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The A_T thermoluminescence band from *Chlamydomonas reinhardtii* and the effects of mutagenesis of histidine residues on the donor side of the Photosystem II D1 polypeptide

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Abstract

A_T thermoluminescence bands were measured from thylakoids and Photosystem II (PS II) particles of wild-type (WT) and site-directed mutants of residues 195 (H195) and 190 (H190) of the PS II D1 polypeptide of *Chlamydomonas reinhardtii.* In wild type thylakoids the peak temperature of the A_T band (pH 6.5) was at -16.2 ± 0.1°C. Maximal thermoluminescence yield was achieved at an illumination temperature of approx. −20°C, as previously observed in spinach PS II particles by Ono and Inoue (FEBS Lett. 278 (1991) 183–186). In contrast to previous observations, we found only a weak pH-dependence of the A_T band intensity, from pH 5.5 to 8 in thylakoids or PS II particles from *C. reinhardtii* or spinach. Conversion of H195 of the D1 polypeptide of PS II to asparagine (H195N), tyrosine (H195Y), or aspartate (H195D) decreased the amplitudes of the A_T bands to approx. 65, 53 and 38% of that of wild type, but did not significantly alter the peak temperatures of the bands. The residual A_T bands in the H195 mutant strains showed illumination temperature- and flash number-dependencies, and pH dependencies similar to those of wild type. Therefore, we suggest that the quantum yield of the luminescence, rather than the energetics of the recombinatory pathway, was modified in the H195 mutants. In other experiments (Roffey, R.A., Kramer, D.M., Govindjee, and Sayre, R.T. (1994) Biochim. Biophys. Acta, in press), we concluded that the equilibrium constant between $Y_Z P^+$ and $Y_Z^+ P$ in Mn-depleted material was dramatically shifted (about 50-fold) in the H195D mutant relative to WT. Since we found no effect of the H195 mutation on the peak temperature of the A_T band, we concluded that Y_Z is probably not the trap for the positive charge involved in the A_T band recombination, in agreement with the conclusion of Ono and Inoue (FEBS Lett. 278 (1991) 183–186). We also conclude that H195 cannot be the redox group responsible for carrying the positive charge for the A_T band recombination. Modification of H190 to phenylalanine completely abolished the A_T band, but also severely affected PS II donor reactions by slowing the electron transfer from tyrosine Y_Z to the oxidized primary donor P_{680}^+ by approx. 100- to 1000-fold. It was not possible to determine whether the loss of the A_T band upon H190 modification was due to the removal of a redox-active histidine or to the secondary effects the modification had on electron transfer reactions. We present a kinetic model that explains many of the data on the A_T band.

Key words: Photosynthesis; Photosystem II; Thermoluminescence; Oxygen evolution; Mutagenesis

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Abbreviations: A_D, a putative alternate electron donor to the oxidized primary electron donor of Photosystem II; Chl, chlorophyll; DCIP, 2,6-dichlorophenolindophenol; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; E_m, the electrochemical midpoint potential; E_{act}, the activation energy for a particular reaction; EPR, electron paramagnetic resonance; H195D, H195N, and H195Y mutant strains of *Chlamydomonas reinhardtii* with aspartate, asparagine and tyrosine, respectively, in place of histidine at position 195 of the D1 polypeptide of Photosystem II; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid; His, histidine; I and I^-, the oxidized and reduced forms of the pheophytin electron acceptor in Photosystem II; Mes, 4-morpholineethanesulphonic acid; P and P^+, the reduced and oxidized forms of the primary chlorophyll donor of Photosystem II; PS II, Photosystem II; Q_A, primary quinone electron acceptor of Photosystem II; Q_B, secondary quinone electron acceptor of Photosystem II; TL, thermoluminescence; Tris, 2-(*N*-morpholino)ethanesulfonic acid; Y_D and Y_Z, endogenous electron donors to P^+, residues tyrosine 160 on the D2 polypeptide and tyrosine 161 on the D1 polypeptide, respectively.

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1. Introduction

The consensus view of electron transfer on the donor side of Photosystem II (PS II) is that, after photo-induced oxidation, the oxidized form of the primary chlorophyll electron donor, P_{680}^{+}, is reduced by a redox active tyrosine residue (Y_{161} on the D1 polypeptide, termed Y_Z), which is, in turn, reduced by the water oxidizing manganese cluster of the oxygen evolving complex (for a review, see [1]). Under special conditions, such as after prolonged dark-adaptation or at low temperatures, the participation of other redox components on the donor side of PS II has been observed. These include the C2-symmetry related tyrosine residue on the D2 polypeptide (Y_{160}, or Y_D), cytochrome *b*-559, and a chlorophyll molecule [2–5]. A histidine (His) residue has also been proposed as a redox-active ligand to the Mn cluster [6]. Support for this suggestion has recently come from electron paramagnetic resonance (EPR) and thermoluminescence (TL) studies, when the normal electron transfer pathway from the Mn cluster to P_{680}^{+} is disrupted by Ca^{2+} depletion from PS II particles [7–9]. It has been suggested that signals from the putative redox-active His residue are only observed in inhibited preparations because, in the intact system, the redox state of the His ligand is expected to be in equilibrium with that of the Mn cluster, and any redox changes are likely to be rapid and transient [6,7].

Thermoluminescence has long been used as a probe of donor and acceptor chemistry in photosynthetic materials (for reviews, see [10–12]), and individual TL bands have been attributed to recombination between electrons trapped on the acceptor side and positive charges trapped on the donor side of PS II. When the Mn cluster of PS II is destroyed (e.g., by washing with high concentrations of Tris), a TL band, termed the A_T band, with peak intensity at around −20°C, can be observed [13]. Ono and Inoue [19] have suggested that the A_T band is due to recombination between an electron on the reduced primary quinone acceptor, Q_A^-, and a positive charge located on a redox-active histidine residue on the donor side of PS II. Ono [20] suggested that this His residue may be the same as that proposed to explain results in Ca^{2+}-depleted PS II particles (see above). Ono suggested that the A_T band His is located close to the Mn cluster and plays an important role in the primary step of photoligation of Mn^{2+} to form the active oxygen evolving complex, by functioning as a redox mediator between P_{680} and exogenous Mn^{2+}. It was further suggested that, in PS II centers depleted of Mn, destruction of the A_T band His is the primary step in photoinhibition. However, these views are not universally accepted, and the assignment of the A_T band components remains controversial (see [1]).

Models of the folding of the D1 polypeptide (e.g., [14–16]) suggest that several His residues are near the special chlorophyll pair and the redox active Y_Z residue and these might be candidates for the A_T band trap. In this work, we have studied the TL properties of *Chlamydomonas reinhardtii*, with site-directed mutations to two of these His residues, H190 and H195 of the D1 polypeptide, with the aim of addressing the assignment of the components involved.

2. Materials and methods

C. reinhardtii strains and site-directed mutagenesis, culture conditions, thylakoid and PS II particle preparation, were as in Roffey et al. [17]. Tris-washing was performed by suspending thylakoids or PS II particles in 0.8 M Tris at pH 8.0 for 40 min on ice in darkness or in subdued room light. Hydroxylamine washing was performed in suspension buffer at pH 6.5 with 2 mM hydroxylamine for 30 min in complete darkness on ice. The Mn extracted preparations were then pelleted at $10\,000 \times g$ for 3 min and re-suspended in suspension buffer (0.3 M sorbitol, 50 mM HEPES, 50 mM Mes, 5 mM $MgCl_2$) at the desired pH (5.0 to 8.5). The centrifugation and re-suspension steps were repeated three times to remove residual Tris or hydroxylamine. The hydroxylamine treatment removed essentially all of the Mn clusters in both thylakoid and PS II particle preparations, as judged by DCIP reduction in the absence of artificial donors, and by the absence of residual B or Q (in the presence of DCMU) thermoluminescence bands (data not shown). Tris treatments of the PS II particle preparations were likewise effective. However, small residual light-induced DCIP reduction rates and Q and B TL bands were observed after Tris treatment of thylakoid preparations. The residual activities could be destroyed by a prior wash with 20 mM NaCl in suspension buffer (pH 7.0), followed by the normal Tris washing. Hydroxylamine washing was found to give more consistent results and so was used for the majority of the experiments.

The TL photometer was constructed in-house. Temperature control was provided by a solid-state heat pump (Peltier cooler, CP1.0–127–06L, Melcor, Trenton, NJ) with the electrical connection side in contact with a copper bar, immersed in liquid nitrogen. Temperature regulation was accomplished by electronically comparing the temperature of the sample, measured via a thermocouple, to the desired temperature, and changing the direction of current flow in the solid state heat pump to either raise or lower the surface temperature to match the set temperature. The desired temperature was set by a digital-to-analog converter on a Data Acquisition and Control Adapter (IBM), controlled from a micro-computer (PC-AT, IBM). The

temperature regulation was approx. ±0.1°C, the maximal cooling rate was >5°C/s and the heating rate could be controlled from 0.01 to above 20°C/sec. Actinic illumination was also computer-controlled. Flash illumination was provided by a saturating single-turnover xenon flashlamp (approx. 3–4 μs duration at half intensity). Constant illumination was provided by a ring of six red light-emitting diodes (LED's, UR3000, Marktek, Inc. 665 nm peak emission, providing approx. 75 $\mu E m^{-2} s^{-1}$) mounted inside the TL photometer housing. Luminescence was detected by a photomultiplier tube (EMI 9592 B with S10 photocathode), mounted 2 cm from the sample surface, protected from actinic illumination by a mechanical shutter. Current from the photomultiplier was converted to a voltage and amplified by an operational amplifier (OPA-111, Burr-Brown) and the output signal was digitized to 12 bits resolution by a Data Acquisition and Control Adapter (IBM). The data was then stored and manipulated using computer programs developed in-house.

For TL curves, 10 μl aliquots of dark-adapted thylakoid or PS II particle suspensions (1 mg/ml chlorophyll) in suspension buffer, were absorbed onto 1.5 cm^2 lens paper (Kodak) strips. Alternatively, Whatman filter paper or Miracloth (Calbiochem) strips were used, though the thickness of the former required that much larger sample volumes be used, and Miracloth was found to emit thermoluminescence at temperatures above about + 40°C. The paper strips were placed in contact with the Peltier cooler surface, and dark-adapted for 120 s at 25°C. The temperature was decreased to −20°C, and the sample was illuminated with red light (75 $\mu E m^{-2} s^{-1}$) for 30 s or with a variable number of saturating single-turnover actinic flashes, given at 300 ms intervals. Immediately after illumination, the temperature was rapidly decreased to −50°C to trap the charge-separated state. The TL curves were recorded from −50 to +30°C at a heating rate of 0.7°C s^{-1}. Curves were smoothed by a 'box-car' algorithm, averaging over ±1°C.

The rise and decay of chlorophyll *a* fluorescence on the microseconds time scale after single-turnover actinic illumination was measured on a laboratory version of the instrument described by Kramer et al. [18] and was constructed in house (Kramer, D.M., Kuo, T-C. and Crofts, A.R., unpublished). Thylakoid or PS II particle samples at 5 mg chlorophyll/ml in suspension buffer at pH 7.5 were used.

3. Results

The A_T thermoluminescence in wild-type C. reinhardtii and Spinach thylakoids and PS II preparations

In order to establish baseline parameters, we compared A_T bands from various preparations of wild-type *C. reinhardtii* and spinach. Very similar data were obtained for thylakoids and BBY-type PS II particle preparations for both species (for preparation details, see Roffey et al. [17]), except that the intensities of the bands emitted from samples of equal chlorophyll concentration were higher in the PS II particle preparations (data not shown) than in thylakoids. This is to be expected, since the number of PS II centers per unit chlorophyll is higher in the PS II particle preparations. We show here only data obtained with thylakoids, on which we performed more detailed studies.

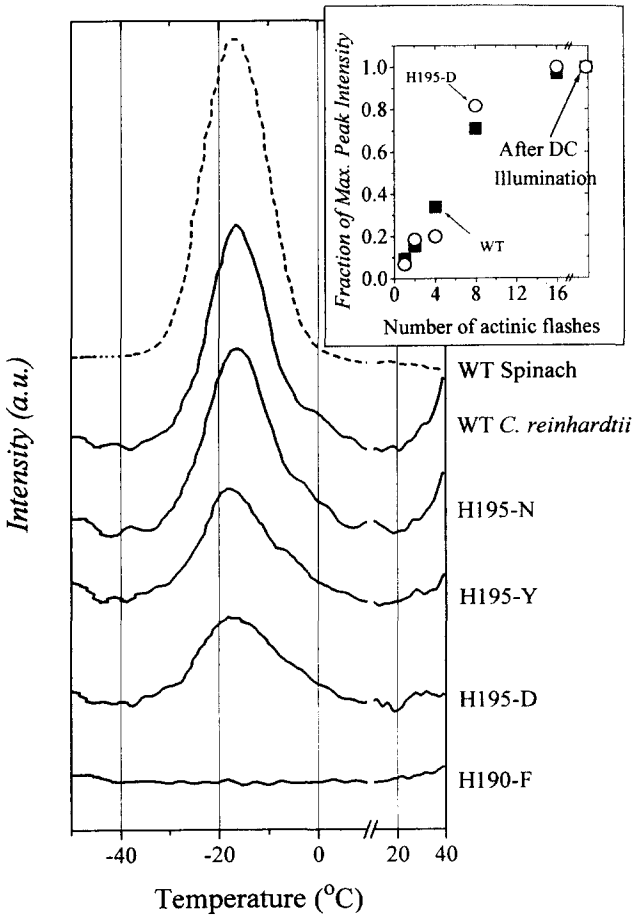


Fig. 1. A_T Thermoluminescence bands from thylakoids of spinach and wild-type and mutant strains of *Chlamydomonas reinhardtii*. The bands were recorded as in Materials and methods. The curves were offset in the Y-axis direction for clarity. The labels to the right of the curves indicate the type of material used in each experiment, from top to bottom: wild-type Spinach; wild-type *Chlamydomonas reinhardtii*; and the mutants: H195N, H195Y, H195D are *Chlamydomonas reinhardtii* thylakoids with the His residue at position 195 of the D1 polypeptide changed to asparagine, tyrosine, and aspartate, respectively. H190F is *Chlamydomonas reinhardtii* thylakoids with the His residue at position 190 of the D1 polypeptide changed to phenylalanine. Inset: The flash-number dependence of the peak amplitude of the A_T band in WT and H195D mutant of *C. reinhardtii*. Peak intensities of A_T TL bands were recorded as above, but after various numbers of saturating, single-turnover actinic flashes (given at 300 ms intervals) instead of continuous illumination.

The TL curves obtained from hydroxylamine-washed thylakoids of spinach and wild-type *C. reinhardtii* are shown in Fig. 1. The peak height and shape of the spinach band was found to be very similar to those reported earlier (e.g., [9,19,20]). The band from *C. reinhardtii* had a peak temperature and band width similar to those of spinach, but had an overall intensity somewhat lower on an equal chlorophyll basis. The relative intensity of the bands as a function of the number of actinic flashes (given at −20°C) was similar for *C. reinhardtii* and spinach thylakoids (the data for *C. reinhardtii* is shown in the inset to Fig. 1, see below). When flashes were given at 300 ms intervals, half-maximal TL intensity was reached after about 4–8 flashes. Increasing or decreasing the power of the actinic flash by approx. 20% had little effect on the saturation behavior. Furthermore, one actinic flash was found to populate approx. 90% of the Q-band (in the presence of DCMU and in thylakoids with intact oxygen evolving systems) leading us to conclude that the flashes we used were close to saturating. Illumination with DC light (30 s at 75 μE m^{-2} s^{-1}, see Materials and Methods) resulted in only a small increase in band intensity. Qualitatively, the saturation behavior we observed was somewhat different from that reported by Ono and Inoue [19], who found that, after 4 actinic flashes, a plateau of band intensity was reached, which represented about 30% of the intensity that was reached after continuous illumination. This discrepancy may be due to differences in illumination, freezing or heating protocols.

The temperature at which the band was most efficiently charged was found to be approx. −20°C and dropped to half intensity at −40 and −5°C for both spinach and *C. reinhardtii* (data not shown), resulting in an asymmetrical bell-shaped curve with a somewhat longer tail towards lower temperatures, similar to that found by Ono (c.f. Fig. 1 of [20]). In preliminary experiments, we found that the band could be nearly fully charged at lower temperatures (e.g., in the region of the low temperature tail of the curve, from −30 to −45°C), but only after extended illumination of several minutes. This suggests that the temperature-dependence is likely the results of two opposing limitations to trapping. It is possible that at higher temperatures, the band can be populated at a relatively high quantum yield but decays before the temperature can be lowered sufficiently to prevent recombination (but see Discussion). At lower temperatures, the quantum yield for population of the band is low, presumably because other processes, such as recombination, are more rapid and out compete the population of the band.

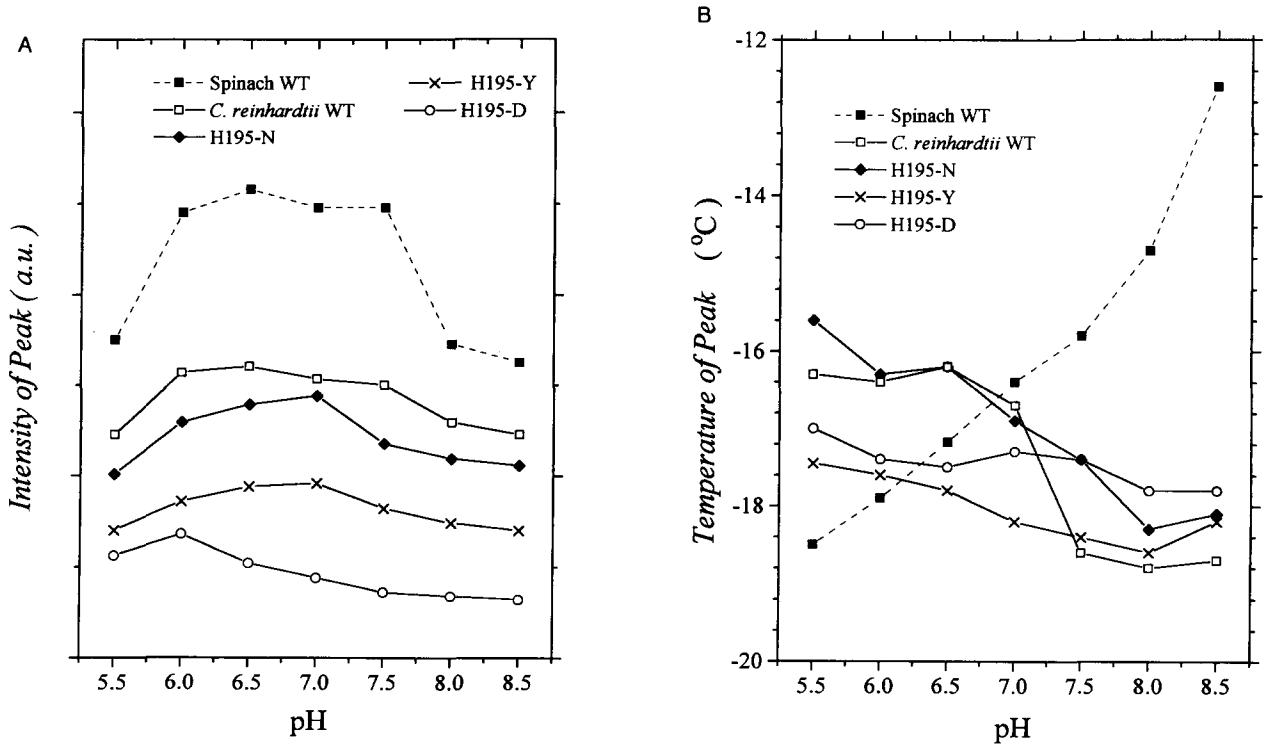


Fig. 2. The pH-dependencies of the A_T band peak intensities and temperatures. Thermoluminescence bands were recorded as in Fig. 1 at a range of pH values from 5.5 to 8.5. (A) and (B) show the pH-dependencies of the peak intensities and peak temperatures, respectively. Thermoluminescence bands were recorded as in Fig. 1. Note that there was a ±0.1°C uncertainty in the temperature regulation (see Materials and Methods).

The A_T bands from mutant strains of C. reinhardtii with modified H195 and H190 residues

Fig. 1 also shows A_T bands, at pH 6.5, from mutant strains of *C. reinhardtii* with modified H195 and H190 residues. We observed bands attributable to the A_T recombination for all three mutants of H195, but were unable to detect a band from the H190 mutant under a wide range of experimental conditions, which included varying the pH from 5.5 to 8.5, the illumination temperature from +20 to −55°C, and the amount of actinic illumination from 1 to 60 s of continuous light (see Materials and Methods) and from 10 to 100 actinic flashes. We note that the number of PS II centers was found to be essentially the same in the mutants and wild-type preparations [27].

The peak TL intensities of the A_T bands in the H195N, H195Y, and H195D mutants were 0.63, 0.53, and 0.38 that of the wild type band, respectively. Although the temperature of the peaks, as well as other properties of the bands were consistent from preparation to preparation, there was, at times, considerable variation in the relative peak amplitudes of the H195Y and H195D mutants with different sample preparations. This was particularly evident in our preliminary experiments, where Tris-washing was used to deplete PS II of Mn. As mentioned above (see Materials and methods), this method was found to be unsuitable for thylakoids from *C. reinhardtii*, since the degree of Mn depletion appeared incomplete and variable. We were able to obtain more consistent results with hydroxylamine-washing, as described in Materials and Methods. Mutation of H195 had only minor effects on the peak temperatures and bandwidths of the A_T bands at pH 6.5. Virtually no change was observed for the H195N mutation, and an approx. 1–2°C lowering of the peak was observed for the H195Y and H195D mutations.

The pH-dependence of the A_T bands

The pH-dependencies for the peak intensities and temperatures of the A_T bands are shown in Figs. 2a and 2b. We found only weak pH-dependencies of the A_T band intensities (Fig. 2a) for both spinach and *C. reinhardtii*. The peak intensity appeared at between pH 6.5–7 for spinach and WT and H195N and H195Y mutants of *C. reinhardtii*. That of the H195D mutant appeared to be shifted to approx. pH 6. There were only small shifts in the temperatures of the peaks with pH (Fig. 2b), especially when compared to the relatively larger shifts observed for the Q and B TL bands (e.g., [21]). For spinach, we found a 6°C increase in the peak temperature as the pH was raised from pH 5.5 to 8.5. On the other hand, there were small *decreases* (about 2°C) in peak temperature over the same pH range for WT and H195N *C. reinhardtii*. The pH-dependencies of the H195D and H195Y TL peak temperatures were flat within the noise level.

It is possible that differences in sample preparation or treatment could have altered the pH-dependence of the luminescence quantum yield and could have yielded the differences between our data and that of Ono [20]. We note, however, that, in preliminary experiments, we found pH-dependencies similar to those shown here for a variety of sample preparations, which included thylakoids and BBY-type PS II particles, both hydroxylamine- and Tris-washed (prepared as in Roffey et al. [17]). We also tested the possibility that a pH-dependent degradation of the PS II centers could have led to the differences in results. After incubating thylakoid preparations in the dark on ice for extended periods of time at a range of pH's, we found that the degradation rate was only moderately pH-dependent and resulted in preferential degradation at alkaline, rather than acid, pH's. After a 12 h incubation, the signal of the pH 8.5 samples had diminished about 50%, whereas that from the samples incubated at pH 5.5 to 7.5 had only diminished by about 20–30%.

4. Discussion

Thermoluminescence from PS II arises from the recombination of positive charges trapped on the donor side with negative charges on the acceptor side in such a way that the excited singlet state of the primary chlorophyll donor, P*, is an intermediate. Though specific traps have been associated with many individual TL bands (for reviews, see [10–12]), those for the A_T band have yet to be assigned. For the A_T band, the trap for the negative charge on the acceptor side is most probably Q_A since addition of DCMU did not inhibit the charging of the band [20]. The assignment of the component responsible for trapping the positive charge is presently the subject of debate. Ono and Inoue [19] have suggested that the trap for the positive charge is a redox-active His residue on the donor side of PS II. They derive support for this assignment from a correlation between the disappearance of the A_T band and the modification of the PS II protein by diethylpyrocarbonate which selectively modifies His [19]. They also point out that their hypothesis is in agreement with suggestions from other groups (e.g., [6–9]) that a His may participate, at least under some conditions, in donor side redox reactions.

As pointed out by Debus [1], the results of Ono [20] do not rule out the possibility that modification of His residues affects A_T band formation through secondary effects. Therefore, the participation of other redox components in A_T band formation cannot be excluded. Debus points out that no EPR or optical signal has yet been shown to be associated with the oxidation of the

putative A_T band His radical. Furthermore, in Mn-depleted PS II preparations, there appears to be only one efficient and rapid donor to P^+, not two as required by the His donor model (but see below), and that this rapid donor has been identified as Y_Z. Debus further suggests that Y_Z would be a more appropriate candidate for the trap and speculates that modification of His by DEPC could have affected the disappearance of the band by structural modification or by removal of hydrogen bond to Y_Z (e.g., from the nearby H190 residue). Ono [20], however, argues that the temperature dependence of formation of the A_T band and signal II_f, excludes the participation of Y_Z in the band. The efficiency of the band formation showed a maximum at around −20°C and dropped off to less than 10% of maximum at 0 and −60°C, whereas the temperature-dependence of II_f was flat above −30° but dropped below −30°. On the contrary, we argue that these temperature-dependencies could be consistent with the participation of Y_Z in the band. The efficiency of populating the band will be a function of the competition between the formation of the charged pair and its destruction through recombination or side-reaction. At high temperatures, formation of the charged pair may have a high quantum efficiency, but the band is likely depleted before the stabilization of the state at low temperature can be accomplished. At low temperatures, the efficiency of formation of the charged pair could be inefficient for kinetic reasons and extended illumination would be required to populate the band.

The crucial question posed by the arguments of Debus [1] is: if we accept that an electron donor other than Y_Z is responsible for the A_T band (e.g., a His residue), should we be able to detect this component by measuring the reduction kinetics of P^+, the oxidation kinetics of Y_Z, or the kinetics of recombination from $Y_Z^+Q_A^-$? Debus argues that the preponderance of data in the literature demands that the efficiency of such an alternate donor would have to be very low. Since the flash-number dependence of A_T band formation reported by Ono and Inoue [19] appears to be high, such a component should have been detected earlier by independent methods. Debus concedes, though, that whereas all experiments demonstrating only one efficient donor to P^+ were performed at room temperature, the A_T band is most efficiently charged at −20°C. One can imagine a situation where differential temperature-dependence of electron transfer rates could result in a temperature-dependent competition between two alternate donors (see below). Furthermore, a quantitative relationship between band peak height (or integrated area under the curves) and the fraction of centers populated with a particular charged pair has by no means been established. In the experiments of Ono and Inoue [19], after one actinic flash the A_T band reached approx. 30% of the intensity achieved after DC illumination. However, the saturation intensity was only 60% of that of the Q band. In our hands, less than 10% of the saturation of the A_T band appears to be populated after one actinic flash (Fig. 1, inset), and the saturation intensity of the band is only about 40% of that of the Q band (data not shown). Though this difference may reflect sample preparation or illumination and freezing procedures, they do suggest a range of efficiencies may be achieved, depending upon experimental conditions.

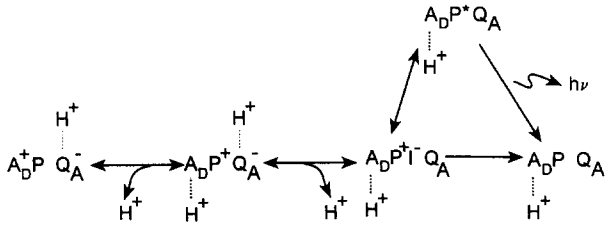
Since we observed nearly WT A_T bands in mutants of *C. reinhardtii* in which the histidine residue at position 195 in D1 has been altered (see Fig. 1), we conclude that H195 of D1 cannot participate in the A_T band recombination. The complete elimination of the A_T band in the H190F mutant is consistent with a possible role in the A_T band recombination. However, since the kinetics of electron transfer from Y_Z to P_{680}^+ and from Q_A^- to Q_B are severely affected in the H190F mutant [27], it is not possible to determine whether the lack of A_T band in this mutant is a direct effect of the removal of H190 or a secondary effect of a compromise in the structure of the complex.

As discussed in detail below, we conclude that, because mutation of H195 did not significantly alter the temperature of the A_T band, the energetics of de-trapping of the charged pair were probably not significantly altered by the mutations. This conclusion has interesting implications for the identity of the component involved in the trapping of the positive charge involved in the A_T band recombination. In parallel experiments on the H195D mutant, we concluded that, in Mn-depleted preparations, there was an approx. 50-fold shift in the equilibrium constant between $Y_ZP^+ \leftrightarrow Y_Z^+P$ with respect to WT and the other H195 mutants (Roffey, Kramer, Govindjee and Sayre, unpublished). This was reflected in an increased rate of recombination from $Y_Z^+Q_A^-$ to Y_ZQ_A, as measured by the decay of the high fluorescence state in the presence of DCMU. We attribute this shift in $Y_ZP^+ \leftrightarrow Y_Z^+P$ equilibrium to a change in the E_m of Y_Z rather in P, in part because the temperature of the A_T band was not shifted in this mutant. If the E_m of P was shifted, we would have expected that the recombination energetics involving any electron donor to P^+ should also have been affected, and therefore, we would have expected that the TL band temperature would have been shifted. By the same reasoning, we conclude that Y_Z is not likely to be the positive charge trap involved in the A_T band recombination, since if it were, the shift in the $Y_ZP^+ \leftrightarrow Y_Z^+P$ equilibrium constant induced by the H195D substitution would be expected to have also shifted the temperature of the A_T band. Instead, we suggest that the trap for the positive charge involved in the A_T band recombination, which we will call A_D, remains at about the same midpoint potential,

with respect to the P^+/P couple in the H195D mutant.

Ono and Inoue [19] have observed that the amplitude of the A_T band decreases at low pH values, suggesting that the appearance of the band was controlled by a group with a pK at about 6.2, close to that expected for a His residue; they concluded that it is the deprotonated His residue that contributes to the A_T band charged pair. However, there are two lines of argument against this interpretation. First, we observed only weak pH-dependencies of the A_T band amplitude in both the WT and mutant strains of *C. reinhardtii* (Fig. 2a). The differences were not due to species, the choice of thylakoids or PS II preparation, or to a pH-dependent degradation of the sample (see Results). Secondly, the variations in amplitude were not accompanied by significant changes in the peak temperature of the band, as would be expected with a change in the energetics of the positive charge trap implied by the involvement of a proton (see below for further discussion of this point).

Besides the relatively weak pH-effects on the peak temperatures of the A_T bands, especially in the H195Y and H195D strains of *C. reinhardtii*, when compared to the relatively larger dependencies of the Q and B bands (e.g., [21]) we also note that the slopes of the pH dependencies of the bands from spinach and *C. reinhardtii* had opposite signs. These phenomena can be understood by considering the reactions in Scheme 1. Recombination involves the oxidation of P by A_D^+ to form P^+ and an oxidation of Q_A^- by P^+ to form P*, followed by its decay with the emission of a photon (i.e., fluorescence or luminescence). In competition with the above, recombination also likely occurs by a non-radiative pathway, possibly involving P^+I^- as the activated intermediate (see [24,25]). To explain the weak pH-dependencies of the A_T bands, we suggest the following two alternate possibilities: 1) no net exchange of protons occurs during the oxidation of P by A_D^+ or during the oxidation of Q_A^- by P^+ (or, if the non-radiative pathway is considered, the oxidation of Q_A^- by I), and 2) an approximately equivalent number of protons are released in one of the steps and rebound during the other. In the latter case, raising the pH would stabilize the positive charge on component A_D^+, which would tend to raise the temperature of the band. This would be offset by a destabilization of the electron on Q_A^-, which would tend to lower the temperature of the band. Small pH dependencies of either sign would be expected if one or more of the protonatable groups had a pK near the pH range used in the experiments, since the difference in proton release on the donor side and binding on the acceptor side could then be fractional. However, experiments to measure the proton binding associated with Q_A reduction [22], the pH-dependence of $Q_A Q_B$ equilibrium [23], and the pH-dependence of the Q TL band [21] are all consistent with a lack of significant protonation associated with Q_A reduction. We suggest that case 1 is a reasonable approximation, but that there is some evidence that weak protonation and deprotonation reactions occur at the acceptor (i.e., Q_A) and donor (i.e., component A_D) sides.



Scheme 1. Diagram of possible protonation and electron transfer reactions involved in the decay of the A_T thermoluminescence band. A_D refers to the 'alternate electron donor' to the primary chlorophyll donor, designated P (see text). Q_A is the primary quinone acceptor of PS II. Recombination occurs in three steps, the reduction of A_D^+ by P, the oxidation of Q_A by the pheophytin, followed by recombination by two alternate competing pathways, one through the first excited singlet of P, P*, resulting in thermoluminescence, and another bypassing P*, resulting in non-radiative decay. To explain our data, we suggest that two weakly pH-dependent equilibria are involved in the recombination reaction. A net binding of a proton to one or more amino acid residues near A_D shifts the equilibrium between $A_D P^+$ and $A_D^+ P$ in favor of $A_D^+ P$, causing the trapped charged pair to be stabilized at low pH's. This is mostly offset by proton binding associated with the reduction of Q_A, which tends to destabilize the charged pair at lower pH's. Both of these effects are expected to be quite small (see text).

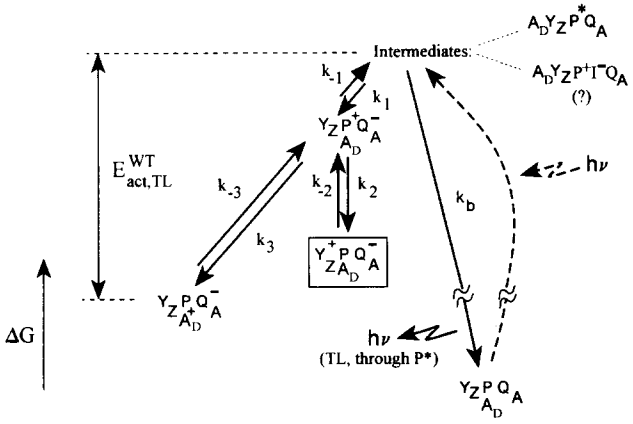
In at least two types of treatments – when the H195 residue was mutated, and when the pH was changed – there was a change in band amplitude with little or no accompanying changes in other band parameters (e.g., temperature, band width, illumination-dependence). Similar phenomena can be seen in previous works, for example see Fig. 1, traces b and c of Vass and Inoue [21], where, upon raising the pH, the amplitude of the B-band was reduced by a large amount with only very small changes in the temperature of the band. The lack of effect on the band temperature leads us to conclude the amplitude changes were not caused by alterations in the energetics of the trapped charge pair, but by alterations in other properties of the system. This is because the temperature and shape of a TL band are determined by the height of the activation barrier, the probability of recombination once the energetic barrier is overcome, and the kinetics of trap depletion, which is affected by flux through both radiative and non-radiative recombinatory pathways (see [10–12,24,25]). The intensity of a band is also a function of these properties, but is, in addition, affected by the yield of charge pair trapping during excitation and the quantum yield of luminescence from the decay of P*. The yield of trapping is determined by the competition

between the formation of charged pairs and their recombination at the illumination temperature and before freezing is completed, and is therefore also determined by the energetics of the recombinatory pathway. A change in band intensity can arise from: 1) a change in the energetics of trapping of charged pairs, 2) a change in the flux through radiative or non-radiative pathways, 3) a change in the number of centers which are active in band trapping, or 4) by a change in the yield of luminescence from P* decay. We expect that 1 and 2 would also give rise to changes in the peak temperatures and shapes of the band.

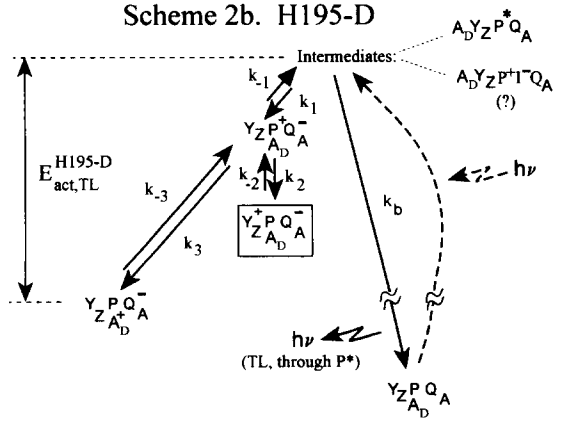
Changes in several properties could conceivably give rise to changes in the number of centers active in A_T band trapping. These include alterations in the ratio of PS II: chlorophyll, changes in the saturation behavior of the band, an increased rate of degradation of PS II centers (or more specifically, an increased rate of degradation of the A_T band components), and an increased sensitivity to photoinhibition. The ratio of PS II centers to chlorophyll content was assayed (see Roffey et al. [17]) and found to be similar in WT and H195 mutants). The flash-number dependence for population of the WT and mutant bands were found to be nearly identical – between 4–8 actinic flashes were required to achieve half of the DC light-induced signal (see Fig. 1, inset). Since PS II preparations with inhibited donor systems are known to be exceedingly sensitive to photoinhibition (e.g., [26]), we tested the possibility that the decreased A_T band intensities in the mutants was caused by increased sensitivities to the actinic illumination. Pre-illumination for up to 60 s with the light used in the TL experiments (75 $\mu E m^{-2} s^{-1}$ red light, see Materials and Methods) at room temperature followed by a two minute dark-adaptation, prior to the normal A_T band experiment, had small and similar effects on the amplitude of A_T bands in WT or H195 mutants (data not shown). This means that photoinhibition of the samples during the TL pre-illumination procedure was probably negligible. We did, however, note that the mutants had lower yields of variable chlorophyll fluorescence [27], and suggest that a change in the yield of luminescence may be responsible for the variation in band intensity.

In order to explain the data we obtained with WT and mutant *C. reinhardtii*, we introduce the kinetic

Scheme 2a. WT



Scheme 2b. H195-D



Scheme 2. A working model for the trapping and emission of the A_T thermoluminescence band. (a) and (b) represent the reactions involved in the WT and H195D mutant bands, respectively. The vertical scale is an arbitrary energy scale. The component A_D is a putative 'alternate' electron donor to the oxidized primary chlorophyll donor, P^+; Y_Z is the redox-active tyrosine residue which is the physiological electron donor to P^+, and Q_A is the primary quinone acceptor of PS II. Centers in the ground state, $A_D Y_Z P Q_A$, are excited by photons to the state $A_D Y_Z P^* Q_A$. Charge separation follows, leading to the formation of the state $A_D Y_Z P^+ Q_A^-$ in a few hundred ps by the reaction governed by the rate constants k_1 and k_{-1}. Reduction of P^+ by Y_Z (i.e., rate constant, k_2) is quite rapid and the majority of centers are converted to the state $A_D Y_Z^+ P Q_A^-$ within a few ms. Centers in $A_D Y_Z^+ P Q_A^-$ are in rapid equilibrium with the state $A_D Y_Z P^+ Q_A^-$ (i.e., $K_{eq} = k_2 / k_{-2}$). $A_D Y_Z^+ P Q_A^-$ is depleted by two competing reactions. The A_T band charge trap, $A_D^+ Y_Z P Q_A^-$, is populated via the reaction controlled by k_3 and the concentration of $A_D Y_Z P^+ Q_A^-$. In competition with this, the back reaction to the ground state is controlled by the equilibrium constant defined by k_1 and k_{-1} and k_b, the intrinsic decay rate from activated intermediates to the ground state. The activated intermediates include $A_D Y_Z P^* Q_A$ (for flux through the radiative pathway), or $A_D Y_Z P^+ I^- Q_A^-$ (for flux through the non-radiative pathway) (see [24,25]). For simplicity, we have combined the intermediates for the radiative and non-radiative pathways, though it should be noted that TL is emitted only from flux through the radiative pathway. After trapping at low temperature, the A_T band luminescence is emitted by recombination from centers in the state $A_D^+ Y_Z P Q_A^-$, with an activation energy of $E_{act,WT}$. Scheme (b) depicts an energy diagram for the mutant H195D band recombination. The midpoint potential of the Y_Z^+ / Y_Z couple is higher than in the wild-type (see text). In this model, the increase in Y_Z midpoint potential accelerates the decay of the state $A_D Y_Z^+ P Q_A^-$ to both the ground state and to $A_D^+ Y_Z P Q_A^-$, but should have little effect on the energetics of the A_T band recombination, since the activation energy for recombination remains the same (i.e., $E_{act,WT} = E_{act,H195D}$).

model diagrammed in Scheme 2a and b. The criteria we used to test whether the model was adequate was that it had to explain the following experimental data. First, changing the redox properties of Y_Z – as occurred in the H195D mutant [27] – does not significantly affect the temperature of the A_T band (see Fig. 1, and discussion above). Therefore, we conclude that Y_Z is not likely to be the positive charge trap for the band, and instead introduce an alternate donor, A_D. Secondly, component A_D does not efficiently reduce P^+ at room temperature. Thirdly, although the back reaction measured from $Y_Z^+Q_A^-$ was about 50-fold more rapid in H195D [27], the flash-number dependence of A_T band charging in this mutant (Fig. 1, inset) was nearly identical to that of WT.

Scheme 2a diagrams a branched model for the electron transfer reactions involved on the donor side of hydroxylamine- or Tris-washed PS II that can account for the majority of the A_T band data. Component A_D is a direct donor to P^+ in competition with Y_Z^+ and has a midpoint potential lower than that of Y_Z. However, reduction of P^+ by A_D is much slower than that by Y_Z (i.e., k_2 is larger than k_3) so that a short time after excitation by a short flash, most centers will be in the state $A_DY_Z^+PQ_A^-$. This state will be in rapid equilibrium with the state $A_DY_ZP^+Q_A^-$. Over a longer time scale, centers will reach $A_D^+Y_ZPQ_A^-$ and $A_DY_ZPQ_A$, since these states are at lower free energy. The fraction of centers that reach the state $A_D^+Y_ZPQ_A^-$, and therefore will display the A_T band, will be determined by the competition between the reaction controlled by k_3 and recombination – determined by the equilibrium between $A_DY_Z^+PQ_A^-$ and $A_DY_ZP^*Q_A$ (i.e., by k_1 and k_{-1}), and by k_b. (Note, that again, we must consider the likelihood that recombination also occurs by a non-radiative pathway; in schemes 2a and 2b, both radiative and non-radiative pathways are combined for simplicity). At room temperature recombination is favored and little $A_D^+Y_ZPQ_A^-$ is accumulated. At −20°C, k_3 can compete more effectively with recombination, since the latter process is expected to require a higher activation enthalpy (i.e., the intermediate for recombination, $A_DY_ZP^*Q_A$, represents a state of higher enthalpy than does the intermediate between $A_DY_ZP^+Q_A^-$ and $A_D^+Y_ZPQ_A^-$) and would therefore be expected to slow more dramatically with decreasing temperature. Thus, the band can be charged at this temperature, though still with low efficiency. Since recombination between $A_D^+Y_ZPQ_A^-$ and $A_DY_ZPQ_A$ would be slow at this temperature, $A_D^+Y_ZPQ_A^-$ can be accumulated upon repetitive flashing. At temperatures lower than −50°C, the formation of both $A_DY_Z^+PQ_A^-$ and $A_D^+Y_ZPQ_A^-$ appear to be kinetically hindered (see above). It is not apparent at which step the block would be expected to occur, though such information may aid in understanding the behavior of the A_T band.

Scheme 2b is a modification of Scheme 2a incorporating the change in Y_Z redox properties induced by the H195D mutation. The fact that such a change in the redox properties of Y_Z had little effect on the A_T band temperature (Fig. 1) is accounted for in the model, since recombination between A_D^+ and Q_A^- should not be significantly affected as long as equilibrium was reached between the donor substates on the time scale of the back reaction. This is because the activation energy required to reach $A_DY_ZP^*Q_A$ from $A_D^+Y_ZPQ_A^-$ would be unaffected. It also accounts for the fact that, despite altering the recombination rate between $A_DY_Z^+PQ_A^-$ to the ground state by a large factor in the H195D mutant [27] the efficiency of A_T band charging appeared little changed (Fig. 1, inset). This is because the fraction of centers reaching the state $A_D^+Y_ZPQ_A^-$ will, as in the WT centers, be determined by the competition between k_3 and recombination; decreasing the equilibrium constant between $A_DY_Z^+PQ_A^-$ and $A_DY_ZP^+Q_A^-$ will speed the processes of A_T band charging and recombination by the same amount, leaving the degree of competition between the branches intact.

The data in this paper lead us to question a linear model, where component A_D is in the electron transfer chain between Y_Z and the Mn complex, as proposed by Ono and Inoue [19]. This is because, in any linear model where A_D reduces Y_Z, a change in the rate of recombination from $Y_Z^+Q_A^-$, as observed with the H195D mutant should also cause a large change in the efficiency of electron transfer from A_D to Y_Z and therefore a change in the efficiency of A_T band trapping.

Though Scheme 2 appears to account for the majority of the data, other models are possible. Also, we are yet unable to identify component A_D. Data from the H190F mutant is consistent with the participation of this His residue in the band, but the severe side-effects of its replacement do not allow us to make a definitive conclusion. Besides a His residue, several established donors to P^+ could be considered for this role, including residual Mn, various amino acid residues and carotenoid or chlorophyll species. Further optical and EPR measurements, conducted under experimental conditions where the formation of the A_T band is favored, may help elucidate the molecular nature of this donor.

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