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Purification and characterization of the photochemical reaction center of the thermophilic purple sulfur bacterium *Chromatium tepidum*

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Pure reaction center preparations from the thermophilic species *Chromatium tepidum* have been obtained by lauryldimethylamine *N*-oxide treatment of chromatophores. The light-induced difference spectrum in presence of 10 mM sodium ascorbate revealed the presence of two high-potential cytochrome *c* hemes (α-band, 555 nm; γ-band, 422 nm). The dithionite-minus-oxidized difference spectrum in the α-band suggests the presence of additional hemes of low potential. These hemes are associated with a single polypeptide (M_r = 36000). The reaction center pigments, probably four bacteriochlorophyll *a* and two bacteriopheophytin *a* molecules, are associated with three polypeptides of apparent molecular weights equal to 33000, 30000 and 22000. A carotenoid molecule is also bound to the reaction center. The three main absorption bands of this molecule are located at 480, 510 and 530 nm at liquid helium temperature. Photochemical activity is found to be stable, even after heating for 10 min at temperatures higher than 60 °C in intact chromatophore membranes. On the other hand, isolated reaction centers or chromatophores treated with 1% lauryldimethylamine *N*-oxide are fully inactivated after heating at temperatures higher than 50 °C. From these results, we propose that lipid–protein interactions are of prime importance in the thermal stabilization of *Chromatium tepidum* reaction centers.

Introduction

A new photosynthetic bacterium has been obtained recently in pure culture by M.T. Madigan [1] from mats collected in a hot spring of Yellowstone National Park. Several properties of this bacterium identify it as a member of the genus *Chromatium* [1,2]. It differs, however, from all other *Chromatium* species in its thermophilic character. Its optimum temperature of development is 48 °C. Based on these characteristics, it has been proposed as a new species, *Chromatium tepidum* [2]. This has opened up the possibility to study and understand the basis of the thermostability of membrane pigment-protein complexes by comparing properties between mesophilic and thermophilic species of the same genus. In a previous work [3], we have analysed the spectroscopic characteristics of the light-harvesting complexes of this new thermophilic bacterium. Two types of light-harvesting complex, B800–855 and B920, are present in the intracytoplasmic membranes [3,4]. Both complexes are thermostable in the intact membrane up to 70 °C, but a good thermostability is

Abbreviations: LDAO, lauryldimethylamine *N*-oxide; PMSF, phenylmethylsulfonyl fluoride; BChl, bacteriochlorophyll; BPh, bacteriopheophytin; TMBZ, 3,3′,5,5′-tetramethylbenzidine.

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observed only for the B800–855 complexes in the isolated state [4]. The present report deals with the isolation, purification of reaction centers of *C. tepidum* and the description of several of its biophysical and biochemical properties. Preliminary accounts of this work have been presented at the Fifth International Symposium on Photosynthetic Prokaryotes [5].

Materials and Methods

C. tepidum was grown as previously described [1]. Cell were harvested after 4–5 days and stored frozen until used. After disruption of the cell walls by two passages through a French press at 110 MPa, chromatophores were obtained as reported in Ref. 3. Sodium dodecylsulfate (SDS) polyacrylamide gel electrophoresis was carried out according to the method of Laemmli [6]. The gel concentration was 12.5%. Reaction centers, chromatophores or molecular weight markers were deposited on the gel in presence of 5% SDS/10 mM PMSF. After migration (3–4 h) the proteins were stained with Coomassie blue R-250. The hemes were detected by addition of H_2O_2 in presence of TMBZ in the dark according to the procedure of Thomas et al. [7]. Absorption spectra were recorded with a Varian 2300 spectrophotometer equipped with a helium gas cryostat (Meric) for low-temperature measurements. Light-induced absorbance changes were measured with a home-built single-beam spectrophotometer. The photodetectors used were a PIN 10 photodiode (UDT) in the near infrared region or a Hamamatsu photomultiplier (R 316) in the visible range. Excitation was provided by YAG laser (Quantel 481 A) coupled with a dye laser (TDL III) or an 800 W quartz halogen lamp filtered appropriately.

Results

Reaction centers were isolated by LDAO (3%, v/v) treatment of chromatophore membranes suspended in the buffer, 10 mM Tris-HCl (pH 8)/1 mM PMSF, with an optical absorbance adjusted to 50 cm^{-1} at 855 nm. After 20 min of incubation in the dark and at room temperature, the suspension was centrifuged at $200\,000 \times g$ for 90 min. Pellets were discarded and the supernatant was subjected to ammonium sulfate precipitation. The reaction centers precipitated at ammonium sulfate concentrations ranging from 30 to 40%.

After a low-speed centrifugation (10 min, $20\,000 \times g$), the floating film, which contained the reaction centers, was resuspended in 0.1% LDAO/10 mM phosphate buffer (pH 7)/1 mM PMSF, and dialysed overnight at 4°C against the same buffer. Some aggregated materials were removed after the dialysis by low-speed centrifugation. The solution was then applied to a hydroxylapatite column equilibrated with 0.05% LDAO/10 mM phosphate buffer (pH 7)/1 mM PMSF. A red-colored band containing reaction centers was eluted between 120 and 160 mM phosphate. After dialysis against 0.05% LDAO/10 mM Tris buffer (pH 8)/1 mM PMSF, the reaction centers can be stored frozen for several weeks before used.

The absorption spectrum recorded at room temperature for a suspension of reaction centers isolated from *C. tepidum* according to the above procedure is depicted in Fig. 1A. In the near infrared region, this spectrum is very similar to the one reported for *C. vinosum* isolated reaction centers [8] or several reaction centers isolated from other BChl-*a*-containing species. The two main absorption bands located at 890 nm and 800 nm are due to BChl *a*, while the 757 nm band is related to the presence of BPh *a*. In the visible part, the 600 nm absorption band can be identified as the Q_x transition of BChl *a*, while the BPh *a* Q_x transitions, which are expected to occur around 540 nm, are not discernible because of the presence of the absorption band of a carotenoid molecule. At low temperature, the long-wavelength band of the BChl dimer shifts to 900 nm (Fig. 1B), while the 757 nm band of the BPh molecules is split into two components (757 and 763 nm). Low temperature allows also the spectral resolution of the carotenoid absorption bands at 480, 510 and 530 nm. Absorption bands of reduced cytochromes are clearly apparent around 422 (γ-band) and 555 nm (α-band).

Although we have not performed a careful determination of the BChl/BPh ratio by solvent extraction, the relative amplitudes of the Q_y transitions of these two chromophores are consistent with this ratio being equal to 2. We therefore

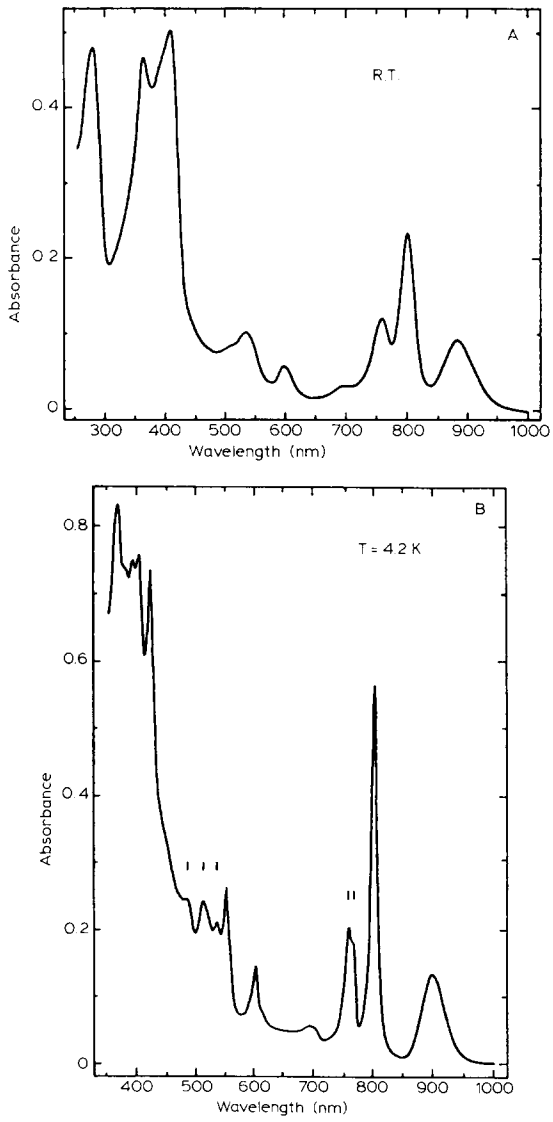

Fig. 1. Absorption spectra of a suspension of purified reaction centers isolated from *C. tepidum*. (A) Record at room temperature. The reaction centers were suspended in 10 mM Tris buffer (pH 8)/0.01% LDAO. (B) Record at liquid helium temperature (4.2 K). The reaction centers were suspended in glycerol/10 mM Tris buffer (pH 8) (2:1, v/v) and 0.05% LDAO in the presence of 10 mM sodium ascorbate.

propose that *C. tepidum* reaction centers contain 4 molecules of BChl *a* and 2 molecules of BPh *a*.

The light-induced difference spectrum following a laser actinic flash is depicted in Fig. 2, for a suspension of isolated reaction centers at ambient redox potential. The flash induces the bleaching of the long-wavelength absorption band of the

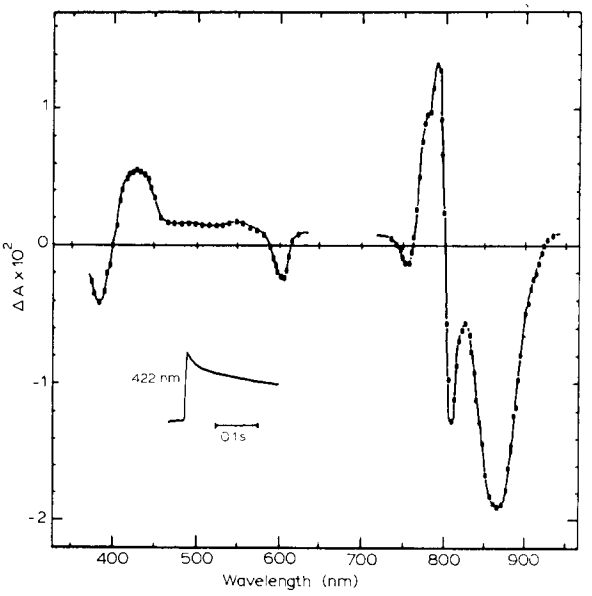

Fig. 2. Light-induced difference spectrum for a suspension of isolated reaction centers of *C. tepidum*. Laser excitation (600 nm) for the region 375–550 and 735–1000 nm. Continuous excitation for the region 550–620 nm. The light-induced absorbance changes obtained with these two types of excitation were normalized at 550 nm. Reaction centers were suspended in 10 mM Tris buffer (pH 8)/0.05% LDAO. The inset represents the absorbance changes measured at 422 nm induced by a single laser flash.

primary donor around 890 nm, an apparent blue-shift of the pigments absorbing at 800 nm and a red-shift of the BPh transition. In the visible part, the 600 nm band is partly bleached and a new band centered around 430 nm appears (Fig. 2). These features are typical of light-induced absorbance changes linked to state P^+Q^-, and very similar to what has been reported for other species of photosynthetic bacteria or for the mesophilic species *C. vinosum*. The rather slow decay observed for the flash-induced absorbance changes ($t_{1/2} \approx 0.5$ s, inset of Fig. 2) suggests that most of the secondary electron acceptor has been retained during the purification and isolation of the reaction centers.

When the redox-potential is lowered by addition of 10 mM sodium ascorbate, two cytochrome hemes are photooxidized upon excitation by two consecutive flashes (inset of Fig. 3). The kinetics are biphasic. The fast phase is shorter than 40 μs and its corresponding light-induced

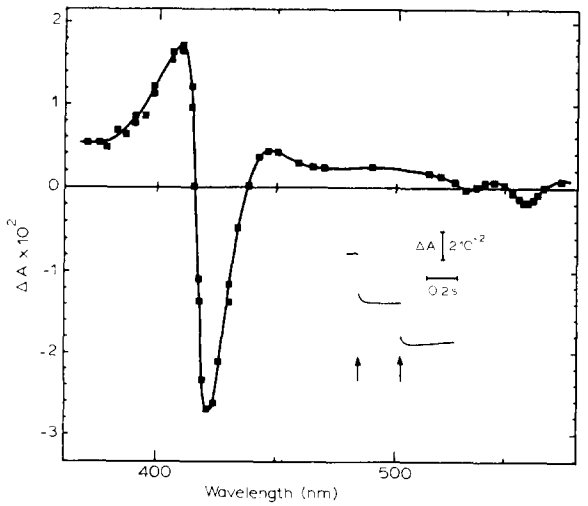

Fig. 3. Light-induced difference spectrum for a suspension of *C. tepidum* reaction centers in 10 mM Tris buffer (pH 8)/0.05% LDAO/10 mM sodium ascorbate. The inset represents the absorbance changes measured at 422 nm following excitation by two laser flashes separated by 250 ms.

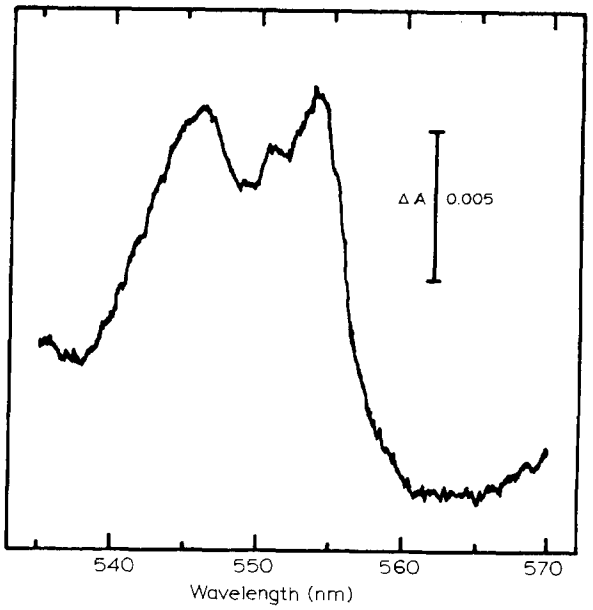

Fig. 4. Reduced (dithionite) minus oxidized (ferricyanide) difference spectrum recorded at 77 K for a suspension of *C. tepidum* reaction centers in the cytochrome α-band region. Reaction centers were suspended in glycerol/10 mM Tris buffer (pH 8) (2 : 1) and 0.05% LDAO.

difference spectrum is shown in Fig. 3. The α-band of the photooxidized cytochrome is peaking at 555 nm while its γ-band is centered at 422 nm. The slow phase ($t_{1/2} \approx 20$ ms) presents a light-induced difference spectrum (not shown) similar to the one observed for state P^+Q^- (Fig. 2). This suggests the presence of reaction centers devoid of cytochrome. In addition to these two high-potential cytochrome hemes, cytochromes of lower redox potential, reducible by addition of dithionite but not by ascorbate, are revealed in the reduced-minus-oxidized difference spectrum recorded at 77 K (Fig. 4) in the 535–570 nm region. The α-band of low-potential cytochromes peaks at 546 nm, while the high-potential cytochromes present a split band, at 554 and 551 nm. The presence of both high and low redox-potential cytochrome is reminiscent of the situation in reaction centers of *C. vinosum* [9].

Coomassie blue staining reveals the presence of three main polypeptides for isolated *C. tepidum* reaction centers after SDS electrophoresis (Fig. 5A, lanes 2 and 4). By comparison with protein standards (Fig. 5A, lane 3), isolated reaction centers from *Rhodopseudomonas viridis* (Fig. 5A, lane 1) and *Rhodobacter sphaeroides* (Fig. 5A, lane 5), these three polypeptides of *C. tepidum* reaction centers are found to have apparent molecular weights of 33000, 30000 and 22000, very similar to what has been observed from the polypeptides of *C. vinosum* reaction centers, 32000, 30000 and 24000 [10] (see, however, Ref. 11). If PMSF is omitted during the gel electrophoresis process, proteolytic digestion of the M_r 30000 polypeptide induces the appearance of three additional bands in the polypeptide profile between 29500 and 27000 (Fig. 5C, lanes 1 and 2). Specific heme staining TMBZ [7] after SDS electrophoresis reveals a stained band (Fig. 5B, lane 1). This stained band has the same apparent molecular weight as the *Rhodopseudomonas viridis* cytochrome polypeptide (36000) (Fig. 5B, lane 2), but is rather faint. No polypeptide of M_r 36000 can be observed in the Coomassie blue staining for *C. tepidum* reaction centers, (Fig. 5A, lanes 2 and 4), while the cytochrome polypeptide was readily discernible in the case of *Rhodopseudomonas viridis* (Fig. 5A, lane 1).

The apparent molecular weight of the bound cytochrome of *C. tepidum* reaction centers M_r (36000) appears to be somewhat smaller than the

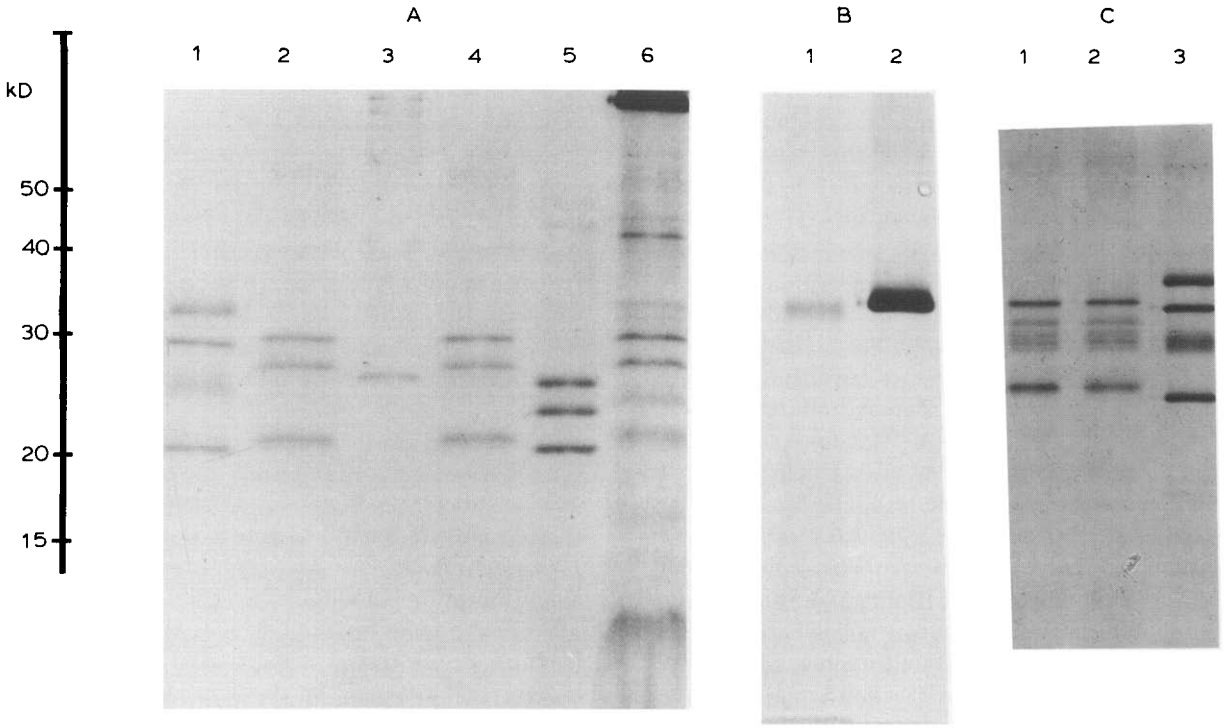

Fig. 5. Electrophoretic pattern on 12.5% polyacrylamide gels. (A) Coomassie blue staining. Lane 1, *Rps. viridis* reaction centers; lanes 2 and 4, *C. tepidum* reaction centers; lane 3, molecular weight standards; lane 5, *Rb. sphaeroides* reaction centers; lane 6, *C. tepidum* chromatophores. (B) Heme staining by TMBZ. Lane 1, *C. tepidum* reaction centers; lane 2, *Rps. viridis* reaction centers. (C) Coomassie blue staining: PMSF has been omitted during the electrophoresis. Lanes 1 and 2, *C. tepidum* reaction centers; lane 3, *Rps. viridis* reaction centers.

one reported for the mesophilic species *C. vinosum*, i.e., between 40000 and 50000 [10,11].

Thermal stability of the photochemical activity has been checked in both intact chromatophore membranes and isolated reaction centers. For that purpose, different samples have been heated at a given temperature for 10 min. The extent of the photooxidizable primary donor was subsequently mesured at 25 °C by flash spectroscopy. For intact membranes, heating at temperature higher than 70 °C was necessary to decrease the quantity of photooxidizable P-890 by a factor of 2 (Fig. 6, circles). On the other hand, photochemical activity of isolated reaction centers disappeared after heating the sample at temperature above 50 °C (Fig. 6, open triangles). If the chromatophore membranes are heated in presence of 1% of the detergent, LDAO (Fig. 6, filled triangles), the situation is then very similar to that encountered for isolated reaction centers.

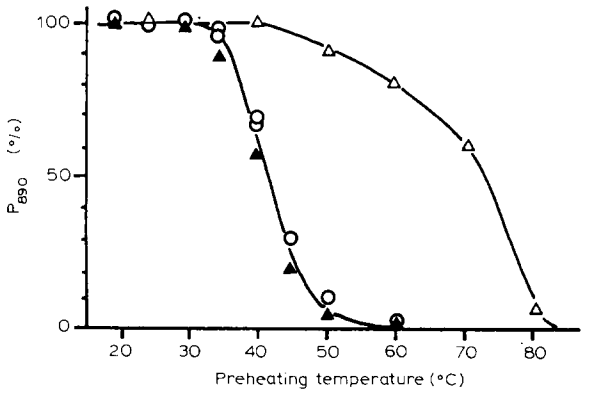

Fig. 6. Percentage of photochemical activity remaining after heating of the different suspensions at the indicated temperatures. For each temperature, a new suspension (○, isolated reaction centers; △, chromatophores, and ▲, chromatophores in presence of LDAO (1%)) was heated for 10 min. The photochemical activity was deduced from a subsequent measurement of the light-induced absorption changes at 900 nm, at room temperature.

Discussion

In spite of the thermophilic character of the *C. tepidum* species, the biochemical properties of the reaction center isolated from that bacterium are very similar to those depicted for the related mesophilic species *C. vinosum*. Firstly, the pigment composition appears to be the same for both species: 4 BChl and 2 BPh molecules. Secondly, *C. tepidum* reaction centers contain three polypeptides of respective molecular weights equal to 33000, 30000 and 22000. Cytochromes are located on a single polypeptide, with an apparent molecular weight of 36000. High- and low-redox-potential hemes are present in the cytochrome of *C. tepidum* reaction centers, again as found in the case of *C. vinosum* [9, 12,13]. Moreover, the isolated reaction centers of *C. tepidum* are thermolabile, as is the case for isolated reaction centers from mesophilic species [14]. This is in contrast to the very good thermostability observed for isolated reaction centers of the thermophilic species, *Chloroflexus aurantiacus* [14]. It is worth recalling that the photochemistry of this green gliding thermophilic bacterium presents many similarities with the one found in purple bacteria. These include the chemical nature of the early electron acceptor, a BPh molecule [15], and the primary electron acceptor, a quinone molecule [16]. On the other hand, differences in pigment and polypeptide composition are observed. Three BChl and three BPh molecules are present in *Chloroflexus aurantiacus* reaction centers instead of four BChl and two BPh for all other purple photosynthetic bacteria. *Chloroflexus aurantiacus* reaction centers contain at most two polypeptides (M_r 30000 and 28000) [14], hardly dissociable [14,17], while reaction centers from purple bacteria contain three (M_r around 21000 (L), 24000 (M) and 28000 (H) plus a polypeptide of higher molecular weight in which are located the hemes for those reaction centers which contain bound cytochromes.

This stresses the importance of the polypeptide composition in the thermal stability of isolated reaction centers [14].

Since the photochemical reaction centers of *C. tepidum* are not thermostable (Fig. 6, circles), how can this bacterium survive at a temperature of 50 °C [1,2]? A partial answer to that question is given in the experiment of Fig. 6, where it is shown that reaction centers embedded in their native lipidic environment present a good thermostability. Upon dislocation of the chromatophore membrane by addition of the detergent LDAO (Fig. 6, filled triangles), the thermostable character of the photochemical activity is lost. This strongly suggests that the thermostability of *C. tepidum* reaction centers is ensured by specific interactions between the protein and the lipids. A similar hypothesis has been postulated by Nozawa et al. [4] concerning the light-harvesting complexes B-920. We therefore conclude that thermostability in the case of an intrinsic membrane protein like the photochemical reaction centers can be achieved in two distinct manners: the reaction center is thermostable in itself, which is the case for *Chloroflexus aurantiacus*, or the reaction center complex is thermostable only in a favorable lipidic environment, which is the case for *C. tepidum*.

During completion of this work, similar results concerning the isolation and purification of reaction centers of *C. tepidum* have been presented by Nozawa and co-workers at the American Biophysical Society meeting held in new Orleans, U.S.A. (February 22–26, 1987) and during the Workshop on Structure, Function and Formation of Membrane-bound Complexes in Phototrophic Bacteria (April 2–5, 1987, Freiburg, F.R.G.) [18].

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