Biochimica et Biophysica Acta, 682 (1982) 21–31
Elsevier Biomedical Press

BBA 41162

THE ROLE OF REAGENTS ACCELERATING THE DEACTIVATION REACTIONS OF WATER-SPLITTING ENZYME SYSTEM Y (ADRY REAGENTS) IN DESTABILIZING HIGH-POTENTIAL OXIDIZING EQUIVALENTS GENERATED IN CHLOROPLAST PHOTOSYSTEM II

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(Received March 30th, 1982)

Key words: ADRY reagent; ESR; Oxygen evolution; Photosystem II; Photosynthesis; (Spinach chloroplast)

A class of compounds, usually referred to as ADRY reagents, destabilize intermediates in the photosynthetic water-oxidizing process. The effects of these species on the reduction kinetics of $Z^{\ddagger}$, the oxidized donor to P-680, have been monitored in Tris-washed chloroplasts by following the decay of EPR Signal IIf. In the presence of ADRY reagents (e.g., sodium picrate, carbonyl cyanide *m*-chlorophenylhydrazone) this process follows an exponential time course, the decay half-time of which decreases as the ADRY reagent concentration increases. From this pseudo-first-order behavior, the second-order rate constants for four commonly used ADRY reagents have been extracted. The ADRY-induced acceleration in $Z^{\ddagger}$ reduction proceeds independently of conditions imposed on the acceptor side of Photosystem II and shows no synergism with exogenous electron donors. These observations are most easily rationalized in terms of a model which proposes direct reduction of $Z^{\ddagger}$ by the ADRY reagent followed by regeneration of the reduced ADRY reagent in a nonspecific reaction with membrane components such as carotenoids, chlorophyll or protein. A comparison of the second-order rate constants we obtain for ADRY reagents in their reaction with $Z^{\ddagger}$ in Tris-washed chloroplasts with those obtained from the literature for the ADRY- reagent induced deactivation of states S_2 and S_3 in oxygen-evolving chloroplasts reveals a close similarity between the two processes. From this observation, a general model for the action of ADRY reagents in destabilizing the high-potential oxidizing equivalents generated in Photosystem II is proposed.

Introduction

Despite the strongly oxidizing potentials generated in chloroplast PS II, most commonly used reductants are unable to compete with water as an electron donor [1]. Only those species which are structurally similar to H_2O (e.g., H_2O_2, NH_2OH) appear to be effective PS II donors in functional chloroplasts. Part of the specificity for water can be attributed to the extremely rapid redox reac-

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Abbreviations: ADRY, acceleration of the deactivation reactions of water-splitting enzyme system Y; ANT-2a, 2-(4-chloro)anilino-3,5-dinitrothiophene; ANT-2p, 2-(3-chloro-4-trifluoromethyl)anilino-3,5-dinitrothiophene; ANT-2s, 2-(3,4,5-trichloro)anilino-3,5-dinitrothiophene; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrozone; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; Mes, 4-morpholineethanesulfonic acid; PS, photosystem.

tions which occur during the transfer of photogenerated oxidizing equivalents from P-680$^+$, through the intermediate carrier, Z, to the oxygen-evolving complex. These reactions proceed with half-times in the submillisecond range and even the slowest step in this process, the actual generation of O_2, occurs with a 1 ms half-time [2–4]. However, the intermediate states of the oxygen-evolving complex are reasonably stable and, under normal conditions, decay with half-times which extend well into the seconds time range [5]. Over the past decade, a class of compounds has been found which accelerates the decay rate of these higher S states [6–9]. Much of this work has been carried out by Renger and co-workers who refer to these species as ADRY reagents. The mechanism by which they destabilize higher S states is uncertain. However, some members of the ADRY class are efficient at S state deactivation at low concentration (approx. 1 per PS II reaction center) and, consequently, models in which ADRY reagents activate a cyclic pathway for the reduction of the oxygen-evolving complex, rather than participate directly in the redox chemistry, appear to be favored. Recently, Renger and co-workers [10,11], using optical techniques to monitor P-680$^+$ decay, have shown that in Tris-washed chloroplasts, ADRY reagents promote the rereduction of $Z^{\dot{+}}$ following flash excitation. Thus, ADRY reagents appear to be general in increasing the rate at which the highly oxidizing equivalents generated by PS II are reduced. In the work described here, we have used EPR spectroscopy to monitor the effect of ADRY reagents on the behavior of $Z^{\dot{+}}$ in Tris-washed chloroplasts. Our results indicate that ADRY reagents act by reducing $Z^{\dot{+}}$ in a bimolecular reaction with second-order rate constants on the order of 10^5–10^7 $M^{-1} \cdot s^{-1}$.

Materials and Methods

Chloroplasts were prepared from market spinach and Tris washing and chlorophyll determinations were carried out as described in Ref. 12. Chlorophyll concentrations in EPR experiments ranged from 3 to 6 mg/ml. The buffer system used in most experiments consisted of 0.4 M sucrose, 0.05 M Hepes, 0.1 M NaCl which was adjusted to pH 7.6. Addition of other reagents is indicated in the figure captions. Ferredoxin and NADP were added as an acceptor system [12] to maintain electron flow under signal-averaging conditions. Standard chemicals were obtained commercially and were of the highest purity available. DCMU was from DuPont and was recrystallized prior to use. ANT-2p was a gift from Dr. G. Renger; ANT-2a was a gift from Dr. R. Blankenship. The organic solvent volume was maintained at less than 1% of the volume of the chloroplast suspension when they were used in order to solubilize the various reagents.

EPR spectroscopy was carried out with a Bruker ER 200 D instrument operating at X-band and interfaced to a Nicolet 1180 computer. The instrument has been modified slightly, primarily in that we increased the effective Q of the phase-sensitive signal detector by imposing either a passive 50 kHz high-pass filter or a PAR 189 selective amplifier between it and the detector diode preamplifier [13]. A Varian TM mode cavity (E238) and Scanlon TM mode flat cell (S-814) were used in the experiments described. In flashing light experiments, a critically damped xenon flashlamp (ILC, Sunnyvale, CA), which provides saturating pulses 14 μs in duration (full width, 1/3 maximum intensity), was used in conjunction with laboratory-constructed capacitor discharge and timing circuitry [13]. Light-induced changes in Signal IIf amplitude were measured by setting the magnetic field value at the low-field maximum of the Signal II spectrum; a modulation amplitude of 4 G was used. The flash repetition rate, instrument time constant and number of passes averaged in specific experiments are detailed in the figure captions.

Results

In oxygen-evolving chloroplasts, $Z^{\dot{+}}$ is rereduced by the oxygen-evolving complex in the submillisecond range following excitation [3]; the free radical state of Z observed under these conditions is referred to as Signal IIvf. If the oxygen-evolving complex is inhibited, electron flow to $Z^{\dot{+}}$ is interrupted and the lifetime of the free radical is extended well into the millisecond time range. Under these conditions, the $Z^{\dot{+}}$ free radical is referred to as Signal IIf and its reduction occurs at the expense of endogenous donors (e.g., ascorbate) in the

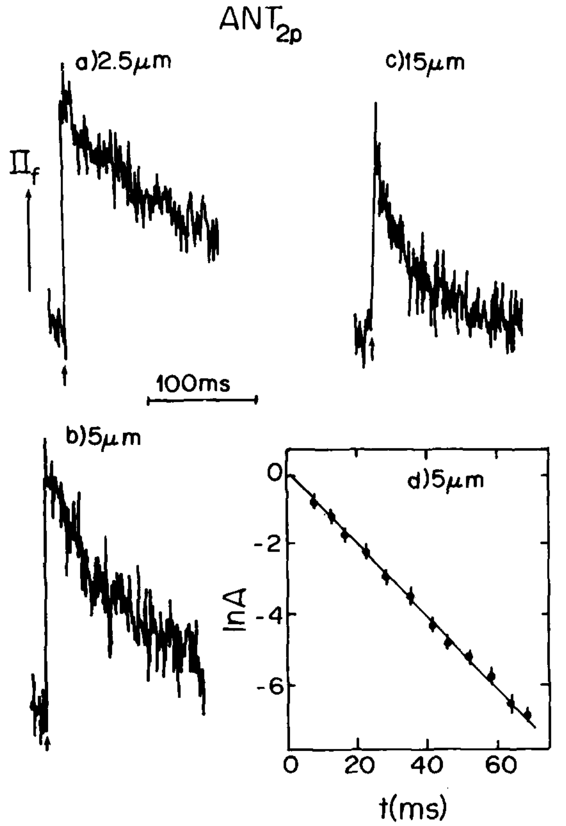

Fig. 1. The effect of ANT-2p on the decay kinetics of Signal IIf in Tris-washed chloroplasts. ANT-2p at the indicated concentrations was used in a–c. A time constant of 500 μs was used in all three experimental traces which are each the average of 180 flashes given at a repetition rate of 0.25 Hz. In d, a semilogarithmic plot of the decay of Signal IIf in b is presented. A hand-drawn curve was fitted to the data and the amplitude of Signal IIf at various times was extracted from this curve.

chloroplast suspension. Upon addition of a reductant, the lifetime of Z^+ is decreased owing to a direct bimolecular reaction of the donor with Z^+ [12]. Fig. 1 shows an analogous experiment where we used the ADRY reagent, ANT-2p, in place of an exogeneous donor. In the absence of the ADRY reagent, Signal IIf exhibits a slow decay ($t_{1/2} \approx 500$ ms). Increasing the ANT-2p concentration from 2.5 through 5 to 15 μM leads to progressively more rapid Signal IIf decay. The linearity of the first-order plot in Fig. 1d shows that the time course of Z^+ reduction in the presence of 5 μM ANT-2p is exponential and hence that the reaction is proceeding under pseudo-first-order conditions.

By plotting k_{obs} for the experimental traces in Fig. 1 vs. the corresponding ANT-2p concentration we can extract the second-order rate constant which describes the decay of Z^+ in the presence of this ADRY reagent (see Fig. 3 and Table I).

We have investigated a number of other ADRY reagents including picrate [14], CCCP and ANT-2a, in their effects on the decay kinetics of Z^+. The data for picrate are shown in Fig. 2 where we show Signal IIf kinetic transients for picrate concentrations between 15 and 180 μM (Fig. 2a–e). The increase in Signal IIf decay rate is apparent as the half-time of exponential decay of Signal IIf decreases progressively from 126 ms in Fig. 2a to 16 ms in Fig. 2e. The picrate experiments were carried out in the presence of 9 mM ferricyanide and 40 mM Ca^{2+} owing to an apparent inhibitory

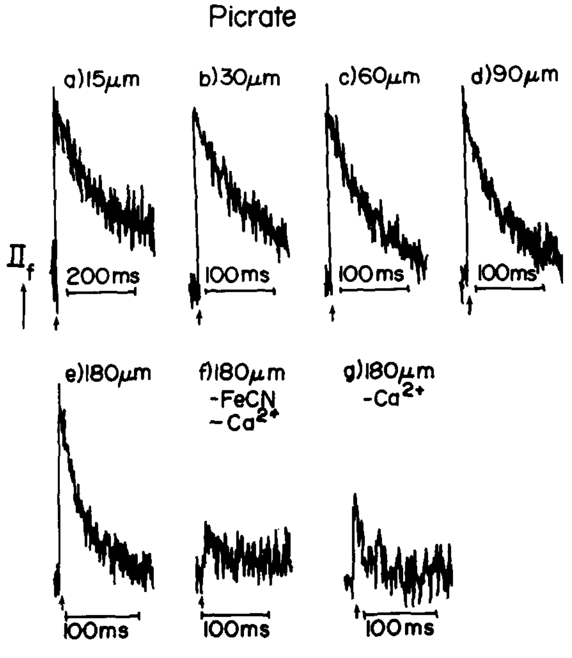

Fig. 2. The effect of picrate addition on the decay kinetics of Signal IIf in Tris-washed chloroplasts. In a–e 9 mM $K_3Fe(CN)_6$ and 40 mM $CaCl_2$ were added to a suspension of Tris-washed chloroplasts at pH 7.6 and picrate at the indicated concentrations was added. Note that the time axis is compressed by a factor of 2 in a compared to the other traces in the figure. In trace f, the $K_3Fe(CN)_6$ (FeCN) and $CaCl_2$ were omitted from the reaction mixture which was 180 μM in picrate; in g 9 mM $K_3Fe(CN)_6$ and 180 μM picrate were present but the $CaCl_2$ was omitted. The instrument time constant was 500 μs; 150 flashes were averaged at a frequency of 0.25 Hz.

effect on the reducing side of PS II by this ADRY reagent, similar to that described for other ADRY reagents [15], which leads to an inhibition of Signal IIf formation at high picrate concentrations (Fig. 2f). Addition of ferricyanide alone relieves this inhibition to some extent (Fig. 2g), but full development of Signal IIf requires both salt addition and ferricyanide. The salt requirement is nonspecific and most likely involves a surface charge shielding effect.

The kinetic data we have obtained for four ADRY reagents are collected in Fig. 3 where we have plotted the observed first-order rate constants (k_{obs}) for Signal IIf decay vs. the corresponding ADRY reagent concentration. The linear plots for all four reagents indicate that the relationship, $k_{obs} = k[\mathrm{ADRY}]$, holds. From the slopes of the lines we can extract values for the second-order rate constant, k, for each of the ADRY reagents. These values are summarized in Table I and indicate that the effectiveness of ADRY reagents in stimulating $Z^{\dot{+}}$ reduction in Tris-washed chloroplasts increases in the order picrate < ANT-2a < CCCP < ANT-2p.

TABLE I

CALCULATED SECOND-ORDER RATE CONSTANTS FOR THE REREDUCTION OF $Z^{\dot{+}}$ IN THE PRESENCE OF VARIOUS ADRY REAGENTS

ADRY reagent	k $(\mathrm{M^{-1} \cdot s^{-1}})(\times 10^{-6})$
ANT-2p	2.7
CCCP	1.9
ANT-2a	0.5
Picrate	0.2

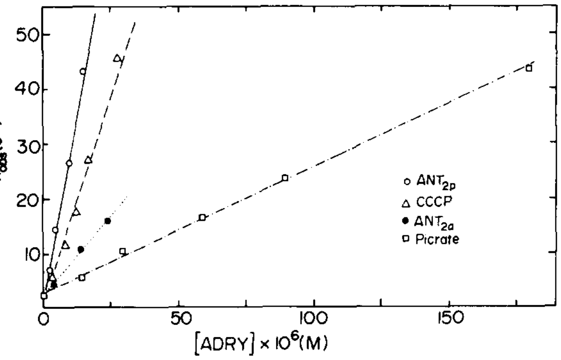

Fig. 3. Graphical determination of the second-order rate constants for the decay of Signal IIf in the presence of various ADRY reagents. The observed rate constant for the decay of Signal IIf in the presence of the indicated ADRY reagent is plotted against the corresponding ADRY reagent concentration. The slope of the straight lines which results is the second-order rate constant. These are tabulated in Table I.

The data of Figs. 1–3 indicate that there is a first-order dependence of the decay rate of Signal IIf upon the ADRY reagent concentration. Two different mechanisms can be proposed to account for this dependence. In the first of these, a direct reduction model, ADRY reagents act in a manner analogous to PS II donors and reduce $Z^{\dot{+}}$ in a direct bimolecular reaction as follows:

$$Z^{\dot{+}} + \mathrm{ADRY(red)} \xrightarrow{k} Z + \mathrm{ADRY(ox)} \tag{1}$$

where ADRY(red) represents the reduced form of the reagent and ADRY(ox) its oxidation product. In this model, the second-order rate constants for the various ADRY reagents in Table I correspond to the rate constant, k, in Eqn. 1 above. To account for the exponential decay of $Z^{\dot{+}}$ even at low ADRY concentration we must also postulate a fast regeneration of ADRY(red) from its oxidation product so that during the course of $Z^{\dot{+}}$ reduction the concentration of this species remains constant, i.e.,

$$\mathrm{ADRY(ox)} + D_r \xrightarrow{\text{fast}} \mathrm{ADRY(red)} + D_o \tag{2}$$

where D_r and D_o represent the reduced and oxidized form of an electron donor in the chloroplast suspension. Taken together, the mechanism proposed in Eqns. 1 and 2 leads to an exponential rate law for $Z^{\dot{+}}$ reduction of the following form:

$$-\frac{d[Z^{\dot{+}}]}{dt} = k_{obs}[Z^{\dot{+}}] \tag{3}$$

where

$$k_{obs} = k[\mathrm{ADRY(red)}] \tag{4}$$

In the second mechanism, a catalytic model, ADRY reagents are not redox active in their interaction with $Z^{\dot{+}}$ but rather catalyze a faster rereduction by other electron donors in the suspension. The following set of reactions can be proposed for this model:

$$Z^{+} + \mathrm{ADRY} \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} Z^{+}\mathrm{ADRY} \qquad \frac{k_1}{k_{-1}} = K \tag{5}$$

$$Z^{+} + \mathrm{D_r} \xrightarrow{k_2} Z + \mathrm{D_o} \tag{6}$$

$$Z^{+}\mathrm{ADRY} + \mathrm{D_r} \xrightarrow{k_3} Z + \mathrm{ADRY} + \mathrm{D_o} \tag{7}$$

The equilibrium in reaction 5 represents the catalytic interaction between Z^{+} and ADRY which results in a more reactive conformation, Z^{+}ADRY. This form of Z^{+} is reduced by the donor D_r more rapidly than is the free form, Z^{+}, i.e., $k_3 > k_2$. For the decay of Signal IIf, which reflects the reduction time course of Z^{+}, we have:

$$-\frac{\mathrm{d}[Z^{+}]}{\mathrm{d}t} = k_2[Z^{+}][\mathrm{D_r}] + k_3[Z^{+}\mathrm{ADRY}][\mathrm{D_r}] \tag{8}$$

To obtain the observed first-order dependence on the ADRY reagent concentration (Figs. 1–3) we must postulate that the equilibrium in reaction 5 is established quickly, i.e. that k_1, $k_{-1} \gg k_2$ or k_3. Then we solve the equilibrium expression in reaction 5 and express [Z^{+}ADRY] in terms of [Z^{+}] and [ADRY] as:

$$[Z^{+}\mathrm{ADRY}] = \frac{k_1}{k_{-1}}[\mathrm{ADRY}][Z^{+}] = K[\mathrm{ADRY}][Z^{+}] \tag{9}$$

and substituting into Eqn. 8 we obtain:

$$-\frac{\mathrm{d}[Z^{+}]}{\mathrm{d}t} = k_{\mathrm{obs}}[Z^{+}] \tag{10}$$

where

$$k_{\mathrm{obs}} = k_2[\mathrm{D_r}] + k_3K[\mathrm{D_r}][\mathrm{ADRY}] \tag{11}$$

In the absence of an ADRY reagent, only the first term in Eqn. 11 survives, whereas in the presence of an ADRY reagent the second term dominates. In this formulation the second-order constants for the various ADRY reagents in Table I correspond to the product $k_3K[\mathrm{D_r}]$. The donor D_r may be a soluble component, either endogenous or exogenous, or it may be a membrane-bound component. In the experiments described below we have attempted to test these two models in order to decide which is more appropriate.

If D_r is a soluble component, then the two models above make different predictions as to the decay of Signal IIf under conditions where both an ADRY reagent and an added donor are present. In the direct reduction model, Z^{+} may be reduced either by the ADRY reagent or by the added donor and thus we expect the observed rate constant to be simply the sum of the rate constants observed when each of the components is present separately. The catalytic model, on the other hand, predicts that an ADRY reagent should increase the rate of Z^{+} reduction when present with added donor owing to the increased concentration of the faster reacting Z^{+}ADRY conformation. Thus, the catalytic model predicts that there should be a synergistic effect when exogenous donor and an ADRY reagent are added to the same chloroplast suspension. Fig. 4 presents the results of two sets of experiments which were carried out to test the contrasting predictions. In Fig. 4a–c, CCCP was used in conjunction with the hydroquinone/ascorbate donor system. In the presence of 5 μM CCCP alone, the decay half-time was 75 ms; with 150 μM hydroquinone alone, the decay half-time was 20 ms. When the ADRY reagent and the exogeneous system were used together, the decay half-time was 21 ms. These results indicate that there is essentially no synergism between the two compounds in the rate at which Z^{+} is reduced. In the second series of experiments (Fig. 4d–h), ANT-2p was used in conjunction with the hydroquinone/ascorbate couple. The conditions were chosen so that Signal IIf decay in the presence of only hydroquinone (20 μM, $t_{1/2} = 190$ ms, Fig. 4d) was slower than that in the presence of ANT-2p alone (10 μM, $t_{1/2} = 38$ ms, Fig. 4e). When the two were used together (Fig. 4f), the observed half-time, 38 ms, again indicates that there is no synergism between an ADRY reagent and a soluble electron donor. Fig. 4g is a control carried out to demonstrate that the decrease in Signal IIf amplitude in Fig. 4e and f relative to that in Fig. 4d is the result of a slight PS II reducing side inhibition induced by ANT-2p, analogous to that observed above for picrate, which can be relieved by addition of ferricyanide and a divalent cation.

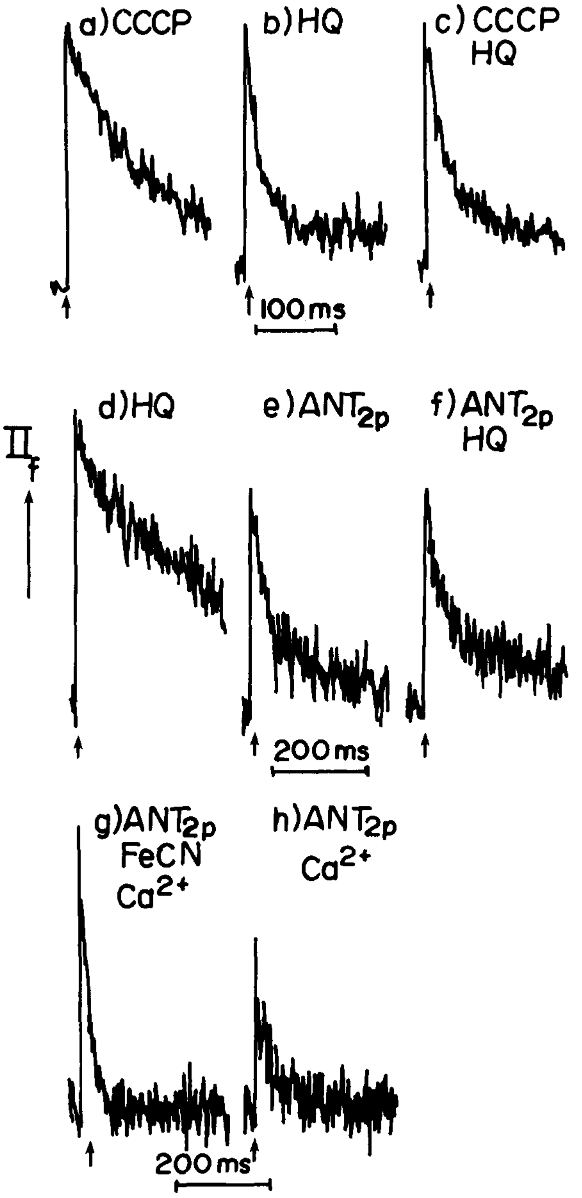

Fig. 4. The effect of an ADRY reagent in conjunction with an electron donor system on the decay kinetics of Signal IIf. In a–c the ADRY reagent, CCCP, and the donor system, hydroquinone/ascorbate, were used. The CCCP concentration in a and c was 5 μM; no CCCP was present in b. Hydroquinone (HQ) (150 μM) and sodium ascorbate (2 mM) were added in b and c, but were absent in a. A time constant of 1 ms was used for traces a–c, each of which is the average of 150 flashes given at a repetition rate of 0.25 Hz. In traces d–h the ADRY reagent, ANT-2p, and the hydroquinone/ascorbate donor system were used. In d and f, the hydroquinone concentration was 20 μM and ascorbate was present at 2 mM; the donor system was omitted in the other traces. The ANT-2p concentration in traces e–h was 10 μM; it was omitted from the chloroplast suspension used in obtaining trace d. In g, 12 mM $K_3Fe(CN)_6$ Fe(CN) and 40 mM $CaCl_2$ were present and in h, 40 mM $CaCl_2$ was added. An instrument time constant of 500 μs was used in obtaining the experimental data in d–h. 180 flashes, given at a rate of 0.25 Hz, were averaged to obtain the final decay curves.

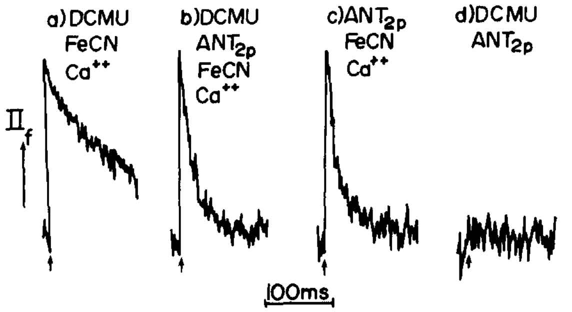

Fig. 5. The effect which conditions on the acceptor side of PS II exert on the decay kinetics of Signal IIf in the presence of an ADRY reagent. The decay of Signal IIf was recorded in Tris-washed chloroplasts in which the final concentration of added reactants was as follows: (a) 100 μM DCMU, 12 mM $K_3Fe(CN)_6$ (FeCN), 40 mM $CaCl_2$; (b) 100 μM DCMU, 12 mM $K_3Fe(CN)_6$, 40 mM $CaCl_2$, 10 μM ANT-2p; (c) 12 mM $K_3Fe(CN)_6$, 40 mM $CaCl_2$, 10 μM ANT-2p; (d) 100 μM DCMU, 10 μM ANT-2p.

Fig. 4h shows that the acceleration in $Z^{\dot{+}}$ decay rate observed in Fig. 4g comes about because of the presence of the divalent cation, most likely because at the pH used in the experiment ANT-2p is largely in its anion form [7] and subject to surface charge effects as we showed previously for the ascorbate monoanion [12,16].

The results of Fig. 4 indicate that if the catalytic role envisioned for ADRY reagents in the second model described above is correct, the source of electrons for the reduction of $Z^{\dot{+}}$ cannot be a soluble reductant. A second potential reductant pool which may be activated by ADRY reagent addition involves reduced components in the electron-transport chain on the acceptor side of PS II [11]. The results of experiments designed to test this hypothesis are shown in Fig. 5. When DCMU is added to Tris-washed chloroplasts. Signal IIf formation and decay are no longer observed under signal-averaged, flashing light conditions [17]. This inhibition can be relieved by addition of ferricyanide and a divalent cation to insure approach of the oxidant to the thylakoid membrane (Fig. 5a, decay half-time = 85 ms). Upon addition of the

ADRY reagent, ANT-2p, a stimulation in the rate of Z^{+} reduction is observed (Fig. 5b, $t_{1/2} = 15$ ms). This result apparently eliminates B, the plastoquinone pool and cytochrome *b*-559 as reductants susceptible to ADRY reagent action owing to the facts that DCMU inhibits transfer of electrons to B and plastoquinone and that in the presence of ferricyanide we expect these species, as well as cytochrome *b*-559, to remain oxidized in the dark. The oxidizing conditions were apparent in that high concentrations of the P-700^{+} free radical were present in these samples. Fig. 5c is a control which shows that the rate of Z^{+} reduction is independent of the presence or absence of DCMU. Fig. 5d shows that if the divalent cation and ferricyanide are omitted, no Signal IIf formation in the presence of DCMU is observed. This experiment indicates that ADRY reagents are unable to catalyze a direct $Z^{+}Q^{-}$ back recombination which agrees with a similar conclusion obtained earlier by Renger et al. [8]. We conclude from the experiments of Fig. 5 that ADRY reagents can exert their accelerating effect on Z^{+} decay independent of the redox state of acceptor-side components and thus the primary mode of ADRY reagent action does not appear to involve catalysis of cyclic flow to Z^{+} from these compounds. Taken together, the results of Figs. 1–5 provide no evidence for the catalytic model of ADRY reagent action, rather they indicate that these reagents are likely to reduce Z^{+} in Tris-washed chloroplasts in a bimolecular reaction as outlined in the direct reduction model above.

Renger has pointed out that effective ADRY reagents possess an acidic proton which, upon dissociation, generate a lipophilic anion. Moreover, the anion form appears to be more effective in stimulating ADRY reagent action than is the neutral form [18]. We have found that similar behavior is exhibited by ANT-2a ($pK_a = 7.0$ [7]) during its reaction with Z^{+}. At pH 5.8, where ANT-2a is over 90% in its protonated form, the decay half-time of Z^{+} is 53 ms. At pH 8.2 and the same total ANT-2a concentration (15 μM), 90% of the ADRY reagent is in the anion form and the decay half-time of Signal IIf decreases to 33 ms (data not shown). Assuming pseudo-first-order behavior and the pK_a value above, one calculates that the anion form of ANT-2a is 1.7-times more effective than the neutral species in Z^{+} reaction. This behavior may not be general, however, in that we find an acceleration in the rate of Z^{+} reduction at constant CCCP concentration when we lower the pH [13].

Discussion

In addition to their uncoupling activity [19] and inhibitory action on the reducing side of PS II [15], ADRY reagents destabilize oxidizing equivalents generated by P-680. States and species which are susceptible to this destabilization effect include the intermediates S_2 and S_3 [8,11], the free radical species, Signal IIs [20] and the electron-transport component Z^{+} (Ref. 10, and this work). Moreover, ADRY reagents alter significantly the behavior of two other species which appear to be linked to PS II function, cytochrome *b*-559 and carotenoids [21–24].

In the work reported here, we have shown that the rate of Z^{+} decay shows a first-order dependence on the concentration of ADRY reagent and have listed in Table I the second-order rate constants for this reaction for four ADRY reagents: ANT-2p, CCCP, ANT-2a and picrate. These data show that the efficiency of these four species in exerting the destabilization effect varies by a factor of about 14 and decreases in the order ANT-2p > CCCP > ANT-2a > picrate. This ordering and the magnitude of the second-order rate constants provide a basis from which the relationship between the effect we observed for ADRY reagents in their interaction with Z^{+} and that observed for their destabilization of S_2 and S_3 can be explored. Renger, Vater and their co-workers [7–11,14,18] have reported S_2 and S_3 decay curves for a variety of ADRY reagents at various concentrations. In general, the decay of the oxygen-evolving complex intermediates in the presence of an ADRY reagent follows an exponential time course (although there are exceptions [14]) and the rate constant for this process increases as the ADRY reagent concentration is increased. This behavior is qualitatively similar to that which we observe for Z^{+} decay in the presence of an ADRY reagent and implies that S_2 and S_3 exhibit pseudo-first-order decay behavior upon ADRY reagent addition. To put this observation in more quantitative terms, we have

TABLE II

CALCULATED SECOND-ORDER RATE CONSTANTS FOR VARIOUS ADRY REAGENTS IN THE DEACTIVATION OF HIGHER FORMAL OXIDATION STATES OF THE OXYGEN-EVOLVING COMPLEX

Reagent	Concentration (M)	$t_{1/2}$ (s)	k_{obs} (s^{-1})	k ($M^{-1} \cdot s^{-1}$)	Ref.
ANT-2p	$1 \cdot 10^{-7}$	0.47	1.5	$1.5 \cdot 10^{7}$	11
	$3 \cdot 10^{-7}$	0.33	2.1	$7 \cdot 10^{6}$	11
	$3 \cdot 10^{-7}$	0.26	2.7	$9 \cdot 10^{6}$	11
	$4 \cdot 10^{-7}$	0.21	3.3	$8.4 \cdot 10^{6}$	7
	$5 \cdot 10^{-7}$	0.18	3.9	$7.7 \cdot 10^{6}$	11
	$1 \cdot 10^{-6}$	0.10	7.0	$7 \cdot 10^{6}$	18
[a]	$1 \cdot 10^{-6}$	1.9	0.4	$0.36 \cdot 10^{6}$	8
				average = $9 \cdot 10^{6}$	
ANT-2s	$2 \cdot 10^{-7}$	0.39	1.8	$9 \cdot 10^{6}$	9
	$4 \cdot 10^{-7}$	0.12	5.7	$1.4 \cdot 10^{7}$	9
	$4 \cdot 10^{-7}$	0.20	3.3	$8.4 \cdot 10^{6}$	7
				average = $10 \cdot 10^{6}$	
ANT-2a	$4 \cdot 10^{-7}$	1.2	0.6	$1.5 \cdot 10^{6}$	7
	10^{-6}	0.43	1.6	$1.6 \cdot 10^{6}$	7
	10^{-6}	1.0	0.7	$0.7 \cdot 10^{6}$	18
	$2 \cdot 10^{-6}$	0.32	2.2	$1.1 \cdot 10^{6}$	7
				average = $1.2 \cdot 10^{6}$	
FCCP	10^{-6}	0.11	6.3	$6.3 \cdot 10^{6}$	9
CCCP	10^{-5}	0.09	7.7	$7.7 \cdot 10^{5}$	9
Picrate	10^{-6}	1.25	0.55	$5.5 \cdot 10^{5}$	14
2,4-Dinitrophenol	10^{-4}	2	0.35	$3.5 \cdot 10^{3}$	14

[a] Carried out at a bare platinum electrode where its redox activity may have led to spurious results [8,11]; not included in determining the average value of the second-order rate constant.

collected data on the ADRY-reagent induced destabilization of S_2 and S_3 which have been reported over the past decade (Table II). The half-times ($t_{1/2}$) were taken from the data reported and converted to pseudo-first-order rate constants by using the relationship, $k_{obs} = \ln 2/t_{1/2}$. The apparent second-order constants, k, were calculated as $k_{obs}/[ADRY]$. The results show that there is remarkably consistent behavior observed for the various species and that the second-order formulation appears reasonable. For ANT-2p, for example, the second-order rate constant varies by no more than a factor of two even when the concentration is varied over an order of magnitude. Moreover, inspection of the ANT-2s and ANT-2a data indicates that this factor of two may be more a function of experimental error (e.g., two separate measurements at $1 \cdot 10^{-6}$ M ANT-2a show a 2-fold variation), than a real concentration dependence in the second-order rate constants. Focusing on the data in Table II for the four ADRY reagents we have studied in their interaction with $Z^{\underset{\cdot}{+}}$, we see that the efficiency of these species declines in the order ANT-2p > ANT-2a > CCCP > picrate. The relative inefficiency of CCCP with respect to ANT-2a in Table II may be an example of experimental variation in that in Ref. 7, Renger indicates the reverse, i.e., that CCCP is more efficient than ANT-2a. In addition, the ADRY reagent efficiencies, as judged by the value of the second-order rate constants, vary by a factor of about 15 as one goes through the series of four. Finally, the magnitudes of the rate constants for a given ADRY reagent in Tables I and II are within a factor of four.

These observations, in conjunction with the data above suggesting that the anion is the more effective form, indicate that the mechanism by which ADRY reagents destabilize S_2 and S_3 in oxygen-evolving chloroplasts is similar to that which we observe for the ADRY-reagent induced rereduction of $Z^{\ddagger}$ in Tris-washed chloroplasts. However, we do not intend to convey the idea that ADRY reagents destabilize S_2 and S_3 by intercepting oxidizing equivalents at $Z^{\ddagger}$ in water-splitting chloroplasts. The data of Table II indicate that S_2 and S_3 destabilization is observed in the hundreds of milliseconds time range under the ADRY reagent concentration conditions usually employed, whereas Z transfers its oxidizing equivalent into the oxygen-evolving complex in the submillisecond range in O_2-evolving chloroplasts [3]. Thus, in uninhibited chloroplasts the site of ADRY reagent action is the oxygen-evolving complex itself, whereas in Tris-washed chloroplasts the much longer lifetime of $Z^{\ddagger}$ makes it susceptible to ADRY reagent attack.

Within the context of the data of Tables I and II, it is worthwhile to consider briefly the effect of ADRY reagents on the decay of Signal IIs. Lozier and Butler [20] originally demonstrated that a number of reagents, including CCCP, destabilized the free radical species which gives rise to this EPR signal so that its decay half-time decreased from hours in the absence of CCCP to seconds in the presence of this reagent. They also showed that subsequent illumination lead to Signal IIs regeneration [20]. Subsequently, Babcock and Sauer [25] confirmed these results and showed that the Signal IIs precursor, which they designated as F, was oxidized to its free radical form by redox interaction with states S_2 and S_3. They also carried out a detailed study of the CCCP concentration dependence of the Signal IIs decay rate and presented data on the decay half-time of Signal IIs which showed that increasing CCCP levels led to increased destabilization of the radical. From the decay half-time data they presented (Fig. 3 in Ref. 25), we obtain an apparent second-order rate constant for the CCCP-induced destabilization of Signal IIs of $6.5 \cdot 10^2\ M^{-1} \cdot s^{-1}$. This value is approximately three orders of magnitude smaller than the second-order rate constant for the CCCP-induced destabilization of $Z^{\ddagger}$ (Table I) or for the destabilization of S_2 and S_3 (Table II). This observation indicates that, although Signal IIs is mechanistically similar to $Z^{\ddagger}$ and to S_2 and S_3 in its susceptibility to the action of ADRY reagents, it is much more sluggish in terms of its response time. Nonetheless, these results show that the action of ADRY reagents appears to be general in that oxidized species generated by the light reaction of PS II are susceptible to destabilization by ADRY reagents and that the decay process which results shows a first-order dependence on ADRY reagent concentration.

Previous considerations of the mechanism of ADRY reagent action [7–11,14] had focused on essentially two alternative possibilities as follows: (1) ADRY reagents are redox-active cofactors and act by reducing the stored oxidizing equivalents directly; (2) ADRY reagents act in a catalytic manner and facilitate a cyclic reaction which accelerates the decay of the stored oxidizing equivalents. The results presented above argue against the catalytic model of ADRY reagent action, but are consistent with the predictions of the direct reduction mechanism. The latter model, however, requires a substrate level reductant (D_r in Eqn. 2) in order to regenerate the reduced form of the ADRY reagent from its oxidation product. An inspection of the literature indicates that this reductant pool is likely to consist of membrane components, such as carotenoids, chlorophyll and protein, which are susceptible to nonspecific oxidation by ADRY(ox). This possibility is indicated by results which showed that addition of the ADRY reagent, CCCP, to either oxygen-evolving or Tris-washed chloroplasts led to the photobleaching of both carotenoids and chlorophylls [22,23]. Similarly, Homan [26,27] has noted photodestruction of PS II in the presence of the ADRY reagents, CCCP or tetraphenylboron, and has suggested that these species funnel oxidizing equivalents generated within the photosystem into destructive side reactions. Finally, it is well established that if reduced cytochrome *b*-559 is available, it is oxidized in the presence of an ADRY reagent and light by a PS II-driven reaction [21,24]. Taken together, these results suggest that a fairly nonspecific oxidation reaction occurs upon illumination of ADRY-treated chloroplasts and that the direct reduction model outlined above may be rewritten as the following two-step sequence:

$$Z^{\ddagger} + \mathrm{ADRY(red)} \xrightarrow{k} Z + \mathrm{ADRY(ox)} \qquad (12)$$

$$\text{ADRY(ox)} + \text{carotenoid/chlorophyll}/b^{2+}\text{-559/soluble reductants} \xrightarrow{\text{fast}} \text{ADRY(red)} + \text{carotenoid}^{+}/\text{chlorophyll}^{+}/b^{3+}\text{-559/soluble oxidation products} \quad (13)$$

Although we cannot provide definitive evidence to eliminate a catalytic mechanism which invokes an ADRY-reagent mediated direct oxidation of carotenoids and chlorophylls by $Z^{\dagger}$, we favor the mechanism outlined in Eqns. 12 and 13 for several reasons. First, it accounts for the exponential decay of Signal IIf we observed in the presence of low concentrations of an ADRY reagent under an extended flash regime without postulating a specialized structural arrangement between $Z^{\dagger}$ and carotenoid, chlorophyll or cytochrome *b*-559. Second, this model accounts for the oxidation of cytochrome *b*-559 in the presence of an ADRY reagent if the cytochrome is in its reduced state prior to initiation of a flash train. However, it does not make the oxidation of this species mandatory for ADRY action. Third, it explains the inhibition of carotenoid bleaching which is observed if an exogenous reductant is added to CCCP-treated chloroplasts [22,23]. Fourth, it accommodates those ADRY reagents which are known to be redox active (e.g., tetraphenylboron, dichlorophenolindophenol). Finally, the model is supported by observation of redox activity for ANT-2s [11], picrate [28] and by our observation of redox activity for several ADRY reagents by cyclic voltammetry (Ghanotakis, D.F. and Babcock, G.T., unpublished results).

The scheme presented in Fig. 6 summarizes the model developed above for the mode of action of ADRY reagents. In O_2-evolving chloroplasts, ADRY reagents act primarily on the states S_2 and S_3 and, in a much slower reaction, on the species $F^{\dagger}$ which gives rise to Signal IIs. In chloroplasts inhibited at the oxygen-evolving complex, the lifetime of $Z^{\dagger}$ is extended significantly and it becomes a site of ADRY reagent action at fairly low ADRY reagent concentrations. The interaction between the ADRY reagent and the PS II oxidant generates the oxidized form of the ADRY reagent, which is able to oxidize the indicated chloroplast components in a fairly nonspecific reaction.

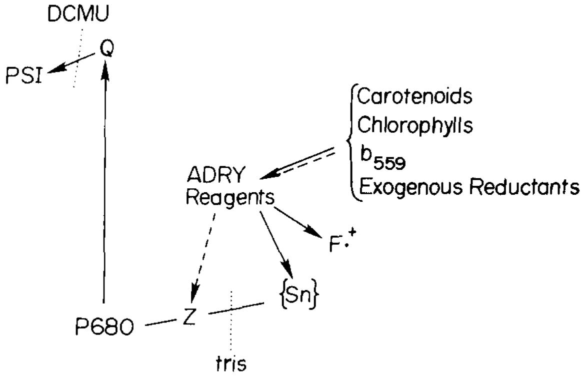

Fig. 6. A model for the role of ADRY reagents in destabilizing oxidizing equivalents generated in PS II. The solid arrows denote proposed reactions in oxygen-evolving chloroplasts, dashed arrows indicate proposed reactions in Tris-washed chloroplasts and the dotted lines indicate inhibitor action. See text for other details. b_{559}, cytochrome *b*-559.

Note added in proof (Received August 17th, 1982)

While this paper was in the press, it came to our attention that a mechanism for ADRY action, similar to that shown in Fig. 6, has been proposed by Sayre and Homann [29] from studies of oxygen consumption induced by addition of these to chloroplasts.

Acknowledgments

This research was supported by the Science and Education Administration of the U.S. Department of Agriculture under Grant No. 59-2261-1-1-631-0 from the Competitive Research Grants Office. Acknowledgment is made to the Donors of the Petroleum Research Fund, administered by the American Chemical Society, for support of this research and to the U.S. National Science Foundation for Grant No. CHE-79-04810 which supported the purchase of the EPR spectrometer.

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