The light-intensity-dependence of the efficacy of 2-(3-chloro-4-trifluoromethyl)-anilino-3,5-dinitrothiophene (Ant2p) to inhibit the photosystem 2 reactions of chloroplasts

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The mechanism by which Ant2p [2-(3-chloro-4-trifluoromethyl)anilino-3,5-dinitrothiophene] inhibits the oxygen evolution capacity of chloroplasts is thought to be due to a rapid reduction of the S_2 and S_3 oxidation states of the oxygen-evolving complex mediated by the oxidation of endogenous donors such as cytochrome b_{559} . The results presented in this paper show that the degree of inhibition by Ant2p of the photosystem 2-supported electron transfer reactions, registered by the light-dependent rate of dichlorophenolindophenol reduction, varies according to the actinic light intensity. Moreover, a similar intensity-dependence can be detected in the extent of the Ant2p-induced cytochrome $b_{559\text{HP}}$ photo-oxidation. We show, however, that the dependence of the cytochrome oxidation is not due to the oxidation *per se*, but reflects changes in the high light-driven re-reduction reaction. The close correlation between the two Ant2p reactions is interpreted as indicating that the effect of Ant2p might be due to an inhibition of the S-state turnovers and not necessarily due to a deactivation process.

ADRY reagents, such as Ant2p, are potent inhibitors of the oxygen evolution capacity of chloroplasts (Homann, 1971; Renger, 1971) which are able to exert their effect at concentrations stoichiometric to the number of photosystem 2 complexes present (Renger, 1972). Although the mechanism of their inhibition remains uncertain, it is generally accepted that ADRY reagents can interfere with the redox reactions on the oxidizing side of the photosystem 2 reaction centre (Renger & Reuter, 1982; Ghanotakis *et al.*, 1982). This activity was first demonstrated in studies which showed that ADRY reagents cause changes in the oxygen yield of flash-activated chloroplasts with a flash frequency-dependence which would be consistent with a fast ($t_1 \sim 250$ ms; Renger & Reuter,

1982) re-reduction of the S_2 and S_3 oxidation states (see Joliot & Kok, 1975) of the oxygen-evolving complex (Renger *et al.*, 1973). Such redox-active behaviour also accounts for the observation that ADRY reagents added to chloroplasts block the back reaction from the bound plastoquinone photosystem 2 acceptor to the S_2 state (Homann, 1972; Renger & Inoue, 1983). This electron donation capability of ADRY reagents is not restricted to the S_2 and S_3 states; for example, in Tris-washed chloroplasts, conditions known to inactivate the oxygen-evolving complex, the re-reduction of the intermediary donor Z (see Bouges-Bocquet, 1980) is accelerated by Ant2p (Ghanotakis *et al.*, 1982; Renger & Reuter, 1982).

The apparent destabilization of the S_2 and S_3 states by ADRY reagents is, however, not believed to be due to a direct reduction of Z^+ , because the measured rates of electron transfer between Z and the oxygen-evolving complex are known to be faster than the deactivation reactions (Ghanotakis *et al.*, 1982). Moreover, since Ant2p is efficacious at low concentrations, the deactivation of the higher S-states cannot result from an irreversible oxidation of the ADRY reagent. ADRY reagents can, however, induce the appearance of a light-

Abbreviations used: Ant2p, 2-(3-chloro-4-trifluoromethyl)anilino-3,5-dinitrothiophene; cytochrome $b_{559\text{HP}}$, the high redox potential form of cytochrome b_{559} ; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DCPIP, 2,6-dichlorophenolindophenol; P_{680} , the reaction centre chlorophyll of photosystem 2; ADRY reagents are a class of photosystem 2 inhibitors denoted for their apparent ability to accelerate the deactivation reactions of the oxygen-evolving complex.

dependent oxidation of cytochrome $b_{559\text{HP}}$ (Ben-Hayyim, 1972; Heber *et al.*, 1979; Maroc & Garnier, 1979) and carotenoid (Velthuys, 1981; Schenck *et al.*, 1982), but there remains no unambiguous proof for a direct role of these endogenous donors in the ADRY effect (see Renger & Eckert, 1981).

Nevertheless, in an attempt to relate these ADRY reagent-induced reactions, several authors have devised electron transfer pathways in which the ADRY reagents mediate the transfer of reducing equivalents from the endogenous donors either to the higher S-states or, under conditions where the oxygen-evolving complex is inactivated, to the oxidized Z (Sayre & Homann, 1979; Ghanotakis *et al.*, 1982). It is envisaged that this alternative pathway can only compete with the oxygen-evolving complex for the oxidizing equivalents generated by the photosystem 2 reaction centre when the individual S-states are long-lived. These models neglect the observation that ADRY reagents can also inhibit the light-dependent reduction of added electron acceptors of photosynthetic electron flow (e.g. Renger *et al.*, 1973). Such behaviour has been interpreted as indicating that the ADRY reagents may induce a cyclic flow around photosystem 2, possibly involving cytochrome $b_{559\text{HP}}$.

In the present report we show that there is a close correlation between the Ant2p-induced inhibition of photosystem 2 photochemical activity and the apparent extent of the photo-oxidation of cytochrome $b_{559\text{HP}}$. We have found that the extent of the photo-oxidation, however, is determined by its rate of DCMU-sensitive re-reduction; a reaction which is promoted at high light intensities under conditions where the inhibition of photosystem 2 by Ant2p is attenuated. These results are discussed in terms of the proposed mechanism of action of ADRY reagents on photosystem 2 activity.

Materials and methods

Intact chloroplasts were obtained from *Pisum sativum* (Feltham First) by standard methods (Chapman *et al.*, 1983), and were suspended at 1–2 mg of chlorophyll/ml concentration in 0.5 mM-Tris/HCl (pH 7.5)/0.33 M-sorbitol/3 mM-MgCl₂ and stored on ice until use.

The light-induced absorption changes of chloroplast suspensions were carried out by using a Perkin-Elmer double-beam dual-wavelength spectrophotometer (model 557) modified to permit side-illumination of the cuvette. The cytochrome absorption changes at 560–540 nm ($\Delta\epsilon = 15 \text{ mM}^{-1} \cdot \text{cm}^{-1}$) were measured in a low-salt buffer medium (150 mM-sucrose/5 mM-NaCl/10 mM-phosphate buffer, pH 7.4) with os-

motically-shocked chloroplasts at a concentration of 50–80 $\mu\text{g/ml}$. Photosystem 2 photochemical activity was assayed by the rate of DCPIP reduction ($\Delta\epsilon_{560-520} = 6.8 \text{ mM}^{-1} \cdot \text{cm}^{-1}$) in the same low-salt buffer supplemented with 0.05 mM-DCPIP and at 10–40 μg of chlorophyll/ml (Packham & Barber, 1983). The actinic light was provided by an Intralux 150 H light source fitted with a Schott RG 658 cut-off filter (100% light intensity of $55 \text{ W} \cdot \text{m}^{-2}$) and the photomultiplier of the spectrophotometer was shielded by a 4 mm Corning 4-96 filter.

Results

Dependence of the inhibition efficacy of Ant2p on the frequency of photosystem 2 turnovers

The ability of Ant2p to act as an inhibitor of photosystem 2 photochemical activity is demonstrated from its effect on the light-limited rate of DCPIP reduction (Fig. 1). At a concentration which is equivalent to about 4 molecules to each photosystem 2, based on a ratio of 600 chlorophyll molecules/photosystem 2 reaction centre, Ant2p causes a severe attenuation (measured as a 70% inhibition) of the DCPIP reduction rate, a result which confirms the previously-noted potency of this ADRY reagent (Renger, 1972). The initial fast transient we observe in the absorption decrease at 560–520 nm, when Ant2p is present, is not the result of a latency in the inhibition of the DCPIP

Fig. 1. Effect of Ant2p on the light-dependent rate of DCPIP reduction

Experimental conditions were as described in the Materials and methods section. The upward- and downward-directed arrows represent the onset and cessation of illumination ($5 \text{ W} \cdot \text{m}^{-2}$). (a) No further additions; the reduction rate corresponds to $18 \mu\text{mol}$ of DCPIP/h per mg of chlorophyll (b) + $0.27 \mu\text{M}$ -Ant2p (3.6 molecules of Ant2p/600 chlorophyll molecules); (c) as (b) + $50 \mu\text{M}$ -DCMU; (d) as (b) + 0.5 mM -diphenylcarbazine.

reduction rate, but may be due to the induction of cytochrome $b_{559\text{HP}}$ photo-oxidation by the inhibitor, a conclusion supported by the fact that the addition of 0.2 mM-ferricyanide removes this transient, probably by causing the chemical oxidation of the cytochrome (see below). The restoration of photochemical activity by diphenylcarbazide (Fig. 1d) indicates that the Ant2p inhibition occurs on the donor electron transfer pathway of photosystem 2 at a site probably close to the catalytic centre of the oxygen-evolving complex (see Packham & Barber, 1983). As Fig. 1(c) shows, both the water-supported and, in the presence of Ant2p, the diphenylcarbazide-supported steady-state rates of DCPIP reduction are abolished by the addition of DCMU.

The sensitivity of the photosystem 2 photochemical activity to Ant2p, as revealed by the concentration dependence, is shown in Fig. 2. The efficacy of Ant2p is markedly affected by the intensity of actinic light; the inhibition is greatest at the lowest light intensities. At an intensity which elicited a maximal reduction rate of $114 \mu\text{mol}$ of DCPIP/h per mg of chlorophyll, the half-maximum inhibition was obtained at $1.1 \mu\text{M}$ -Ant2p. With a lower intensity, which promoted a maximal reduction rate of only $18 \mu\text{mol}$ of DCPIP/h per mg of chlorophyll, $0.18 \mu\text{M}$ -Ant2p was sufficient to produce the same degree of inhibition (Fig. 2a).

The correlation between the Ant2p-induced inhibition and the photosystem 2 turnover rate is examined in more detail in Fig. 2(b) where the inhibition caused by the addition of different levels of Ant2p is plotted against the rate of DCPIP reduction in untreated chloroplasts, obtained by adjusting the light intensity over a wider range. Again, it is clear that relatively high concentrations of Ant2p (>18 molecules/600 chlorophyll molecules) are necessary for inhibition under conditions where the photosystem 2 turnover rate is fast, an observation also detected when diphenylcarbazide was present as an electron donor. A similar intensity-dependence of Ant2p inhibition was observed when the photosystem 2 photochemical activity was monitored by measuring the rate of oxygen evolution with 1 mM-benzoquinone and 1 mM-ferricyanide as electron acceptors.

The inhibition of the DCPIP reduction rate by Ant2p at limiting light conditions can be partially reversed by the addition of cations to the suspension medium. For example, incubation of the chloroplasts with 4 mM- MgCl_2 was found to result in only a 10% inhibition by Ant2p (concentration of 1.7 molecules/600 chlorophyll molecules), compared with an inhibition value of 35% obtained under low-salt conditions. In addition it was also found that the control rates were stimulated by

Fig. 2. Inhibition by Ant2p of the water-to-DCPIP electron transfer

(a) Concentration dependence. The control rates in the absence of inhibitor were measured as 114.4 and $18.2 \mu\text{mol}$ of DCPIP/h per mg of chlorophyll for the high (HL, $55 \text{ W} \cdot \text{m}^{-2}$) and low (LL, $5 \text{ W} \cdot \text{m}^{-2}$) light intensities, respectively. (b) Turnover-rate dependence. The degree of inhibition caused by the addition of Ant2p was plotted against the uninhibited control rate for samples illuminated at different light intensities: 1, $0.09 \mu\text{M}$ -Ant2p (1.8 Ant2p molecules/600 chlorophyll molecules); 2, $0.18 \mu\text{M}$ -Ant2p; 3, $0.87 \mu\text{M}$ -Ant2p; 4, $4.6 \mu\text{M}$ -Ant2p.

4 mM- Mg^{2+} (from 45 to $94 \mu\text{mol}$ of DCPIP/h per mg of chlorophyll). Although such a cation effect has not been carefully documented in terms of species and valency dependency, our preliminary

Fig. 3. Inhibition by Ant2p of the hydroxylamine-supported DCPIP reduction

Experimental conditions were as for Figs. 1 and 2, except for the addition of 5 mM-hydroxylamine (pH 7). (a) Concentration dependence. The control rates were measured as 137 and 74 μmol of DCPIP/h per mg of chlorophyll at two light intensities. (b) Turnover dependence. The inhibition caused by 0.6 μM -Ant2p (16.3 molecules/600 chlorophyll molecules) was plotted against the uninhibited control rate for samples illuminated at different light intensities.

results suggest that it may involve electrostatic screening of the membrane surface charges. It is probable that the presence of cations brings about a decrease in energy transfer to photosystem 1, and thereby augments the amount of excitation energy which arrives at the photosystem 2 reaction centre at limiting light intensities (Barber, 1980). Thus, the decreased potency of Ant2p in the presence of 4 mM- Mg^{2+} may arise from the increased turnover rate of photosystem 2.

Fig. 3 shows the effect of Ant2p on the DCPIP reduction supported by hydroxylamine oxidation. The addition of 5 mM-hydroxylamine is sufficient to abolish the oxygen-evolution capacity of chloroplasts, but since reducing equivalents supplied by hydroxylamine can enter the donor pathway the photochemical activity of photosystem 2 is not adversely affected. We have found that the DCPIP reduction rate under these conditions is attenuated by high concentrations of Ant2p (Fig. 3a), with a half-maximal inhibition at 1.2 μM . The inhibition by Ant2p is not, however, increased at low light intensities, as it was in Fig. 2(a) where water was the donor. Indeed, Fig. 3(b) shows that at very low light intensities inhibition is less than that observed at high light. Such a result emphasizes the difference between water and hydroxylamine acting as donors in photosystem 2. It should, however, be noted that the sensitivity of the hydroxylamine-supported rate to Ant2p concentrations is comparable with that of the water-to-DCPIP rate when measured at high light intensities.

The pre-treatment of chloroplasts with 0.125%

Fig. 4. Effect of glutaraldehyde on the intensity-dependent Ant2p inhibition

Experimental conditions were as for Figs. 1 and 2, except that the chloroplasts were incubated with 0.125% glutaraldehyde for 2 min before the assay of activity. The degree of inhibition of the DCPIP reduction rate caused either by (a) 0.09 μM (0.6 Ant2p molecules/600 chlorophyll molecules) or (b) 0.46 μM (2.8 Ant2p molecules/600 chlorophyll molecules) was measured at different actinic light intensities.

glutaraldehyde, which has little effect on the rate of DCPIP reduction supported by water oxidation, did not protect the photosystem 2 complexes from inhibition by Ant2p. Also, as Fig. 4 shows, after glutaraldehyde treatment the inhibition by the ADHY reagent is not increased as the light

intensity is decreased, as was found in Fig. 2(b). This result suggests that the turnover rate-dependence of the Ant2p inhibition of water-to-DCPIP electron transfer may involve conformational changes in the donor chains of photosystem 2.

Ant2p-induced photo-oxidation of cytochrome $b_{559\text{HP}}$

As shown in Fig. 1, the addition of Ant2p introduces a relatively fast absorption decrease at 560 nm, which is not thought to be associated with the light-dependent reduction of DCPIP. This absorption change is examined in more detail in Fig. 5, where no DCPIP has been added. The absorption decrease is strictly dependent on the

presence of Ant2p (Fig. 5b) and low actinic light intensities, and is abolished by pre-treatment of the chloroplasts with 0.2 mM-ferricyanide (Fig. 5c). From the light-induced difference spectrum (Fig. 6b) and from the sensitivity of the signal to mild oxidants, we identify the absorption decrease to register the photo-oxidation of the high potential form of cytochrome b_{559} . Although the extent of the oxidation can correspond to 2 haem/600 chlorophyll molecules, such maximal changes are detected only in chloroplasts supplemented with 0.2 mM-hydroquinone.

The dependence of the photo-oxidation of cytochrome $b_{559\text{HP}}$ on the concentration of Ant2p is shown in Fig. 7. Both the extent and the kinetics of the oxidation increase as the Ant2p concentration is raised. A maximal oxidation of the cytochrome was observed at an Ant2p concentration well below the estimated number of photosystem 2 centres (less than 0.3 Ant2p molecules per 600

Fig. 5. Ant2p-induced cytochrome $b_{559\text{HP}}$ absorption changes

Experimental conditions were as described in the Materials and methods section. The absorption changes at 560–540 nm invoked by low (LL, $5\text{ W}\cdot\text{m}^{-2}$) or high (HL, $55\text{ W}\cdot\text{m}^{-2}$) light intensities were measured. (a) No further additions; (b) $+0.91\text{ }\mu\text{M}$ -Ant2p (5.9 Ant2p molecules/600 chlorophyll molecules); (c) as (b) $+0.2\text{ mM}$ -ferricyanide; (d) as (b) $+50\text{ }\mu\text{M}$ -DCMU.

Fig. 6. Light-induced difference absorption spectrum of cytochrome $b_{559\text{HP}}$

Conditions were as for Fig. 5(b). The extents of the (a) high (HL, $55\text{ W}\cdot\text{m}^{-2}$) and (b) low (LL, $5\text{ W}\cdot\text{m}^{-2}$) light-induced absorption increase and decrease, respectively, were plotted against the measuring wavelength.

Fig. 7. Effect of Ant2p on the photo-oxidation of cytochrome $b_{559\text{HP}}$

Experimental conditions as for Fig. 5, except for a chlorophyll concentration of 0.18 mg/ml. The upward- and downward-directed arrows represent the onset and cessation of low-intensity ($5\text{ W}\cdot\text{m}^{-2}$) light, respectively. (a) No addition; (b) $+0.05\text{ }\mu\text{M}$ -Ant2p (0.15 Ant2p molecules/600 chlorophyll molecules); (c) $+0.1\text{ }\mu\text{M}$ -Ant2p (0.3 Ant2p molecules/600 chlorophyll molecules); (d) $+1.0\text{ }\mu\text{M}$ -Ant2p (3.0 Ant2p molecules/600 chlorophyll molecules).

chlorophyll molecules), indicating perhaps that the inhibitor has sufficient lateral mobility in the membrane to visit several centres within the duration of the illumination period. The oxidation has first-order kinetics, with a half-time at saturation levels of Ant2p of 1.5s.

Figs. 5 and 6(a) show that when high actinic intensities were used, the oxidized cytochrome $b_{559\text{HP}}$ was re-reduced. This reaction is completely suppressed by the addition of DCMU (Fig. 5d), which implicates the plastoquinone pool in the reduction process. Such a conclusion is supported by the observation that the high light-dependent reduction of cytochrome $b_{559\text{HP}}$ was also inhibited in chloroplasts treated with ferricyanide (Fig. 5c), a condition where the plastoquinone pool would be maintained in an oxidized state.

The addition of 0.5 mM-diphenylcarbazide was found to have little effect on either the extent or the kinetics of the light-dependent cytochrome $b_{559\text{HP}}$ absorption changes (results not shown). Treatment of the chloroplasts with 5 mM-hydroxylamine, however, abolishes the photo-oxidation. It is

Fig. 8. Intensity-dependence of the cytochrome $b_{559\text{HP}}$ photo-oxidation

The extent of the net absorption change at 10s after the onset of illumination was plotted against the actinic light intensity ($100\% = 55\text{ W}\cdot\text{m}^{-2}$). (a) $+0.1\text{ }\mu\text{M}$ -Ant2p (0.24 Ant2p molecules/600 chlorophyll molecules); (b) $+1.0\text{ }\mu\text{M}$ -Ant2p (2.4 Ant2p molecules/600 chlorophyll molecules); (c) $+10.0\text{ }\mu\text{M}$ -Ant2p (24 Ant2p molecules/600 chlorophyll molecules).

probable that the loss of the signal under these conditions is due to the conversion by hydroxylamine of the high-potential cytochrome b_{559} to its denatured low-potential form (see Bendall, 1982).

The intensity dependence of the cytochrome $b_{559\text{HP}}$ redox reactions is examined in detail in Fig. 8. At reduced light intensities a low concentration of Ant2p (0.24 molecules/600 chlorophyll molecules) is essentially as effective as high concentrations (>1 molecule/600 chlorophyll molecules) in promoting the oxidation of cytochrome b_{559} . At high light intensities, however, high concentrations of Ant2p are necessary in order to detect a photo-oxidation. Upon the addition of DCMU, the extent of photo-oxidation of cytochrome b_{559} is halved (Fig. 5d) and relatively insensitive both to the amount of added Ant2p and to the actinic light

Fig. 9. Correlation between the Ant2p inhibition of the DCPIP reduction rate and the extent of the cytochrome *b*_{559HP} re-reduction

The degree of inhibition caused by 0.18 μ M-Ant2p on the DCPIP reduction rate (as for Fig. 2b) and the extent of the re-reduction of cytochrome *b*₅₅₉ (as for Fig. 5b) were measured at several actinic light intensities.

intensity. These results suggest that the extent of the apparent photo-oxidation is determined by a DCMU-sensitive re-reduction which becomes saturated at light intensities higher than those necessary for the oxidation of cytochrome *b*_{559HP}.

The extent of the cytochrome *b*_{559HP} oxidation is also attenuated by the addition of divalent cations (results not shown), in line with our observation that similar conditions decrease the efficacy of the Ant2p inhibition of photosystem 2 photochemical activity. Fig. 9 shows, however, that the extent of the re-reduction of the photo-oxidized cytochrome *b*_{559HP} does not show a linear correlation with the degree of inhibition of the DCPIP reduction rate, but rather fits a sigmoidal relationship. Even so, under conditions which cause a half-maximal inhibition of photosystem 2 activity, we detect a net oxidation of one haem/600 chlorophyll molecules.

Discussion

In this report we have shown that the addition of low concentrations of Ant2p to chloroplasts can cause the inhibition of photosystem 2-supported DCPIP reduction and promote the photo-oxidation of the high potential form of cytochrome *b*₅₅₉. The location of the inhibition caused by low concentrations of Ant2p is presumed to be the oxygen-evolving complex, since diphenylcarbazide, which can donate electrons to the intermediary carrier Z (Babcock & Sauer, 1975), is able to overcome the effect.

The inhibition of the DCPIP reduction rate was found to depend not only on the concentration of Ant2p, but also on the actinic light intensity. The efficacy of low concentrations of Ant2p was increased at light intensities where the photosystem 2 turnover was rate limiting. Although such low Ant2p concentrations did not affect the hydroxylamine-supported reduction rate, the inhibition caused by higher concentrations was found not to increase as the actinic light intensity was decreased. Since water oxidation, unlike hydroxylamine oxidation (Bouges-Bouquet, 1973), involves charge accumulation, these results implicate the higher S-states in the turnover-rate dependence of the Ant2p inhibition and suggest that the behaviour of low concentrations of Ant2p observed under steady-state illumination is akin to the ADRY reagent effect detected in single-turnover studies (Renger, 1972). At high levels of Ant2p, however, other effects may occur, since not only is the water oxidation inhibited in bright light but also the ability of hydroxylamine and diphenylcarbazide to maintain photosystem 2 activity. The apparent extent of the cytochrome *b*_{559HP} photo-oxidation is also affected by the actinic light intensity; the amplitude of the oxidation increased as the intensity decreased. This intensity-dependent effect has also been reported by Heber *et al.* (1979) and Maroc & Garnier (1979). Such a result might suggest that the oxidation of the haem is coupled to the degree of inhibition of photosystem 2 caused by Ant2p. The amplitude of the photo-oxidation of cytochrome *b*_{559HP} in the presence of DCMU, however, was insensitive to the actinic light intensities employed in this study. Moreover, the re-reduction of the photo-oxidized cytochrome *b*_{559HP} is inhibited by DCMU, suggesting that the intensity dependence is determined by the re-reduction reaction and not by the oxidation *per se*. We have found a close correlation between the extent of the re-reduction of the photo-oxidized haem and the degree of inhibition of photosystem 2 activity, but the precise relationship is not linear.

The observation that ADRY reagents inhibit the light-dependent reduction of photosystem 2 electron acceptors does not seem to fit with the models which suggest that the deactivation of the S-states is coupled to the oxidation of endogenous donors (Sayre & Homann, 1979; Ghanotakis *et al.*, 1982). Although such models readily explain the loss of the oxygen evolution capacity of chloroplasts, the light-driven electron transfer reactions from the site of water-splitting to the reaction centre and beyond are presumed to be unaffected. In view of our observation that the DCPIP reduction rate is impaired by Ant2p, we might suggest that the ADRY reagent stimulates a cyclic process around photosystem 2 involving cyto-

chrome $b_{559\text{HP}}$. The DCMU sensitivity of the re-reduction of the photo-oxidized cytochrome $b_{559\text{HP}}$ would indicate that this cyclic process involves the plastoquinone pool, a possibility already considered by Renger *et al.* (1973). Our observation that ferricyanide abolishes the light-dependent reduction of cytochrome b_{559} (see Fig. 5) suggests that a simple cyclic mechanism around photosystem 2, induced by Ant2p inhibition, may not operate in the presence of added oxidants of the plastoquinol pool. A possible alternative explanation to account for the inhibition of DCPIP reduction is to propose that Ant2p inhibits the formation of the high S-states rather than promotes their deactivation. This would inhibit electron flow to the photo-oxidized P_{680}^+ , and the re-reduction of cytochrome $b_{559\text{HP}}$ would be impeded due to the lack of reducing species. With bright light the inhibition caused by ADRY reagents is somewhat relieved, resulting in an increased rate of electron flow and a re-reduction to cytochrome $b_{559\text{HP}}$.

Support for this proposal has recently come from a study of the re-reduction of P_{680}^+ using flash spectroscopy (N. K. Packham & R. C. Ford, unpublished work). Using isolated membranes enriched in photosystem 2 and treated with low concentrations of Ant2p, it was found that the amplitude of the fast phase was decreased as the flash frequency was lowered, a result that might indicate that the electron flux to P_{680}^+ is inhibited at low turnover rates.

A turnover-rate dependency of Ant2p inhibition of photosystem 2 activity is similar to the effect produced by the addition of cation chelators to inverted thylakoid membranes (Packham & Barber, 1983, 1984). In these earlier studies, the intensity-dependence of the inhibition was explained in terms of a structural reorganization of the donor chains of photosystem 2 incurred by the removal of a loosely bound Mn^{2+} pool (Packham & Barber, 1984). The turnover dependence was assumed to arise from the competition between the back reactions and diffusion-limited forward reactions to re-reduce the photooxidized P_{680}^+ . At low light intensities the back reaction is favoured (t_1 about 0.5 ms; Van Best & Mathis, 1978), and the apparent degree of inhibition caused by chelators was increased. It is interesting to speculate that a similar mechanism might apply for the inhibition caused by ADRY reagents. Indeed, the observation that glutaraldehyde treatment of chloroplasts prevents the turnover rate-dependence of the Ant2p inhibition indicates that conformational changes are possibly involved. It could be that

these conformational changes are due to the displacement of Mn^{2+} in analogy with the action of chelators. Further work to investigate this possibility is needed.

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