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Pigment organization and energy transfer in green bacteria. 3. Picosecond energy transfer kinetics within the B806–866 bacteriochlorophyll *a* antenna complex isolated from *Chloroflexus aurantiacus* *

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The B806–866 antenna protein complex from *Chloroflexus aurantiacus* has been isolated using the detergents lauryldimethylamine *N*-oxide (LDAO) and Deriphat-160. These preparations have been characterized spectroscopically. While the B806–866 complex was unstable for longer periods in 0.1% LDAO a high stability was observed when 0.1% Deriphat-160 was used as a detergent. In order to study the energy-transfer processes between the 806 and 866 nm absorbing excited states, fluorescence decays were measured with picosecond resolution in the detection wavelength range from 800 to 910 nm upon excitation at 790 nm. A fast energy transfer from the 806 nm to the 866 nm absorbing states was resolved, and was found to have an equilibration time of 5 ± 1 ps. This equilibration time is in line with previous estimates from steady-state fluorescence. Due to the fast equilibration time, resolution of this energy-transfer process has not been achieved in the past when measuring whole cells of *C. aurantiacus*.

Introduction

Chloroflexus aurantiacus is a phototrophic bacterium whose membrane-located photosynthetic apparatus resembles that of green and purple bacteria. It contains as extramembranous antenna the BChl-*c*-containing chlorosomes [1], a structure which is similar to the main antenna system of the green sulfur and nonsulfur bacteria [2]. The intramembranous B806-866 antenna complex and the reaction center are similar to the B800–850 antenna complex and reaction center of purple bacteria, respectively [3]. Several groups have presented evidence for a sequential energy transfer scheme in *C. aurantiacus* [4–8]. According to this scheme, energy is transferred from the chlorosome to the BChl a_{790}-complex and then via the B806-866 complex to the reaction center. We have recently presented a detailed time-resolved fluorescence study of whole cells which is also in line with such a sequential scheme [9]. Despite the fact that many details of the energy migration scheme were resolved in our data from whole cells, we noticed during those measurements that the kinetics of whole cells are too complex and we were unable to resolve the energy transfer within the B806-866 antenna complex. Likewise, Mimuro et al. [5] were unable to resolve this transfer process when studying whole cells. In a recent paper by Causgrove et al. [8] the time given in a kinetic scheme was 'fast' for the relaxation within that complex.

Although the main antenna system of *Chloroflexus*, the so-called chlorosomes, has been studied extensively, little is known about the structure and function of the B806-866 antenna complex. Analogous to the antenna complexes of purple bacteria, it is composed of two small polypeptides which have been sequenced by Zuber and co-workers. The α-polypeptide has a molecular weight of 4900 and shows an amino-acid identity of 27–39% to the light-harvesting α-polypeptide of the B870 (B890) antenna complex of purple bacteria [10]. The amino acid identity to the α and β polypeptides of the purple bacterial B800–850 complex

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Abbreviations: SPT, single-photon timing; BChl, bacteriochlorophyll; B806-866, bacteriochlorophyll *a* 806-866 protein complex; LDAO, lauryldimethylamine *N*-oxide; DAS, decay-associated spectrum.

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is much lower (14–23% and 9–14%, respectively). However, the β-polypeptide with a molecular weight of 6300 shows a high amino-acid identity to the β polypeptides of antenna complexes of purple bacteria [11]. Thus, one may assume, taking into account also the spectral similarities, that the B806-866 antenna complex has a similar function in *C. aurantiacus* as the B800-850 antenna complex in purple bacteria.

Spectroscopic studies of the isolated B806-866 complex are scarce so far. Only an absorption spectrum has been published by Feick et al. [12] which was limited to the NIR region. Data available for comparison of the whole spectrum were presented by Vasmel et al. [7] who studied the isolated cytoplasmic membrane of *C. aurantiacus*. In the linear dichroism spectrum of the long-wavelength absorption region they found substantial differences as compared to the corresponding region where the B800-850 complex of purple bacteria absorbs [7]. The reported energy transfer efficiencies are 40% for carotenoid → BChl a energy transfer and about 100% for B806 → B866 energy transfer. Estimates for the rate constants based on measured fluorescence quantum yields gave a maximal lifetime of about 6 ps for the B806 state (pigment) [7,13].

We report here the first time-resolved measurements on the isolated B806-866 antenna complex from *C. aurantiacus*. These results are important for a better understanding of the complex kinetics observed for the energy migration in whole cells. Along with the results obtained from isolated reaction centers [14] and isolated chlorosomes [9,15,16] we have now characterized all individual components taking part in the photosynthetic energy transfer chain in *C. aurantiacus*. This organism is now therefore one of the best characterized systems concerning energy migration in photosynthetic bacterial organisms. The data on all these isolated fragments will be used in the future to establish an energy-transfer model for the intact organism.

Materials and Methods

C. aurantiacus was grown in 1 liter batch cultures and membranes were prepared as described [17]. The isolation of the antenna complex was performed by extracting the membranes (adjusted to $A_{865} = 14$) with 0.7% LDAO (Fluka) at 4°C for 1 h. The extract was centrifuged in a Beckmann Ti70 rotor for 1.5 h at 45000 rpm. Along with the B806-866 antenna complexes the supernatant contained reaction centers, cytochromes, free pigments and several non-coloured proteins. This supernatant was transferred to a DEAE-Sephacel column (300 × 26 mm) which was equilibrated with 20 mM Tris-HCl (pH 9.0). The column was washed with 20 mM Tris-HCl (pH 8.0) containing 0.3% LDAO until the eluate was colourless. The same buffer with 30 mM NaCl and 0.1% LDAO added was used to remove further proteins. The reaction centers can be obtained subsequently by increasing the NaCl concentration to 60 mM [14,18]. The B806-866 complex was eluated together with cytochrome *c*-554 with 150 mM NaCl. The isolated complex contained no reaction centers or chlorosomes. This complex was used for a first series of fluorescence lifetime measurements (B806-866–LDAO). We noted, however, that it is quite unstable over periods exceeding about 1 day. Further purification along with a high stability were achieved by the following procedure. The crude B806-866–LDAO was diluted 1:1 with 20 mM Tris-HCl (pH 8.0) 0.1% Deriphat-160 (30% stock solution obtained from Serva) and subjected to a 16 × 200 mm column with DEAE-Sepharose CL-6B (Pharmacia) which had been equilibrated with the dilution buffer. Cytochromes and contaminating proteins were removed with buffer containing 350 mM NaCl. The B806-866 complex was eluted with 450 mM NaCl. The latter complex can be stored for several weeks without spectral changes at 4°C. Further dilution down to 200 mM NaCl with Deriphat buffer is possible without affecting the stability. Absorption spectra and stationary fluorescence spectra were obtained as described [17].

Picosecond fluorescence decays were recorded on a single-photon timing (SPT) apparatus [19] under magic-angle polarization conditions at 22°C. Without magic-angle conditions the fast energy transfer described in this paper was not resolved, probably due to interference with intramolecular depolarization processes. A fast near-infrared sensitive microchannel-plate detector (R2809U-05, Hamamatsu) was installed for high resolution measurements between 800 nm and 910 nm. Measurements were performed until 20000–30000 counts were accumulated in the peak channel. A channel resolution of 2.0 ps was used. The system temporal response function was tuned to be 30–35 ps (FWHM), which results in a time-resolution of about 3 ps [14]. The fluorescence decays were detected at several emission wavelengths (spectral bandwidth ≈ 12 nm). The sample was excited by a cavity dumped dye laser (using Styryl 8 as the laser dye) at a wavelength of 790 nm. During the measurements the sample was purged with nitrogen and slowly pumped through the measuring cuvette. The data were analysed both by single decay analysis and global lifetime analysis of the fluorescence decays [19]. A sum of exponentials is assumed as the model function. The results of the global analysis are plotted as decay-associated spectra (DAS).

Results and Discussion

Steady-state spectra

The absorption spectra of the two isolated B806-866 complexes are shown in Fig. 1A and B. For the B806-

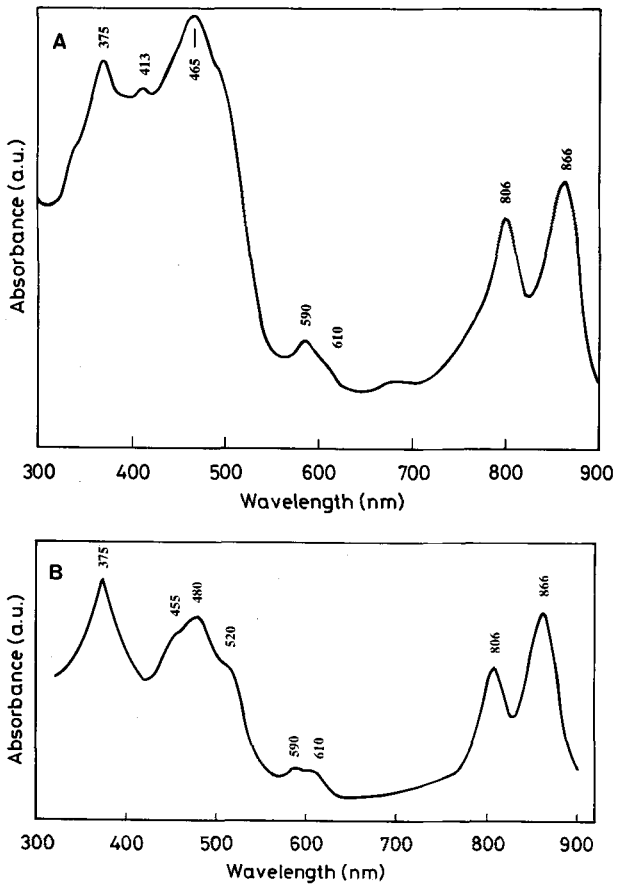

Fig. 1. Absorption spectrum of the B806-866 complex isolated with LDAO as detergent (A) and with Deriphat (B).

866–LDAO complex we observe a broad carotenoid absorption with absorption bands at 440, 465 and 500 nm and an additional absorption band at 413 nm from cytochrome c_{554} (Fig. 1A). A pronounced absorption shoulder at 756 nm due to BPheo *a* of the reaction center as seen in Ref. 7 is absent, as expected for the pure antenna complex. We find an absorption ratio of 1.2:1 for the bands at 866 and 806 nm in the B806-866–LDAO preparations. Feick et al. [12] have reported an absorbance ratio $A_{866}:A_{806}$ of 1.6:1. This difference may be explained partly by free BChl *a* with a broad absorption around 770 nm which typically for LDAO preparations increases the apparent amplitude of the 806 nm absorption band. Taking into account excitonic interactions between the B806 and B866 components also a somewhat modified geometry might be an explanation. Cytochromes and free pigments were removed in the B806-866–Deriphat preparations. The ratio $A_{866}:A_{806}$ in this complex is about 1.4 (Fig. 1B). Also a shift of the carotenoid absorption bands at 455, 480 and 520 nm is observed as compared to the LDAO complex. In acetone solution these bands are shifted to 435, 464 and 492 nm. Since these values differ from the ones reported by Halfen et al. [20] for isolated carotenoids of *Chloroflexus*, we conclude that a mixture of several different carotenoids is probably bound to the B806-866 complex. We have not tried to isolate and identify them.

The steady-state fluorescence excitation and emission spectra (Fig. 2) are comparable for both isolated complexes except that fluorescence from free pigments is much stronger for the B806-866–LDAO complex. It should be noted that the excitation band at 862 nm in Fig. 2 might be slightly distorted due to the anomalies in the excitation monochromator at long wavelengths. Fluorescence emission spectra excited at 375 nm (BChl *a*), 485 nm (carotenoids) and 790 nm (B806) show the same features: the main emission is detected at 885 nm (Fig. 2). Excitation at 485 nm and 790 nm results in spectra where this emission band was the only one detectable at wavelengths above 600 nm. Excitation at 375 nm gave rise to two additional emission bands at about 677 nm (free BChl *c*) and 790 nm (free BChl *a*) especially with B806-866–LDAO complexes (not shown). The emission of these additional components was low as compared to the main emission around 885 nm. To further characterize the 677 nm and 790 nm emission bands, excitation spectra were run with these emission wavelengths as well (data not shown). These experiments confirmed that these two bands arise from free BChl pigments. The maxima found for carotenoid excitation ($\lambda_{em} = 880$ nm, data not shown) at 460 nm, 489 nm and at 524 nm correspond well with those reported by Vasmel et al. [7] at 461 nm, 485 nm and at 522 nm. The main excitation maximum of the carotenoids is shifted to 489 nm as compared to the absorption maximum at 465 nm for the B806-866–LDAO complex (Fig. 1A). For the B806-866–Deriphat complex the shape of the carotenoid absorption and excitation is very similar, indicating the contribution of

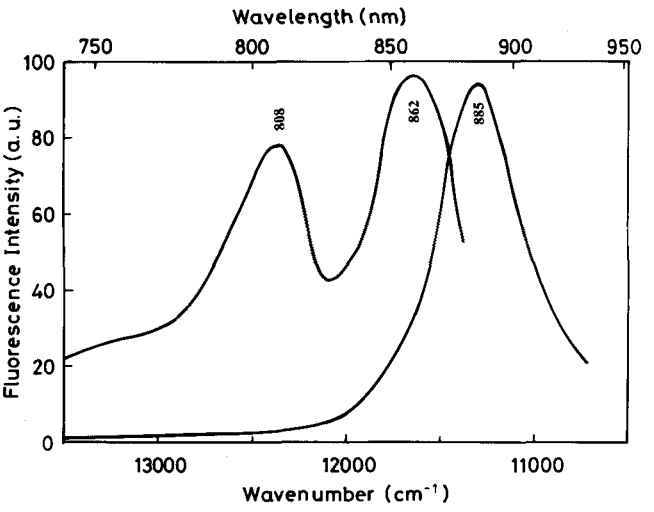

Fig. 2. Corrected fluorescence excitation ($\lambda_{em} = 900$ nm) and emission spectra ($\lambda_{exc} = 375$ nm) of the B806-866–Deriphat complex at 22°C.

TABLE I

Fluorescence lifetimes obtained by analyzing the data with single decay (λ_{em} = 865 nm) and global analysis procedures

The τ_1 amplitude is negative (rise-term) in all data for λ_{em} = 865 nm. Errors are ±1 ps for τ_1 and ±10% for the other lifetimes in global analysis. In single-decay analysis the errors are larger.

	τ_1 [ps]	τ_2 [ps]	τ_3 [ps]	τ_4 [ps]	τ_5 [ns]	χ^2
Global analysis						
B806-866-Deriphat						
3-components	4	–	–	538	1.63	1.48
4-components	5	–	165	729	2.62	1.15
Single-decay analysis						
B806-866-LDAO						
2-components			225	730		1.18
3-components	5		222	730		1.17
4-components	7	15	253	748		1.09
B806-866-Deriphat						
2-components			191	758		1.38
3-components	4		145	741		1.23
4-components	5	17	238	791		1.06

all these pigments to energy transfer. Estimates based on a comparison of the absorption and fluorescence excitation spectra (Fig. 2) for the Q_y absorption bands with an emission at 900 nm result in a calculated energy transfer efficiency of more than 90% for the transfer of energy from the 806 nm to the 866 nm absorbing pigments or states. The calculated energy transfer efficiency for carotenoid → B866 energy transfer was about 80% when setting the transfer efficiency as 100% for excitation at 370 nm (BChl *a*) for the Deriphat complex. This value is higher than the approx. 40% reported for cytoplasmic membranes [7]. The latter value is more in line with the efficiency found by us for the LDAO complex. In this complex we have calculated maximal energy transfer efficiencies of 50% for the carotenoid → BChl *a* energy transfer. Additional excitation bands at 590 and 612 nm (BChl *a* Q_x transitions) were also close to those reported by Vasmel et al. [7].

Time-resolved fluorescence spectra

Fluorescence decays have been measured for both B806-866 complexes at several emission wavelength upon excitation at 790 nm. Due to the free pigment contaminations of the B806-866-LDAO complex (free BChl and carotenoids) we will focus our interest on the B806-866-Deriphat complex, since this system is clearly in a more native state than the LDAO complex. This was realized after studying the time-resolved spectra of the B806-866-LDAO complex. The lifetimes obtained by single-decay analysis (c.f. Table I, λ_{em} = 865 nm) are very similar for both complexes, but the amplitudes of the approx. 16 ps and 240 ps components are much lower for the B806-866-Deriphat complex (13.6% and 13.4% for the LDAO and 5.8% and 7.2% for the Deriphat complex, respectively). We thus attribute these lifetimes as indications for somewhat damaged antenna complexes. In addition, the global analysis procedure was complicated at short emission wavelengths in the B806-866-LDAO complex due to free BChl *a* with a long lifetime (≈ 2.3 ns). Thus, we conclude from this that the B806-866-LDAO complex can not be considered to be undisturbed with respect to the kinetics of the native antenna complex.

Single decay analysis of the B806-866-Deriphat complex gave less complex results due to the higher stability and purity of this complex. For the emission at 865 nm we found four lifetimes with significant amplitudes. These lifetimes are approx. 790 ps, 240 ps, 17 ps and 5 ps. The last component has a negative amplitude (rise-term) and thus is attributed to energy transfer from the B806 to B866 excited states. The relative amplitudes of the components with 240 and 17 ps are quite low (7.2% and 5.8%, respectively, λ_{em} = 865 nm) and thus may represent some quenched B866 pigments in not fully intact complexes. Clearly, it was not possible to obtain reasonable fits without taking into account a short component, thus indicating a dominant role of this component in the B806-866-Deriphat complex (Table I).

With single-decay analysis using only a short analysis time-window (0.66 ns) a smaller number of lifetime components is required (Fig. 3). The analysis window is independent on the actual measurement range since the time window used for measurement is in the case of 2.0 ps/channel resolution about 8 ns in all cases. This approach was used to demonstrate the existence and influence of the short-living component on the residuals more clearly. In this shorter analysis window three components are sufficient to obtain acceptable fits at 880 nm with lifetimes of about 820 ps, 240 ps and 6 ps. The χ^2 value was 1.2 for this analysis. Fitting

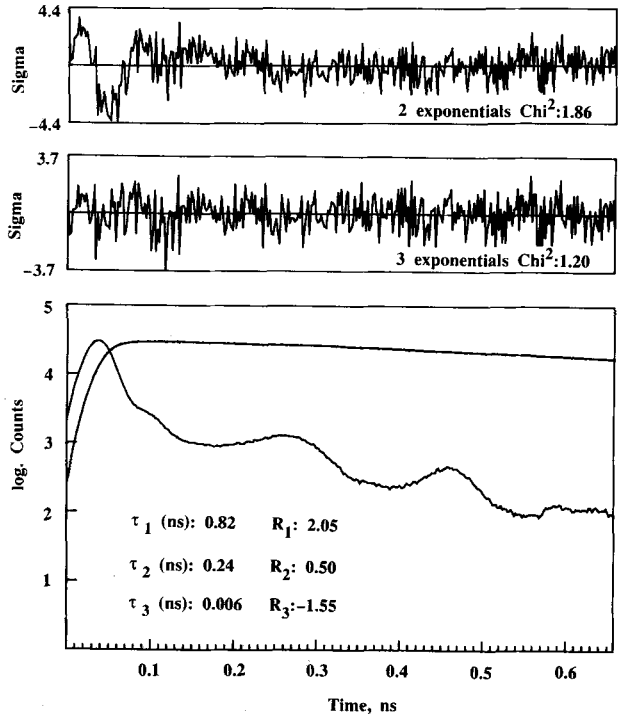

Fig. 3. Single-decay analysis of a fluorescence decay of the B806-866-Deriphat complex detected at 880 nm fitted with three components, $\lambda_{exc.} = 790$ nm. The weighted residuals above the spectrum are for the same decay with and without the 6 ps component. The lower curve in the measurement is the instrument response function (prompt), the upper curve represents the overlapping measured fluorescence decay and the fitted function. Using two components the lifetimes found are 0.77 and 0.26 ns.

this decay without the short-lived rise-term component of about 5 ps resulted in unacceptable fits with strong deviations in the residuals during the first 100 ps and an increased χ^2 value of 1.9.

From these results it is clear that we are able to resolve an approx. 5 ps component in all cases. However, the errors in the lifetimes obtained by single-decay analysis are higher and in the following we will use only the values obtained by the global analysis procedure. Nevertheless, we consider the single-decay analysis results as important for two reasons: (i) They give important hints as to the validity or limitations of a simple kinetic model with two states. In view of the lack of detailed information on the structure and the number of coupled pigments in a B806-866 complex we can not be sure about the adequate kinetic model for energy transfer. (ii) It is important to check by single-decay analysis the identity of the ultrafast lifetime in a wavelength region where the amplitude is positive (decay) and in a wavelength region where the amplitude is negative (rise).

The result of a global analysis procedure from lifetime measurements with 2.0 ps (B806-866-Deriphat) channel resolution is shown in Fig. 4. The results of several fits are also compiled in Table I. Four components are essentially sufficient to obtain acceptable fits. In analogy to the B806-866-LDAO complex (results not shown) a component with a lifetime of larger than 2 ns is needed but its amplitude is very low (see Fig. 4). Another minor component has a lifetime of 165 ps but a spectrum almost identical to that of the major 730 ps component, indicating some quenched B866 excited states as the origin for this component. The shortest-lived component (4–5 ps) is the most revealing one in the DAS, since at long emission wavelengths it has a negative amplitude. This feature with positive amplitudes at short emission wavelengths and negative amplitudes at longer emission wavelengths is a direct proof for an energy transfer process between two pigment pools and/or a relaxation between two excitonically coupled excited states. The zero crossing of this DAS occurs at about 850 nm. The maximum observed at longer wavelength corresponds well with the fluorescence emission maximum. The short-lived 4–5 ps component is essential for obtaining a good fit, as is indicated by the residual plots (Fig. 5). The component with positive amplitudes only, a 730 ps lifetime and a peak at about 880 nm in the DAS is interpreted as the overall relaxation time of the native B806-866-Deriphat complex.

Fig. 5 demonstrates that acceptable fits without the 5 ps component can not be obtained for the B806-866-Deriphat complex. The analysis window was a relatively short time-window of 0.55 ns in this case and thus it is difficult to resolve properly the long-lived components. However, this method is useful for demonstrating the short-lived components more clearly. Three lifetime components of about 5 ps, 436 ps and 3.4 ns were sufficient in this case. Fitting the decays with only two components gave rise to a large deviation

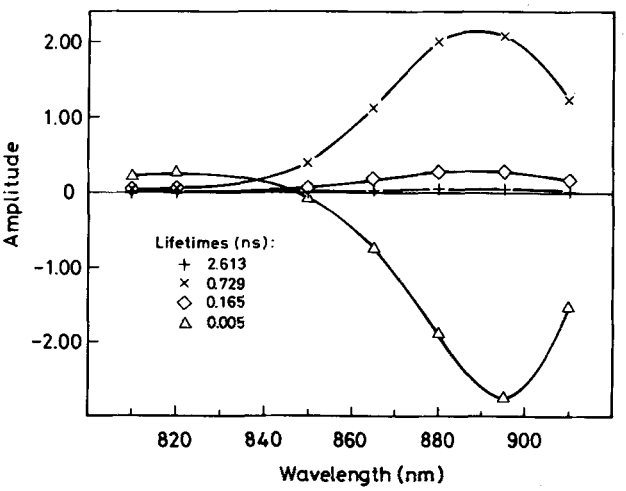

Fig. 4. DAS obtained by global analysis of all measured decays for the B806-866-Deriphat complex at room temperature. Four lifetime components were sufficient for a good description of the B806-866-Deriphat complex, $\lambda_{exc} = 790$ nm. The analysis range is 1.2 ns.

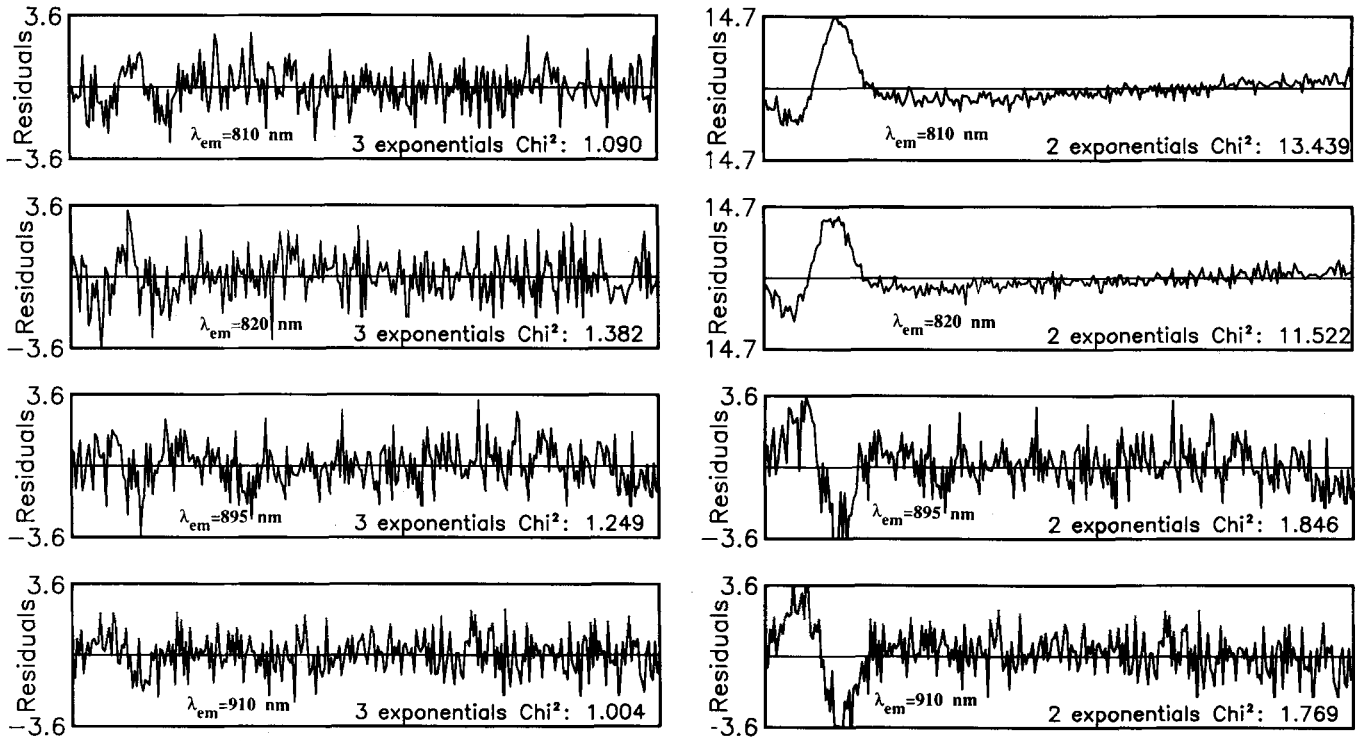

Fig. 5. Residual plots of global analyses using two and three exponentials for the B806-866-Deriphat complex, $\lambda_{exc} = 790$ nm. The global analyses were performed only over a small analysis range (0.55 ns), which gave rise to some uncertainty on the lifetimes of the long-living components. With two exponentials the obtained lifetimes were 436 ps and 3.4 ns (fixed), with three exponentials 5 ps, 436 ps and 3.4 ns (all lifetimes and shifts free). It was not possible to obtain acceptable fits without the 5 ps component.

of the residual plots, especially at short wavelength (810 nm, 820 nm), where the 5 ps component is the only one with high amplitude. The deviations are not as strong in the region where the long-lived components have high amplitudes (895 nm, 910 nm). Note that the deviation in the upper two residuals is approximately a mirror image of that in the lower two residuals. This corresponds to the fact that the shortest-lived component is a decay time in the wavelength range of the BChl *a* 806 fluorescence and a rise time in the wavelength range of the BChl *a* 866 fluorescence.

One model of interpreting the ultrafast 4–5 ps component consists in an energy transfer process between pigments (states) absorbing around 806 nm and pigments (states) absorbing around 866 nm. An alternative explanation invoked also for an ultrafast 5 ps component in isolated chlorosomes [15] consists in a model where it has to be interpreted as the equilibration time of two excitonically strongly coupled states. The reported values of two previous studies based on steady-state fluorescence [7,13] are in agreement with the energy transfer time of about 5 ps.

Our results indicate that stationary methods do not provide sufficient information to decide whether or not an isolated antenna complex is native. On the basis of stationary spectra alone both the B806-866-LDAO and -Deriphat complexes would appear to be native. Nevertheless, we observed a much higher heterogeneity in the B806-866-LDAO complex by time-resolved spectroscopy. In contrast to the LDAO complex, the B806-866-Deriphat complex seems to be native, since only two kinetic components with high amplitudes were observed, with lifetimes of 5 ps and 730 ps, and only minor amplitudes for other components. In the most simple model we would expect only two lifetime components with significant amplitudes for two coupled exited states.

The energy-transfer processes within the antenna complexes isolated from purple bacteria have been studied previously (see Ref. 21 for a review). For the B800-850 antenna complex, whose absorption and fluorescence spectra are quite similar to those of the B806-866 complex, an energy transfer time of about 1 ps was resolved by transient absorption measurements at room temperature [22] and 1–2 ps at 77 K [23]. The lifetime resolved for the energy transfer in the B806-866 complex is substantially longer. However, in a more recent study the energy transfer time in the B800-850 complex was determined by transient absorption femtosecond spectroscopy to be 2.5 ps [24].

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