A New-Type Photosystem II Inhibitor which Blocks Electron Transport in Water-Oxidation System

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The mode of action of three types of conjugated enamine compounds was investigated by means of thermoluminescence measurement. Cyanoacrylate and 2-(1-ethoxyethylaminomethylidene)-4-dodecyl-5,5-dimethyl-cyclohexane-1,3-dione (ACm12) converted the B-band (30 °C) arising from $S_2Q_B^-$ charge recombination to a downshifted 6 °C-band. This band was proved to be identical with the DCMU-induced Q-band (6 °C) arising from $S_2Q_A^-$ recombination, indicating that these two compounds block Q_A to Q_B electron transport. 3-(1-dodecylaminopropyridene)-6-methyl-2H-pyran-2,4-dione (APp12), on the other hand, induced an abnormal band peaking at 15 °C between the Q-band and B-band. From the gradual downshift of its peak temperature in titration experiments, this band was assigned to arise from a modified $S_2Q_B^-$ charge pair, in which the properties of either Q_B^- or S_2 is altered. The 15 °C-band showed normal oscillation during the first 2 flashes, but the oscillation was interrupted thereafter. Another thermoluminescence analysis by use of post flash low temperature illumination protocol revealed that APp12 affects neither Q_A to Q_B nor Q_B^{2-} to PQ electron transport, but specifically blocks S_3 to S_0 transition. These results indicate that APp12 is a new-type PS II inhibitor.

Introduction

A number of herbicides have been reported to block the electron transport in photosystem (PS) II, and extensive knowledge about their mode of action has been accumulating [1–3]. By means of photoaffinity labeling, some of these herbicides were shown to bind to D1 protein, a reaction center component of PS II, and thereby interrupt the electron flow from Q_A^- to Q_B [4, 5].

Recently several conjugated enamine compounds (Fig. 1) were introduced to be potent in inhibiting PS II electron transport [6–8]. Since all these compounds have a HN–C= moiety, which is a putative binding and/or activity determinant common to all urea/triazine type herbicides as pointed out by Trebst [3], they are expected to affect Q_A to Q_B electron transport. In fact, some of their derivatives are more active in inhibiting the PS II electron transport than the standard herbicide like DCMU [6–8].

Abbreviations: ACm12, 2-(1-ethoxyethylaminomethylidene)-4-dodecyl-5,5-dimethyl-cyclohexane-1,3-dione; APp12, 3-(1-dodecylaminopropyridene)-6-methyl-2H-pyran-2,4-dione; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; PS, photosystem; Q_A and Q_B, primary and secondary quinone acceptors, respectively.

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In this study, we comparatively investigated the effects of three conjugated enamine compounds on thermoluminescence, expecting a common mode of action among them. It was found that cyanoacrylate and ACm12 act as DCMU-type inhibitors. Contrary to our expectation, however, APp12 induced an abnormal thermoluminescence B-band which arises from an $S_2Q_B^-$ (or $S_3Q_B^-$) charge pair with modified properties. From the oscillatory behavior of the APp12-induced B-band under single turnover flashes, we concluded that the inhibition site by APp12 is S_3 to S_0 transition.

Materials and Methods

ACm12 and APp12 were synthesized and purified as reported in [7, 8]. Spinach thylakoids were prepared as in [9] and stored in darkness at −80 °C until use. Inhibition of PS II electron transport was assayed by DCIP photoreduction monitored spectrophotometrically at 600 nm as described in [10]. Thylakoids for thermoluminescence measurements were diluted (0.25 mg Chl/ml) with 25% (v/v) glycerol, 10 mM $MgCl_2$ and 50 mM HEPES-NaOH (pH 7.0), illuminated with orange light for 45 sec, and then dark-adapted for 5 min at room temperature to afford constant initial ratios of $S_1:S_0$ and $Q_B:Q_B^-$ unless otherwise stated. Thermoluminescence was measured as described in [11]. The samples were il-

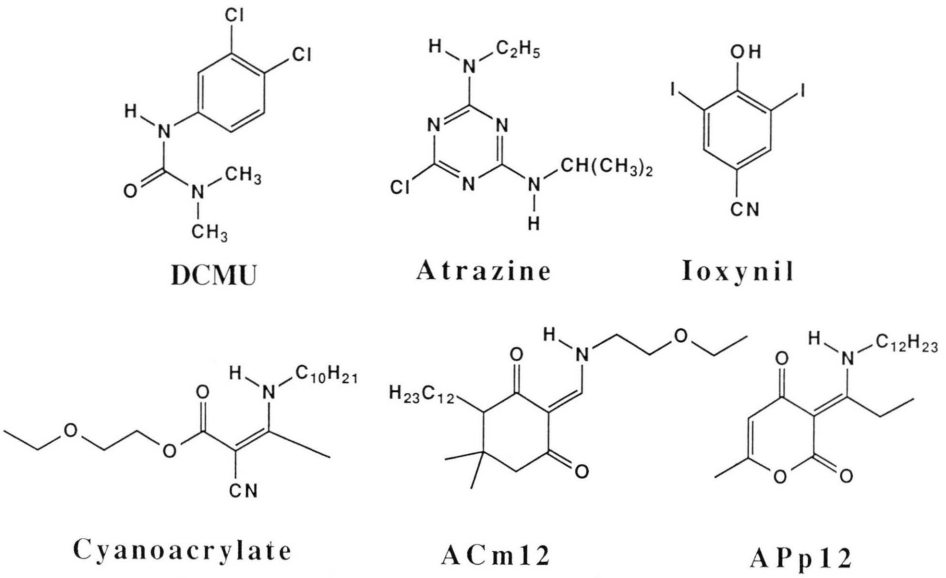

Fig. 1. Chemical structures of cyanoacrylate, ACm12 and APp12 as compared with those of DCMU, atrazine and ioxynil.

luminated with flashes (5 μs, 4 J, white light) from a xenon strobe, and in some experiments with continuous red light (> 630 nm, 0.7 mW/cm^2). The heating rate was 0.8 °C/sec.

Results

Through previous structure-function relationship studies [7, 8], cyanoacrylate, ACm12 and APp12 have been shown to be the most potent inhibitors among their respective derivatives. Table I lists the potencies of these compounds in inhibiting PS II electron transport in comparison with those of standard herbicides, DCMU, atrazine and ioxynil. The concentrations of the three compounds for half inhibition are one order below those of atrazine or ioxynil and close to or even lower than that of DCMU, indicating that all of these three are highly potent in inhibiting the electron transport of PS II.

Table I. Inhibitory effects of cyanoacrylate ACm12 and APp12 on DCIP-Hill reaction in spinach thylakoids as compared with those of DCMU, atrazine, and ioxynil.

Inhibitors	Concentrations for 50% inhibition [M]
Cyanoacrylate	1.2×10^{-8}
APp12	4.0×10^{-8}
ACm12	1.0×10^{-8}
DCMU	3.7×10^{-8}
Atrazine	2.5×10^{-7}
Ioxynil	1.0×10^{-7}

The effects of these inhibitors on thermoluminescence glow curve are depicted in Fig. 2. The glow curve of untreated thylakoids after a single flash excitation shows the B-band at around 30 °C (bottom curve), which has been assigned to arise from charge recombination of $S_2Q_B^-$ charge pair [11, 12]. In the presence of an inhibitor which interrupts Q_A to Q_B electron transport, the same single flash excitation yields an $S_2Q_A^-$ charge pair, which recombines more easily than $S_2Q_B^-$ to give the Q-band at a lower temperature of −10 to 6 °C [11–13]. Through the pioneering work by Droppa *et al.* [13], it has been shown that the peak temperature of the Q-band varies appreciably depending on the species of inhibitors used to block the electron transport from Q_A to Q_B. As shown in Fig. 2, the Q-bands induced by DCMU and atrazine peak at 6 °C and 3 °C, respectively, whereas the Q-band induced by ioxynil peaks at an appreciably lower temperature of −10 °C [13, 14]. These phenomena are interpreted as follows: all these inhibitors bind to D1 protein in the vicinity of Q_B-binding site [15], but the amino acid residues interacting with respective herbicide molecules differ slightly depending on herbicide species, and these differences influence over the D2 protein to give rise

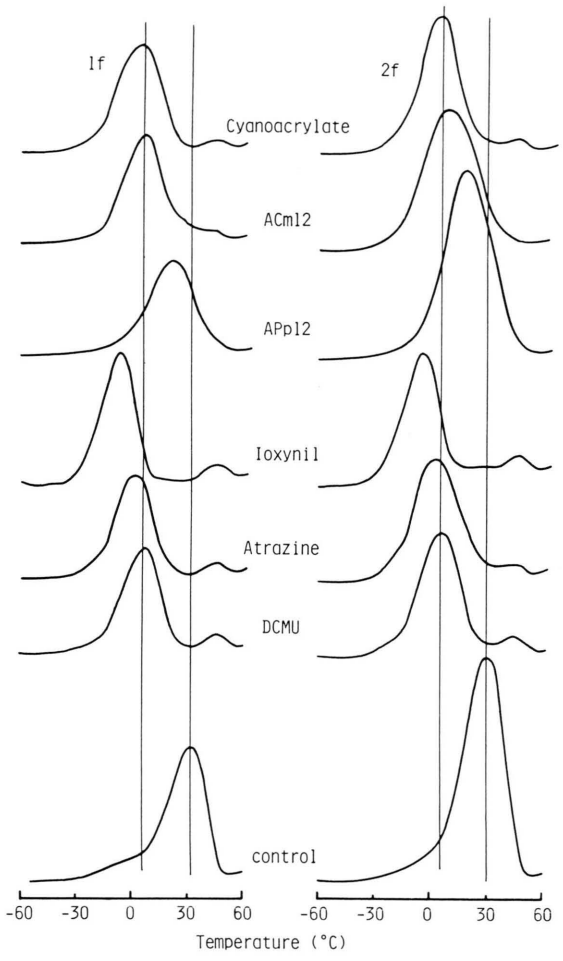

Fig. 2. Effects of cyanoacrylate, ACm12 and APp12 on thermoluminescence glow peak as compared with those of DCMU, atrazine and ioxynil. Sample thylakoids treated with 10 μM each of the inhibitors were illuminated with one (left column) or two (right column) flashes at −15 °C, rapidly cooled to 77 K, and then gradually warmed in darkness (0.8 °C/sec). Thermoluminescence during warming was recorded against sample temperature.

to a change in microenvironment of the Q_A-binding site, which results in variously modulated redox potential of Q_A^- as detected by herbicide-type-specific peak positions of the Q-band. Base on these phenomena, Droppa *et al.* [13] have proposed that the above phenomena can be used to classify the action of various herbicides. When we apply this method to our newly synthesized inhibitors, cyanoacrylate and ACm12 are classified into a urea/triazine type herbicide, since both of them induced a Q-band peaking at 6 °C, which is identical with the DCMU-induced Q-band.

In contrast to the above two inhibitors, the glow peak induced by APp12 was found at around 15 °C, which is intermediary between the peak positions of the B-band and Q-band. As will be shown later, the 15 °C-band is not a Q-band due to $S_2Q_A^-$ recombination but a type of B-band due to a modified $S_2Q_B^-$ charge pair. In fact the height of the 15 °C-band was nearly doubled on illumination with the 2nd flash as opposed to the Q-band induced by the other herbicides, indicative of formation of S_3 state (see later). It is of note that the concentration of APp12 employed in this experiment (10 μM) is sufficient enough to completely block the electron transport under continuous illumination, showing only a two to three e^- equivalent reduction of the acceptor pool when measured by fluorescence induction method (data not shown). It is also of note that APp12 does not affect at all the duroquinol-supported photoreduction of methylviologen [16], indicative of no effect on the electron transport in PS I and Cyt b_6/f complex. These results suggest that APp12 inhibition allows electron flow of at least two e^- equivalents in PS II even under the conditions of complete inhibition. This is clearly different from the inhibition mode by other herbicides in which only one equivalent of electron transport through PS II reaction center is allowed.

Fig. 3 shows the thermoluminescence glow curves of thylakoids treated with different concentrations of herbicides. In untreated thylakoids, one flash illumination generated a single B-band peaking at 30−35 °C. In the presence of 0.5 μM DCMU, the Q-band peaking at 6 °C was induced at the expense of the B-band, resulting in a double-peaked glow curve, and at 5 μM DCMU the B-band was completely replaced by the Q-band. This indicates that DCMU affects the PS II centers in all-or-none mode: the centers occupied by herbicide show the Q-band while the remaining centers show the B-band. Similarly, ACm12 at 0.5 μM slightly replaced the B-band with Q-band and completely at 5 μM. Thus, ACm12 also acts in all-or-none mode like DCMU, although its concentration needed for complete replacement is slightly higher than that of DCMU.

As to APp12, the situation is very different. With APp12 at 0.5 μM, the glow peak temperature was slightly downshifted with no change in peak height, and much more at 5 μM to give an intermediate peak

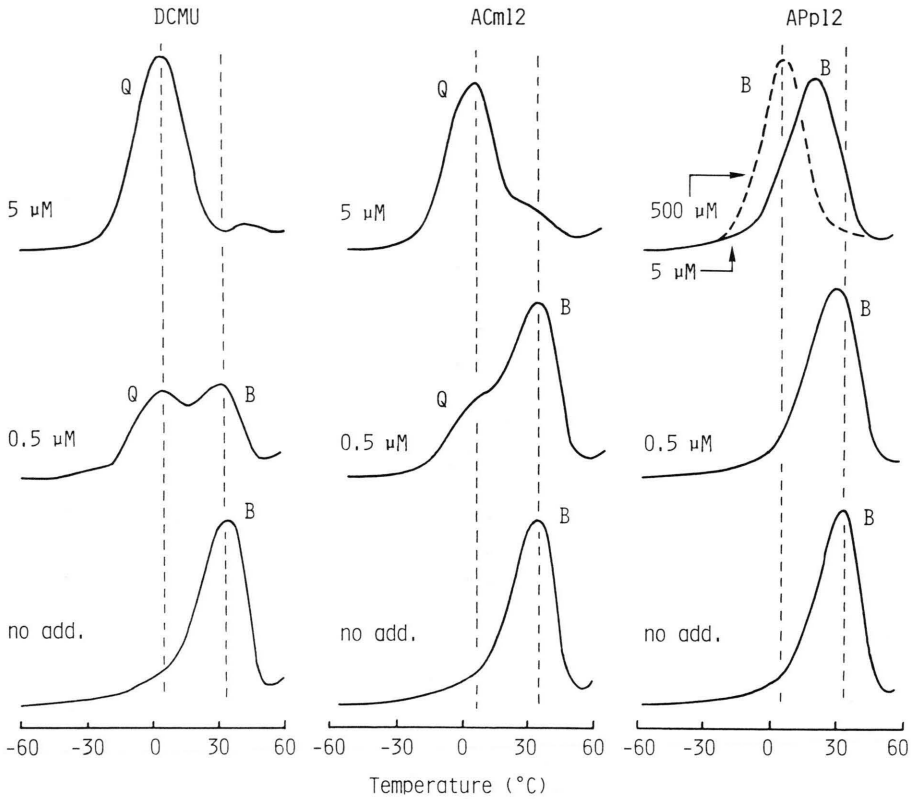

Fig. 3. Glow curve changes during titration with DCMU (left), ACm12 (middle) and APp12 (right). Sample thylakoids were treated with indicated concentrations of respective inhibitors, illuminated with a single flash at −15 °C, and then subjected to thermoluminescence measurement.

temperature (15 °C) between the Q-band and B-band. On increasing the concentration to 500 μM, the peak temperature was further downshifted to 7 °C with no significant decrease in emission intensity. Since both the bands at 15 °C (at 5 μM) and 7 °C (at 500 μM) were appreciably intensified by the 2nd flash by factors of 2 and 1.5, respectively, indicative of formation of $S_3Q_B^-$ pair (see Fig. 4, upper panel), the negative charge responsible for this band cannot be Q_A^- (single electron carrier) but Q_B^- (two electron carrier). Thus we may conclude that the APp12-induced downshifted band is due to recombination of an $S_2Q_B^-$ charge pair with modified properties. According to the general theory of thermoluminescence [17, 18], the downshift of peak temperature is ascribed to either lowered oxidation potential of Q_B^- or raised oxidation potential of S_2. Unfortunately, however, the present experiments do not give a convincing answer to this question.

Fig. 4 depicts the peak temperature and emission intensity of the APp12-induced glow peak titrated against APp12 concentration. The peak temperature after one or two flashes was gradually downshifted with no discrete break with increasing APp12 concentration (lower panel). It is of note that the glow peak temperature after the 2nd flash ($S_3Q_B^-$) was slightly lower (by 4 °C) than that after the 1st flash ($S_2Q_B^-$). This indicates that despite the marked downshift in peak temperature, the APp12-modified $S_3Q_B^-$ pair recombines at a lower temperature than the modified $S_2Q_B^-$, in agreement with the similar relationship known for normal $S_2Q_B^-$ and $S_3Q_B^-$ charge pairs [11]. In contrast to these responses of peak temperature, the peak heights after the 1st and 2nd flashes were not much affected by 500 μM APp12 which is 10 times higher than the concentration needed for complete inhibition. These results indicate that PS II turns over at least twice under the

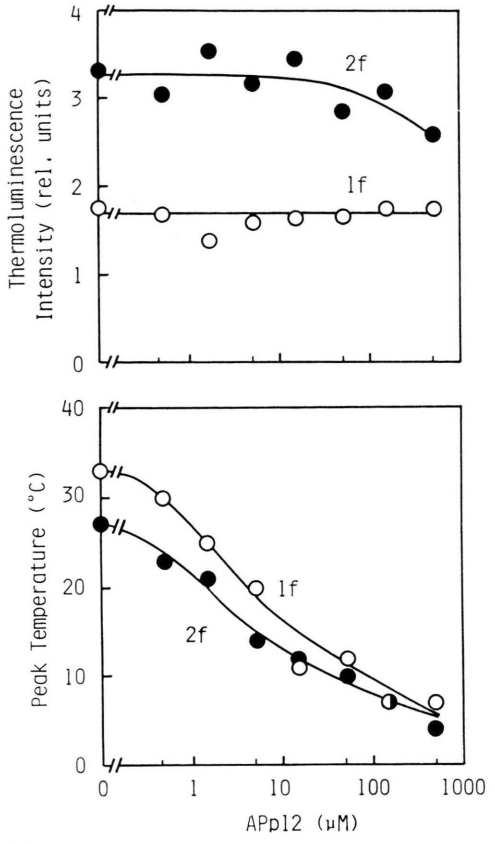

Fig. 4. APp12 titration curves of thermoluminescence peak height (upper panel) and glow peak temperature (lower panel). Sample thylakoids were treated with varying concentrations of APp12, illuminated with one (○) or two (●) flashes at −15 °C, and then subjected to thermoluminescence measurement.

condition where the electron transport is completely inhibited by APp12, and in turn that the negative charge responsible for the APp12-induced thermoluminescence band is not Q_A^- but Q_B^-.

The inhibition site of APp12 was investigated by measuring the oscillation pattern of thermoluminescence band. When thylakoids were illuminated with continuous light for 45 sec and then dark-adapted for 5 min, the PS II acceptor side is known to relax to a $Q_B:Q_B^-$ ratio of 50:50, while the donor side to an $S_1:S_0$ = 75:25 (ref. [11, 12]). When thus dark-adapted thylakoids were illuminated with a series of flashes, the B-band showed a quadruple oscillation with a maximum at the 2nd and a minimum at the 4th flash (Fig. 5A1 and A2, broken lines). In the presence of APp12 at 15 μM, the intensity of the 15 °C-band increased up to the 2nd flash but did not vary any more to show oscillation, indicating that oscillation was interrupted after the 2nd flash (Fig. 5A2). After a longer dark-adaptation of thylakoids, the acceptor side relaxes to a different ratio of $Q_B:Q_B^-$ = 75:25 with no change in $S_1:S_0$ ratio [11, 19]. When such thylakoids were illuminated with a series of flashes, the B-band height showed an oscillation pattern as depicted by the broken line in Fig. 5B2, which exhibited maxima after the 1st and 5th flashes and a minimum after the 4th flash in agreement with our previous report [19]. In the presence of APp12, the height of the 15 °C-band oscillated in a similar manner as the control samples up to 2nd flash, but stopped oscillation thereafter (Fig. 5B1 and B2 continuous lines). It is thus inferred that the APp12-inhibited PS II reaction center turns over twice but stops turning over thereafter consistent with the results in Fig. 5A1 and A2.

In general consideration, the interruption of electron transport after two turnovers can be interpreted by the following alternatives: (i) S_3 to S_0 transition on donor side is blocked, (ii) Q_B^{2-} to PQ electron transport on acceptor side is blocked. According to the present interpretation of the mechanism of thermoluminescence oscillation, the oscillation pattern of Fig. 5B2 excludes the latter possibility: In normal untreated thylakoids, the major (62.5%) redox species in dark-adapted centers are S_1Q_B, which are converted by the 1st flash to $S_2Q_B^-$, a thermoluminescent pair, and by the 2nd flash to S_3Q_B, a non-thermoluminescent pair, since the $S_3Q_B^{2-}$ generated by the 2nd flash reacts with PQ to lose the negative charge, and thereby decreases the thermoluminescence peak height after the 2nd flash. If we assume that APp12 blocks Q_B^{2-} to PQ electron transport, the redox pair will be expected after the 2nd flash in major (62.5%) centers is $S_3Q_B^{2-}$. Although we have no data at present about the thermoluminescence properties of the charge pair involving Q_B^{2-}, this pair is reasonably assumed to be thermoluminescent, since it has both positive and negative charges. Assuming these, the thermoluminescence intensity after the 2nd flash should be comparable or even higher than that after the 1st flash. Obviously, the pattern depicted by Fig. 5B2 (solid line) is not the case, and indicates that APp12 does not affect Q_B^{2-} to PQ electron transport. This conclusion agrees with the observation that APp12 does not inhibit diphenylcarb-

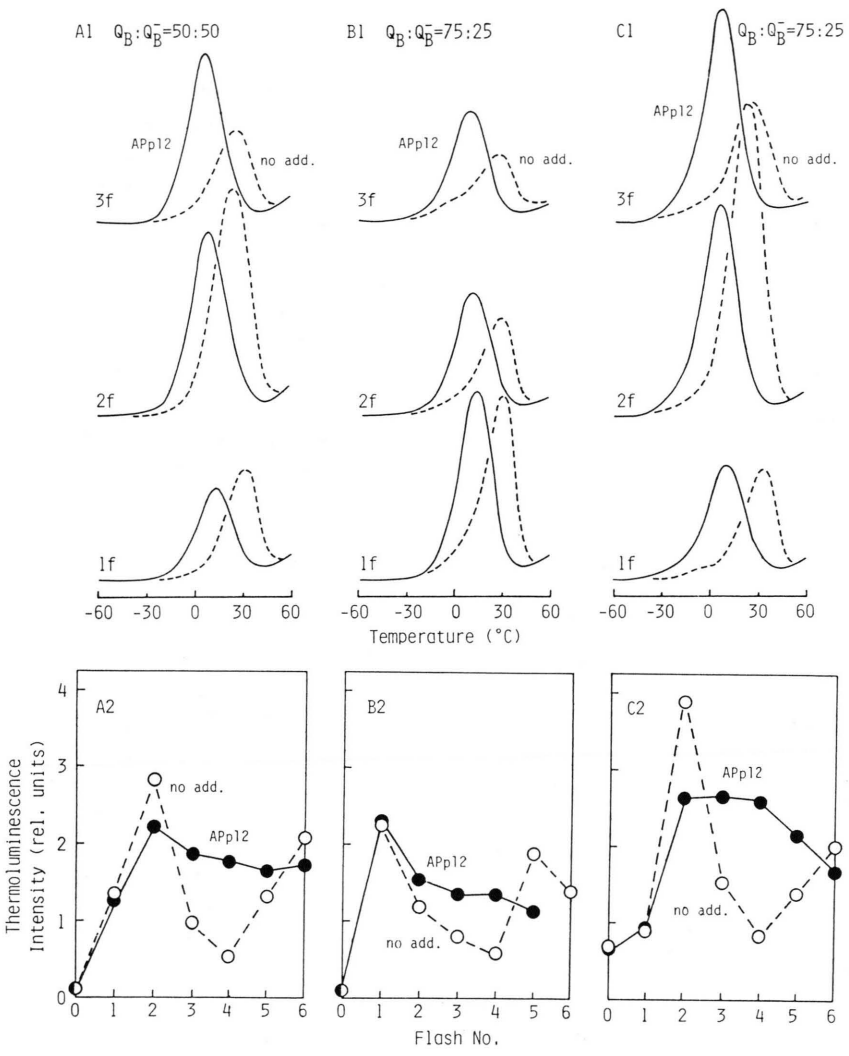

Fig. 5. Effects of APp12 on thermoluminescence oscillation under three different conditions. (A) Short-time (5 min) dark-adapted thylakoids ($Q_B:Q_B^-$ = 50:50; $S_1:S_0$ = 75:25) were illuminated at 0 °C with a series of flashes. (B) Long-time (3 h) dark-adapted thylakoids ($Q_B:Q_B^-$ = $S_1:S_0$ = 75:25) were illuminated as in (A). (C) The same as (B) except that the flash-illuminated thylakoids were further illuminated at 77 K with continuous light for 45 sec. (Post flash low temperature illumination protocol. See text and Fig. 6 for details.) Upper panel shows the glow curves after the first three flashes, and lower panel show the oscillations during the first six flashes. Solid and broken curves are data for APp12-treated and untreated control thylakoids, respectively.

azide photooxidation: the half inhibition concentration for diphenylcarbazide-supported photoreduction of DCIP by NH_2OH-treated thylakoids was 0.32 μM. This concentration is 8 times higher than the half inhibition concentration for DCIP-photoreduction with water as electron donor (40 nM) by untreated thylakoids (Table I).

The above conclusion was reinforced by Fig. 5C experiments, in which a "post flash 77 K continuous illumination protocol" [11] was employed. This protocol consists of a flash preillumination at room temperature followed by rapid cooling and illumination with continuous light at 77 K. In this protocol, the positive charges are the S_2 or S_3 state created by flash preillumination at room temperature, whereas the negative charges are the sum of electrons on Q_B^- generated by preflashes at room temperature and those on Q_A^- generated by 77 K illumination, since 77 K illumination transfers one extra electron from Cyt b_{559} to Q_A without affecting the S-state [19]. If we assume a blocking of Q_B^{2-} to PQ electron transport by APp12, the charge pairs will be formed from the major (62.5%, S_1Q_B) centers in initial dark-adapted condition are $S_2Q_B^{2-}$ and $S_3Q_A^-Q_B^{2-}$ after the 1st and 2nd (pre)flashes, respectively (Fig. 6). Since both pairs can be assumed to be thermoluminescent, we should expect strong luminescence after both the 1st and 2nd (pre)flashes. On the contrary, if we do not

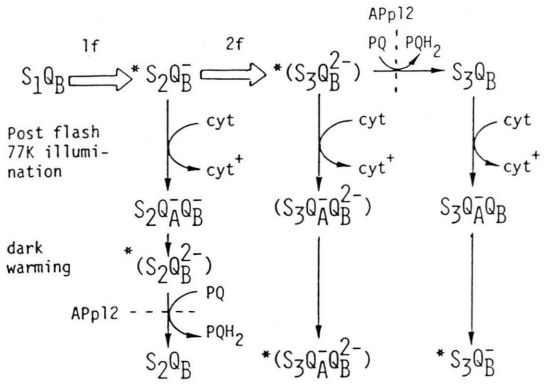

Fig. 6. Charge pair changes in the initial dark-adapted major (62.5%) S_1Q_B centers after excitation with post flash 77 K illumination protocol, with or without APp12 inhibition of Q_B^{2-} to PQ electron transport. Charge pairs with parentheses are those expected by assuming inhibition of Q_B^{2-} to PQ electron transport and those without are the pairs by assuming no inhibition. Charge pairs with and without * stand for thermoluminescent and non-thermoluminescent pairs, respectively. Cyt and Cyt^+ stand for reduced and oxidized cytochrome b_{559}, and dark warming is given during thermoluminescence measurement. Note that 77 K illumination delivers one extra electron to Q_A by oxidation of Cyt, and Cyt^+ does not function as positive charge in thermoluminescence emission [11].

assume the blocking, the expected major (62.5%) charge pairs after the 1st and 2nd (pre)flashes are S_2Q_B and $S_3Q_B^-$, respectively (Fig. 6). The former pair is non-thermoluminescent whereas the latter is thermoluminescent, so that the oscillation pattern should be zero after the 1st flash and high after the 2nd flash.

When we investigated the effect of APp12 by employing this protocol, the results depicted in Fig. 5C were obtained. In untreated thylakoids, the B-band height after the 1st flash preillumination (followed by 77 K illumination) was lower as compared with the height with no post 77 K illumination. After the 2nd (pre)flash, the B-band was markedly enhanced and then markedly lowered after the 3rd (pre)flash (Fig. 5C1, broken lines), showing a sharp maximum at the 2nd flash and a minimum at the 4th flash (Fig. 5C2, broken line). The weak luminescence at flash number zero is a contribution by the small amount of S_2 formed in competition with Cyt b_{559} oxidation during 77 K illumination. In the presence of APp12 the B-band height after the 1st (pre)flash was low but was markedly increased after the 2nd (pre)flash to ca. 70% of the control B-band height. After the 3rd (pre)flash, however, the B-band height remained at the same level as that after the 2nd (pre)flash (Fig. 5C1, solid lines), indicating that the oscillatory behavior was abolished after the 2nd (pre)flash.

The oscillatory pattern after the first two (pre)flashes in the presence of APp12 is a clear low-high pattern, and matches the pattern predicted by assuming no inhibition of Q_B^{2-} to PQ electron transport. Note that the weak thermoluminescence after the 1st (pre)flash is a contribution by minor (12.5%) $S_1Q_B^-$ centers in the initial dark-adapted state, which are converted to S_2Q_B (non-luminescent) by the 1st (pre)flash and then to $S_2Q_B^-$ by 77 K illumination *via* $S_2Q_A^-Q_B$ state. Thus the possibility of blocking by APp12 of Q_B^{2-} to PQ electron transport can be excluded. After the 3rd (pre)flash, the oscillation was interrupted, showing a monotonous decline after the 4th to 6th (pre)flashes, and the interruption can now be attributed to the inhibition on the donor side. Based on these results we conclude that APp12 inhibits S_3 to S_0 transition in the water oxidation system. The mode of action of APp12 is totally different from that of regular PS II inhibitors and APp12 should be classified as a new-type inhibitor.

Discussion

The three conjugated enamine compounds examined in this study exhibited high potency in inhibiting PS II electron transport. Despite the similarity in their chemical structure (conjugated enamine system), their mode of action was not the same. Two of them, cyanoacrylate and ACm12 induced a thermoluminescence glow peak identical with the DCMU-induced Q-band in all-or-none mode (Fig. 2 and 3), indicating that they bind to the Q_B-site in D1 protein to block Q_A to Q_B electron transport. In sharp contrast to these, APp12 induced an unusual thermoluminescence glow peak located between the Q-band and B-band (Fig. 2). From the titration (Fig. 4) and oscillation (Fig. 5) experiments, it was inferred that APp12 induces a modified charge pair ($S_2Q_B^-$ and $S_3Q_B^-$ after the 1st and 2nd flashes, respectively), and thereby interrupts the S-state turnover by blocking S_3 to S_0 transition. These results clearly show that APp12 and its derivatives are a new-type inhibitors which specifically affect the electron transport in the water-oxidizing enzyme system.

Several cases are reported in which an inhibitor of Q_A to Q_B electron transport additionally affects the electron transport on donor side. Based on DCMU-induced decrease in F_{max} fluorescence yield in PS II enriched membrane fragments, Carpentier *et al.* [21] proposed that DCMU has an additional influence on the donor side electron transport. Jursinic and Stemler [22] found a decrease in atrazine-binding affinity on removal of PS II Mn, and suggested a cooperation between donor and acceptor sides in herbicide binding. Based on delayed fluorescence analyses, Pfister and Schreiber [23] proposed that ioxynil affects water oxidation in addition to inhibition of Q_A to Q_B electron transport. In view of the recent membrane spanning model of D1 protein [1] in PS II reaction center, these phenomena may be ascribable to the fact that D1 protein affords not only the Q_B-site on acceptor side but also the Mn-site on donor side. Probably, a part of the effects brought about by herbicide binding to the Q_B-site spans through the membrane to effect some structural modification of the water-oxidizing enzyme.

As to APp12, we have at present no information about its binding site. Based on the similarity in chemical structure, one may assume that APp12 binds to the Q_B-site. However, this is very unlikely in view of the fact that the two electron gate functioning of Q_B is well preserved under complete inhibition of O_2 evolution, which clearly conflicts with the recent concept of herbicide binding to the Q_B-site, replacement of Q_B quinone by herbicide molecule [1, 2]. Judging from our observations that APp12 preferentially affects the water-oxidation system, we favor to assume its binding site on the donor side of PS II, which gives rise to a downshift of the glow peak temperature of $S_2Q_B^-$ (or $S_3Q_B^-$) through an increase in oxidation potential of S_2 (or S_3), and thereby interrupts S_3 to S_0 transition [24].

In the above hypothesis, we assumed the inhibition of S-state transition by APp12 as resulted from its effects on oxidation potential of S_2 (or S_3). However, as has been briefly mentioned in result part, the cause for downshift in glow peak temperature can be alternatively ascribed to an altered property of Q_B^- instead of S_2 (or S_3) state, according to the general theory of thermoluminescence [17, 18]. In this case, we have to assume that whichever part of PS II (donor side or acceptor side) APp12 binds to, its effects influence over both sides of the thylakoid membrane, resulting in interruption of S_3 to S_0 transition on the donor side, and modulation of Q_B^- environment on the acceptor side to lower its redox potential. Photoaffinity labeling experiments with APp12 are expected to provide crucial information about these alternative hypotheses.

Apart from these considerations, our data in this paper clearly show that APp12 is a unique inhibitor which preferentially affects the functioning of water oxidation enzyme.

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