Biochimica et Biophysica Acta 806 (1985) 210–220
Elsevier

BBA 41685

On the mechanism of ADRY agent interaction with the Photosystem II donor side

B. Hanssum, G. Dohnt and G. Renger *

Max Volmer Institut für Biophysikalische und Physikalische Chemie der Technischen Universität, Strasse des 17. Juni 135, D-1000 Berlin 12 (F.R.G.)

(Received July 9th, 1984)

Key words: Water splitting; Electron transport; ADRY agent; Photosystem II; (Spinach chloroplast)

The effect of 2-(3-chloro-4-trifluoromethyl)anilino-3,5-dinitrothiophene (ANT 2p), known to be the most powerful compound among ADRY agents (Renger, G. (1972) Biochim. Biophys. Acta 256, 428–439), on the decay kinetics of the redox states S_2 and S_3 of the water-oxidizing enzyme system and on the donor component D_1 in Tris-washed chloroplasts has been investigated. The following was found. (a) In isolated spinach chloroplasts without ANT 2p, the decay of S_3 and S_2 can be completely described by a sequence of one-electron-transfer steps: $S_3 \rightarrow S_2 \rightarrow S_1$. (b) In the dark state, S_0 is slowly auto-oxidizable with a half-life of more than 5 min in isolated spinach chloroplasts without ANT 2p. (c) Only one ANT 2p molecule per 40 reaction centers of system II is sufficient to induce a significant acceleration of the S_2 and S_3 decay. (d) The dependence on ANT 2p concentration of S_2- and S_3-decay rates can be consistently described by a simple kinetic scheme, assuming that ANT 2p acts as a very mobile catalyst within the thylakoid membrane reacting with the water-oxidizing enzyme system independent of its redox state forming a transient complex. The S_2 and S_3 decay induced by ANT 2p occurs via a one-electron-transfer sequence in normal chloroplasts. (e) The numerical evaluation of the scheme reveals a high ANT 2p affinity for the water-oxidizing enzyme system, with a dissociation constant of the order of $1 \cdot 10^{-7}$ M^{-1}. This value closely corresponds with an estimation based on thermoluminescence measurements (Renger, G. and Inoue, Y. (1983) Biochim. Biophys. Acta 725, 146–154). The kinetic parameters also suggest a rapid exchange of ANT 2p between the binding sites. (f) Fluorescence measurements obtained in normal chloroplasts in the presence of DCMU led to rate constants for the S_2 decay which correspond to values derived from oxygen yield data. The ANT-2p-induced reduction of D_1^{ox}, the donor to P-680$^+$, was inferred to occur with a larger rate constant than the S_2 decay. Based on these data, ANT 2p is confirmed to be the most efficient reactant with the water-oxidizing enzyme system known so far which allows a very specific kinetic labeling of redox states S_2 and S_3.

Introduction

Photosynthetic water oxidation by oxidizing redox equivalents generated via photoreaction at P-680 within the reaction center complex occurs in a four-step univalent redox reaction sequence,

* To whom all correspondence should be addressed.

Abbreviations: DCMU, 3-(3′,4′-dichlorophenyl)-1,1′-dimethylurea; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; PS II, Photosystem II; Chl, chlorophyll; Mes, 4-morpholineethanesulphonic acid; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; ANT 2p, 2-(3-chloro-4-trifluoromethyl)anilino-3,5-dinitrothiophene.

catalyzed at a mangano-protein referred to as a water-oxidizing enzyme system (for a recent review see Ref. 1). The redox states of this enzyme system symbolized by S_i (i represents the number of oxidizing equivalents stored in the water-oxidizing enzyme system) are kinetically well-characterized by their lifetimes, but the chemical identification still remains to be clarified. Surprisingly, in dark-adapted chloroplasts, the water-oxidizing enzyme system was found to attain almost exclusively the S_1 redox state, whereas the higher states S_2 and S_3 relax in the dark down to S_1 with half-lifetimes of 40–80 s, including a fast decay of 1–2 s in 20% of all systems [2]. S_4 has a comparatively short lifetime of about 1 ms and, in isolated chloroplasts, S_0 becomes very slowly oxidized in the dark with kinetics in the 10 min time domain (for a review see Ref. 3). The lifetimes of states S_2 and S_3 are markedly shortened by chemicals referred to as ADRY agents [4], whereas S_1 is not significantly affected [5]. Based on measurements of average lifetimes as a function of ADRY concentration, the active substances were assumed to act as mobile catalysts for S_2 and S_3 decay by an unknown endogeneous electron donor [6]. Recent studies of the effect of ANT 2p on thermoluminescence confirmed a mobile catalyst mechanisms [7]. ADRY agents do not only accelerate the decay of S_2 and S_3 but also increase the relaxation rate of flash-induced signal II_f [8] and 820 nm absorption changes [9] in Tris-washed chloroplasts completely deprived of their oxygen-evolving capacity. This result led to the conclusion that ADRY agents directly react as redox-active species with the oxidized radical form of the donor component D_1 [8] which, in normal chloroplasts, mediates the electron transfer between the water-oxidizing enzyme system and P-680. This raises the question whether ADRY agents in normal chloroplasts primarily attack D_1 rather than interfere directly with the states S_2 and S_3. Stoichiometric investigations revealed that ADRY agents do not act as conventional PS II electron donors but become rapidly regenerated, if they really participate in redox reactions. Therefore, regardless of the molecular mechanism, ADRY agents should be considered as catalysts for S_2 and S_3 decay rather than as PS II electron donors. In this sense, ADRY agents are modifiers which can be used for a unique kinetic labeling of the S_2 and S_3 states in the water-oxidizing mechanism. The present study was performed in order to clarify the reaction mechanism of ADRY agents and to confirm by direct oxygen yield measurements that ANT 2p represents the most specific substance so far known for the modification of S_2 and S_3 lifetimes.

Materials and methods

Isolation of thylakoids from spinach (*Spinacea oleracea*) was performed as in Ref. 2 except for the centrifugation procedure. Thylakoids were centrifuged at $8000 \times g$ for 30 s in both steps. The number of intact water-oxidizing enzyme systems was found to be one per approx. 500 chlorophylls, as determined by conventional flash-induced oxygen yield measurements [4].

Oxygen was detected with an unmodulated Joliot-type oxygen electrode [10]. The thylakoid suspension (1 mg Chl/ml) on the Pt electrode was separated from the upper buffer compartment, containing the Ag/AgCl reference electrode, by a single dialysis membrane. Thylakoids were isolated, stored on ice and transferred to the electrode in darkness. After 5 min of equilibration on the electrode, the thylakoids were illuminated with a series of saturating flashes (10 μs halfwidth) using a Stroboslave 1539-A (General Radio). The signals were digitally recorded with a Nicolet Explorer III storage oscilloscope. The amplitudes were taken to indicate the amount of oxygen produced. For calculations on relative S-state concentrations, the Kok-model was used (for details see Appendix). In order to prevent effects due to insufficient diffusion through the membrane, thylakoid suspension and buffer solution were directly mixed with ANT 2p. The buffer solution comprised of: 50 mM Tricine-NaOH (pH 7.6)/10 mM NaCl/5 mM $MgCl_2$/0.3 M sorbitol. To monitor Q_A^- oxidation in the presence of DCMU by fluorescence yield changes, fluorescence was excited by a pulsed, green light-emitting diode (1024 pulses, 5 μs wide at varying repetition rates) and monitored by an avalanche photodiode (RCA 30872). A digital storage oscilloscope (Nicolet Explorer III) recorded the fluorescence pulses synchronously in the external address advance mode, thus yielding the time-course of Q_A^- oxidation. Q_A was reduced

by a single saturating flash (15 μs halfwidth, EG& G Flashlite). Chlorophyll concentration was 20 μg/ml, DCMU concentration 2 μM.

From fluorescence yield changes, relative Q_A^- concentrations were deduced, accounting for energy transfer between photosynthetic units by a transfer parameter of 0.5 [11].

Results

Oxygen yield measurements

Despite ample evidence presented for a univalent redox reaction sequence $S_3 \xrightarrow{k_{i,3}} S_2 \xrightarrow{k_{i,2}} S_1$ (for a review see Ref. 3), the deactivation mechanism of S_2 and S_3 in the absence of ADRY agents is still a matter of debate, because recent results on chlorella favor a more complex pattern including two 2-electron transfer steps $S_3 \rightarrow S_1$ and $S_2 \rightarrow S_0$, coupled via an unknown component C [12]. In order to test this idea, the S_2 redox state population as a function of dark time after two preillumination flashes, spaced 300 ms part, has been analyzed (for numerical evaluation of the data see Appendix). The results depicted in Fig. 1 clearly show that at rather short dark times, the S_2 redox state population increases probably due to reductive formation from S_3 in the dark (experiments at pH 6.0 are especially appropriate to reveal the effect clearly, because, under these conditions, the S_3 decay is faster than the S_2 decay so that an intermediate accumulation takes place).

The S_2 values (calculated from experimental data according to Eqn. A-4) produced via consecutive reaction from S_3 were fitted according to Eqn. A-6 deduced in the Appendix. Rate constants for S_2 and S_3 decay obtained from this fit corresponded to rate constants for S_2 and S_3 decay determined in separate experiments (see Ref. 2).

This favors a univalent redox reaction sequence for S_3 decay. To corroborate this assumption, the time dependence of S_2 was calculated on the basis of this reaction scheme with rate constants $k_{i,3}$ and $k_{i,2}$ determined directly from S_3 and S_2 decay after two and one preillumiation flash(es), respectively. The theoretical curve nicely fits the experimental data so that at least in isolated spinach thylakoids the univalent redox reaction sequence dominates the decay of S_3 and S_2. A two-electron donation to S_3 and S_2, however, could be possible to a minor extent, if the unknown donor component D, responsible for the fast decay of about 20% of S_2 and/or S_3 [2], is able to react in this manner. However, a previous numerical analysis favored D as a one-electron donor only [2] and, therefore, rules out this possibility. Hence, it seems likely to assume that ADRY agents also accelerate the S_2 and S_3 decay via a univalent redox sequence. To confirm this, analogous experiments were performed in the presence of ANT 2p, which is known as the most powerful ADRY agent [4]. The

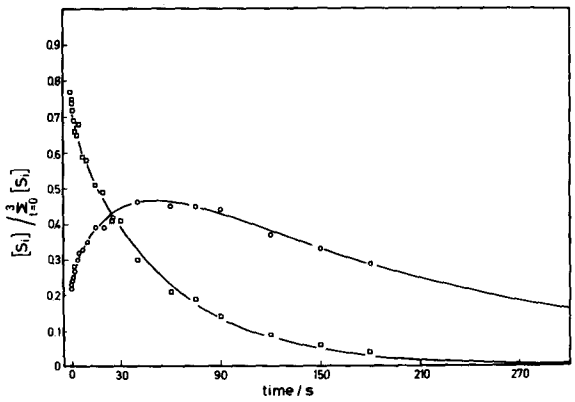


Fig. 1. Decay kinetics of S_3 and S_2 in thoroughly dark-adapted spinach thylakoids after two preflashes. The relative population probabilities of the S states were calculated according to equations derived from the Kok-scheme (see Appendix). Flash frequency was 3.3 Hz; 1 mg Chl/ml at pH 6.0 using Mes instead of Tricine (see Materials and Methods). ○, time-course of S_2; □, S_3 decay.

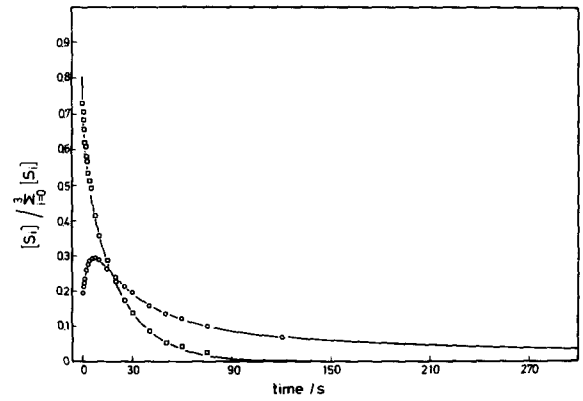


Fig. 2. Decay kinetics of S_3 and S_2 in thoroughly dark-adapted spinach thylakoids after two preflashes with 100 nM ANT 2p at pH 7.6. ○, time-course of S_2; □, S_3 decay.

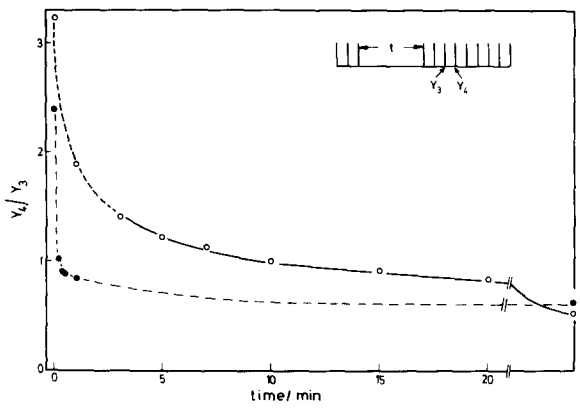
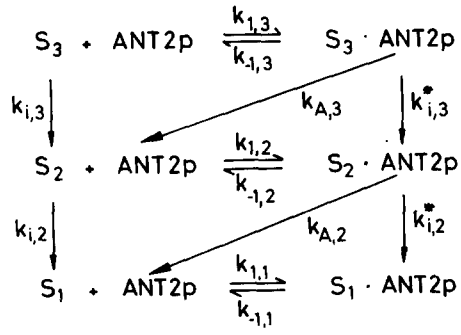


Fig. 3. Ratio of the oxygen yield in the fourth and third flash of a flash sequence after three preflashes as a function of the dark time between preflashes and sequence to monitor the oxidation of S_0 to S_1 in spinach thylakoids, in the absence and presence of 1 μM ANT 2p.

data in Fig. 2 strongly support the suspected mechanism. Previous data led to the conclusion that ADRY agents do not affect the S_1 redox state [5]. In order to analyze this phenomenon more thoroughly, chloroplasts were preilluminated with three flashes giving rise to S_0 accumulation. After a definite dark time, a flash sequence was fired and the ratio of oxygen yields Y_4/Y_3 (Y_3 and Y_4 are the yields caused by the 3rd and 4th flash of sequence) was measured as a function of dark time following the preillumination flashes. The data of Fig. 3 show that Y_4/Y_3 decreases with increasing dark time. The steep decline in the presence of ANT 2p during the first minute is almost exclusively due to accelerated S_3 and S_2 decay, giving rise to increased S_1 population. After this decay, only a slight variation is observed. Therefore, ANT 2p does not affect the S_1 state markedly. The present data do not permit to resolve any possible effects on S_0 autoxidation. On the basis of these data, the mechanism described by Scheme I was used for the kinetic analysis of the ADRY effect. This mechanism implies the following assumptions: (a) ANT 2p is highly mobile within the thylakoid membrane so that the ANT 2p molecules rapidly exchange between the different water-oxidizing enzyme systems, (b) if ANT 2p acts as a redox-active catalyst, its recovery rate is much faster than its possible oxidation so that the ANT 2p concentration remains constant, (c) for the sake of simplicity the affinity of ANT 2p to the water-oxidizing enzyme system is considered independent of the redox state of the water-oxidizing enzyme system, (d) the internal decay rate constants, $k_{i,3}$ and $k_{i,2}$, are invariant to ANT 2p binding. In order to analyze the ANT 2p effect within this scheme, the S_2 and S_3 decay kinetics were measured as a function of ANT 2p concentration. The maximum ANT 2p concentration was less than 1 ANT 2p molecule per water-oxidiz-



Scheme I

Model for ANT 2p interaction with the redox states of the water-oxidizing enzyme system. k_i denotes the internal rate constants for S_3 and S_2 decay, respectively; k_1 and k_{-1} denote the rate constants for binding and release of ANT 2p; k_A describes the decay of the ANT 2p complexed S state into the dissociated form of the corresponding lower S state.

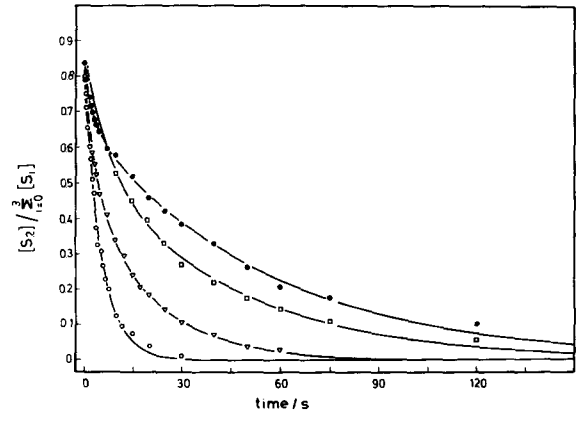


Fig. 4. Decay kinetics of S_2 after one preflash at different ANT 2p concentrations. Symbols represent the experimental data, the curves were calculated on the basis of Scheme I by using a Runge-Kutta-algorithm as described in the Appendix. ●, Control; □, 50 nM; ▽ 200 nM; ○, 500 nM ANT 2p, respectively.

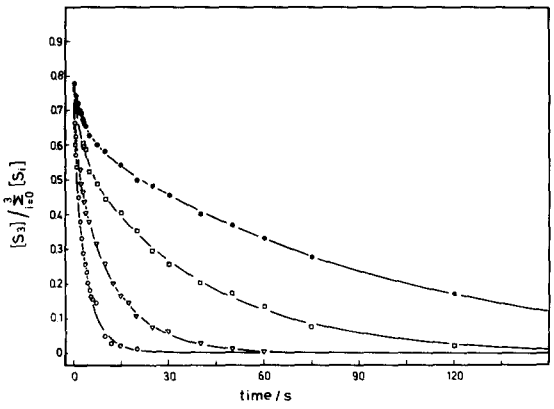


Fig. 5. Decay kinetics of S_3 after two preflashes at different ANT 2p concentrations. Symbols represent the experimental data, the curves were calculated on the basis of Scheme I by using a Runge-Kutta-algorithm as described in the Appendix. ●, Control; □, 50 nM; ▽, 200 nM; ○, 500 nM ANT 2p, respectively.

ing enzyme system. The data obtained are depicted in Figs. 4 and 5 (it should be emphasized that equilibration of ANT 2p through the dialysis membrane of the Joliot-type electrode is rather slow so that chloroplasts have been preincubated with definite amounts of ANT 2 p concentration). The experimental data for the ANT 2p-induced accelerated S_2 and S_3 decay cannot be described by single exponential kinetics. After suitable numerical parameter fit for one ANT 2p concentration, the constants k_1, k_{-1} and k_A were obtained (for the sake of simplicity, $k_{A,3} = k_{A,2} = k_A$, $k_{1,3} = k_{1,2} = k_{1,1} = k_1$ and $k_{-1,3} = k_{-1,2} = k_{-1,1} = k_{-1}$ are assumed).

All other S_3 and S_2 decay curves with different ANT 2p concentrations could be fitted with these constants varying only the ANT 2p concentration if the parameters are allowed to vary in small intervals:

$5 \cdot 10^6\ M^{-1} \cdot s^{-1} < k_1 < 1 \cdot 10^7\ M^{-1} \cdot s^{-1}$ *

$2\ s^{-1} < k_{-1} < 3\ s^{-1}$

$0.65\ s^{-1} < k_A < 0.70\ s^{-1}$

This variation of parameter is small enough in order to be consistent with the model, especially if it is taken into account that the set of differential equations was solved numerically.

Therefore, this scheme implies an appropriate mechansim for the ADRY effect. It reveals that ANT 2p is an extremely effective and rather mobile species influencing the water-oxidizing enzyme system (see Discussion).

Fluorescence measurements

It has been shown previously that different ADRY agents like ANT 2p [7], ANT 2s [5] and CCCP [13] highly retard the recovery of variable fluorescence in DCMU-blocked chloroplasts. This effect has been explained by the assumption that ADRY agents compete with the internal back-reaction between S_2 and Q_A^-, thus leading to state $Q_A^- S_1$ which cannot act as a fluorescence quencher. Therefore, fluorescence measurements should provide an independent method for the analysis of the ADRY effect that is also applicable to Tris-washed chloroplasts completely deprived of their oxygen-evolving capacity. In the simplest case, the reaction pattern in the presence of DCMU and ANT 2p can be described by the following schemes for normal (Eqn. 1, see Ref. 7) and Tris-washed (Eqn. 2) chloroplasts:

$$\underset{\|Q_A S_1}{\text{DCMU}} \underset{k_{i,S_2}}{\overset{h\nu}{\rightleftharpoons}} \underset{\|Q_A^- S_2}{\text{DCMU}} \underset{k_{A,n}}{\overset{\text{ANT 2p}}{\rightarrow}} \underset{\|Q_A^- S_1}{\text{DCMU}} \qquad (1)$$

$$\underset{\|Q_A D_1}{\text{DCMU}} \underset{k_{i,D_1^{ox}}}{\overset{h\nu}{\rightleftharpoons}} \underset{\|Q_A^- D_1^{ox}}{\text{DCMU}} \underset{k_{A,T}}{\overset{\text{ANT 2p}}{\rightarrow}} \underset{\|Q_A^- D_1}{\text{DCMU}} \qquad (2)$$

These equations imply that the ANT 2p concentration remains constant, because the recovery kinetics are much faster than any possible ANT 2p oxidation.

Using this simple scheme, one has to take into account additionally that the flash-induced fluorescence yield change is not completely reversible, i.e., the fluorescence yield does not return to the level before the flash. Thus, apart from the voluntarily added donor (in this case, ANT 2p), another endogeneous component has to be regarded to compete with the back-reaction and electron injection by ANT 2p.

Depending on the homogeneity of all Photosystems II, generally two different mechanisms could

* These constants are related to the bulk phase ANT 2p concentration.

be responsible for the above-mentioned partial irreversibility of the fluorescence decay. If all Photosystems II are homogeneous, this phenomenon is due to competitive electron donation, so that in the case of ANT 2p, a three-fold parallel reaction scheme has to be solved. Accordingly, one obtains for the experimentally determined ratio of the oxidized and reduced form of Q_A at sufficiently long dark time after flash, $[Q_A^-]_\infty/[Q_A]_\infty$, the relation:

$$\frac{[Q_A^-]_\infty}{[Q_A]_\infty} = \frac{k_{A,j}}{k_{i,j}}[\text{ANT 2p}] + \frac{k_D}{k_{i,j}} \tag{3}$$

where k_D is the rate constant for electron donation by an unknown internal component D which statistically competes in all reaction centers with Q_A^- for S_2 and/or D_i^{ox} as an ultimate oxidant; $k_{A,j}$ and $k_{i,j}$ as defined by Eqns. 1 and 2 with $j = n$ or T, respectively. For Eqn. 3 as well as the following, Eqn. 4, the total amount of Q_A, i.e., $[Q_A^-] + [Q_A]$, has been normalized to 1. On the other hand, in the case of an extreme heterogeneity, one could assume the existence of two types of PS II, with only one fraction containing a donor for fast D_i^{ox} reduction. Therefore, only these systems are responsible for the irreversible fluorescence relaxation, because D reacts much faster than Q_A^- with S_2 and/or D_i^{ox}. If this reaction remains almost unaffected at moderate or low ANT 2p concentrations, the kinetic analysis leads to:

$$\frac{1}{[Q_A^-]_\infty} = 1 + \frac{k_{i,j}}{k_{A,j}} \frac{1}{[\text{ANT 2p}]} \tag{4}$$

Fig. 6 shows that in the absence of ANT 2p in Tris-washed chloroplasts, Q_A^- becomes reoxidized to an extent of 70–80% via a two-phase decay of the order of 10–20 ms (20% of total amplitude) and 200–250 ms (50–60% of total amplitude), while about 20–30% remain reduced, probably due to an alternative donor which competes with Q_A^- for D_i^{ox} as the ultimate oxidant. The shape of the fluorescence decay qualitatively corresponds with previous findings [14]. The reoxidation of Q_A^- in Tris-washed chloroplasts in the presence of DCMU is somewhat faster than in normal chloroplasts, but these differences will not be discussed here. The evaluation of the data in Fig. 6 according to Eqn. 3 is presented in Fig. 7 and shows that in the case of Tris-washed chloroplasts, the experimental data fit a straight line as predicted by the model: $k_{A,T} = k^0_{A,T}$ [ANT 2p]. The slope in Fig. 7 represents the ratio $k^0_{A,T}/k_{i,T}$ where $k_{i,T}$ is the internal rate constant for Q_A^- reoxidation by D_i^{ox} in the absence of ANT 2p. If only the dominant phase with $t_{1/2} = 200$–250 ms is considered to provide a sufficient approximation of $k_{i,T}$, one obtains $k^0_{A,T} = 3 \cdot 10^8\ M^{-1} \cdot s^{-1}$. Analogously, a linear relation is also obtained for $1/[Q_A^-]_\infty$ as a function of

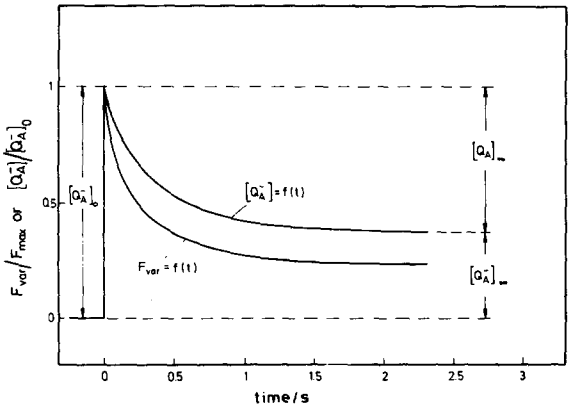


Fig. 6. Time-course of fluorescence yield and Q_A^- oxidation after one saturating flash in DCMU-treated Tris-washed spinach thylakoids in order to determine the proportion of permanently reduced centers. (chlorophyll concentration 20 μg/ml; DCMU concentration 2 μM).

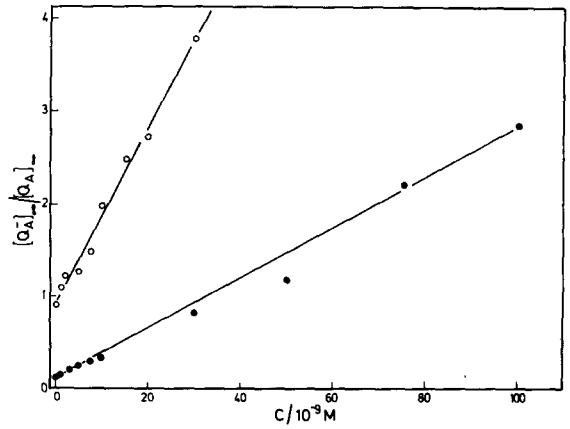


Fig. 7. Ratio of reduced and oxidized acceptor Q_A as a function of ANT 2p concentration in order to determine the second-order rate constant for the reaction between ANT 2p and the donor side in normal (●) or Tris-washed (○) thylakoids.

1/[ANT 2p] (data not shown). Taking the slope of this curve and using the same $k_{i,T}$ value as in Eqn. 3, one obtains $k^0_{A,T} = (4–5) \cdot 10^7\ M^{-1} \cdot s^{-1}$. Both methods give $k^0_{A,T}$ values, which differ by almost one order of magnitude. A comparison of the values shows that $k_{A,T}$ obtained by Eqn. 4 fits markedly better with data obtained by ERP measurements [8]. This might be a hint to favour a heterogeneous mechanism. However, the present kinetic data do not permit an unambiguous conclusion. Regardless of these details, the rate constants determined in this study are higher than those reported in Ref. 8. It remains to be analyzed whether or not the quite different experimental conditions (e.g., difference in the thylakoid concentration of more than two orders of magnitude, presence of DCMU) account for this phenomenon. The analogous experiment performed in normal chloroplasts also gives a straight line.

From the slope, a value of $k^0_{A,n} = 1.2 \cdot 10^7\ M^{-1} \cdot s^{-1}$ is derived which closely resemble the apparent half-life times obtained by oxygen yield measurements. A similar value is obtained by using Eqn. 4 (data not shown). The results provide additional evidence for the idea that in normal DCMU-treated chloroplasts, Q_A^- becomes predominantly reoxidized via S_2. Furthermore, these data show that DCMU binding to the acceptor does not markedly affect the interference of ANT 2p with the donor side of PS II, which agrees with previous conclusions based on thermoluminescence measurements [7].

Discussion

The present study shows that in isolated spinach chloroplasts the decay of S_2 and S_3 occurs predominantly via a one-electron redox sequence in contrast to recent data reported for algae [12]. This difference might be due to a lack of component C in class II chloroplasts that is claimed to be required for the coupling of two-electron transfer steps between S_3 and S_1, and S_2 and S_0, respectively. Further experiments are required to clarify this point.

In agreement with recent data [2], the present results confirm that S_1 is the most stable redox state of dark-adapted isolated spinach chloroplasts. Redox state S_0 was shown to become autooxidized with a half-life time of 5–10 min which slightly accelerates if the negative polarization of the electrode is switched off during dark adaptation. These data clearly show that the redox transition $S_0 \rightarrow S_1$ markedly differs from the other redox transitions in the water-oxidizing enzyme system with regard to its redox potential as originally reported by Bouges-Bocquet [15]. Therefore, this property should be kept in mind for all models proposed for the mechanism of photosynthetic water oxidation. ANT 2p does not interfere with the properties of the $S_0 \rightarrow S_1$ transition, except for the differences in transition kinetics.

The ANT 2p-catalyzed S_2 and S_3 decay can be consistently described by a reaction scheme that includes only one-electron transfer steps. It is further assumed that ANT 2p specifically reacts with the water-oxidizing enzyme system Y to form a complex in a reaction independent of the redox state S_i. The latter idea is based on thermoluminescence measurements revealing that ANT 2p, added to dark-adapted chloroplasts (i.e., being in state S_1) and frozen to $-15°C$ before flash-induced S_2 generation, also accelerates the S_2 decay [7]. In this case, biphasic decay kinetics are observed. This biphacicity supports the idea that at $-15°C$ the mobility of ANT 2p within the thylakoid membrane is drastically reduced so that only systems Y which were complexed with ANT 2p in the dark-adapted S_1 state exhibit an enhanced S_2-decay rate after flash-induced S_2 formation. The S_2 and S_3 decay kinetics as a function of ANT 2p concentration can be satisfactorily described in Scheme I by numerical evaluation of the same set of kinetic parameters. This analysis leads to interesting conclusions about the dynamics of ANT 2p action: (a) ANT 2p complexes the water-oxidizing enzyme system (including donor D_1) with a rather high affinity, but in contrast to the dynamics of many herbicides that bind to the PS II acceptor side [16], ANT 2p rapidly exchanges between different systems Y. (b) The dissociation rate constant of the ANT 2p system Y complex exceeds that of S_2 and S_3 decay, indicating that the ANT 2p effect is an activation-limited rather than a diffusion-limited reaction. (c) The scheme tacitly implies that in the case of ANT 2p being directly invovled in a redox reaction, the molecule becomes rapidly restored by an unknown donor so that

ANT 2p actually functions as a catalyst for S_2 and S_3 decay. Therefore, this study confirms our previous conclusion that ADRY agents act as mobile catalysts for S_2 and S_3 decay by an unknown electron donor [6]. That statement did not imply any specific mechanism and especially does not exclude a redox-type action (as extensively discussed in Ref. 17 for the catalytic process, it only means that ADRY agents are not irreversibly consumed as PS II electron donors. Furthermore, it was inferred, that no specific endogeneous electron donor is required for the ADRY agent-catalyzed S_2 and S_3 decay (see Ref. 6). Recently, it was inferred that EPR measurements 'argue against the catalytic model of ADRY reagent action' [8]. This statement, however, appears to be misleading, because it refers to the details of the catalytic mechanism rather than provide a proof to discard the catalytic function of ADRY agents. The present study shows that only one ANT 2p molecule per 40 PS II is sufficient to affect the S_2 and S_3 decay significantly. If one excludes the cooperation of 40 water-oxidizing enzyme systems Y with regard to the lifetimes of S_2 and S_3 (vide infra), the substoichiometric action can be explained only by the assumption that if ANT 2p really acts as reductant for oxidizing equivalents at the PS II donor side, it becomes restored rapidly enough by an endogeneous electron source. This is the typical reaction scheme for a catalytic mechanism where the catalyst (ANT 2p) undergoes a transient redox change. However, it should be emphasized that the postulation that ANT 2p participate in a redox cycle is not at all essential for the basic conclusion that ANT 2p acts as a catalyst for the S_2 and S_3 decay with an unknown endogeneous donor component (or components?). It is quite clear (see Refs. 5, 7, 13) that ANT 2p and other ADRY agents do not affect the internal decay mechanism of S_2 via recombination with Q_A^-, because in DCMU-blocked chloroplasts, the ANT 2p-catalyzed reduction of S_2 competes with the above-mentioned reaction (see also Fig. 7).

Despite recent evidence for ADRY-induced oxidation of cytochrome *b*-559 and carotenoids (for details see Ref. 8), the nature of the ultimate internal donor component for the ANT 2p-catalyzed S_2 and S_3 decay remains to be identified unambiguously. It was found that at only one ANT 2p molecule per PS II (500 chlorophylls) the ADRY effect still remains unimpaired, even after 200–300 single turnover flashes. This effect readily excludes cytochrome *b*-559 for stoichiometrical reasons as terminal electron acceptor and also carotenoids, because they are only transiently oxidized.

Another mechanistically important point should be mentioned. The ADRY effect has been sometimes related to lipophilic anions and therefore tetraphenylboron was suggested as 'super ADRY agent' [18]. However, it should be kept in mind that tetraphenylboron becomes irreversibly consumed [19], whereas ADRY agents function as (redox) catalysts in a completely reversible manner at sufficiently low concentrations. Latest data confirm this different behaviour also at very low concentrations of ANT 2p and tetraphenylboron, respectively (Hanssum, B. and Renger, G., unpublished data).

The effect of ANT 2p on the dark-reduction of D_1^{ox} in Tris-washed chloroplasts raised another question about the mechanism of ADRY agents (see Ref. 9): Do ADRY agents react directly with S_2 and S_3 in normal chloroplasts or do they catalyze S_2 and S_3 reduction indirectly via D_1? In the latter case, two alternatives could be distinguished: (a) ADRY agents cause S_2 and S_3 decay via D_1^{ox} reduction without affecting the redox equilibrium between D_1/D_1^{ox} and S_1/S_2 and S_2/S_3, respectively. (b) ADRY agents simultaneously shift the above-mentioned redox equilibrium and catalyze D_1^{ox} reduction by an internal donor other than Q_A^-. In principle, both mechanisms should be distinguishable by kinetic measurements. Unfortunately, a possible D_1^{ox} attack by ADRY agents in normal chloroplasts cannot be detected directly. on the other hand, a comparison of the rates of D_1^{ox} reduction in Tris-washed chloroplasts with the S_2 and S_3 decay in normal chloroplasts does not permit unambiguous conclusions, because the reactivity of ANT 2p with D_1^{ox} could be different in both types of chloroplasts due to the loss of at least three surface-exposed polypeptides at the system II donor side in Tris-washed chloroplasts [20]. Therefore, further experiments are requried to clarify the mechanistic details of the ADRY effect.

A last point should be briefly discussed. The effect of substoichiometric ANT 2p concentrations

could be also explained by an alternative mechanism to the mobile-catalyst model described by Scheme I. If one assumes that a large number of water-oxidizing enzyme systems share a sequestered domain, which affects the lifetimes of S_2 and S_3, an ANT-2p-induced modification of this domain by only a few ANT 2p molecules (at the extreme of only one molecule) would be sufficient to change the S_2 and S_3 lifetime of all water-oxidizing enzyme systems Y. Evidence for sequestered proton and chloride domains around the water-oxidizing enzyme systems Y have been reported recently [21–23]. However, an ANT 2p attack on these proton domains appears very unlikely as the cause of the observed ADRY effect. It is known that gramicidin equilibrates the sequestered domain [23] with the bulk phase. In contrast to that, the lifetimes of S_2 and S_3 remain invariant to rather high gramicidin concentrations that include a drastic increase of H^+ permeability in the thylakoid membrane [24] and therefore should also permit the significantly slower equilibration of the sequestered domains [23].

These arguments, of course, do not totally exclude a domain mechanism for the ADRY effect, but for the time being a mobile catalyst mechanism appears more attractive, because it describes the experimental data consistently. Regardless of the mechanistic details of the ADRY effects, one might speculate, that the sequestered domains if they really exist in class-II particles facilitate the ANT 2p diffusion between different water-oxidizing enzyme systems. Another important implication of the present study should be emphasized: conclusions drawn from substoichiometric titration experiments have to be considered with care unless the mode of action of the reagents which were used for the experiment is unambiguously resolved in its mechanistic details.

Appendix

Determination of S_2 and S_3 decay kinetics

The S_2 and S_3 population can be generally calculated from experimental data according to Kok's scheme using the recursion equations:

$$Y_n = (1-\alpha)S_{3,n-1} + \beta S_{2,n-1} \tag{A-1}$$

and:

$$\boldsymbol{S}_n = \mathbf{K}\boldsymbol{S}_{n-1} \tag{A-2}$$

Where Y_n is the O_2 yield caused by the nth flash of a train, $S_{2,n-1}$ and $S_{3,n-1}$ is the probability of S_2 and S_3 population just before the nth flash, $\boldsymbol{S}_n$ and $\boldsymbol{S}_{n-1}$ are state vectors and $\mathbf{K}$ the transformation matrix of the S_i states (for details, see Ref. 25), α and β represent the probability of misses and double hits, respectively. After two preillumination flashes, the population of state S_0 is rather low and can be neglected. Furthermore, if a first-order approximation for α and β terms is applied, the following equations are obtained:

$$S_3(t_d) = \frac{0.25}{1-\alpha}\left[Y_1(t_d) - \frac{\beta Y_2(t_d)}{1-\alpha}\right] \tag{A-3}$$

$$S_2(t_d) = \frac{0.25}{1-2\alpha-\beta}\left[Y_2(t_d) - \frac{\alpha Y_1(t_d)}{1-\alpha} - \frac{\beta Y_3(t_d)}{1-3\alpha}\right] \tag{A-4}$$

with $S_2(t_d)$ and $S_3(t_d)$ the normalized population of S_2 and S_3, respectively, at dark time t_d after two preillumination flashes, and $Y_i(t_d)$ the oxygen yield caused by the ith flash of the train starting at t_d after two preillumination flashes ($i = 1, 2, 3$).

The numerical calculation was performed with values $\alpha = 0.11$, $\beta = 0.03$ and by definition of the steady-state flash yield:

$$\tfrac{1}{10}\sum_{i=1}^{10} Y_i = 0.23$$

The decay kinetics of S_2 and S_3 were calculated on the basis of the one-electron transfer sequence:

$$S_3 \overset{k_3}{\rightarrow} S_2 \overset{k_2}{\rightarrow} S_1$$

and taking into account the fast S_2 and S_3 decay arising in a fraction (symbolized by 'a') of the centers by an as yet not identified internal donor component (see Ref. 2):

$$S_3(t_d) = S_3(0)[a\cdot\exp(-k_{3,\text{fast}}\cdot t_d) + (1-a)\cdot\exp(-k_{3,\text{slow}}\cdot t_d)] \tag{A-5}$$

$$S_2(t_d) = S_3(0)\left\{\frac{a\cdot k_{3,\text{slow}}}{k_{2,\text{slow}} - k_{3,\text{fast}}}[\exp(-k_{3,\text{fast}}\cdot t_d)\right.$$

$$-\exp(-k_{2,\text{slow}}t_d)] + \frac{(1-a)k_{3,\text{slow}}}{k_{2,\text{slow}} - k_{3,\text{slow}}}$$

$$\times [\exp(-k_{3,\text{slow}}t_d) - \exp(-k_{2,\text{slow}}t_d)]\Big\}$$

$$+ S_2(0)[a \cdot \exp(k_{2,\text{fast}}t_d) - (1-a) \cdot \exp(k_{2,\text{slow}}t_d)] \quad \text{(A-6)}$$

For the sake of simplicity, the small percentage of donor consumption between the first and second preillumination flash has been neglected. The kinetic parameters $k_{2,\text{fast}}$, $k_{2,\text{slow}}$, $k_{3,\text{fast}}$ and $k_{3,\text{slow}}$ as well as factor 'a' were experimentally determined as described in Ref. 2.

Numerical evaluation according to Scheme I of S_2 and S_3 decay kinetics as a function of ANT 2p concentration

Scheme I implies the following set of differential equations for S_3 and S_2 decay in the presence of ANT 2p:

$$\frac{d[S_3]}{dt} = -(k_{i,3} + k_{1,3}[\text{ANT 2p}])[S_3] + k_{-1,3}[S_3 \cdot \text{ANT 2p}] \quad \text{(A-7)}$$

$$\frac{d[S_3 \cdot \text{ANT 2p}]}{dt} = k_{1,3}[S_3][\text{ANT 2p}] - (k_{-1,3} + k^*_{i,3} + k_{A,3})[S_3 \cdot \text{ANT 2p}] \quad \text{(A-8)}$$

$$\frac{d[S_2]}{dt} = k_{i,3}[S_3] + k_{-1,2}[S_2 \cdot \text{ANT 2p}] + k_{A,3}[S_3 \cdot \text{ANT 2p}] - (k_{i,2} + k_{1,2}[\text{ANT 2p}])[S_2] \quad \text{(A-9)}$$

$$\frac{d[S_2 \cdot \text{ANT 2p}]}{dt} = k_{1,2}[S_2][\text{ANT 2p}] + k^*_{i,3}[S_3 \cdot \text{ANT 2p}] - (k_{-1,2} + k^*_{i,2} + k_{A,2})[S_2 \cdot \text{ANT 2p}] \quad \text{(A-10)}$$

$$\frac{d[S_1]}{dt} = k_{i,2}[S_2] + k_{-1,1}[S_1 \cdot \text{ANT 2p}] + k_{A,2}[S_2 \cdot \text{ANT 2p}] - k_{1,1}[S_1][\text{ANT 2p}] \quad \text{(A-11)}$$

$$\frac{d[S_1 \cdot \text{ANT 2p}]}{dt} = k_{1,1}[S_1][\text{ANT 2p}] + k^*_{i,2}[S_2 \cdot \text{ANT 2p}] - k_{-1,1}[S_1 \cdot \text{ANT 2p}] \quad \text{(A-12)}$$

$$\frac{d[\text{ANT 2p}]}{dt} = -(k_{1,1}[S_1] + k_{1,2}[S_2] + k_{1,3}[S_3])[\text{ANT 2p}] + (k_{-1,3} + k_{A,3})[S_3 \cdot \text{ANT 2p}] + (k_{-1,2} + k_{A,2})[S_2 \cdot \text{ANT 2p}] + k_{-1,1}[S_1 \cdot \text{ANT 2p}] \quad \text{(A-13)}$$

The initial concentration of [ANT 2p] after addition to dark-adapted chloroplasts is obtained by using mass balance equation:

$$[\text{ANT 2p}]_0 = [\text{ANT 2p}]_{\text{free}} + [S_1 \cdot \text{ANT 2p}]$$

and:

$$[S_1] + [S_1 \cdot \text{ANT 2p}] = 1 \quad \text{(A-14)}$$

as well as the equilibrium condition:

$$\frac{k_{-1}}{k_1} = \frac{[S_1][\text{ANT 2p}]}{[S_1 \cdot \text{ANT 2p}]} \quad \text{(A-15)}$$

The numerical evaluation of this set of differential equations was performed on a Hewlett-Packard 9825 B desk calculator using a Runge-Kutta-algorithm with dynamic subintervalls. A 20% fraction the S_2 and S_3 population was considered to decay via fast internal kinetics (see Ref. 2) during the course of integration.

Acknowledgements

The authors would like to thank A. Schulze for drawing the figures. The financial support by the Deutsche Forschungsgemeinschaft is gratefully acknowledged.

References

1 Renger, G. and Govindjee (1985) Photosynth. Res., Vol. 6, issue 1, in the press
2 Vermaas, W.F.J., Renger, G. and Dohnt, G. (1984) Biochim. Biophys. Acta 764, 194–202
3 Joliot, P. and Kok, B. (1975) in Bioenergetics of Photosynthesis (Govindjee, ed.), pp. 387, Academic Press, New York

4 Renger, G. (1972) Biochim. Biophys. Acta 256, 428–439
5 Renger, G., Bouges-Bocquet, B. and Delosme, R. (1973) Biochim. Biophys. Acta 292, 796–807
6 Renger, G. (1973) Biochim. Biophys. Acta 314, 113–116
7 Renger, G. and Inoue, Y. (1983) Biochim. Biophys. Acta 725, 146–154
8 Ghanotakis, D.F., Yerkes, C. and Babcock, G.T. (1982) Biochim. Biophys. Acta 682, 21–31
9 Renger, G. and Reuter, R. (1982) Photobiochem. Photobiophys. 3, 317–325
10 Joliot, P. (1972) Methods Enzymol. 24, 123–134
11 Joliot, P. and Joliot, A. (1964) Compt. Rend. Acad. Sci. Paris 2850, 4622–4625
12 Lavorel, J. and Maison-Peteri, B. (1983) Physiol. Veg. 21, 509–517
13 Homann, P.H. (1971) Biochim. Biophys. Acta 245, 129–143
14 Etienne, A.L., Boussac, A. and Lavergne, J. (1981) in Proceedings of the 5th International Congress on Photosynthesis (Akoyunoglou, G., ed.), Vol. 2, pp. 405–413, Balaban Internaional Science Services, Philadelphia, PA
15 Bouges-Bocquet, B. (1980) Biochim. Biophys. Acta 594, 85–103
16 Vermaas, W., Dohnt, G. and Renger, G. (1984) Biochim. Biophys. Acta 765, 74–89
17 Renger, G. (1972) Eur. J. Biochem. 27, 259–269
18 Velthuys, B.R. (1981) in Proceedings of the 5th International Congress on Photosynthesis (Akoyunoglou, G., ed.), Vol. 2, pp. 75–85, Balaban International Science Services, Philadelphia, PA
19 Erixon, K. and Renger, G. (1974) Biochim. Biophys. Acta 333, 95–106
20 Larsson, C., Jansson, C., Ljungberg, U., Åkerlund, H.-E. and Andersson, B. (1984) in Advances in Photosynthesis Research (Sybesma, C., ed.), Vol. I, pp. 363–366, Martinus Nijhoff/Dr. W. Jung Publishers, The Hague
21 Johnson, J.D., Pfister, V.R. and Homann, P.H. (1983) Biochim. Biophys. Acta 723, 256–265
22 Theg, S.M. and Junge, W. (1983) Biochim. Biophys. Acta 723, 294–307
23 Dilley, R.A., Prochaska, L.J., Baker, G.M., Tandy, N.E. and Millner, P.A. (1982) Curr. Top. Membrane Transport. 116, 345–369
24 Renger, G., Bouges-Boquet, B. and Büchel, K.H. (1973) J. Bioenerg. 4, 491–505
25 Lavorel, J. (1976) J. Theor. Biol. 57, 171–185