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LETTERS

Direct Measurement of the Effective Rate Constant for Primary Charge Separation in Isolated Photosystem II Reaction Centers

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Transient absorption measurements of the pheophytin *a* anion band and Q_x band bleach region using preferential excitation of P680 are performed on isolated photosystem II reaction centers to determine the effective rate constant for charge separation. A novel analysis of the Q_x band bleach region explicitly takes the changing background into account in order to directly measure the rate of growth of the bleach. Both spectral regions reveal biphasic kinetics, with a ca. $(8\ ps)^{-1}$ rate constant for the faster component, and a ca. $(50\ ps)^{-1}$ rate constant for the slower component. We propose that the faster component corresponds to the effective rate constant for charge separation from within the equilibrated reaction center core and provides a lower limit for the intrinsic rate constant for charge separation. The slower component corresponds to charge separation that is limited by slow energy transfer from a long-wavelength accessory chlorophyll *a*.

Introduction

The nature of the primary charge separation reaction in the isolated photosystem II (PSII) reaction center (RC) is an area of considerable interest and controversy (see reviews in refs 1 and 2). On the basis of analogy to bacterial RCs and modeling of the results of studies of larger-sized PSII complexes,[3] an intrinsic rate constant of ca. $(3\ ps)^{-1}$ is anticipated. A quite similar rate constant of $(2\ ps)^{-1}$ has been well established for PSII RCs at liquid He temperatures by hole-burning studies[4] and is supported by low-temperature transient absorption measurements.[5,6] Unlike hole-burning experiments, which for this system provide a direct measurement of the intrinsic rate constant, time-resolved experiments can only measure the effective rate constant for charge separation. The effective (i.e., observed) rate constant may be significantly slower than the intrinsic rate constant due to slow energy transfer processes and/or "equilibration" (i.e., bidirectional subpicosecond energy transfer) of the excitation energy between the primary donor P680 and other pigments. (Unless explicitly stated otherwise, all further discussion of rate constants will refer to the effective rate constant.) Several studies have concluded ca. $(3\ ps)^{-1}$ to be the rate constant for charge separation near room temperature,[7−13] whereas others have determined it to be ca. $(21\ ps)^{-1}$.[14−17]

There are several reasons for the difficulty in establishing the rate constant for primary charge separation in PSII RCs by time-domain techniques, which are the only methods available above liquid He temperatures. Virtually all of the fluorescence measurements have used the time-resolved single photon

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counting technique, which is hampered by the purported $(3\ \text{ps})^{-1}$ rate constant being close to an order of magnitude faster than the experimental instrument response. The difficulty with using transient absorption techniques is due to the lack of a simple unambiguous indicator of charge separation. The groups that currently propose a ca. $(3\ \text{ps})^{-1}$ rate constant generally base their assertion upon data in the Q_y band region. This band has been extensively examined[5−8,11,13,14,16−19] (see also review in ref 2), but distinguishing between charge separation and energy transfer processes with that data is very difficult, due to the severe overlap of the six chlorophyll *a* (Chl) and two pheophytin *a* (Pheo) pigments in that band, and even further complicated by the small Stokes shift of the stimulated emission. Both we and the group from Imperial College[17] feel that other spectral regions are better suited for the determination of the rate constant for charge separation.

We have examined the bleach of the Pheo Q_x band at 543 nm using a novel analysis in which the effect of the changing "background" is taken into account. This analysis is supported by data obtained at the Pheo anion band at ca. 455 nm. Together the data provide strong evidence that charge separation is biphasic upon excitation of the red side of the Q_y band. We propose that the formation of $P680^+ - Pheo^-$ from the equilibrated RC core occurs with a rate constant of $(8\ \text{ps})^{-1}$, and charge separation limited by slow energy transfer from a red accessory Chl occurs with a rate constant of $(50\ \text{ps})^{-1}$.

Experimental Section

Complete details of the experimental setup for the femtosecond transient absorption measurements and the sample preparation procedure are given in ref 20. Briefly, sub-200-fs excitation pulses at 687 nm (∼6 nm bandwidth) were provided by an optical parametric amplifier (OPA).[21] The polarization of the white light continuum probe was at the magic angle with respect to the pump. Pump and probe spot sizes were ∼250 μm (diameter). Excitation energies ranged from 7.5 to 100 nJ, low enough to avoid multiple excitation of a significant number of individual RCs[20] (*vide infra*). PSII RCs were isolated from spinach using a modified Nanba-Satoh preparation that substitutes dodecyl β-maltoside for Triton X-100.[22] These RCs were ca. six Chl preparations and were kept at a temperature of 5 °C during the measurements. Samples had a Q_y peak absorbance of 1.6 in a 1-cm cell; the absorbance was 0.5 at the pump wavelength. The sample of the bacterial reaction center of *Rhodobacter sphaeroides* R26 was prepared as described in ref 23.

By analogy to the bacterial RC, the PSII RC is commonly thought to consist of a "core" that contains four Chls and two Pheos (i.e., pigment quantities identical to the bacterial RC) and two additional accessory Chls that are somehow peripheral to the core.[1] It is believed that the core pigments are linked by subpicosecond energy transfer.[18,19] The excitation wavelength of 687 nm was chosen to selectively excite the pigments on the red side of the composite Q_y band, i.e., P680 and the active Pheo. This minimizes the effects on the measured charge separation rate constant due to slow energy transfer from the accessory Chls that absorb on the blue side of the band (see ref 2), although there will still be absorption by an accessory Chl near 681−684 nm.[24,25]

Results

The Pheo Q_x band is a sensitive indicator of charge separation, as charge separation cannot occur without the bleaching of this narrow, well-defined band. However, several things complicate the interpretation of the kinetics of this band, such as the inability

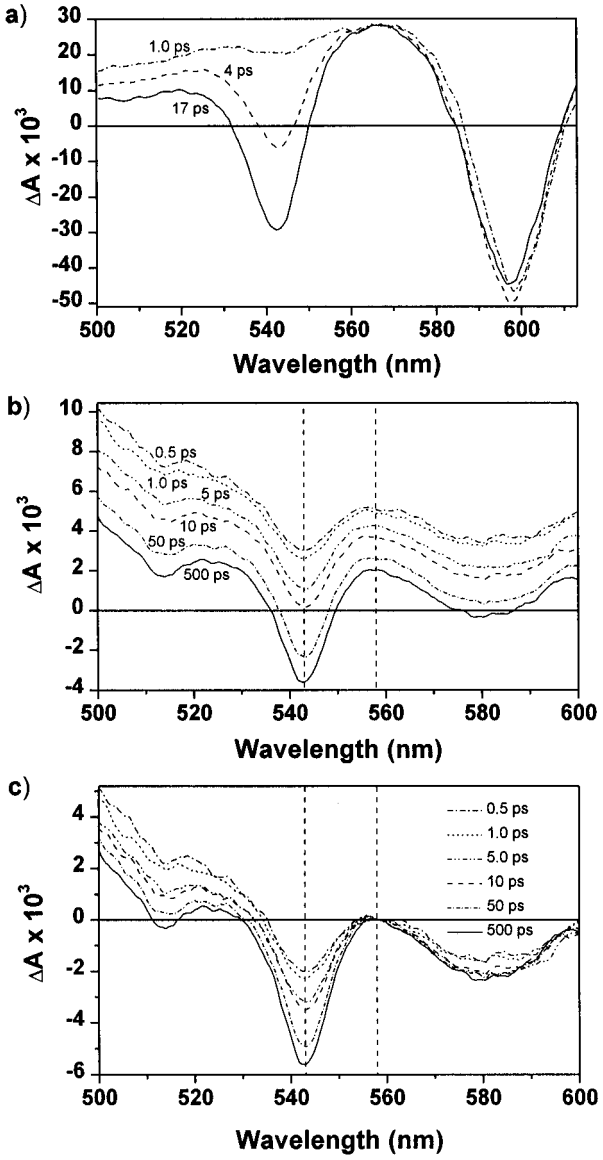

Figure 1. (a) Transient absorption spectra of the RC of the photosynthetic bacteria *Rb. sphaeroides* R26 at 1.0, 4, and 17 ps after the 870-nm, 200-nJ excitation pulse. (b) Transient absorption spectra of the isolated PSII RC recorded at various time delays between 0.5 and 500 ps after the 100-nJ, 687-nm excitation pulse. Dashed vertical lines show the 543 and 558-nm probe wavelengths used for the bleach-growth analysis. (c) Same data as in (b), but with the data vertically shifted to set $\Delta A_{558\ \text{nm}} = 0$ at all six delay times.

to distinguish $Pheo^-$ from $^{1*}Pheo$ on the basis of data solely from this band (*vide infra*). Before examining the Pheo Q_x band bleach region of the PSII RC, it is instructive to look at the behavior of the better-understood RC of photosynthetic bacteria. Figure 1a shows transient absorption spectra of the RC of *Rb. sphaeroides* R26 at room temperature pumped with 870-nm pulses, which results in direct excitation of the special pair. The depth of the bleach of the bacteriopheophytin *a* (BPheo) Q_x band increases from near zero at 1.0 ps to a maximum value at ∼17 ps. The transient absorption signal to the red side of the bleach, however, does not change on this time scale. It is clear that a kinetic measurement probing at the peak of the bleach band (∼543 nm) should be a direct, valid measurement of the bleaching of the BPheo ground state.

Figure 1b shows the same spectral region of the isolated PSII RC recorded at six times between 0.5 and 500 ps after the 687-nm excitation pulse. Over this time range, the transient absorption signal changes substantially at all wavelengths (not

just at the Pheo Q_x bleach), in sharp contrast to the case of the bacterial RC. The origin of this changing "background" is unclear; it could be due to the effects of energy transfer or charge separation or both. Its magnitude, however, is clearly quite substantial, accounting for almost half of the amplitude of the decay at the bleach maximum (543 nm). Thus, a kinetic measurement at 543 nm may be severely contaminated with phenomena unrelated to and occurring with a different rate constant than charge separation.

It is really the depth of the Pheo Q_x bleach that is the measure of Pheo ground state depletion, and therefore it is the rate of *growth* of the bleach, free from the changing background, that corresponds to the rate of charge separation. This growth can be directly measured by subtracting the kinetics of the background from the kinetics at the bleach maximum. The problem is how to best account for the changing background *at the bleach maximum*, where the background cannot be directly measured.

What wavelength should then be used to estimate the background kinetics at the Pheo Q_x bleach maximum? Clearly, the closer we get to the Pheo Q_x bleach band (without being in the band), the better. We decided not to use the blue shoulder at ca. 523 nm as it may suffer from overlap with the Pheo Q_x vibrational side band centered at 513 nm; this effect can be clearly seen in the case of the bacterial RC (see Figure 1a). Instead, we have used the red shoulder at 558 nm for this purpose. To within a few nanometers, there is certainly nothing unique about this wavelength. However, at five nanometers to either the blue or red side, one is clearly within the Pheo and Chl Q_x bands, respectively. Figure 1c shows same spectra as Figure 1b, but with all six spectra vertically shifted to set $\Delta A_{558\ nm} = 0$. Essentially, what we have done is removed the effect of the changing background (at 558 nm) from all of the spectra. The growth of the Pheo Q_x band is now quite clear; one can even see what appears to be a small growth in the Chl Q_x band centered at ~580 nm. The data now look more reminiscent of the case of the bacterial RCs, including the behavior of the blue side of the Pheo Q_x band.

Intrinsic in the manipulation of the data used to generate Figure 1c is the assumption of the wavelength independence of the background. Over a large wavelength range, this assumption is certainly open to question. Quantitatively, however, we are only really interested in how well the data at 558 nm simulates the background 15 nm further to the blue. In the 500−600 nm wavelength region, the transient absorption signal is the sum of the excited state absorption spectra of the transient species (chlorin excited singlet and ion radical states) and the bleaches of the chlorin Q_x bands. The changing background is clearly not due to growth of the narrow Q_x bleaches, so it must be due to excited state absorption. In general, the absorption spectra of both chlorin excited singlet states[20,26] and ion radical states[27,28] are rather broad and featureless throughout this part of the visible spectrum. It is therefore highly unlikely that the behavior of the background would change significantly between 543 and 558 nm.

Figure 2a shows the kinetics at 543 and 558 nm; the 543-nm minus 558-nm difference data can be seen in Figure 2b. In effect, the subtraction allows us to measure the Pheo Q_x bleach kinetics of Figure 1c, rather than the kinetics of Figure 1b. The subtraction data are fit to an instrument response-limited component and an ensuing biexponential rise of 9 ± 2 and 54 ± 8 ps. The instantaneous component represents the partial depletion of the Pheo ground state during the excitation pulse (*vide infra*) and contains (41 ± 5)% of the final bleach amplitude. The subsequent increase in bleach amplitude beyond this level presumably corresponds to charge separation; the fast

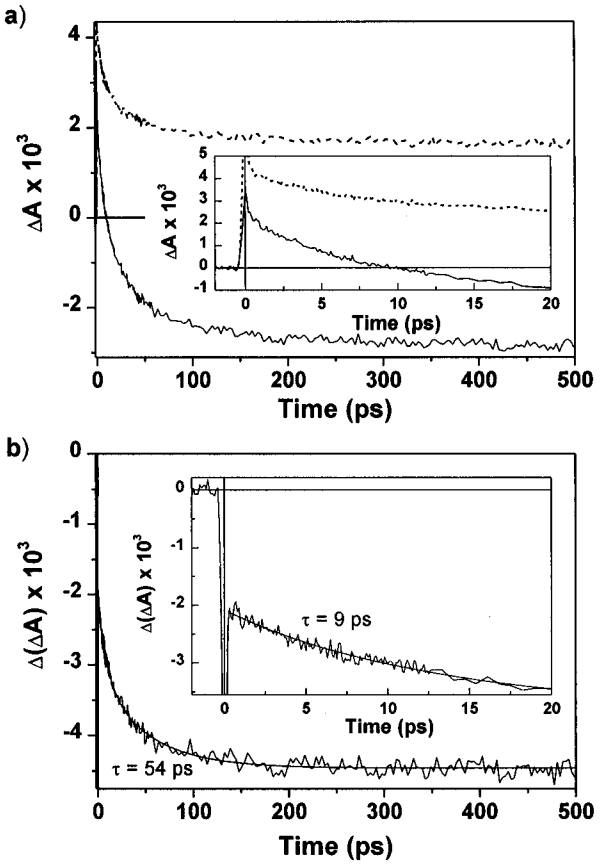

Figure 2. (a) Transient absorption kinetics at 543 and 558 nm (solid and dashed lines, respectively) for the isolated PSII RC (λ_{exc} = 687 nm, 100 nJ). Inset shows the data at early time. (b) 543 nm minus 558-nm kinetics, showing the growth of the Pheo Q_x band bleach, with fit to 9 and 54-ps components. Inset shows early time behavior; the spike at $t = 0$ is due to the strong solvent response at 558 nm.

and slow components of this rise contain (28 ± 6)% and (31 ± 4)% of the total amplitude of the transient, respectively. A single-wavelength measurement at 543 nm, by comparison, requires three kinetic components to fit the data,[20] with none of these components matching either of the time constants determined from the bleach growth analysis.

The amplitude of the Pheo Q_x bleach may not necessarily correspond solely to charge separation, however, as the bleach due to ^{1*}Pheo is indistinguishable from that due to Pheo$^-$. This is clearly not a hypothetical issue: the substantial fraction (41%) of the final bleach amplitude that is formed on a subpicosecond time scale is undoubtedly due to ^{1*}Pheo rather than subpicosecond charge separation.[19] We have therefore examined the Pheo anion band region to determine if the inability of the Q_x bleach to distinguish ^{1*}Pheo from Pheo$^-$ could affect the ability of the bleach band to properly measure the rate constant for charge separation. Figure 3 shows transient absorption spectra of the Pheo anion band recorded at seven times between 0.5 and 500 ps. The large signal at 0.5 ps is due to excited state−excited state absorption of the chlorin pigments, while the increase in the signal beyond that level is due to the formation of Pheo$^-$. Kinetics measured at 456 nm reveal an instrument-response-limited component, followed by a biexponential rise of 7.0 ± 0.6 ps and 48 ± 3 ps (see Figure 4). The fast and slow components contain (28 ± 2)% and (29 ± 2)% of the amplitude of the rise, respectively, with the remainder ((43 ± 2)%) due to the instantaneous formation of chlorin excited singlet states.

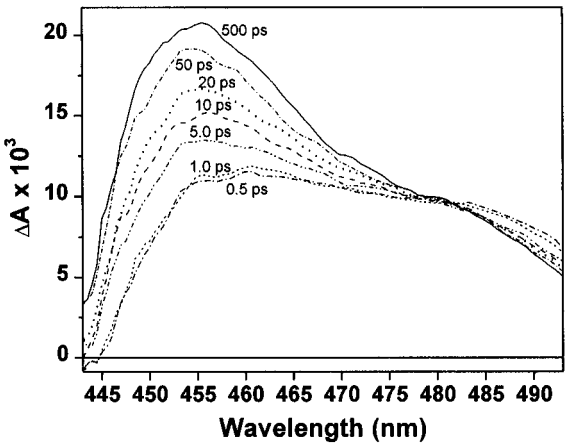

Figure 3. Transient absorption spectra of the Pheo anion band region of the isolated PSII RC recorded at various delays between 0.5 and 500 ps after the 100-nJ, 687-nm excitation pulse.

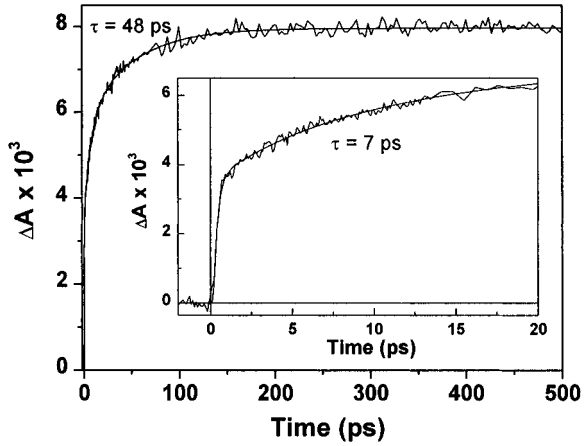

Figure 4. Transient absorption kinetics of the isolated PSII RC at the Pheo anion band at 456 nm, with fit to 7 and 48-ps components (λ_{exc} = 687 nm, 30 nJ). Inset shows early time behavior, including instrument-response-limited rise due to the instantaneous formation of chlorin excited singlet states upon optical excitation.

Discussion

There is no *a priori* reason why the rate constant for the charge-separation process can be determined by a single-wavelength kinetic measurement of the anion band when a dual wavelength measurement is necessary in the Pheo Q_x bleach region. However, the signal level in the Pheo anion band is substantially higher, which would diminish the effect of a changing background if its amplitude were wavelength independent. Moreover, there is evidence that the amplitude of the changing background becomes greatly diminished in the blue spectral region: examination of Figure 3 reveals what appears to be an isobestic point at ca. 480 nm, where the amplitude of the transient absorption signal is invariant with time between 500 fs and 500 ps. The similarity of the direct kinetic measurement at the Pheo anion band to the kinetics of the growth of the Pheo Q_x bleach using our background subtraction analysis provides compelling evidence that both experiments are measuring the same phenomena. Not only are the rate constants identical (to within the error bars) but so are the ratios of the relative amplitudes of the slow and fast components: 1.1 $\pm$ 0.1 and 1.1 $\pm$ 0.3 for the anion band and bleach growth experiments, respectively.

The biphasic nature of the charge separation process in the isolated PSII RC suggests the following kinetic model for selective excitation of the red pigment pool. The faster component (8 ps) corresponds to charge separation from within the fully equilibrated RC core upon direct excitation of the core. Although the faster component is not limited by slow energy transfer, it does not represent the intrinsic rate constant for charge separation. Rather, it is the effective or "equilibration-limited" rate constant, with the intrinsic rate constant being masked by the details of subpicosecond energy transfer among the core pigments. The slower component (50 ps) corresponds to charge separation upon excitation of the red accessory Chl that also absorbs on the red side of the Q_y band and therefore reflects slow energy transfer-limited charge separation. We cannot, however, rule out the possibility that the measured biphasic kinetics for charge separation is due to heterogeneity in the intrinsic rate constant for charge separation, as might be caused by a distribution of electron transfer coupling constants and hence rate constants.

Our results differ from previous work on isolated PSII RCs. No comparison to results based on transient absorption measurements of the Q_y bleach will be made; as mentioned earlier, both we and others believe that this region is not well-suited to differentiate between charge separation and energy transfer processes. We will therefore limit ourselves to discussing experiments performed in the same wavelength regions as in this work. In the case of the Pheo anion band region, our results differ from those of the one previous experiment because of the higher quality of our data. In that earlier study, a single-exponential rise of 21 ps following the instrument-response-limited component was measured.[14] That study was unable to discern the biexponential nature of the kinetics as the data extended out to only 80 ps and had a fairly low signal-to-noise (S/N) ratio. Our data can also be fit to a 21-ps single-exponential rise if the data is truncated at 150 ps, although the fit results in poor residuals.

However, our current 543-nm data (see also ref 20) is not fundamentally different than the data of other groups at that same wavelength.[13,14,16,17] It is our background-subtraction analysis that provides the different results and which we believe enables us to obtain more accurate kinetics for charge separation. Previous endeavors at measuring the rate constant for charge separation on the basis of the Pheo Q_x bleach have treated the changing background in one of two ways. Two of the studies have kinetic data (in this region) only at the peak of the bleach and therefore have *de facto* completely ignored the effect of the changing background.[8,16] The other studies have data at many wavelengths[13,14,17] and use a global analysis method to analyze the data. As this method is applied, the data at multiple wavelengths are fit simultaneously to a sum-of-exponentials function, with the time constants set to be wavelength-independent but with the amplitudes allowed to vary as a function of wavelength. Only the more recent of these studies have had sufficiently good S/N to discern the multiexponential kinetics in this wavelength region.[13,17] However, only Klug *et al.*[17] cover a broad enough wavelength region to clearly show the existence of a changing background, as the Q_x region data of Müller *et al.* extended only from 535 to 555 nm.[13]

The global analysis method makes the intrinsic assumption that the time constant(s) for the charge separation process are a subset of those actually identified by the global analysis. There is no consensus, however, as to even how *many* exponentials are required to fit the Pheo Q_x bleach region, with recent studies giving one,[16] two,[17] three,[20] and even four[13] components in the picosecond time regime. The four-exponential global analysis reported by Müller *et al.* includes data from both the Pheo Q_x region and the chlorin Q_y region, with signal levels in the later region reported to be ca. 150 times greater than in the former.[13] To the best of our knowledge, Müller *et al.* did not perform a

separate global analysis on the Pheo Q_x region alone. Given their S/N ratio of ca. 10:1 in the Q_x region, we find it difficult to believe that a four-exponential decay is justified here. Furthermore, we believe that doing the global analysis over both regions has the danger of strongly biasing the time constants to the those of the Q_y region, and thus their global analysis makes the further assumption that the time constants are identical in the two regions. This is not a frivolous concern, as detection-wavelength-dependent kinetics have been observed in the bacterial reaction center.[29−31]

The extreme difficulty of reliably fitting three or more exponential components coupled with the low S/N of the data due to the weak extinction coefficients in the Q_x region and the low pump levels required to avoid multiple excitations[16,20,32] makes it somewhat questionable that a "true" fit will *ever* be reached. In addition, the assumption that the charge separation rate constant(s) are a subset of the "true" global analysis rate constants may not even be correct, as our own data suggests. Our subtraction method makes no such assumptions, as it directly gives the bleach growth information. We feel that this model-independent nature of our method is a powerful advantage and that the legitimacy of the method is confirmed by the agreement of the bleach growth results with those of the anion band.

Several recent publications have addressed the issue of the intensity dependence of the PSII RC kinetics using the transient absorption technique.[16,20,32] The kinetics at the anion band were measured with excitation energies between 7.5 and 100 nJ, while the energies used to examine the Pheo Q_x bleach band were between 30 and 100 nJ. There was no statistically significant variation as a function of pump energy in the ranges examined in either the rate constants or the amplitude ratios of any of the kinetic components in any of the wavelength regions discussed in this Letter. At 30 nJ, we estimate 0.055 absorbed photons per RC, which corresponds to $<3\%$ of the excited RCs experiencing multiple excitations[20] (presumably resulting in annihilation in those RCs). We have compared our 30-nJ level with the excitation conditions used in ref 13 by comparing the Q_y peak optical densities and the number of absorbed photons/cm^2 in the two studies: our conditions result in almost a factor of 2 lower excitation level. We consider the small-but-finite degree of multiple excitations in our experiments to be completely inconsequential to the results presented above.

Conclusion

Using a novel analysis of the Pheo Q_x bleach region along with data from the Pheo anion band, we have determined that charge separation in the isolated PSII RC occurs with two rate constants upon excitation of the red side of the Q_y band: $(8\ ps)^{-1}$ and $(50\ ps)^{-1}$. The faster rate constant is proposed to correspond to charge separation from the fully-equilibrated RC core and the slower rate constant to charge separation that is limited by slow energy transfer from a long-wavelength accessory Chl. Our results for the effective rate constant for charge separation differ from the $(21\ ps)^{-1}$ rate constant previously proposed on the basis of data in the same spectral regions for two reasons: (a) substantially better signal-to-noise in the Pheo anion band and (b) our unique analysis of the Pheo Q_x region.

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