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Connectivity of Photosystem II Is the Physical Basis of Retrapping in Photosynthetic Thermoluminescence

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ABSTRACT Energy transfer between photosystem II (PSII) centers is known from previous fluorescence studies. We have studied the theoretical consequences of energetic connectivity of PSII centers on photosynthetic thermoluminescence (TL) and predict that connectivity affects the TL Q band. First, connectivity is expected to make the Q band wider and more symmetric than an ideal first-order TL band. Second, the presence of closed PSII centers in an energetically connected group of PSII centers is expected to lower the probability that an exciton originating in a recombination reaction becomes retrapped. The latter effect would shift the Q band toward lower temperature, and the shift would be greater the higher the percentage of closed PSII centers at the beginning of the measurement. These effects can be generalized as second-order effects, as they make the Q band resemble the second-order TL bands obtained from semiconducting solids. We applied the connected-units model of chlorophyll fluorescence to derive equations for quantifying the second-order effects in TL. To test the effect of the initial proportion of closed reaction centers, we measured the Q band with different intensities of the excitation flash and found that the peak position changed by 2.5°C toward higher temperature when the flash intensity was lowered from saturating to 0.39% of saturating. The result shows that energy transfer between reaction centers of PSII forms the physical basis of retrapping in photosynthetic TL. The second-order effects partially explain the deviation of the form of the Q band from ideal first-order TL.

INTRODUCTION

Thermoluminescence (TL) is light emitted by systems in which radiation causes a separation of charges, and so formed semistable charge pairs recombine when heated, liberating the stored energy in the form of light. Photosynthetic TL (for reviews, see (1–5)) reflects several different charge recombination reactions in photosystem II (PSII). The best characterized photosynthetic TL bands are the Q and B bands that originate in charge recombination reactions between the S_2 and S_3 states of the oxygen-evolving complex and the reduced quinone acceptors Q_A and Q_B of PSII. The Q band originates in recombination reactions $S_{2/3}Q_A^- \rightarrow S_{1/2}Q_A$ and the B band in the reactions $S_{2/3}Q_B^- \rightarrow S_{1/2}Q_B$. A TL quantum is emitted if the recombination reaction produces an excited state of the primary donor and the excitation energy becomes dissipated radiatively.

The Q band is routinely measured from thylakoid samples by using 3,4-dichlorophenyl-(1′,1′)-dimethylurea (DCMU), which blocks electron transport from Q_A to Q_B. In a typical TL experiment, a thylakoid sample is illuminated with a short flash at low temperature and then heated at a constant rate. The Q band peaks at 5–20°C, the exact peak temperature depending on the sample material and experimental conditions. The B band, peaking at 35–40°C, is measured similarly but in the absence of DCMU.

The Q band of TL has often been found to be wider and more symmetrical than an ideal first-order TL band (6,7). In this study, we tested whether this deviation from first-order form could be due to second-order effects caused by the energetic connectivity of PSII. Experimental data indicate that energetic connectivity of PSII affects the peak position and the relative width of the Q band as predicted by the calculations.

THEORY

TL was first theoretically treated by Randall and Wilkins (8). They calculated that if a single type of a charge-separated state (a) decays in a first-order reaction producing luminescence, then the intensity of the luminescence (I_L) is directly proportional to the decay rate of a, or

$$I_L = -c\,\frac{d[a]}{dt}\,, \tag{1}$$

where c is a positive proportionality constant linking the reaction rate and luminescence intensity and $[a]$ is the (time-dependent) concentration of a. During heating at constant rate β, $d[a]/dt$ can be described with the differential equation

$$\frac{d[a]}{dt} = -s_0 e^{-\frac{E_a}{k_B(T_0+\beta t)}}\,[a]\,, \tag{2}$$

where t is time, s_0 is a constant (frequency factor or preexponential factor), T_0 is temperature at $t = 0$, E_a is the activation energy of the charge recombination reaction, and k_B is Boltzmann's constant.

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If we use the Eyring theory for reaction rates (9) and dissect E_a into the enthalpy of activation ($\Delta H^{\ddagger}$) and the entropy of activation ($\Delta S^{\ddagger}$), the equation for $d[a]/dt$, and thus for I_L (via Eq. 1), takes a slightly more complicated form ((10); see also (5)):

$$\frac{d[a]}{dt} = -\frac{k_B(T_0 + \beta t)}{h} e^{\frac{\Delta S^{\ddagger}}{k_B}} e^{-\frac{\Delta H^{\ddagger}}{k_B(T_0+\beta t)}}[a], \qquad (3)$$

where h is Planck's constant. The transmission coefficients of the Eyring theory are assumed to have the value 1. In both Eqs. 2 and 3, the intensity of first-order TL is always proportional to $[a]$. Equation 3 cannot be integrated in a closed form. TL bands are conventionally shown as plots of I_L versus temperature (T), where $T = T_0 + \beta t$.

TL bands may deviate from the shape of an ideal first-order band. The simplest possible reason for deviation from first-order behavior in TL is that several different charge recombination reactions produce luminescence in the sample or that one charge recombination occurs via multiple pathways (11–13). If the different reactions or pathways have similar but not identical activation parameters, then the resultant TL band may assume a complex shape. Another possible reason for deviation from first-order behavior is that the TL band reflects a single reaction but the activation parameters of this reaction are not exact, i.e., they have a distribution (5). Distributed thermodynamic parameters might be found if the reactants are complex molecules, and the presence of multiple recombination pathways would increase the complexity. Deviation from first-order behavior also occurs if the TL phenomenon actually is of second order. In this case, the intensity of recombination luminescence depends on temperature according to the equation

$$I_L = ck[a]^2, \qquad (4)$$

where the rate constant k is the same as in Eq. 3, i.e., $k = (k_B/h)(T_0 + \beta t)\exp\{\Delta S^{\ddagger}/k_B - \Delta H^{\ddagger}/k_b(T_0 + \beta t)\}$. It has also been suggested that the exponent in Eq. 4 might have a fractional value (for review, see (14)). This so-called "general-order kinetic model" is an empirical convenience rather than an analytical tool (14) and was not considered here because fractional values of the exponent lead to severe conflicts with physical models describing TL (15).

Second-order TL bands have a wider and more symmetrical form than first-order bands. The physical basis of second-order TL has been elucidated for semiconducting solids (16). In a semiconducting solid, an electron is said to be trapped when it is lifted from a low-energy luminescence center to the conduction band and then drops to a semi-stable trap state that has energy between the luminescence center and the conduction band. A luminescence photon is emitted when thermal energy lifts an electron from a trap back to the conduction band, and the electron returns to a luminescence center. The rate of flow of trapped electrons to the conduction band naturally depends on the concentration of trapped electrons, $[a]$. Once the electron has entered the conduction band, the electron may either find an empty luminescence center or become retrapped by an empty trap. The probability that an electron on the conduction band finds an empty luminescence center is directly proportional to the concentration of empty luminescence centers. Because empty luminescence centers are created by the trapping process, their concentration is equal to $[a]$. Thus, luminescence intensity is proportional to $[a]^2$, and Eq. 4 applies.

Let us now consider DCMU-treated thylakoids. Excitation energy transfer between different PSII units forms an equivalent of the conduction band by mediating reactions of the type (5)

$$\{S_2Q_A^-\}_a + \{S_1Q_A\}_b \rightarrow \{S_1Q_A\}_a + \{S_2Q_A^-\}_b, \qquad (5)$$

where subscripts "a" and "b" denote two different PSII reaction centers. It should be noted that when recombination has produced the singlet excited state of the primary donor, formation of $S_2Q_A^-$ may occur in the same reaction center. However, this local retrapping that does not involve energy transfer between PSII reaction centers does not cause deviation from first-order kinetics in TL (5,14).

Let us now consider Q-band TL in a system in which the reaction of Eq. 5 is possible. The rate of formation of the excited state of the primary donor (P_{680}*) from $S_2Q_A^-$ is proportional to the concentration of the $S_2Q_A^-$ states. Once formed via the recombination reaction $S_2Q_A^- \rightarrow S_1Q_A$ that produces P_{680}*, the exciton would be injected to the antenna system. Due to the energetic connectivity between PSII centers, the exciton might soon be found in another PSII unit. If this other PSII center is closed, then the exciton ends up as a luminescence photon with high probability, but otherwise the exciton would most probably become retrapped via the re-formation of the $S_2Q_A^-$ state. Thus, the concentration of the luminescence substrate ($[S_2Q_A^-]$) would affect both the formation of excited chlorophyll via recombination and the probability of radiative decay of the so formed excited chlorophyll. However, Eq. 4 does not apply as such, because the probability of radiative decay of an exciton in PSII is not zero even if the reaction center is in a photochemically open state (e.g., S_1Q_A). To calculate the effect of $[S_2Q_A^-]$ on luminescence yield, we need to consider energy transfer between PSII reaction centers in detail.

The emission spectrum of TL is roughly similar to the emission spectrum of PSII fluorescence (17), suggesting that photons originating from recombination reactions are emitted by the same chlorophylls as fluorescence photons. We will therefore assume that the relationship between the proportion of closed reaction centers and the yield of radiative decay of an exciton is the same for TL and chlorophyll *a* fluorescence. However, because the recombination reaction by definition opens a reaction center, the radiative yield must be calculated for excitons originating in open reaction centers only. For the analysis of the effect of energetic connectivity, we chose the connected units model of Lavergne and Trissl ((18); see also (19)). In this model, a PSII unit consists of a reaction center and associated antenna molecules. Excitons are in equilibrium within a unit and excitation energy can also be transferred between units (rate constant k_{con}). In the model, reaction centers can be in two states, open and closed. The model contains a rate constant for photochemistry, including other reaction-center losses occurring in open reaction centers (k_{o}), a rate constant for losses occurring in a closed reaction center (k_{c}), and a rate constant for losses from the antenna (k_{l}). In analysis of energetic connectivity, an experimentally accessible connectivity parameter, J (18), is used instead of the intrinsic rate constant k_{con}.

Using the equations of the connected-units model (18), the fluorescence yield for an exciton originating in a PSII unit with an open reaction center is

$$\Phi_f^{\mathrm{o}} = k_{\mathrm{rad}} \frac{k_{\mathrm{c}} + k_{\mathrm{l}} + k_{\mathrm{con}}}{(k_{\mathrm{c}} + k_{\mathrm{l}} + qk_{\mathrm{con}})(k_{\mathrm{o}} + k_{\mathrm{l}}) + (k_{\mathrm{l}} + k_{\mathrm{c}})(1-q)k_{\mathrm{con}}}, \tag{6}$$

where k_{rad} is the rate constant for radiative decay of an exciton in the antenna and q is the fraction of open PSII reaction centers.

Rearranging the definition of the connectivity parameter J (18,19), we get

$$k_{\mathrm{con}} = \frac{(k_{\mathrm{o}} + k_{\mathrm{l}})J}{F_{\mathrm{M}}/F_0 - 1 - J}, \tag{7}$$

where F_{M}/F_0 is the ratio of the chlorophyll a fluorescence yield measured with all reaction centers closed to the yield measured with all centers open, and noting that (19)

$$k_{\mathrm{c}} + k_{\mathrm{l}} = F_0/F_{\mathrm{M}}(k_{\mathrm{o}} + k_{\mathrm{l}}), \tag{8}$$

we get

$$\Phi_f^{\mathrm{o}} = \frac{k_{\mathrm{rad}}}{k_{\mathrm{o}} + k_{\mathrm{l}}} \frac{\frac{F_0}{F_{\mathrm{M}}} + \frac{J}{F_{\mathrm{M}}/F_0 - 1 - J}}{\frac{F_0}{F_{\mathrm{M}}} + q\frac{J}{F_{\mathrm{M}}/F_0 - 1 - J} + \frac{F_0}{F_{\mathrm{M}}}(1-q)\frac{J}{F_{\mathrm{M}}/F_0 - 1 - J}} = \frac{k_{\mathrm{rad}}}{k_{\mathrm{o}} + k_{\mathrm{l}}} \frac{1 + J}{1 + qJ}. \tag{9}$$

Fig. 1 shows how Eq. 9 behaves with different values of J. The quantity $(1 + J)/(1 + qJ)$ is equal to the ratio $\Phi_{\mathrm{p}}(q)/[q\Phi_{\mathrm{p}}(1)]$, where $\Phi_{\mathrm{p}}(q)$ is the photochemical yield as a function of q (19). If $J = 0$, the fluorescence yield of an exciton found in an open reaction center simply represents the F_0 yield of isolated reaction centers, and accordingly, Eq. 9 no longer depends on q.

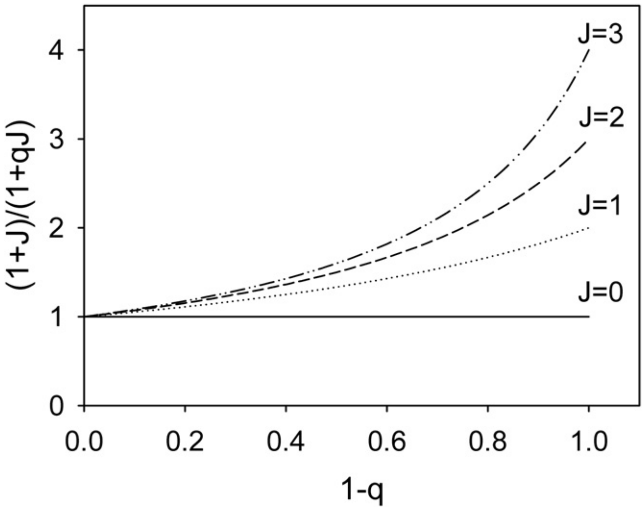

FIGURE 1 Relative fluorescence yield for excitons originating in open reaction centers as a function of the proportion of closed reaction centers $(1 - q)$, calculated from Eq. 9 with J values of 0–3, as indicated.

To use Eq. 9 as a correction factor, we need to multiply Eq. 3 by Eq. 9. Two modifications will be introduced at the same time. First, we incorporate the constant $k_{\mathrm{rad}}/(k_{\mathrm{o}} + \mathrm{k}_{\mathrm{l}})$ in the arbitrary constant c (see Eq. 3) and change the symbol of the arbitrary constant to d. Second, because Q-band measurements are always done without referring to the actual concentration of the recombining charge pairs, we replace the concentration of the TL substrate, $[\mathrm{S_2Q_A^-}]$ or $[a]$ in Eqs. 1–4, by the proportion of closed reaction centers, $(1 - q)$. The q parameter can have all values from 0 to 1, and the value of q increases in the course of the recombination reaction. Because of the presence of the factor q also in Eq. 9, the kinetics calculated after the correction is not exactly first order, and therefore, the rate constant should be considered to be calculated for a formal initial concentration, e.g., $[\mathrm{S_2Q_A^-}]_{t=0} = 1$. With these modifications, we get the following expression for Q-band TL intensity:

$$I_{\mathrm{L}} = -d \frac{k_{\mathrm{B}}(T_0 + \beta t)}{h} e^{\frac{\Delta S^{\ddagger}}{k_{\mathrm{B}}}} e^{-\frac{\Delta H^{\ddagger}}{k_{\mathrm{B}}(T_0 + \beta t)}}(1 - q) \times \frac{1 + J}{1 + qJ}. \tag{10}$$

For the B band, the situation is, in principle, more complex than for the Q band, as the TL substrate (a of Eq. 3) is $\mathrm{S_2Q_B^-}$, whereas the q parameter of Eq. 9 is the ratio $[\mathrm{Q_A}]/([\mathrm{Q_A^-}] + [\mathrm{Q_A}])$. However, the recombination reaction $\mathrm{S_2Q_B^-} \rightarrow \mathrm{S_1Q_B}$ can be measured only when essentially all PSII centers are open. Thus, Eq. 3 applies to the B band without corrections for energy transfer between PSII centers. No flash intensity dependence was found for the B band in an earlier study (20).

MATERIALS AND METHODS

TL bands were simulated with the differential equations using the MODELMAKER 4 software (ModelKinetics, Oxfordshire, UK).

A homemade luminometer was used to measure the TL curves. The detector is an EMI9558B photomultiplier tube operated with an ORIEL 70705 (Newport, Stratford, CT) power source. After preamplification and offset adjustment with homemade devices, the TL signal is fed to a PG8112 A/D card (National Instruments, Austin, TX) and digitized with 12-bit resolution. The photomultiplier is protected with an electronic shutter (VS25S1T0, Vincent Associates, Rochester, NY). To allow the use of a light guide as an alternative illumination method, the photomultiplier is attached on the sample in a slightly tilted position. The sample is cooled and heated with a water-cooled single-stage Peltier module (TB-127-1,0-0,8; Kryotherm, Carson City, NV) capable of adjusting the temperature between −20 and +70°C. The maximum heating rate is 1°C/s. The temperature is controlled with fuzzy-logic-based software via two analog outputs from the PG8112 card. The sample cuvette is thin 20-mm-diameter copper well, and the temperature is measured with a flat thermocouple (CO2-K, Omega Engineering, Manchester, UK) glued to the bottom of the cuvette.

Pumpkin (*Cucurbita maxima*) was grown in a research greenhouse at 20°C in a 16 h light/8 h dark rhythm under the photosynthetic photon flux density of 150 μmol m^{-2} s^{-1} during the light phase. Spinach (*Spinacia oleracia*) was grown in a phytotron in otherwise similar conditions except that the light phase was 12 h long. Thylakoid membranes were isolated as described earlier (21) and stored at −80°C. For the TL measurements, thylakoids were diluted to 1 mg chlorophyll/ml in a medium containing 14 mM HEPES-KOH, pH 7.6, 0.3 M sorbitol, 5 mM $MgCl_2$, 4 mM NaCl, 0.7 M glycine betaine, 20 μM DCMU, and 30% glycerol. The thylakoid sample (120 μL) was dark adapted for at least 15 min and pipetted in the dark on an 18-mm-diameter disk of filter paper placed at the bottom of the TL cuvette. For Q-band measurements, the temperature was lowered to −15°C or to −10°C, as indicated. When the minimum temperature was reached, heating at the rate of 1°C/s was initiated, and after 1 s of heating, the sample was flashed once with a FX-200 xenon flash lamp (EG&G, Gaithersburg, MD) placed on top of the sample at a distance of 1 cm. TL recording was started 4 s after the flash. The flash energy was set to 1.0 J, and 50% absorbing neutral density filters (Lee Filters, Hampshire, United Kingdom) were used to adjust the intensity. In one set of experiments, as indicated, the flash energy was 4.55 J, more neutral density filters were used to lower the flash intensity, and a 30-s delay was applied between the flash and the beginning of the heating ramp.

The TL signal was digitized at the rate of 10 samples/s (equal to 10 samples/°C) and smoothed by calculating a moving average using a window of 35 data points (3.5°C), and three replicate TL curves were always averaged. A strongly smoothened background signal, measured as an average from three nonflashed samples, was routinely subtracted from each TL curve. Baseline curves, obtained by applying the heating protocol to an empty cuvette, were used to evaluate the background signals. Before calculating the relative width of the TL band, each band was normalized by dividing by the maximum value.

RESULTS

The thermoluminometer was found to operate reliably, and replicate samples produced very similar TL curves (Fig. 2). The increase in the temperature during heating was linear, as the mean of the squared difference between the temperature calculated by assuming linear heating and that measured by the thermocouple was only $0.08 \pm 0.004(°C)^2$ ($n = 4$) during heating at 1°C/s.

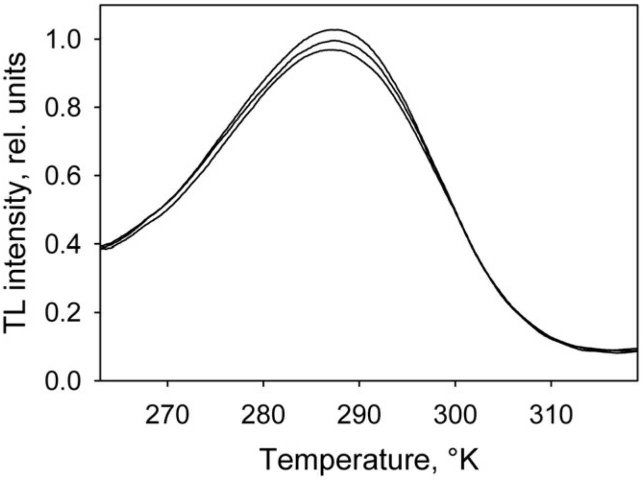

FIGURE 2 TL Q-band measurements from three replicate samples. Pumpkin thylakoids (120 μL, 120 μg Chl) were dark adapted for 10 min in the presence of 20 μM DCMU and 30% glycerol, temperature was lowered to −14°C, heating at 1°C/s was commenced, and a saturating xenon flash was fired after 1 s of heating. The original TL curves were smoothened with moving average using a 3.5°C window.

To test whether the effect of the energetic connectivity of PSII centers explains the form of the Q band, we compared the experimental Q band to bands obtained from the first-order kinetic model (Eq. 3) and from a model that takes into account the energetic connectivity of PSII (Eq. 10). Small changes in $\Delta S^{\ddagger}$ and $\Delta H^{\ddagger}$ can cancel each other's effect on the TL curve. Therefore, we chose to run the simulations using the value $\Delta S^{\ddagger} = -0.525$ meV/K. At 298 K, this $\Delta S^{\ddagger}$ value would be equivalent with $s_0 = 1.4 \times 10^{10}$ s^{-1} in the Arrhenius equation (Eq. 2); a similar value ($s_0 = 1.1 \times 10^{10}$ s^{-1}) was used in an earlier study (12).

The first-order TL band was clearly a bad approximation of the Q band (Fig. 3). TL bands calculated using Eq. 10 better resembled the experimental results, and at $J = 0.8$, the simulated band overlapped well with the experimental Q band except at low temperatures (Fig. 3). The maximum value of J, corresponding to energetic connectivity between all PSII centers via a common antenna (lake model), is equal to $F_M/F_0 - 1$ (19), and thus the maximum connectivity value for plant PSII with the F_M/F_0 value of 5 would be $J = 4$. The value $J = 4$ is equivalent to the p value (22) of 0.8. However, J values >0.8 made the band wider than the experimental data. Although both the first-order band and the band obtained from Eq. 10 peak at the same temperature, different activation parameters were used in the two simulations. In the simulated first-order band, the activation enthalpy $\Delta H^{\ddagger}$ was 636 meV, whereas Eq. 10 was simulated with the $\Delta H^{\ddagger}$ value of 645.5 meV. At 298 K, these values correspond to activation free energy values of 793 and 802 meV, respectively.

Different activation parameters were thus required to fit TL bands obtained from Eqs. 3 and 10 using the same experimental data, suggesting that the peak position of the band obtained from Eq. 10 depends on J. A simulation of the

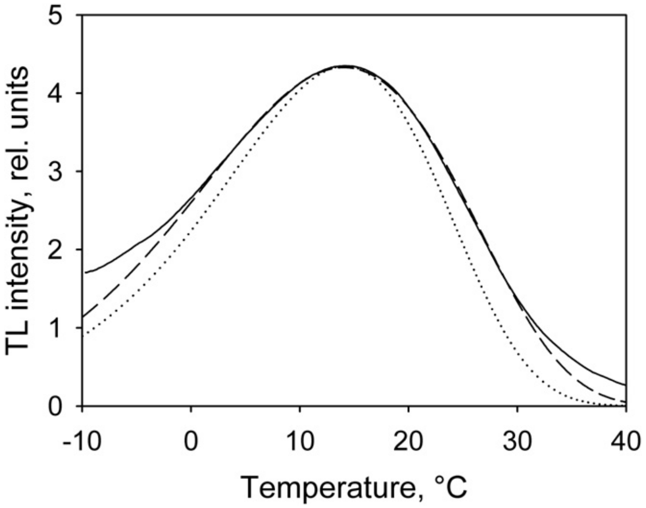

FIGURE 3 Comparison of an experimental Q band from pumpkin thylakoids (*solid line*) with TL curves obtained from Eq. 3 (*dotted line*, first-order kinetics, $\Delta H^{\ddagger} = 636$ meV) and from Eq. 10 (*dashed line*) ($\Delta H^{\ddagger} = 645.5$ meV, $J = 0.8$, $(1 - q)_{t=0} = 1.0$). In both simulated curves, $\Delta S^{\ddagger}$ was -0.525 meV/K. TL was measured as in Fig. 2.

behavior of Eq. 10 confirms this conclusion (Fig. 4 *A*). At $J = 0$, Eq. 10 produces a first-order TL band, and increasing J values lead to gradual widening and symmetrization of the TL band. At the same time, the peak shifts toward lower temperature (Fig. 4 *A*). Because this shift resembles the dependence of the peak temperature of a second-order TL band on the initial number of trapped states ($[a]_{t=0}$ in Eq. 4; see (16)), we also calculated the effect of varying the initial number of closed PSII centers $(1 - q)_{t=0}$ in Eq. 10. With the J value of 3.0 and $\Delta H^{\ddagger}$ value of 644.5 meV, the peak shifted by 6.2°C toward higher temperature when $(1 - q)_{t=0}$ was varied from 1 to 0.2 (Fig. 4 *B*). The smaller the J value, the smaller was the shift in the peak temperature (Fig. 4 *C*), and at the J value of 0.8, the shift was 3.3°C. Fig. 4 *D* shows that not only the peak temperature but also the form of the TL band calculated from Eq. 10 depends on $(1 - q)_{t=0.}$ A smaller fraction of initially closed PSII centers leads to a narrower TL band, and the low-temperature side of the band is affected more than the high-temperature side (Fig. 4 *D*).

To test the effect of the initial fraction of closed PSII reaction centers experimentally, we measured the Q band in pumpkin thylakoids using different intensities of the excitation flash that is fired before the heating begins. The flash energy was constant and neutral density filters were used to lower the amount of light passing to the sample. The resulting Q-band measurements (Fig. 5 *A*) show that the TL peak moved toward higher temperature when the flash intensity was lowered from saturating intensity to 0.39% of saturating. In this flash intensity range, the area of the TL peak varied from 100% to 3.2% of the area obtained with a saturating flash (Fig. 5 *B*). The full extent of the peak shift effect, obtained from the zero point of the derivative of each TL curve, was ~2.5°C (Fig. 5 *B*).

The small magnitude of the peak shift suggested that the value of J was <0.8, which produced a good fit for the curve obtained with a saturating flash (Fig. 3). Numerical simulations of Q-band TL, using Eq. 10 with the J value of 0.4 and activation enthalpy of 644 ± 9 meV ($\Delta G^{\ddagger} = 800$ meV at 25°C), showed similar behavior for peak temperature as the experiments (Fig. 5 *B*). We applied a 1.4% (9 meV) standard deviation for the activation enthalpy (5) in the simulation to better match the experimental curve form. We also tested whether varying the intensity of the flash has the expected effect on the width of the TL band. Fig. 4 *D* shows that connectivity widens the rising edge of TL curves measured using a strong excitation flash, compared to the ideal first-order form, and the first-order form is approached

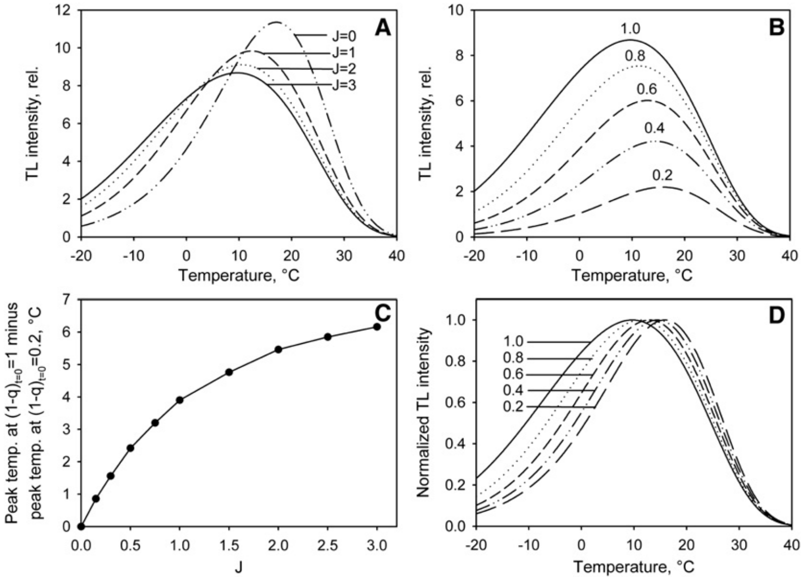

FIGURE 4 TL curves obtained by simulating Eq. 10. (*A*) Effect of varying the J value between 0 and 3, as indicated, with $(1 - q)_{t=0} = 1$. (*B*) Effect of varying the initial proportion of closed reaction centers, $(1 - q)_{t=0}$, between 0.2 and 1.0, as indicated, with $J = 3$. (*C*) The difference between the temperature of the TL peak value obtained with $(1 - q)_{t=0} = 0.2$ and $(1 - q)_{t=0} = 1.0$, plotted as a function of J, calculated from Eq. 10. (*D*) The same curves as in *B*, normalized so that the peak amplitude is 1.0. In all curves, $\Delta H^{\ddagger} = 643$ meV and $\Delta S^{\ddagger} = -0.525$ meV/K.

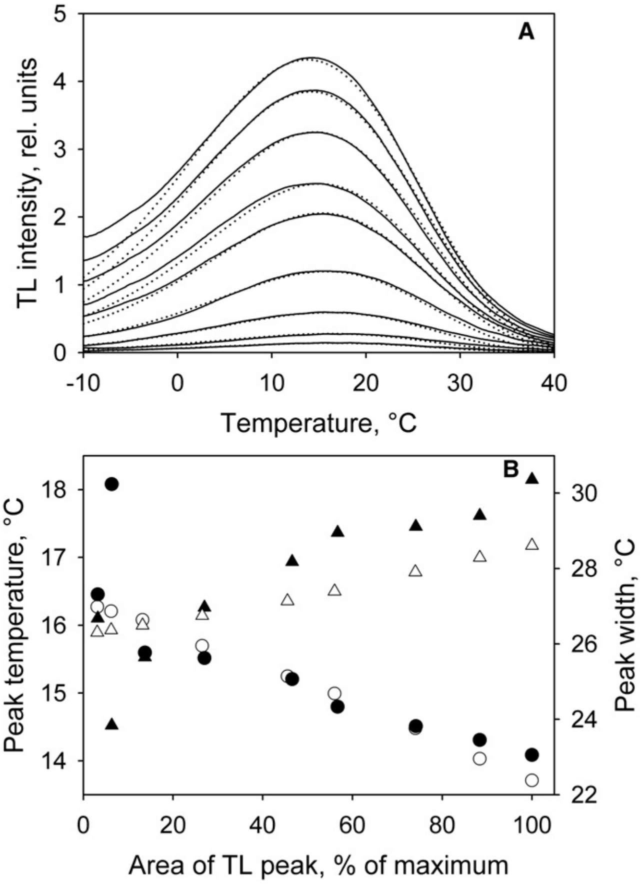

FIGURE 5 Effect of the flash intensity on the Q band. (*A*) TL curves from pumpkin thylakoids measured using (*uppermost* to *lowest curves*, respectively) a 100% saturating actinic flash and flash intensities lowered with neutral density filters to 50%, 25%, 12.5%, 6.25%, 3.13%, 1.6%, 0.78%, and 0.39% of the saturating intensity. The dotted lines show simulated TL curves with $\Delta H^{\ddagger} = 644 \pm 9$ meV, $\Delta S^{\ddagger} = -0.525$ meV/K and $J = 0.4$. (*B*) Peak position of the Q band (*circles*) and width of the normalized Q band at half-maximum height (*triangles*) from experimental data (*solid symbols*) and from simulations (*open symbols*). In *B*, all values are plotted as a function of the area of the TL band, integrated from −10°C to 40°C. The flash was fired at −14°C during heating at 1°C/s. Each experimental curve represents an average of three measurements.

when the flash intensity is lowered. Fig. 5 *B* shows that the width of the normalized TL band at half-maximum decreased by ~3.5°C when the flash intensity was lowered from saturating to 0.39% of saturating and the narrowing occurred so that the rising edge shifted toward higher temperature. Simulations showed that the width of the normalized Q band depends on flash intensity, as expected for $J = 0.4$, except that the simulated curves were narrower than the experimental ones (Fig. 5). These data confirm that the second-order effects in Q-band TL are due to energy transfer between PSII centers.

To make sure that the observed second-order effects in the Q band are not specific to one species, we measured the Q band from spinach thylakoids by using flash intensities spanning the range of 20% of saturation to full saturation. A similar shift of the peak position was found in spinach thylakoids as in pumpkin thylakoids, and simulations suggested the *J* value of 0.4 (Fig. 6).

The finding that the peak temperature and relative width of the Q band depend on flash intensity naturally depends on the presence of relatively large differences in the proportion of closed PSII reaction centers at the beginning of the heating ramp during the TL assay. If temperature-independent reactions decrease the number of closed reaction centers even at low temperature, then slow heating, starting the assay at very low temperature, or a long delay between illumination and heating, would diminish the differences between Q-band measurements done using different flash intensities. We tested the effect of a long delay between the actinic flash and the beginning of the heating ramp. The flash was fired at −10°C and the subsequent heating at 1°C/s was started only after 30 s. The delay resulted in lower TL bands than in our standard procedure, indicating that the substrate of TL was depleted during the delay. Consequently, the signal/noise ratio was not as good as in curves in which the actinic flash was fired during heating (Figs. 5 *A* and 7 *A*). Fig. 7 *A* shows that consistent differences in the peak temperature could not be seen if a 30-s delay was applied between flash and heating. Furthermore, curves obtained with low-intensity flashes were only marginally narrower than curves measured with high-intensity flashes (Fig. 7 *B*). These data show that the decrease in the number of closed reaction centers during a long delay at −10°C leads to virtual disappearance of some of the apparent second-order effects. However, even if the dependence of the peak position on

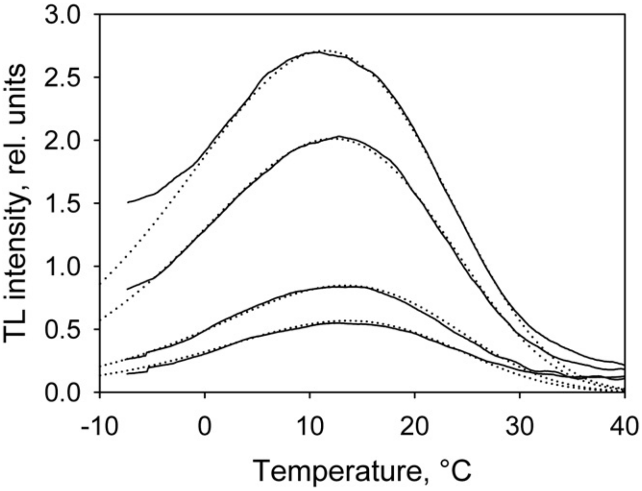

FIGURE 6 Dependence of Q-band position on actinic flash intensity in spinach thylakoids. TL was measured using (*uppermost* to *lowest curves*, respectively) a saturating flash and using lower flash intensities of 25%, 1.5%, and 0.78%. The flash intensity was lowered with neutral density filters. The flash was given at −10°C during heating at 1°C/s. The dotted lines show simulated TL curves with $\Delta H^{\ddagger} = 638.5 \pm 9$ meV, $\Delta S^{\ddagger} = -0.525$ meV/K and $J = 0.4$. Each experimental curve represents an average of three measurements.

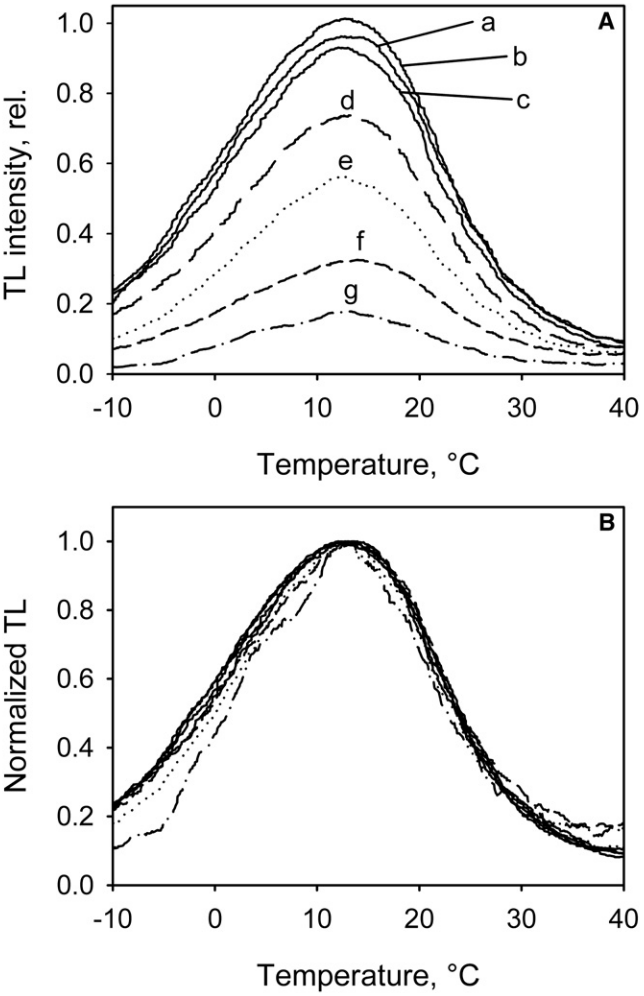

FIGURE 7 (*A*) Q-band measurements from spinach thylakoids applying a 30-s delay between the actinic flash and the beginning of the heating ramp. The energy of the flash was 4.55 J, and the light intensity was lowered with 2-10 layers of neural density filter foil with 50% transmittance. Flash intensity obtained with two neutral density filters was defined as 100% intensity, and thus the flash intensities were 100%, 50%, 25%, 12.5%, 6.25%, 3.13%, 1.56%, 0.78%, and 0.39% for curves *a*–*i*, respectively. Each curve is an average of three independent measurements. (*B*) The experimental TL curves after normalization by dividing by the maximum TL intensity.

flash intensity disappeared, the form of the Q band was still far from the ideal first-order form, confirming that energy transfer alone is not sufficient to explain the form of the Q band.

DISCUSSION

Exchange of excitation energy between PSII reaction centers has been used to explain the finding that the increase in the quantum yield of fluorescence in dark-adapted photosynthetic material is not exponential but sigmoidal (18,23–24). Assumptions about energetic connectivity have profound effects on the analysis of fluorescence data, including the decay of fluorescence yield after a single turnover flash (5), analysis of the OJIP transient (25), and analysis of photochemical quenching (26). The results of this study indicate that energetic connectivity also has consequences for the analysis of photosynthetic TL.

We chose the connected-units model (18) for the analysis of energetic connectivity, because this model is mathematically simple and requires no assumptions about the number of PSII units that can transfer energy between each other. Use of a model in which PSII units form domains and energy transfer occurs within a domain but not between domains (27) would obviously give an equation that would differ from Eq. 10. However, the predicted effects on the Q band would remain qualitatively similar, because they depend on the existence rather than the extent of the energetic connectivity.

Application of the connected-units model to the analysis of the Q band of photosynthetic TL leads to a simple correction to the equation describing the intensity of recombination luminescence as a function of time in a system that is heated at a constant rate (Eq. 10). After the correction for energy transfer between PSII centers, the simulated Q band shows second-order characteristics, including a widened form of the TL peak and dependence of the peak temperature and the band form on the initial fraction of closed PSII centers (Fig. 4). Experimental data confirmed both effects (Fig. 5).

For quantification of energetic connectivity, we used the parameter J (18), related to the widely used connectivity parameter p (22) as $J = p/(1 - p)$. The J values obtained from the dependence of the peak temperature on the intensity of the actinic flash were 0.4 for both pumpkin and spinach thylakoids, corresponding to a respective p value of 0.29. This value is similar to the value $p \approx 0.25$ obtained from the analysis of fluorescence induction for leaves of higher plants (25) but lower than the J value of ~1.5 (corresponding to $p = 0.6$) obtained from the analysis of fluorescence rise in the presence of DCMU in spinach thylakoids suspended in buffer containing 2–7 mM $MgCl_2$ (24). J values obtained for PSIIα alone in turn fall to the clearly higher range of 1.88–2.51 ($p = 0.65$–0.72) (18). Taking into account that J values determined with TL may be lowered due to the presence of several routes for the recombination reaction, some of which deplete the luminescence substrate even at low temperatures (11–13; see also Fig. 7), these data indicate that the energetic connectivity of PSII is responsible for the second-order effects found in TL. The second-order effects in TL are caused by retrapping, and energetic connectivity thus provides the physical basis for the effects of retrapping in photosynthetic TL.

Although the dependence of the peak position and relative width of the Q band on flash intensity (Fig. 5) are caused by energetic connectivity of PSII, the form of the Q band cannot be fully explained by the second-order effects (Figs. 3 and 7) even if the Q band is allowed to widen by assuming that the activation enthalpy has a 1.4% standard deviation (5).

Multiple reaction routes (11–13) are apparently needed to explain the behavior of the Q band, especially at the low-temperature side where the deviation from the simulated band is strongest (Fig. 5).

TL measurements are typically used to calculate the activation parameters of the recombination reactions. In photosynthetic TL, the activation (free) energy is a direct measure of the amount of energy that was lost due to stabilization of the charge separation in PSII. In most of the earlier analyses of the Q band (1–5), the Arrhenius equation (Eq. 2) was used to obtain E_a and a preexponential factor s_0. Usually, s_0 is in the range 10^9–10^{10} s^{-1}, corresponding to $\Delta S^{\ddagger}$ values in the range −0.55 to −0.75 meV/K at 298 K in Eq. 3. The value of $\Delta G^{\ddagger}$ (at 298 K) obtained from our analysis using Eq. 10 (800 meV) is slightly higher than an earlier estimation of the free energy difference between $P_{680}{}^{*}Q_A$ and $S_2P_{680}Q_A^-$, 780 meV (11). Because the peak temperature is mainly determined by $\Delta G^{\ddagger}$ (equal to $\Delta H^{\ddagger} - T\Delta S^{\ddagger}$), not by the enthalpy or entropy contribution alone, different combinations of $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ lead to TL curves peaking at the same temperature. We chose a negative $\Delta S^{\ddagger}$ and a corresponding low value of $\Delta H^{\ddagger}$ to match earlier analyses in the literature. A negative $\Delta S^{\ddagger}$ is compatible with the fact that the charge-separated state designated as $S_2P_{680}Q_A^-$ actually represents an equilibrium of several states, containing a combination of S_1 or S_2, P_{680} or $P_{680}{}^{+}$, Q_A or Q_A^-, tyrosine Z as either Z or Z^+, and pheophytin either as Pheo or $Pheo^-$. Reaching $S_1P_{680}{}^{*}Q_A$ by recombination would thus require a decrease of entropy. A positive $\Delta S^{\ddagger}$ contribution in the range 0.4–0.45 meV/K would in turn be caused by mixing of the excited state of P_{680} with excitation of one out of 100–200 antenna chlorophylls, as this mixing lowers the $\Delta G^{\ddagger}$ required to reach $P_{680}{}^{*}$ by 120–140 meV. Our TL data cannot unequivocally tell whether $\Delta S^{\ddagger}$ is positive or negative, but the conclusions about the effect of energetic connectivity remain essentially unchanged if we assume that $\Delta S^{\ddagger}$ is positive and $\Delta H^{\ddagger}$ has a high value (see Fig. S1 in Supporting Material).

The activation energy, obtained from a standard first-order fit of the TL curve, is sensitive to the position of the TL peak. Therefore, the finding that the peak position of the Q band depends on the initial fraction of closed PSII reaction centers must be taken into account in an analysis of TL. For example, a mutant in which only a small fraction of Q_A becomes reduced by an actinic flash may have the Q band at a higher temperature than the wild-type because the wild-type displays the second-order effects caused by energy transfer between PSII centers. A similar artifact may also be expected in a mutant that is resistant to the herbicide used in the measurement of the Q band.

The effect of energetic connectivity on TL predicts that connectivity also affects delayed light emission from photosynthetic samples. In fact, the kinetics of the decay of delayed light emission has often been found to show second-order effects, and hyperbolic rather than exponential equations have therefore often applied to fit the time course of delayed light emission (28–31). Hyperbolic fits have also been found useful for analyzing the decay of chlorophyll *a* fluorescence yield after a single turnover flash in the presence of DCMU (32). These second-order-like phenomena may be partially explained by retrapping of the excitation energy during recombination reactions.

SUPPORTING MATERIAL

One figure is available at http://www.biophysj.org/biophysj/supplemental/S0006-3495(09)00566-9.

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