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Spectroscopic properties of antenna complexes of *Rhodobacter sphaeroides* in vivo

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Intact membranes of antenna mutants of *Rhodobacter sphaeroides* obtained by chemical mutagenesis containing only the B800–850 or the B875 reaction center complex were used to study the spectral properties of these antenna complexes separately in vivo. Wild-type spectral characteristics were restored to each mutant, following complementation by the relevant gene. It is shown that the absorption spectra of recombinant strains and of wild-type *Rhodobacter sphaeroides* can be analyzed in terms of those of the separate complexes as observed in the mutants. Distinct differences occur between the spectra of the antenna complexes isolated by means of detergent solubilization of the membrane and those of the mutants. Measurements of absorption and flash-induced absorption difference spectra and of linear dichroism and fluorescence polarization spectra at low temperature indicate that in the intact membrane the previously characterized bacteriochlorophyll Q_y absorption bands near 800, 850 and 875 nm display an optical inhomogeneity and that they all contain a relatively weak transition at longer wavelength, the orientation of which is more parallel to the membrane plane than the orientation of the main transitions. Rapid and efficient energy transfer to the long-wave component ($BChl_{870}$) in the B800–850 complex could be demonstrated. Some of the long-wave transitions are also observable at room temperature. They may reflect the mode of aggregation of the complexes in their lipid environment and, by increased overlap between donor emission and acceptor absorbance, serve to facilitate energy transfer within the antenna system.

Introduction

The pigments of photosynthetic purple bacteria are bound to proteins which are embedded in the intracytoplasmic membrane. The majority of these pigments serve as an antenna to funnel the excitation energy towards the photochemical reaction centers. The antenna of *Rhodobacter sphaeroides* consists of two different pigment protein complexes, B800–850 and B875. The B800–850 complex is characterized by two strong absorption bands of BChl *a* in the near infrared with maxima near 800 and 850 nm. The long-wavelength complex B875 with a major absorption band at 875 nm supposedly surrounds and interconnects the

Abbreviations: BChl, bacteriochlorophyll; B800–850 and B875, antenna complexes identified by their near-infrared absorption maxima; CD, circular dichroism; LD, linear dichroism.

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reaction centers [1,2]. In addition to BChl *a*, membranes of *Rb. sphaeroides* contain carotenoids which transfer their excitations to BChl *a* [3,4].

Both pigment-protein complexes have been reported to contain two different subunits, the α and β polypeptides [5,6], which form large aggregates in the membrane [2,7]. The primary struc-ture of these polypeptides has been determined and it was found that both subunits contain a hydrophobic stretch [5,6] which presumably is α-helical and traverses the membrane [8]. The antenna complexes of *Rb. sphaeroides* have been solubilized by disrupting the membrane by means of detergents and purified by various fractionation procedures [9–11].

For a quantitative understanding of the light-harvesting properties of these complexes and of their function in transferring excitation energy to the reaction center detailed information on their spectroscopic properties is essential. The properties of the isolated complexes have been studied extensively [9–13], but it may be assumed that the spectral characteristics of these antenna complexes are not only affected by pigment–pigment and pigment–protein interactions, but also by the aggregation state of the polypeptides and by the lipid environment [14]. One must therefore expect that solubilization of the antenna complexes would cause some significant changes in their spectroscopic properties, but until now it was not possible to study B800–850 and B875 separately in situ.

Recently, mutants of *Rb. sphaeroides* have been obtained that contain only one of the two antenna complexes. In this report a spectral characterization of these mutants will be presented. It will be shown that the spectral properties of the antenna of *Rb. sphaeroides* in its native environment are in some respects significantly different from those of the isolated complexes.

Materials and Methods

Mutant M21, which lacks the B800–850 antenna complex, and mutant NF57, which lacks the reaction center and the B875 complex, were isolated following chemical mutagenesis of *Rb. sphaeroides* NCIB 8253 with *N*-nitrosoguanidine. The wild-type absorbance spectrum was restored to M21 by *puc* A, B genes encoding the α and β polypeptides of the B800–850 complex, which are borne on plasmid pMA81 [15]. The recombinant strain is designated M21[MA81]. The wild-type absorbance spectrum and photosynthetic competence were restored to mutant NF57 by a clone pNH1491 which contains the gene encoding the reaction center H-subunit [16] carried on plasmid pNH1491 (Hunter, C.N., unpublished results). The recombinant strain is designated NF57[NH1491]. Gene transfers were carried out using a conjugation procedure based on the transfer functions of RP1 (Hunter, C.N., unpublished results). Cells of NF57 were grown aerobically in the dark, whereas those of M21 were grown photosynthetically in the absence of oxygen. Membranes were prepared by sonication of the cells, followed by purification of the crude cell extract on a sucrose step gradient (15% and 40% w/w sucrose). After 4 h of centrifugation at $100\,000 \times g$ membranes were collected from the interface. For all experiments the membranes were suspended in a 10 mM Tris buffer (pH = 8.0) containing 66% (v/v) glycerol to obtain clear samples upon cooling. Data on the isolated complexes, prepared from wild-type *Rb. sphaeroides*, were taken from Refs. 12 and 13.

Both mutants were tested for reaction center activity by monitoring the reversible photobleaching at 868 nm under continuous illumination at room temperature (in the absence of glycerol). Assuming equal in vivo extinction coefficients for the antenna BChl *a* and for the primary electron donor P-870 at this wavelength, we calculated the size of the photosynthetic unit to be 40 BChl a molecules for M21. This number agrees well with the number of B875 molecules per reaction center found in wild-type *Rb. sphaeroides* and a number of other strains and species of purple bacteria [17]. No reversible bleaching was observed in NF57, indicating that this mutant contains less than one reaction center per 10 000 BChl *a* molecules. Fluorescence induction was also measured for both mutants. In M21 the fluorescence yield increased by a factor of 2.6 upon closing of the reaction centers, whereas no fluorescence induction was observed in NF57.

The apparatus used to measure absorption, fluorescence emission, excitation and fluorescence polarization spectra is described in Ref. 18. Linear

and circular dichroism spectra were recorded with the apparatus described in Ref. 19. For measurement of linear dichroism the samples were oriented by biaxial pressing of a polyacrylamide gel [20]. The anisotropy ratio (LD/A) was obtained by measuring the absorption and linear dichroism of the same oriented sample. The picosecond absorbance difference measurements were performed with the spectrometer described in Ref. 21 equipped with an optical multichannel analyzer for detection [22]. Excitation in the infrared was obtained using a parametric oscillator.

Results

Absorption spectra

Fig. 1 shows the absorption spectra of the NF57 and M21 mutants of *Rb. sphaeroides* measured at 4 K. The spectrum of NF57 (Fig. 1A) shows the characteristic bands of the B800–850 complex at 797 and 852 nm. Neither the absorption spectrum nor its second nor its fourth derivative gave any evidence for a band or shoulder near

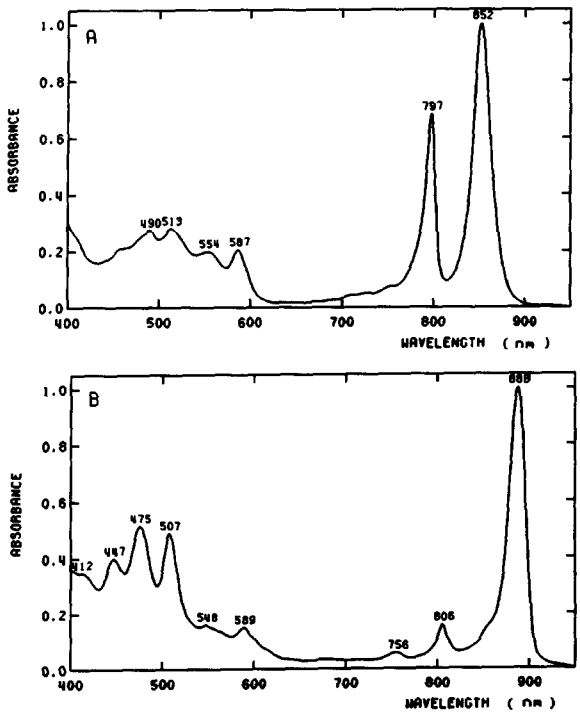

Fig. 1. Absorption spectra of membranes of the mutants NF57 (A) and M21 (B) recorded at 4 K.

890 nm, indicating the virtual absence of the B875 complex in this mutant. In the visible region both the Q_X transition of BChl *a* at 587 nm and the bands of carotenoid can be seen. The bands at 554 and 490 nm are probably due to spheroidenone, whereas the shoulder at 476 nm and, in part, the band at 513 nm may be attributed to spheroidene. The formation of spheroidenone is presumably due to the aerobic growth conditions used [23].

The spectrum of M21 (Fig. 1B) is dominated by the Q_y band of the B875 complex, located at 888 nm. Around 850 nm a weak shoulder is observed that can be attributed to some residual B800–850. The height of this band varied for different batches of cells with an upper limit of 3% of the absorbance at 888 nm of the B875 complex. Reaction center bands can be discerned at 756 and 806 nm. The second derivative spectrum showed two bacteriopheophytin bands located at 753 and 759 nm. In the carotenoid region the major contribution is from spheroidene with maximum at 447, 475 and 507 nm. Only a weak band of spheroidenone is observed at 548 nm. The band at 412 nm presumably arises from cytochrome absorption.

Fig. 2 compares the absorption spectra of membranes of the mutants in the near-infrared region with those of the purified complexes isolated by means of detergent solubilization, as measured by Kramer et al. [12,13]. The bands near 800 and 850 nm of NF57 (Fig. 2A) are considerably broader than of the isolated complex and the maximum of the latter band is shifted by 3 nm to longer wavelengths. The opposite is the case for M21 (Fig. 2B), where the infrared absorption band is narrower than that of the isolated B875 complex and somewhat blue-shifted. These observations thus show that there are distinct differences between the spectroscopic properties of the antenna complexes contained in the membrane, as observed in the mutants, and those of the isolated complexes.

An attempt was made to fit the 4 K absorption spectrum of the recombinant strain M21[MA81] with a linear combination of the spectra of both antenna complexes using a least-square approximation (Fig. 3). In this strain the B800–850 complex is restored (see Materials and Methods and Ref. 15). In the first case (Fig. 3A) the spectra of

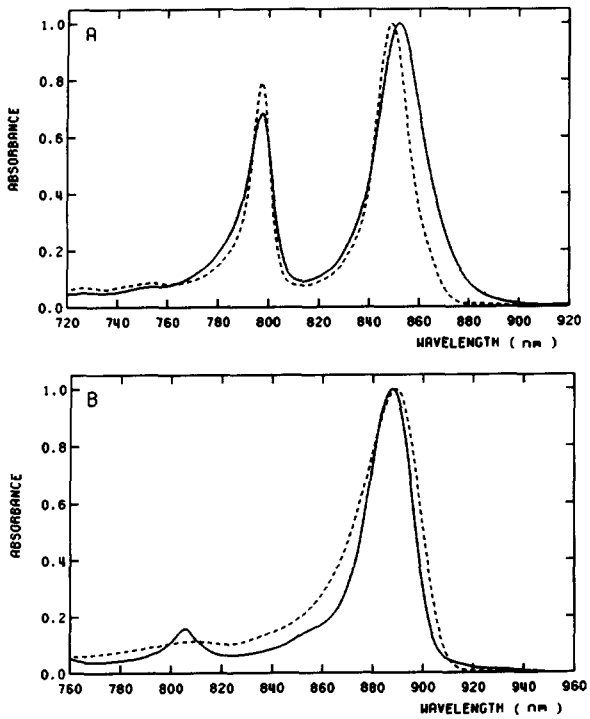

Fig. 2. Q_y region of the 4 K absorption spectra of NF57 (A) and of M21 (B) as indicated by the solid lines. The dashed lines give the spectra of the isolated B800–850 complex (A) and of the isolated B875 complex (B) (see text).

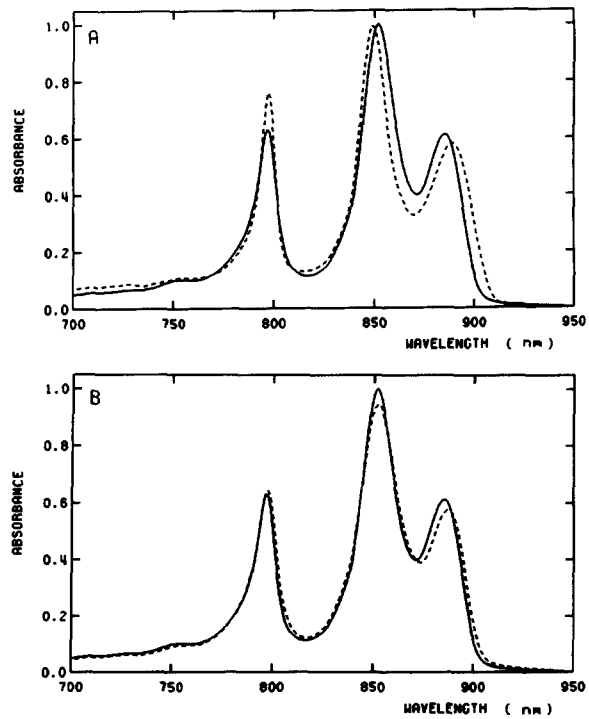

Fig. 3. Spectral analysis of the Q_y region of the 4 K absorption spectrum (———) of the recombinant strain M21[MA81]. The dashed lines give a linear combination of the spectra of the isolated B800–850 and B875 complexes (A) and of the spectra of the mutants NF57 and M21 (B).

the isolated B800–850 and B875 complexes were used, whereas in the second case (Fig. 3B) the spectra of NF57 and M21 were used. It is clear that a much better fit was obtained in the latter case: large deviations with respect to widths and locations of the $BChl_{850}$ and $BChl_{875}$ bands were observed when the absorption spectrum was fitted with the spectra of the isolated complexes. Similar results were obtained with membranes from recombinant strain NF57[NH1491] and with wild-type *Rb. sphaeroides*. We thus conclude that the spectra of the complexes in vivo in the wild type and in the mutants of *Rb. sphaeroides* are very similar and clearly different from those of the isolated complexes.

Gaussian deconvolution

Over the region 810–930 nm the 4 K absorption spectra were deconvoluted into Gaussian-shaped components. It can be seen from Fig. 4A that the spectrum of NF57 cannot be accounted for by a single band, but a good fit was obtained with two additional components, located at 832 and 870 nm. The position of these three bands agreed well with those in the fourth-derivative spectra (not shown). The band at 832 nm can be ascribed to a vibrational sub-band of $BChl_{850}$, but the component at 870 nm clearly demonstrates a spectral inhomogeneity of the 850-nm band. Deconvolution of the absorption spectra of wild-type *Rb. sphaeroides* and of the recombinant strains NF57[NH1491] and M21[MA81] also yielded an additional component at 870 nm (not shown). We shall refer to the bacteriochlorophyll responsible for the band near 870 nm as $BChl_{870}$.

The second- and fourth-derivative spectra of M21 showed the presence of two components with about equal strengths with maxima at 883 and 891 nm. However, a satisfactory deconvolution into Gaussian components was only obtained with two additional minor components (Fig. 4B). The band near 899 nm accounts for about 10% of the in-

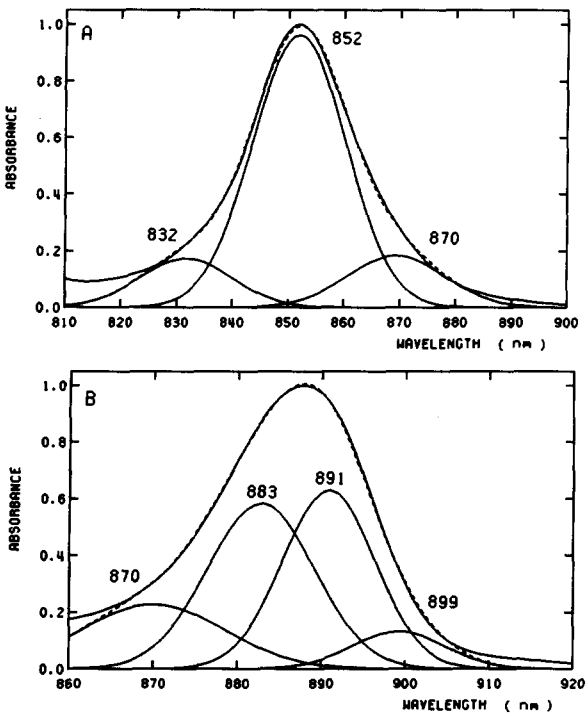

Fig. 4. Gaussian deconvolution of the 4 K absorption spectra of NF57 (A) and of M21 (B). The dashed lines give the sum of the Gaussian components.

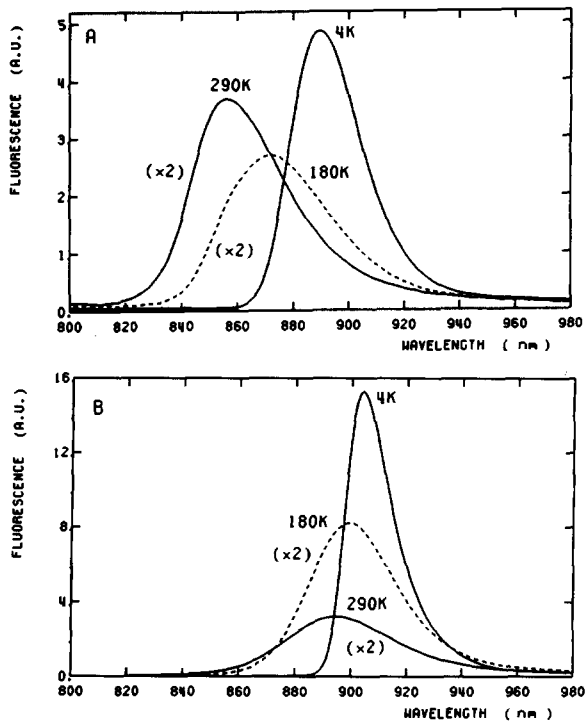

Fig. 5. Emission spectra of NF57 (A) and of M21 (B) measured at various temperatures and excited at 590 nm.

tegrated absorption. The presence of a minor long-wavelength component, called $BChl_{896}$ has been suggested in the literature [13,24]. Its existence is also indicated by linear dichroism and fluorescence polarization measurements (see below) and time-resolved polarized absorption [25]. The rather broad band near 870 nm which is needed to obtain a good fit presumably consists of several vibrational sub-bands.

Fluorescence

The emission spectra of NF57 and M21 measured at various temperatures are shown in Fig. 5. At room temperature the fluorescence maximum of NF57 (Fig. 5A) is situated at 856 nm, whereas a maximum at 889 nm was observed at 4 K. At intermediate temperatures (210–120 K) the second derivative spectra showed the presence of two emission bands. At 180 K these were located at 858 and 872 nm; the first one can be discerned as a shoulder on the main emission band (broken line). The intensity of the long-wavelength component in the second derivative spectra increased upon cooling, indicating a shift in the equilibrium between the two states giving rise to both emissions. Above 210 K only the short-wave emission could be discerned, whereas below 120 K most emission occurred from the long-wavelength transition. Although the emission band at 4 K was significantly broader than of the isolated complex [12], the short-wave component could not be discerned at this temperature. However, for cells from a different batch of culture this component was found to be clearly present in the 4 K fluorescence [2].

Both the absence of significant emission from $BChl_{800}$ and the excitation spectrum of the fluorescence detected at 905 nm (not shown) indicated efficient energy transfer from $BChl_{800}$ to $BChl_{850}$ in agreement with earlier results obtained with isolated complexes [12]. Furthermore, the excitation spectrum also indicated that the excitation are efficiently transferred from $BChl_{850}$ to $BChl_{870}$. The average efficiency of energy transfer from carotenoids to BChl *a* was found to be 65%.

The emission spectrum of M21 (Fig. 5B) at room temperature had a maximum at 897 nm. Upon cooling this band shifted to 905 nm and closely resembled the B875 emission as observed in wild-type *Rb. sphaeroides* [4]. No evidence for the existence of more than one emission band was found in this case. Above 850 nm and in the Q_X region the 4 K excitation spectrum of M21 closely followed the BChl *a* absorption, while a value of 50% was found for the transfer efficiency from the carotenoids. An efficiency of less than 20% was obtained upon excitation in the reaction center bands at 756 and 806 nm. A similar low efficiency was earlier observed for a number of purple bacteria at room temperature [26] and for the green filamentous bacterium *Chloroflexus aurantiacus* at 4 K [27]. The small amount of residual B800–850 present in M21 transferred its excitation energy with an efficiency of about 60%.

The polarization of the fluorescence measured at 4 K is shown in Fig. 6. In the case of NF57 the 920 nm emission due to $BChl_{870}$ was strongly depolarized when excited in the major absorption band at 850 nm, resulting in a *p*-value of only 0.06. Upon excitation at longer wavelengths the *p*-value rose to 0.14 at 875 nm. The degree of polarization of the B875 emission in M21, measured at 940 nm, was low between 850 and 880 nm (0.07), but rose with increasing wavelengths to a value of 0.42 at 905 nm, in agreement with the results of Kramer et al. [13], with the isolated complex and with chromatophores of wild-type *Rb. sphaeroides* and mutant R26, indicating that upon direct excitation of the long-wavelength component ($BChl_{896}$) no depolarization of fluorescence occurs.

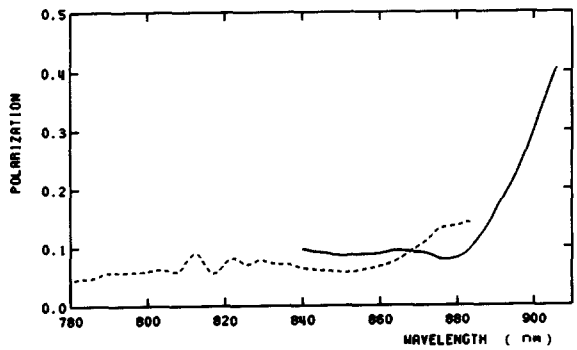

Fig. 6. Fluorescence polarization spectra of M21 (———) and of NF57 (- - - - -) detected at 940 and 920 nm, respectively, and measured at 4 K.

Circular dichroism

Circular dichroism spectra of NF57 and M21 measured at 77 K are shown in Fig. 7. NF57 showed a strong CD signal with positive and negative bands at 853 and 868 nm, respectively. In contrast to the isolated B800–850 complex [12] the CD signals around 800 nm were relatively weak (Fig. 7A). The band due to B875 and to the reaction center in the spectrum of M21 (Fig. 7B) were located at 892–903 nm and 805–816 nm, respectively. The CD signal of B875 was significantly narrower than in the isolated complex [13]. The strength of the CD signal at 855–870 nm in this mutant corresponds to a B800–850 content of 3% relative to B875, in agreement with the absorption spectrum (Fig. 1B). In the spectra of the recombinant strains NF57[NH1491] and M21[MA81] (not shown) CD bands of B800–850

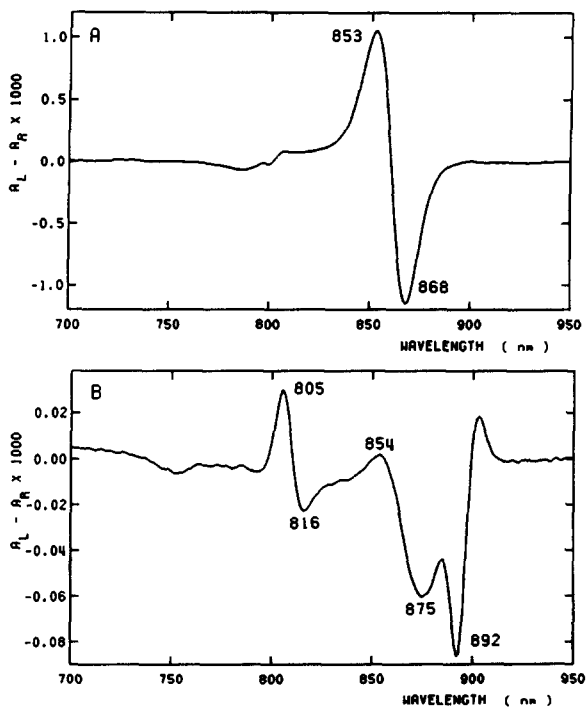

Fig. 7. Circular dichroism spectra of NF57 (A) and M21 (B) measured at 77 K. The absorbance was 1.00 at 850 and at 875 nm, respectively (293 K).

were observed at 854 and 863 nm, and at 856 and 869 nm, respectively, together with weaker CD signals due to B875 and the reaction center.

Linear dichroism

Fig. 8 shows the LD spectra of NF57 and M21 measured at 4 K. The membranes were oriented by biaxial pressing [20]. As these membranes probably orient with the plane of the membrane parallel to the orientation axis it follows that the Q_y transitions of $BChl_{800}$, $BChl_{850}$ and $BChl_{875}$ are oriented preferentially parallel to the plane of the membrane [28], in agreement with earlier work with the wild strain. The polymerization process used in preparing the gel had some effect on the spectral properties of NF57. The intensity of the band at 800 nm, which is known to be somewhat unstable [29], was reduced by 45% and the band near 850 nm was red-shifted by 4 nm. The LD spectrum of NF57 (Fig. 8A) is similar to that published for the isolated B800–850 complex [12] with positive signals in the Q_y region and negative

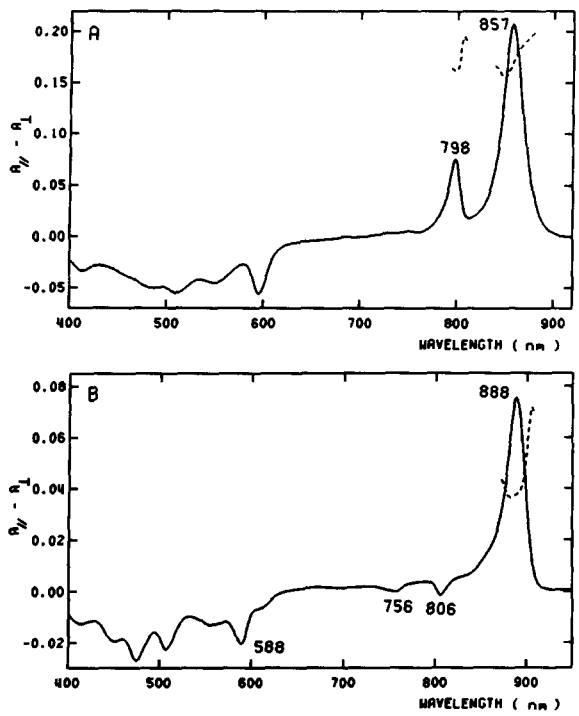

Fig. 8. Linear dichroism ($A_{\parallel} - A_{\perp}$) spectra of NF57 (A) and of M21 (B) measured at 4 K. The broken lines show the anisotropy ratios ($\Delta A/A$) on an arbitrary scale. The absorbance was 0.40 at 850 and at 875 nm, respectively (293 K).

bands for Q_x transitions and the carotenoids. The anisotropy ratio (LD/A) is given by the dashed line and shows an increase across the band at 850 nm. This indicates that the long-wavelength pigment ($BChl_{870}$) makes a smaller angle with the plane of the membrane than $BChl_{850}$, in agreement with the relatively low polarization value in the region below 860 nm (Fig. 6). A similar increase is also observed for the band at 800 nm. In the region between 560 and 620 nm the minimum at 595 nm indicates a largely perpendicular orientation for the Q_x transition of $BChl_{850}$, whereas the maximum at 583 nm reflects the more in-plane orientation of $BChl_{800}$ in agreement with Refs. 30, 31 and 12. The approximately constant anisotropy ratio in the carotenoid region indicates that spheroidene and spheroidenone have the same average orientation.

The LD spectrum of M21 (Fig. 8B) shows a strong positive band at 888 nm, negative bands for the reaction center components at 756 and 806 nm, the Q_x transition at 588 nm and the carotenoid molecules. The rise of the anisotropy ratio across the Q_y band can again be explained by a more parallel orientation of the long-wavelength pigment ($BChl_{896}$). It should be noted that the spectral inhomogeneities of the 850-nm band in NF57 and of the 875-nm band in M21 were also observed in the LD spectra at room temperature (not shown).

In contrast to what was observed in the LD spectrum of isolated reaction centers of *Rb. sphaeroides* [32,33] both reaction center bands of M21 have a negative sign, corresponding to an angle of less than 55° between the corresponding transitions and the normal to the membrane, which normal approximately coincides with the C_2 axis of the reaction center [34]. The positive LD measured in the BChl *a* band near 800 nm in isolated reaction centers may be due to a different orientation of these particles as compared to those of the membranes. An analysis of the spectrum of the reaction center of *C. aurantiacus* in fact indicates that the orientation axis in a pressed gel may differ considerably from the C_2 symmetry axis [35].

Picosecond absorbance-difference spectroscopy

The formation of excited bacteriochlorophyll

was also studied by measurement of the flash-induced absorbance changes in the picosecond time range. Fig. 9 shows the absorbance-difference spectrum of membranes of NF57, measured at 77 K with coinciding 35 ps probe and excitation pulses. The wavelength of excitation was 800 nm. At wavelengths above 854 nm the spectrum shows a broad negative signal, with a minimum at 865 nm and a shoulder at approx. 875 nm. Below 854 nm an absorbance increase is seen with a maximum at 847 nm.

The general shape of the difference spectrum is similar to those observed by Nuijs et al. [36,37] upon excitation of membranes of purple bacteria and of the green bacterium *Chloroflexus aurantiacus*, which spectra were explained in terms of a bleaching of BChl due to ground-state depletion upon formation of the singlet excited state, together with a blue shift of the absorption band of several neighboring BChl molecules. The spectrum of Fig. 9 can be explained in a similar way, with the assumption that the shoulder near 875 nm is due to the formation of excited $BChl_{870}$. These results indicate that energy transfer from $BChl_{800}$ to $BChl_{850}$, and hence to $BChl_{870}$, is essentially completed within the duration of the flash, and proceeds with a time constant of less than 35 ps, in general agreement with conclusions obtained from measurements of fluorescence yields and picosecond spectroscopy on wild type *Rb. sphaeroides* [2,4,25].

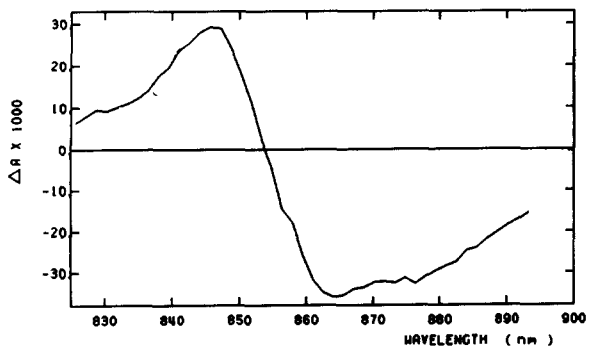

Fig. 9. Absorbance-difference spectrum of NF57 with coinciding probe and excitation pulses (35 ps), measured at 77 K. Excitation at 800 nm. The absorbance was 0.80 at 850 nm (77 K).

Discussion

The two light-harvesting complexes of *Rb. sphaeroides*, B800–850 and B875, have been studied extensively in the isolated form [9–13,30]. However, little attention has been given to the possible effects of the isolation techniques on the spectroscopic properties of the complexes. The use of membranes of the mutants NF57 and M21 provided us with an opportunity to study the characteristics of the antenna complexes in the absence of detergent-induced artefacts. The use of these mutants as a probe for the characteristics of B800–850 and B875 in vivo is justified by the fact that the optical properties of the wild type and of the recombinant strains can be analyzed in terms of those of the separate complexes as observed in the mutants. Such an analysis was not possible on the basis of the spectral properties of the isolated complexes.

Analysis of the absorption, LD and fluorescence polarization spectra of mutant NF57 indicates the presence of a minor long-wave transition, which accompanies the main Q_y band near 850 nm. Gaussian deconvolution of the absorption spectrum indicates that the transition is located at 870 nm at 4 K and comprises about 15% of the total absorption of the 850 nm band. It appears to be absent in the isolated B800–850 complex [12,31], although it should be noted that Bolt and Sauer [30] reported an inhomogeneity in the LD spectrum of their preparation. The increase in the anisotropy ratio observed by us across the 800 nm band in NF57 suggests the presence of a minor long-wave component near 806 nm, but this component could not be resolved in the absorption spectrum.

The fluorescence spectra show that at room temperature most emission occurs from $BChl_{850}$, but upon cooling energy transfer to $BChl_{870}$ becomes predominant and at 4 K most fluorescence is emitted from this long-wave species. The excitation spectrum at this temperature indicates that the excitation energy is efficiently transferred from $BChl_{800}$ and $BChl_{850}$ to $BChl_{870}$. This is confirmed by the absorbance-difference spectrum measured at a picosecond time-scale which indicates that energy transfer to $BChl_{870}$ is essentialy complete within 35 ps. It seems therefore reason-

able to assume that $BChl_{870}$ functions as an intermediate to funnel excitations from B800–850 to B875.

Our experiments with M21 similarly indicate the presence of a minor component absorbing at 899 nm at 4 K, which may account for 10% of the total Q_y absorption of the B875 complex, and is presumably identical to $BChl_{896}$ observed in the wild-type, mutant R26 and in the isolated complex [13]. In addition to this a Gaussian deconvolution of the 4 K absorption spectrum showed the presence of two major transitions of about equal intensity at 883 and 891 nm.

Several models [12,31,38–40] have been proposed for the antenna structure of purple bacteria. These models have recently been reviewed and discussed in terms of the spectral properties of the antenna complexes by Pearlstein [41]. The models of Kramer et al. [12] and Breton et al. [31] and of Scherz and Parson [38] concern the pigment-pigment interactions within the 'basic units' of the B800–850 complex, and in their present form do not readily explain the existence of the minor long-wave transitions ($BChl_{806}$ and $BChl_{870}$). As a matter of fact our results indicate that at least for the B800–850 complex the long-wave absorption bands may reflect the mode of aggregation of the antenna complexes in the photosynthetic membrane.

Models for the supramolecular organization of the antenna, mainly based on the primary structures of the polypeptides, have been proposed by Loach et al. [39] and Zuber and coworkers [40]. The model of Zuber et al. assumes a "cyclic unit structure" for the arrangement of the B800–850 and B875 subunits. In spite of the overall symmetry of such an arrangement, the local environment may vary significantly for different BChl *a* molecules in such a system, giving rise to a spectral red shift of their absorption bands. Measurements of excitation annihilation [2] indicate that $BChl_{870}$ is arranged in clusters of approx. eight pigment molecules. This suggests that these local assymetries may affect groups of neighboring BChl *a* molecules.

We conclude that the presence of a long-wave absorbing BChl associated with the major spectral forms is a general property of the light-harvesting system of *Rb. sphaeroides* and possibly other species of purple bacteria. Two of these ($BChl_{870}$ and $BChl_{896}$) are also observable at room temperature. It seems probable that $BChl_{870}$ has an important role in energy transfer by facilitating the transfer of excitation energy from the B800–850 complex to B875 by an increased overlap of donor emission and acceptor absorption. $BChl_{896}$ may serve to funnel excitation energy towards the reaction center [24,25,42,43].

Acknowledgements

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