THE INDUCTION OF A HIGH RESISTANCE TO 3-(3,4-DICHLOROPHENYL-1,1-DIMETHYLUREA (DCMU) OF OXYGEN EVOLUTION IN SPINACH CHLOROPLASTS BY TRYPSIN TREATMENT

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1. Introduction

DCMU, the most widely used poison in present photosynthesis research [1,2], is known to inhibit very effectively the electron transfer from the reaction centers of system II, C_{II}, into the plastoquinone pool [3,4] without affecting the functional integrity of C_{II} itself [5,6]. Hence, with the aid of DCMU-type inhibitors a functional isolation of the oxygen evolving capacity from the overall electron transport should be possible, if the electrons produced at C_{II} are transferred to exogenous acceptors. However, substances which are able to mediate a DCMU-insensitive oxygen evolution are almost unavailable. Therefore, it has been inferred, that the primary electron acceptor of photosystem II, X 320 (see ref. [1,7]), is covered up by a barrier which hinders the electron transfer to external electron acceptors [8]. If this assumption is correct, then a suitable modification or the removal of the X320 barrier should be sufficient to allow the mediation of a DCMU-insensitive oxygen evolution by exogeneous electron carriers of sufficiently high redox potential.

Indeed, it was found that in special cell free preparations of *Phormidium luridum* a DCMU resistant oxygen evolution can be obtained with $K_3[Fe(CN)_6]$ as the electron acceptor [9,10]. Furthermore, electron acceptors like silicomolybdate which are also effective as structural modifiers [11] were shown to mediate a system II electron transport insensitive to DCMU [12].

Recently, it was found by Regitz and Ohad [13] that digestion of *Chlamydomonas reinhardi* chloroplasts with soluble trypsin removes the DCMU-inhibition site of photosystem II. However, as the water-splitting enzyme system was reported to be concomittantly destroyed, Regitz and Ohad did not investigate the possibility of the induction of a DCMU-insensitive oxygen evolution. On the other hand, I found that under selected conditions a DCMU-resistant oxygen evolution can be obtained in spinach chloroplasts by mild trypsin treatment [14]. In the present communication it is shown, that trypsination of spinach chloroplasts under suitable conditions leads to the induction of resistance to DCMU of $K_3[Fe(CN)_6]$-mediated oxygen evolution. Furthermore, in contrast to normal chloroplasts, the rates of oxygen evolution and of the reoxidation of $X\,320^-$ in trypsinated chloroplasts, become strongly dependent on the nature and concentration of the exogeneous electron acceptor.

2. Materials and methods

The chloroplasts were prepared from market spinach according to the method of Winget et al. [15], except that 10 mM ascorbate was present in the grinding medium. For storage in liquid nitrogen 5% dimethylsulfoxide was added. The oxygen evolution activity of the stored chloroplasts after thawing was nearly the same as that of freshly prepared chloroplasts.

The reaction mixture contained: chloroplasts (50 μM and 100 μM chlorophyll for the measurements of oxygen and of the absorption changes, respectively). 10 mM KCl, 2 mM $MgCl_2$, 20 mM Tricine–NaOH,

pH 7.0. The addition of electron acceptors is indicated in the figures.

Chloroplasts suspensions containing 50 μg/ml trypsin (commercially available from Boehringer) were incubated in the dark for 5 min at room temperature, then the measurements were performed without addition of trypsin inhibitor (see also ref. [16]).

Oxygen was measured with a Clark-type electrode [17] by a repetitive flash excitation technique (see ref. [18]). The absorption changes due to the turnover of X 320 were detected by the method described in ref. [19], except that for excitation longer flashes (~ 20 μs) have been used. The maximum of the X 320 absorption change is located around 325 nm, but the absorption changes were measured at 334 nm, because at this wavelength there exists an emission band of the ultraviolet-mercury lamp (Hanovia), with which a higher signal/noise ratio was obtained. The same results were found at 325 nm. All measurements were made at room temperature (20°C).

3. Results and discussion

The rate of photosynthetic oxygen evolution is determined by two factors: (a) the total number of functional active water-splitting enzyme systems Y, N_Y, and (b) the rate limiting step of the overall electron transport from H_2O to the terminal acceptor. Both parameters can be experimentally obtained by measurements of the average oxygen yield under repetitive flash excitation conditions. If the time t_d between the flashes is long in comparison to the electron transfer time of the rate limiting step, τ_{lim}, then the average oxygen yield per flash, M_{O_2} (t_d), directly reflects N_Y. On the other hand, if t_d is short in comparison to τ_{lim}, then M_{O_2} (t_d) indicates the kinetics of the rate limiting step. Accordingly, in the present study, the rate and N_Y have been determined by measurements of M_{O_2} (t_d = 2 ms) and M_{O_2} (t_d = 500 ms), respectively.

Figure 1 shows the average oxygen yield per flash at t_d = 500 ms, M_{O_2} (t_d = 500 ms), in normal and in trypsinated chloroplasts as a function of the concentrations of $K_3[Fe(CN)_6]$ and of DCMU. These results indicate: (1) the number of intact watersplitting enzyme systems Y, N_Y, is only slightly affected by trypsin treatment under our experimental conditions and in the presence of $K_3[Fe(CN)_6]$ as electron acceptor, (2) the electron transport of system II per se becomes highly resistant to DCMU in trypsinated chloroplasts and (3) N_Y is practically independent of

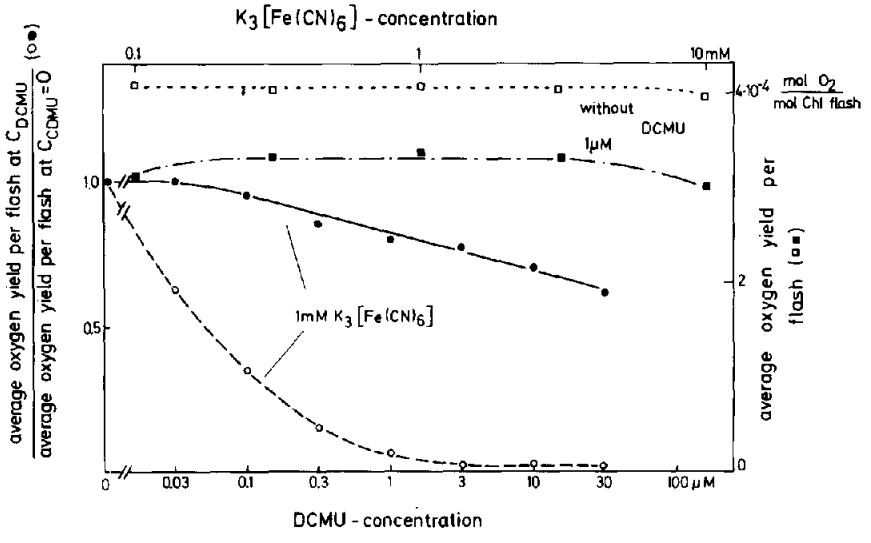

Fig.1. Average oxygen yield per flash at t_d = 500 ms as a function of the concentrations of DCMU and $K_3[Fe(CN)_6]$, respectively, in normal (open symbols) and in trypsinated (closed symbols) chloroplasts. Addition of DCMU and $K_3[Fe(CN)_6]$ as indicated in the figure, other experimental details as described in Materials and methods.

the $K_3[Fe(CN)_6]$ concentration in the range of 0.1–10 mM in normal as well as in trypsinated chloroplasts.

If this effect of trypsin is really caused by the removal of the accessibility barrier to external redox agents of the primary electron acceptor of photosystem II, X 320, then a different pattern is expected for the rate of oxygen evolution in normal and trypsinated chloroplasts, respectively. The rate limiting step of the linear electron transport from H_2O to exogenous class-I-acceptors (see ref. [20]) in normal chloroplasts was shown to be the endogenous reoxidation of plastohydroquinone of the pool, which is dependent on the coupling state of the thylakoid membrane (for review see ref. [1]). Thus, the overall rate of oxygen evolution should be independent of the concentration of class-I-acceptors like $K_3[Fe(CN)_6]$, provided that secondary effects (uncoupling, inhibition) of these agents can be excluded.

On the other hand, trypsination of chloroplasts was shown to interrupt the endogenous electron transport between X 320 and the plastoquinone pool, irrespective of the presence of DCMU [14,16]. Therefore, the electron transfer from $X\,320^-$ to the exogeneous acceptors probably becomes the rate limiting step, and the rate of oxygen evolution may therefore be expected to be strongly dependent on the concentration of the exogenous electron acceptor. Figure 2 shows the average oxygen yield per flash at t_d = 2 ms, M_{O_2} (t_d = 2 ms), as a function of the $K_3[Fe(CN)_6]$-concentration in normal and in trypsinated chloroplasts. As the number of intact water-splitting enzyme systems Y, N_Y, was shown to be constant (see fig.1), the amount of M_{O_2} (t_d = 2 ms) reflects exclusively the rate of the limiting step of the overall electron transport from H_2O to the exogenous acceptor. The results indicate, that the basal rate (absence of uncouplers or phosphorylating conditions) of oxygen evolution in normal chloroplasts at pH 7.0 is independent of $K_3[Fe(CN)_6]$-concentration in the range of 0.1–10 mM. On the other hand, in trypsinated chloroplasts (in the presence of 5 μM DCMU) the rate of oxygen evolution is strongly dependent on the $K_3[Fe(CN)_6]$-concentration. This favors the assumption that in trypsinated chloroplasts the electron transfer rate to the exogenous acceptor becomes rate limiting for oxygen evolution. In the simplest case the overall rate would

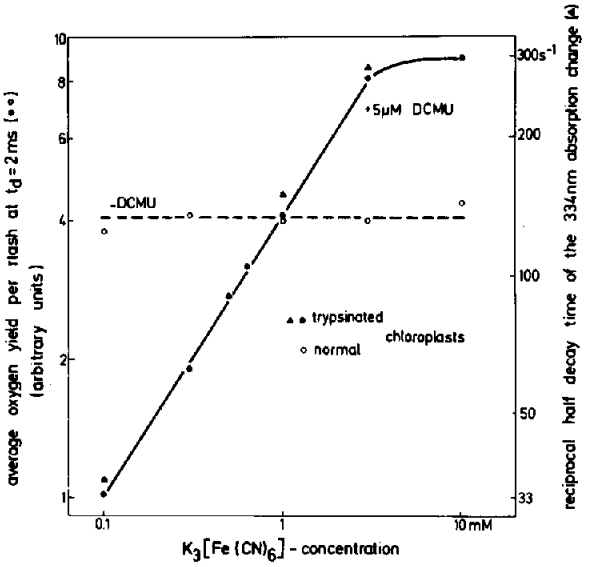

Fig.2. Dependency on the $K_3[Fe(CN)_6]$ concentration of the average oxygen yield per flash at t_d = 2 ms in normal and in trypsinated chloroplasts and of the reciprocal half decay time of the 334 nm absorption change in trypsinated chloroplasts. Double logarithmic plot.

be given by the diffusion current of the discharge of $[Fe(CN)_6]^{3-}$ at the negatively charged sites ($X\,320^-$) of the outer surface of the thylakoid membrane. Therefore, the rate of oxygen evolution should be linearly related to the $K_3[Fe(CN)_6]$-concentration. However, from the linear part of fig.2 the following relation is obtained:

$$\text{rate}_{O_2} \sim M_{O_2}\ (t_d = 2\text{ ms}) = a \cdot \left[K_3[Fe(CN)_6]\right]^{0.6} + b \qquad (1)$$

where a and b are constant and $\left[K_3[Fe(CN)_6]\right]$ denotes the $K_3[Fe(CN)_6]$ concentration. As the exponent of the $K_3[Fe(CN)_6]$ concentration term is not an integer, a more complex mechanism is inferred to be responsible for the rate limitation of oxygen evolution in trypsinated chloroplasts.

If the overall rate of system II electron transport in trypsinated chloroplasts is really determined by the heterogeneous electron transfer step from $X\,320^-$ to $K_3[Fe(CN)_6]$ at the outer thylakoid/aq. solution interface, then the reoxidation kinetics of $X\,320^-$ must be strongly dependent on the concentration of

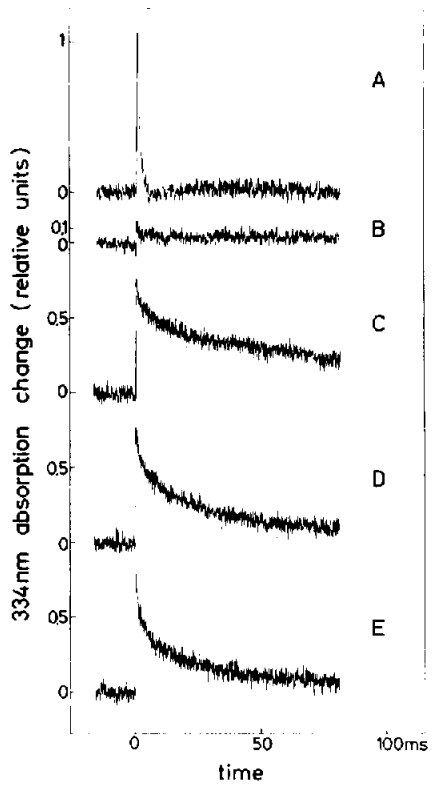

Fig.3. Absorption changes at 334 nm as a function of time in chloroplasts A: normal chloroplasts, 0.3 mM $K_3[Fe(CN)_6]$ as electron acceptor, B: as A, but with 5 μM DCMU, C–F: trypsinated chloroplasts in the presence of 5 μM DCMU with $K_3[Fe(CN)_6]$-concentrations of 0.3 mM, 1 mM and 3 mM, respectively. 512 signals were averaged in a FABRI-TEK, Mod. 1062. Time t_d between the flashes: 500 ms. Other experimental conditions as described in Materials and methods.

$K_3[Fe(CN)_6]$, in contrast to normal chloroplasts, where the X 320 recovery should remain unaffected. The $X^{\cdot}320$ turnover kinetics were measured via the absorption changes at 334 nm (see Materials and methods). The results are depicted in fig.3. In normal chloroplasts (fig.3A) a fast $X\,320^-$ reoxidation with $\tau_{1/2}$ = 600 μs [1,21] occurs, which is independent of the $K_3[Fe(CN)_6]$-concentration (data not shown). This reaction is completely blocked by DCMU (fig.3B). A markedly different pattern appears in trypsinated chloroplasts, characterized by two features (see fig.3C–E) : (a) the reoxidation of $X\,320^-$ is significantly slowed down and b) the kinetics of the $X\,320^-$ reoxidation becomes strongly dependent on the $K_3[Fe(CN)_6]$-concentration, whereas the initial amplitude remains unaffected. A kinetic analysis of the data showed that the reoxidation of $X\,320^-$ in trypsinated chloroplasts cannot be described by a simple second order reaction or by a mono- or two-phase exponential decay. This might be caused by heterogeneity of the sample (different degrees of trypsination and distribution of exogenous electron acceptor at system II) as well as by a more complex intrinsic kinetics of the electron transfer step from $X\,320^-$ to $K_3[Fe(CN)_6]$ which could include also the special plastoquinone molecule B, interconnecting in vivo X 320 with the pool ([22], see also discussion in ref. [14]). Further experiments are required to clarify this point. However, despite these difficulties the reciprocal half decay time of the 334 nm absorption change can be used as a crude measure for the rate of electron withdrawing from $X\,320^-$. These values as a function of the $K_3[Fe(CN)_6]$concentration are included in fig.2. It is seen that the dependence on the $K_3[Fe(CN)_6]$concentration is practically the same for the reciprocal half decay time of the 334 nm absorption change as for M_{O_2} (t_d = 2 ms). Thus, it can be inferred that the reoxidation of $X\,320^-$ by $K_3[Fe(CN)_6]$ at the outer surface of the thylakoid membrane is the rate limiting step of oxygen evolution in trypsinated chloroplasts.*

On the basis of this conclusion two further effects are expected to appear. First, taking into account also the increase of membrane permeability by trypsin [14], the rate of oxygen evolution should be nearly independent of uncouplers, in contrast to normal chloroplasts. Indeed, it was found that 2-(3-chloro-4-trifluoromethyl)anilino-3,5-dinitrothiophene (ANT 2p), a very potent ADRY-type uncoupler [23,24], leads to a 4-fold increase of M_{O_2} (t_d = 2 ms) in normal chloroplasts, whereas in trypsinated chloroplasts no stimulatory effect is observed, but rather a decrease at higher $K_3[Fe(CN)_6]$-concentrations (data not shown).

Secondly, in trypsinated chloroplasts system II electron transport should be mediated only by

* However it must be emphasized that a direct quantitative correlation between M_{O_2} (t_d = 2 ms) and the reciprocal half decay time can not be made, because of the different excitation conditions (t_d = 2 ms versus 500 ms) giving rise to different situations for $K_3[Fe(CN)_6]$ diffusion to the outer surface of the thylakoids.

electron acceptors with a redox potential high enough for oxidation of X 320⁻. However, it was shown very recently [14], that the redox potential alone is not a sufficient criterium. Therefore, several electron acceptors have been tested for their ability to mediate a DCMU insensitive oxygen evolution in trypsinated chloroplasts. Measurements of M_{O_2} (t_d = 500 ms) show, that Toluidene blue and *p*-benzoquinone are ineffective, $KMnO_4$ and *m*-cresolindophenol are poor acceptors (10–20% activity of control), wheras 2,6-dichlorophenolindophenol (DCIP) has an activity which is only slightly below to that of $K_3[Fe(CN)_6]$. With respect to the rate of the oxygen evolution the best results were obtained with 0.4 mM diaminodurene + 5 mM $K_3[Fe(CN)_6]$, a class-III-acceptor couple [20].

These marked differences in the ability of various redox agents with a positive midpoint potential to support oxygen evolution in trypsinated chloroplasts in the presence of DCMU suggests that a specific reaction mechanism is required for the electron transfer from X 320⁻ to exogenous acceptors in trypsinated chloroplasts. The details of this mechanism remain to be clarified. The effect of trypsin is strongly dependent on the pH of the chloroplast suspension. In the presence of 5 μM DCMU and with 1 mM $K_3[Fe(CN)_6]$ as electron acceptor the maximal rate of oxygen evolution in trypsinated chloroplasts was obtained at pH 7.0, in contrast to normal chloroplasts with a maximum above pH 8.5.

At pH values above 7.0 a rapid time dependent decline of the rate was observed in trypsinated chloroplasts and at pH 8.5 the oxygen evolution completely disappeared. This effect (which might be mainly due to the pH-dependency of the enzymic activity of trypsin) shows that well defined conditions are required in order to get a high (DCMU-insensitive) rate of oxygen evolution by trypsin treatment. Therefore, the apparently contradictory results of Regitz and Ohad [13], who reported a severe inhibition by trypsin of system II electron transport with $K_3[Fe(CN)_6]$ as acceptor, can be explained, because their experimental conditions ($K_3[Fe(CN)_6]$-concentration, pH) were far from the optimum in addition to the use of different biological material.

The present results show, that mild trypsin treatment leads to a selective modification of the surface of the thylakoid membrane without severe destruction of the watersplitting enzyme system. Therefore, such modified preparations can be used for both the functional separation of system II electron transport and for the study of its structural and functional organization (see also ref. [14]).

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References

[1] Witt, H. T. (1971) Quart. Rev. Biophys. 4, 365–477.
[2] Avron, M. (1967) in: Current Topics in Bioenergetics (Sanadi, R., ed) Vol. II, pp. 1–22, Academic Press, New York.
[3] Duysens, L. N. M. and Sweers, H. E. (1963) Microalgae and Photosynthetic Bacteria, pp. 353–372, University of Tokyo Press, Tokyo.
[4] Joliot, P. (1965) Biochim. Biophys. Acta 102, 116–134.
[5] Bennoun, P. (1970) Biochim. Biophys. Acta 216, 357–363.
[6] Renger, G. and Wolff, Ch. (1975) Z. Naturforsch. 30c, 161–171.
[7] van Gorkum, H. J. (1974) Biochim. Biophys. Acta 347, 439–442.
[8] Wolff, Ch., Büchel, K. H. and Renger, G. (1975) Z. Naturforsch. 30c, 172–182.
[9] Diner, B. A. and Mauzerall, D. C. (1971) Biochim. Biophys. Acta 226, 492–497.
[10] Piccioni, R. G. and Mauzerall, D. C. (1976) Biochim. Biophys. Acta 423, 605–609.
[11] Ben Hayyim, G. and Neumann, J. (1975) FEBS Lett. 56, 240–243.
[12] Giaquinta, R. T. and Dilley, R. A. (1975) Biochim. Biophys. Acta 387, 288–305.
[13] Regitz, G. and Ohad, I. (1976) J. Biol. Chem. 251, 247–252.
[14] Renger, G. (1976) Biochim. Biophys. Acta 440, 287–300.
[15] Winget, G. D., Izawa, S. and Good, N. E. (1965) Biochem. Biophys. Res. Commun. 21, 438–443.
[16] Renger, G., Erixon, K., Döring, G. and Wolff, Ch. (1976) Biochim. Biophys. Acta (in press).
[17] Clark, L. C. (1956) Trans. Amer. Soc. Artif. Internal Organs 2, 41–58.
[18] Renger, G. (1972) Biochim. Biophys. Acta 256, 428–439.

[19] Renger, G. and Wolff, Ch. (1976) Biochim. Biophys. Acta 423, 610–614.
[20] Saha, S., Onitrakul, R., Izawa, S. and Good, N. (1971) J. Biol. Chem. 246, 3204–3209.
[21] Vater, J., Renger, G., Stiehl, H. H. and Witt, H. T. (1968) Naturwiss. 55, 220–221.
[22] Bouges-Bocquet, B. (1973) Biochim. Biophys. Acta 314, 250–256.
[23] Renger, G., Bouges-Bocquet, B. and Büchel, K. H. (1973) J. Bioenergetics 4, 491–505.
[24] Renger, G. (1975) FEBS Lett. 52, 30–32.