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STUDIES ON THE ADRY AGENT-INDUCED MECHANISM OF THE DISCHARGE OF THE HOLES TRAPPED IN THE PHOTOSYNTHETIC WATERSPLITTING ENZYME SYSTEM Y

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SUMMARY

The effect of 2-(3-chloro-4-trifluoromethyl)anilino-3,5-dinitrothiophene (ANT 2p) on the oxygen evolution, fluorescence and delayed light emission of spinach chloroplasts has been investigated. It was found that;

1. ANT 2p strongly accelerates the deactivation of states S_2 and S_3 of the water-splitting enzyme system Y.

2. In DCMU-poisoned chloroplasts ANT 2p prevents the back reaction of the electrons located at the primary acceptor, Q, with the holes (positive charges) stored in the water-splitting enzyme system Y.

3. In chloroplast suspensions without artificial electron acceptors, the fluorescence rise in weak actinic light vanishes in the presence of ANT 2p. The fluorescence yield in DCMU-inhibited chloroplasts is not significantly changed by ANT 2p.

4. The intensity of the delayed light emitted after excitation with one short flash is remarkably decreased by ANT 2p.

5. In weak actinic light the reduction rate of the artificial electron acceptor methyl viologen is suppressed in the presence of ANT 2p.

From these experimental results it is concluded that ANT 2p induces a cycle within the electron transport chain, leading to a dissipative recombination of the holes stored in the water-splitting enzyme Y with the electrons of an as yet unknown donor.

Two possibilities for the mode of action of this cycle are discussed.

INTRODUCTION

The oxidation of water to molecular oxygen in the photosynthetic apparatus induced by chlorophyll *a* of Photosystem II (chlorophyll a_{II}) is performed by the

Abbreviations: chlorophyll a_{II}, active chlorophyll *a* of Photosystem II; ADRY, Acceleration of the Deactivation Reactions of the water-splitting enzyme system Y; ANT 2p, 2-(3-chloro-4-trifluoromethyl)anilino-3,5-dinitrothiophene; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone.

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cooperative reaction of four positive charges (the so-called trapped holes, see ref. 1), which are stored in the water-splitting enzyme system Y[1]. With respect to the trapped hole cooperation, the Y systems are functionally independent units[2,3]. Therefore, the cooperativity of the trapped holes can be realized only by the stepwise generation (*via* chlorophyll a_{II}, see refs 4 and 5) and accumulation of four trapped holes in each system Y. Because of this mechanism by which trapped holes accumulate in the water-splitting enzyme system Y there exist different activation states, symbolized by Kok *et al.*[3] as S_0, S_1, S_2 and S_3. The oxidation of water takes place if S_4 is produced:

$$S_4 + 2H_2O \rightarrow S_0 + O_2 + 4H^+ \quad (1)$$

Only states S_0 and S_1 are stable in the dark. Trapped holes stored by S_2 and S_3 are discharged by slow deactivation reactions in the dark[6–8].

Recently it was found[9,10] that a number of substances [the agents of the Acceleration of the Deactivation Reactions of the water-splitting enzyme system Y (ADRY), see ref. 10] decrease the stability of the trapped holes. This ADRY effect was explained by the assumption, that a cyclic electron flow is induced by the ADRY agents either through conformational changes or by direct participation of ADRY molecules as electron carriers[1,11].

Although it was recently proved unequivocally[11] that in chloroplasts the ADRY effect really decreases the stability of the trapped holes, it remained to be clarified, whether this effect influences all of the active states in the water-splitting enzyme system Y in the same way or whether there exist differences in the action of the ADRY agents on the different states. Furthermore, with respect to the mechanism of the ADRY effect, two questions need to be answered;

(1) Which carrier in the electron transport chain provides the electrons for the discharge of the trapped holes?

(2) Does the ADRY agent-catalyzed discharge of the trapped holes occur *via* a true back reaction, *i.e.* does this reaction lead to the production of excited electronic states in chlorophyll a_{II} by the recombination of holes and electrons?

The results presented in this paper show that the ADRY agent ANT 2p accelerates the deactivation of the active states S_2 and S_3 in the water-splitting enzyme system Y to nearly the same degrees. Furthermore, it was concluded that the primary electron acceptor of System II, the quencher Q (which is assumed to be the component X in the electron transport chain, see ref. 12), seems to be unable to provide the electrons for the ADRY effect, which means that a short cycle from Q^- to the oxidizing side of System II induced by ADRY agents is improbable. On the other hand, fluorescence measurements in chloroplasts in the absence of an electron acceptor prove that ADRY agents induce a cyclic electron flow in the electron transport chain from an unknown donor to the system Y.

Results obtained for methyl viologen reduction indicate that the electrons are captured by the cycle which is induced by ADRY agents in preference to capture by the exogeneous electron acceptor.

From the luminescence experiments it was concluded that the discharge of the trapped holes by 2-(3-chloro-4-trifluoromethyl)anilino-3,5-dinitrothiophene (ANT 2p) does not generate excited electronic states.

In the light of these facts, possible mechanisms for the ADRY effect are discussed.

MATERIALS AND METHODS

Preparation of chloroplasts

The chloroplasts were prepared according to the method of Winget *et al.*[14], except that 10^{-2} M ascorbate was present during the grinding of the spinach. For storage in liquid nitrogen, 5% dimethylsulfoxide was added. After thawing, the activity of the stored chloroplasts was nearly the same as that of freshly prepared chloroplasts.

Reaction mixtures

The experiments were performed at pH 7.5. The reaction mixtures contained $5 \cdot 10^{-2}$ M Tris–HCl buffer, 10^{-2} M NaCl, 0.4 M saccharose. As electron acceptors 10^{-4} M $NADP^+$ and $5 \cdot 10^{-7}$ M ferredoxin were used, as well as, in Fig. 4, 10^{-4} M methyl viologen. The chlorophyll concentration of the chloroplast suspension and the amounts of ANT 2p and 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) added are indicated in the legends to the figures.

Measurements

The deactivation of the active states in the water-splitting enzyme system Y was analyzed in the same way as described by Joliot *et al.*[2]. For the measurements of the oxygen yield of each flash the highly sensitive oxygen polarographic method developed by Joliot *et al.*[15,16] was used.

The fluorescence measurements were carried out with an apparatus described by Delosme[17].

For the analysis of the delayed luminescence decay after illumination with a short flash, the method described in ref. 18 was used.

The rate of methyl viologen reduction with modulated red light (650 nm) was measured with the polarographic device mentioned above, except that the polarisation of the platinum cathode was +600 mV.

RESULTS

The influence of ANT 2p on the time course of deactivation

The deactivation kinetics of the active states S_2 and S_3 of the water-splitting enzyme system Y were obtained by the method of Joliot *et al.*[2]. The decay of S_3 can be directly determined experimentally by the measurement of the oxygen yield of the first flash, Y_1, in a sequence of short flashes after preillumination with two flashes. Since the probability of double hits, β, was negligible, S_3 is given by:

$$S_3 = \frac{1}{1-\alpha} Y_1 \qquad (2)$$

where α is the probability of missing (see refs 2 and 3). Hence, S_3 is directly proportional to Y_1 after preillumination with two flashes, so that the decay of S_3 and Y_1 are exactly the same. In Fig. 1a the oxygen yield Y_1 is shown as a function of the

time Δt between the flash sequence and the two preillumination flashes, both in the absence and in the presence of 10^{-6} M ANT 2p. It is seen that the decay rate of S_3 is enhanced in the presence of 10^{-6} M ANT 2p by at least one order of magnitude.

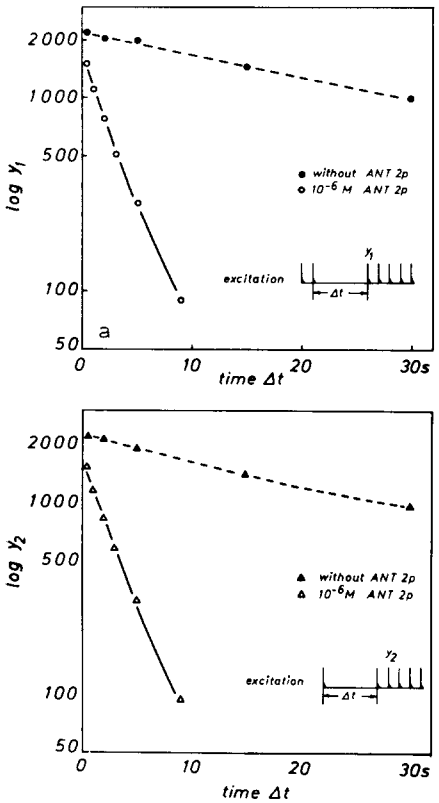

Fig. 1. (a) Oxygen yield of the first flash Y_1 in a sequence of short flashes as a function of the time Δt between the flash sequence and the second flash of two preillumination flashes in chloroplasts. The time between the two preillumination flashes was 160 ms in the presence of ANT 2p, and 320 ms in its absence. Also, between the flashes of the sequence a time of 160 ms was used in the presence of ANT 2p and 320 ms in its absence. The ANT 2p content of the buffer solution flowing through the cuvette is indicated in the figure. Chlorophyll concentration: $4 \cdot 10^{-4}$ M. Other experimental conditions are as described in Materials and Methods. Ordinate: logarithmic. (b) Oxygen yield of the second flash Y_2 in a sequence of short flashes as a function of the time Δt between the flash sequence and one preillumination flash in chloroplasts. Experimental conditions are as in (a).

In a similar way, the decay of S_2 can be obtained by the oxygen yield of the second flash, Y_2, of a short flash sequence after preillumination with one flash. Again neglecting the double hit probability β, S_2 can be evaluated by;

$$S_2 = \frac{1}{(1-\alpha)^2} Y_2 \tag{3}$$

In Fig. 1b the oxygen yield Y_2 as a function of the time Δt between the preillumination

flash and the flash sequence is depicted. A similar acceleration as for S_3 is observed for the ANT 2p induced decay of S_2.

These results clearly show that the ADRY agent ANT 2p accelerates the deactivation of the active states S_2 and S_3 of the water-splitting enzyme system Y in chloroplasts.

It should be mentioned that, in contrast to chloroplasts, algae (*Chlorella*) showed a decrease in the deactivation rate of S_2 and S_3. This effect was not investigated in detail. After showing that the decay of S_2 and S_3 is strongly accelerated in chloroplasts by the ADRY agent ANT 2p, the next step of the investigation was to search for the mechanism of this effect.

Since the states S_2 and S_3 represent 2 and 3 trapped positive charges, respectively, electrons are required for a deactivation of these states. Therefore it was interesting to look for the source of these electrons in the ADRY effect. The excitation of Photosystem II by a photon leads ultimately to a charge separation giving rise to the reduced primary acceptor (quencher Q^-) and to the oxidized primary electron donor D^+ (or designated as Z^+).

It is known that in DCMU-inhibited chloroplasts, where the reoxidation of Q^- by the electron pool is blocked, a back-reaction between Q^- and D^+ occurs, accompanied by the emission of light. With respect to the ADRY effect it was interesting to see whether this back reaction is influenced by ANT 2p.

The inhibition of the back reaction between D^+ and Q^- by ANT 2p

Information about the rate of the back reaction is obtainable by fluorescence measurements, which reflect the degree of reduction of the primary electron acceptor of System II[19,20]. DCMU-inhibited chloroplasts were excited by a short strong flash. Immediately after this flash the quencher Q is completely reduced. The reoxidation of Q^- in DCMU poisoned chloroplasts is only possible by the back reaction. At time Δt after the strong flash, the amount of the quencher remaining in the reduced state Q^- was indicated by the fluorescence yield $\Phi(\Delta t)$ in weak detecting light[19,20]. If we suppose, that $\Phi(\Delta t)$ is linearly related to Q^-, then the function $\Phi(\Delta t)$ reflects the reoxidation of Q^- by D^+.

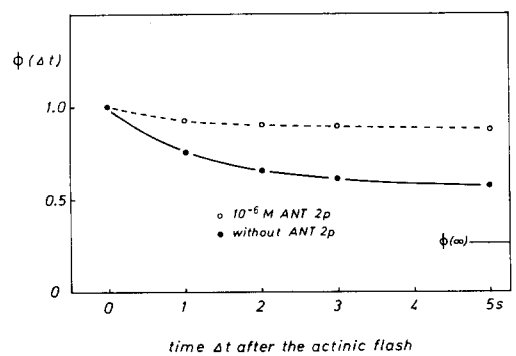

Fig. 2. Fluorescence yield $\Phi(\Delta t)$ in weak detecting light as a function of the time Δt after a saturating short flash in chloroplasts. Reaction mixture: chloroplasts ($2 \cdot 10^{-4}$ M chlorophyll), 10^{-5} M DCMU, ANT 2p added as indicated in the figure. Other substances and the experimental conditions were as described in Materials and Methods. Ordinate: arbritary units, $\Phi = 1$ is the maximal fluorescence yield reached after the saturating flash.

In Fig. 2 the fluorescence yield $\Phi(\Delta t)$ as a function of the time Δt between the strong short flash and the fluorescence measurement in weak detecting light is given. The results clearly show, that ANT 2p strongly inhibits the back reaction.

This is in agreement with experimental data obtained by Homann[21] for the ADRY agent carbonyl cyanide *m*-chlorophenylhydrazone (CCCP).

A similar effect was found for the artificial System II electron donor hydroxylamine[22]. However, this substance acts in the water-splitting enzyme system Y in a completely different way[11,23].

From this result it can be concluded that the ADRY agent ANT 2p discharges the trapped holes, but does not act as an irreversible electron donor like hydroxylamine[11]. Hence, it is reasonable to assume, that a cyclic electron flow can be induced by ANT 2p. To test this mode of action, the fluorescence induction in chloroplasts was investigated in the absence of an electron acceptor under weak and strong light excitation conditions.

The influence of ANT 2p on the fluorescence rise in the absence of electron acceptors in chloroplasts

In Fig. 3a the normal fluorescence rise in chloroplasts is shown. This rise disappears in the presence of 10^{-6} M ANT 2p, (Fig. 3b). However, at a higher intensity of actinic light the fluorescence increase is essentially normal, even in the presence of ANT 2p (Fig. 3c). On the other hand, it is shown in Figs 3d and 3e that,

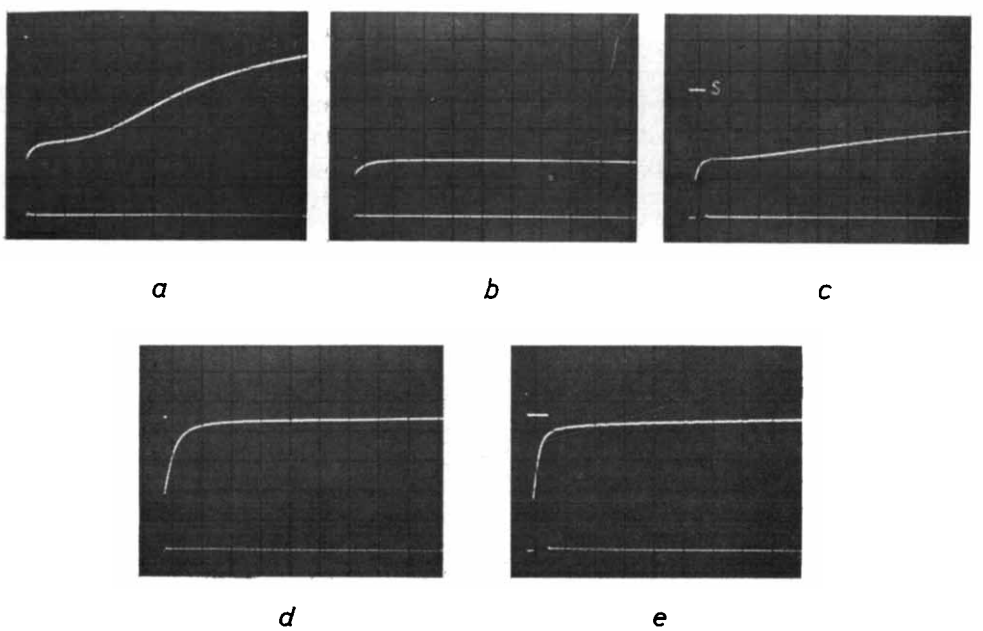

Fig. 3. Fluorescence rise in chloroplasts in continuous actinic light. The reaction mixture was the same as in Fig. 2, except for the omission of DCMU in (a), (b) and (c). (a) Weak actinic light (10^4 ergs·cm^{-2}·s^{-1}). (b) Weak actinic light as in (a), 10^{-6} M ANT 2p. (c) Strong actinic light ($2 \cdot 10^5$ ergs·cm^{-2}·s^{-1}), 10^{-6} M ANT 2p. (d) Weak actinic light as in (a), 10^{-6} M ANT 2p, 10^{-5} M DCMU. (e) Strong actinic light as in (c), 10^{-6} M ANT 2p, 10^{-5} M DCMU. S= steady-state level of fluorescence. Time scale: weak light 0.5 s per division, strong light 0.05 s per division.

in DCMU-blocked chloroplasts, ANT 2p does not significantly change the fluorescence level at different intensities of actinic light.

From these fluorescence induction measurements the conclusion can be drawn that ANT 2p induces a cyclic electron flow, which leads at low light intensities to a fast complete reoxidation of the reduced primary electron acceptor Q^-, so that the fluorescence cannot develop. At higher light intensities the reduction rate of the quencher Q is faster than the reoxidation rate of Q^-, so that an appreciable amount of the quencher becomes reduced, giving rise to the stronger fluorescence. If this cycle is really responsible for the ADRY effect, then one would expect that, in the presence of exogeneous electron acceptors, the ADRY-induced cycle competes with the artificial electron acceptors for the electrons produced by the light reactions.

The influence of ANT 2p on the rate of the methyl viologen reduction in chloroplasts

The rate of the methyl viologen reduction induced by excitation with modulated light was measured for two light intensities and at different concentrations of ANT 2p.

In Fig. 4 the stationary rate of the methyl viologen reduction is depicted. In the presence of ANT 2p a remarkable supression of the reduction rate is observed. Kok, B. (personal communication) showed that this reduction is almost one order of magnitude more rapid than the deactivation of S_2 and S_3 in the presence of 10^{-6} M ANT 2p. The inhibition of methyl viologen reduction by ANT 2p suggests that the electron source for the deactivation of S_2 and S_3 in the presence of ANT 2p is located between the two photosystems. When 10^{-6} M ANT 2p is added in the dark, there is no transitory methyl viologen reduction at the beginning of the illumination period. This effect can be explained by the assumption that either ANT 2p oxidizes P700 in the dark or that some other inhibition of System I takes place. Hence it is possible that ANT 2p can withdraw electrons from System I. Therefore, a long cycle from System I to the water-splitting enzyme system Y, induced by ANT 2p, cannot be excluded (see ref. 1), although the kinetic data mentioned above favor a shorter cycle.

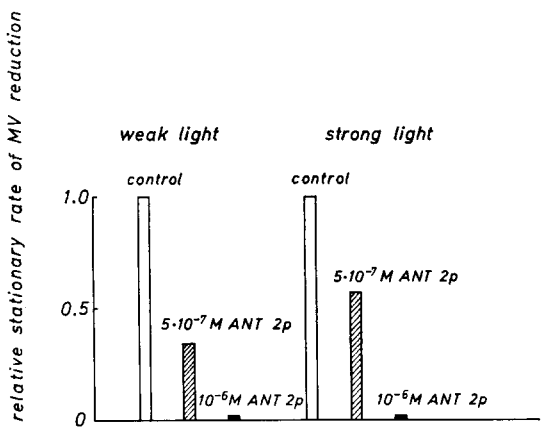

Fig. 4. Dependence of the stationary rate of the methyl viologen (MV) reduction in weak and in strong actinic modulated light (λ= 650 nm) on the ANT 2p added. Chlorophyll concentration: $4 \cdot 10^{-4}$ M. 10^{-3} M NH_4Cl was added as uncoupler, other experimental conditions are as described in Materials and Methods. Control rates: 320 in weak modulated light ($5 \cdot 10^3$ ergs·cm^{-2}·s^{-1}) 1780 in strong modulated light ($4 \cdot 10^4$ ergs·cm^{-2}·s^{-1}).

The influence of ANT 2p on the delayed light emission

It was shown[18] that normal deactivation in System II produces excited electronic states giving rise to a delayed light emission. Therefore, the question arises whether the ANT 2p-induced cycle generates electronic excited states also. The delayed light emitted after excitation with a short flash was measured in the time range of 1 ms to 80 s in the absence and in the presence of 10^{-6} M ANT 2p. At times shorter than 10 ms the artefact caused by the short exciting flash was significant. Therefore, in Fig. 5 the ratio of the luminescence intensities observed in the presence of 10^{-6} M ANT 2p and in its absence L(ATN 2p)/L(normal) is given only for $t \geqslant 10$ms. The results show that with increasing time t the ratio decreases until a nearly steady state is reached. A small increase in the ratio occurs at times $t > 10$ s.

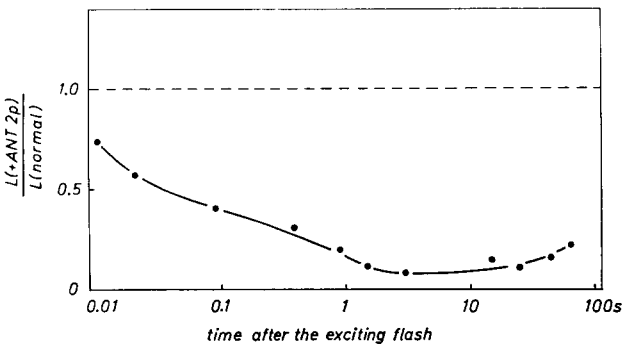

Fig. 5. Ratio of the delayed luminescence intensity L(ANT 2p)/L(normal) emitted after a short flash in the presence of 10^{-6} M ANT 2p and in its absence as a function of the time after the flash. The reaction mixture was the same as in Fig. 2, except for the omission of DCMU. Time scale: logarithmic.

According to Joliot *et al.*[2] the luminescence is given by:

$$L = k[\text{ox}]\cdot[\text{red}]\cdot r\cdot\varphi \qquad (4)$$

where k is the rate constant; [ox] and [red] are the concentrations of the primary oxidant and reductant of System II, respectively; r is the probability for the generation of an excited singlet state by the normal deactivation process; and φ is the probability for photon emission from the excited state.

From the above-mentioned fluorescence measurements in the presence of DCMU, it can be concluded, that ANT 2p does not significantly change the fluorescence yield of the excited chlorophyll singlet state itself. If we additionally assume that in the absence of DCMU the state of the quencher Q after one exciting short flash is nearly the same in the absence and in the presence of 10^{-6} M ANT 2p, φ can be suggested as being constant in a first order approximation. Since the half time for the reoxidation of Q is about 0.6 ms[24–26] this assumption is reasonable for the above mentioned time range of $t > 10$ ms. Furthermore, one can propose that the concentrations of [ox] and [red] produced by the single short exciting flash are practically the same irrespective of the presence or absence of ANT 2p. But, in the presence of 10^{-6} M ANT 2p a much faster decay of S_2 and S_3 is observed (Fig. 1).

This indicates an accelerated reduction of oxidizing equivalents stored on S_2 and S_3 by electrons of an unknown source, *i.e.*

$$(k \cdot [ox] \cdot [red])_{ANT\,2p} > (k \cdot [ox] \cdot [red])_{normal}$$

is valid. Hence, the delayed light intensity — especially in the time range where the ADRY effect becomes significant — is only explainable by a new deactivation process with a low probability for the generation of excited electronic states.

DISCUSSION

The results obtained in the present study show that the ADRY agent ANT 2p accelerates the deactivation of the states S_2 and S_3 in the water-splitting enzyme system Y. This corroborates earlier results obtained by an indirect method[11]. It should be mentioned that the values obtained here for the deactivation rate of S_2 and S_3 in the presence of 10^{-6} M ANT 2p are smaller than those which would be expected from earlier results[10]. These quantitative differences in the degree of the ADRY effect are explainable by the fact that ANT 2p is partially transformed to an inactive form at the blank platinum electrode.

As in normal chloroplasts in the absence of ADRY agents, there are no differences in the stability of S_2 and S_3 in the presence of ANT 2p.

The results which indicate that the stability of S_1 is not influenced by ANT 2p in the same way (unpublished) provide further evidence for the fact that S_1 is completely different in its nature in comparison to S_2 and S_3. Hence, it is reasonable to assume that S_1 is at a much lower potential than S_2 and S_3.

Whether the mechanism for deactivation of the trapped holes in the presence of ANT 2p is a one step deactivation in the form

$$S_3 \rightarrow S_2 \qquad S_2 \rightarrow S_1$$

or a two step deactivation

$$S_3 \rightarrow S_1 \qquad S_2 \rightarrow S_0$$

cannot be decided by the present experimental data.

Similar observations to those made by Joliot *et al.*[2] lead to the conclusion that in the presence of ANT 2p, as well as in its absence, the deactivation reactions in the water-splitting enzyme system Y are neither pure one step nor pure two step processes.

It was found by Joliot *et al.*[2] that the long-time delayed fluorescence decay (>1 s after the flash) is correlated with the rate of the decrease of S_3. Hence, it was concluded that the deactivation of the states S_2 and S_3 is accompanied by the generation of excited electronic states. The present results show that the strong acceleration of the decay rates of S_2 and S_3 leads to a nearly 10-fold decrease of the delayed luminescence emission in the same time range. Therefore, the ADRY effect is not a pure catalytic effect in the sense that it only decreases the activation energy barrier of the natural deactivation processes of the states S_2 and S_3. The ADRY effect avoids the generation of excited electronic states.

The difference in both mechanisms (the normal and the ADRY-induced de-

activation) is also shown by the fact that the ADRY agent ANT 2p inhibits the back reaction between D^+ and Q^- in DCMU-poisoned chloroplasts.

Hence, one can conclude that whereas in normal chloroplasts the back reaction between D^+ and Q^- is preferred, the ADRY effect opens a pathway for funneling electrons from another electron source to the system Y. This electron donor is much more effective than the reduced primary acceptor Q^-. The open question to be answered now is: Which substance is this effective electron donor?

The possibility of an irreversible electron donor function of ADRY agents was rejected for stoichiometrical reasons[11]. Therefore, only an endogeneous electron donor can be responsible for the discharge of the trapped holes. The present fluorescence measurements in the absence of electron acceptors prove that the ADRY agent ANT 2p induces an electron cycle. Through the methyl viologen reduction experiment it was shown that the ANT 2p-induced cycle suppresses very effectively the electron transport to System I electron acceptors. Kinetic arguments (fast reduction of methyl viologen by P700 in the absence of ANT 2p) favor the location of the electron donor for the ADRY effect between the primary acceptor of System II (Q) and P700. However, an electron donor located on the reducing side of System I cannot be totally excluded.

For a discussion of possible pathways of the ADRY agent-induced cycle, the structural organization of the electron transport chains in the thylakoid membrane must be taken into account. Although the detailed structure of the thylakoid membrane remains to be elucidated, two important facts seem to be clear;

(1) The electron transport chains are not functionally isolated entities[27,28].

(2) The electron transport pathway is anisotropically arranged within the thylakoid membrane[29,30].

If we take into account these facts, then irrespective of the details of the 3-dimensional array of the electron transport carriers within the thylakoid membrane, three possible pathways for the discharge of the trapped holes stored in the water-splitting enzyme system Y have to be considered (Fig. 6);

(a) A short circuit between Q^- and the holes in system Y. This way is symbolized by "A".

(b) A longer circuit from an electron donor located between Q and P700 to the system Y. This pathway is symbolized by "B" in Fig. 6.

(c) It is also possible that the discharge occurs through an energy barrier E which separates the reducing side of System I from the oxidizing side of System II within the 3-dimensional array of the whole thylakoid membrane. This discharge possibility of the trapped holes is indicated by "C" in Fig. 6.

If one envisages an electron transport chain system connected by a common plastoquinone pool[27] as a functional entity, then in respect to this entity the pathways A and B can be considered as "intra-transport-chain" dissipative pathways, whereas C, which connects different electron transport chain entities, can be suggested as being an "inter-transport-chain" dissipative pathway.

From the fact that the ADRY agent ANT 2p inhibits the back reaction in DCMU-treated chloroplasts one can conclude that the reaction pathway A is not responsible for the ADRY effect. Therefore, ANT 2p reduces either the potential barrier E for the inter-transport-chain dissipation C, or the wall W for the intra-transport-chain dissipative pathway B.

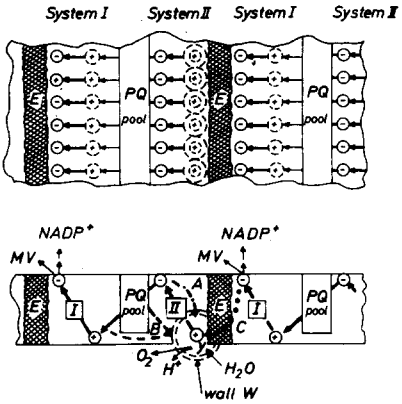

Fig. 6. Scheme of a matrix array of electron transport chains within the thylakoid membrane. Upper part: top view. Lower part: side view. The negative and the positive charges represent the primary electron acceptor and the primary electron donor of both light reactions, respectively. E= energy barrier between the System I acceptor side of one array of electron transport chains (connected by a common plastoquinone pool) with the donor side of System II of a contiguous array of electron transport chains. W= potential wall surrounding the storage device of the holes within the water-splitting enzyme system Y. A represents the back reaction of System II. B symbolizes an artificial intra-transport-chain induced by ANT 2p. The electron donor for this cycle is located between plastoquinone and P700. C symbolizes an artificial inter-transport-chain across the barrier E induced by ANT 2p. The intermediates between the light reactions and the plastoquinone pool (*e.g.* plastocyanin and cytochrome *f*) are omitted in the scheme. MV, methyl viologen; PQ, plastoquinone.

If pathway C exists, then the ADRY effect could be envisaged as an electrical short circuit within the whole thylakoid membrane.

Further investigations are required to explore these two possibilities and to show the nature of the electron donor for the discharge of the trapped holes more clearly.

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