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REGULAR PAPER

Probing binding site of bacteriochlorophyll *a* and carotenoid in the reconstituted LH1 complex from *Rhodospirillum rubrum* S1 by Stark spectroscopy

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Abstract Stark spectroscopy is a powerful technique to investigate the electrostatic interactions between pigments as well as between the pigments and the proteins in photosynthetic pigment–protein complexes. In this study, Stark spectroscopy has been used to determine two nonlinear optical parameters (polarizability change $\mathrm{Tr}(\Delta\boldsymbol{\alpha})$ and static dipole-moment change $|\Delta\boldsymbol{\mu}|$ upon photoexcitation) of isolated and of reconstituted LH1 complexes from the purple photosynthetic bacterium, *Rhodospirillum* (*Rs.*) *rubrum*. The integral LH1 complex was prepared from *Rs. rubrum* S1, while the reconstituted complex was assembled by addition of purified carotenoid (all-*trans*-spirilloxanthin) to the monomeric subunit of LH1 from *Rs. rubrum* S1. The reconstituted LH1 complex has its Q_y absorption maximum at 878 nm. This is shifted to the blue by 3 nm in comparison to the isolated LH1 complex. The energy transfer efficiency from carotenoid to bacteriochlorophyll *a* (BChl *a*), which was determined by fluorescence excitation spectroscopy of the reconstituted LH1 complex, is increased to 40%, while the efficiency in the isolated LH1 complex is only 28%. Based on the differences in the values of $\mathrm{Tr}(\Delta\boldsymbol{\alpha})$ and $|\Delta\boldsymbol{\mu}|$, between these two preparations, we can calculate the change in the electric field around the BChl *a* molecules in the two situations to be $\mathbf{E}_\Delta \approx 3.4 \times 10^5$ [V/cm]. This change can explain the 3 nm wavelength shift of the Q_y absorption band in the reconstituted LH1 complex.

Keywords Bacteriochlorophyll *a* · Binding-site · Carotenoid · LH1 complex · Purple photosynthetic bacterium · Reconstitution · *Rhodospirillum rubrum* · Stark spectroscopy

Abbreviations

BChl *a*	Bacteriochlorophyll *a*
LDAO	*N,N*-lauryl dimethyl aminooxide
LH1	Light-harvesting 1 complex
RC	Reaction centre
Rs. rubrum S1	*Rhodospirillum rubrum* strain S1

Introduction

Reconstituting carotenoids into carotenoidless photosynthetic pigment–protein complexes has proved to be a powerful approach for the study of carotenoid function. Both carotenoidless reaction centres and light-harvesting complexes have been successfully reconstituted with a range of carotenoids (Frank 1999). Reconstitution can only be used in these types of experiments when the pigments occupy the same binding-site in the reconstituted and wild-type pigment–protein complexes. Moreover, they must adopt similar structures and interact with the protein in the same way as in the wild-type complexes. This has been tested only in the case of reaction centres from the

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carotenoidless mutant of *Rhodobacter sphaeroides* strain R26.1, by comparing the X-ray crystal structure of wild-type carotenoid-containing reaction centres with those reconstituted with a synthetic carotenoid, 3,4-dihydrospheroidene (Roszak et al. 2004). There have been several studies involving reconstituting carotenoids into the LH1 complex from *Rhodospirillum* (*Rs.*) *rubrum*. Unfortunately it has not yet been possible to crystallize the LH1 complex from *Rs. rubrum*. Therefore other indirect spectroscopic methods must be applied to investigate whether, carotenoids reconstituted into carotenoidless LH1 complexes faithfully recreates the wild-type situation.

Reconstitution of the LH1 complex in vitro was first reported by Davis et al. (1995). They isolated the component molecules of the LH1 complex from *Rs. rubrum* (carotenoids, BChl *a*, LH1-α and LH1-β polypeptides) and performed the reconstitution in the presence of the detergent, β-octylglucoside (β-OG). These reconstituted LH1 complexes had a Q_y absorption maximum at 880 nm at 4°C, which is consistent with that of the LH1 complexes in chromatophores. However, in their preparation, the vibrational structure of the $S_0 \rightarrow S_2$ absorption band of the carotenoids was not very clear, since they used a mixture of carotenoids extracted from *Rs. rubrum* S1. Recently, reconstitutions were reported into the LH1 complex from *Rs. rubrum* using a range of well-defined carotenoids (Akahane et al. 2004; Fiedor et al. 2004; Fiedor and Scheer 2005). These studies used a series of purified carotenoids with different extents of π-conjugation. This series of reconstituted LH1 complexes was used to study the singlet-singlet energy-transfer processes from carotenoid to BChl *a* (Akahane et al. 2004). They have also been used to investigate the triplet-triplet energy-dissipation processes (Kakitani et al. 2007).

In all the above cases, the authors used absorption spectroscopy as the main criterion to evaluate the quality of the reconstituted LH1 complexes. However, this is rather insensitive to small changes of the reconstituted complexes. We have used Stark spectroscopy, which is more sensitive to changes in the pigments' local environment, to more carefully evaluate the quality of the reconstituted complexes. Stark spectroscopy has a well-established theoretical basis and can detect changes in the intermolecular interactions in pigment–protein complexes (Yanagi et al. 2004, 2005). In this study, we have reconstituted the LH1 complex from *Rs. rubrum* S1 by addition of purified all-*trans*-spirilloxanthin to the monomeric subunit of the LH1 complex. Stark spectroscopy has been used to compare the electrostatic environments of carotenoids and bacteriochlorophylls in the isolated LH1 complex from *Rs. rubrum* S1 and the reconstituted LH1 complex. The data show that there is substantial difference of the electrostatic field around the bacteriochlorophylls between the reconstituted and purified complete LH1 complexes, although that around carotenoid is essentially the same.

Materials and methods

Growth of the cells of *Rs. rubrum* S1 and preparation of chromatophores

Cells of *Rs. rubrum* strain S1 were grown anaerobically in the light in C-succinate media (modified from Cohen-Bazire et al. 1957) at 29°C, harvested by centrifugation (18,800*g* × 10 min at 4°C), resuspended in 20 mM Tris–HCl buffer (pH 8.0) and stored in a freezer (–20°C) until use. Chromatophores were prepared as described separately (Nakagawa et al. 2007).

Isolation and purification of all-*trans*-spirilloxanthin from *Rs. rubrum* S1

A crude spirilloxanthin extract (a mixture of carotenoids which has spirilloxanthin as its main component) was isolated from cells of *Rs. rubrum* S1 according to the method of Furuichi et al. (Furuichi et al. 2002). The cells were suspended in methanol, stirred and centrifuged (5,000*g* × 10 min at 4°C). A major fraction of BChl *a* was removed by this process. The pellet obtained after centrifugation was resuspended in acetone, stirred and centrifuged (5,000*g* × 10 min at 4°C). This removed most of the lipids. The pellet was resuspended in benzene, stirred, and centrifuged (5,000*g* × 10 min at 4°C); the benzene supernatant contained the carotenoids. The benzene extraction was repeated three times in order to collect most of the carotenoids. The pooled benzene fractions were dried over anhydrous Na_2SO_4, and then rotary evaporated to dryness.

Pure all-*trans*-spirilloxanthin was obtained by Alumina column chromatography (Fujii et al. 2001). The crude spirilloxanthin was dissolved in a mixture of *n*-hexane and diethyl ether (95:5 v/v). This solution was then subjected to column chromatography (5.5-cm inner diameter) using Alumina (Merck, Aluminium Oxide 90) as the solid phase. The concentration of diethyl ether in the mobile phase was gradually increased. Pure all-*trans*-spirilloxanthin was eluted at a diethyl ether concentration of 25% in *n*-hexane. This fraction was rotary evaporated, and the dried spirilloxanthin was redissolved in 10 ml of benzene. All-*trans*-spirilloxanthin was crystallized from this solution by addition of a small amount of *n*-hexane. The crystallized spirilloxanthin was collected, and rinsed 10 times with *n*-hexane to remove any contamination with *cis*-isomers. All these protocols were performed under dim light. The all-

trans configuration of the product, spirilloxanthin, was confirmed by 600 MHz ^{1}H-NMR spectroscopy.

Reconstitution of the LH1 complexes

Reconstitution of the LH1 complexes was performed according to the method developed by Fiedor et al. (2004). Chromatophores of *Rs. rubrum* S1 were homogenized in 20 mM Tris–HCl (pH 8.0) buffer solution, and then freeze-dried. These dried chromatophores were suspended in benzene, stirred for 30 min and centrifuged (5,000*g* × 10 min at 4°C) in order to remove the carotenoids. This procedure was repeated several times until the pellet (chromatophores) became blue; these carotenoidless chromatophores were dried in vacuo. About 100 mg of these dried, carotenoidless chromatophores were solubilized at 4°C with 8 ml of 40 mM Tris–HCl (pH 8.0) buffer containing 10 mM Na-ascorbate and 0.3% LDAO. Applying this solubilization protocol, monomeric subunits of the LH1 complex are produced. This sub-complex contains LH1-α and LH1-β polypeptides and BChl *a*. Following centrifugation (7,000*g* × 40 min at 4°C) of this extract, the supernatant containing the major fraction of LH1 monomeric subunits, was stored in a freezer at –80°C until use.

About 0.5 ml of the above solution of the LH1 monomeric subunit was diluted with 15 ml of 40 mM Tris–HCl (pH 8.0) buffer. After addition of 300 µl of 5% [v/v] LDAO, the solution was further diluted with 35 ml of 40 mM Tris–HCl (pH 8.0) buffer. A freshly prepared acetone solution of all-*trans*-spirilloxanthin (OD_{498} = 1–1.2) was gradually added to the LH1 monomeric subunit solution under N_2 atmosphere at 0°C; absorption spectra of the reconstituted complexes were recorded at 4°C after each addition. As the concentration of spirilloxanthin was increased, the Q_y absorption band of BChl *a* shifted to longer wavelengths. When the maximum absorption of the Q_y band reached to 880 nm, the addition of the spirilloxanthin solution was stopped and the final solution containing reconstituted LH1 complexes was stored in a refrigerator for one night in order to stabilize the reconstituted complexes.

The reconstituted LH1 complexes were purified by ion-exchange chromatography using DEAE-cellulose DE52 (Whatman). The DE52 column (1.5-cm inner diameter) was equilibrated with 20 mM Tris–HCl (pH 8.0) buffer containing 0.025% Triton X-100. The crude reconstituted LH1 complexes were loaded on to the column. The excess amount of spirilloxanthin molecules, which are not properly bound to the LH1 complexes, were eluted with the 20 mM Tris–HCl (pH 8.0) buffer containing 0.045% Triton X-100 and 50 mM NaCl. Then the major purified fraction of the reconstituted LH1 complexes was eluted using 20 mM Tris–HCl (pH 8.0) buffer containing 0.025% Triton X-100 and 175 mM NaCl. The NaCl was removed afterwards by dialysis against 20 mM Tris–HCl (pH 8.0) containing 0.025% Triton X-100. All these protocols were performed at 4°C in darkness.

Preparation of LH1 complexes from *Rs. rubrum* S1

LH1 complexes of *Rs. rubrum* S1 were prepared as described separately (Nakagawa et al. 2007).

Spectroscopic analysis

Absorption, circular dichroism (CD), fluorescence excitation spectra were recorded as described separately (Nakagawa et al. 2007). Details of the Stark spectroscopic measurements and the analysis are described separately (Nakagawa et al. 2007).

Results and discussions

Figure 1(a) compares the absorption spectra of the reconstituted LH1 complexes (solid black line) and the LH1 complexes isolated from *Rs. rubrum* S1 (solid red line). These spectra have the same peak wavelengths in the Soret (376 nm) and Q_x (589 nm) of BChl *a* absorption bands. However, a difference is observed in their Q_y absorption bands. The reconstituted LH1 has its Q_y absorption maximum at 878 nm. This is 3 nm blue-shifted in comparison to the isolated LH1 (881 nm).

In the 435–570 nm spectral region, three absorption peaks, ascribable to the vibrational structure of $S_0 \rightarrow S_2$ absorption band of the carotenoids, can be clearly observed. In the case of the reconstituted LH1 complexes the absorption maxima of the carotenoids are 1–2 nm blue-shifted relative to those of the isolated complexes. In addition, the relative intensity of the Soret band, with respect to that of the carotenoid bands, is always stronger in the reconstituted LH1 complex. A possible reason for these additional differences is contamination with aggregated carotenoids present in the solution of the reconstituted LH1 complexes. This hypothesis has been tested by taking a difference absorption spectrum between the reconstituted and isolated LH1 complexes (see Fig. 1 b). In the region between 300 and 600 nm, this difference spectrum is compared with that of a spectrum of an aggregate of spirilloxanthin (broken line in Fig. 1b). Clearly, especially in the Soret region part of the difference spectrum can be attributed to the absorption of the carotenoid aggregate. Also, there seems to be less carotenoid

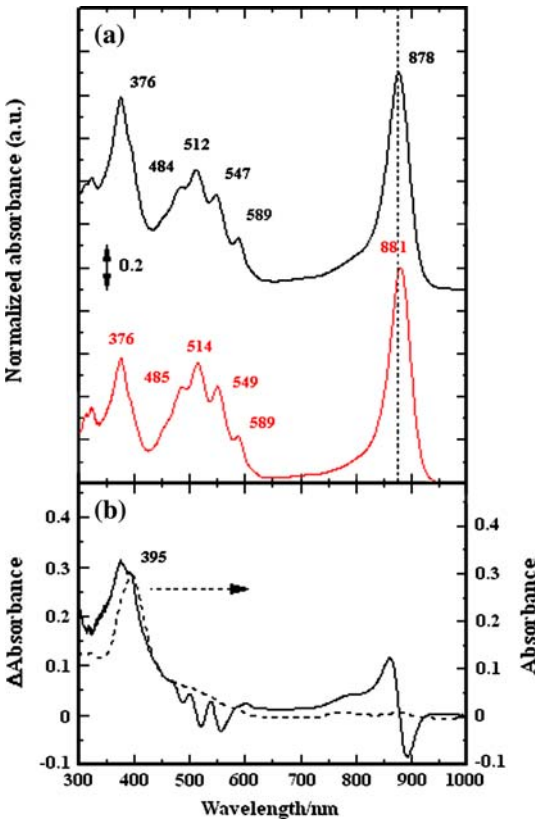

Fig. 1 (**a**) Normalized (Q_y-band) absorption spectra of the reconstituted LH1 complex (solid black line), and of the purified LH1 complex (solid red line) isolated from *Rs. rubrum* S1 in 50 mM Tris–HCl (pH 8.0) buffer containing 0.025% Triton X-100 at 4°C. (**b**) Difference absorption spectra (solid black line) of the reconstituted LH1 minus the purified complete LH1 complexes. Broken line shows the absorption spectrum of the aggregates of spirilloxanthin in 50 mM Tris–HCl (pH 8.0) buffer containing 0.045% Triton X-100 at 4°C

present in the reconstituted complex, than in the isolated one.

Figure 2 compares the CD spectra of the reconstituted (solid black line) and isolated (red black line) LH1 complexes. The CD spectra show distinct differences in the 400–500 and 800–850 nm regions. Therefore, we have applied the Stark spectroscopy for the purpose of more sophisticated analysis.

Figure 3 compares the Stark spectra of the reconstituted (solid black line) and isolated (solid red line) LH1 complexes. Interestingly, the Stark spectra in the carotenoid absorption region are essentially identical in both preparations. In the BChl *a* Q_y absorption region, however, the preparations exhibit quantitative differences. The fact that Stark spectra are similar in the region of the carotenoid absorptions indicates that the carotenoid aggregates, if present, are not very sensitive to the applied electric field. This fact can be explained if we assume that the aggregates of spirilloxanthin are not bound to the protein; the intensity of the Stark signals of carotenoids is severally, strongly

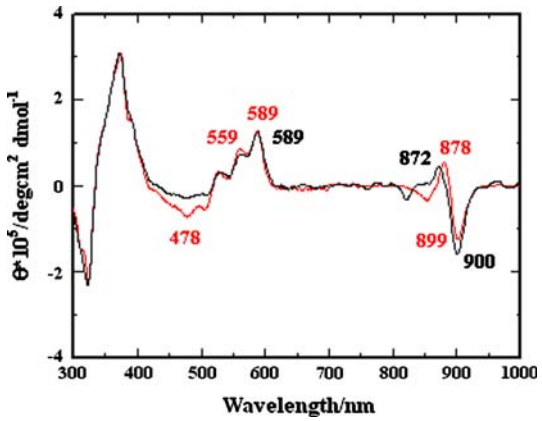

Fig. 2 Circular dichroism (CD) spectra of the reconstituted LH1 (solid black line), and of LH1 (solid red line) complexes isolated from *Rs. rubrum* S1 in 50 mM Tris–HCl (pH 8.0) buffer containing 0.025% Triton X-100 at 4°C

enhanced when they are bound into appropriate binding site of the pigment–protein complexes (Yanagi et al. 2004, 2005). Table 1 summarizes the nonlinear optical parameters (Tr($\boldsymbol{\Delta\alpha}$) and $|\boldsymbol{\Delta\mu}|$) of the carotenoid and the BChl *a* Q_y absorption bands of the reconstituted and isolated LH1 complexes. The values of these two parameters are identical in the case of carotenoid absorption band. The Tr($\boldsymbol{\Delta\alpha}$) values of the BChl *a* Q_y absorption band are the same also, within the limit of experimental errors. However, the $|\boldsymbol{\Delta\mu}|$ values of BChl *a* Q_y band show a small but reproducible difference between the reconstituted and isolated LH1 complexes. The difference of the $|\boldsymbol{\Delta\mu}|$ was determined to be 0.6 [D/*f*]. This small but significant change reflects a subtle difference in the electrostatic environment around BChl *a* in these two preparations.

Here, we determine the difference of the magnitude of the local electric field $\mathbf{E}_\Delta$ felt by the BChl *a* molecules in

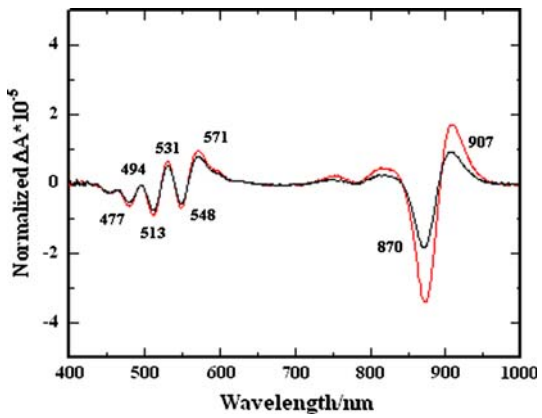

Fig. 3 Normalized Stark spectra in PVA matrices at room temperature of the reconstituted LH1 (solid black line), and of LH1 (solid red line) complexes isolated from *Rs. rubrum* S1. Normalization was performed against the magnitude of the externally applied electric field as well as the concentrations of the samples

Table 1 The nonlinear optical parameters (Tr($\Delta\alpha$) and $|\Delta\mu|$) of the carotenoid and BChl *a* Q_y absorption bands of the reconstituted and purified complete LH1 complexes from *Rs. rubrum* S1 determined by Stark spectroscopy

LH1 complex from *Rs. rubrum* S1	Carotenoid		BChl *a* (Q_y)	
	Tr($\Delta\alpha$) (Å^3/f^2)	$\|\Delta\mu\|$ (D/f)	Tr($\Delta\alpha$) (Å^3/f^2)	$\|\Delta\mu\|$ (D/f)
Reconstituted LH1	1,400 ± 100	7.5 ± 0.2	350 ± 20	4.9 ± 0.1
Purified complete LH1	1,400 ± 100	7.2 ± 0.2	390 ± 30	5.5 ± 0.1

the reconstituted and isolated LH1 complexes. The total electric field $\mathbf{E}_n$ that the BChl *a* in the reconstituted LH1 complex experiences, can be described by Eq. 1.

$$\mathbf{E}_n = \mathbf{E}_a + \mathbf{E}_S + \mathbf{E}_\Delta, \tag{1}$$

where, $\mathbf{E}_a$ is the externally applied electric field and $\mathbf{E}_s$ is the local electric field around the BChl *a* in the complete LH1 complex that is due to its surroundings. In this case, the Stark shift ($\Delta\nu_{ge}$) of the BChl *a* Q_y absorption band can be described by Eqs. 2 and 3 (see Yanagi et al. 2005), where $\Delta\boldsymbol{\mu}_{rec}$ is the static dipole-moment change of the BChl *a* Q_y band of the reconstituted LH1 complex.

$$h\Delta\nu_{ge} = -\Delta\boldsymbol{\mu}_{rec}\cdot\mathbf{E}_a - \frac{1}{2}\mathbf{E}_a\cdot\Delta\boldsymbol{\alpha}\cdot\mathbf{E}_a - (\Delta\boldsymbol{\mu}+\mathbf{E}_S\cdot\Delta\boldsymbol{\alpha})\cdot\mathbf{E}_\Delta - \frac{1}{2}\mathbf{E}_\Delta\cdot\Delta\boldsymbol{\alpha}\cdot\mathbf{E}_\Delta - \Delta\boldsymbol{\mu}\cdot\mathbf{E}_s - \frac{1}{2}\mathbf{E}_s\cdot\Delta\boldsymbol{\alpha}\cdot\mathbf{E}_s \tag{2}$$

$$\Delta\boldsymbol{\mu}_{rec} = \Delta\boldsymbol{\mu} + \Delta\boldsymbol{\alpha}\cdot(\mathbf{E}_S + \mathbf{E}_\Delta) \tag{3}$$

Based on these equations, the difference in the transition energies between the BChl *a* Q_y absorption band of the reconstituted LH1 and that of the complete LH1 can be calculated as shown in Eq. 4.

$$h\Delta\nu_{ge}^{\mathbf{E}_\Delta} = -(\Delta\boldsymbol{\mu}+\Delta\boldsymbol{\alpha}\cdot\mathbf{E}_S)\cdot\mathbf{E}_\Delta - \frac{1}{2}\mathbf{E}_\Delta\cdot\Delta\boldsymbol{\alpha}\cdot\mathbf{E}_\Delta \tag{4}$$

In this study, it has been demonstrated that the reconstituted LH1 and the isolated LH1 show similar values of Tr($\Delta\alpha$) (see Table 1). In this case, it is possible to determine the magnitude of $|\mathbf{E}_\Delta|$ using Eqs. 5 and 6.

$$\Delta(\Delta\boldsymbol{\mu}) = \Delta\boldsymbol{\mu}_{rec} - \Delta\boldsymbol{\mu}_S = \Delta\boldsymbol{\alpha}\cdot\mathbf{E}_\Delta \tag{5}$$

$$|\mathbf{E}_\Delta| \approx \frac{\Delta(\Delta\boldsymbol{\mu})}{\mathrm{Tr}(\Delta\boldsymbol{\alpha})}, \tag{6}$$

where, it is supposed that the $\Delta\boldsymbol{\alpha}$ of the BChl *a* Q_y is parallel to the direction of the Q_y transition dipole-moment (He et al. 2001). Therefore, using Eq. 6, $|\mathbf{E}_\Delta|$ can be determined as 3.4×10^5 [V/cm]. In addition, using Eq. 4, the value of the Stark shift ($\Delta\nu_{ge}^{\mathbf{E}_\Delta}$), due to the presence of $|\mathbf{E}_\Delta|$, can also be calculated; it is equal to 3.6 meV (see He et al. 2001, and Kjellberg et al. 2003 for supportive information).

Based on the above results it can be suggested that the presence of this $\mathbf{E}_\Delta$ is responsible for the high energy (blue) shift of the BChl *a* Q_y absorption band of the reconstituted LH1 complex compared with the complete LH1 complex. A 3 nm blue-shift of the BChl *a* Q_y absorption band, as shown in Fig. 1, would correspond to a 3 meV shift in energy. Our analysis, based on Stark spectroscopy, therefore, is a good prediction of the direction and magnitude of the observed shift of the BChl *a* Q_y absorption band.

Figure 4 compares the absorption and fluorescence excitation spectra of both the reconstituted and complete LH1 complexes. Comparing the area under the curve of the

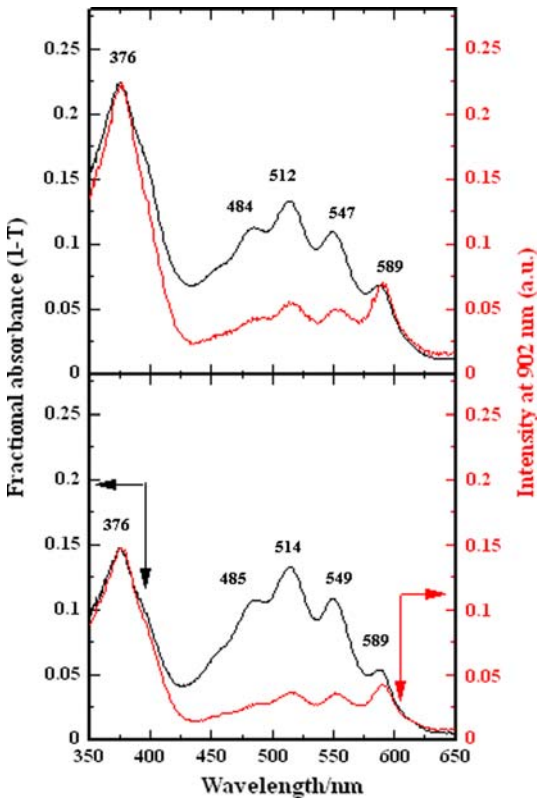

Fig. 4 Fractional absorption (solid black line) and fluorescence excitation spectra (solid red line) of (**a**) reconstituted LH1 (upper panel) and (**b**) LH1 complexes isolated from *Rs. rubrum* S1, (lower panel) in 50 mM Tris–HCl (pH 8.0) buffer containing 0.025% Triton X-100 at 4°C

fractional absorbance and the fluorescence excitation spectra, the singlet-singlet energy-transfer efficiency of carotenoid to BChl *a* was determined to be 28% for the complete LH1 complex. This value is in good agreement with the previously reported values (Akahane et al. 2004; Cogdell et al. 1992; Rademaker et al. 1980). The efficiency of this energy-transfer process is higher (40%) in the reconstituted LH1 complex. It is interesting to speculate whether this pronounced difference could be due to the changes in the local electric field surrounding the BChl *a* molecules. Further experiments are required to test this idea. This study does, however, illustrate the power of Stark spectroscopy to prove the effects of subtle changes in the local environment of pigments in their proteinaceous binding sites.

Reconstitution experiments are useful to investigate the physiological functions of the pigment molecules bound to the pigment–protein complexes. In these experiments, however, it is always assumed that the reconstituted pigment–protein complexes have exactly the same structure as the original, unmodified pigment–protein complexes. It appears that Stark spectroscopy provides a much more sensitive assay to test this assumption than standard absorption spectroscopy.

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