Analysis of the Absorption Spectrum of Photosystem II Reaction Centers: Temperature Dependence, Pigment Assignment, and Inhomogeneous Broadening[†]

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ABSTRACT: In this study a model for decomposition and pigment assignment of the low-temperature (10 K) absorption spectrum of the photosystem II reaction center (D_1-D_2-cytochrome b_{559} complex, PSII-RC) is developed. It is based on theoretical calculations of the line shapes of the inhomogeneously broadened pigment spectra, taking into account electron−phonon coupling. The analysis is performed under the hypothesis that exciton coupling is weak, except for the P_{680} special pair. In this way a detailed decomposition of the absorption spectrum is obtained. Within the model the temperature dependence of the spectrum can be well explained. It is mainly caused by the temperature-dependent changes of the homogeneous absorption spectra of the individual pigments in the PSII-RC. In addition, slight changes in the inhomogeneous distribution functions have to be taken into account. Two slightly different parameter sets are found. We prefer one of these parameter sets which indicates that an accessory chlorophyll (Chl) is the lowest energy pigment in the RC core and that the two antenna Chls have their spectral maxima at 667.7 and 677.9 nm, respectively. The relationship between the shape of the absorption spectrum and the pigment stoichiometry of the sample (ratio of chlorophyll *a*:pheophytin *a*), which was noticed by comparison of a variety of different independently prepared samples, can be explained by the presence of "additional" Chl molecules which are nonstoichiometrically bound to part of the PSII-RCs. These Chls can be grouped into three spectrally distinguishable pools. One of them has its absorption maximum at about 683 nm and is responsible for the prominent shoulder that is present in the 10 K absorption spectra of most PSII-RC preparations. Our results suggest that the Chl content of the samples has been underestimated in many spectroscopic studies on the PSII-RC.

The thylakoid membranes of higher plants contain the reaction centers (RCs)[1] of photosystem (PS) I and PSII, where solar light is used as energy source to drive a photochemical charge separation. Both of these RCs are coupled to pigment−protein complexes which serve as light-harvesting systems. In the PSII complex, water is used as electron donor and molecular oxygen is released [for a review see Dau (1994)]. In 1987 the RC of PSII (PSII-RC) was isolated for the first time (Nanba & Satoh, 1987), and since then it has been the subject of numerous spectroscopic and biochemical studies [for reviews see Renger (1992) and Barber (1994)]. This pigment−protein complex is composed of the D_1 and D_2 proteins, the α- and β-subunits of cytochrome b_{559} (*psbA*, *-D*, *-E*, and *-F* gene products, respectively), and the small *psbI* gene product, and it is known as the D_1-D_2-cytochrome b_{559} complex. The peripheral Chl-binding proteins, the oxygen-evolving complex and both quinone electron acceptors are lost during the isolation procedure (Chapman et al., 1989). The amino acid sequences of the D_1 and the D_2 subunits are homologous to the L and M subunits, respectively, of the RC from purple bacteria, of which the X-ray structure has been solved at atomic resolution (Deisenhofer et al. 1985; Michel & Deisenhofer, 1988). The L and M subunits of bacterial RCs bind four bacteriochlorophylls (BChls), two bacteriopheophytins (BPheos), and one carotenoid. Two of the BChls are arranged to form a "special pair", which constitutes the primary electron donor of the RC. In contrast to that, largely varying pigment stoichiometries have been reported for the PSII-RC (Nanba & Satoh, 1987; Montoya et al., 1991; Aured et al., 1994; Chang et al., 1994; Kobayashi et al., 1990; Dekker et al., 1989). However, most recent studies report pigment ratios of about 6 Chl:2 Pheo:1−2 β-carotene (Kobayashi et al., 1990; Gounaris et al., 1990). The detailed structure of the PSII-RC is not known, but it is widely assumed that the arrangement of the cofactors is similar to that of the bacterial RC and that it contains two "extra" Chls which are more loosely coupled to the core and function as kind of "antenna Chls". At present there also occurs an intense debate about the nature of P_{680}, the primary electron donor in the PSII-RC. It most likely involves two Chls which are arranged to form a special pair (Barber, 1993, 1994; Renger, 1992). It should be noted here that PSII-RC preparations with a stoichiometry of about 4 Chl:2 Pheo:1 β-carotene have also been reported (Montoya et al., 1991; Yruela et al., 1994; Chang et al., 1994). However, more recent data indicate a higher Chl:Pheo ratio of such preparations [Yruela and Holzwarth, results to be published; see also Eijckelhoff and Dekker (1995)].

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[1] Abbreviations: BChl, bacteriochlorophyll; BPheo, bacteriopheophytin; Chl, chlorophyll *a*; HPLC, high-performance liquid chromatography; IDF, inhomogeneous distribution function; P_{680}, primary electron donor in photosystem II; Pheo, pheophytin *a*; PSII-RC, reaction center of photosystem II (D_1-D_2-cytochrome b_{559} complex); PW, phonon wing; RC, reaction center; rt, room temperature; THF, tetrahydrofuran; ZPL, zero phonon line.

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Understanding of the absorption spectrum of the PSII-RC in terms of the spectral contributions of the individual chromophores is crucial for all kinds of spectroscopic and functional studies. Of special interest, in particular for time-resolved experiments, are the Q_y ($S_0 - S_1$) transitions of the Chls and Pheos in the PSII-RC. Most of these experiments are complicated by the pronounced spectral congestion of this region. As a result, at room temperature only one single absorption band at about 676 nm appears. More spectral details can be resolved when the samples are cooled to low temperatures. Due to this situation, the assignment of the various chromophores in the PSII-RC to their spectral positions within the absorption spectrum is still a matter of debate, which has been addressed in a number of studies: Gaussian decomposition has been carried out on spectra taken at liquid helium temperatures (van Kan et al., 1990; Otte et al., 1992), at 77 K (Montoya et al., 1993), and at room temperature (Garlaschi et al., 1994). By this method it is easily possible to get excellent fits for the shape of the PSII-RC absorption spectra. However, gaussian bands are at best only very crude estimates of the shapes of single pigment spectra and thus the results of such a gaussian decomposition have to be considered quite unreliable, given the large number of chromophores in the PSII-RC. Spectral hole burning is a method that can provide quite detailed information about the absorption properties of the chromophores in a pigment−protein complex. For PSII-RCs this method has been extensively used by Small and co-workers (Tang et al., 1990, 1991; Jankowiak et al., 1989; Chang et al., 1994). The parameters that describe the electron−phonon coupling (Huang−Rhys factors and mean phonon frequencies) have been measured for P_{680}, Pheo, and some "antenna Chls". Besides that, the hole-burning experiments showed that the spectra of the pigments in the PSII-RC are strongly inhomogeneously broadened. It was possible to identify the approximate spectral maxima of one Pheo and of P_{680}. According to these hole-burning data, at low temperatures the P_{680} absorption spectrum (Q_y band) consists of two exciton bands, one peaking at around 667 nm and the other at around 681 nm, with the low-energy band carrying more than 90% of the oscillator strength of the dimer (Chang et al., 1994). These results are in good agreement with the triplet-minus-singlet absorption difference spectra of Kwa et al., (1994a) and the circular dichroism (CD) measurements of Braun et al., (1990) and Otte et al. (1992). The Pheo that could be identified in the hole-burning experiments of Small and co-workers (Tang et al., 1990) was nearly isoenergetic with the lower exciton band of P_{680} and was assumed to be the active Pheo in electron transfer. Kwa et al. (1994b) carried out fluorescence site-selection experiments and interpreted their data in a way that, in addition of P_{680} and at least one Pheo, there exists a long-wavelength Chl absorbing at around 680 nm.

Tetenkin et al., (1989) first pointed out that it might be unwarranted to describe the PSII-RC absorption spectrum as a sum of individual pigment spectra due to fairly strong excitonic interactions between the majority of the pigments. A similar view is taken in a recent paper by Durrant et al., (1995), who describe the PSII-RC core as a coupled multimer of pigments. All these spectroscopic studies could not unequivocally clarify the structure of P_{680}. Nevertheless, most studies so far can be interpreted in a way that P_{680} is a Chl dimer, although this is not the only possible interpretation. However, there is experimental evidence that the structure of this putative Chl dimer would differ significantly from that of the special pair in purple bacteria (Kwa et al., 1994a).

The main aim of this paper is to undertake a new effort—based on theoretical line shape calculations—to develop a physically reasonable and consistent picture of the PSII-RC absorption spectrum. It is crucial that this analysis should incorporate as many of the above mentioned experimental results as possible. Such a model will have to address explicitly the phenomenon of inhomogeneous broadening and explain the substantial temperature dependence of this spectrum on the basis of first principles. As an important side aspect, the pigment stoichiometry of several different PSII-RC samples will be related quantitatively to the observed spectral changes. This model as a working hypothesis assumes that, with the exception of the putative P_{680} Chl dimer, the pigments are only weakly coupled. We will at the end discuss the validity and limits of this concept.

MATERIALS AND METHODS

Sample Preparation. The PSII-RC samples were isolated from spinach leaves according to the method of van Leeuven et al. (1991). Comparing the results from many independent isolation runs, we noted a substantial variability in the Chl/2 Pheo ratio, ranging from about 6.2 Chl/2 Pheo up to 8 Chl/2 Pheo (see below). Some of the obtained PSII-RC samples, in particular those with high Chl/2 Pheo ratios, were further purified following the procedure of Kwa et al., (1992). This method includes a further incubation step of the isolated PSII-RCs in a buffer containing 2.5% Triton X-100 and 1.5% taurine. The starting material for this purification had a pigment ratio of about 8 Chl/2 Pheo. In the following we will refer to these methods as method 1 and method 2, respectively. The samples were shock-frozen and stored at liquid nitrogen temperatures, without adding glycerol, until use.

Determination of the Chl:Pheo Ratio. For pigment extraction, the PSII-RC samples in buffer (20 mM Bis-Tris, pH 6.5, 0.03% dodecyl β-maltoside, 20 mM $MgCl_2$, 5 mM $CaCl_2$, 100 mM $MgSO_4$, and 200 mM sucrose) were diluted with tetrahydrofuran (THF) in a ratio of 1/1 (v/v). This mixture was shaken and then sonicated for 5 min in a Bransonic 220 ultrasonic bath. Aggregated proteins were removed by centrifugation for 5 min at 3000 rpm in a Beckmann TJ-6 centrifuge. The resulting supernatant was analyzed by HPLC using a Nucleosil C-18 column (diameter 4.6 mm, length 125 mm) and a MeOH−MeOH/THF gradient (100:0−100:10). At a wavelength of 420 nm all three pigments, Chl, Pheo, and β-carotene, could be detected (using a Shimadzu SPD-6AV detector). To obtain the Chl/2 Pheo ratio of the PSII-RC samples, the relative areas under the Chl and the Pheo peaks were divided and the resulting number was multiplied by a calibration factor of 2.38. This calibration factor was determined in the following way: A stock solution of Chl (Sigma) in THF was divided into two parts, and one of them was converted into pure Pheo by adding a small drop of concentrated hydrochloric acid (10 M), thus producing Chl and Pheo solutions with exactly the same concentration (neglecting the small error caused bythe volume of the added acid which was less than 0.1%). Either of these two pigment solutions was mixed with buffer in a

1:1 ratio to reproduce the conditions used for the chromatography of the RC pigments. To obtain the calibration factor, aliquots of these solutions were injected into the HPLC column and the chromatographic areas of both compounds were compared. It should be noted that this procedure does not require any prior determination of the absorption coefficients of Chl or Pheo, respectively.

The protein pellet obtained after the extraction still contained a small amount of pigment as indicated by its slightly green color. We confirmed that a second extraction resulted in the same pigment ratio within the error limits as the first one. We note that if the solution of the extracted pigments was stored for several hours in the dark, the result of the pigment analysis was substantially altered due to Pheo degradation (resulting in a higher Chl/2 Pheo ratio), whereas no degradation could be detected for Chl during this time period. By performing several independent meaurements of the Chl/2 Pheo ratio for each sample, we estimated the statistical error of this method to be about ±0.2 Chl/2 Pheo. Typically at least three measurements were averaged. With this method of pigment analysis the ratio of β-carotene/Chl or β-carotene/Pheo could not be determined quantitatively; only relative ratios could be compared between different samples.

Recording of Absorption Spectra. The absorption spectra were measured on a rapid-recording spectrophotometer (Omega 10, Bruins Instruments, München, Germany). The slit width of the instrument was set to 0.2 mm, corresponding to a spectral resolution of about 0.7 nm. Typically 120 scans were averaged for each spectrum. To eliminate baseline artifacts due to scattering and stray light, a cutoff filter (OG 515, Schott) was placed in the detection and reference beams. The 10 K absorption spectra were measured by using a liquid helium flow cryostat (Leybold Heraeus). For temperatures of 77 and 150 K another cryostat (Oxford Instruments, Model DN 704) with liquid nitrogen cooling was used. Before the measurements the RC samples (in buffer) were rapidly mixed with 60% (v/v) distilled glycerol. Spectra at 277 K were recorded with samples made anaerobic by the addition of a glucose/glucose oxidase/catalase system in a water-cooled cuvette without adding glycerol. The optical path length of the cuvette was 1 cm in each case. Plastic cuvettes (Ratiolab) were used for low-temperature measurements.

RESULTS

Pigment Content and Absorption Spectra. The absorption spectrum of the PSII-RC undergoes a substantial change in shape when the temperature is lowered from 277 to 10 K. For the main band of the Q_y region this is shown in Figure 1 for a sample with 6.4 Chl/2 Pheo. Note that the spectra in this figure are scaled differently for reasons of better presentation. At 277 K the absorption band appears almost structureless with a single maximum at 675.5 nm. At a temperature of 150 K the region near the maximum of the spectrum starts to flatten, and at 77 K two single peaks are resolved. This structure becomes much more pronounced when the temperature is lowered to 10 K. Then the spectral positions of the two peaks are located at about 672 and 678 nm, respectively. This characteristic temperature dependence has also been described by other workers [see, e.g., van Kan et al. (1990)] but has not been explained so far. Closer inspection of the 10 K spectrum reveals some more details

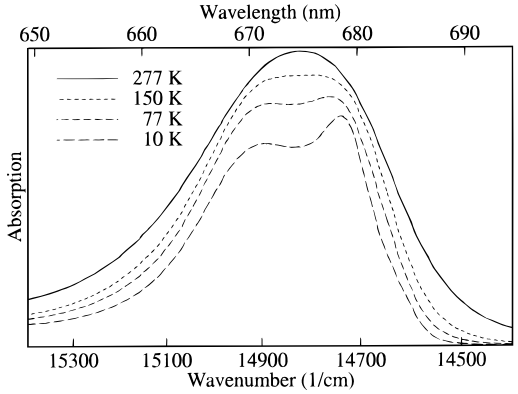

FIGURE 1: Absorption spectrum of a PSII-RC sample at various temperatures. The sample used for this measurement had a pigment composition of 6.4 ± 0.2 Chl/2 Pheo. Note that the spectra are not scaled equally for better presentation.

which give rise to characteristic features in the second derivative spectrum. These details are described later (see Figure 3). Similar spectral changes with temperature were observed for all the different PSII-RC samples measured.

In order to correlate the pigment composition of different PSII-RC preparations with their absorption spectra, we extracted the pigments of samples obtained from independent preparations and determined the Chl/2 Pheo ratios by HPLC. The statistical error in the determination of the molar ratio of Chl to Pheo was estimated to be about ±0.2 Chl/2 Pheo. We compared a variety of samples which had been prepared according to two different isolation procedures: Method 1 is a standard method in our laboratory following the procedure of van Leeuven et al. (1991). Such samples have been used by us for spectroscopic studies on PSII-RCs in the past, in particular for fluorescence lifetime measurements (Gatzen et al., 1992; Holzwarth et al., 1994; Gatzen et al., 1995). Method 2 involved a further purification step (see Materials and Methods). Samples prepared according to method 1 mostly had Chl/2 Pheo ratios between 6.2 and 6.5, but in some preparations also higher ratios of up to 8.0 Chl/2 Pheo were found, despite the fact that to the best of our knowledge all steps had been carried out in the same way. By applying one further purification step (method 2) the Chl/2 Pheo ratio of these samples could be lowered. Pigment ratios between 6.0 and 6.4 Chl/2 Pheo resulted when the samples before the second purification step typically had a pigment ratio of about 8 Chl/2 Pheo. None of the samples we produced by these two methods had a pigment ratio of less than 6.0 Chl/2 Pheo within the experimental uncertainty (using the same HPLC procedure).

A striking result of the comparison of many independent PSII-RC preparations is that one mostly finds Chl/2 Pheo ratios that are definitely not integer numbers, even if the experimental error is considered. This indicates that most of the samples should contain a fraction of pigments which are bound in a nonstoichiometric way. Consequently most samples in fact represent an inhomogeneous mixture of RCs with different pigment composition.

A critical comparison shows that the shape of the absorption spectra depends not only on the temperature but also on the Chl/2 Pheo ratio of the samples. This can be seen from Figure 2, where the 10 K absorption spectra from six different samples are shown together with their corresponding Chl/2 Pheo ratios as determined by HPLC. For this

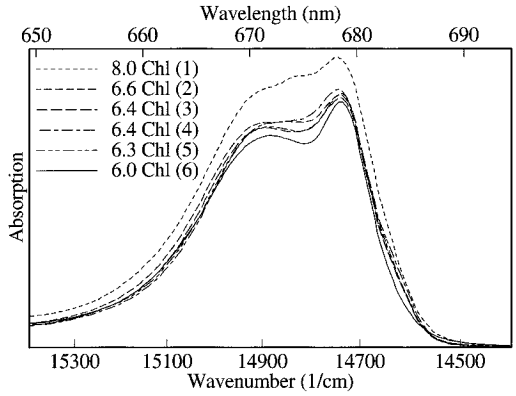

FIGURE 2: The 10 K absorption spectra of different PSII-RC preparations; also shown are the Chl/2 Pheo ratios as determined by HPLC and the identification which is referred to in the text. The area under the spectra is normalized to the measured pigment content.

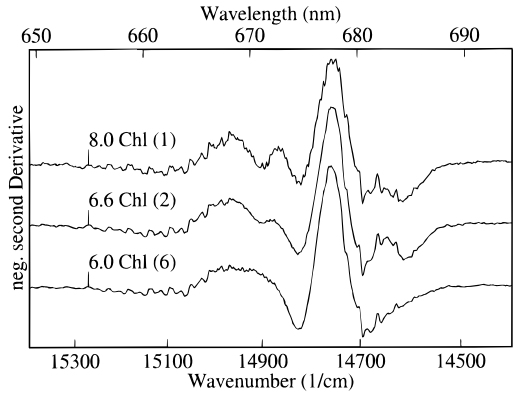

FIGURE 3: Second derivatives of three absorption spectra from Figure 1. The curves are multiplied by a factor of −1. Also indicated are the sample numbers and the Chl/2 Pheo ratios as measured by HPLC.

comparison, samples covering the whole range of absorption differences observed have been selected. Also shown are the identification numbers of the samples (in parentheses) which we will refer to in the following text. Samples 1, 2, 4, and 5 were prepared according to method 1, and samples 3 and 6 according to method 2. The area under the curves is normalized to the total Chl and Pheo content by assuming that each PSII-RC spectrum contains the oscillator strength of two Pheo plus that of the number of Chl found by HPLC. The oscillator strength of Pheo was taken as $^2/_3$ that of a Chl (Chang et al., 1994; Kwa et al., 1994b). As a result of this normalization the spectrum of sample 1 with 8.0 Chl/2 Pheo has the highest relative area, and that of sample 6 with 6.0 Chl/2 Pheo has the lowest relative area. However, irrespective of the normalization procedure the shapes of the absorption spectra differ significantly between the six samples: One trend is that the valley between the two main peaks is filled up with increasing Chl/2 Pheo ratio. In sample 6, which has a Chl/2 Pheo ratio of 6.0, these two peaks are well separated. Going to higher Chl/2 Pheo ratios the valley vanishes, and in the spectrum of sample 1 with 8.0 Chl/2 Pheo it is even converted into a peak. The presence of this spectral feature rising between the two peaks shows up also very clearly in the (negative) second derivatives of the absorption spectra (see Figure 3). In the 670-nm region of these derivative curves, sample 6 with a Chl/2 Pheo ratio of 6.0 shows a single peak. With increasing Chl content this peak starts to show a dip at around 672 nm and finally for sample 1 with 8.0 Chl/2 Pheo it is split up into two distinct peaks. Another characteristic spectral feature—different for the various samples—is a shoulder on the low-energy side of the absorption spectra which gives rise to a peak at around 683 nm in the negative second derivative (Figure 3). All but one of the samples we compared show this feature, but to a largely varying extent. A spectrum without this shoulder is that of sample 6, with the lowest Chl content of all samples measured here (6.0 Chl/2 Pheo). This indicates that pigments responsible for the red spectral feature can be removed by a further purification step, which implies that the low-energy shoulder in the absorption spectrum of the PSII-RC is not an intrinsic property of that complex. It could be argued that the red shoulder results from an excitonic coupling between a β-carotene and one of the chlorins in the PSII-RC and that the shoulder could be removed by extracting β-carotene from the PSII-RCs. As described above, our method of pigment analysis does not allow us to determine the absolute molar ratio of β-carotene/2 Pheo, but we are able to reliably compare the relative changes between different samples. We can thus rule out that β-carotene influences the Q_y region in such a way since sample 6 has a higher β-carotene/2 Pheo ratio than some of the other samples which show the shoulder in their absorption spectrum.

From Figure 2 another important point can be noticed: It is evident that samples with identical Chl/2 Pheo ratios still can have absorption spectra that substantially differ from each other. For example this can be seen by comparing the spectra from samples 3 and 4. This means that there exists no simple relationship between the shape of the absorption spectrum and the Chl/2 Pheo ratio of the samples.

Quantitative Model for the Absorption Spectra of PSII-RCs. It is our aim to construct a quantitative spectral model that should fulfill several purposes: First, it should avoid a pure curve-fitting procedure based on some assumed but questionable line shape. The individual spectra should have a shape that is physically reasonable. Second, it should explain the observed temperature dependence of the spectra well. Both of these aims demand a procedure that involves the calculation of proper spectral line shapes and their temperature dependence based on a well-established theory involving descriptions of electron−phonon coupling and inhomogeneous broadening. Furthermore the model should be consistent with the observed pigment contents of the different preparations and explain quantitatively the relationship between the pigment composition and the overall spectral shapes. While the spectral positions of the individual pigments within the absorption spectrum are still not clear, at least for some of the pigments there exists some evidence concerning their approximate absorption properties: At low temperatures the spectrum of P_{680} consists of a strong exciton band at around 681 nm and a weak one at around 667 nm (Kwa et al., 1994a; Chang et al., 1994). At least one of the Pheos absorbs at a wavelength of about 680 nm (Tang et al., 1990). These positions should be maintained as closely as possible in the model. The spectral positions of the remaining pigments have to be determined by some suitable procedure.

Model Assumptions. In this section we describe the hypotheses and theoretical procedures used for the construction of the model. The hypotheses made here are based

partly on our own experimental results, on the findings of other groups, and on the homology of the PSII-RC to the RC of purple bacteria. Some of these points will be justified a posteriori in a later section.

In the introduction it was pointed out that the RC of purple bacteria can be, with some modifications, considered as a suitable starting model for the organization of the PSII-RC. We therefore assume that each PSII-RC contains a primary electron donor P_{680} which is a dimer of two Chls, that it contains two further Chls ("accessory Chls") and two Pheos. These four Chls and two Pheos form a structure which is homologous to the bacterial RC and we expect the respective pigments to be present in each single PSII-RC particle. In the following we will therefore refer to these chromophores as "core pigments". Nevertheless, these core pigments do not necessarily have the same spatial organization as the cofactors in the bacterial RC. Furthermore we assume that the PSII-RCs contain two "antenna Chls" which can be partially removed during the isolation procedure. Since the minimum ratio of Chl/2 Pheo which we found in our samples is 6.0 ± 0.2, these two pigments also should be present in the majority of the PSII-RCs. To explain Chl/2 Pheo ratios of more than 6.0 we need the presence of "additional Chls" which are bound to the PSII-RCs in a nonstoichiometric way. Thus in our model the differences between the individual spectra of different preparations are attributed to variations in the content of the antenna Chls and of the additional Chls. We further assume that there exists no correlation between the contents of these Chls. This explains the fact that there does not seem to exist a straightforward relationship between the pigment stoichiometry on the one hand and the exact shape of the spectra on the other hand. It is important to note that in our model all the spectral parameters for the corresponding pigments in different preparations are assumed to be exactly the same, with the only exception of a variation in the amplitudes of the antenna and additional Chls. This includes the demand that the bacterial-like core RC is identical for all different preparations. In other words, all the different absorption spectra of PSII-RCs consist of a constant part, which is the same for each sample, plus a sample-specific variable part.

One important question concerns the extent of exciton coupling between the pigments in the PSII-RC. If a number of pigments is subject to a strong exciton interaction, then the spectrum of the whole system can no longer be described as a sum of individual pigment spectra. How relevant the effects of exciton coupling are for the PSII-RC core is currently a matter of debate (Durrant et al., 1995; Tetenkin et al., 1989). Direct experimental evidence for an exciton splitting exists in our view so far only for the spectrum of the primary donor P_{680} (Chang et al., 1994; Kwa et al., 1994a). In our model we assume exciton interaction to be of minor importance between most of the pigments in the PSII-RC, with the exception of the putative P_{680} dimer (see Discussion). We thus describe the total absorption spectrum as a sum of undisturbed Chl and Pheo spectra plus the spectrum of a coupled P_{680} Chl dimer. The temperature-dependent shapes of the individual pigment spectra have to be calculated on the basis of experimental data and a suitable theory.

Calculation of Pigment Spectra. Like in other pigment−protein complexes, the absorption spectrum of the PSII-RC is inhomogeneously broadened (Chang et al., 1994; Jankowiak et al., 1989; Tang et al., 1990, 1991). Every single pigment has a homogeneous absorption spectrum $B(\omega)$ that is determined (i) by the coupling of the electronic transition to intramolecular localized vibrations of the chromophore and (ii) by the coupling of the transition to lattice vibrations (phonons) of the surrounding protein matrix. The absolute spectral positions of these homogeneous absorption lines can be shifted specifically and stochastically due to coupling of the surrounding medium to each single chromophore. The stochastic effects lead to the phenomenon of inhomogeneous broadening. An inhomogeneously broadened spectrum $I(\omega)$ is obtained by a convolution integral of the homogeneous absorption spectrum $B(\omega)$ with an inhomogeneous distribution function IDF(ω) (assumed to be Gaussian-shaped). The latter represents the combined effects of the environment on the pigments.

$$I(\omega) = \int_{-\infty}^{\infty} IDF(\omega_0)B(\omega - \omega_0)\, \mathrm{d}\omega_0 \qquad (1)$$

Once the shape of the homogeneous spectrum is given, the experimentally observed inhomogeneously broadened spectrum is completely determined by the parameters of the Gaussian IDF: spectral position, half-width, and amplitude. The IDF is a measure for the probability to find the purely electronic transition (zero phonon line, see below) of a specific pigment at a certain spectral position. It should be noted that the maximum of the IDF in general is not identical to the maximum of the inhomogeneously broadened pigment spectrum.

To obtain a homogeneous absorption profile $B(\omega)$ the linear Franck−Condon approximation for the electron−phonon coupling can be used [see Rebane (1970) for the underlying theory]. The exact details of the procedure used here have been described by Pullerits et al. (1995, 1994) and will be summarized here only very briefly.

If in a first step the coupling of the optical transition to the intramolecular vibrations is neglected, a temperature-dependent homogeneous spectral band $b(\omega)$ consists of a narrow Lorentzian zero phonon line (ZPL) $\Phi_0(\omega)$ and a broad phonon wing (PW) $\Phi(\omega)$:

$$b(\omega) = \Phi_0(\omega) + \Phi(\omega) \qquad (2)$$

The PW $\Phi(\omega)$ can be calculated by using the following expressions:

$$\Phi(\omega,T) = \sum_{m=1}^{\infty} \Phi_m(\omega,T) \qquad (3)$$

$$\Phi_m(\omega) = \frac{1}{m!}\int_{-\infty}^{\infty} d\nu_1 \ldots \int_{-\infty}^{\infty} d\nu_m f(\nu_1,T) \ldots f(\nu_m,T) \times \Phi_0(\omega - \Omega + \nu_1 + \ldots + \nu_m,T) \qquad (4)$$

Here Ω denotes the frequency of the ZPL, ν the phonon frequency of the respective mode, and T the absolute temperature. The phonon function $f(\nu,T)$ can be expressed as the sum of the Stokes and the anti-Stokes parts of the respective one-phonon side band. This function depends on the strength of the coupling to the lattice vibrations, e.g., the Huang−Rhys factors S for the phonons. The higher the Huang−Rhys factor, the stronger is the relative contribution of the PW in eq 2.

Table 1: Parameters Used for the Calculation of the Homogeneous Spectral Line Shape for Different Chromophores in the PSII-RC

chromophore	Huang−Rhys factor S	mean phonon frequency ω_m (cm^{-1})	ref
P_{680}	1.9	26	Jankowiak et al., (1989)
Chl	0.8	30	Jankowiak et al., (1989)
Pheo	0.7	20	Chang et al., (1994)

To include intramolecular vibrations of the chromophore into the calculation, the same formalism can be applied. Instead of the ZPL $\Phi_0(\omega)$ the homogeneous spectral band $b(\omega)$ has to be used in eq 3. Thus finally the homogeneous absorption profile $B(\omega)$ can be calculated which, after convolution with an appropriate IDF (eq 1), yields the inhomogeneously broadened spectrum $I(\omega)$ that can be observed experimentally. It should be noted that throughout this work the electron−phonon coupling is described only by one mean phonon frequency because more detailed experimental data are not available at present (see below).

For calculating the homogeneous spectra $B(\omega)$ in the framework of the above described formalism, one requires the frequencies for the localized intramolecular vibrations of each chromophore type and their respective Franck−Condon factors. For Chl these data have been taken from the literature (Gillie et al., 1989; Avarmaa & Rebane, 1985). In order to properly describe the tails of the vibrational sidebands for the decomposition of the Q_y region of the PSII-RC absorption spectrum, vibrational frequencies up to approximately 1000 cm^{-1} were considered in the calculations of the homogeneous spectra. For the description of the phonon side bands one furthermore requires the Huang−Rhys factors and the mean phonon frequencies. For PSII-RCs these data have been measured for P_{680}, Chl, and Pheo by Small and co-workers (Jankowiak et al., 1989; Chang et al., 1994). Unfortunately, for Pheo the exact Franck−Condon factors are not available in the literature to our knowledge. For this reason we made the simplifying assumption of using the Chl vibrational structure also for the calculation of the Pheo spectra, but with the important difference that the Q_y region of the Pheo absorption spectrum carries only $^2/_3$ of the Chl oscillator strength (Chang et al., 1994; Kwa et al., 1994b). We furthermore use the specific Huang−Rhys factor and mean phonon frequency for Pheo as determined by hole-burning (Chang et al., 1994). Due to the lack of more detailed information, a further simplification is that all Chls (with the exception of the P_{680} special pair Chls) in the PSII-RC are described by the same set of parameters with regard to the electron−phonon coupling. The same holds for both Pheos. For P_{680} we assume the oscillator strength of two Chl molecules (distributed over two exciton bands) since in our model it consists of a pair of excitonically coupled Chls. The exact parameters used are collected in Table 1.

An an example Figure 4 shows the calculated homogeneous absorption spectra for Chl at different temperatures. As required by theory (Rebane, 1970) these spectra show a very pronounced temperature dependence which is mainly due to the temperature dependence of the electron−phonon coupling. At 10 K the spectrum consists of a prominent zero phonon line (ZPL) and a relatively weak phonon wing (PW) on the high-energy side. With increasing temperature the amplitude of the ZPL decreases and that of the PW increases

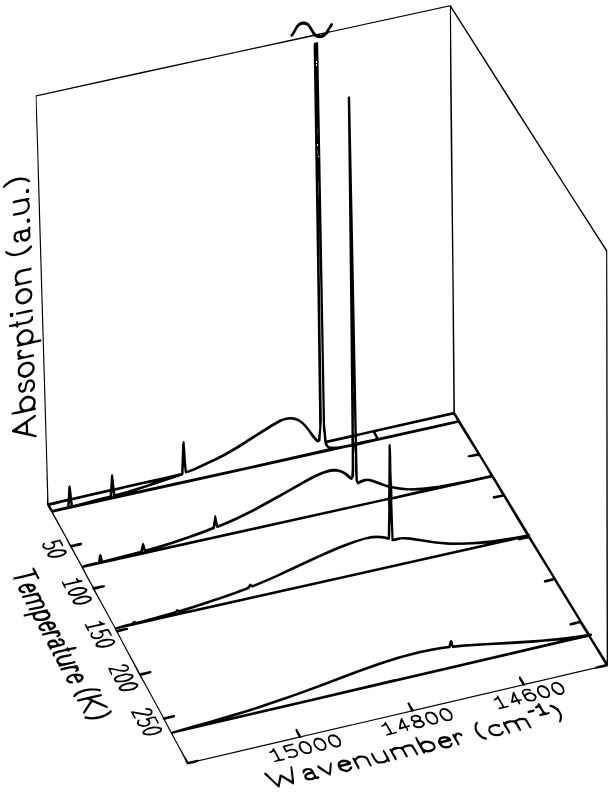

FIGURE 4: Calculated homogeneous absorption spectra for Chl *a* at different temperatures. These spectra are calculated as described in the text. Note that the ZPL of the 10 K spectrum is cut off at about $^1/_3$ of its height.

while the PW also extends more and more to the low-energy side. Finally at 277 K the homogeneous absorption spectrum is completely dominated by the PW and the ZPL has only a minor contribution to the overall spectrum. The homogeneous absorption spectrum of Pheo shows a similar temperature dependence whereas the effects are more pronounced for P_{680}, in accordance with its higher Huang−Rhys factor (data not shown). In the case of inhomogeneously broadened pigment spectra one has to be aware of the possibility that also the width and the spectral position of the IDF might undergo some changes, depending on the temperature-dependent properties of the pigment surroundings (see below).

Global Data Analysis. According to our model in the fitting procedure the absorption spectra of the PSII-RC samples are described as a sum of an invariant part (RC core pigments) plus a sample-specific variable part (antenna and additional Chls). For the spectral decomposition the amplitudes, half-widths (FWHM) and positions of the IDFs have to be optimized for every single pigment. The parameters are coupled across different samples in the following way: (i) The spectral positions and half-widths of the IDFs have to be the same for all corresponding pigments of the different PSII-RC samples. (ii) The IDF amplitudes of the core pigments have to be the same across all samples included in the analysis. A problem like this can be solved numerically by using a method known as "global analysis". Originally this method has been established for the analysis of time-resolved fluorescence data (Beechem et al., 1985) and in this field the method is now commonly used (Holzwarth, 1995). The basic principle of this procedure is that a set of fitting parameters is determined by simultaneous analysis of several

independent and different data sets. The important point is that at least some parameters must be assumed to be the same across all these data sets in order to gain an advantage as compared to single-curve analysis. Some other parameters may be specific for each single data set. In the case of fluorescence decay curves the former are, for example, the fluorescence lifetimes and the latter are the amplitudes of the corresponding exponentials. The main advantage of global analysis as compared to single-curve analysis is that the number of fitting parameters per data set is reduced and thus the accuracy and reliability of the fitting parameters is increased substantially. This concept can be easily adapted to similar problems like the analysis of a set of different absorption spectra. Following our spectral model, then the IDF half-widths and IDF positions of the corresponding pigments have to be the same across all different PSII-RC absorption spectra included in the fitting procedure. The same holds for the amplitudes of the core pigments. Only the amplitudes of the antenna and additional Chls are allowed to vary between the different experimental spectra. For the optimization of this parameter set a genetic algorithm was used (Holland, 1992). Among other advantages, this fitting procedure has the important property that it does not suffer from falling into local minima. All calculations were performed on a wavenumber scale. The main criterion for the quality of the resulting fit is the weighted mean square deviation between the experimental and the fitted spectrum. However, some additional restrictions are included in our spectral model that have to be taken into account to judge the quality of the fits: The Chl/2 Pheo ratios were required to be the same as those found by HPLC within experimental error. For the core pigments, given the oscillator strengths, the area ratios of P_{680}:Chl:Pheo had to be very close to 2:1:0.66. Finally, the spectral positions of the P_{680} bands and of one Pheo had to be consistent with the experimental data (Chang et al., 1994; Tang et al., 1990; Kwa et al., 1994a).

It should be noted that, taking into account all these restrictions described above, the parameter space is very much restricted and the whole procedure is far from being a free curve-fitting procedure. In addition, the use of global analysis further restricts the fitting space to a large extent.

Description of the PSII-RC Absorption Spectra in the Framework of Our Model. A set of PSII-RC absorption spectra from samples with different Chl/2 Pheo ratios was analyzed in the way described above. First the results for the spectra recorded at a temperature of 10 K will be described. We found that the shape of these spectra can be very well explained within our model given all the restrictions mentioned above. Five different sample spectra (samples 2−6) were included in the fitting procedure. Taking into account all the restrictions mentioned above, two about equally good—within the error limits—parameter sets for the spectral decomposition were obtained.

Figure 5 shows the results of parameter set I for the 10 K absorption spectrum of sample 3 (6.4 Chl/2 Pheo). The experimental spectrum is depicted together with the corresponding fitted curve and the individual spectral components. The amplitudes of the pigment spectra marked with V are different for each single PSII-RC absorption spectrum (variable part), whereas the remaining parameters are exactly the same for each sample. The variable part contains two antenna Chls, one of them contributing to the high-energy

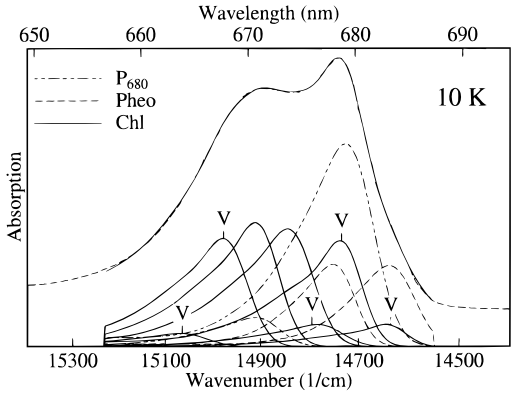

FIGURE 5: Results of a global spectral decomposition (parameter set I), which includes the experimental 10 K absorption spectra of five different samples as described in the text. In this figure the result for sample 3 (6.4 ± 0.2 Chl) is depicted. The experimental spectrum is given (uppermost dashed line) together with the fitted spectrum (uppermost solid line) and the individual spectra used for the fit. Note that the experimental spectra and the fit are not scaled equally to the single components and additionally a baseline shift has been introduced for reasons of better presentation. The amplitudes of single components marked with V are allowed to vary between different samples; the remaining components form the spectral "core" that is identical for each 10 K spectrum included in the fit.

and one to the low-energy peak of the PSII-RC absorption spectrum. Furthermore, it contains three spectrally different pools of additional Chls. The minimum number of additional Chls that had to be included in the fit was three. When only two of them were allowed, then the quality of the fit was substantially worse. It has to be emphasized that in the spectral decomposition according to parameter set I (Figure 5) the absorption on the very red side of the spectrum is dominated by a Pheo (dashed line).

The weighted mean square deviation of the second decomposition that was found by the global fitting algorithm is slightly worse as compared to the first data set (1.77×10^{-3} vs 1.29×10^{-3}). It should be noted that this difference in χ^2 is within the error limits of the whole procedure. Clearly also parameter set II provides a satisfactory description of the 10 K absorption spectra as can be seen from Figure 6a (again for sample 3). The only essential difference between the two decompositions is that the redmost-absorbing Pheo in parameter set I is exchanged with a Chl of the RC core. Thus the absorption at the red edge of the spectrum now is dominated by a Chl rather than a Pheo. The spectral parameters of the remaining pigments differ only slightly between the two data sets. On the basis of the fit of the absorption spectra alone, it is not possible to decide between the two parameter sets. For this reason we give the data for both data sets in Table 2. The hypothetical absorption spectrum of the RC core can be calculated; it is shown in Figure 8 together with its spectral decomposition (using parameter set II).

Of special importance are the fitting results for the absorption spectrum of sample 6. This sample had the lowest Chl/2 Pheo ratio (6.0) and its spectrum differed very much from those of the other samples, especially on the red side. The decomposition for this sample is shown in Figure 7 (using data set II). Out of the three additional Chl pools only the pool on the high-energy side of the absorption spectrum has a substantial amplitude. The other two additional Chls in this PSII-RC spectrum are almost zero.

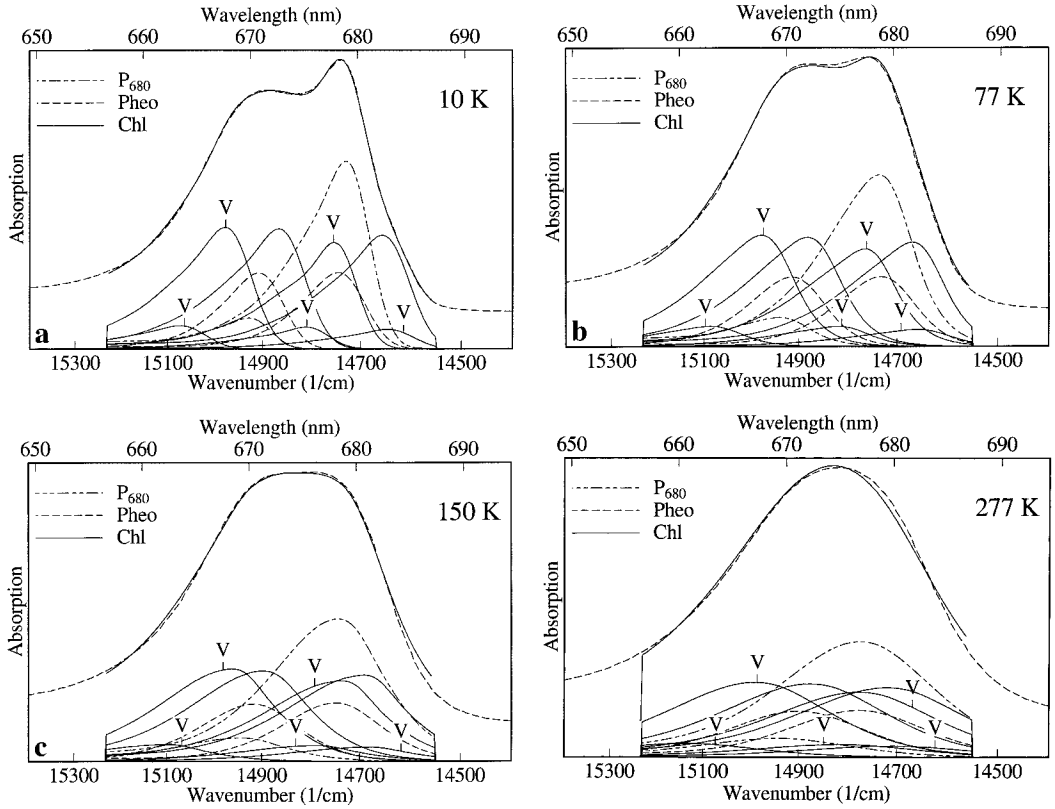

FIGURE 6: (a) Spectral decomposition of the PSII-RC absorption spectrum of sample 3 recorded at 10 K according to parameter set II. In contrast to the decomposition of parameter set I (shown in Figure 5) the redmost-absorbing pigment of the core here is a Chl, rather than a Pheo. Note that the variable spectra in this decomposition are very similar to those shown in Figure 5. (b−d) Results of global spectral decompositions for temperatures of 77, 150, and 277 K, respectively. The analysis of these three data sets was performed analogously to that of the 10 K spectra, except that the amplitudes of the fitting spectra were taken to be the same as in the 10 K decomposition.

Table 2: Fitting Parameters Obtained from the Global Decomposition of the 10 K Absorption Spectra of Five Different PSII-RC Preparations

assignment	spectral maximum (nm, cm^{-1})	maximum of IDF (nm, cm^{-1})	FWHM of IDF (cm^{-1})	amplitude of IDF (au)
		(A) Parameter Set I		
P_{680} (−)	679.1, 14725	680.1, 14703	104	1.59
P_{680} (+)	670.6, 14912	671.5, 14891	96	0.23
$Pheo_1$	677.8, 14754	678.8, 14732	82	0.65
$Pheo_2$	683.2, 14637	684.1, 14616	95	0.64
Chl_{acc1}	673.6, 14846	674.5, 14826	100	0.93
Chl_{acc2}	670.7, 14910	671.3, 14896	93	0.98
Chl_{ant1}	667.7, 14977	668.4, 14960	97	0.71−0.85[b]
Chl_{ant2}	678.6, 14736	679.3, 14722	86	0.72−1.00[b]
Chl_{add1}	663.6, 15069	664.5, 15048	106	0.05−0.18[b]
Chl_{add2}	676.4, 14784	677.3, 14764	98	0.00−0.35[b]
Chl_{add3}	682.9, 14643	683.3, 14634	81	0.00−0.30[b]
		(B) Parameter Set II		
P_{680} (−)	678.8, 14732	679.8, 14710	90	1.53
P_{680} (+)	669.8, 14930	670.6, 14913	85	0.25
$Pheo_1$	678.2, 14745	679.3, 14721	100	0.62
$Pheo_2$	670.1, 14923	671.8, 14884	95	0.62
Chl_{acc1}	672.7, 14865	673.5, 14847	103	0.98
Chl_{acc2}	682.3, 14656	683.2, 14637	103	0.93
Chl_{ant1}	667.7, 14977	668.5, 14958	104	0.83−0.95[b]
Chl_{ant2}	677.9, 14751	678.6, 14737	90	0.75−0.91[b]
Chl_{add1}	663.4, 15074	664.3, 15053	104	0.14−0.27[b]
Chl_{add2}	675.2, 14810	675.9, 14795	91	0.02−0.33[b]
Chl_{add3}	682.7, 14648	683.7, 14627	101	0.00−0.28[b]

[a] Chl_{acc}, accessory chlorophyll; Chl_{ant}, antenna chlorophyll; Chl_{add}, additional chlorophyll. [b] The exact value of this parameter is specific for every single absorption spectrum.

Here it becomes evident that the redmost-absorbing of the additional Chl pools is mainly responsible for the characteristic 683-nm peak in the second derivative of most PSII-RC samples: Sample 6 does not contain this additional Chl and consequently does not show that peak (see Figure 3).

Can One of the Parameter Sets Be Ruled Out? We now return to the question of the two different parameter sets that both fit the absorption spectra about equally well. According to parameter set II (Figure 7) the redmost-absorbing pigment in sample 6 is the low-energy accessory

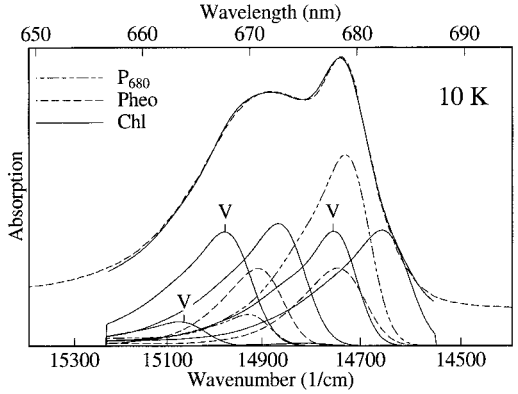

FIGURE 7: Same experiment as in Figure 6a (parameter set II) but for a sample containing 6.0 ± 0.2 Chl/2 Pheo (sample 6 from Figure 1). Note in particular the absence of the ~683-nm additional Chl pool.

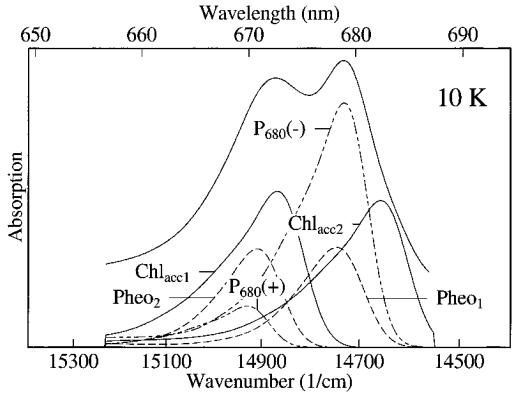

FIGURE 8: Calculated 10 K absorption spectrum of the core PSII-RC resulting from the spectral decomposition given in Figures 6a and 7. (parameter set II) Also shown is the decomposition of this calculated curve into the spectra of $P_{680}(+)$ and $P_{680}(-)$ (upper and lower exciton bands of the primary electron donor), two accessory Chls, and two Pheos.

Chl of the RC core. On the basis of the spectral decomposition of parameter set I (Figure 5), the redmost-absorbing pigment of that sample would be a Pheo. If this were correct, a fairly pronounced Pheo-type fluorescence should be expected after exciting the sample at the red edge of its absorption spectrum (Kwa et al., 1994b). Our fluorescence site-selection experiments show that at 10 K the pigment with the lowest energy in samples similar to 6, i.e., lacking the red-most additional Chl, is indeed a Chl and not a Pheo (Konermann and Holzwarth, manuscript in preparation). *We thus can safely rule out the spectral decomposition according to parameter set I (Figure 5).* For this reason we will in the following only discuss in detail the implications derived from parameter set II, although most of the general features are quite similar for both parameter sets.

It should be noted that the description of the PSII-RC absorption spectra within this model only holds for samples with up to approximately 7 Chl/2 Pheo. If the spectra of samples with higher Chl/2 Pheo ratios were also included, then no good fit could be obtained—at least not under the assumption of only three additional Chl pools. For this reason the absorption spectrum of sample 1 with 8.0 Chl/2 Pheo has not been considered for this data analysis.

Temperature Dependence. To describe the temperature dependence of the PSII-RC absorption spectra the homogeneous absorption profiles for the respective temperature have

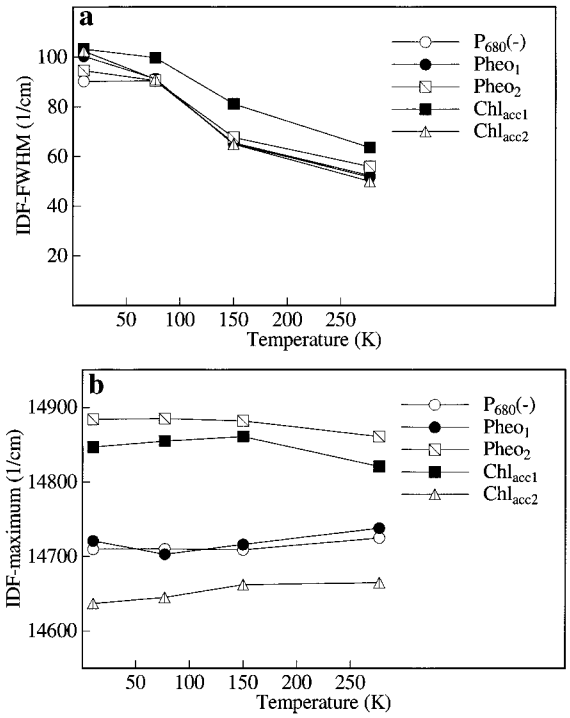

FIGURE 9: Temperature dependence of the IDF widths (a) and IDF maxima (b) for the core pigments of the PSII-RC (see also Figure 6) for parameter set II. A similar dependence is found for parameter set I (data not shown).

to be used (see Figure 4). If, however, only the homogeneous spectra were replaced and the IDF parameters from the 10 K decomposition were directly used, the resulting calculated curves did not fit the experimental absorption spectra very well over a large temperature range (data not shown). While the 77 K spectra could still be described quite well by such a procedure, there was no good agreement for temperatures of 150 and 277 K. For the latter temperatures the experimental spectra are somewhat narrower than the calculated ones. Thus the replacement of the homogeneous spectra alone is not sufficient to explain the temperature dependence of the entire PSII-RC absorption spectra. However, if the IDF parameters are allowed to vary slightly as a function of temperature, all the absorption spectra can be described quite well. We thus carried out further global fitting procedures with absorption spectra recorded at 77, 150, and 277 K, with the important restriction that the IDF parameters had to be as close as possible to the values found for the 10 K data set (parameter set II). Figure 6b−d shows the fits for sample 3 at temperatures of 77, 150, and 277 K, respectively. To compensate for the broadening of the calculated spectra when the temperature is increased, the IDF positions had to be slightly shifted and the widths of the IDFs had to be reduced, whereas the amplitudes, which mainly reflect the pigment content, were directly taken from the 10 K decomposition for all temperatures. At 277 K the IDFs have a width that is about half the values obtained for the 10 K spectra. The maximal difference in the respective IDF positions for 10 and 277 K is about 35 cm^{-1}. This temperature dependence of the IDF parameters is depicted for the pigments of the RC core in Figure 9. The IDF widths and positions of the remaining pigments show a quite similar temperature dependence (data not shown). Finally, it should be mentioned that also with the spectral decomposition according to parameter set I, the tem-

perature dependence of the spectra could be explained equally well and the corresponding IDF parameters show a temperature dependence that is quite similar to the one shown here for parameter set II.

DISCUSSION

Shape of Absorption Spectra and Chl/2 Pheo Ratio. In this work we compared the pigment stoichiometry and the absorption spectra at various temperatures of PSII-RC samples that were prepared according to two different methods. The Chl/2 Pheo ratio is lower for method 2 samples because method 2 includes the procedures of method 1 plus a further purification step. The resulting samples show a variability in their Chl/2 Pheo ratios ranging from 6.2 to 8.0 Chl/2 Pheo for method 1 and from 6.0 to 6.4 Chl/2 Pheo for method 2. If one were to take the Chl/2 Pheo ratio as a measure for the purity of the sample, then the purest samples with 6.0 Chl/2 Pheo could only be obtained by method 2. The reason for the differences between independent preparations following the same method is not clear. They might, at least partly, be due to differences in the starting material used for the preparations (spinach was bought from the local market at different seasons). In any case this shows that one cannot in general rely on the full reproducibility of the established isolation procedures for PSII-RCs. As an important consequence, since PSII-RCs with different pigment composition have different spectral properties, the pigment stoichiometry of every single batch should be determined by HPLC and also the low-temperature absorption spectrum should be recorded before the sample is used for spectroscopical experiments where the exact details may be important.

Another important result of the pigment determinations of many different batches in our laboratory is that the Chl/2 Pheo ratios that can be found are in general not integer numbers, in agreement with results from other laboratories (J. Dekker, personal communication; J. Barber, personal communication). This shows that the corresponding samples consist of mixtures of PSII-RCs with different pigment stoichiometry. If it were possible to measure the Chl/2 Pheo ratios of individual PSII-RCs in a sample, then a distribution of different integer Chl/2 Pheo ratios would be found. By HPLC, not the width but only the mean value of this distribution can be determined. This is unfortunate since the exact details of this distribution may substantially influence the results of kinetic measurements, hole-burning experiments, etc.

At room temperature the Q_y band of the chlorins in the PSII-RC are heavily congested and as a consequence the absorption spectrum does not show a detailed structure like, for example, that of the RC from purple bacteria (Jean et al., 1988). It is at least partly possible to overcome this problem when the samples are cooled to low temperatures, as is shown in Figure 1. Then also the absorption spectrum of the PSII-RC reveals some characteristic and pronounced features.

Our data show that the shape of the 10 K absorption spectra strongly depends on the Chl/2 Pheo ratio of the samples, but interestingly samples with comparable pigment ratios still can have substantially different absorption spectra. Within our model this can be explained easily as follows: During the isolation procedure the PSII-RC is peeled out of the entire PSII complex by removing proteins and pigments. When individual isolation procedures are compared to each other then it might happen that more loosely bound pigments are not always removed to the same extent. Sometimes more red-absorbing and sometimes more blue-absorbing external Chls are removed. When the detergent incubation is stopped, then in most cases the removal of these external pigments is not really complete. Some of the particles still can contain additional Chls as a remainder of the PSII core antenna or even in nonnative binding sites that are now exposed to the outside but were originally saturated by protein−protein interactions. It might also happen that some PSII-RCs lose one or even both of their antenna Chls. With such a sample it is possible to measure a Chl/2 Pheo ratio of 6.0 even when it still contains considerable amounts of additional Chls.

Notwithstanding the above-mentioned situation, the approximate Chl/2 Pheo ratio of the samples can be estimated to some extent from the shape of the low-temperature (10 K) absorption spectrum even without performing an HPLC analysis. Of special importance in this respect is the region around 670 nm in the negative second derivative of the spectrum. Here samples with about 6 Chl/2 Pheo show a single peak. At higher Chl/2 Pheo ratios this peak develops a dip at 672 nm, and finally at a pigment ratio of up to 8 Chl/2 Pheo it is split up into two well-separated peaks. Another significant feature that is present in most of the 10 K absorption spectra is the shoulder on the low-energy side of the absorption spectrum, which in the negative second derivative shows up as a prominent peak near 683 nm. Only sample 6 with a Chl/2 Pheo ratio of 6.0 does not show this feature. The origin of this band has been discussed in the literature before. It has been suggested that it should represent an exciton band of P_{680} (van Kan et al., 1990) or belong to a spectrally different pool of P_{680} which is present in part of the PSII-RCs (Kwa et al., 1994a). Our data strongly suggest that this shoulder has a different origin: Since it can be removed by a further purification step it is very likely that it is not an intrinsic property of the PSII-RC and especially it is not a part of P_{680}. In our view this shoulder results from a red-absorbing Chl that is bound to a fraction of the PSII-RCs and that is only partly removed during the isolation procedure. This interpretation is in full accordance with the recent findings of Chang et al., (1994, 1995b). On the basis of temperature-dependent hole-burning spectra, the authors of that study also drew the conclusion that a fraction of the PSII-RCs contains a Chl absorbing at around 684 nm, which is connected to P_{680} via energy transfer.

For the discussion of the relationships between pigment stoichiometry and spectral shape it is also important to include the low-temperature absorption spectra of PSII-RCs from other laboratories. The question of the Chl/2 Pheo ratio in PSII-RCs and the relationship between pigment stoichiometry and absorption spectrum was recently addressed in a paper of Chang et al., (1994). In that paper three different kinds of samples were compared with Chl/2 Pheo ratios determined to be about 4, 5, and 6. When the low-temperature absorption spectra of those samples are compared to the spectra of our samples (Figure 2), it seems likely that the Chl/2 Pheo ratio in that study is systematically underestimated. The spectrum of their "4 Chl preparation" looks very similar to one of our samples with 6.0−6.5 Chl/2 Pheo. In the spectrum of their "5 Chl preparation" the valley

between the main absorption peaks is not clearly resolved, and for their "6 Chl preparation" the peaks are not separated at all. According to our results this clearly indicates that the Chl/2 Pheo ratio of the latter samples is substantially higher than determined by the authors of that study. This view is also supported by a recent study of Eijckelhoff and Dekker (1995). However, we agree with the conclusion of Chang et al., that part of the "extra" Chl bound to the PSII-RC absorbs on the low-energy side of the spectrum (see above).

For the spectroscopic studies of van Kan et al. (1990) and Otte et al. (1992), the samples were prepared by a different method than our PSII-RCs (Chapman et al., 1988). The second derivative of the low-temperature absorption spectrum from those samples looks very similar to that of our samples with about 7 Chl/2 Pheo. This is especially true for the region around 670 nm, which can be taken as an approximate measure for the Chl/2 Pheo ratio (see above). In the work of Otte et al., the Chl/2 Pheo ratio of the PSII-RCs was not measured, but it was only stated that those samples were assumed to contain about 6 Chl/RC.

The situation is similar for the samples used by Kwa et al., (1994a,b, 1992). Here also the Chl/2 Pheo ratio was not determined exactly, but it was again assumed that those RCs contained about 6 Chls. Also in this case the 670-nm region of their absorption spectrum clearly reveals the presence of some additional Chls, although the corresponding features are less pronounced than in the spectrum of van Kan et al. Moreover, the spectrum extends about 2 nm more to the red side than the spectra of our samples; i.e., the spectrum has an intense red tail. This has to be interpreted in a way that those samples also have a higher Chl content; in particular, they contain a substantially increased amount of "red Chl" as compared to our samples.

It has to be noted that the 683-nm peak in the second derivative of the absorption spectra from our samples is about 1 nm blue-shifted as compared to the corresponding feature in the spectra of the above-mentioned laboratories. Most likely this difference is also due to the higher amount of red Chl in those samples. Such differences in samples may become quite important if the results of various spectroscopic experiments are compared.

Model Parameters. On the basis of a theoretical calculation of homogeneous line shapes for the chromophores in the RC and the assumption of weak coupling between pigments (except for the special pair), we were able to construct a model that describes the temperature dependence on the one hand and the relationship between the varying pigment stoichiometry and the shape of the PSII-RC absorption spectrum on the other hand very well. In the following the resulting model parameters will be discussed and finally a justification for the assumptions underlying this spectral model with the presented.

Since the structure of the PSII-RC is not known, a very important point in our model is the assumption of a pigment homology of the PSII-RC to the reaction center from purple bacteria (Michel & Deisenhofer, 1988). According to present understanding the RC core of PSII consists of two Chls constituting the primary electron donor P_{680}, two accessory Chls, and two Pheos (Seibert, 1993). Since these pigments are located in the interior of the protein complex, they can probably not be removed during the isolation procedure without destroying the whole complex. Therefore it is reasonable to assume a constant part in the absorption spectrum which results from the presence of these core pigments. The minimal pigment content found in our samples is about 6 Chl/2 Pheo. In order to rationalize this finding it is assumed that there exist two more antenna Chls which are bound relatively tightly to the periphery of the PSII-RC proteins. Time-resolved fluorescence experiments indicate the presence of two relatively slow energy-transferring Chls (Roelofs et al., 1991, 1993; Holzwarth et al., 1994; Gatzen et al., 1995) which most likely have a relatively large distance of ≥ 20 Å to the core pigments (Schelvis et al., 1994; Deisenhofer & Michel, 1991). If these antenna Chls are not hidden deeply in the protein matrix like the core pigments, then it might be possible to remove a certain percentage of them during the isolation procedure. This structural picture of the PSII core is consistent with the results of computer modeling studies (Ruffle et al., 1992; Nugent et al., 1993), which indicate that the histidines 118 on both the D_1 and D_2 protein are located probably on the outside of the PSII-RC and that these residues could act as binding sites for antenna Chls. Since in most of the samples the Chl/2 Pheo ratio is even higher than 6 (but often a noninteger number), it follows that some of the PSII-RCs in the samples do bind some additional Chls to a varying extent. As discussed above, these Chls might be remainders of the PSII antenna complex which could not be completely removed during the isolation procedure. Another possibility would be that they are attached at some nonnative binding sites at the periphery of the PSII-RC, thus constituting the picture of a "sticky" protein surface.

For the global data analysis procedure we included the absorption spectra of several different PSII-RC samples and fitted them simultaneously to get the IDF amplitudes, spectral positions, and FWHMs of the individual transitions. As a result of this procedure, all spectra can very well be described as the sum of a core spectrum, which is constant across all samples, plus a sample-specific variable part. The constant part consists of the contributions of the two exciton bands of P_{680}, two accessory Chls, and two Pheos (see Figure 8). Our way of analyzing the data included some important restrictions. One of those restrictions was that the position of the P_{680} exciton bands has to be close to the values found in hole-burning experiments (Chang et al., 1994) and singlet-minus-triplet absorption difference spectra (Kwa et al., 1994a). Another restriction was that at least one of the Pheos should have its absorption maximum around 680 nm (Tang et al., 1990). Furthermore, the ratios of the oscillator strengths between the core pigments were fixed in the framework of our model and the Chl/2 Pheo ratios of the fits had to be identical to the values determined by HPLC within the error limits. All these demands are fulfilled by two different data sets, which mainly differ in the assignments of the redmost-absorbing pigment of the RC core (Chl or Pheo). On the basis of recent site-selection experiments (Konermann and Holzwarth, manuscript in preparation) we could rule out the decomposition which contains Pheo as the redmost-absorbing core pigment. This leaves us with the spectral decomposition shown in Figure 6a (parameter set II). In our view this data set is in good agreement with the experimental data of several laboratories. In particular, this decomposition predicts one accessory Chl and one additional Chl pool which both absorb at lower energies than P_{680} and can therefore act as excitation traps at low

temperatures. This is in very good agreement with temperature-dependent measurements of the fluorescence quantum yield (Groot et al., 1994) and also with fluorescence site-selection data from the literature (Kwa et al., 1994b).

To explain the differences between the various absorption spectra it was necessary to vary slightly the amplitudes of the antenna Chls. This finding served as a criterion for the assignment of the antenna Chls, which are located with their spectral maxima on the blue and on the red side of the spectrum. It was also necessary for a good global fit to allow for three spectrally different pools of additional Chls, also present with varying amplitudes (Table 2). It cannot be decided whether these three pools of additional Chls really represent distinct Chl-binding sites with different spectral properties. Another possibility would be to assume only one binding site which can be in different conformational states. The fact that the amplitudes of the antenna and additional Chls are sample-specific in any case shows that they can be partially removed during the isolation procedure and are thus probably located more on the outside of the reaction center. This means that especially pigments which absorb on the blue (around 670 nm) and the red side (around 680 nm) of the spectrum are responsible for the relatively slow energy transfer toward the RC core pigments, as found in time-resolved fluorescence measurements (Holzwarth et al., 1994; Roelofs et al., 1991, 1993; Gatzen et al., 1995). This is also supported by a recent work (Vacha et al., 1995) where it was shown that it is possible to specifically remove a Chl absorbing at around 670 nm from a PSII-RC with >6 Chl/2 Pheo. The resulting "5 Chl sample" no longer shows the above-mentioned slow energy-transfer component on the blue side of the spectrum. It has to be noted that this sample is obtained by using a Cu affinity column which is an important difference from the other isolation procedure described in the literature. The absorption spectrum of that sample looks very similar to what is obtained after subtracting the contribution of the blue antenna Chl band from our calculated absorption spectra.

Temperature Dependence of IDF Parameters. The temperature dependence of the PSII-RC absorption spectra is mainly caused by the changing shapes of the homogeneous spectra as caused by electron−phonon coupling due to the much higher Huang−Rhys factors for phonons as compared to the ones for localized vibrations (see Figure 4). Whereas the changes in the IDF spectral positions are relatively small (less than 35 cm^{-1}) over a very large temperature range from 10 to 277 K, the widths of the IDFs have to be modified from a mean value of about 95 cm^{-1} at 10 K to a mean value of about 91 cm^{-1} at 77 K and 65 cm^{-1} at 277 K. It should be pointed out that the predicted IDF widths at 10 K in the range of 80−100 cm^{-1} are in excellent agreement with the data from hole-burning experiments (Jankowiak et al., 1989; Tang et al., 1990, 1991). Most of the slight spectral shifts could well be explained by changes in the refractive index of the protein with temperature. It has been shown experimentally that the volume of proteins can be reduced by lowering the temperature (Fields et al., 1994). If the protein contracts then the effects of the surrounding matrix on the pigments will be more pronounced, and thus the IDF widths may be expected to be larger at lower temperatures. This interpretation gains some additional support from the high-pressure hole-burning studies of Chang et al., (1995a), where the width of the IDF from P_{680} increases substantially with increasing pressure. The relatively large changes predicted by our fits in the IDF FWHMs for temperatures above 100 K are nevertheless unexpected, however. The temperature-dependent transition from a static distribution to a dynamic distribution will occur around the glass transition point of the protein, which might well change the width of the distribution. There are good reasons, however, why our model predicts substantially smaller IDF FWHMs at higher temperatures. First of all, we cannot expect that the simple linear electron−phonon coupling theory will hold for the whole temperature range from 10 up to 277 K (Rebane, 1970). Second, the mean phonon frequency and the Huang−Rhys factor cannot be considered to be temperature-independent over the whole range. Also, it would be required to include the coupling of electronic transitions to more than one lattice vibration mode at higher temperatures. As a result of these limitations, our calculated homogeneous spectra for temperatures above 100 K probably provide too large homogeneous FWHMs. For example, the FWHM of the calculated homogeneous Chl *a* spectrum at 277 K is about 18 nm, whereas it is known experimentally that in nonpolar solvents the experimental width of a Chl *a* spectrum, which already includes some inhomogeneous broadening, is also about 18 nm (data not shown). Thus the effect of a too large homogeneous bandwidth is simply compensated for in our calculation at higher temperatures by narrower IDFs. We thus suspected that our model will be formally quite good up to temperatures of about 100 K. The fact that even the 277 K spectra do fit quite well (although the IDF FWHMs should not be taken as such) is quite understandable easily on the basis of the mathematical compensation effects between inhomogeneous and homogeneous widths (see Figure 9a).

It would be possible to make some reasonable compensation in the temperature dependence of the Huang−Rhys factors and the phonon frequencies in order to arrive at more realistic FWHMs at higher temperatures. However, since there are no reliable experimental data available for the required temperature-dependent parameters, any such calculations at present would be too approximative and we choose to use the same parameter set over the whole temperature range.

How Important Is Excitonic Interaction? We now turn to the key question of how important excitonic interactions between the pigments of the PSII-RC are for the understanding of its absorption spectrum. In the case that such interactions between the chromophores in the RC core should be at least intermediately strong, individual pigments could not be assigned to specific absorption bands. In our spectral model exciton coupling was neglected with the exception of the putative P_{680} dimer. This point will now be justified. The interaction energies between the single chromophores in a complex depend on their distances (R^{-3} dependence) and the orientations of their transition dipole moments relative to each other (Pearlstein, 1991). A strong excitonic interaction can only occur if the respective pigments are in close proximity (typically <15 Å) and have a favorable orientation relative to each other. From this we can already conclude that this kind of interaction can be neglected for the antenna and additional Chls in our model (Durrant et al., 1995) since the distances are probably too large. However, this might be different in the case of the six pigments in the RC core.

It has been suggested that the spectral properties of the RC core are completely governed by such intermediate to strong excitonic interactions (Tetenkin et al., 1989). The problem was also addressed in a recent paper by Durrant et al. (1995). In that study the structure of the bacterial RC was superimposed on the PSII-RC in order to calculate the interaction energies between the pigments in the RC core. If this is done, the calculated dipolar coupling energy between the primary donor Chls comes out to be about 400 cm^{-1}, a value that is nearly 4 times larger than the one observed experimentally for P_{680} (Kwa et al., 1994a). Thus, at least in the case of the two special pair Chls, the orientation and/or the distance of the chromophores in the PSII-RC has to be assumed to be substantially different from that of the bacterial RC. In order to account for this fact, Durrant et al. assumed a larger distance for these two Chls constituting the special pair, as compared to the bacterial RC. Nevertheless, they left the positions of the remaining chromophores unchanged. Accordingly, the absorption spectrum of the RC core pigments has to be described as that of a coupled system resulting from the substantial excitonic interactions (about 100 cm^{-1}) between the majority of the pigments. In their model the primary electron donor P_{680} is not considered to be a "dimer" but a "multimer" of excitonically coupled pigments, extended over the whole core and including the active Pheo.

In our view such a "multimer model" of excitonically coupled RC core at present is in conflict with a variety of experimental observations. For example, hole-burning spectroscopy (Tang et al., 1990) has shown that it is possible to bleach one Pheo selectively and essentially without changing the remaining part of the spectrum or introducing substantial band shifts. The same is true for the spectrum of the primary donor. Both oxidation of P_{680} and triplet formation introduce relatively little band shifting but only the disappearance of a band and the appearance of a Chl monomer band (van Kan et al., 1990). A simple disappearance of a band is also observed upon reduction of Pheo (Tang et al., 1990). All these observations are in contradiction with the picture of a strongly excitonically coupled multimer RC core. Furthermore, the calculated spectrum of the multimer (Durrant et al., 1995) has almost all its oscillator strength in the 680-nm band and very little in the 670-nm band, in contrast to the observed experimental spectrum and also in contrast to our calculated RC core spectrum (Figure 8). A severe disadvantage of the multimer model consists also in the fact that a temperature dependence cannot be calculated due to lack of a suitable theory. This fact makes an experimental check of many of its predictions impossible. One further criticism to the model of Durrant et al. is the following: There exists a general agreement that the orientation of the special pair Chls is different in purple bacterial RCs and in PS II RCs. Taking this for granted, it is very likely that also the positions of the remaining pigments are different in the two RCs. Since the interaction energies between the chromophores are strongly dependent on their distances and relative orientations, it is unjustified that only the structure of the special pair region is changed for the exciton calculations. Indeed, taking all these arguments into account, it seems likely that excitonic interactions do not play such an important role in the RC core as suggested by Durrant et al. Given the poor information we presently have on the PS II RC structure, it is quite possible that a spatial arrangement significant different from that of the bacterial RC is realized which could well result in much lower interaction energies between the pigments than those calculated on the basis of the purple bacterial RC structure. In summary, in our opinion at present there exists more experimental evidence against relatively strong coupling than in favor of it. Considering these arguments we fell quite justified in neglecting to a first approximation the excitonic interactions between the majority of the pigments in the framework of our model. Nevertheless, we are well aware of the fact that this also represents only an approximative description of the electronic properties of the PSII-RC. Finally, it has to be pointed out that even if the X-ray structure of a pigment−protein complex is known and exciton theory is used to calculate the absorption spectrum of this complex, the result might be only a relatively poore description of the experimental spectrum [see the review by Pearlstein (1991)].

However, it has to be noted that our spectral model is not necessarily in complete contradiction to the picture of a core reaction center with excitonic interaction among all six pigments. If one were to accept this view, the results of our spectral decomposition only need a different interpretation: From the absorption spectra of the different samples we extracted an invariant spectral core which we described as the sum of spectra from a Chl dimer P_{680}, two accessory Chls, and two Pheos (Figure 8). If only the overall shape of this spectral core is considered, it could also be interpreted as the spectrum of an excitonically coupled multimer system. Also, the relationship between spectral shapes and pigment stoichiometry in our model would not be affected by this point of view. If, however, this interpretation were used, the assignment of RC core pigments to their specific spectral positions and the calculation of temperature-dependant single pigment spectra, as done in our study, would be unwarranted.

CONCLUSIONS

Despite the fact that our model is based on some assumptions and simplifications, it provides a satisfactory description of the PSII-RC absorption spectrum. The relationship between pigment ratio and the shape of the absorption spectrum can be explained, and moreover the temperature dependence of the spectrum is described on the basis of well-established theories, which in our view provides strong support to the validity of the approach. At present the model is furthermore in agreement with a substantial amount of experimental data from several research groups. Most importantly, however, this model makes a variety of specific predictions and assignments that in the future can be tested in detail experimentally. In our view it presently represents a very useful working hypothesis that allows us to design new experiments which eventually will be suitable to either support or disprove the underlying assumptions of the model. It will be particularly important to what extent the predictions from this model will be consistent with the results of time-resolved experiments. Such tests are presently in progress in our laboratory.

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