The susceptibility of the *p*-benzoquinone-mediated electron transport and atrazine binding to trypsin and its modification by $CaCl_2$ in thylakoids and PS II membrane fragments

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Comparative studies in thylakoids and oxygen-evolving Triton X-100 PS II membrane fragments reveal: (i) There exists one high-affinity atrazine binding site per PS II in both preparations. The affinity is reduced in PS II membrane fragments. (ii) The susceptibility to tryptic attack on atrazine binding and p-BQ-mediated electron transport is markedly reduced by $CaCl_2$ in PS II membrane fragments, but no protection is observed in thylakoids. NH_2OH and $K_3[Fe(CN)_6]$ both reduce the binding affinity in PS II membrane fragments. The action of NH_2OH is invariant to $CaCl_2$ addition. The implications of these findings for functional studies with PS II membrane fragments are discussed.

Photosystem II membrane fragment *Atrazine binding* *Trypsin effect* *$CaCl_2$ protection* *Hydroxylamine effect* *Potassium ferricyanide effect*

1. INTRODUCTION

Photosynthetic water cleavage by visible light into dioxygen and bound hydrogen (in the form of plastoquinol) takes place in system II of cyanobacteria and green plants. Many mechanistic problems of the reaction sequence and questions about the structural array of functional groups within their apoproteins remain unresolved (reviews [1,2]). Essential progress towards the possibility of more refined analysis has been achieved by preparative methods for isolation of PS II membrane fragments that are virtually free of PS I activity and still fully competent in oxygen evolution [3–7]. All these procedures imply a detergent treatment that leads to fractionation of thylakoids. The use of detergents could have important consequences because special lipids were reported to be of functional relevance for PS II activity [8,9]. Therefore, a substitution of these components by detergent molecules might imply mechanistic consequences for the reaction pattern of PS II. This point is especially interesting for the acceptor side because: (i) the electron transfer to exogenous acceptors is the rate-limiting step for the overall reaction and (ii) the two-electron gating mechanism leading to plastoquinol formation (review [10]) essentially depends on the stability of the semiquinone form of the bound plastoquinone Q_B.

This study is an attempt to analyze modifications at the PS II acceptor side by measurements of the effects of trypsin, $CaCl_2$, NH_2OH and $K_3[Fe(CN)_6]$ on oxygen evolution and herbicide binding in Triton X-100 PS II membrane

Abbreviations: ABP, atrazine-binding protein; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; Mes, 4-morpholineethanesulfonic acid; Q_A, primary plastoquinone acceptor of PS II; Q_B, secondary plastoquinone acceptor of PS II; p-BQ, *p*-benzoquinone; Ph-p-BQ, phenyl-*p*-benzoquinone; PS II, photosystem II

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fragments and thylakoids. The data reveal significant differences between both types of preparations.

2. MATERIALS AND METHODS

Thylakoids and Triton X-100 PS II membrane fragments were prepared according to Winget et al. [11] and a modified procedure of Berthold et al. [4], respectively, as described in [12]. Trypsin treatment was performed as in [12] in a reaction medium containing: thylakoids or PS II membrane fragments (50 μM chlorophyll), 15 mM NaCl, 5 mM $MgCl_2$ and 20 mM Mes-NaOH (pH 6.0); other additions as indicated in figure legends. [^{14}C]Atrazine binding was measured according to Tischer and Strotmann [13]. The binding studies were performed in the dark at 5 min equilibration with [^{14}C]atrazine before centrifugation.

The average oxygen yield per flash was determined with a Clark-type electrode as described in [14].

3. RESULTS AND DISCUSSION

The reaction pattern of the PS II acceptor side is strongly affected by modifications of the atrazine-binding 32 kDa polypeptide, referred to as 32 kDa ABP (review [15]). To test possible structural differences of functional relevance between thylakoids and PS II membrane fragments, the effect of trypsin on the average oxygen yield per flash was measured in the presence of p-BQ as exogenous electron acceptor. The data obtained are depicted in fig.1. In thylakoids the activity rapidly drops as a function of incubation time with trypsin. This effect is due to interruption of electron transport between Q_A and Q_B [16,17] and the failure of p-BQ to act as an electron acceptor for Q_A^- [16,18]. $CaCl_2$ does not affect this phenomenon. Interestingly enough, a different pattern is observed in PS II membrane fragments. Interruption of the electron transport to p-BQ requires an almost 10-times longer trypsin incubation (in the presence of $K_3[Fe(CN)_6]$ as acceptor the activity remains almost constant in this time domain; see [19]. These quantitative differences between both sample types might be indicative of structural modifications of acceptor side polypeptides in Triton X-100 PS II membrane fragments.

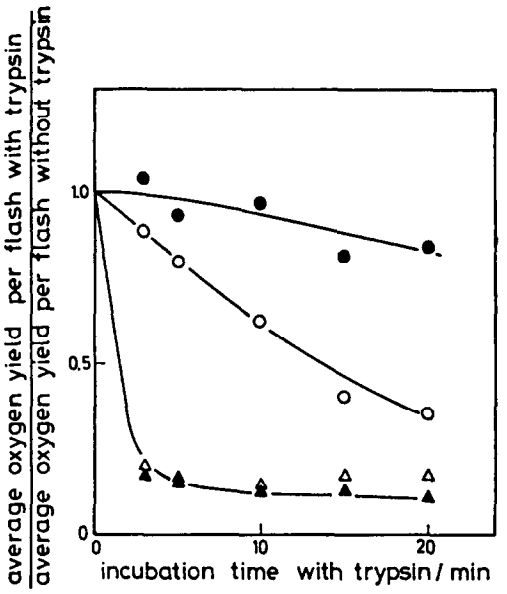

Fig.1. Average oxygen yield per flash normalized to the control value (at t_{inc} = 0) as a function of incubation time (t_{inc}), with trypsin in thylakoids (△, ▲) and PS II membrane fragments (○,●). The reaction medium contained thylakoids or PS II membrane fragments (50 μM chlorophyll), 10 mM NaCl, 20 mM Mes-NaOH (pH 6.0), 50 μg trypsin/ml and 200 μM p-BQ. Addition of 10 mM $CaCl_2$ is indicated by closed symbols (●, ▲).

More direct evidence for changed susceptibility to trypsin attack is provided by the remarkable effect of Ca^{2+}. If Ca^{2+} is present during trypsinization of PS II membrane fragments the deleterious effect almost completely disappears (fig.1). However, Ca^{2+} addition to the assay medium after trypsin treatment does not provide protection (not shown). This failure of Ca^{2+} to stimulate flash-induced oxygen evolution is clearly related to the ability of p-BQ to function as a PS II electron acceptor in trypsinized PS II membrane fragments. Further evidence for a Ca^{2+} effect at the acceptor side was found in PS II membrane fragments trypsinized at pH 7.5 (oxygen-evolving capacity completely eliminated).

In these samples a part of the relaxation kinetics of 320 nm absorption changes became accelerated by 10 mM $CaCl_2$, whereas those at 830 nm (reflecting $P680^+$ reduction) remained invariant [20]. In addition to acceptor side effects, a Ca^{2+}-induced specific reconstitution does exist that is located at

the PS II donor side (Völker, M., Eckert, H.J. and Renger, G., in preparation). The results in fig.1 lead to the conclusion that in PS II membrane fragments polypeptides are modified which play a functional role for the acceptor side electron transport. Mild trypsin treatment of thylakoids markedly affects herbicide binding [21,22]. Accordingly, similar differences to those for the p-BQ-mediated electron transport depicted in fig.1 could arise for atrazine binding in thylakoids and PS II membrane fragments, respectively. To test this idea, the effects of Ca^{2+} and trypsin treatment on atrazine binding were analyzed. Nontreated control samples contain one high-affinity atrazine-binding site per PS II (thylakoids: 550 chlorophylls per atrazine-binding site vs 500 chlorophylls per oxygen-evolving PS II; the corresponding values in PS II membrane fragments were 320 vs 300, respectively) with ~2-fold higher affinity in thylakoids compared with PS II membrane fragments (not shown). Fig.2 shows the results obtained in treated samples. Trypsinization in the absence of Ca^{2+} markedly decreases the number of binding sites whereas the effect on the affinity of the remaining binding sites is not as pronounced. Qualitatively similar results were obtained in thylakoids. Remarkable protection of PS II membrane fragments is observed if Ca^{2+} is present during the trypsin treatment.

This Ca^{2+} protection of the 32 kDa ABP to proteolytic attack (pH 6.0) is confirmed by immunodecoration experiments kindly performed by N. Nelson (unpublished). If, on the other hand, Ca^{2+} is added after trypsinization of PS II membrane fragments the effect of proteolysis on atrazine binding is not impaired but seems to be even slightly stimulated.

The qualitative correspondence of the effect of Ca^{2+} and trypsin on atrazine binding and p-BQ mediated PS II electron transport suggests that both phenomena are related to structural changes of the same polypeptide. It should be emphasized that Ca^{2+} affects only the susceptibility of the 32 kDa ABP towards trypsin attack, whereas atrazine binding in control PS II membrane fragments is not modified by Ca^{2+} addition (not shown).

Another important aspect that has to be analyzed is the possibility of structural modifications by molecules that are frequently used for electron transport studies in thylakoids and PS II membrane fragments. NH_2OH is often applied as a tool for selective elimination of the oxygen-evolving capacity [23,24].

A thorough analysis of the experimental data obtained sometimes requires detailed knowledge about additional effects that could arise at the PS II acceptor side (see [25]). The results depicted in fig.4 exhibit a marked impairment of atrazine binding by 1 mM NH_2OH in PS II membrane fragments. Similar data were obtained in thylakoids (not shown) confirming recent findings [26]. The NH_2OH effect was found to be practical-

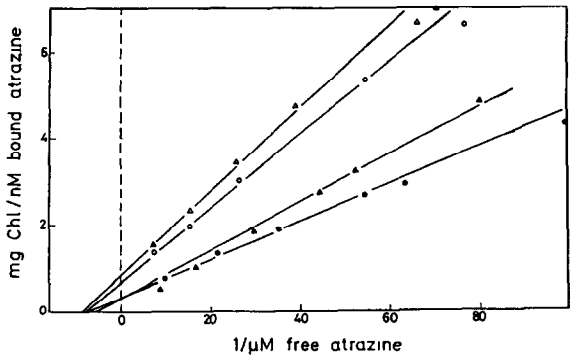

Fig.2. Double-reciprocal plot for binding of [^{14}C]atrazine to PS II membrane fragments. (●) Control (with or without 10 mM $CaCl_2$); (▲) trypsinized (see section 2) in the presence of 10 mM $CaCl_2$; (○) trypsinized in the absence of $CaCl_2$; (△) trypsinized in the absence of $CaCl_2$, but addition of 10 mM $CaCl_2$ in the assay medium for atrazine binding.

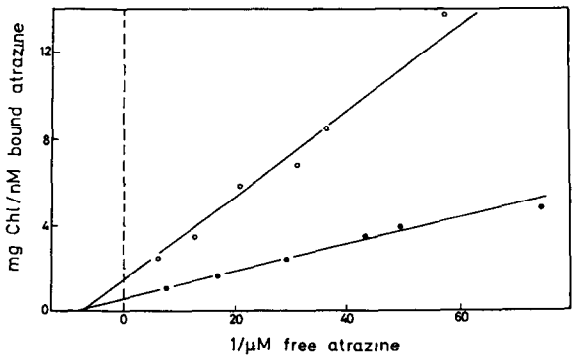

Fig.3. Double-reciprocal plot for binding of [^{14}C]atrazine to PS II membrane fragments in the absence (●) or presence of 1 mM NH_2OH (○).

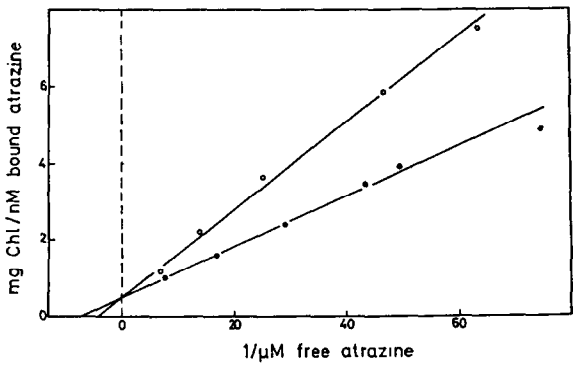

Fig.4. Double-reciprocal plot for binding of [^{14}C]atrazine to PS II membrane fragments in the absence (●) or presence of 0.5 mM $K_3[Fe(CN)_6]$.

ly independent of the presence of 10 mM $CaCl_2$ (not shown).

Binary flash-induced oscillations of [^{14}C]DCMU binding were observed in isolated spinach thylakoids [27]. Likewise, a remarkable affinity decrease of [^{14}C]bromoxynil binding was observed in pea thylakoids under reducing conditions [28]. These effects are clearly related to the redox state of Q_B. Another redox active component of functional relevance for the PS II acceptor side is C-400 that becomes oxidized by $K_3[Fe(CN)_6]$ at sufficiently high redox potentials [29,30]. Recently evidence has been presented for the identification of C-400 with the high-spin Fe^{2+} [31] that is associated with Q_A and Q_B [32,33]. Based on comparative studies a model has been proposed for the microenvironment of the $Q_A...Fe^{2+}...Q_B$ segment within the polypeptide matrix [34]. If one accepts the above-mentioned substantiation of C-400, $K_3[Fe(CN)_6]$ should oxidize Fe^{2+} to Fe^{3+}. This reaction implies a change of the electrostatic interaction due to Fe^{3+} within the microenvironment of Q_A and Q_B that could also give rise to changes of the affinity to herbicide binding. To test this idea experiments were performed in the presence of 0.5 mM $K_3[Fe(CN)_6]$ which oxidizes C-400 to a large extent [25]. Fig.5 shows that 0.5 mM $K_3[Fe(CN)_6]$ leads to a significant decrease of the affinity for atrazine binding. This effect is entirely related to C-400, because oxidation of some remaining Q_B^- by $K_3[Fe(CN)_6]$ should lead to an increased affinity rather than to the observed decrease. In contrast to the findings in PS II membrane fragments atrazine binding remained totally unaffected by $K_3[Fe(CN)_6]$ in thylakoids. The latter result is in perfect agreement with a recent study which showed that in thylakoids $K_3[Fe(CN)_6]$ did not modify atrazine binding, but reduced the affinity to DCMU [35]. The $K_3[Fe(CN)_6]$ effect again reveals that marked differences exist at the PS II acceptor side between thylakoids and Triton X-100 PS II membrane fragments.

4. CONCLUSION

Our results show that the preparation of PS II membrane fragments by using Triton X-100 treatment causes structural modifications in the polypeptides that are of functional relevance. It is assumed that substitution of functionally essential lipids by detergent molecules is responsible for the observed phenomena. As a consequence of these findings it is inferred that unambiguous conclusions based on rate measurements of oxygen evolution in PS II membrane fragments can be drawn only if possible interfering acceptor side effects are carefully analyzed and properly separated.

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REFERENCES

[1] Renger, G. and Govindjee (1985) Photosynth. Res. 6, 33–45.
[2] Andersson, B. and Akerlund, H.E. (1986) in: Topics in Photosynthesis (Barber, J. ed.) Elsevier, Amsterdam, New York, in press.
[3] Kuwabara, T. and Murata, N. (1982) Plant Cell Physiol. 23, 533–539.
[4] Berthold, D.A., Babcock, G.T. and Yocum, C.F. (1981) FEBS Lett. 134, 231–234.
[5] Dunahay, T.G., Staehelin, L.A., Seibert, M., Ogilvie, P.D. and Berg, S.P. (1984) Biochim. Biophys. Acta 364, 179–193.

[6] Ikeuchi, M., Yuasa, M. and Inoue, Y. (1985) FEBS Lett. 185, 316–322.
[7] Ghanotakis, D. and Yocum, C.F. (1986) FEBS Lett. 197, 224–248.
[8] Gounaris, K. and Barber, J. (1985) FEBS Lett. 188, 68–72.
[9] Gounaris, K., Whitford, D. and Barber, J. (1983) FEBS Lett. 163, 230–234.
[10] Crofts, A.R. and Wraight, C.A. (1983) Biochim. Biophys. Acta 726, 149–185.
[11] Winget, G.D., Izawa, S. and Good, N.E. (1965) Biochem. Biophys. Res. Commun. 21, 438–443.
[12] Völker, M., Ono, T., Inoue, Y. and Renger, G. (1985) Biochim. Biophys. Acta 806, 25–34.
[13] Tischer, W. and Strotmann, H. (1977) Biochim. Biophys. Acta 460, 113–125.
[14] Renger, G. (1972) Biochim. Biophys. Acta 256, 438–439.
[15] Renger, G. (1986) Physiol. Veg. 24, in press.
[16] Renger, G. (1976) Biochim. Biophys. Acta 440, 287–300.
[17] Renger, G. and Weiss, W. (1982) FEBS Lett. 137, 217–221.
[18] Renger, G. (1979) Z. Naturforsch. 34c, 1010–1014.
[19] Renger, G., Rutherford, A.W. and Völker, M. (1985) FEBS Lett. 185, 243–247.
[20] Weiss, W. and Renger, G. (1986) Biochim. Biophys. Acta 850, in press.
[21] Trebst, A. (1979) Z. Naturforsch. 34c, 986–991.
[22] Tischer, W. and Strotmann, H. (1979) Z. Naturforsch. 34c, 992–995.
[23] Cheniae, G.M. and Martin, I.F. (1971) Biochim. Biophys. Acta 253, 167–181.
[24] Tamura, N. and Cheniae, G.M. (1985) Biochim. Biophys. Acta 809, 245–259.
[25] Renger, G. and Weiss, W. (1986) Biochim. Biophys. Acta 850, in press.
[26] Jursinic, P. and Stemler, A. (1983) Plant Physiol. 73, 703–708.
[27] Laasch, H., Urbach, W. and Schreiber, Cl. (1983) FEBS Lett. 159, 275–279.
[28] Vermaas, W.J.F., Renger, G. and Arntzen, C.J. (1984) Z. Naturforsch. 39c, 368–373.
[29] Ikegami, I. and Katoh, S. (1973) Plant Cell Physiol. 14, 829–836.
[30] Bowes, J.M., Crofts, A.R. and Itoh, S. (1979) Biochim. Biophys. Acta 547, 320–335.
[31] Petrouleas, V. and Diner, B.A. (1986) Biochim. Biophys. Acta 849, 193–202.
[32] Nugent, J.H.A., Diner, B.A. and Evans, M.C.W. (1981) FEBS Lett. 124, 241–244.
[33] Rutherford, A.W. and Mathis, P. (1983) FEBS Lett. 154, 328–334.
[34] Trebst, A. (1986) Z. Naturforsch. 41c, 240–245.
[35] Wraight, C.A. (1985) Biochim. Biophys. Acta 809, 320–330.