In search of a putative long-lived relaxed radical pair state in closed photosystem II

Kinetic modeling of picosecond fluorescence data

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ABSTRACT The concept of a relaxed radical pair state in closed photosystem (PS) II centers (first quinone acceptor reduced) is critically examined on the basis of chlorophyll fluorescence decay data of the green alga *Scenedesmus obliquus*. Global analysis resulting in the decay-associated fluorescence spectra from closed PS II centers reveals a new PS II lifetime component ($\tau \approx 380$ ps) in addition to two PS II components ($\tau \sim 1.3$ and 2.1 ns) resolved earlier. Particular emphasis was given to resolve a potential long-lived (~ 10 ns) component of small amplitude; however, the longest lifetime found is only 2.1 ns. From comparison of experimental and simulated data we conclude that the maximum relative amplitude of such a potential long-lived component must be <0.1%. The PS II kinetics are analyzed in terms of a three-state model involving an antenna/reaction center excited state, a primary radical pair state, and a relaxed radical pair state. The rate constants for charge separation and presumed radical pair relaxation as well as those for the reverse processes are calculated. Critical examination of these results leads us to exclude the formation with high yield (>15%) of a long-lived ($\tau \geq 3$ ns) relaxed radical pair in closed PS II. If at all distinguishable kinetically and energetically from the primary radical pair, a relaxed radical pair would not live longer than 2–3 ns in green algae. The data suggest, however, that the concept of a long-lived relaxed radical pair state is inappropriate for intact PS II.

INTRODUCTION

The processes of charge separation, charge stabilization, and charge recombination in photosystem (PS) II[1] have recently been studied by several groups using laser spectroscopy in the nanosecond and picosecond time ranges (for reviews see references 1–5). It is now generally accepted that in PS II the primary electron transfer takes place from the excited donor P* to the primary electron acceptor I, a pheophytin (1, 6–8). In open PS II centers the electron on I^- is transferred to the primary quinone acceptor Q_A within ~300–500 ps (7–9). In closed PS II centers, where Q_A is reduced beforehand, this second electron transfer process is not possible. The decay of the photoinduced radical pair P^+I^- in this case can occur via several channels, including charge recombination to the ground state and/or to singlet excited states as well as triplet radical pair formation. A long fluorescence lifetime component of 1–3 ns (3) is generally associated with closed PS II, thus increasing the fluorescence quantum yield from F_0 to the maximum level F_{max} by a factor of 4–5. It was proposed originally by Klimov and co-workers that the long-lived emission was delayed luminescence due to charge recombination from the radical pair in closed PS II centers (1, 6).

Holzwarth and co-workers recently proposed a detailed kinetic model that describes the processes of charge separation, charge recombination, and charge stabilization in both open and closed PS II centers (10). The model predicts biexponential decay kinetics for the equilibrated excited antenna chlorophyll (Chl)/reaction center state. Correlated measurements of picosecond absorption transients and picosecond fluorescence kinetics on PS II particles isolated from the cyanobacterium *Synechococcus species* (~80 Chl/P_{680}) (8) provided the experimental basis for a determination of the rate constants related to these electron transfer processes in PS II. It was concluded that reduction of Q_A (closing the reaction centers) causes a decrease in the rate constant for primary charge separation by a factor of ≈6. In contrast, the rate constant for charge recombination is only slightly affected. The reduced charge separation rate leads to a corresponding lengthening of the Chl excited state lifetime which is the main cause for the Chl fluorescence lifetime component of 1–3 ns in higher plant chloroplasts and green algae (2, 5) or ~1.3 ns for the PS II particles studied by Schatz et al. (8). A further consequence of the reduced charge

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[1] *Abbreviations used in this paper:* Chl, chlorophyll; CSS, charge separated state(s); DAS, decay-associated spectrum; DCMU, 3-(3,4-dichlorophenyl)1,1'-dimethylurea; FWHM, full width at half maximum; I, pheophytin; PRP, primary radical pair; PS II, photosystem II; RRP, relaxed radical pair.

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separation rate is a decrease of the maximal transient concentration of radical pair from 53% in open reaction centers to 23% in closed reaction centers (10). It is important to note that the amount of radical pair formed depends on the antenna size. It can be estimated from these data that the maximum amount of radical pair formed in the closed state should not exceed ~15% in intact systems with PS II antenna sizes of ~200–250 Chl/P_{680} (10). This estimate is in line with recent photovoltage measurements of charge separation in PS II by Trissl et al. (11) and Leibl et al. (12). The maximum concentration of the radical pair formed with Q_A reduced was ~30% of that measured with oxidized Q_A. Thus, the relative transient concentration of radical pair in closed centers was ~10% (12). Similar conclusions on a drastic reduction of radical pair yield were drawn by Eckert et al. (13) on the basis of transient absorption measurements.

Results conflicting with the above conclusions were recently reported by two groups. From flash photolysis experiments with PS II particles of different sizes and origins, Hansson et al. (14) reported radical pair lifetimes in closed PS II ranging from 4 to 36 ns with transient radical pair yields varying from 28 to 67% (depending on the interpretation). Schlodder and Brettel (15), working with the same PS II particles as Schatz et al. (8) reported from closed PS II centers a high yield of radical pair with a long lifetime of ~11 ns. The amplitude of this component was as high as that of the faster (i.e., ≤1 ns) component(s). A transient radical pair concentration amounting to up to 60% of that found in open centers was reported (15). In absolute terms this means a relative transient radical pair concentration of 51–57% (depending on the assumed radical pair yield in open reaction centers, i.e., 85–95%). In contrast, Schatz et al. (8) did not find any such long-lived component(s) in absorption transients from the same particles. Furthermore, such a long-lived component (>4 ns) was found neither by Eckert et al. (13), nor by Nuijs et al. (7) in absorption experiments with closed PS II centers from higher plants, nor by Trissl and co-workers (11, 12) in photovoltage experiments with various PS II samples. The long lifetime (11 ns) was assigned by Schlodder and Brettel (15) to a relaxed radical pair (RRP) state that was assumed to be formed out of the shorter-lived primary radical pair (PRP). It was suggested that such a relaxation process might involve reorganization of the surrounding protein, in response to charge separation (15). This interpretation required an extension of the kinetic model proposed by Schatz et al. (10) to represent the RRP state. This procedure followed in some respects the one applied in the interpretation of the fluorescence kinetics from bacterial reaction centers (16) and chromatophores (17) where a long-lived RRP state was also proposed.

In view of these strongly conflicting reports with respect to the lifetime and yield of radical pair state(s) in closed PS II centers and the possible occurence of a long-lived RRP state, we present here a study of fluorescence kinetics designed specifically to clarify this issue. It was the aim of our measurements to resolve unambiguously a potential ~10-ns component with relative amplitude of a few percent or less, which could be related to recombination luminescence from the presumed RRP state. Our measurements were carried out on whole cells of the green alga *Scenedesmus obliquus*. At first glance, and in view of the previous experiments discussed above, it would appear more reasonable to carry out such a study on isolated PS II particles rather than on intact green algae. However, so far it has been impossible to isolate PS II particles (which normally requires the use of detergents) without functionally disconnecting at least a small part of the antenna Chls. This disconnected Chl, with expected lifetimes in the range of 3–6 ns (18), would interfere with any highly sensitive measurement of the radical pair kinetics by delayed luminescence. Despite the high quality of the PS II preparations such interference was observed in previous measurements (8) and was even more pronounced in measurements with a highly increased S/N ratio (T. A. Roelofs, unpublished results), as is required for the resolution of a weak RRP luminescence with ~10-ns lifetime.

To eliminate this problem we therefore decided to study intact cells from homocontinuous cultures of the green algae *Scenedesmus obliquus* for several reasons. First, no disconnected Chl was found in previous studies in this system (19, Bittersmann, E., and A. R. Holzwarth, manuscript submitted for publication). Second, it is expected that all PS II centers are intact and functional in this exponentially growing culture. With respect to these factors, the system seems to be ideal for analyzing potential long-lived PS II fluorescence components of low amplitude. It is obvious, however, that studying intact green algae adds some further complexity to the analysis. First, a PS I fluorescence component will be present. This is not considered to create a serious problem because the PS I lifetime and its decay-associated spectrum can be separated sufficiently well from the PS II contributions, particularly under conditions of closed PS II centers (F_{max}), as has been shown earlier (19, 20, Bittersmann, E., and A. R. Holzwarth, manuscript submitted for publication). A second more serious problem arises from the proposed α,β heterogeneity of PS II in higher plants and green algae (21). This will be discussed in detail below. Despite these problems, we consider the homocontinuous culture of this green alga to represent the best available sample to address the problem of the PS II radical pair kinetics by fluorescence decay measurements.

MATERIALS AND METHODS

Homocontinuous cultures of *Scenedesmus obliquus* were grown as will be described elsewhere (Bittersmann, E., and A. R. Holzwarth, manuscript submitted for publication). For the measurements the cell suspension was diluted with growth medium to ~6 μg Chl/ml. The Chl a/b ratio was ~3.8. For closing the PS II centers (F_{max}) 20 μM DCMU and 10 mM hydroxylamine were added and the sample (pumped at 350 ml/min) was illuminated with moderately intense white light just before entering the flow measuring cuvette (for further experimental details see reference 19). Control experiments were carried out under F_0 conditions (open PS II centers) without additions on dark-adapted samples. The picosecond fluorescence measurements were carried out with the same single-photon timing system and in the same way as described previously (19) unless stated otherwise. The channel resolution was 11 ps/channel and the system response function had a FWHM of 140 ps. All decays measured with ~65,000 counts in the peak channel to obtain high S/N ratios were submitted to a global analysis procedure and the results presented as decay-associated spectra (DAS) (19, 22, Bittersmann, E., and A. R. Holzwarth, manuscript submitted for publication).

RESULTS

Fluorescence kinetics

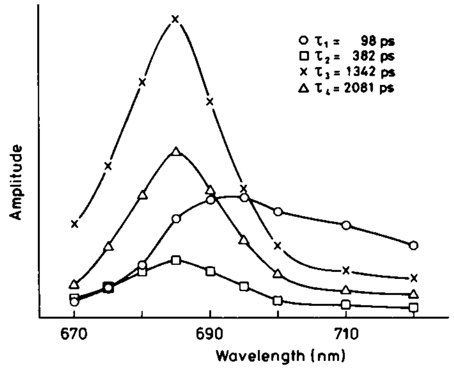

FIGURE 1 DAS of *Scenedesmus* cells at the F_{max} level excited at 650 nm, as calculated from the corrected amplitudes for the four lifetime components in the global analysis. The curves between the data points are interpolated with third-order polynomials.

Fluorescence decays with closed PS II centers (F_{max}) were recorded in the range between 670 and 720 nm at 5 or 10 nm intervals with excitation at 650 nm. In a global fitting procedure four components were needed to describe the fluorescence kinetics adequately, as judged from the weighted residual plots and the χ^2 values. The amplitudes of the different components plotted vs. the wavelength of detection give the DAS shown in Fig. 1. The fastest component (τ_1 = 98 ps) has a different spectral shape and a red-shifted emission maximum relative to the other components and has earlier been attributed to PS I emission (19). The other components (τ_2 = 380 ps, τ_3 = 1.34 ns, and τ_4 = 2.08 ns) all have very similar DAS with maxima at ~685 nm. For this reason we attribute them all to PS II. The 380-ps component, with a minor amplitude (~9%, cf. Table 1), is new as compared with previous results of the F_{max} state (19). The highly improved counting statistics in this work (viz., 65,000 counts in the peak channel in all decays) has enabled us to resolve this component. Control experiments under F_0 conditions (open centers) were carried out at the same excitation wavelength and with the same counting statistics. They gave results basically identical to those described earlier (19) (data not shown). However, only the F_{max} experiments are of interest in this work. The presence of an ultrafast exciton equilibration component ($\tau \sim$ 15 ps) reported recently (19a) is also indicated in the data but has been ignored in the present analysis (as these data are not suitable for complete resolution of this component because of the time resolution limit, i.e., 11 ps/channel and a FWHM of system response function of 140 ps). This does not have any influence on the conclusions drawn here because this lifetime is shorter by one order of magnitude than the time scale and lifetimes of the processes considered here (see below).

In the analysis described above the decays were fitted over a time window of 6 ns, appropriate for ~2 ns being the longest lifetime found. As it was our aim to resolve a possible long-lived component (τ > 5 ns), we extended the fitting window to 18 ns on the same data. In Table 1 we compare the results found with both fitting windows. With the extended fitting window a good fit is likewise obtained with four components. The lifetimes and the relative amplitudes at 685 nm are identical within the error limits as compared with the shorter fitting window. The complete DAS (not shown) are also virtually identical. Even when allowing for five lifetime components, no longer-lived (τ > 2.1 ns) component could be resolved in any of the analyses (Table 1). Instead, we resolve a very fast component (τ ~15–25 ps), indicative for the exciton

TABLE 1 Lifetimes τ (ps) and relative amplitudes A (at 685 nm) from the global analyses of the fluorescence decay data from whole cells of *Scenedesmus obl.* at F_{max} (see Materials and Methods)

6-ns fitting window		18-ns fitting window		18-ns fitting window	
τ	A	τ	A	τ	A
				22 ps	−12.5%
98 ps	16.0%	97 ps	15.7%	92 ps	20.2%
380 ps	9.2%	360 ps	8.9%	393 ps	8.3%
1.34 ns	48.0%	1.32 ns	47.4%	1.35 ns	47.3%
2.08 ns	26.8%	2.08 ns	28.0%	2.11 ns	24.2%

The decays were fitted as a sum of four and five exponentials over time windows of 6 and 18 ns, respectively.

equilibration in the antenna system (as mentioned above).

The independence of the lifetimes on the variation of the fitting window points to the fact that a significant long-lived component ($\tau > 5$ ns) is not present in the data within the resolution limit. Moreover, even if the four component fits should actually represent a more complex mixture of lifetimes, the absence of any long-lived component is clear.

Simulated decays

It is important for our considerations to find the quantitative upper limit for the relative amplitude of a possible long-lived component (i.e., $\tau \approx 10$ ns) that should be resolvable under the present conditions by the global analysis procedure. To find that limit we performed data analyses also on sets of simulated decay data. Decay curves at the different detection wavelengths were generated as a sum of five exponential terms, four of them being identical to the experimentally found components (cf. Table 1). The relative amplitudes of these lifetime components were chosen according to the results of the analyzed experimental DAS (cf. Fig. 1). The fifth component, with a lifetime of 10 ns and a DAS similar to those for the other PS II components, was added with various relative amplitudes in the range of 0.02–1.5%. The curves were convoluted with a generated system response function that had the same FWHM as experimentally obtained (i.e., ≈ 140 ps), and Poissonian distributed noise (23) corresponding to the experimentally obtained counting statistics (65,000 counts in the peak channel) was added to the decays. Such sets of simulated data were then analyzed globally as a sum of four and five exponentials by the same procedure as applied to the experimental curves, including a fitting window of 18 ns. Table 2 compiles the results for the longest lifetime component from the four and five component analyses on data sets with different relative amplitudes of the added 10-ns component. Four component global analysis of the control simulation, i.e., with no additional 10-ns component recovered the four lifetimes as well as the corresponding DAS with a maximum error of 10% (cf. Table 2). It is interesting that analysis of any single simulated decay did not provide a complete recovery of the four components. This shows that one needs global data analysis to resolve these kinetics. Table 2 shows that down to 0.02% relative amplitude, the 10-ns lifetime is recovered within 10% accuracy whereas its relative amplitude is underestimated by 50% at this limit. Allowing for only four components, we see that down to 0.1% of the total amplitude, the longest lifetime is lengthened significantly (from 2.1 to 5.5 ns) although the amplitude is overestimated (because of mixing with the 2.1-ns component). For relative amplitudes ≥ 0.05% of the 10-ns component a significant difference is observed in the global χ^2 values between four and five component analyses. Thus, we can safely use 0.1% as an upper limit for the relative amplitude of a 10-ns component that could have been resolved in our experimental fluorescence decays. Because no such component was found, however, we must conclude that a 10-ns component, if present at all, must have a significantly smaller amplitude.

TABLE 2 Results of global analyses on simulated data sets. The decay-associated spectra were simulated with the same lifetimes (τ_1 = 98 ps, τ_2 = 380 ps, τ_3 = 1.34 ns, τ_4 = 2.08 ns) and relative amplitudes as found in the experimental DAS (Fig. 1). A varying amount of a 10-ns component was added with the same spectrum as the other PS II components.

A* (%)	1.5	1.0	0.5	0.2	0.1	0.05	0.02	0.00
τ_5 (ns)‡	10.3	10.3	10.5	10.7	10.9	11.0	10.3	2.39
A_5 (%)‡	1.41	0.93	0.46	0.17	0.08	0.04	0.01	−28.0**
$\chi^{2,§}$	0.993	0.993	0.994	0.996	0.999	1.002	1.006	1.015
τ_4 (ns)‖	9.74	9.62	9.21	7.83	5.48	2.61	2.20	2.05
A_4 (%)‖	1.57	1.08	0.60	0.34	0.46	8.35	20.7	30.7
$\chi^{2,§}$	1.066	1.072	1.081	1.090	1.093	1.066	1.019	1.016

*Theoretical rel. amplitude of an additional 10-ns component.

‡Longest lifetime (τ_5) and corresponding relative amplitude (A_5) at λ_{em} = 685 nm in a global five-component analysis of the simulated decays. The four other lifetimes and rel. amplitudes are recovered within 3 and 10% accuracy, respectively.

§Global χ^2 value for the five and four component global analyses, respectively.

‖Longest lifetime (τ_4) and corresponding relative amplitude (A_4) at λ_{em} = 685 nm in a global four component analysis of the simulated decays. The lifetimes components τ_2 and τ_3 are also distorted by mixing with the 10-ns component (τ_2: 950 → 475 ps and τ_3: 1.80 → 1.44 ns by reducing the relative amplitude of the 10-ns component from 1.5 to 0.02%). The fastest component (τ_1 ~ 100 ps) is modified only slightly.

**This component compensates the increased amplitude (from 27 to 53%) of τ_4 = 2.27 ns.

DISCUSSION

The fluorescence kinetics from *Scenedesmus obl.* at the F_{max} level are characterized by four exponential components in the present data. The fastest component, with a lifetime in the 90–100-ps range, has been attributed in previous work to PS I, based on its time resolved excitation and emission spectra (19, 20, Bittersmann, E., and A. R. Holzwarth, manuscript submitted for publication). For this reason and because we are concerned here only with the PS II kinetics, we shall ignore this component in the following discussion. The three other components have very similar spectra and they are all ascribed to PS II. In the past, PS II fluorescence kinetics of *Scenedesmus* cells at the F_{max} level have been described by two exponentials only, which were assigned in terms of the PS II heterogeneity (21), one component resulting from PS II_{α} and the other from PS II_{β} (19). Because of the improved S/N ratio in the present data we have been able to resolve a third PS II component of ~380 ps for the first time. The observation of these three PS II components allows at least two different models for interpretation. The first involves a homogeneous PS II population and will be discussed here in detail. The second one is an extension of our PS II model presented previously (10) by including the heterogeneity of the PS II pool (α and β centers; see also below). In principle, models that assign one of the observed PS II component ($\tau \geq 350$ ps) at the F_{max} level to antenna energy transfer could also be considered. However, such models have been rejected earlier as being inconsistent with the data (19, 24, Bittersmann, E., and A. R. Holzwarth, manuscript submitted for publication), and they will therefore not be discussed here any further.

An important result of the analysis of the experimental data is the absence, within the resolution limit of 0.1%, of a lifetime component longer than 2.1 ns. This result is completely independent of any kinetic model and of any further assumptions. The absence of a long-lived ($\tau \geq 5$ ns) component already points to either a very low transient concentration of a putative relaxed radical pair or, alternatively, to an energetically very low-lying relaxed radical pair state which then could have a long lifetime. A detailed quantitative analysis of the data, in terms of the model discussed below, is more revealing, however.

Kinetic model

A model predicting biexponential fluorescence kinetics for a homogeneous PS II pool was proposed earlier (10). In the present work the previous model is extended to a three-exponential model by considering a relaxed radical pair state that presumably is formed from the primary radical pair. The kinetic scheme is shown in Fig. 2. It is the aim of this work to examine whether the PS II fluorescence in intact green algae with closed PS II centers can be described in terms of such a model. The elements of this scheme, denoted *A–D*, are represented by different states of the various pigments involved. These are the primary donor P, the primary acceptor pheophytin I, the quinone Q_A, and the antenna chlorophylls Chl_n. The excited states of the antenna and of the donor P are considered to equilibrate on a time scale much faster than the primary charge separation. Recently, experimental evidence for this so-called trap-limited model has been reported (8, 19a). The trap-limited model for exciton decay in the antenna allows us to describe the excited states in the antenna and the reaction center by the generalized state *B* (see reference 10 for a further justification). As a result of this, k_1 is the ***apparent*** rate constant for charge separation. The ***intrinsic*** rate constant for charge separation k_1^{int}, that is the rate constant for charge separation in the isolated reaction center without any antenna pigments, is proportional to k_1. The proportionality constant is given by the partition factor for the exciton between the antenna and the primary donor P* (10, 25)

$$k_1^{int}/k_1 = N \cdot \exp[-hc/kT(\lambda_{Chl}^{-1} - \lambda_P^{-1})], \quad (1)$$

where N is the number of chlorophyll molecules in the antenna with maximal absorption at λ_{Chl}, whereas the primary donor absorbs maximally at λ_P. For PS II with an antenna size of 240 Chls this partition factor is ~113.

A second feature of the model is a reversible energy partition between the excited state of the donor and the

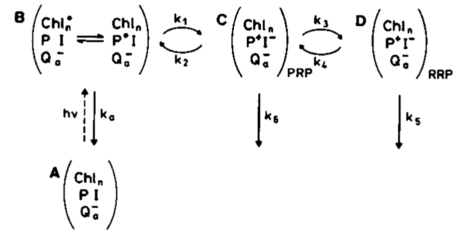

FIGURE 2 Kinetic model for the primary processes in closed PS II reaction centers. The given rate constants describe the following processes: k_a is the sum of radiative plus radiationless decay of excited states in the antenna (i.e., intrinsic antenna decay); k_1 is the apparent rate constant for primary charge separation; k_2 describes charge recombination back to the excited state of the donor, P*; k_3 is the rate constant for the presumed relaxation of the primary radical pair (i.e., formation of the relaxed radical pair); k_4 describes the recombination of the relaxed radical pair back to the primary radical pair; k_5 and k_6 are the deactivation rate constants for the relaxed and primary radical pair, respectively, to the ground and/or triplet states (see Discussion).

primary radical pair described by the rate constants k_1 and k_2, as pointed out earlier (26, 27). A relaxed radical pair state RRP, formed (with rate constant k_3) from the primary radical pair PRP, has been proposed by Schlodder and Brettel (15). These authors did not take into account, however, the reversibility of such a relaxation process. This is the most crucial point. We do not see any argument for ignoring the reverse process. The rate constant of back reaction from the putative RRP state should be the result of the data analysis rather than resulting from an unjustified a priori assumption. Therefore, we also consider the back reaction in our scheme (rate constant k_4). Furthermore, rate constants k_5 and k_6 describe the depopulation of radical pairs, i.e., the additional deactivation processes from the RRP and PRP states, respectively, back to the ground states. For simplicity the spin dephasing processes occurring in the radical pair states which lead to triplet radical pairs and population of $^3P^*$ (28) are also contained in k_5 and k_6. The rate constant k_a gives the intrinsic antenna decay, i.e., the sum of the radiative and radiationless decay processes of excited states in the antenna back to the ground state.

The set of coupled differential equations describing this model has been solved by application of Laplace transforms. In Appendix A equations are derived (eqs. A4–A6) that give the temporal development of the concentrations of the three states involved: Chl* in the antenna in equilibrium with P* (state B), primary radical pair PRP (state C), and relaxed radical pair RRP (state D), (cf. Fig. 2). In short, the model predicts for all three states a three-exponential time function for the concentration development with the same lifetimes τ_1, τ_2, and τ_3, but with different relative amplitudes. Chlorophyll fluorescence thus decays with a three-exponential time course. The primary radical pair is populated with τ_1 and decays with τ_2, and τ_3, whereas the presumed relaxed radical pair builds up with τ_2 and is depopulated with lifetimes τ_1 and τ_3. The solution of this kinetic model in principle (provided we have seven independent input parameters) enables us to find the set of seven rate constants that describe the experimentally found kinetics for each of these three states. From the concentration developments we can calculate the maximal transient concentration c_{max} for the different states. In the case of multiexponential decay kinetics this parameter is more useful than the commonly used term "yield," which is calculated from the initial relative amplitude of the longest exponential lifetime component for each respective state (15). It is also important to know the development of the total concentration of charge separated states (CSS), i.e., the sum of the concentrations of both radical pair states. This parameter allows, inter alia, for a comparison of the fluorescence decay data with time-resolved photovoltage measurements (11, 12) The determination of the rate constants also enables us to calculate the changes in standard free energy accompanying primary charge separation ($\Delta\Delta G^0_{cs}$) and the presumed radical pair relaxation process ($\Delta\Delta G^0_{rel}$). In Appendix B the corresponding formulae for the calculation of $\Delta\Delta G^0$ are given, assuming that equilibrium thermodynamics applies (eqs. B1).

The kinetic model predicts a three-exponential decay for Chl fluorescence. The analysis of the experimental data therefore yields only five independent parameters, i.e., three lifetimes and two amplitudes, because the measured amplitudes are only relative. With no other reliable data available, two additional assumptions must therefore be made to reduce the number of rate constants in the model to be fitted to the experimental data. First we decided to use fixed values for k_a. A range of $k_a = 0.3–0.6$ ns^{-1} seems reasonable (see below for a detailed discussion on the k_a values). The second simplification we make is to assume that $k_5 = k_6$, i.e., the recombination processes in both radical pair states to any long-lived states (triplets and/or ground states) occur with the same rate constants. The solutions are thus parametrically dependent on k_a, whereas the second assumption $k_5 = k_6$ is considered to be not very restrictive, as will be justified below.

We now apply this model to the experimentally measured PS II fluorescence kinetics, considering also a potential 10-ns component with a maximum amplitude of 0.1% relative to the total fluorescence (see Results). Four triexponential combinations are possible, i.e., cases I–IV. By composing each one of the cases I–III that include the potential long-lived component, we leave out one of the experimentally found components. By doing so, we imply, for the sake of the argument, that the component which is ignored does not originate from the same PS II pool as the other three components. Only Case IV describes the actually found PS II kinetics, assuming that all three components come from the same homogeneous PS II pool. We intentionally consider all possible combinations explicitly, despite the fact that some combinations give rather pathological results and/or do not appear to be reasonable a priori. Note that in all four cases the amplitudes of the three components considered in the analysis are renormalized to 100%. The four cases are thus characterized the the following way:

Case I: 380 ps (16%), 1.34 ns (84%), 10 ns (0.18%);
Case II; 380 ps (26%), 2.08 ns (74%), 10 ns (0.28%);
Case III; 1.34 ns (64%), 2.08 ns (36%), 10 ns (0.13%);
Case IV: 380 ps (11%), 1.34 ns (57%), 2.08 ns (32%).

The relative amplitudes given in parentheses refer to the maxima of the DAS at $\lambda_{em} = 685$ nm, but are very similar over the whole DAS. The calculated rate constants and further derived quantities for the four cases I–IV, based on the kinetic model shown in Fig. 2, are collected in Table 3.

We shall judge the results of these analyses in terms of three main criteria. The first criterion is $\Delta\Delta G^0_{tot} \equiv (\Delta\Delta G^0_{cs} + \Delta\Delta G^o_{rel}) \geq -60$ meV. This lower limit corresponds to the difference in standard free energy between the excited donor P* and the primary radical pair in open reaction centers, calculated from the F_0 data (Bittersmann, E., and A. R. Holzwarth, manuscript submitted for publication) (cf. Fig. 3). Our calculation assumes that the two PS II components with lifetimes of ~300 ps (60%) and ~550 ps (40%) at F_0 would originate from a homogeneous PS II population. The value of $\Delta\Delta G^0_{tot} \geq -60$ meV is both consistent with a more detailed analysis in terms of a heterogeneous $PSII_{\alpha,\beta}$ model (29) as well as with recent

TABLE 3 Kinetic and thermodynamic analysis of the four cases I–IV of three-exponential combinations of lifetimes (τ_i) and corresponding rel. amplitudes (in parentheses). The amplitudes refer to λ_{em} = 685 nm. Rate constants k_1–k_5 refer to Fig. 2. The rate constant for the primary charge separation in the "isolated reaction center" is given by k_1^{int}. C_{max} is the calculated maximal transient concentration (at time t_{max}))Tof the PRP, RRP, and the sum of PRP and RRP, i.e., CSS.

k_a	$k_{1\text{-}5}$	$\Delta\Delta G^0_{cs}$	$\Delta\Delta G^0_{rel}$	PRP	RRP	CSS	$1/k_1^{int}$
ns^{-1}	ns^{-1}	*meV*		c_{max} at t_{max}	c_{max} at t_{max}	c_{max} at t_{max}	*ps*
Case I: τ_1 = 380 ps (16%), τ_2 = 1.34 ns (84%), τ_3 = 10 ns (0.18%)*							
0.3	0.75 0.64 1.6 0.0061 0.10	−4	−140	0.17 at 675 ps	0.44 at 3.57 ns	0.48 at 2.77 ns	12
0.4	0.65 0.74 1.5 0.0065 0.099	3	−137	0.15 at 675 ps	0.36 at 3.57 ns	0.39 at 2.70 ns	14
0.5	0.55 0.87 1.3 0.0072 0.099	12	−132	0.13 at 675 ps	0.27 at 3.57 ns	0.30 at 2.58 ns	16
0.6	0.45 1.1 1.2 0.0084 0.097	22	−124	0.10 at 675 ps	0.19 at 3.57 ns	0.22 at 2.35 ns	20
Case II: τ_1 = 380 ps (26%), τ_2 = 2.08 ns (74%), τ_3 = 10 ns (0.28%)*							
0.3	0.73 1.2 0.77 0.0038 0.099	13	−134	0.19 at 795 ps	0.29 at 4.56 ns	0.35 at 2.81 ns	12
0.4	0.63 1.4 0.58 0.0050 0.098	20	−120	0.16 at 795 ps	0.19 at 4.56 ns	0.25 at 2.04 ns	14
0.5	0.53 1.7 0.32 0.0091 0.094	29	−90	0.14 at 795 ps	0.09 at 4.56 ns	0.17 at 1.18 ns	17
0.6	0.43 2.0 0.028 0.10 0.000005	39	32	0.11 at 795 ps	0.006 at 4.55 ns	0.11 at 0.82 ns	21

TABLE 3 (continued)

k_a	k_{1-5}	$\Delta\Delta G^0_{cs}$	$\Delta\Delta G^0_{rel}$	PRP	RRP	CSS	$1/k_1^{int}$
Case III: τ_1 = 1.34 ns (64%), τ_2 = 2.08 ns (36%), τ_3 = 10 ns (0.13%)*							
0.3	0.35 0.047 0.42 0.011 0.10	−50	−91	0.21 at 1.69 ns	0.26 at 5.91 ns	0.37 at 3.34 ns	25
0.4	0.25 0.066 0.40 0.012 0.099	−34	−89	0.15 at 1.69 ns	0.17 at 5.91 ns	0.26 at 3.26 ns	36
0.5	0.15 0.11 0.36 0.013 0.098	−8	−83	0.09 at 1.69 ns	0.09 at 5.91 ns	0.15 at 3.06 ns	59
0.6	0.050 0.33 0.15 0.031 0.080	48	−40	0.03 at 1.69 ns	0.01 at 5.91 ns	0.04 at 2.16 ns	179
Case IV: τ_1 = 380 ps (11%), τ_2 = 1.34 ns (57%), τ_3 = 2.08 ns (32%)							
0.3	0.57 0.69 1.1 0.13 0.53	5	−53	0.14 at 750 ps	0.14 at 2.13 ns	0.24 at 1.39 ns	16
0.4	0.47 0.84 0.97 0.15 0.51	15	−47	0.12 at 750 ps	0.10 at 2.13 ns	0.19 at 1.32 ns	19
0.5	0.37 1.1 0.78 0.19 0.47	27	−36	0.09 at 750 ps	0.06 at 2.13 ns	0.13 at 1.21 ns	24
0.6	0.27 1.5 0.49 0.30 0.36	43	−12	0.07 at 750 ps	0.03 at 2.13 ns	0.08 at 1.03 ns	33

*This amplitude corresponds to 0.1% of the total fluorescence, including all the other lifetime components.

photovoltage experiments by Leibl et al. (12). The underlying assumption made is that the standard free energy of P* is not influenced by the redox state of Q_A. A more negative value of $\Delta\Delta G^0_{tot}$ would imply that the relaxed radical pair in closed centers (Q_A reduced) was lower in free energy than the radical pair in open centers with Q_A oxidized, as illustrated in Fig. 3. We consider this to be a physically unreasonable situation taking into account the Coulombic interaction energy between I^- and Q_A^- in the radical pair state in closed centers, which is not present in open centers. To account for this electrostatic interaction between I^- and Q_A^-, the free energy of the

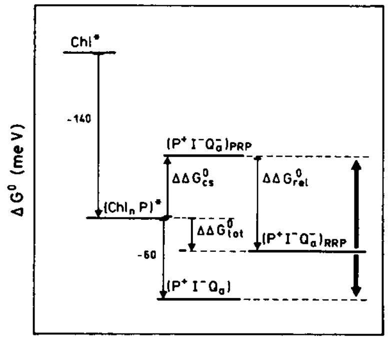

FIGURE 3 Scheme of the standard free energy content of the different excited and charge separated states in open and in closed PS II centers with an antenna size of ~240 Chl/P_{680}. The decrease in ΔG^0 caused by exciton delocalization over the whole antenna is calculated as $\Delta\Delta G^0 = -kT \cdot \ln(240) = -140$ meV. The change in ΔG^0 for the primary charge separation in open centers is extracted from data to be published (Bittersmann and Holzwarth, manuscript submitted for publication). This span in ΔG^0 sets a lower limit to the free energy content of the relaxed radical pair as discussed in the text.

RRP should be significantly higher, so that the value of −60 meV represents the absolute lower limit. It is difficult to give an exact number for this increase, however, because neither the distances between these chromophores nor the local dielectric constant is known. In view of these difficulties we will operate in the following discussion with the lower limit of −60 meV, keeping in mind, however, that a substantial rise in standard free energy of a putative RRP state as compared with this level is in order.

The second criterion is the maximal transient concentration of CSS c_{max} formed. According to Trissl and co-workers (11, 12) based on photovoltage measurements that determine directly the concentration of CSS, this maximum concentration should be <15%. These measurements were carried out on BBY PS II preparations (12, 30) and on swollen chloroplasts (11), i.e., on PS II systems with antenna sizes similar to those found in green algae. We therefore set the limit c_{max} of CSS to ≤15%. Basically the same value can be estimated from an extrapolation of our previous data on PS II particles (10) to antenna sizes of ~240 Chls/P_{680}.

As a third criterion we evaluate the rate of primary charge separation in the isolated reaction center k_1^{int}, calculated according to Eq. 1. It was estimated (10) that at the F_0 level $1/k_1^{int}$ would be ~2.7 ps. Very recently Wasielewski et al. (31) performed transient absorption measurements on isolated PS II reaction center D_1D_2-cyt-b_{559} complexes and found a value of 3 ps for $1/k_1^{int}$. From the analysis of the data on PS II particles it is known that k_1, and accordingly also k_1^{int}, decrease by a factor of 6 upon reduction of Q_A (10). Reduction of k_1 by a factor of 3 results from the photovoltage measurements (12). This sets an upper limit for $1/k_1^{int}$ of 16–18 ps. Therefore, values of $1/k_1^{int} \geq 18$ ps must be rejected as inconsistent with the data available on several PS II systems.

The problem of determining k_a

At this point it is appropriate to discuss the adequate value for k_a because, for the reasons pointed out above, k_a must be fixed to solve for the other rate constants. The results of the analysis therefore become parametrically dependent on k_a. This dependence is only weak for the F_0 case but quite pronounced for the situation at F_{max}. Because k_a is the sum of the radiative and nonradiative rate constants, its magnitude cannot be determined with straightforward methods. Two different approaches can be used to overcome this problem.

The first approach uses the lifetimes of isolated Chl protein antenna complexes without reaction centers. This limits the range k_a to 0.3–0.9 ns^{-1} (32–34). It is interesting that an upper limit for k_a is set by the fluorescence kinetic data themselves in conjunction with the kinetic scheme. Notably it turns out that values of $k_a > 0.6$ ns^{-1} are excluded because this leads to negative values for at least one of the other rate constants, which is unacceptable for physical reasons. In fact, even a value of $k_a = 0.6$ ns^{-1} leads to a pathological situation in several of the analyses discussed below in the sense that they are very close to the point where at least one rate constant would become negative. Thus, one might want to exclude the case $k_a = 0.6$ ns^{-1}. We have nevertheless included it in our analyses shown in Table 3 because the tendency is such that higher k_a values lead to both higher values for $\Delta\Delta G^0_{tot}$ and lower radical pair concentrations, which tends to be in better agreement with two of our exclusion criteria. However, we consider values in the range k_a = 0.3–0.5 ns^{-1} to be the most reasonable ones.

The second approach to solve all the rate constants in the kinetic scheme, including k_a, would be to use knowledge about some other quantity, e.g., the amount of CSS formed. This approach has been followed by us previously (10). By making the conservative assumption of a lower limit for the overall yield of $P^+Q_A^-$ formation in open PS II centers of 85%, we arrived at $k_a = 0.9$ ns^{-1}. Any higher value for this yield, which is in fact very likely, would have resulted in a lower k_a value. Analogously, the upper limit for k_a at F_{max} conditions was determined by assuming a maximum yield of ≤15% for any long-lived (triplet and/or relaxed radical pair) state(s). Problems with negative rate constants did not occur in this case of the smaller isolated PS II particles because the longest lifetime at F_{max} was only ~1.3 ns. In any case, however, this second approach is not useful in our present analysis because it is our aim to *determine* the maximal concentration of CSS and not to *assume* it beforehand. In view of these difficulties we present the results of our analysis for the range of k_a = 0.3–0.6 ns^{-1}, which is both reasonable and physically allowed.

The kinetic analyses

The results of the kinetic analyses of cases I–IV and the range of k_a values, in terms of the model discussed above, are given in Table 3. The table lists the rate constants, the free energy changes, the maximal transient radical pair concentrations, and the intrinsic charge separation time. We may safely exclude any case in these results that does not simultaneously meet all three criteria presented above because these limits were set very conservatively. It follows that both the free energy criterion as well as the c_{max} limit of 15% are violated in all the analyses for case I, as well as with k_a = 0.3 and 0.4 ns^{-1} in case II and for k_a = 0.3–0.5 ns^{-1} in case III. For this latter case, we have additionally very high $1/k_1^{int}$ values caused by the relatively long τ_1 (1.34 ns). This criterion excludes all these analyses, in particular that for k_a = 0.6, as being unreasonable. The results for k_a = 0.6 in case II can be rejected by the value for $\Delta\Delta G_{rel}^0$ being positive (this is a pathological situation as it does not describe a relaxation process). The situation for k_a = 0.5 ns^{-1} in case II is on the borderline with respect to all three criteria. However, this case can be excluded by another argument: the concept of a relaxed radical pair state evidently involves a higher maximal transient concentration of the RRP than of the PRP. The situation is just opposite, however, insofar as c_{max} for the RRP is considerably smaller than c_{max} for the PRP. This argument can also be applied to most of the situations in cases II and III with k_a = 0.5–0.6 ns^{-1} in addition to the other three criteria discussed above. It follows that none of the cases that include a 10-ns component in an amount still compatible with the experimental data (i.e., all the analyses for cases I–III) can be accepted in terms of the three criteria and the general implications of a relaxed radical pair concept. We now discuss the remaining case IV which simply describes the three experimentally found PS II lifetimes. This would a priori seem to be the most reasonable case in terms of the relaxed radical pair concept. The free energy criterion is fulfilled for the whole range of k_a values. This is not surprising if one realizes that the relative amplitude of the recombination fluorescence component from the RRP is ~30%. For k_a values of 0.3 and 0.4 ns^{-1}, c_{max} of CSS is well above the limit of 15%, which excludes these two cases. For the remaining two situations the value for $1/k_1^{int}$ is significantly higher than the 18-ps limit. In addition, the above-mentioned argument on the ratio of c_{max} in the PRP and RRP applies to these two analyses as well. We thus have excluded all analyzed cases as being incompatible with the limits set by the experimental data.

The kinetic analyses in Table 3 also show that the assumption on the irreversibility of the radical pair relaxation, as made by Schlodder and Brettel (15), turns out to be unjustified. In all cases the rate constant for the back reaction, k_4, has values that differ from zero significantly. In case IV, k_4 is even of the same order of magnitude as the other rate constants.

For the analyses presented in Table 3 we assumed that $k_5 = k_6$ (see Fig. 2), i.e., the additional deactivation processes from both radical pair states to any long-lived state (triplet and/or ground state) occur with the same rate constant. In addition, we also performed the modeling with various other k_6/k_5 ratios to check for the nonrestrictive character of this assumption. If we set k_6 = 0, then all the results tend to violate the three criteria applied even more severely than they do in Table 3. If, on the other hand, we increase the k_6/k_5 ratio from 1 to 4, all the situations can be disqualified by the three main criteria and/or by the argument on the ratio of c_{max} in PRP and RRP. Even increasing k_6/k_5 to 10 does not result in any acceptable situation, whereas many of them have become pathological ($\Delta\Delta G_{rel}^o > 0$). So, it is obvious that the conclusions of our kinetic modeling are not restricted by the assumption $k_6/k_5 = 1$.

Judgement of the analyses presented here now becomes a matter of discussion on how hard or soft the assumed limits of $1/k_1^{int}$, c_{max} (CCS), and $\Delta\Delta G_{tot}^o$ are. In our view all our assumptions are rather conservative, and we therefore think that the experimental data are inconsistent with the concept of a second, i.e., relaxed radical pair state. If one were nevertheless to accept this concept, one clearly would be forced to conclude that this additional radical pair then would live for only ~2 ns (case IV) and not for ~10 ns as proposed by Schlodder and Brettel (15). Because these authors ignored entirely the back reaction, and thus the free energy criterion, they came to accept such a long-lived radical pair state with high yield. Clearly our own data obtained on isolated PS II particles are inconsistent with a high yield of long-lived radical pair state (8) as are other measurements (7, 13). It is interesting in this connection that long-lived components were observed in PS II particles by transient absorption after strong illumination cycles but not in fresh, unirradiated samples (13). One might argue that, in cases where long-lived components with lifetimes ≥5 ns were observed, they might be due to triplet states. However, this possibility has been excluded by the authors on several grounds (14, 15). An alternative explanation for the long-lived components could be that they originate from damaged PS II centers (14).

Our rejection of the relaxed radical pair concept asks for an alternative interpretation of the three PS II fluorescence components observed in intact algae. The two-state model proposed earlier (10), which considers only one radical pair state, predicts biexponential fluorescence kinetics for a homogeneous PS II pool. Our discussion so far has ignored the results of previous studies that the components with lifetimes of ~1.3 and ~2.1 ns have different excitation wavelength dependence (20), which was one of the reasons that they were ascribed to different types of PS II centers, viz.,α and β centers with different antenna pigment composition. This creates a further strong argument against any combination of these components (cases III and IV) in terms of a RRP model which requires that all involved fluorescence components originate from the same antenna pool. We can therefore exclude the RRP concept also from an entirely different point of view.

In a forthcoming paper we shall show that the data presented here can be consistently interpreted in terms of the two-state model proposed originally (10) without the necessity to include a relaxed radical pair state (as already presented in ref. 29). Instead, when the α/β heterogeneity is taken into account, with each type of PS II center decaying with biexponential kinetics, in principal four PS II fluorescence components result. Whether or not one can resolve all these four components by application of global lifetime analysis depends on the lifetime values, their relative amplitudes, and their spectral shapes.

CONCLUSIONS

We have questioned the concept of two distinguishable radical pair states in closed PS II, i.e., a primary one and a long-lived (~10 ns) relaxed one, and have concluded that this concept is inconsistent with our own experimental data as well as with those of several other groups. One may ask the question, however, as to the nature of the radical pair state in closed PS II that has a lifetime of ~2 ns and is formed with low yield. Should it be called a *relaxed* or an *unrelaxed* state? It would seem reasonable in our view to think of this state in terms of a relaxed state in the sense that the proteins have undergone some conformational adaptation to the new charge distribution reached in the radical pair. One could well imagine that such a supramolecular structure passes through several intermediate conformations in a time much shorter than 2 ns. Such intermediates could simply be inaccessible to the experimental methods applied so far. Our results clearly do not exclude the possibility that the reaction center proteins undergo further functionally important conformation relaxations. Such relaxations would have to occur, however, on a time scale longer than the lifetime of the radical pair state (>2 ns) found here.

APPENDIX A

Kinetics

The kinetic model, given in Fig. 2, is characterized by the following set of coupled differential equations:

$$\begin{aligned}
&(k_1 + k_a) \cdot B + \mathrm{d}B/\mathrm{d}t - k_2 \cdot C = 0\\
&\quad - k_1 \cdot B + (k_2 + k_3 + k_6) \cdot C + \mathrm{d}C/\mathrm{d}t - k_4 \cdot D = 0\\
&\qquad - k_3 \cdot C + (k_4 + k_5) \cdot D + \mathrm{d}D/\mathrm{d}t = 0. \qquad \text{(A1)}
\end{aligned}$$

Here B, C, D represents the concentrations in the corresponding states. Using Laplace transforms (s) one can eliminate the time derivatives. With the boundary conditions $B(t = 0) = B_0$, $C(t = 0) = D(t = 0) = 0$ (at zero time there are only excited states in the antenna present, i.e., state B), the following set of coupled linear equations results:

$$\begin{aligned}
&(k_1 + k_a + s) \cdot L(B) - k_2 \cdot L(C)\\
&\quad - k_1 \cdot L(B) + (k_2 + k_3 + k_6 + s) \cdot L(C) - k_4 \cdot L(D) = 0\\
&\qquad - k_3 \cdot L(C) + (k_4 + k_5 + s) \cdot L(D) = 0. \qquad \text{(A2)}
\end{aligned}$$

This set of equations can be solved for all three variables, as shown below for B:

$$L(B) = B_0 \frac{s^2 + p \cdot s + q}{s^3 + a_2 \cdot s^2 + a_1 \cdot s + a_0}$$

with:

$$\begin{aligned}
a_2 &= k_1 + k_2 + k_3 + k_4 + k_5 + k_6 + k_a\\
a_1 &= q + k_1 \cdot (k_3 + k_4 + k_5 + k_6) + k_a \cdot p\\
a_0 &= k_1k_3k_5 + k_1k_4k_6 + k_1k_5k_6 + k_2k_4k_a\\
&\quad + k_2k_5k_a + k_3k_5k_a + k_4k_6k_a + k_5k_6k_a\\
p &= a_2 - k_1 - k_a\\
q &= k_5 \cdot (k_2 + k_3 + k_6) + k_4 \cdot (k_2 + k_6). \qquad \text{(A3)}
\end{aligned}$$

Before inverse Laplace transformation can be performed, the denominator in this expression must be solved. This third order polynomial has been solved numerically, yielding the real roots $-\lambda_1$, $-\lambda_2$, and $-\lambda_3$. Inverse Laplace transformation yields (35):

$$\begin{aligned}
B = B_0 \Bigg(&\frac{(-\lambda_1^2 + p\lambda_1 - q)}{(\lambda_1 - \lambda_2) \cdot (\lambda_3 - \lambda_1)} e^{-t/\tau_1}\\
&+ \frac{(-\lambda_2^2 + p\lambda_2 - q)}{(\lambda_1 - \lambda_2) \cdot (\lambda_2 - \lambda_3)} e^{-t/\tau_2} + \frac{(-\lambda_3^2 + p\lambda_3 - q)}{(\lambda_2 - \lambda_3) \cdot (\lambda_3 - \lambda_1)} e^{-t/\tau_3} \Bigg)
\end{aligned}$$

with:

$$\tau_1 = 1/\lambda_1, \quad \tau_2 = 1/\lambda_2, \quad \tau_3 = 1/\lambda_3. \qquad \text{(A4)}$$

As the measured chlorophyll fluorescence intensity is proportional to $B(t)$, this model predicts triexponential decay kinetics for Chl fluorescence. For the primary radical pair and its relaxed form (states C and D, respectively) the concentration developments are also predicted to be triexponential, with the same τ values, but with different relative amplitudes:

$$C = B_0 \cdot k_1 \left(\frac{(\lambda_1 - k_4 - k_5)}{(\lambda_1 - \lambda_2) \cdot (\lambda_3 - \lambda_1)} e^{-t/\tau_1} + \frac{(\lambda_2 - k_4 - k_5)}{(\lambda_1 - \lambda_2) \cdot (\lambda_2 - \lambda_3)} e^{-t/\tau_2} + \frac{(\lambda_3 - k_4 - k_5)}{(\lambda_2 - \lambda_3) \cdot (\lambda_3 - \lambda_1)} e^{-t/\tau_3} \right) \qquad \text{(A5)}$$

$$D = B_0 \cdot k_1 k_3 \left(\frac{-1}{(\lambda_1 - \lambda_2) \cdot (\lambda_3 - \lambda_1)} e^{-t/\tau_1} + \frac{-1}{(\lambda_1 - \lambda_2) \cdot (\lambda_2 - \lambda_3)} e^{-t/\tau_2} + \frac{-1}{(\lambda_2 - \lambda_3) \cdot (\lambda_3 - \lambda_1)} e^{-t/\tau_3} \right) \qquad \text{(A6)}$$

We normalize the concentration functions to B_0, so that the calculated maximal concentrations for the different states are relative ones.

APPENDIX B

Energetics

For a reversible process the change in standard free energy is given by $\Delta\Delta G^0 = -kT \cdot \ln(K)$, with K being the equilibrium constant, k the Boltzmann constant, and T the absolute temperature. In situations where the two involved states are in thermal equilibrium with their environment, the equilibrium constant K is equal to the ratio of the two rate constants for the forward and backward process, respectively. Thus, we obtain:

$$\Delta\Delta G^0_{\text{cs}} = -\,kT \cdot \ln(k_1/k_2)$$
$$\Delta\Delta G^0_{\text{rel}} = -\,kT \cdot \ln(k_3/k_4). \qquad \text{(B1)}$$

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