Pigment Assignment in the Absorption Spectrum of the Photosystem II Reaction Center by Site-Selection Fluorescence Spectroscopy†

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Received January 23, 1997; Revised Manuscript Received March 31, 1997⊗

ABSTRACT: The steady state fluorescence properties of the photosystem II reaction center (D_1−D_2−cyt-b_{559} complex, PSII-RC) have been investigated by site-selection spectroscopy. The pattern of the vibronic bands in the emission spectra is used to identify the fluorescing species that have their absorption maxima on the red edge of the spectrum (at around 682 nm). At 10 K, even samples with a low content of red absorbing chlorophyll *a* (Chl) show pure Chl emission upon excitation at 685 nm, whereas at 77 K the fluorescence of the PSII-RCs is contributed to by Chl and pheophytin *a* (Pheo) in a ratio of roughly 8:2. These results allow an unequivocal distinction between two different spectral decompositions that were recently suggested for the absorption spectrum of the PSII-RC [Konermann, L., & Holzwarth, A. R. (1996) *Biochemistry* 35, 829]. Only one of these decompositions is compatible with the experimental data presented here according to which the absorption on the red edge of the spectrum is dominated by an accessory Chl.

Since the reaction center of photosystem II (PSII-RC)[1] was isolated for the first time in 1987 (Nanba & Satoh, 1987), it has been subject to numerous biochemical and spectroscopical studies [for reviews, see Renger (1992) and Barber (1993)]. According to present understanding, this pigment−protein complex contains about six chlorophylls *a* (Chl), two Pheophytins *a* (Pheo), and one to two β-carotenes (Eijckelhoff & Dekker, 1995; Gounaris et al., 1990). Upon optical excitation, energy equilibration occurs among the various chromophores which is followed by an electron transfer from the primary donor P_{680} to one of the Pheo molecules (Durrant et al., 1992; Holzwarth et al., 1994; Müller et al., 1996). However, the interpretation of spectroscopic experiments on the PSII-RC is strongly hampered by the fact that the Q_y absorption and fluorescence bands of the Chls and Pheos in the complex very strongly overlap. The assignment of these single pigments to distinct positions in the absorption spectrum is not a straightforward task and has been the subject of a considerable number of studies [see, e.g., van Kan et al. (1990), Garlaschi et al. (1994), Tang et al. (1991), and Konermann and Holzwarth (1996)]. Tetenkin et al. (1989) pointed out that excitonic interactions between the chromophores could be crucial for the understanding of the PSII-RC, so that it would be unwarranted to describe its absorption spectrum as a sum of single pigment spectra. Directly related to this problem is the question as to the nature of P_{680}, which according to a number of publications can be described as a dimer of two molecules Chl (van Kan et al., 1990; Noguchi et al., 1993; Michel & Deisenhofer, 1988; Kwa et al., 1994a). This view is in contrast to a work of Durrant et al. (1995), who considered P_{680} as a "multimer" which comprises contributions from more than two chromophores and also includes the Pheo electron acceptor.

We recently proposed a detailed model that describes the absorption properties of the PSII-RC (Konermann & Holzwarth, 1996). This model assumes *weak* excitonic interactions between the chromophores (with the exception of the putative P_{680} dimer) so that the absorption spectrum basically can be described as the sum of individual pigment spectra. It takes into account electron−phonon coupling and the inhomogeneous broadening of the pigment spectra. The temperature dependence of the PSII-RC absorption spectrum, the relationship between varying pigment stoichiometry, and the shape of the spectrum were all explained very well. It was thus possible to assign specific absorption bands to the individual chromophores in the complex. Unfortunately, two different spectral decompositions were obtained that described the experimental absorption spectra about equally well. According to the first of these decompositions, the very red edge of the absorption spectrum ($\lambda \geq 684$ nm) would be dominated by Pheo, whereas for the second one it would be dominated by Chl. Thus, to safely rule out one of these possibilities, detailed information about the absorption properties of this spectral region are required.

The data available in the literature are not sufficient to make a definite decision between the two decompositions: Tang et al. (1990) interpreted their hole-burning data on the PSII-RC in a way that at 4 K one of the Pheo molecules absorbs almost isoenergetically with P_{680} at about 682 nm. The authors concluded that this should be the Pheo which is active in charge separation. Kwa et al. (1994b) used site-selection fluorescence spectroscopy to obtain information about the low energy pigments at 4 K. In that study, the

† This work was supported in part by the Deutsche Forschungsgemeinschaft (Sonderforschungsbereich 189, Heinrich-Heine-Universität, Düsseldorf) and Max-Planck-Institut für Strahlenchemie, Mülheim a.d. Ruhr). It is part of the Ph.D. thesis of L.K. at the Heinrich-Heine-Universität, Düsseldorf.

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⊗ Abstract published in *Advance ACS Abstracts,* May 15, 1997.

[1] Abbreviations: Chl, chlorophyll *a*; Chl/2 Pheo ratio, stoichiometrical ratio of chlorophyll *a* per two molecules of pheophytin *a*; P_{680}, primary electron donor of photosystem II; Pheo, pheophytin *a*; PSII-RC, reaction center of photosystem II (D_1−D_2−cyt-b_{559} complex); ZPL, zero-phonon line.

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vibrational region of the fluorescence spectrum upon selective excitation was used as a "fingerprint" to identify the emitting species. From their data, Kwa et al. concluded that there is a third long wavelength Chl present (besides the two Chls constituting P_{680}) and they estimated the absorption maximum of this Chl to be about 679−682 nm. However, these site-selection fluorescence spectra did not support the presence of a long wavelength absorbing Pheo that was claimed by Tang et al. (see above). The interpretation of the data of Kwa et al. is complicated by the fact that the samples used for that study had a Chl/2 Pheo ratio that was not very well determined and was probably in the range of 6.5 (Konermann & Holzwarth, 1996). Thus, a possible Pheo emission could have been masked by the fluorescence of "additional" Chls.

Here we report on fluorescence site-selection experiments analogous to those of Kwa et al. but at two different temperatures and by using PSII-RC samples with precisely known Chl/2 Pheo ratio. As a result of this study, we can unequivocally rule out the spectral decomposition with a "red" Pheo and support the one with a Chl on the low energy side of the absorption spectrum.

EXPERIMENTAL SECTION

PSII-RCs were prepared according to the method of van Leeuwen et al. (1991) with slight modifications. Instead of incubating the samples for two times in a Triton X-100-containing buffer, the incubation was carried out three times. The pigment stoichiometry of the samples was measured by HPLC as described previously (Konermann & Holzwarth, 1996). The samples were stored in liquid nitrogen until further use.

Chl (Sigma) was dissolved in buffer (10 mM Bis-Tris, pH 7.2) containing 2% Triton X-100. To obtain a Pheo solution, such samples were acidified by one drop of HCl (10 M) per 3 mL of sample volume. Complete pheophytinisation was checked by HPLC. For spectroscopic measurements, all samples were mixed with 60% Glycerol. Absorption spectra of the samples were recorded as described previously (Konermann & Holzwarth, 1996). For absorption and fluorescence measurements, the optical density of the samples was adjusted to about 0.8 per 1 cm at the maximum of the Q_y band.

Fluorescence spectra were recorded on a home-built spectrometer. The samples were excited by a cw Dye Laser (Spectra Physics, model 375 B) using DCM (Radiant Dyes) laser dye pumped by an Ar^+-Laser (Spectra Physics, model 2030). The sample was placed in a liquid helium flow cryostat (Leybold Heraeus), and the fluorescence light was detected at an angle of 90° to the excitation beam. The emission was focussed onto the entrance slit of a spectrograph (Jobin-Yvon, HR 250) by a lens (f = 150 mm). Fluorescence spectra were recorded by a CCD camera system (Chromex), which was controlled by a personal computer. Each emission spectrum was corrected for the wavelength dependence of the CCD camera sensitivity. The overall spectral resolution of this setup was about 1 nm.

RESULTS

Absorption Spectra. The measurements for this study have been carried out on two PSII-RC samples, which have been prepared according to the same method (see previous section). The Chl/2 Pheo ratio has been determined by HPLC to be 6.0 ± 0.2 in both cases. Nevertheless, the spectral properties of these two samples differ from each other. Figure 1 shows the long-wavelength range of the absorption spectra at a temperature of 10 K. Sample 2 shows a shoulder at about 684 nm in its absorption spectrum, whereas this feature is almost absent in the spectrum of sample 1.

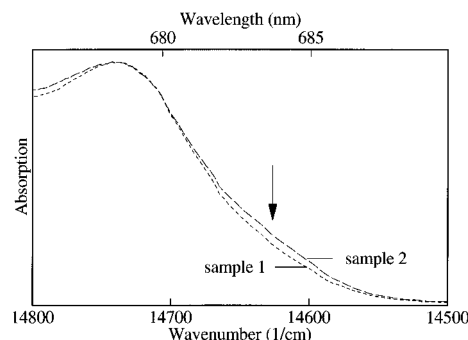

FIGURE 1: Long-wavelength range of the absorption spectra of the two different PSII-RC samples used in this study. The spectra were normalized to their maximum. The arrow indicates the shoulder at around 684 nm in the spectrum of sample 2.

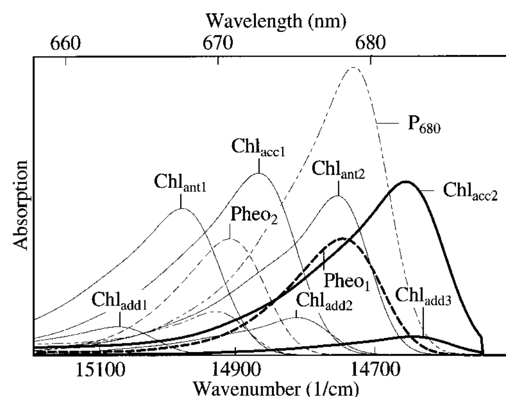

FIGURE 2: Decomposition of the absorption spectrum of PSII-RC sample 2 for a temperature of 10 K according to [Konermann and Holzwarth (1996)]. For details, see text.

The absorption spectra of both samples were analyzed in the framework of the spectral model presented recently (Konermann & Holzwarth, 1996); for this purpose the second decomposition given in that work was used. The result of this analysis for the 10 K spectrum of sample 2 is given in Figure 2. The spectrum of this sample is described as the sum of spectral bands assigned to the primary electron donor (P_{680}), two Pheos, two accessory Chls (Chl_{acc}), and two antenna Chls (Chl_{ant}). Furthermore, three minor spectral bands of additional Chls (Chl_{add}) were included in the analysis, which are assigned to "pools" of nonstoichiometrically bound Chl. According to the spectral model, the redmost absorbing of these three pools (Chl_{add3}) is largely responsible for the shoulder on the low-energy side of the PSII-RC absorption spectrum. The decomposition for the absorption spectrum of sample 1 gave very similar results as the one for sample 2. The only important difference is that for sample 1 the Chl_{add3} pool is missing.

Site-Selection Fluorescence Measurements. The vibronic region of the PSII-RC fluorescence spectrum was measured at 77 K upon selective excitation in the Q_y region. Figure 3 shows the dependence of the emission spectra on the excitation wavelength for sample 1. The respective spectra for sample 2 are virtually identical to the ones shown here

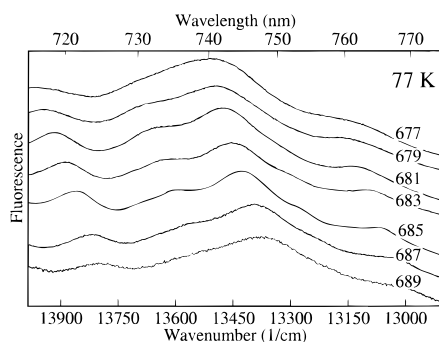

FIGURE 3: Vibronic region of the emission spectra of PSII-RC sample 1 measured at a temperature of 77 K. Numbers at the spectra denote the different excitation wavelengths.

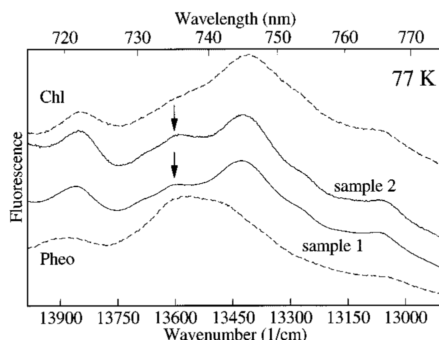

FIGURE 4: Vibronic region of the emission spectra of the PSII-RC samples 1 and 2 excited at 685 nm at a temperature of 77 K. Also shown are the respective emission spectra for Chl (excited at 685 nm) and Pheo (excited at 681 nm). The spectrum of Pheo has been shifted on the wavenumber scale to align it to the remaining spectra. The band that has been marked with an arrow indicates a contribution of Pheo to the emission of the two PSII-RC samples.

(see also Figure 4). The peak positions of the vibronic bands shift with the excitation wavelength. This is a manifestation of site selection, which arises due to the selective excitation into the ZPLs of the inhomogeneously broadened absorption spectrum (Personov, 1983; Kwa et al., 1994b). Closer inspection of the spectra in Figure 3 reveals that the structure of the vibronic bands is most pronounced when the samples are excited at 685 nm.

To identify the pigments that are responsible for the observed vibronic emission bands, the spectra of the PSII-RCs (excited at 685 nm) were compared with those of Chl and Pheo (Figure 4). Chl was also excited at 685 nm whereas for the Pheo sample an excitation wavelength of 681 nm had to be chosen to obtain a spectrum with good signal/noise ratio. In order to compensate for the different excitation wavelengths (685 nm vs 681 nm) and to allow a direct comparison of all four spectra, the Pheo spectrum in Figure 4 was shifted by a value determined according to the excitation wavelength difference on the wavenumber scale, i.e., by 85 cm^{-1} (Kwa et al., 1994b).[2] As can be seen, the vibronic bands of both PSII-RC samples closely resemble the Chl spectrum and not that of Pheo. However, the pronounced shoulder at 735 nm in both PSII-RC spectra is coincident with the peak position of the main vibrational band of Pheo and it has no corresponding counterpart in the Chl spectrum. From these data we conclude that upon excitation at 685 nm at a temperature of 77 K the emission of both PSII-RCs is contributed to by both Chl and Pheo. The relative contributions of both pigment types can be roughly estimated to be about 80 and 20%, respectively, from a

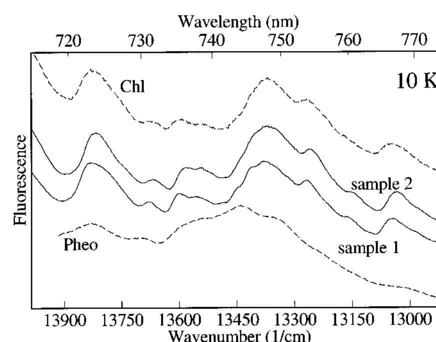

FIGURE 5: Vibronic region of the fluorescence spectra of the PSII-RC samples 1 and 2, Chl and Pheo as in Figure 4 but for a temperature of 10 K.

decomposition of the spectrum into the composite spectra.

Also, at a temperature of 10 K, the peak positions of the vibronic bands shift with the excitation wavelength, indicating site-selective excitation conditions. The data we obtained at 10 K are very similar to those measured earlier by Kwa et al. (1994b) (data not shown). The PSII-RC spectra obtained upon excitation at 685 nm are depicted in Figure 5. They show a more pronounced structure than at 77 K and again are very similar for both samples. The figure also shows the comparison between the PSII-RC spectra and those of Chl and Pheo (as in Figure 4). Inspection of the two PSII-RC spectra reveals that they now almost perfectly resemble the spectrum of Chl whereas there is no indication for a Pheo contribution at this temperature. This shows that at 10 K and upon excitation on the low energy side of their absorption spectra, the emission of both PSII-RC samples is solely due to Chl.

DISCUSSION

The PSII-RC samples investigated in this study were prepared according to the same method and both had a Chl/2 Pheo ratio of 6.0 ± 0.2. Nevertheless, their absorption spectra clearly differ from each other. In a preceeding work, a comparable variability also was observed for other isolation methods (Konermann & Holzwarth, 1996). Whereas the spectrum of sample 2 showed a shoulder at around 684 nm, this feature was not present in the spectrum of sample 1 (see Figure 1). This shoulder is a typical feature in the spectra of most PSII-RC samples. We have previously demonstrated that it is possible to remove the pigments responsible for this spectral feature by choosing a special isolation procedure (Konermann & Holzwarth, 1996). The present work shows that such PSII-RCs (like sample 1) can also be obtained by using the method described in the Experimental Section.

The site-selection fluorescence spectra presented in this work for a temperature of 10 K (Figure 5) are very similar

[2] We note that the alignment in the work of Kwa et al. (1994) was performed on the vibronic band at 723.5 nm in the PSII-RC spectra (for $T = 4$ K). Such kind of alignment on a vibrational band has in principle an element of arbitrariness. We note however that there is no necessity to align on a particular peak in order to compare the spectra among various samples or excitation wavelengths within one sample since, in principle, the only correction required is that for different excitation wavelengths, i.e., the important parameter in site selection spectroscopy when measuring the frequency of a vibronic band is only the difference (in cm^{-1}) to the excitation frequency. In the present work, thus the only shifting correction applied is that for the difference in excitation frequency, i.e., there is no subjective or arbitrary shift correction implied in our analysis.

to the ones presented by Kwa et al. (1994b). These show that at such a low temperature a Chl-type pigment acts as excitation trap in the PSII-RC and thus is the terminal emitting species. However, the samples used by Kwa et al. presumably contained a higher amount of red-absorbing additional Chl than the samples used in this work (Konermann & Holzwarth, 1996) so that the emission in their study could have been caused by these additional Chls not actually belonging to the PSII-RC. In contrast, the samples used in this work had a Chl/2 Pheo ratio of about 6.0. Especially for sample 1, one can rule out the possibility that the emission at 10 K is mainly caused by a red-absorbing Chl contamination since according to the spectral analysis this sample is devoid of the Chl_{add3} pool. We agree with the argumentation of Kwa et al. (1994b) that the observed Chl emission cannot be caused by P_{680} since recombination fluorescence is expected to be very weak at such low temperatures and direct fluorescence from P* is also extremely weak. This is confirmed quantitatively by a recent work where we analyzed the low temperature fluorescence kinetics of the PS II-RC in detail (Konermann et al., 1996). Moreover, according to our spectral analysis, the inhomogeneously broadened absorption spectrum of P_{680} has its maximum at around 679 nm (for $T = 10$ K) so that at 685 nm this pigment is excited only to a very minor extent (Konermann & Holzwarth, 1996), (cf. also Figure 2).

Evidence in favor of a Pheo absorbing on the low-energy side of the PSII-RC spectrum comes from a number of studies. On the basis of their absorption detected magnetic resonance data at a temperature of 1.2 K, van der Vos et al. (1992) found a bleaching due to a Pheo triplet at around 680 nm. Chang et al. (1994) reported on a long-wave absorbing pigment around 683 nm in a hole-burning study and attributed it to an external (linker or antenna) Chl. This assignment was reinforced in a more recent paper (Chang et al., 1995). In their Gaussian decomposition of the PSII-RC absorption spectrum for 72 K and at rt, Garlaschi et al. (1994) and Cattaneo et al. (1995) also reported on this long-wave band but also found a band centered at 680 nm that was attributed to a Pheo. Kwa et al. (1994b) performed the first fluorescence site-selection study on the long-wave pigment(s) and attributed them mainly to Chl but also to Pheo. The spectral position for the Pheo band mentioned above roughly agrees with the absorption maximum of the low energy Pheo (678.2 nm at 10 K) that we suggested on the basis of our spectral model (Konermann & Holzwarth, 1996). We cannot exclude that the absorption spectrum of that Pheo may be at slightly longer ($\leq$680) wavelength although such a red-shift as compared to our spectral decomposition would probably cause some deviations in the time-resolved data (Konermann et al., 1997). Our finding clearly disagrees however with the interpretation of Tang et al. (1990) according to which this Pheo should have its absorption maximum at even lower energies (681−682 nm) for a temperature of 4.2 K. If this were the case, it should be the red-most absorbing pigment in a large part of the PS II-RCs and thus should significantly contribute to the emission. For samples with a relatively high amount of low energy Chls (like, e.g., sample 2 used in this work), the Pheo emission could possibly be masked by the fluorescence of a more of less pronounced Chl_{add3} pool. However, the present work shows that even sample 1 (which lacks these additional red-absorbing Chls) does not show any indications for Pheo emission at 10 K. We can thus safely conclude that the low energy Pheo must absorb at shorter wavelengths than suggested in the work of Tang et al. (1990). The fact that a substantial Pheo emission is observed only at temperatures above about 50 K is quite in line with a significant energy difference between the Pheo and the Chl excited states.

We now return to a problem that arose in our previous work on the absorption spectrum of the PSII-RC (Konermann & Holzwarth, 1996). Two decompositions that both fitted the experimental absorption spectra about equally well were found. According to the first decomposition at 10 K, the absorption on the red edge of the spectrum was dominated by a Pheo, according to the second one by an accessory Chl. *The site-selection spectra presented in this work speak definitely in favor of the second decomposition which is shown in Figure 2*. Accordingly, the level ordering on the low-energy side of the spectrum is such that Chl_{acc2} and $Pheo_1$ have their absorption maxima at 682.3 and 678.2 nm, respectively. For the first decomposition, one would expect a pronounced Pheo-type emission upon excitation on the red edge of the absorption spectrum. This is not observed experimentally. In the previously mentioned work of Tang et al. (1990), a broad (120 cm^{-1}) hole centered at 681−682 nm was observed at 4.2 K upon burn wavelengths around 670 nm. Because of the appearence of an additional hole in the Pheo Q_x region at 545 nm and based on the results of dithionite reduction experiments, it was concluded that the hole at 681−682 nm was contributed to by the Pheo active in charge separation. In our view, these results have to be interpreted in a slightly different way. The spectral decomposition shown in Figure 2 suggests that only the high-energy side of this hole was contributed to by Pheo whereas it was mainly caused by the bleaching of Chl_{acc2}. $Pheo_1$ in the spectral decomposition can thus be identified with the one active in charge separation. It should be pointed out that the spectral position of $Pheo_2$ at around 670 nm in the PSII-RC that is predicted by the decomposition is in excellent agreement with the results of a work by Mimuro et al. (1995). From their low temperature fluorescence data, the authors of that study concluded that this Pheo would be the inactive one, which is in line with the interpretation presented above. In a recent hole-burning study, Groot et al. (1996) detected trap pigments in the PSII-RC which had a lifetime of roughly 4 ns and an absorption maximum around 682 nm at liquid helium temperatures. In the framework of our spectral model these pigments can be identified primarily with Chl_{acc2} and to some minor extend with Chl_{add3}.

On the basis of the spectral decomposition model (as shown in Figure 2), we recently presented a detailed analysis of the low temperature fluorescence kinetics of the PSII-RC (Konermann et al., 1996). The kinetic model predicted an almost pure Chl emission (about 99%) for temperatures $\leq$20 K upon excitation at 685 nm. This is in full accordance with the data shown in Figure 5. At a temperature of 77 K, however, the kinetic model predicts that the Pheo contribution in the stationary emission spectrum increases to 14% for sample 1 and 12% for sample 2, respectively. This prediction is fully confirmed by the site-selection spectra shown in Figure 3 where a Pheo contribution of about this percentage clearly shows up. Due to the increase in temperature, the thermal equilibrium is shifted slightly more to the pigments having their ZPLs at higher energies so that Chl_{acc2} no longer traps the excitation energy so efficiently.

The quite small difference in the Pheo contributions of samples 1 and 2 predicted by the model is in line with the experimental observation that the site-selected fluorescence spectra of both samples used in this study are almost identical.

Our model for the absorption spectrum of the PSII-RC (Konermann & Holzwarth, 1996) was based on the assumption that excitonic interactions between the chromophores can be neglected (with the exception of the putative P_{680} Chl dimer). This view is in contradiction to the multimer model of Durrant et al. (Durrant et al., 1995), who suggested that such interactions are crucial for the understanding of the spectral properties of the PSII-RC. The results presented in this work point toward the validity of the concept used for our spectral model. In the view of Durrant et al., the active Pheo and the accessory Chls are part of a spectral multimer, whereas our data clearly indicate that one can assign distinct monomer type absorption and emission spectra to these pigments. In the case of a mixed strongly coupled aggregate (consisting of Chls and Pheos), we do not expect that the excitonic bands would show the vibronic pattern of either a monomeric Chl or a monomeric Pheo.

ACKNOWLEDGMENT

We thank S. Völker from the University of Leiden for sending us the manuscript of Groot et al. (1996) prior to publication. The authors also would like to thank M. Reus for preparing the PSII-RC samples. I. Y. acknowledges a fellowship from the European Community (Human Capital and Mobility Program).

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BI9701484