THE ACTION OF 5-CHLORO-3-tert. BUTYL-2′-CHLORO-4′-NITRO–SALICYLANILIDE AND α,α′-BIS(HEXAFLUOROACETONYL)ACETON ON THE WATER-SPLITTING ENZYME SYSTEM Y IN SPINACH CHLOROPLASTS

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Received 20 January 1975

1. Introduction

During the last years a number of chemicals including derivatives of carbonylcyanidephenylhydrazones, of 2-anilino-3,5-dinitrothiophenes and of phenoles have been found to destabilize the oxidizing equivalents which are stored in the photosynthetic water-splitting enzyme system Y [1]. This phenomenon has been designated as ADRY-effect* [2]. It has been shown that the agents which induce an ADRY-effect are characterized by their ability to act as uncouplers of phosphorylation and by the presence of an acidic NH- or OH-group in their molecular structure [1,3]. The frequently used uncouplers 5-chloro-3-tert. butyl-2′-chloro-4′-nitrosalicylanilide (S 13) and α,α′-bis(hexafluoroacetonyl)aceton (1799) contain an acidic NH- and OH-group, respectively. Hence, it could be anticipated, that these substances act not only as uncouplers, but also as ADRY-agents.

In the present paper it will be shown, that indeed both substances exert an ADRY-effect. This effect occurs in nearly the same concentration range as the uncoupling effect.

* *Abbreviations*: ADRY: Acceleration of the deactivation reactions of the watersplitting enzyme system Y. CCCP: carbonylcyanide-*m*-chlorophenylhydrazone. FCCP: carbonylcyanide-*p*-trifluoromethoxyphenylhydrazone. S13: 5-chloro-3-tert.butyl-2′-chloro-4′-nitrosalicylanilide, 1799: α,α′-bis(hexafluoroacetonyl)aceton. Tricine: *N*-tris-(hydroxymethyl)-methylglycine.

2. Materials and methods

Spinach chloroplasts were prepared from market spinach according to the method of Winget et al. [4]; 5% dimethylsulfoxide was added as protective agent for storage in liquid nitrogen.

The oxygen was measured with a Clark-type electrode [5] by a repetitive technique as has been described in [2]. The complete reaction mixture contained: chloroplasts (50 μM chlorophyll), 0.25 mM K_3 [$Fe(CN)_6$] + 0.25 mM K_4 [$Fe(CN)_6$] as electron acceptor, 10 mM KCl, 2 mM $MgCl_2$, 20 mM Tricine–NaOH, pH = 7.0. Temperature: 21°C. Photosynthesis was excited with saturating short flashes ($\tau_{1/2} \approx$ 20 μsec).

3. Results

It has been shown earlier that the rate of the deactivation of the higher trapped hole accumulation states S_2 and S_3 (s. Kok-model, [6]) of the water-splitting enzyme system Y is correlated with the decrease of the relative average oxygen yield per flash, $\varphi(t_d)$, with increasing time t_d between the flashes [7]. $\varphi(t_d)$ directly reflects the relative steady state concentration of $S_3(t_d)$ under repetitive flash excitation conditions [7].

In fig.1 $\varphi(t_d)$ as a function of the time t_d between the flashes in the absence and in the presence of 30 μM S 13 and of 0.6 mM 1799, respectively, is depicted. The results clearly show a significant acceleration of

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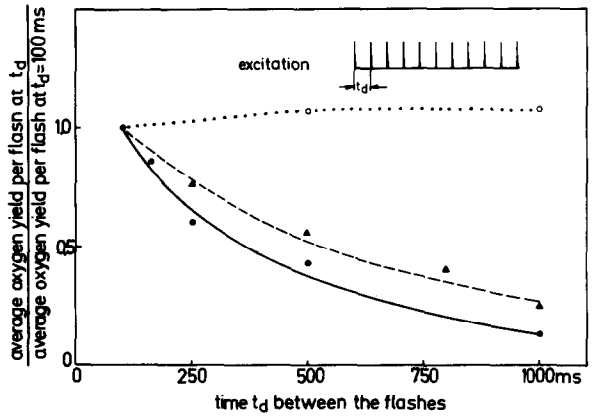

Fig.1. Relative average oxygen yield per flash $\varphi(t_d)$ as a function of the time t_d between the flashes in the absence (o···o···o) and in the presence of 30 μM S 13 (●---●—●) or of 1799 (x–x–x) in spinach chloroplasts.
Experimental conditions were as described in Materials and methods.

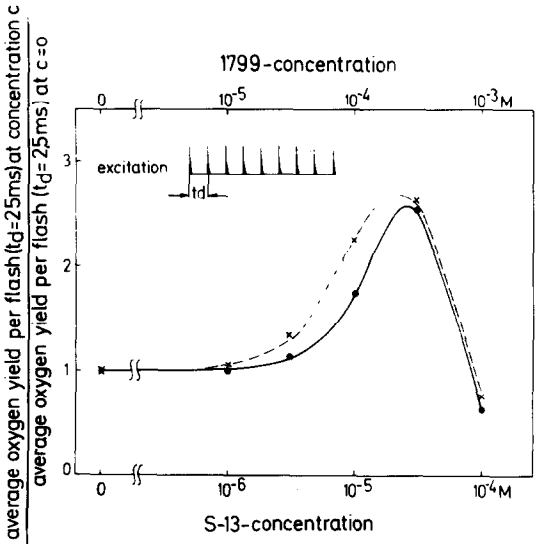

Fig.2. Average oxygen yield per flash at t_d = 2.5 msec as a function of the concentration of S 13 or of 1799, normalized by the average oxygen yield per flash at t_d = 2.5 msec in the absence of these substances, in spinach chloroplasts. Addition of S 13 (●—●—●) or of 1799 (x–x–x) as indicated by the abscissae. Other experimental conditions were as described in Materials and methods.

$\varphi(t_d)$ with half times of 250 msec (30 μM S 13) and 450 msec (0.6 mM 1799), respectively. At lower concentrations the accelerating effect is much weaker or absent, whereas at higher concentrations inhibitory effects arise (results not shown here). Hence, both substances acting, a suitable concentration range, as powerful ADRY-agents. It is seen that for the induction of approximately the same ADRY-effect a more than 20-fold higher concentration of 1799 in comparison to S 13 is required.

In order to investigate the mode of action of a special substance as an ADRY-agent firstly the question arises whether there exists for each ADRY-agent any form of correlation between its activity as an uncoupler and the ADRY-effect. Hence, the concentrations of S 13 and 1799, respectively, remain to be determined, which are necessary for the induction of the uncoupling effect.

The uncoupling effect can be simply measured by the stimulation of the rate of basal electron transport in continuous light of saturating intensity or in repetitive flashes at dark times between the flashes, t_d, which are short in comparison to the half time of the rate limiting step of the linear electron transport chain [8].

In fig.2 the average oxygen yield per flash at t_d = 2.5 msec as a function of S 13- or 1799-concentration, normalized by the corresponding value in the absence of an uncoupler, is given. The results indicate that qualitatively the uncoupling effect, reflected by an increase of the normalized average oxygen yield per flash at t_d = 2.5 msec, rises in nearly the same concentration range as the ADRY-effect. However, there does not seem to exist an exact quantitative correlation between both effects with respect to the dependence on concentration (unpublished results). Further experiments are required to clarify the quantitative relation between uncoupling activity and ADRY-effect for ADRY-agents with different chemical structure.

The decrease observed at higher concentrations is caused by inhibitory effects.

4. Discussion

The present results confirm earlier findings which indicate that uncouplers containing NH- or OH-groups act additionally as ADRY-agents, i.e. they decrease the

lifetimes of the higher trapped hole accumulation states S_2 and S_3 of the water-splitting enzyme system Y. The rough correlation between ADRY-effect and uncoupling activity could indicate that the stability of S_2 and S_3 is strongly related to the proton gradient across the thylakoid membrane. However, this possibility has been already excluded on the basis of results which show that some uncouplers like NH_4Cl or gramicidin do not act as ADRY-agents [1]. Hence, with respect to the biological function two types of uncouplers have to be distinguished: the 'normal-type' uncouplers and the 'ADRY-type' uncouplers. The present results show that, at least for the substances S 13 and 1799, the uncoupling activity and the ADRY-effect occur in nearly the same concentration range. Hence, if 'ADRY-type' uncouplers are applied, their bifunctional action has to be taken into consideration for the interpretation of all experimental results of those biological activities which not only depend on the energization of the thylakoid membrane, but in addition are related to the charge accumulation state S_i ($i = 0,...4$) of the water-splitting enzyme system Y. Phenomena of this kind are the oxygen evolution [9], the H^+-release by system II with water as electron donor [10] and the delayed fluorescence [11]. In this respect it is interesting to note, that in spinach chloroplasts luminescence triggered by organic solvents [12] was totally blocked by 'ADRY-type' uncouplers CCCP and FCCP, whereas 'normal-type' uncouplers (NH_4Cl, gramicidin, valinomycin) remain practically without influence.

Because the ADRY-effect is characterized by comparitive slow kinetics, this type of action becomes prominent only at continuous light of low intensity or with repetitive flashes at times $t_d > 25 - 50$ msec, depending on the concentration and on the specific power of the 'ADRY-type' uncoupler used.

Acknowledgements

The author is very indebted to Dr W. A. Cramer, Purdue University, West Lafayette, for the gift of S 13 and 1799. He wishes to thank Mrs Hohm-Veit for her valuable technical assistance. The financial support of Deutsche Forschungsgemeinschaft is gratefully acknowledged.

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