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STUDIES ON THE MECHANISM OF ADRY AGENTS (AGENTS ACCELERATING THE DEACTIVATION REACTIONS OF WATER-SPLITTING ENZYME SYSTEM Y) ON THERMOLUMINESCENCE EMISSION

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The effect of 2-(3-chloro-4-trifluoromethyl)anilino-3,5-dinitrothiophene (ANT-2p), known to be the most powerful ADRY agent (Renger, G. (1972) Biochim. Biophys. Acta 256, 428–439), on thermoluminescence has been investigated. Two thermoluminescence bands were analyzed: (a) the emission peaking at about 20–30°C caused by warming up of untreated chloroplasts, illuminated with a single 5 μs flash at room temperature and frozen rapidly to 77 K; and (b) the band emitted in the range of −10 up to 10°C after warming of chloroplast suspensions containing 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) which were illuminated with a single 5 μs flash at −15°C and frozen rapidly at 77 K. These bands were attributed to the recombination of the $B^-S_2(S_3)$ and X-320 $^-S_2$ states, respectively (Rutherford, A.W., Crofts, A.R. and Inoue, Y. (1982) Biochim. Biophys. Acta 682, 457–465). It was found that: (1) The $B^-S_2(S_3)$ band is markedly diminished at very low ANT-2p concentrations of less than one molecule per 2000 chlorophylls. (2) The inhibition of the X-320 $^-S_2$ band requires significantly higher concentrations of ANT-2p (50% peak reduction at one ANT-2p molecule per 100 chlorophylls). (3) Preflashing at room temperature before cooling to −15°C diminishes the X-320 $^-S_2$ band significantly in the presence of ANT-2p, while almost no effect is observed in its absence. (4) The state X-320 $^-S_2$ decays monoexponentially with a half-lifetime of 2 min at −15°C in the absence of ANT-2p. In the presence of one ANT-2p molecule per 800 chlorophylls the decay becomes biphasic with half-lifetimes of 0.5 and 2 min and an amplitude ratio of 2:3, respectively. The results obtained can be explained consistently by the function of ANT-2p as an ADRY agent acting as a mobile species within the thylakoid membrane at room temperature. At subzero temperatures, a 'fixed-place' mechanism appears to be operative. The implications for the ADRY effect and thermoluminescence are discussed.

Introduction

The essential reactions for photosynthetic water cleavage by visible light occur within PS II. The overall process at PS II can be summarized as water photolysis into hydrogen bound to plastoquinone (PQH_2) and molecular oxygen. Its realization requires the transformation of an electronically excited state located at a special chromo-

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Abbreviations: ADRY agents, agents accelerating the deactivation reactions of water-splitting enzyme system Y; Chl, chlorophyll; PS, photosystem; PQ, plastoquinone, DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; ANT-2p, 2-(3-chloro-4-trifluoromethyl)anilino-3,5-dinitrothiophene; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; NMANT-2p, *N*-methyl-ANT-2p.

phore into a ground-state biradical complex of sufficient oxidizing and reducing power and a stability high enough to assure efficient subsequent reactions with water. It was found that this 'stable' biradical complex consists of a photooxidized Chl *a* cation and a plastosemiquinone anion (For a recent review, see Ref. 1). The formation of oxygen requires cooperation of four oxidizing redox equivalents, whereas two-electron cooperation leads to plastoquinone formation. This mechanism gives rise to peculiar oscillation patterns of the redox sequences at the donor and acceptor side of the reaction center complex with periodicities of four and two, respectively. The formation of plastoquinol involves a special plastoquinone molecule [2,3], referred to as B, with plastosemiquinone as stable intermediary redox state. B becomes reduced by electron transfer from the primary plastoquinone in its semiquinone form, $X\text{-}320^-$.

The intermediary states of water oxidation have not yet been substantiated (for theoretical consideration, see Refs. 4 and 5), but the lifetime of the states corresponding to two and three oxidizing redox equivalents, referred to as S_2 and S_3, are known to be of the order of seconds, while S_1 (representing one trapped oxidizing equivalent) is extremely stable (for reviews, see Refs. 6 and 7). Oxidizing and reducing redox equivalents, trapped at the donor and acceptor side of the reaction center complex, recombine under light emission of low quantum yield (for a review, see Ref. 8). This gives rise to delayed light emission [9] and thermoluminescence [10]. Accordingly, thermoluminescence appears to be a very useful tool for analysis (via the detrapping mechanisms) of the nature of oxidizing and reducing redox equivalents stored in PS II, as shown recently [11]. It has been known for a long time that the states S_2 and S_3 of the water-splitting enzyme system Y can be destabilized selectively by substances referred to as ADRY agents [12,13]. However, the molecular mechanism of ADRY effect has not yet been clarified. Accordingly, it seems worthwhile to study the effect of ADRY agents on thermoluminescence emission. The present study shows that the most powerful ADRY agent, ANT-2p, is a very potent inhibitor of thermoluminescence caused by the detrapping of electrons from B^- and holes from S_2 or S_3. It also diminishes the thermoluminescence due to the recombination of the reduced primary plastoquinone acceptor, $X\text{-}320^-$, and S_2 at room temperature as well as subzero temperatures. The implications of these findings for the mechanism of the ADRY effect will be discussed.

Materials and Methods

Chloroplasts were prepared from market spinach by standard methods [14]. Chloroplasts were suspended at 4–5 mg Chl/ml in 0.4 M sucrose, 10 mM NaCl, 2 mM $MgCl_2$ and 50 mM Hepes (pH 7.0) and stored on ice (for daily use) or frozen in a cold box and defrosted for measurements.

Thermoluminescence experiments were carried out on diluted chloroplasts (700–800 μg/ml Chl) as described previously [15] with the sample on filter paper. The heating rate was either 1 or 0.4°C/s. Chloroplasts were illuminated either at room temperature or at −15°C with saturating flashes and frozen in liquid nitrogen immediately after illumination or after a time as indicated in the figure legends.

Fluorescence induction curves were measured on highly diluted samples (5 μg/ml Chl) in the presence of 1 μM DCMU. A light-pipe system was used so that fluorescence emission was detected from the surface of the cuvette which was illuminated with actinic light. Chloroplasts were illuminated with blue light (projector lamp, Schott filter BG 28) and fluorescence emission was detected with a photomultiplier.

Results

Fig. 1 depicts typical traces of thermoluminescence signals induced by illumination with a short flash (xenon lamp) at room temperature, in the absence or presence of ANT-2p or the related but inactive derivative NMANT-2p. It shows that ANT-2p, at concentrations of less than one molecule per reaction center, almost completely suppresses thermoluminescence emission in the range 20–30°C, which was ascribed to S_2B^- and S_3B^- detrapping [11]. On the other hand, the *N*-methylated derivative NMANT-2p, which is inactive as an ADRY agent [16], does not affect the thermo-

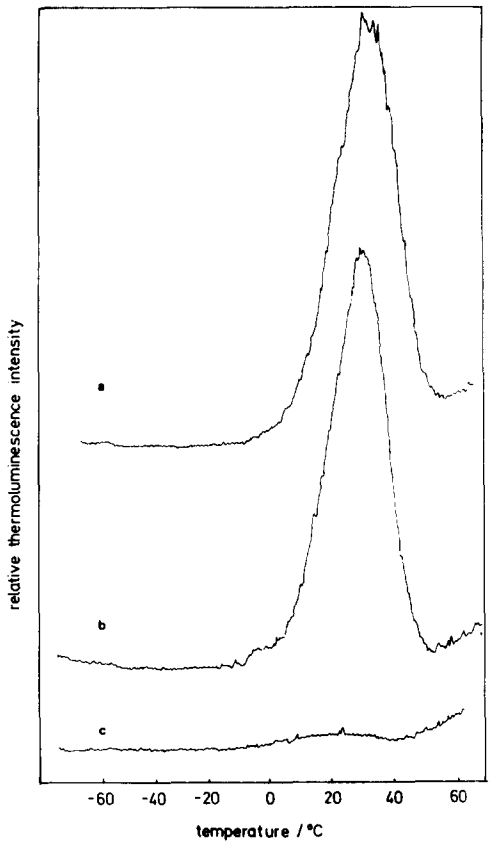


Fig. 1. The effect of ANT-2p and NMANT-2p upon the thermoluminescence bands. Dark-adapted chloroplasts were illuminated at room temperature with one 5 μs flash (white light, 4.5 J) and frozen rapidly to 77 K. Thermoluminescence was recorded upon warming the sample at a rate of 1°C/s. (a) No addition, (c) in the presence of 1 μM ANT-2p, (b) in the presence of 2 μM NMANT-2p.

luminescence signal. This result indicates that the observed inhibition by ANT-2p is caused by the ADRY effect and not by an unspecific quenching.

Fig. 2 describes the extremely high efficiency of ANT-2p. One molecule per 2000 Chl molecules is sufficient to decrease the thermoluminescence signal to 30% of its control value. The data can be explained only with the assumption that ANT-2p acts as a mobile catalyst for the S_2 and S_3 decay, and therefore confirm previous conclusions about the mechanism of ADRY agents [17]. Accordingly, in frozen samples a different reaction pattern is expected, if one assumes that under these conditions the lateral diffusion of ADRY agents is

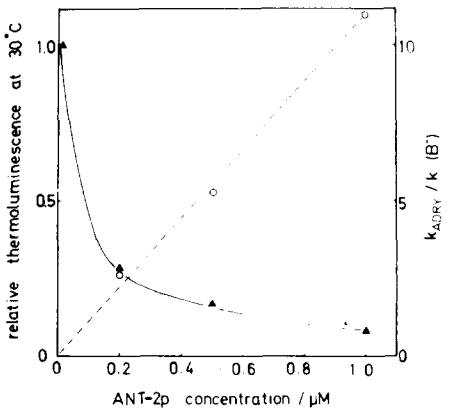


Fig. 2. The effect of ANT-2p concentration upon the amplitude of the thermoluminescence (▲) band at 30°C. Experimental conditions as in fig. 1. The ratio $k_{ADRY}/k_i(B)$ (○) was calculated according to Eqn. 5 (see Discussion).

significantly reduced. In order to be able to measure thermoluminescence emitted at subzero temperatures by hole detrapping from S_2 (or S_3), the electron detrapping must arise from the reduced primary plastoquinone, $X\text{-}320^-$ [11]. As the redox equilibrium $X\text{-}320^-B \rightleftharpoons X\text{-}320B^-$ is inclined towards $X320B^-$ even at temperatures below that of the $X\text{-}320^-S_2$ thermoluminescence emission [11], the following experiments were performed in the

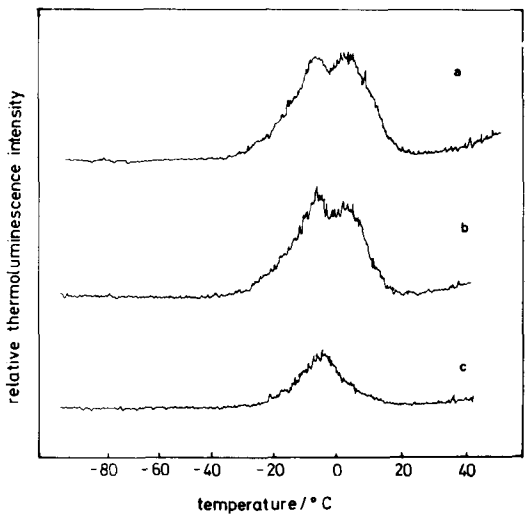


Fig. 3. The effect of ANT-2p upon thermoluminescence bands in the presence of 30 μM DCMU. Dark-adapted chloroplasts were illuminated at −15°C with one 5 μs flash and frozen rapidly to 77 K. Thermoluminescence was recorded at a warming rate of 0.4°C/s. (a) No addition, (b) in the presence of 1 μM ANT-2p, (c) in the presence of 10 μM ANT-2p.

presence of DCMU. Typical traces of thermoluminescence emission in DCMU-inhibited chloroplasts are depicted in Fig. 3. The emission temperature and shape of the control signal are practically identical with those reported previously [11]. There is a characteristic shape for emission in the range from −10 up to 10°C, with a dip around 0°C as a result of the solid-liquid transition of water (for a detailed discussion, see Ref. 11). The signal is partly inhibited by 1 μM ANT-2p, corresponding to one ANT-2p molecule per 800 Chl molecules. The effect is much less pronounced than that observed in chloroplasts without DCMU. The modification of the thermoluminescence band and the significantly smaller effect of ANT-2p are obviously caused by a markedly reduced mobility of ANT-2p at lower temperatures and by competition of the much faster reduction of S_2 by the reduced primary plastoquinone, X-320$^-$ (vide infra).

If one assumes that the mobility of ANT-2p is essential for suppression of thermoluminescence due to ANT-2p-induced enhanced S_2 decay, the signal shape should be changed characteristically, because the ANT-2p effect is expected to become more pronounced at higher temperatures, so that the emission at subzero temperatures is less affected than above 0°C. This should give rise to an apparent peak shifting towards lower temperatures. The data of Fig. 3. confirm this to be the case.

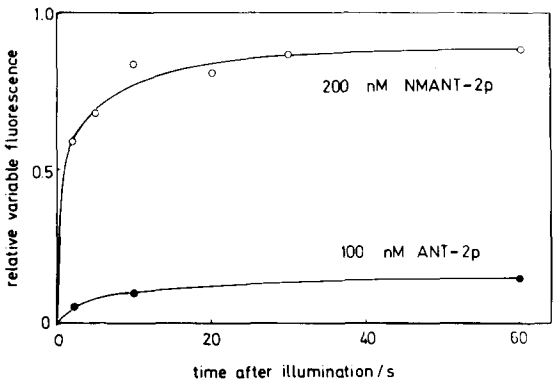


Fig. 4. The effect of ANT-2p and NMANT-2p upon the recovery of variable fluorescence after illumination. Dark-adapted chloroplasts were illuminated in the presence of 1 μM DCMU and either 100 nM ANT-2p or 100 nM NMANT-2p. After the indicated dark time the fluorescence induction was recorded.

In order to prove that the ANT-2p effect on thermoluminescence in DCMU-inhibited chloroplasts is exclusively caused by accelerated S_2 decay, it has to be shown that ANT-2p does not affect the redox state of X-320$^-$. Therefore, measurements of the variable fluorescence were performed as a function of dark time after illumination with continuous light. The data in Fig. 4. indicate that the variable fluorescence, which reflects the amount of photoreducible X-320, is highly suppressed by ANT-2p even after rather long dark times after continuous illumination, whereas NMANT-2p does not affect the comparatively fast reoxidation of X-320$^-$ by S_2. This result confirms previous findings that illumination of DCMU-inhibited chloroplasts in the presence of ADRY agents transforms PS II into the highly fluorescent state [13], X-320$^-S_1$ (at higher concentrations ANT-2p acts as a fluorescence quencher by an effect not related to its function as an ADRY agent). Accordingly, ANT-2p does not act as a catalyst, giving rise to faster recombina-

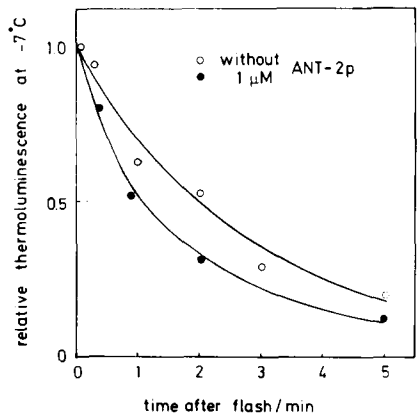


Fig. 5. The effect of dark time after illumination with a 5 μs flash upon the amplitude of the thermoluminescence band at −7°C in the absence and presence of ANT-2p. Dark-adapted chloroplasts were illuminated with a single 5 μs flash in the presence of 30 μM DCMU and kept at −15°C in the dark. After the indicated time the sample was frozen rapidly to 77 K. Thermoluminescence was recorded at a warming rate of 0.4°C/s. (a) No addition, (b) in the presence of 1 μM ANT-2p. The fitting of the experimental thermoluminescence data can be achieved by the function: $TL(t_d) = TL(0) \cdot \Sigma a_j \exp(k_j t)$, with $a = 1$, $k_i(\text{X-320}^-) = 5.8 \cdot 10^{-3}$ s^{-1} (control) and $a_1 = 0.4$, $k_{ADRY} = 2.3 \cdot 10^{-2}$ s^{-1}, and $a_2 = 0.6$ and $k_i(\text{X-320}^-) = 5.8 \cdot 10^{-3}$ s^{-1}; $TL(t_d)$ = thermoluminescence of the sample kept in darkness at −15°C for time t_d after the actinic flash before freezing to 77 K. TL(0) = thermoluminescence of the sample frozen to 77 K immediately after the flash.

tion of X-320$^-$ and S_2, but selectively enhances the decay rate of S_2. The decrease of thermoluminescence by ANT-2p in DCMU-inhibited chloroplasts (see Fig. 3), therefore, is due to the accelerated decay of S_2 through the ADRY effect. It further shows that the donor molecule for this S_2 decay (either the ANT-2p molecule itself or and endogenous donor) in its oxidized state, does not store holes which can be detrapped for thermoluminescence in the observed temperature range.

In order to analyze the mechanism of the ADRY effect at subzero temperatures, DCMU-inhibited chloroplasts were illuminated at −15°C by a short flash, and the thermoluminescence was measured after keeping the chloroplasts for a certain time at −15°C in the dark before rapid freezing at 77 K. In chloroplasts without ANT-2p, the decrease of the thermoluminescence emission is determined by the kinetics of the back-reaction between X-320$^-$ and S_2. The data depicted in Fig. 5 show that this reaction has a half-lifetime of approx. 2 min at −15°C and, therefore, is about 100 times slower than at room temperature. Interestingly enough, a similar ratio of S_2 decay rate at room temperature and −15°C has been observed in the absence of DCMU, although the absolute rates are at least 10-times slower [18]. This might suggest that S_2 is predominantly reduced by B$^-$, involving X-320$^-$ as intermediary carrier. A similar conclusion has been drawn very recently on the basis of fluorescence data [19]. If ANT-2p is able to compete for S_2 decay with X-320$^-$ even at subzero temperatures (−15°C), a faster decline of thermoluminescence is anticipated to arise. Fig. 5 indicates that this really does occur, because the decrease is steeper in the presence of 1 μM ANT-2p.

However, the striking difference between both curves is the type of decay kinetics. In DCMU-inhibited chloroplasts, in the absence of ANT-2p, the thermoluminescence decrease can be described by a monophasic exponential decay, while in the presence of ANT-2p biphasic kinetics arise (see Fig. 5). The slow phase has practically the same kinetics as the decay in DCMU-inhibited chloroplasts without ANT-2p, whereas the faster kinetics are characterized by an almost 4-times shorter half-lifetime. This result can be explained by the assumption that only a fraction of PS II contains bound ANT-2p sufficiently close to the water-splitting enzyme system Y to induce a faster S_2 decay, while the other PS II do not bind ANT-2p. A comparison of the stoichiometry of approx 0.5 ANT-2p per PS II with the amplitude ratio for the thermoluminescence decrease in Fig. 5 (40% fast and 60% slow kinetics suggests a rather specific binding of ANT-2p close to the water-splitting enzyme system Y (see Discussion).

If the thermoluminescence decrease, observed in Fig. 5, is really caused by competitive S_2 decay with X-320$^-$ and ANT-2p as reactive species, the same effect should occur also at room temperature, Since the recombination rate between X-320$^-$ and S_2 is comparatively fast under these conditions [20,21], another type of experiment was performed. Chloroplasts were illuminated at room temperature in the presence of 30 μM DCMU with flashes spaced by a dark time of 10 s. After cooling to −15°C, an actinic flash was applied and the chloroplasts rapidly frozen to 77 K. The data depicted in fig. 6 show that, in the presence of ANT-2p, the amplitude of the thermoluminescence in DCMU-inhibited chloroplasts strongly depends on the number of preillumination flashes, while, in the absence of ANT-2p, only a very small effect is observed. The experimental dependence on the number of flashes in the presence of ANT-2p can be sufficiently described by the assumption

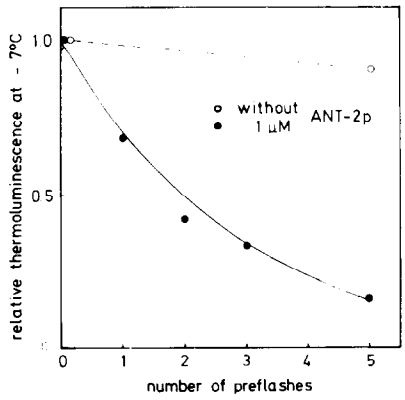


Fig. 6. The effect of preillumination flash number upon the amplitude of the thermoluminescence band at −7°C. Dark-adapted chloroplasts were illuminated at room temperature in the presence of 30 μM DCMU with the indicated number of preillumination flashes spaced at 10 s dark time. 10 s after the last flash, the sample was cooled to −15°C, illuminated by a 5 μs flash and frozen rapidly to 77 K. Thermoluminescence was recorded at a warming rate of 0.4°C/s. (a) No additions, (b) in the presence of 1 μM ANT-2p. Theoretical curve (———) calculated according to Eqn. 2 with $k_i(\text{X-320})/k_{\text{ADRY}} = 2.3$.

of competitive S_2 decay. If, for the sake if simplicity, only two reactions are taken into account, the following scheme arises:

$$\begin{array}{ccc} {}^{DCMU}\|X\text{-}320\cdot Chl\ a_{II}S_1 & \underset{k_i(X\text{-}320^-)}{\overset{flash}{\rightleftharpoons}} & {}^{DCMU}\|X\text{-}320^-\cdot Chl\ a_{II}S_2 \\ & & \downarrow k_{ADRY} \\ & & {}^{DCMU}\|X\text{-}320^-\cdot Chl\ a_{II}S_1 \end{array} \quad (1)$$

where k_i (X-320$^-$) is the rate constant for the internal reduction of S_2 by X-320$^-$ and k_{ADRY} that of the ADRY-catalyzed S_2 decay (with an as yet unidentified ultimate electron donor).

Assuming that, under these conditions, exclusive electron-hole detrapping from the state X-320$^-\cdot$ Chl $a_{II}S_2$ and subsequent charge recombination leads to thermoluminescence, the dependence of thermoluminescence (TL) peak intensity on the number of preillumination flashes, n, is given by:

$$(TL)_n = \left(\frac{k_i(X\text{-}320^-)}{k_i(X\text{-}320^-)+k_{ADRY}}\right)^n \quad (2)$$

The data of Fig. 6 can be described by Eqn. 2 as shown by the theoretical curce obtained for $k_i(X\text{-}320^-)/k_{ADRY} = 2.3$. The slight decrease of thermoluminescence in the absence of ANT-2p might indicate that the actual scheme is more complex, probably due to other internal S_2 decay reactions (the possibility of competitive S_2 decays by endogenous reductants other than the primary and secondary plastoquinone in the reduced semiquinone form and in the absence of ADRY agents will be discussed elsewhere; Rutherford, A.W., Renger, G., Koike, H. and Inoue, Y., unpublished data).

The comparatively less pronounced effect of

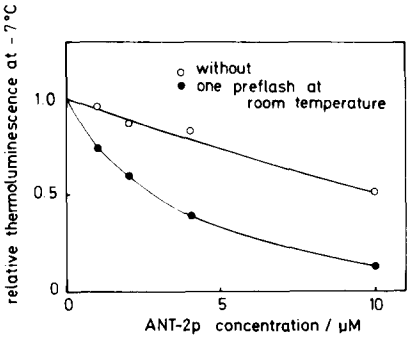


Fig. 7. The effect of ANT-2p concentration upon the amplitude of the thermoluminescence band at −7°C without and with one preflash at room temperature. Experimental conditions as in Fig. 6.

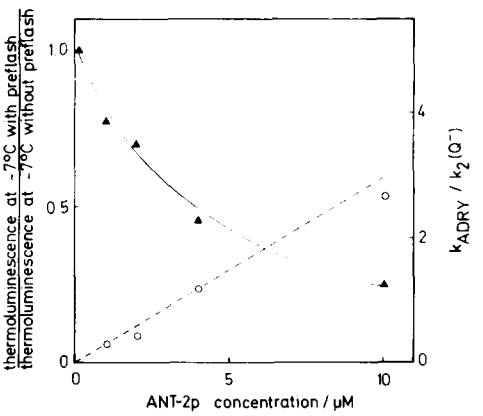


Fig. 8. The effect of ANT-2p concentration upon the amplitude ratio of the thermoluminescence band at −7°C with and without preflash, respectively. Experimental data from Fig. 7. The ratio $k_{ADRY}/k_i(X\text{-}320^-)$ was calculated by using Eqn. 2.

ANT-2p in DCMU-inhibited chloroplasts is caused by the efficient decays of X-320$^-\cdot$ Chl $a_{II}S_2$ via the back-reaction ($k_i(X\text{-}320^-)$). Accordingly, at higher concentrations of ANT-2p, the decrease of thermoluminescence should be more pronounced. This effects is really observed, as shown in Fig. 7 for the action of one preillumination flash as a function of ANT-2p concentration. Since at higher concentration the thermoluminescence also decreases without a room temperature preflash, the data were normalized to the control curve. The results obtained are depicted in Fig. 8. They confirm increasing efficiency at higher ANT-2p concentrations.

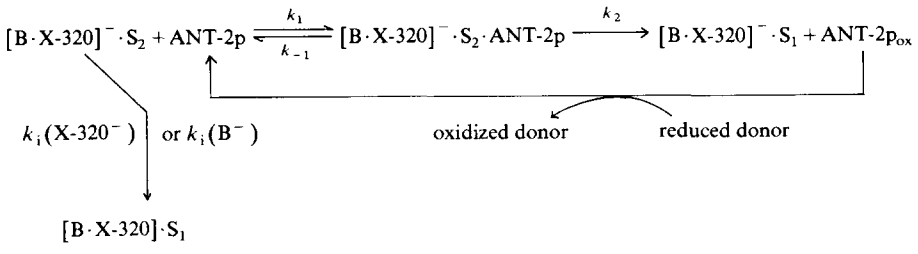
Discussion

The results presented in this study confirm the idea that thermoluminescence emitted from DCMU-inhibited chloroplasts illuminated by a flash at −15°C is caused by recombination of electrons and holes thermally detrapped from X-320$^-$ and S_2, respectively. Therefore, the effect on thermoluminescence indicates that ANT-2p is efficient in competing with the X-320$^-S_2$ recombination, even at −15°C. Assuming a mobile ADRY mechanism to be unlikely at this temperature, only those PS II reaction centers should reveal an ANT-2p-induced accelerated S_2 decay which contain the ANT-2p molecule closely bound to the water-splitting enzyme system Y (fixed-place mechanism).

Accordingly, despite experimental scatter, the data of Fig. 5 should permit a rough estimation of the dissociation constant for ANT-2p binding to system Y. If system Y is assumed to be the only specific binding unit for ANT-2p, the dissociation constant for processes restricted to the thylakoid membrane, $K_T = [\text{ANT-2p}]_{T,\text{free}} \cdot [Y]/[Y \cdot \text{ANT-2p}]$ can be expressed as follows:

$$K_T = \frac{\left(1 - p\frac{[Y]}{c_T}\right)(1-p)}{p} c_T \qquad (3)$$

where c_T is the total ANT-2p concentration within the thylakoid membrane and p the probability of ANT-2p binding to system Y, i.e., $p = [Y \cdot \text{ANT-2p}]/[Y]_{\text{total}}$. The data in Fig. 5 reveal that $p = 0.4$, provided that the relative amplitude of the fast decay directly reflects the binding probability p. The experiments were performed at an overall stoichiometric ratio of 1 ANT-2p molecule per two systems Y, calculated on the basis of 400 Chl molecules per PS II. If, for the sake of simplicity, all ANT-2p molecules are assumed to be located within the thylakoid membrane, the upper limit of the K_T value is evaluated: therefore, $K_T \leqslant 0.3 c_T$. However, for practical purposes, dissociation constants of binding agents are related to the total suspension volume. They are defined as the free agent concentration in the suspension where half of the binding sites are occupied at infinite thylakoid dilution. Unfortunately, the assay conditions in the present study do not allow determination of an exact K value. Again, an upper limit is obtained if we simply neglect the distribution of the free ANT-2p between thylakoid and aqueous phase. Accordingly, the experimental data lead to the conclusion that K has to be smaller than 0.3 μM. Regardless of this detail, the present data indicate that ANT-2p binds specifically to the water-splitting enzyme system Y, with a binding constant comparable to those of well known inhibitors of the acceptor side (such as DCMU). DCMU binds to the herbicide-binding protein [22], thereby interrupting electron transfer between the primary and secondary plastoquinone. Different mechanisms have been proposed for the DCMU effect: (a) a direct competition of DCMU and plastoquinone for the same binding site [23] or (b) an allosteric type of inhibition [24]. In any case, DCMU does not function as a redox-active species. A different mechanism should, therefore, be taken into consideration for the ADRY effect, because these agents appear to be redox active [25]. On the basis of theoretical considerations, S_2 and S_3 have been substantiated as a binuclear bridge of $H_3O_2^-$ and H_2O_2, respectively, between two manganese centers, referred to as 'cryptohydroxyl' and 'cryptohydrogenperoxide', respectively [26]. Accordingly, ANT-2p could bind close to these functional complexes, thereby destabilizing either via direct electron transfer or via an allosteric reduction with an unknown endogenous electron donor. The data in Fig. 5 also indicate that binding of ANT-2p to system Y appears to be rather independent of the redox state, S_i, because binding occurs before S_2 formation at -15°C. It has to be emphasized that the binding constant does not necessarily correspond to that at room temperature, because the suspension was frozen to -15°C rather slowly, before S_2 was formed by flash excitation. A different mechanism is assumed to be responsible for the ADRY effect at room temperature , because ANT-2p is expected to act as a mobile species [17]. In the simplest case, involving only the internal species X-320$^-$ and/or B$^-$ (rate constants k_i(X-320$^-$) and k_i(B$^-$), respectively) and ANT-2p as reactants, the decay of S_2 can be described by the following scheme:



(4)

A complete analytical solution of the differential equations describing the kinetics of S_2 decay according to the scheme depicted by Eqn. 4 is impossible. However, if one assumes that ANT-2p recovery is very fast compared to S_2 decay, two limiting situations can be considered: (a) $k_2 \ll k_1$ and (b) $k_2 \gg k_1$. In the first case, a complex nonlinear dependence of the phenomenological rate constant k_{ADRY} on the total ANT-2p concentration arise, whereas in the latter case an almost linear relation is obtained. From the data in Fig. 8, the concentration dependence of k_{ADRY} can be calculated. On the basis of the scheme for competitive S_2 decay given by Eqn. 1 and the assumption that the ADRY-catalyzed S_2 decay prevents thermoluminescence (TL), one obtains with:

$$r = \frac{(TL)^{preflash}_{ADRY}}{(TL)^{0}_{ADRY}} = \frac{k_i(X\text{-}320^-)}{k_i(X\text{-}320^-) + k_{ADRY}}$$

$$k_{ADRY} = \left(\frac{1}{r} - 1\right) k_i(X\text{-}320^-) \qquad (5)$$

where r is the ratio of thermoluminescence after one preilluminating flash related to the corresponding signal without preflash. The ratio, $k_{ADRY}/k_i(X\text{-}320^-)$, depicted in Fig. 8, reveals a linear relationship with the ANT-2p concentration. Therefore, the data favor the idea of a diffusion controlled ANT-2p-induced S_2 decay. Taking $k_i(X\text{-}320^-)$ to be 0.6 s^{-1} [20,21], k_{ADRY} is calculated to be of the order of 0.25 s^{-1} at $C_0 = 1$ μM ANT-2p. These values are of the same order of magnitude as the ANT-2p-induced S_2 and S_3 decay obtained by oxygen-yield measurements in the absence of DCMU (Dohnt, G. and Renger, G., unpublished data). Accordingly, the ADRY effect does not seem to be modified drastically by DCMU attached to the herbicide-binding protein (vide infra). This, again, shows that ADRY agents rather specifically catalyze the S_2 and S_3 decay.

In the absence of DCMU, the ANT-2p effect on thermoluminescence was found to be much more pronounced (see Fig. 2). This phenomenon is easily understandable, because the internal decay of S_2 characterized by $k_i(B^-)$ is much slower in the absence than in the presence of DCMU. Accordingly, the internal decay of S_2 cannot compete as effectively with the ADRY effect as the back-reaction between $X\text{-}320^-$ and S_2. Substituting, in Eqn. 5, $k_i(X\text{-}320^-)$ by $k_i(B^-)$ and r by the thermoluminescence band at 30°C normalized to the corresponding control value, a linear dependence on the ANT-2p concentration is also found in the absence of DCMU (see Fig. 2). Based upon the half-lifetime of S_2 and S_3 of 20–30 s in the absence of DCMU [13], the data in Fig. 2 show that $k_{ADRY} = 0.25$ s^{-1} at 1 μM ANT-2p, a value which nicely fits the value obtained from Figs. 8 and 6.

The data in Figs. 2,6, and 8 obtained under very different experimental conditions lead to practically the same k_{ADRY} value and, therefore, can be explained by the same underlying mechanism.

The results presented in this study additionally show that thermoluminescence is an appropriate tool for analysis of the redox state of the water-splitting enzyme system Y. A detailed analysis, however, requires information about the redox state of the primary and secondary plastoquinones, because both species in their semiquinone form act as suitable traps for electrons which give rise to thermoluminescence after detrapping and recombination with detrapped oxidizing redox equivalents from system Y.

Furthermore, the data confirm the conclusion that ANT-2p acts as a mobile species which effectively enhances the decay of S_2 and S_3. In the frozen state, a fixed-place mechanism appears to be more plausible, as shown by the biphasic decay of thermoluminescence after a flash at −15°C.

The close correspondence of the rate constants derived from thermoluminescence data with those directly obtained from oxygen measurements reveals that the effect of ANT-2p on thermoluminescence can be completely explained by its function as ADRY agent. Therefore, it can be concluded that appropriate ADRY agents, like ANT-2p, do not affect the quantum yield of exciton formation via recombination of S_2 and S_3 with either $X\text{-}320^-$ or B^-. Likewise, unspecific quenching of excitons does not occur under our experimental conditions. Accordingly, ANT-2p is a practically ideal ADRY agent, which accelerates very specifically the decay of S_2 and S_3. Concentrations of less than one molecule per reaction center are sufficient to induce a significant ADRY effect. For these reasons, ANT-2p appears to be the most specific agent of

the water-splitting enzyme system Y so far known. The implications of specific ANT-2p binding to system Y are currecntly being analyzed and will be presented in a forthcoming paper.

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