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MEASUREMENTS OF $S_2Q_B^-$ RECOMBINATION BY DELAYED THERMOLUMINESCENCE REVEAL HETEROGENEITY IN PHOTOSYSTEM II ENERGETICS*

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Key Word Index—Photosynthesis; photosystem II; recombination; thermoluminescence; energetics; conformational substates; S-states; plastoquinone; *Spinacia oleracia*; Spinaceae.

Abstract—The measurement of recombination kinetics from the $S_2Q_B^-$ state of photosystem II (PS II) in spinach thylakoids using a thermoluminescence technique is described. Such measurements reveal that PS II exists in at least two substates with distinct kinetic and thermodynamic behaviors. Since it is not affected by treatments to alter the formal redox state of the PS II centers it was concluded that the heterogeneity is probably due to the existence of at least two conformational substates of the PS II proteins. The observed heterogeneity persists on a time scale or minutes at physiological temperatures, and thus is likely to be important for PS II function *in vivo*. © 1997 Elsevier Science Ltd. All rights reserved

INTRODUCTION

Photosystem II (PS II) is central to our understanding of plant biochemistry. Its unique ability to extract electrons from water has provided our ecosystem with an endless supply of reducing power, extending the frontiers of life and dramatically altering the chemistry of our atmosphere as well as the overall energy and nutrient cycles of the planet [1, 2]. At the same time, PS II may play a large role in limiting photosynthetic life to narrow environmental conditions. It is thought to be the primary site of photoinhibition induced by a wide range of environmental stresses [3, 4] and is one of the first apparent sites of heat-induced inhibition of photosynthesis [5]. In addition, PS II electron transfer appears to be a primary site of chilling damage in chill-sensitive plants [6, 7] and the interquinone electron transfer reactions of PS II have been shown to freeze out, limiting photosynthesis in many mesophilic plants at temperatures below about 5° C [8–10]. Furthermore, PS II represents a major control point for the entire photosynthetic chain and thus its activity is highly regulated [11].

A large number of studies have culminated in a basic model for the mechanism of PS II [1, 2, 11, 12]. Photons are captured by the antenna complex and delivered to the primary electron donor in PS II, the special pair of chlorophylls known as P_{680}, forming the excited singlet state, P^*_{680}. The energized electron on P^*_{680} is transferred to a bound pheophytin molecule (Pheo), leaving the highly oxidizing P^+_{680} species. The oxygen evolving complex (OEC) of PS II acts as a charge accumulator, storing up four oxidizing equivalents via its so-called S-state transitions, to oxidize two water molecules, releasing one molecule of O_2 and four protons. The highly reducing electron on $Pheo^-$ is transferred to a series of two PQ electron acceptors, collectively known as the "two electron gate" because it acts as an interface between the one electron chemistry of P_{680} and Pheo, with the two electron chemistry of the mobile plastoquinone (PQ) pool. The primary PQ acceptor, Q_A, acts as an $n = 1$ carrier and transfers the electron to the secondary PQ acceptor Q_B. The properties of the PQ at the Q_B site are modified by the D1 protein to stabilize the semiquinone form, thus permitting it to act both as a one- and a two-electron acceptor. Upon two turnovers of P_{680}, Q_B is reduced to plastoquinol (PQH_2) which migrates out of the Q_B site to be oxidized by the cytochrome b_6f complex.

Further progress in understanding the mechanism of PS II and the role it plays in determining the survival of plant has been accelerated in recent years by the study of site-directed mutant forms of the PS II proteins as well as the forms from organisms adapted to extreme environments. However, these resources can only be fully exploited with detailed analysis of the properties of PS II in these strains. In the past

*This work is dedicated to C. A. "Bud" Ryan on the occasion of his 65th birthday.

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decade a battery of assays has been developed to probe the formation and decay of specific PS II redox states in standard laboratory strains. However, our early investigations showed that some of these assays, particularly those probing states with Q_B in the semiquinone form (Q_B^-), could not be applied to a large number of site-directed mutants of *Chlamydomonas reinhardtii* [A. Kanazawa, D. M. Kramer and A.R. Crofts, in preparation] or to species, such as the psychrotrophic green algae *Chlorella antarctica* [13] with modified Q_B redox properties. It was therefore necessary to extend the available techniques or to develop new ones that are more universally applicable.

Thermoluminescence (TL), the measurement of light emitted from a photoactivated sample upon heating, has been used for some time to qualitatively characterize the energetics of photosynthetic materials [14, 15–19]. In most photosynthesis experiments, samples are activated with pulsed or continuous light during, or just prior to, cooling to low temperatures to trap the charge-separated states. The cooled samples are then heated at a constant rate and thermal activation of the system allows recombination of charge pairs via P_{680}^*. The exciton on P_{680}^* can then be transferred to the antenna chlorophyll where it decays by non-radiative or fluorescence pathways. Curves of luminescence intensity against temperature are called TL or "glow" curves, the peaks of which reflect the activation enthalpy, intrinsic rate constant and entropy for recombination [15, 18, 20]. In the case of PS II, specific TL bands have been empirically assigned to the decay of specific charged pair species and have thereafter been used to probe the redox state of PS II centers under a variety of conditions [14, 19, 20]. Past efforts to use TL curves to directly quantify energetic or kinetic parameters for reaction centers have been unsuccessful, possibly due to the complexity of their charge separated states (see below).

In this work, we introduce a technique, which we term "delayed TL" (DTL), that utilizes TL to quantify the amounts of centers in certain redox states, and thus to measure recombination rates of these states. The technique is useful in probing the behavior of states involving Q_B^-, particularly in strains with defective or modified Q_B sites or OECs. When results from DTL are combined with those from other assays, a detailed thermodynamic picture of PS II electron transfer can be drawn. We show that the DTL technique can also reveal hitherto unexplored heterogeneity in PS II energetics. We propose that this heterogeneity is due to the existence of at least two conformational substates of PS II proteins, as previously observed for other proteins [21] and for bacterial reaction centers [22–25]. In the case of PS II, the effects of these conformational substates persist at physiological temperatures over time scales important for catalytic turnover. We discuss possible importance of these conformational substates on the interpretation of TL bands and PS II function.

RESULTS AND DISCUSSION

Since the area under the TL curve should be proportional to the number of centers recombining during the experiment, we have used it as a reporter for recombination kinetics. To follow the decay of PS II redox states with DTL, saturating single-turnover actinic flashes were given to thylakoid samples, and variable time delay allowed before cooling to 0° and initiation of the TL heat ramp. The variable dark delay allowed for fractions of PS II centers to recombine before the TL curve was taken, and thus a plot of TL area against delay time yielded the kinetics of recombination. A similar approach was taken by Demeter et al [26] and Johnson et al. [27] for measuring the decay of the high temperature C-band in thylakoids. Because the C-band decays over many minutes, this type of experiment was feasible with conventional instruments. However, in order to apply the technique to lower temperature TL bands more precise timing and rapid temperature control were necessary. The instrument we have constructed to achieve these requirements is described in the Experimental section.

To test the validity of the DTL approach, we studied recombination from the $S_2Q_B^-$ state which results in the emission of the so-called B-band [14] in standard spinach thylakoid preparations in which this reaction has been characterized using previously available techniques. Figs. 1 and 2 show B-band curves and their integrated areas as a function of delay time, during which the $S_2Q_B^-$ states recombined. The half-

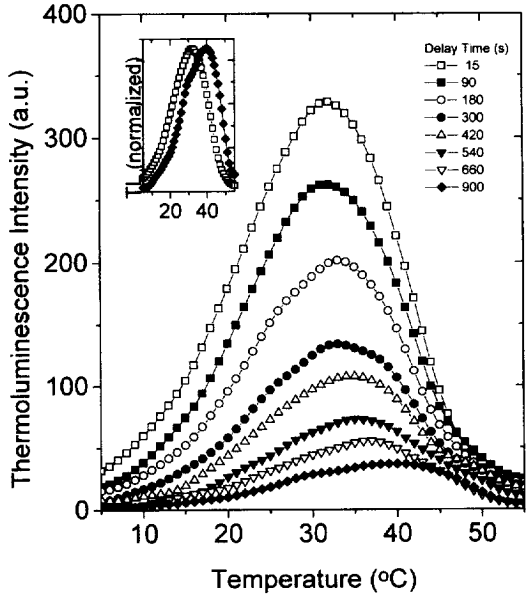

Fig. 1. Dependence of thermoluminescence B-band curves on delay time between flash excitation and temperature ramp. Delay times are given in the legend. Inset: B-bands after 15 and 900 s delay times normalized to their maximum intensities.

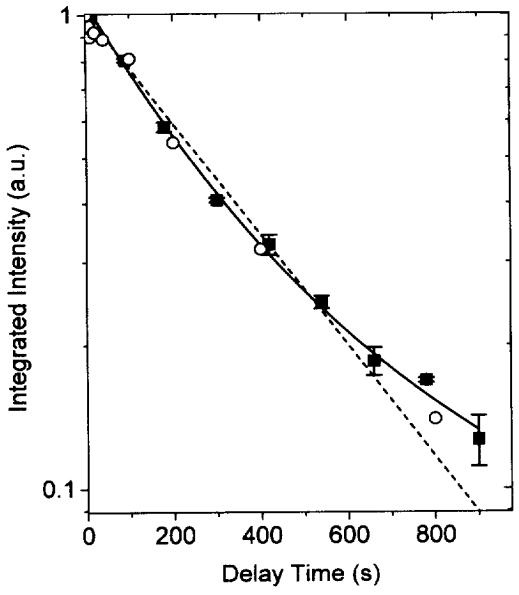

Fig. 2. Decay kinetics of the $S_2Q_A^-$ state of photosystem II measured by DTL as in Fig. 1 (closed squares) and rephasing of binary oscillations (open circles). The dashed and continuous lines represent best fits to the DTL data by one or two first-order decay components respectively.

time for the decay of the $S_2Q_B^-$ state measured by DTL was ca. 275 sec at 15°C.

For comparison, we measured the decay kinetics of $S_2Q_B^-$ under the same conditions by an alternate technique developed by Robinson and Crofts [28]. This technique takes advantage of the fact that, at least in some strains, the electron transfer from Q_A^- to fully oxidized Q_B is between 150 and 200 μsec, whereas that from Q_A^- to the Q_B^- state is between 350 and 400 μsec. Thus, the yield of chlorophyll *a* fluorescence (which reflects the fraction of centers in the Q_A^- state [29]) measured at 250 μs after each actinic flashes is sensitive to the fraction of centers in the Q_B and Q_B^- states. Since the redox state of Q_B alternates between the quinone and semiquinone states as the two-electron gate turns over, a series of single-turnover actinic flashes will produce a period-two oscillation in the fluorescence yield at 250 μs after each flash. The phase and extent of this oscillation will reflect the redox state of Q_B before the flash series. Starting with all Q_B sites fully oxidized, the fluorescence yield 250 μs after the flashes will be low–high–low–high reflecting the flash-induced alteration in oscillation in Q_B redox state from $Q_B \Rightarrow Q_B^- \Rightarrow Q_B \Rightarrow Q_B^-$. When a single pre-flash is given before the flash series, forming the Q_B^- state, the phase of the oscillation will be inverted (high-low-high-low) reflecting the following series of Q_B redox states: $Q_B^- \Rightarrow Q_B \Rightarrow Q_B^- \Rightarrow Q_B$. In the Robinson and Crofts technique, a variable delay is given after the pre-flash to allow recombination of a fraction of the Q_B^- state before the flash series. The extent of oscillation, estimated by taking the normalized difference in the fluorescence yield after the first two flashes, is plotted against the delay time, yielding an estimate for the Q_B^- recombination kinetics. Figure 2 (open circles) shows the kinetics of $S_2Q_B^-$ recombination measured by the Robinson and Crofts technique. Although the rephasing technique has a lower signal-to-noise ratio, the decay curve was similar to that measured by the DTL technique, indicating that the same phenomenon is most likely being measured by both techniques.

The DTL technique fills a critical gap in our ability to characterize PS II centers with modified Q_A to Q_B electron transfer properties. Both the binary oscillation rephasing experiments [28] as well as the pulsed oxygen electron technique [30, 31] require a fully-active Q_B site. The rephasing experiments additionally require that the rates of Q_A^- oxidation depend significantly on the redox state of Q_B. Both of these requirements in large part restrict the application of these techniques to wild type centers from standard laboratory species. DTL can in principle be applied to PS II centers without fully active Q_B or OEC site, making it a more universally applicable technique for probing PS II energetics.

Possible disadvantages of the technique include the requirement for a fresh sample for each time point of the measured decay curve—since the PS II centers are likely to be disturbed by the higher temperatures of the curve—along with the associated variability in data caused by sample-to-sample variations, and the requirement for a specialized TL photometer. Nevertheless, we feel that the increased utility of the DTL the technique far outweighs these difficulties.

PS II heterogeneity revealed by DTL

Two aspects of the DTL data suggest that $S_2Q_B^-$ decay is complex. Firstly, the decay kinetics of the integrated area of the B-band did not fit well to a single first-order component (Fig. 2). A reasonable fit was only obtained with two first-order components, with time constants for the fast and slow phases of 220 and 1000 s with relative amplitudes of about 0.8 and 0.2, respectively, suggesting that at least two kinetic processes are involved. Secondly, there was a noticeable shift in the peak temperature of the B-band as depletion progressed (see Figs. 1 and 3). The effect is most easily observed in the normalized traces in the inset to Fig. 1. The dependencies of peak position and band width are plotted in Fig. 3. After a short delay time of 15 sec. (see Fig. 1), the B-band temperatures were similar to those previously found for this band by other laboratories [32]. However, after a long (900 sec.) delay, the peak had shifted to *ca* 37°, i.e. a 6.2° shift in the peak occurred concurrent with an ca. 85% decrease in peak area.

The shift in B-band temperatures may reflect a quasi-second order reaction of the excitons with reaction centres. It has long been known that a PS II antenna system can deliver excitons to more than one reaction centers [33]. Thus an exciton emitted into the pigment bed by one PS II centre can be recaptured by

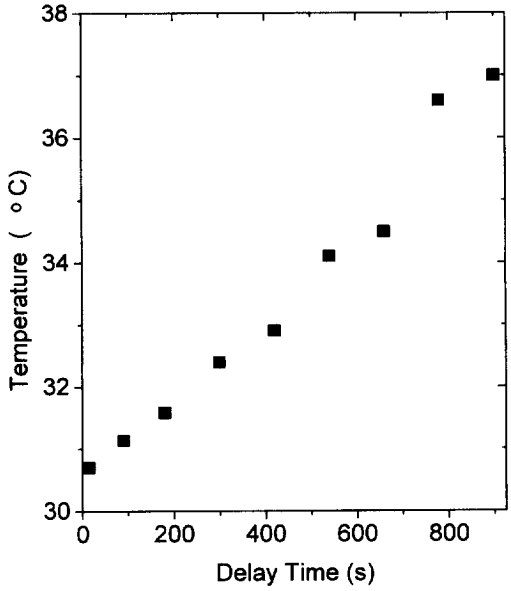

Fig. 3. Dependence of B-band temperature on delay time. The band temperature was estimated as described in the Experimental section.

another, provided that it is in an open (active) state. The irreversible processes of fluorescence and non-radiative exciton decay compete with recapture, eventually pulling the reaction towards recombination. The efficiency of exciton recapture should thus depend upon the number of connected centres in open states, with the chances of recapture increasing as the fraction of open centres increases. One might

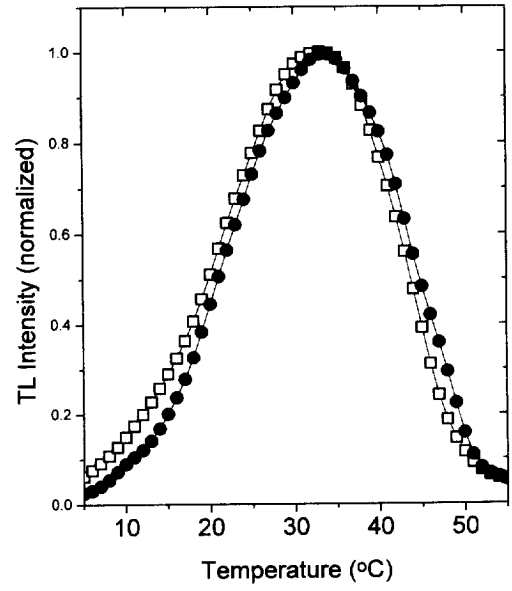

Fig. 4. Dependence of B-band shape on the intensity of the excitation flash. The curves represented by the open squares and closed circles represent B-bands taken after flashes that excited essentially all and ca. 15% of PS II centers, respectively. Curves were normalized to their maximum intensities.

therefore expect that the effective rate constant for recombination should decrease as recombination progresses, slowing the decay curve and shifting the TL band to higher temperatures. To test for this possibility, we compared B-bands after excitation with saturating or weak flashes. Figure 4 shows that the peak temperature of the B-band after weak excitation (resulting in ca. 15% activation) was ca. 1° higher than that after a saturating flash. This difference is small compared to 6–7° change observed upon depletion of the 85% of the band by recombination (Figs. 1 and 3), implying that the energetic connectivity of PS II centres via their antenna is only a minor factor in the observed shift of the TL bands during recombination.

Since a homogeneous population of charge-separated PS II centres should emit a single TL band, as well as yield a single exponential decay curve, the above results point to the existence of heterogeneous populations of PS II centres with different energetics of their charge-separated states. Heterogeneity of this type could arise from different formal charge-separated states. Upon dark adaptation, the OEC is found in its two stable S-states, S_0 and S_1, in the proportion of ca. S_0:S_1 = 1:3 [35]. The Q_B site is likewise found its in two possible redox states, with a ratio of Q_B:Q_B^- of about 7:3 [36, 37]. We thus expect a single flash to produce mainly the $S_2Q_B^-$ state, but also small fractions of the $S_1Q_B^-$, $S_1Q_B^{2-}$, and $S_2Q_B^{2-}$ [37]. Plastoquinol formed in the latter two states will be exchanged with pool PQ on the msec time scale [38, 39], producing the S_1Q_B and S_2Q_B states. Photosystem II centers with S_0, S_1 or oxidized Q_A and Q_B do not produce TL bands in the temperature range we have studied [40] so one might conclude that the bands observed in Fig. 1 should have arisen solely from decay of the $S_2Q_B^-$ state. On the other hand, it is conceivable (although unlikely, given the short duration of the xenon flash) that a small number of double hits may have led to the formation of a fraction of $S_3Q_B^-$ which, at lower pH values, can produce a TL band ca. 3-5° colder than that from $S_2Q_B^-$ [40]. Thus, we consider the possibility that $S_3Q_B^-$ recombination contributed to the peak shift observed in Fig. 1.

To test for contributions from $S_3Q_B^-$ recombination we used a pre-flash technique to alter the dark-adapted population of S-states [34]. Samples were dark adapted for 5 min. and illuminated with a saturating single-turnover flash, which clocked the S-states forward by one step, producing a distribution of S_1:S_2 = 0.25:0.75. A further dark adaptation of 5 min. resulted in the decay of S_2 states to S_1. The S_1 state is stable under these conditions leading to a nearly homogeneous population of S_1. Figure 5 compares B-bands taken after the pre-flash procedure after simple dark adaptation. The area under the pre-flash treated sample was ca. 12% larger, consistent with an increase in the fraction of centres in the S_1 state before the TL assay. However, the shape and peak temperatures of the two curves were nearly identical, and thus we

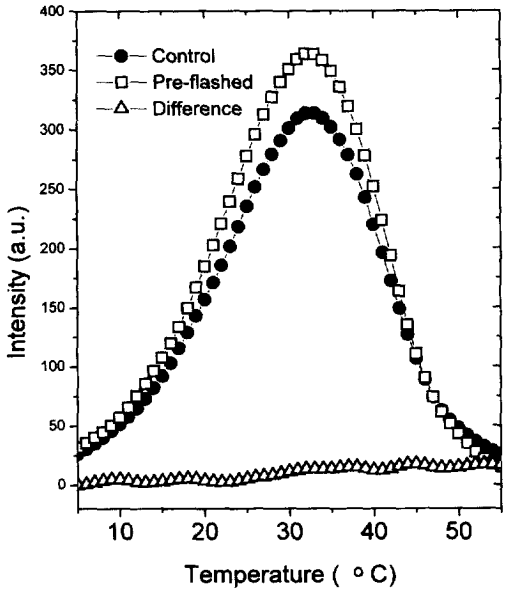

Fig. 5. The effects of altering the distribution of PS II S-states on the position of the B-band. Samples were either given the pre-flash treatment described in Results and Discussion (open squares) or simply dark-adapted (closed circles). The curve represented by the open triangles is the difference between the two curves after normalization to their respective maximum intensities.

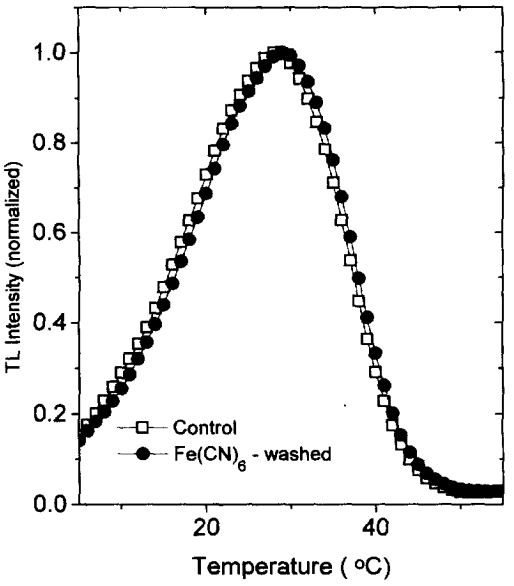

Fig. 6. The effects of ferricyanide washing on the B-band. Samples were either incubated in resuspension buffer (open squares) or with a 100 M solution of sodium ferricyanide in resuspension buffer (closed circles) and washed as described in Results and Discussion.

conclude that the distribution of the OEC S-states most probably did not contribute to observed shift in the TL bands.

In order to oxidize residual Q_B^- in the sample, thylakoid membranes were incubated in the dark in a 100 μM sodium ferricyanide solution (in resuspension buffer) for 10 min., followed by three cycles of washing with resuspension buffer [14]. This treatment has been shown to oxidize Q_B^- without significantly oxidizing the non-heme iron (Q_{400}), which requires significantly higher concentrations of ferricyanide [14]. The shapes and peaks of B-bands taken after ferricyanide treatment were nearly identical (peaks were within 1°C) to those similarly washed with just buffer (Fig. 6). We therefore conclude that the observed shifts in TL bands were most likely not due to alterations in the formal redox states of either the acceptor or donor sides of PS II.

An alternate explanation for the heterogeneity is that recombination occurs from the same formal charge-separated state (i.e. $S_2Q_B^-$), but that PS II exists in different conformational substates each with distinct energetic parameters for recombination. This possibility is consistent with the emerging view of protein structure which states that, rather than being homogeneous, proteins exist in a large number of conformational substates defined by different backbone and side chain positions which equilibrate over a wide range of time scales [21]. Each of the different conformational substates can have distinct biophysical properties, inducing heterogeneity in many spectroscopic and enzymatic properties of enzymes. There is evidence for two types of heterogeneity in photosynthetic bacterial reaction centers (RCs) [22–26], one that is induced by charge-separation and another that is static on the time scale of recombination events. Illumination of RCs produces charge separated states which generate large electric fields within the protein. In response, the protein appears to relax to conformations that better solvate the charges [23]. This relaxation, in turn, affects the energetics of charge recombination processes, lengthening their kinetics into a stretched exponential function. There is also evidence for heterogeneity in RCs that persist over a time scale of msecs are thus not induced by charge separation, but are static on the time scale of most electron transfer reactions in the RC [25]. These have been interpreted as reflecting conformational substates that interconvert slowly, even at physiological temperatures. Recently, Vassiliev *et al.* [42] have provided evidence that similar conformational substates exist in isolated photosystem I reaction centres. We suggest that the DTL technique reveals the existence of similar conformational substates in PS II.

At present, the simplest possible model is that PS II exists in two conformational substates and that the population distribution between these two states is static or interconvertable only on the many minutes time scale (i.e. longer than the decay time of the $S_2Q_B^-$ state). The energetics of $S_2Q_B^-$ recombination in the two putative conformations are reflected in the two first-order decay components (220 and 1000 sec) derived from Fig. 2. If the difference is due solely to a change in the activation energy for recombination,

the difference in charge-pair trap depths in the two conformations would be *ca* 40 mV, which is significant compared to the measured $\Delta E'$ for $Q_A^- Q_B \Leftrightarrow Q_A Q_B^-$ of about 80 mV [12]. Thus, we expect that about 20% of the PS II centres have a substantially more stable $S_2Q_B^-$ state and that this difference in energetics will have relatively large effects on their function *in vivo*.

Implications for interpretation of TL band properties

In principle, TL bands can reveal information about the enthalpies of activation, the intrinsic rate constants, and entropic factors for charge recombination [19]. However, previous attempts by other groups to quantify thermodynamic parameters solely from the shapes of TL bands have been only partially successful, probably due to the complexity of the electron transfer reactions involved [15, 18, 19]. The technique presented here provides one method of deriving quantitative data from TL curves. It also allows the resolution of TL bands into components representing distinct substates of PS II. Shifts observed in TL bands have in the past been attributed to changes in the midpoint potentials of PS II electron donors and acceptors. However, our results show that such shifts may alternatively represent interconversion of substates or preferential depletion of the charge-separated state from one or more of the subpopulations. It is thus apparent that great care must be taken in interpreting such TL data, but that careful analysis may reveal important new information.

Possible physiological implications

One of the remarkable features of the observed PS II heterogeneity is that it persists at physiological temperatures and over long time scales (several to many minutes) and is thus likely to be relevant to the *in vivo* function of PS II. The many observations that PS II is functionally heterogeneous are thus relevant, and several possible sources of heterogeneity should be considered, including PS II centres in various stages of assembly or disassembly, spatially-distinct populations of PS II centres (i.e. in the stroma and grana), PS II α- and β-centres (that appear to differ in their associated antenna and electron transfer properties) and non-B centres (that are incapable of reducing Q_B) [43]. It is noteworthy that TL bands have not yet been associated with any of these alternate forms of PS II and we suggest that further investigation of the present phenomenon may yield critical clues as to the energetic basis of these physiologically-important phenomena.

EXPERIMENTAL

Preparation of thylakoids. All experiments were performed on *Spinacia oleracia* (spinach) thylakoids prepd as in [44] and resuspended to a concn of 2.5 mg chlorophyll ml^{-1} in media containing 330 mM sorbitol, 10 mM KCl, 1 mM ethylenediaminetetraacetic acid (EDTA), 5 mM $MgCl_2$ and 50 mM *N*-[2-hydroxyethel]piperazine-*N'*-[2-ethanesulfonic] acid (HEPES) buffer at pH 7.3.

Construction of the TL photometer. Typical TL experiments begin with sample illumination by flashes or constant light followed by trapping of the excited, or charge-sepd, states by cooling. Samples are then heated at constant rates in the dark and luminescence is measured by a photodetector. Conventional instruments, such as that described by Ichikawa [45] require the user to transfer illuminated samples to a liquid N_2 bath for cooling. This does not allow the precise and rapid timing between illumination and cooling events required for the type of experiments performed here. We have therefore developed a new photometer in which both rapid cooling and reheating of the sample are under precise computer control.

Figure 7 is a schematic diagram of the present instrument. The temp. of the sample is controlled using a solid-state (Peltier-effect) heat pump. The heat pump can act both to cool and heat the sample and can generate maximum temp. differences of *ca* −30° and 200° in the cooling and heating modes, respectively. One side of the heat pump is in contact with a copper bar which functions as a heat sink and is immersed in a cooling liquid (usually liquid N_2 or ice H_2O, depending upon experimental requirements) contained in an insulated dewar. The other side is in contact with the sample holder. Chloroplast samples (usually 50 μl) are introduced into the instrument on a 1 cm × 1 cm square sheet of fine serigraphy nylon mesh (20 holes per cm, 0.02 mm thickness). The nylon mesh is in contact with a thin layer of aluminium foil (0.0125 mm thickness) attached to the exposed side of the heat pump by an aluminium oxide heat conducting paste (Wakefield Engineering). The nylon mesh acts to spread the sample evenly over the foil surface by capillary action (very thin samples are required for rapid and even temp. control). The temp. of the sample is measured by a narrow (0.2 mm) diameter K-type thermocouple junction held in place between the aluminium foil layer and the ceramic surface of the heat pump. The thermocouple voltage is linearized by an integrated circuit and the temp. value is digitized by the control computer (a 486 processor-based personal computer). Temp. regulation is achieved by feed regulating of the direction of current flow to the heat pump. A temp. set point is output by the control computer by a digital-to-analogue converter on an analogue input–output extension card in the control computer. An analogue comparator is then used to determine whether the measured temperature is higher or lower than the set temp. The output of the comparator then controls a set of power transistors that allow control the direction of current flow through the heat pump. The heat pump can accurately, precisely, and rapidly regulate the temp. of the sample. Surface temps, measured with an independent thermocouple junction in contact with the liquid sample, were ±0.1°

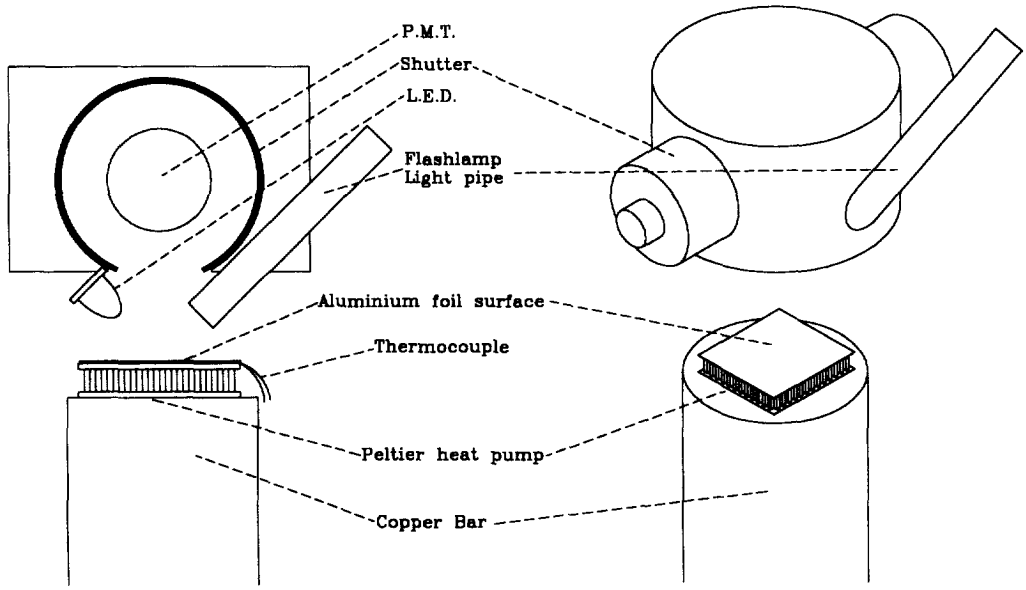

Fig. 7. Schematic diagram of the thermoluminescence photometer used in these studies. See the Experimental section for construction details.

from the set temp. with variation across the surface of *ca* $\pm 0.1°$. Cooling rates with liquid N_2 as the cooling substrate were faster than $10°\ sec^{-1}$ in the temp. range between $+30$ and $-20°$, but slowed to *ca* 3° sec as the temp. approached $-50°$. Maximum heating rates were significantly more rapid. In conventional instruments, since heat is added to the sample at a constant rate, discontinuities in the both the kinetics of temp. change and TL intensity are normally observed at the ice-water phase transition. However, in the present instrument, since the sample temp.is directly regulated, this artefact is largely eliminated. Thermoluminescence is measured during the heating ramp by a near infrared-sensitive photomultiplier (PMT, R446UR, Hamamatsu). To achieve high signal-to-noise ratios, the PMT was placed within 1.5 cm of the sample, allowing high collection of the (diffuse) luminescence. The PMT was protected from actinic illumination by a home-built cylindrical shutter that fits as a sleeve around the PMT housing. Rotation of the sleeve shutter closes or exposes the PMT to the sample. To prevent temp. changes to the detector caused by the heat pump or condensation of sample liquid, the PMT was covered by a layer of clear plastic (cut from a Petri plate). The output of the PMT is converted into a current by a low-noise amplifier with variable gain and a time constant of 100 msec. The outputs of the PMT amplifier and the sample temp. were continuously digitized by a 12-bit resolution analogue-to-digital converter on an analogue input–output extension card in the control computer. Data was accumulated continuously and stored for later manipulation. Flash actinic illumination was provided by a pulsed xenon (FX801, EG&G) lamp powered by discharge of a 1 μF capacitor charged to between 300 and 1500 V. The half time for the flash discharge was *ca* 3.5 μs. The timing of xenon discharges was computer controlled via the analogue input–output extension card, allowing precise delay times between illumination and initiation of TL experiments. More details and a set of schematic and mechanical diagrams are available from the authors.

Thermoluminescence experiments. For the experiments performed here an ice-water bath was used as the cooling medium for the copper bar, allowing rapid cooling to *ca* $-20°$. The sample holder temp. was set to 15° and chloroplast samples (50μl of a 2.5 mg ml^{-1} chlorophyll suspension) in the presence of 1 μM gramicidin D were placed in the photometer as described above. In most experiments samples were dark-adapted in the TL sample holder for 5 min at 15°, illuminated by a saturating single-turnover flash and again dark adapted for 5 min before proceeding with the TL experiment. This dark adaptation was performed to coordinate the PS II O_2 evolving complexes into the S_1 state (see above). The heating rate was set to $0.5°\ sec^{-1}$ and incremented by the program at 1 sec intervals. A fresh, dark-adapted thylakoid sample was used for each TL trace. The peak temp. of the TL curves were estimated by taking the average of the half height temps.

Fluorescence decay kinetics. A microsecond time-resolution pulse-probe kinetic fluorimeter, based on that of Kramer *et al.* [46, 47], was used. Thylakoid samples were diluted to a concn of 5 μg chlorophyll ml^{-1} in resuspension buffer (above) with 1 μM gramicidin. 10 μM *p*-benzoquinone was added to set the redox state of PS II so that all Q_B would be in the quinone form. Samples were dark adapted for a minimum of 5 min, followed by a series of single-turnover

actinic flashes at 300 msec intervals, provided by a pulsed xenon flashlamp (halftime for flash discharge of *ca* 3 μsec). Each actinic flash was followed by a series of weak probe pulses from a series of light-emitting diodes (LEDs, peak emission at 640 nm, pulse half width of 2 μsec), exciting *ca* 0.1% of centres per pulse. The fluorescence emission upon each LED pulse was measured by a photodiode detector protected by a glass colour filter which transmits light above 695 nm.

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