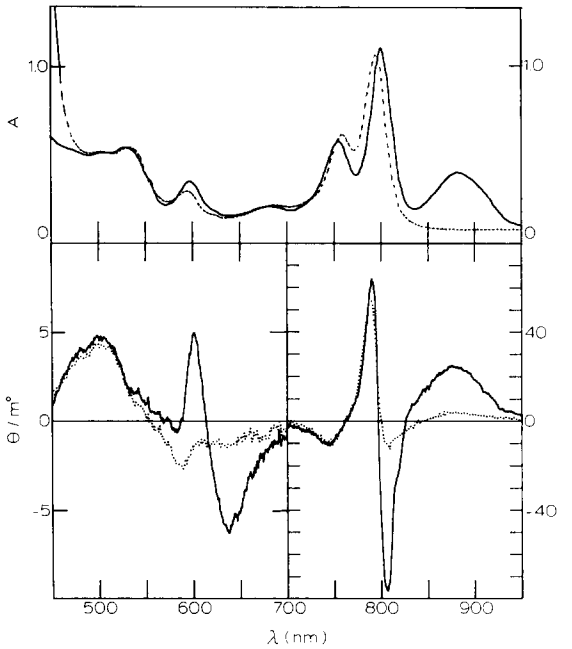

Fig. 9. The visible and near-infrared absorption and CD spectra of the cytochrome-containing reaction center from *C. tepidum* in 20 mM Tris/0.05% LDAO (pH 8.5) at 4°C in 2 mm optical length cell. Solid line is sample with ascorbate and the dashed line is sample with ferricyanide.

purple bacteria [33–35]. A couplet-type CD was observed for the reduced state in the Q_x region of BChl *a* (580–680 nm). The couplet-type CD was converted to a single-signed CD in the oxidized state.

The far-ultraviolet portion of the ultraviolet CD spectrum (Fig. 10a) is consistent with the X-ray crystallographic data of *R. viridis* [16] and infrared spectra [36] predicting that the reaction center is composed predominantly of α-helical structures. The tertiary structure (helix-to-helix interactions or the amino-acid residue interactions) is reflected in the 250–300 nm region CD spectrum (Fig. 10b). The presence of CD in this region indicates that many of the aromatic amino-acid residues have rigid structures in the native reaction center polypeptide.

Absorption and CD spectra of *C. tepidum* reaction centers that have been treated with sodium borohydride are shown in Fig. 11. This treatment, introduced by Pearlstein and co-workers [25], is believed to lead to the selective removal of one of the 800-nm-absorbing BChl molecules. The borohydride treatment had little effect on the 885 nm absorption band, but significantly decreased the absorption in the 800 nm region, similar to the

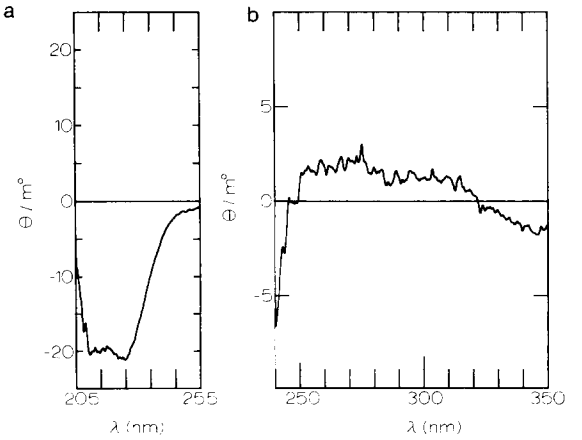

Fig. 10. The ultraviolet CD spectrum of the cytochrome-containing reaction center from *C. tepidum* in 20 mM Tris/0.05% LDAO (pH 8.5) at 4°C in 2 mm optical length cell. (a) In the 210–250 nm region (0.06 mm path length); (b) in the 240–350 nm region.

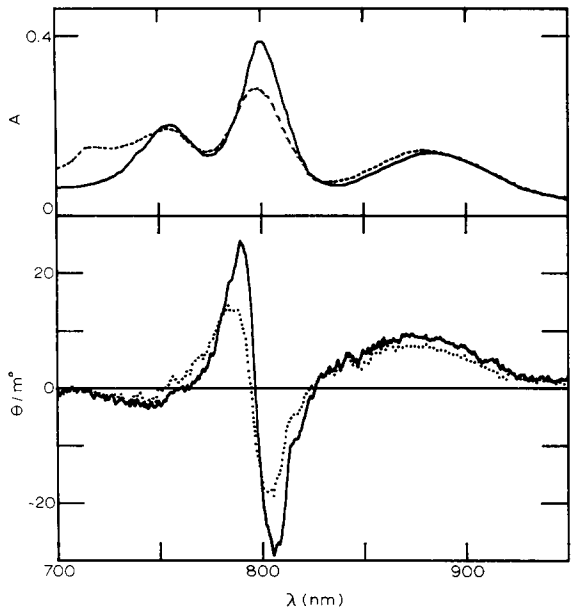

Fig. 11. The near-infrared absorption and CD spectrum of the cytochrome-containing reaction center from *C. tepidum* in 20 mM Tris/0.05% LDAO (pH 8.5) at 4°C in 2 mm optical length cell. Solid line, no addition; dashed line, same sample 1.5 h after exposure to $NaBH_4$.

results observed in *R. sphaeroides* [25]. The absorption and CD at 810 nm, which has been interpreted as arising from the higher-energy exciton band of P-870 [37], are affected more by the borohydride treatment than is the absorption at 885 nm. This observation, as well as other recent results [38], seem to argue against the view that 800 nm absorption is due mainly to an exciton band of P-870, because the two halves of an exciton pair would be expected to behave in a similar manner in such an experiment. Our results suggest that the 800 nm absorption is due mainly to the accessory BChls (Nozawa, T., unpublished data) and that the 806 nm CD is due to a mixture of excited states of the accessory BChls and the higher-energy exciton half of the special pair. This is in agreement with the calculations of Vasmel et al. [39].

Discussion

The most interesting feature of the reaction center from *C. tepidum* is the observation that complexes both with and without tightly bound cytochrome can be isolated in an active form. Similar results were reported for *C. vinosum* by Romijn and Amesz [10]. The cytochrome-containing reaction centers have slightly longer wavelength BChl absorption bands in the near-infrared than do the cytochrome-free samples. Furthermore, the effect is larger (10 nm shift) in the P-870 absorption band than it is on the B-800 absorption band (1 nm). No effect is observed on the BPh absorption at 755 nm. If the orientation of the cytochrome in *C. tepidum* is similar to that found in *R. viridis*, then the tightly bound cytochrome is closest to P-870 and farthest from the BPh, consistent with the order of the magnitude of the effect observed in *C. tepidum*. This result gives an indication of the effect of the bound cytochrome on the rest of the reaction center structure. Whether these small shifts in energy of the BChl absorption bands result from minor structural changes induced by the binding of the cytochrome or an electrochromic effect of charges in the cytochrome on the pigment absorption cannot be determined at this time. A change in the redox state of the cytochrome does not appear to induce any further shifts in the absorption spectra of the pigments (data not shown).

The relative amounts of the two populations of reaction centers appeared relatively invariant with time during the course of the purification procedure. Attempts to dissociate the cytochrome from the cytochrome-containing reaction center samples by incubation with high detergent concentrations and elevated temperatures were only marginally successful, suggesting that the two populations possibly existed in the membrane prior to detergent extraction. Additional work will be required to establish whether this is indeed the case. The cytochrome-free reaction centers unexpectedly had a similar absorbance at 280 nm as the cytochrome-containing samples. In many samples, additional absorbance in the 250–500 nm region was observed in the cytochrome-free reaction centers compared to the cytochrome-containing samples. The pigment responsible for this absorption has not yet been identified; it could be separated from the cytochrome-free reaction centers only by multiple DEAE-HPLC runs.

In conclusion, we have isolated and partially characterized the reaction center from a thermo-

philic purple sulfur bacterium, *Chromatium tepidum*. The reaction center has a tightly bound cytochrome *c* which is a single peptide with a molecular mass of 44 kDa. The reaction center can be prepared both with and without the cytochrome attached. The cytochrome probably contains four hemes, two copies of both high-potential hemes (*c*-556, hydroquinone-reducible) and low potential hemes (*c*-553, dithionite-reducible). The low-potential heme, *c*-553, can be photooxidized at 77 K. In addition to the cytochrome, the reaction center contains three polypeptides, with molecular masses of 22, 28 and 33 kDa. The reaction center of *C. tepidum* is overall very similar to that of the mesophilic purple sulfur bacterium, *C. vinosum* [7–10,31].

Acknowledgements

Supported by grants from the Competitive Research Grants office of the U.S. Department of Agriculture (84-CRCR-1-1523), the Herman Frasch Foundation, the Japanese Ministry of Education (Japan-U.S. Cooperative Photoconversion and Photosynthesis Research Program), and by a Biomedical Research Support Grant (S07 RR07112) awarded to Arizona State University by the Biomedical Research Support Grant Program, Division of Research Resources, National Institutes of Health. We thank Professor M. Madigan for supplying a culture of *C. tepidum* and for advice on growth conditions, and Drs. U. Manz and S. Weber, of F. Hoffman-La Roche, for a gift of menaquinone standards.

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