REQUIREMENT OF AN ACIDIC PROTON IN SUBSTANCES WHICH ACT AS ACCELERATORS OF THE DEACTIVATION REACTIONS IN THE WATER-SPLITTING ENZYME SYSTEM OF PHOTOSYNTHESIS

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1. Introduction

Recently it was found [1, 2], that the positive charges generated by photosystem II, which are responsible for water oxidation (these charges are designated as trapped holes, cf. [3]), can be destabilized by a class of substances, designated ADRY-agents* [4]. The molecular mechanism of the ADRY effect remains to be elucidated, but in the last year results were obtained leading to the conclusion that the acidic NH-group, present in all ADRY-agents so far known, is an important functional element [4]. In this paper it will be shown that substances which contain an acidic OH-group instead of an acidic NH-group, can act as ADRY agents.

Furthermore, it was found that the *N*-methylderivatives of the ADRY agents ANT 2a and ANT 2p do not act as ADRY substances. From these results it is concluded that the presence of an acidic group in the ADRY substances is probably an indispensible functional element for their action as ADRY agents.

Measurements of the ADRY effect as a function of the pH of the chloroplasts suspension lead to the conclusion that the negatively charged anion form is important for the ADRY effect.

* Abbreviations:

ADRY:	Acceleration of the deactivation reactions in the 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;
NMANT 2a:	2-*N*-methyl-(4-chloro)anilino-3,5-dinitrothiophene;
NMANT 2p:	2-*N*-methyl-(3-chloro-4-trifluoromethyl)anilino-3,5-dinitrothiophene;
DNP:	2,4-dinitrophenol;
MES:	morpholinoethanesulfonate.

2. Materials and methods

Spinach chloroplasts were prepared from market spinach according to the method of Winget et al. [5]; 5% dimethylsulfoxide was added as protective agent for storage in liquid nitrogen.

The oxygen was measured polarographically with a Clark-type electrode [6] by a repetitive technique as described elsewhere [4]. The complete reaction mixture contained: chloroplasts (50 μM chlorophyll), 0.1 mM $K_3[Fe(CN)_6]$ + 0.1 mM $K_4[Fe(CN)_6]$ as electron acceptor, 10 mM KCl, 2 mM $MgCl_2$, 20 mM MES-NaOH pH ≈ 6.5. Temperature: 21°. Photosynthesis was excited by saturating white short flashes ($\tau_{\frac{1}{2}} \approx 20$ μsec).

3. Results and discussion

It had been shown earlier (cf. [4]) that the rate of the deactivation reactions in the water-splitting enzyme system is correlated with the decrease of the relative average oxygen yield per flash $\varphi(t_d)$ with increasing time t_d between the flashes. Furthermore,

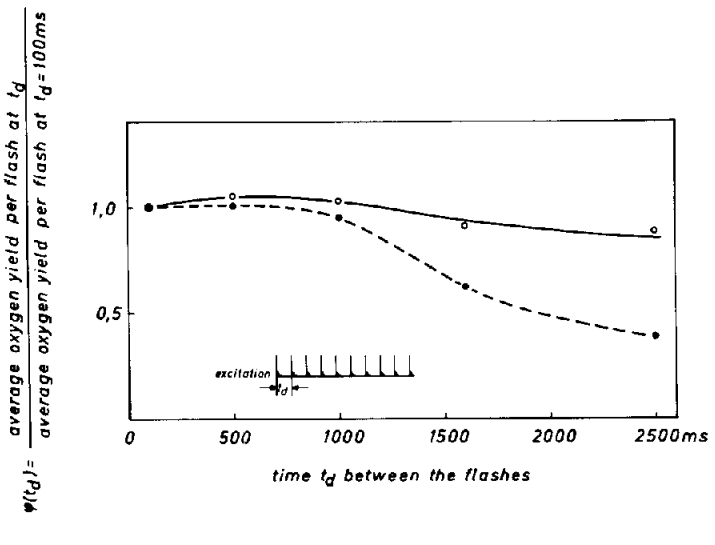

Fig. 1. Relative average oxygen yield per flash $\varphi(t_d)$ as a function of the time t_d between the flashes in the absence (o–o–o) and in the presence of 1 mM DNP (•–•–•) in chloroplasts. Experimental conditions were as described in Materials and methods.

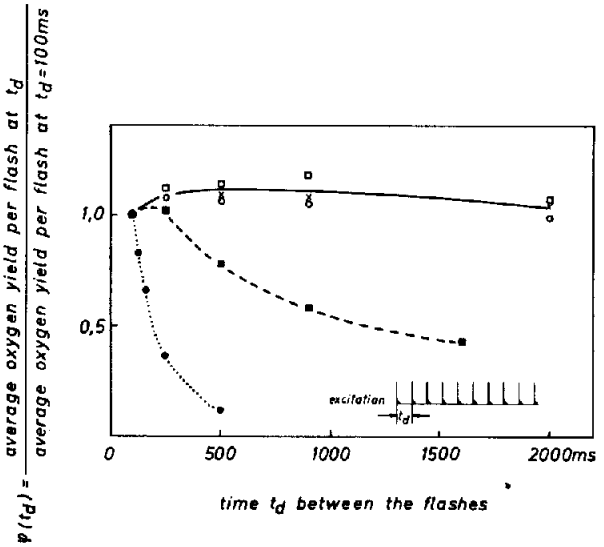

Fig. 2. Relative average oxygen yield per flash $\varphi(t_d)$ as a function of the time t_d between the flashes in the absence (x–x–x) and in the presence of various derivatives of 2-anilinothiophene in chloroplasts. Anilinothiophene concentration: 1 μM. (o–o–o) NMANT 2p, (•–•–•) ANT 2p, (□–□–□) NMANT 2a, (■–■–■) ANT 2a. Other experimental conditions were as described in Materials and methods.

it was found that the rate of the ADRY effect can be described approximately by first order kinetics (cf. [4]).

In fig. 1 $\varphi(t_d)$ as a function of the time t_d between the flashes is depicted in the absence and presence of 1 mM DNP. The results clearly show, that DNP experts an ADRY effect. DNP is distinguished from the ADRY-agents so far known by the presence of an acidic OH-group instead of the acidic NH-group. The ADRY-effect of substances containing an acidic OH-group has also been demonstrated by Vater [7] for the indophenols in more detail.

Therefore, it can be inferred that the acidic NH-group is not an indispensible functional element of the ADRY-agents, but rather it seems important that molecules acting as ADRY-agents contain an acidic proton.

The role of an acidic proton for the ADRY effect was tested by comparing the action of well known ADRY-agents to that of their *N*-methyl derivatives on the function $\varphi(t_d)$. The method for the synthesis of the *N*-methyl derivatives will be described elsewhere (G. Renger and K.H. Buchel, in preparation). In fig. 2 the effect of different derivatives of 2-anilino-3,5-dinitrothiophene on $\varphi(t_d)$ is depicted. It seen that the *N*-methyl derivatives of ANT 2a and ANT 2p do not lead to an acceleration of the natural decrease rate of $\varphi(t_d)$, i.e. they do not act as ADRY-agents. As was already shown [4], ANT 2a and ANT 2p exert an ADRY effect. From these results it is concluded that an acidic proton is probably an indispensible functional element of all ADRY-agents.

It remains to investigate whether the protonated or the non-protonated form of the ADRY-agents or both are the active species.

In order to answer this question the reciprocal half time $(\tau_{h\frac{1}{2}})^{-1}_{app}$ (cf. [4]) of the decrease of $\varphi(t_d)$ was determined as a function of the pH-value of the chloroplast suspension in the pH range of 5–8 at a constant total amount of ADRY-agent. In this way the anion concentration can be varied, dependent on the pH value according to:

$$[ADRY^-]_k = \frac{[K_{ADRY}]_k}{[H^+]_k + [K_{ADRY}]_k} \cdot [ADRY_{tot}]_k \quad (1)$$

$[ADRY_{tot}]$ = total amount of the ADRY-agent,

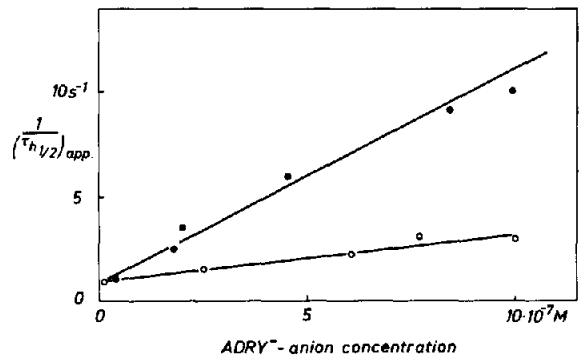

Fig. 3. Reciprocal half time $(\tau_{h\frac{1}{2}})^{-1}_{app}$ of the apparent first order decay of $\varphi(t_d)$ as a function of the ADRY-agent-anion concentration at a constant total amount (0.2 or 1 μM) of the ADRY-agents in chloroplasts. The $ADRY^-$ concentration was determined according to eq. (3). (○–○–○) ANT 2a, (●–●–●) ANT 2s. Buffers used: 10 mM MES-NaOH in the pH range of 5–7, and 10 mM Tricine-NaOH in the range of 7–8. Other experimental conditions were as described in Materials and methods.

K_{ADRY} = dissociation constant of the acidic proton, index k refers to the k-th phase of the complex system of a chloroplast suspension.

Since the ADRY-agents act in the water-splitting enzyme system which is postulated to be localized near the inner phase of the thylakoid [8,9], it is assumed that the ADRY-agent concentration of the inner aqueous phase is relevant for the ADRY-effect. Therefore, only a two-phase system will be considered, with $k = i$ or o (i = inner, o = outer aqueous phase of the thylakoid, respectively).

For the evaluation of $[ADRY_{tot}]_i/[ADRY_{tot}]_o$ the neutral form of the ADRY-agents is considered as to be freely permeable through the thylakoid membrane (this assumption is compatible with results obtained for different uncouplers (cf. [10–12]). Then one obtains:

$$\frac{[ADRY_{tot}]_i}{[ADRY_{tot}]_o} = \left(\frac{[K_{ADRY}]_i + [H^+]_i}{[K_{ADRY}]_o + [H^+]_o}\right) \cdot \frac{[H^+]_o}{[H^+]_i} \cdot P_{ADRY} \qquad (2)$$

P_{ADRY} = partition coefficient of the neutral form of the ADRY-agent between the inner and the outer phase of the thylakoid. Assuming aqueous phases of similar ionic strength:

$$[K_{ADRY}]_i \approx [K_{ADRY}]_o \text{ and } P_{ADRY} \approx 1 \qquad (3a)$$

If one additionally assumes, that

$$[H^+]_i \approx [H^+]_o \qquad (3b)$$

(because of the uncoupling activity, cf. [13]), then it follows:

$$[ADRY_{tot}]_i \approx [ADRY_{tot}]_o \qquad (3c)$$

and therefore from eq. (1):

$$[ADRY^-]_i \approx [ADRY^-]_o \qquad (4)$$

In fig. 3 the reciprocal half time $(\tau_{h\frac{1}{2}})^{-1}_{app}$, indicating the rate of the ADRY-effect, is depicted as a function of the $ADRY^-$ concentration, determined according to eq. (1) with the pK-values of [13] for different pH values of the chloroplast suspension.

The obtained results show that the ADRY-effect increases with increasing $ADRY^-$ concentrations at a constant total ADRY amount. Hence, it is concluded that the anion form of the ADRY-agents is important for the observed ADRY-effect. Further conclusions about the mechanism of the ADRY-effect cannot be drawn because of the complexity of $(\tau_{h\frac{1}{2}})^{-1}_{app}$ (cf. [4]) and the restrictions introduced into the discussion, as expressed by eqs. (2), (3a) and (3b).

Summarizing, it can be concluded that an acidic proton is probably an indispensible functional element of ADRY-agents, and that their anion form is important for the ADRY-effect.

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1. *Note added in proof*

The importance of an acidic NH-group in biological effectors was clearly shown for the ability of agents to

act as potent uncouplers of oxidative phosphorylation by K.H. Büchel and F. Korte, Angew. Chemie 77 (1965) 814. A similar role of the NH-group was found for the action of benzimidazole-derivatives as inhibitors of photosynthetic reactions, K.H. Büchel, W. Draber, A. Trebst and E. Pistorius, Z. Naturforsch. 21b (1966) 247.

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