A Bacteriochlorophyll *a* Antenna Complex from Purple Bacteria Absorbing at 963 nm[†,‡]

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ABSTRACT: A recently isolated species of the photosynthetic purple sulfur bacteria, provisionally called strain 970, was investigated with respect to its antenna function by means of various spectroscopic techniques, including fluorescence and pump−probe absorption difference spectroscopy. The bacterium contains bacteriochlorophyll *a* and an as yet unidentified carotenoid, perhaps 3,4,3′,4′-tetrahydrospirilloxanthin. It has a single antenna complex of the LH1 type, with a Q_y absorption band situated at the unusually long wavelength of 963 nm at room temperature and 990 nm at 6 K. In contrast to many other species, the reaction center showed two well-separated absorption bands of bacteriopheophytin at 6 K, located at 747 and 762 nm. The primary electron donor showed a bleaching band centered at 925 nm upon photooxidation. Thus, the energy gap between LH1 and the primary electron donor is quite large in this strain: 425 cm^{-1}. Nevertheless, trapping occurred with a time constant of 65 ± 5 ps, similar to the rates observed in other purple bacteria. As in other species, no back-transfer from the reaction center to the antenna was observed. Our results show that strain 970 is a unique subject for the study of antenna and reaction center function and organization.

Purple bacteria possess two types of light harvesting complexes, called LH1 and LH2, which are located in the cytoplasmic membrane. Both are ring-like multimeric complexes of dimers of α- and β-polypeptides (*1*). Each α,β-dimer is thought to bind either two or three bacteriochlorophyll (BChl)[1] molecules, usually BChl *a*. A few species contain BChl *b* instead of BChl *a*.

The LH1 complex is thought to enclose the reaction center (*2*, *3*) and is therefore called the core antenna complex. The LH2, or peripheral, antenna complexes are assumed to be adjacent to the LH1 complex. Not all of the approximately 80 known species of (anaerobic) photosynthetic purple bacteria contain an LH2 complex: at least 12 species are known to possess only LH1. Six of these contain BChl *b* and are, in fact, the only BChl *b* containing species known to date. Two well-known species which contain LH1 only are *Rhodospirillum* (*Rsp.*) *rubrum* and *Blastochloris* (*Blc.*; formerly *Rhodopseudomonas*[2]) *viridis*, with BChl *a* and BChl *b*, respectively.

In solvents such as acetone and diethyl ether the BChl *a* Q_y band is situated near 770 nm (13000 cm^{-1}), but in the antenna complexes of purple bacteria it is strongly red shifted. This is in particular true for the LH1 complex, which for most species shows an absorption band near 880 nm (corresponding to a red shift of 1630 cm^{-1}). Occasionally the band is located above 900 nm, as in *Roseospirillum* (*Rss.*) *parvum* [909 nm, corresponding to a red shift of 1990 cm^{-1} (*8*, *9*)] and *Thermochromatium* (*Thc.*; formerly *Chromatium*) *tepidum* [918 nm, a red shift of 2090 cm^{-1} (*10*)]. Even larger red shifts have been observed in BChl *b* containing purple bacteria, such as *Blc. viridis* and *Thiococcus* (*Tcc.*; formerly *Thiocapsa*) *pfennigii*. BChl *b* in diethyl ether has an absorption maximum at 791 nm, whereas the main LH1 band in these bacteria absorbs around 1020 nm, corresponding to an energy difference of 2850 cm^{-1}.

The cause of the much larger shifts in BChl *b* containing bacteria is not understood. However, the evidence to be presented below shows that the effect is not unique to BChl *b*. By applying a similar illumination-dependent enrichment method as described for *Rss. parvum* (*8*), a purple sulfur bacterium was isolated from the surface layer of marine mud. This strain showed an absorption spectrum with a maximum at 963 nm and was found to contain BChl *a*. The bacterium has not been formally described yet, but its spectral and photochemical properties are presented in this paper. The

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[1] Abbreviations: BChl, bacteriochlorophyll; *Blc.*, *Blastochloris*; BPhe, bacteriopheophytin; HPLC, high-pressure liquid chromatography; LH, light harvesting; P870, primary electron donor; *Rba.*, *Rhodobacter*; *Rbi.*, *Rhodobium*; *Rsp.*, *Rhodospirillum*; *Rss.*, *Roseospirillum*; *Rvb.*, *Rhodovibrio*; *Tcc.*, *Thiococcus*; *Thc.*, *Thermochromatium*.

[2] Recently, many genera of purple bacteria were rearranged; see refs *4*−*7*.

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bacterium is provisionally called strain 970.

MATERIALS AND METHODS

Strain 970 was isolated from a ditch on the shore of the North Sea island of Baltrum, Germany. Rose-red patches in the surface layer of the bottom sediment were collected in tubes; the tubes were sealed without headspace and transported back to the laboratory. After isolation, cultures were grown in CR medium containing 1.25 mM sulfide and 3 mM acetate (*8*). Analysis of the 16S rRNA showed that the isolate is a member of the γ-Proteobacteria and is most closely related to *Thiorhodovibrio winogradskyi* DSM 6702^T (J. Overmann et al., in preparation). Membrane fragments were prepared by sonication of cells suspended in a buffer containing 50 mM Tris-HCl and 1 M NaCl (pH 8.3) and subsequent centrifugation for 5 min at 15000*g*. The supernatant contained the membrane fragments.

For pigment analysis, the suspensions of membrane fragments were extracted at 4 °C with a 100-fold excess of a mixture of methanol and acetone (1/1 v/v). The extracts were analyzed by reversed-phase HPLC on a C18 silica column (Chrompack Spherisorb 5 ODS2, 250 × 4.6 mm i.d.) using a mixture of methanol and acetone (9/1 v/v) as eluent at a flow rate of 1 mL/min. Pigment elution was monitored by means of a Jasco MD-915 diode array detector. Pigments were identified on the basis of their absorption spectra and retention times.

Room temperature and low-temperature absorption and fluorescence spectroscopy was performed with a single beam spectrophotometer described by Otte (*11*). The spectral resolution was 0.5 nm for the absorption measurements and 3 nm for room temperature and 2 nm for low-temperature fluorescence measurements. Picosecond absorption changes were measured with a home-built amplified dye laser system, operating at 10 Hz, as described earlier (*12*). Excitation was with a broad band between 850 and 950 nm. Measurements at cryogenic temperatures were performed with a Utrecs-LSO (Tartu, Estonia) or an Oxford Instruments helium flow cryostat. Glycerol (66% v/v) was added to obtain clear samples at low temperature.

RESULTS

Pigment Composition. The pigment composition of membrane fragments of strain 970 was determined by HPLC analysis. Whereas the location of the 963 nm absorption band at first suggested the presence of BChl *b*, the chromatogram clearly showed only BChl *a* to be present (Figure 1). This was esterified with phytol (peak 3), but as in other species (*13*, *14*) small amounts (less than 4%) of metabolic precursors of phytol were also found (peaks 1 and 2). Bacteriopheophytin (BPhe) *a* eluted after 23 min (peak 4). From the areas of the peaks, the ratio of BChl *a* to BPhe *a* was calculated to be 17; with 2 BPhes and 4 BChls per reaction center, this implies 30 antenna BChls per reaction center. This number is somewhat higher than that found for other LH1-only species of purple bacteria as determined by Francke and Amesz (*14*) by the same method. Still, it is lower than the number of 32 that would follow from the measurements of Karrasch et al. (*2*). There is increasing doubt, however, if the reaction center is completely surrounded by an LH1 ring (*15*−*17*), and this would lower the pigment to reaction center ratio.

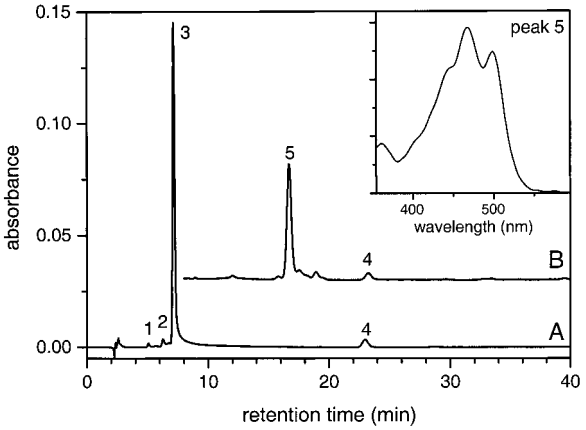

FIGURE 1: Reversed-phase HPLC elution pattern of membrane extracts of strain 970 measured at 760 nm (A) and at 450 nm (B). Chromatogram B was two times enlarged. Numbered peaks: BChl *a* esterified with (1) geranylgeraniol, (2) tetrahydrogeranylgeraniol, (3) phytol; (4) BPhe *a*; (5) carotenoid, possibly 3,4,3′,4′-tetrahydrospirilloxanthin. Inset: absorption spectrum of peak 5 in the elution solvent methanol/acetone (9/1 v/v).

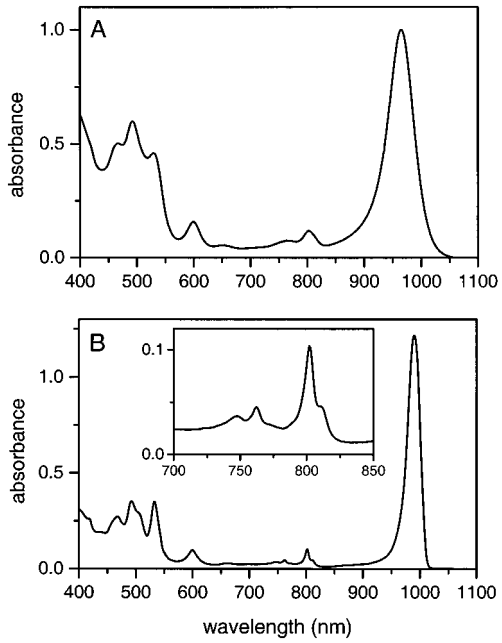

FIGURE 2: Absorption spectrum of membranes of strain 970 measured at room temperature (A) and at 6 K (B). Inset: the region of reaction center BPhe *a* and accessory BChl *a* at 6 K. The membranes were suspended in a buffer of 50 mM Tris-HCl (pH 8.3); for the low-temperature measurement 66% glycerol (v/v) was added.

The main carotenoid eluted after 17 min (peak 5), virtually the same retention time as of spirilloxanthin in this system (*9*). The absorption spectrum, however, resembled that of rhodopin (Figure 1, inset). This carotenoid is possibly 3,4,3′,4′-tetrahydrospirilloxanthin, which has a spectrum similar to that of rhodopin and which as yet has only been found in *Tcc. pfennigii* and in *Rhodospira trueperi*, two BChl *b* containing purple bacteria (*18*, *19*).

Absorption Spectra. Strain 970 has a unique absorption spectrum when compared to other photosynthetic bacteria. At room temperature the absorption maxima in the infrared wavelength region are located at 766, 802, and 963 nm (Figure 2A), the latter being the major peak in the spectrum and obviously due to LH1. The other two bands can be

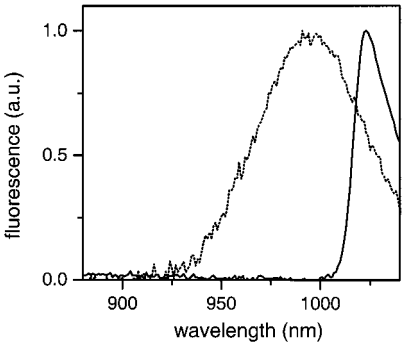

FIGURE 3: Fluorescence emission spectra of membranes at room temperature (dotted line) and at 6 K (solid), measured in buffer and in buffer with 66% glycerol (v/v), respectively. The excitation wavelength was 590 nm. The spectra were normalized at their maxima.

ascribed to reaction center BPhe *a* and BChl *a*, respectively. There is no evidence for the presence of an LH2 antenna complex. In the visible region, the BChl Q_x transition was located at 600 nm at room temperature, while carotenoid bands are seen at 464, 491, and 530 nm.

Upon cooling to 6 K the band at 963 nm narrowed considerably and shifted even further into the infrared, to 990 nm (Figure 2B). In the second derivative of the spectrum two closely spaced maxima were observed at 989 and 995 nm. A similar splitting of the LH1 peak at low temperature has been observed in *Blc. viridis* (*20*). The maxima in the carotenoid region were located at 468, 492, 507 (shoulder), and 533 nm. At 6 K, the reaction center showed four bands in the region 700−850 nm (Figure 2B, inset). Two clearly separated BPhe *a* bands at 747 and 762 nm are present. Inspection of unpublished data of C. Francke (see also ref *21*) shows that the low-temperature absorption spectra of *Rhodobium* (*Rbi.*) *marinum* (formerly *Rhodopseudomonas marina*) and *Rhodovibrio* (*Rvb.*) *sodomensis* (formerly *Rsp. sodomense*) likewise show two BPhe *a* bands. Species that clearly do not show such a double band include *Rsp. rubrum*, *Rss. parvum*, *Rhodomicrobium vannielii*, *Rhodobacter* (*Rba.*) *sphaeroides*, and *Rhodocyclus tenuis* (see also refs *9* and *21*). The absorption region of the accessory BChls *a* around 800 nm has a similar shape as, e.g., encountered in isolated reaction centers of *Rba. sphaeroides* (*22*), and in membranes of *Rbi. marinum*, with a band at 802 nm and a shoulder at 812 nm. The latter is absent in *Rsp. rubrum* and *Rvb. sodomensis* (unpublished and ref *21*).

Energy Transfer and Trapping. The pigment system of strain 970 was further characterized by fluorescence and time-resolved absorption difference spectroscopy. At room as well as at low temperature, the fluorescence emission spectrum showed a single band due to LH1, located at 994 nm at room temperature and at 1023 nm at 6 K (Figure 3). Strain 970 thus displays a rather large Stokes shift of 320 cm^{-1} at 6 K. This appears to be larger than for some other purple bacteria (*23*) and suggests strong electron−phonon coupling. The structural cause of this phenomenon is not yet known. It may be noted that for *Thc. tepidum* a similar Stokes shift has been observed (*23*).

Both at room temperature and at 6 K, the BChl Q_x and Q_y bands in the excitation spectra (Figure 4) matched the absorption ($1 - T$) spectrum, except for the reaction center bands near 760 and 800 nm that were completely lacking in

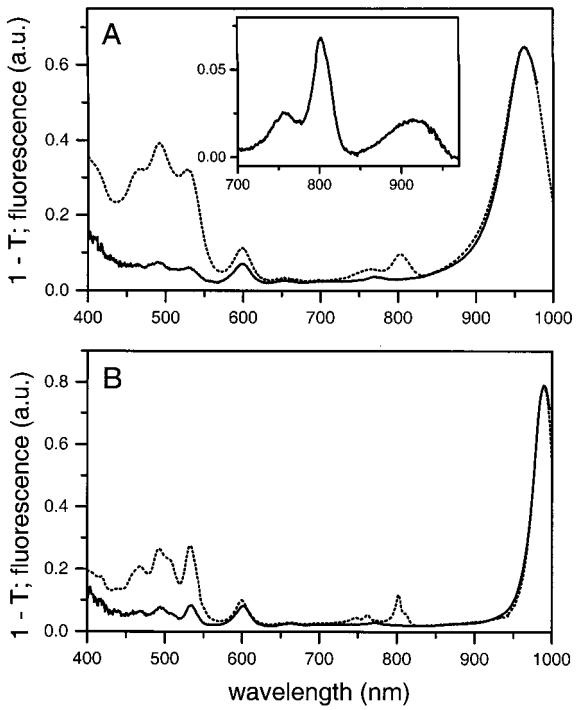

FIGURE 4: Fluorescence excitation (solid lines) and $1 - T$ spectra (dotted) at room temperature (A) and at 6 K (B). Detection wavelengths were 998 and 1015 nm, respectively. The spectra were normalized at the longest wavelength maxima. Inset: difference between the room temperature $1 - T$ and excitation spectra. Conditions as for Figure 3.

the excitation spectra. At room temperature, the difference spectrum (Figure 4A, inset) also showed a band at 920 nm, which may be ascribed to P870 (we shall use the designation P870 for the primary electron donor irrespective of the location of its maximum absorbance). Although experiments with other purple bacteria already demonstrated the absence of back-transfer to LH1 upon excitation of the accessory BChls and the BPhes (*23*−*25*), the present study now for the first time shows that this applies also for direct excitation of P870. At low temperature, however, the P870 band was not resolved in this measurement (Figure 4B). The weak band at 770 nm in the excitation spectra is probably due to a vibrational band of the antenna BChl *a*. The carotenoids show a low efficiency of excitation energy transfer to antenna BChl *a* of 25−30%, which is independent of temperature. This efficiency is in the same range as that found for a number of purple sulfur bacteria containing spirilloxanthin (*26*), to which 3,4,3′,4′-tetrahydrospirilloxanthin is structurally and metabolically related.

Time-resolved absorption difference spectra in the femtosecond and picosecond time region are shown in Figure 5. The resulting difference spectra showed a maximum bleaching at 987 nm and an excited-state absorption band around 940 nm, indicative of exciton interaction (*27*). The signal developed in a few hundred femtoseconds, presumably determined by the time resolution of our apparatus, and subsequently decayed again. The rate of decay was dependent on the detection wavelength, showing that the band has an inhomogeneous structure, which may be related to the double peak observed in the low-temperature absorption spectrum. On the blue side of the bleaching band a rapid decay component of about 0.5 ps was present. This may be ascribed

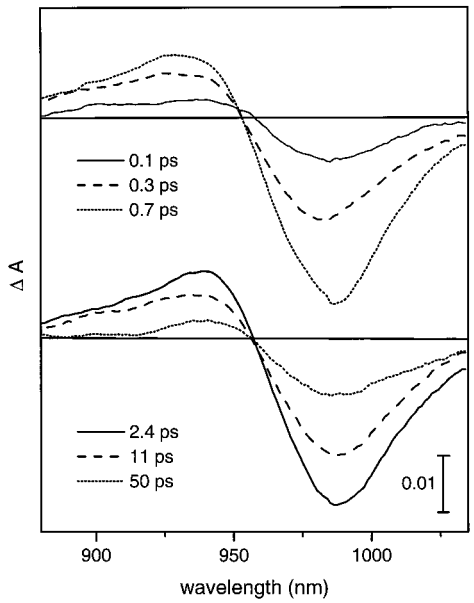

FIGURE 5: Time-resolved absorption difference spectra of membranes measured at 275 K upon excitation with a broad band between 850 and 950 nm. 10 mM ascorbate and 20 μM *N*-methylphenazonium methosulfate were added to keep P870 reduced between excitation pulses.

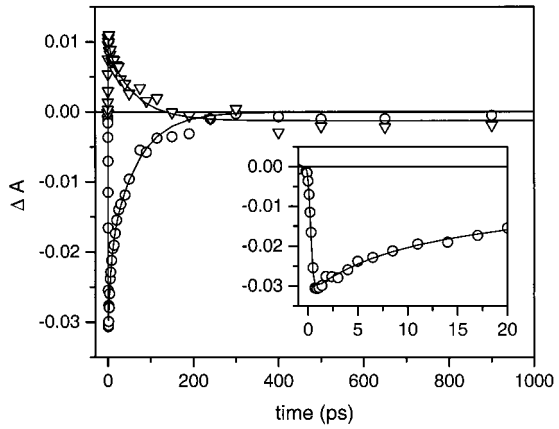

FIGURE 6: Kinetics of absorbance changes averaged from 920 to 945 nm (triangles) and from 975 to 1000 nm (circles), derived from the difference spectra. Inset: Kinetics at 975−1000 nm on an expanded time scale. The lines show biexponential fits of the decay with time constants for both wavelengths of 6.4 and 64 ps and relative amplitudes of 0.2 and 1 at 920−945 nm and of 0.5 and 1 at 975−1000 nm.

to an excitonic relaxation within the transition manifold, giving rise to a rapid red shift of the bleaching band (*28*−*30*). This rapid decay component was absent at 990 nm, near the maximum of the bleaching, where two components of 6.4 and 64 ps were seen (Figure 6). The same components were present in the excited-state absorption band around 940 nm. At 1000 nm the time constants had increased to 10.8 and 80 ps, respectively. The 64 ps decay component is in the same range as observed for other purple bacteria (*9*, *25*, *31*−*34*), and it may be ascribed to trapping by the reaction center. The 6.4 ps component is not so easy to explain. It did not appear to be due to exciton annihilation, since lowering the intensity of the excitation pulse did not influence the contribution of this fast component.

At 400 ps after excitation the signal had decayed into a broad bleaching band around 925 nm (Figure 7). This band

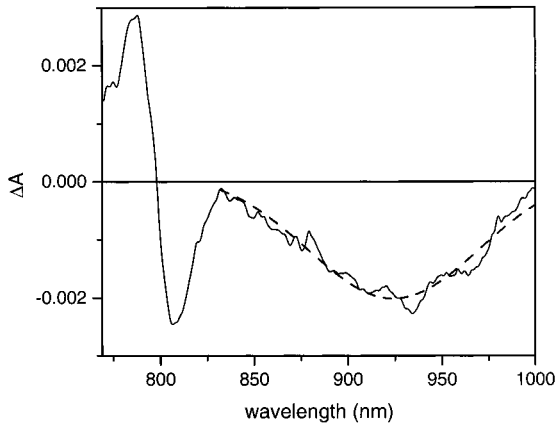

FIGURE 7: Absorbance difference spectrum of membranes, recorded as for Figure 5 and averaged between 400 and 1200 ps. The dashed line is a Gaussian fit of the bleaching band between 830 and 1000 nm.

was accompanied by a blue shift centered at 800 nm, as is typical for photooxidation of P870. As earlier observed with other species of purple bacteria (*9*, *25*, *31*, *35*), the P870 band was much smaller than that of excited LH1, even if one allows for stimulated emission in the latter. This is another indication for exciton interaction between the BChls *a* of the LH1 antenna (*31*). The bleaching band of P870, however, was at a much longer wavelength than observed for other BChl *a* containing purple bacteria and was also unusually broad (see also Figure 4A, inset). Nevertheless, the low-energy band of P870 in this species is still at considerably higher energy (425 cm^{-1}) than the LH1 antenna band.

DISCUSSION

Strain 970 belongs to the relatively few species of purple bacteria that have only an LH1 antenna complex. Its Q_y absorption band is located at 963 nm at room temperature. This complex thus constitutes by far the longest wavelength absorbing BChl *a* antenna known to date. In fact, it represents the longest absorption wavelength for BChl *a* in any known system [the red shift is almost 200 nm (2570 cm^{-1}) as compared to BChl *a* in diethyl ether]. The *Thc. tepidum* LH1 antenna previously held the record with an absorption maximum at 918 nm (*10*). The red shifts in the BChl *b* containing species can be even larger, to around 1020 nm (*18*, *36*), corresponding to a shift of 2850 cm^{-1} compared with BChl *b* in diethyl ether.

Our observations address two important aspects regarding the antenna function and organization. The first one, mentioned already, is the strong red shift of the LH1 BChl *a* Q_y band, which is almost as large as in most BChl *b* containing bacteria. This indicates that the molecular structure of BChl *b* cannot be the only factor that determines these red shifts. Possible causes include enhanced exciton interaction between the BChls (the structures of the LH1 complexes involved are essentially unknown) and specific interactions with the protein, like hydrogen bond formation (*37*). Data on the primary structure of the polypeptides of strain 970 might shed some light on this question. The other explanation, enhanced interaction between the BChls *a*, will be difficult to prove without accurate three-dimensional structures of the various LH1 complexes. Our pump−probe

absorption difference spectra (Figure 5) provide clear evidence for significant exciton interaction, but a comparison with model spectra (*30*, *37*) does not give decisive information on the extent of interaction and the so-called delocalization length.

Second, our data reinforce the notion that the rate constant of trapping in purple bacteria is essentially independent of the location of the long-wavelength absorption bands of P870 and of the LH1 antenna: about $(50\ \text{ps})^{-1}$ for a variety of BChl *a* containing species and for the BChl *b* containing species *Blc. viridis* (*9*, *25*, *31*−*34*). This also means that the efficiency of light harvesting upon excitation of LH1 is about the same in these species. For strain 970 the yield φ can be calculated to be 94% from the well-known equation $\varphi = k_0/(k_0 + k_1)$, where k_0 is the rate constant of trapping and k_1 that for the "intrinsic" decay of excited LH1 without reaction center, estimated at $10^{-3}\ \text{ps}^{-1}$ (*39*).

Relatively few difference spectra of P870 oxidation have been measured of cytoplasmic membranes; most studies have been done with isolated reaction centers. The few difference spectra, however, that have been measured with membranes give the impression that the absorption band of P870 is normally located at a somewhat shorter wavelength than that of the LH1 antenna (*10*, *25*, *34*, *36*, *40*−*42*). Relatively large energy gaps of several hundreds of inverse centimeters between the absorption maxima of P870 and LH1 have been observed in *Thc. tepidum* ($240\ \text{cm}^{-1}$) and *Rss. parvum* ($285\ \text{cm}^{-1}$) and in the BChl *b* containing species *Blc. viridis* ($350\ \text{cm}^{-1}$) (*9*, *10*, *36*). For strain 970 we find an even larger gap of $425\ \text{cm}^{-1}$.

The remarkable uniformity of the trapping constant is hard to explain, no matter whether the trap-limited or the transfer-to-trap-limited model for energy conversion is used (*30*, *42*). The absence of back-transfer from the reaction center to the antenna (*23*−*25*) gives clear evidence that the trap-limited model does not apply to purple bacteria anyway. Moreover, in this model the large energy gap in strain 970 (and also in *Blc. viridis*) would effectively prevent charge separation by shifting the equilibrium toward the excited antenna BChls.

The transfer-to-trap-limited model also poses serious problems, but this model at least offers the advantage that one does not have to violate the laws of thermodynamics to make it fit the observations. From a comparison of Figures 3 and 7 it can be seen that there is actually some overlap between the antenna BChl fluorescence and the P870 absorption band, due to the strong broadening of both bands. However, reverse energy transfer to the antenna would be heavily favored, unless the lifetime of excited P870 (P870*) would be reduced by some relaxation process (*36*). Perhaps a rapid delocalization of the excitation energy of P870* as proposed by Fischer and Scherer (*43*) could be involved.

Finally, the position of the LH1 absorption band of strain 970 by itself raises some questions with respect to the effectiveness of light harvesting under natural conditions. The absorption maximum is located near the first infrared absorption maximum of water at 975 nm (*44*). The absorbance of water at 963 nm is $0.46\ \text{cm}^{-1}$ (*44*). This means that a layer of 5 cm already reduces the irradiation to 0.5% of the incident intensity. The competitive advantage of absorbing light at longer wavelengths than other BChl *a* containing purple bacteria thus rapidly turns into a disadvantage below the water surface. Recently, indications were found for bacteria absorbing around 980 nm in microbial mats from Antarctica (*45*). It would be of interest to determine the nature of these bacteria, whether they represent strain 970 or yet another species of purple bacteria. It should also be noted that no purple bacteria are known with major BChl *b* absorption bands at shorter wavelengths than 986 nm. Possibly, in strain 970 the maximum for the absorption wavelength of BChl *a* is reached and BChl *b* is needed to get to longer wavelengths.

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