A possible model for the organic prosthetic group of the water-splitting complex in the cyanobacterium *Anacystis nidulans*

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O_2-evolving photosystem II complexes, which were isolated from the cyanobacterium *Anacystis nidulans* as described previously [(1986) Biochim. Biophys. Acta 849, 203–210], were further purified on a Sepharose 6B column. These complexes retain the L-amino acid oxidase protein. Moreover, various types of photosystem II inhibitors were tested for their effect on L-amino acid oxidase activity. The results show that chlorpromazine, 2,4,6,2′,4′,6′-hexanitrodiphenylamine, and *o*-phenanthroline inhibited photosynthetic O_2 evolution as well as L-amino acid oxidase activity, while 3-(3,4-dichlorophenyl)-1,1-dimethylurea and 4-hydroxy-3,5-diiodobenzonitrile only inhibited photosynthetic O_2 evolution. These results further support a functional role of the L-amino acid oxidase protein in photosynthetic O_2 evolution. We suggest that a flavin-derived molecule – possibly a 10,10ª-ring-opened derivative of FAD – is the organic prosthetic group of the water-splitting complex in *A. nidulans* and that this flavin derivative is able to chelate mangenese.

Oxygen evolution *Photosystem II* *L-Amino acid oxidase* *Flavoprotein* (Anacystis nidulans)

1. INTRODUCTION

Almost identical PS II reaction center complexes have been isolated from various oxygenic photosynthetic organisms indicating that this supramolecular complex exists as a stable structural and functional unit in thylakoid membranes. Advances in our understanding about the polypeptide composition of such PS II complexes have been paralleled by the research on the inorganic cofactors (Mn^{2+}, Ca^{2+} and Cl^-) required for photosynthetic O_2 evolution [1–4]. However, the identity of the organic prosthetic group 'Z' of the water-splitting complex has remained unclear. The redox couple 'Z/Z^+' has been examined by absorbance difference spectra and by EPR studies. Based on these results, several groups have suggested that Z is likely to be a plastoquinone-like molecule [5–10]. However, recent experiments by De Vitry et al. [11] have indicated that there is not enough plastoquinone-9 in PS II complexes from *Chlamydomonas rheinhardtii* that both acceptor and donor of PS II could be a plastoquinone-9.

We have previously shown that highly active O_2-evolving PS II preparations from the cyanobacterium *Anacystis nidulans* contain a flavoprotein with L-amino acid oxidase activity, and we have suggested that this protein has a functional role in photosynthetic O_2 evolution. However, such PS II preparations did not contain the expected stoichiometric amount of 'authentic oxidized FAD' [12]. To exclude the possibility that the L-amino acid oxidase protein has remained accidentally in the complex, we have further purified

Abbreviations: PS, photosystem; chl, chlorophyll; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; ioxynil, 4-hydroxy-3,5-diiodobenzonitrile; DP 15, 2,4,6,2′,4′,6′-hexanitrodiphenylamine; LDAO, lauryldimethylamine *N*-oxide

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such complexes and show by SDS-polyacrylamide gel electrophoresis and immunological studies that the L-amino acid oxidase protein remained present in such purified complexes. Moreover, we have examined various inhibitors of PS II reactions for their inhibitory effect on L-amino acid oxidase activity, since we wanted to see whether structural features of those inhibitors which inhibited both reactions would support our hypothesis that a flavin-derived molecule is functional in PS II reactions.

2. MATERIALS AND METHODS

The growth of *A. nidulans* (*Synechococcus leopoliensis*) and preparation of PS II complexes were the same as described [12]. These PS II complexes, which were obtained after sucrose density gradient centrifugation, were further purified on a Sepharose 6B column. 5 ml of the PS II complex, which was obtained after gradient centrifugation and to which sucrose was added to give a final concentration of 0.5 M, were placed on a Sepharose 6B column (28 × 2 cm) which was equilibrated with 0.05 M Hepes-NaOH, pH 6.5, containing 0.03 M $CaCl_2$, 25% glycerol, and 0.5 M sucrose. Elution was done with the same buffer and fractions of 2 ml were collected. The green PS II complex (fractions 11–14) which had O_2-evolving activity and L-amino acid oxidase activity eluted ahead of residual blue material. Small amounts of L-amino acid oxidase activity were also found in fractions 29–33. Before measuring L-amino acid oxidase activity, the samples were dialyzed against 0.02 M Hepes-NaOH, pH 6.5.

The activity assays for photosynthetic O_2 evolution and L-amino acid oxidase activity were done as in [12]. Fluorometric measurements were also performed according to [13]. Excitation was achieved by light filered through a blue filter (BG 38) from Schott (Mainz) and fluorescence was measured at 686 nm. The reaction mixture contained in 1 ml: 54 mM Hepes-NaOH, pH 6.5, 54 mM $CaCl_2$, 27 mM sucrose and the PS II complex after gradient centrifugation containing 5.2 μg chl.

L-Amino acid oxidase was purified as described in [14]. When the purified enzyme was assayed, the reaction mixture contained in a total volume of 1.85 ml: 54 mM Hepes-NaOH, pH 7, 1.6 mM L-arginine (adjusted with Hepes to pH 7), 500 μg catalase, and L-amino acid oxidase (0.4 μg protein, spec. act. 100). In all experiments in which chlorpromazine was tested, Mes-NaOH buffer instead of Hepes-NaOH buffer and phenyl-*p*-benzoquinone instead of potassium ferricyanide were used, because in the presence of Hepes and ferricyanide chlorpromazine precipitates.

SDS-polyacrylamide gel electrophoresis was performed as in [12]. The antibody against the purified L-amino acid oxidase was the same as used in previous experiments [15]. This antibody inhibits the activity of the isolated L-amino acid oxidase to about 80%, but does not inhibit the enzyme when it is still bound to the membrane [15]. The PS II complex which was used for the Ouchterlony test was dialyzed overnight against 20 mM Hepes-NaOH, pH 6.5, and then incubated with 1% Triton X-100 in ice for 2 h.

Ioxynil was a kind gift from Professor A. Trebst and DP 15 from Professor W. Oettmeier, Ruhr-Universität Bochum.

3. RESULTS AND DISCUSSION

3.1. *Presence of L-amino acid oxidase protein in purified PS II complexes*

Previously we have isolated a PS II complex with the detergent LDAO from thylakoid membranes of the cyanobacterium *A. nidulans* [12]. This complex was further purified by passing the complex through a Sepharose 6B column. This procedure gave one main fraction which contained the PS II complex and a smaller fraction (clearly separated from the first fraction) which contained small amounts of blue material and of solubilized L-amino acid oxidase. The rates of photosynthetic O_2 evolution and L-amino acid oxidase activity of the PS II complex before and after column chromatography are given in table 1. It should be emphasized again that it is difficult to obtain a good correlation between the two measured activities because L-amino acid oxidase activity is suppressed by all three inorganic cofactors required for photosynthetic O_2 evolution, and in addition the 'detectable' L-amino acid oxidase activity is also dependent on the degree to which the hydrophilic substrate, L-arginine, can reach the enzyme in the PS II complex. This, of course, is strongly influenced by the lipids and the

Table 1

Photosynthetic O_2 evolution and L-amino acid oxidase activity in purified PS II complexes

	Photosynthetic O_2 evolution	L-Amino acid oxidase activity (O_2 uptake)
	(μmol $O_2 \cdot$ mg chl$^{-1} \cdot h^{-1}$)	
After sucrose gradient	1160	35
After Sepharose 6B column	1322	15

Details are given in section 2

detergent in the complex and underlies changes during the purification procedure. The PS II complex after column chromatography consisted of six major peptides of 57, 49, 43, 36, 27 and 10 kDa. Mainly a 13 kDa peptide, which is most likely a phycobili protein [12], had disappeared after column chromatography. However, the 49 kDa peptide, which is identical in molecular mass to the subunit of the L-amino acid oxidase [14], continued to be a prominent band (fig.1). Moreover, an antibody raised against the purified L-amino acid oxidase still gave a precipitation band with the purified complex (fig.1).

3.2. *Effect of inhibitors*

Since previous experiments [12] indicated that a modified FAD (and not authentic oxidized FAD) could possibly be functional in PS II reactions, we have examined different types of PS II inhibitors [16–20] for their effect on L-amino acid oxidase activity. The results in table 2 show that three types of substances inhibited photosynthetic O_2 evolution as well as L-amino acid oxidase activity. These were chlorpromazine [19,20], the substituted diphenylamine DP 15 [17], and *o*-phenanthroline [18], while DCMU and ioxynil [16] only inhibited photosynthetic O_2 evolution. Chlorpromazine and DP 15 clearly caused a reduction of the chl *a* fluorescence, while in the case of *o*-phenanthroline the more lipophilic bathophenanthroline [21] had to be used to show that a reduction of the chl *a* fluorescence could be obtained (fig.2). A reduction of the chl *a* fluorescence is generally interpreted as an inhibition on the donor side of PS II. On the other hand, those substances which inhibited only O_2 evolution (DCMU and ioxynil), increased the chl *a* fluorescence (only shown for DCMU). A 10-fold increase or decrease of the DCMU concentration did not cause a decrease in chl *a* fluorescence, as seen, for example, with spinach PS II preparations [22].

Kinetics of the inhibition of the purified L-amino acid oxidase by these three types of PS II inhibitors were quite similar and were all very complex as previously published for chlorpromazine [23]. Lineweaver-Burk plots showed that the inhibition decreased with increasing substrate concentration for all three substances, but that the inhibition was apparently partly noncompetitive (not shown).

There are some difficulties in comparing these inhibitors for their effect on the two examined reactions. A lipophilic character of a substance contributes to inhibition of the photosynthetic reactions. On the other hand, L-amino acid oxidase becomes water-soluble when removed from the membrane and then the more lipophilic substances do not bind well to the enzyme, especially in the presence of the substrate. We have therefore used DP 15 for comparison, because among the available diphenylamines [17] this compound is the most water-soluble.

3.3. *Hypothetical model*

We believe that it is justified to assume that those inhibitors which inhibit photosynthetic O_2 evolution as well as L-amino acid oxidase activity have structural similarities with the structure of the 'unknown' prosthetic group of the water-splitting complex. Phenothiazines, e.g. chlorpromazine, have structural similarities to isoalloxazine, and it has long been known that a number of phenothiazines inhibit flavoenzymes, such as amino acid oxidases [24]. Substituted diphenylamines, such as DP 15, show a structural similarity to a 10,10^a-ring-opened flavin derivative which has absorbance maxima in the 390 and 600 nm regions and which has been suggested to be an intermediate in oxygenase reactions [25]. DP 15 also inhibited other amino acid oxidases, e.g. the kidney D-amino acid oxidase (not shown). The third substance which inhibited both reactions was the metal chelator *o*-phenanthroline. However, metal chelation does not seem to be the only reason for its inhibition of PS II reactions, since

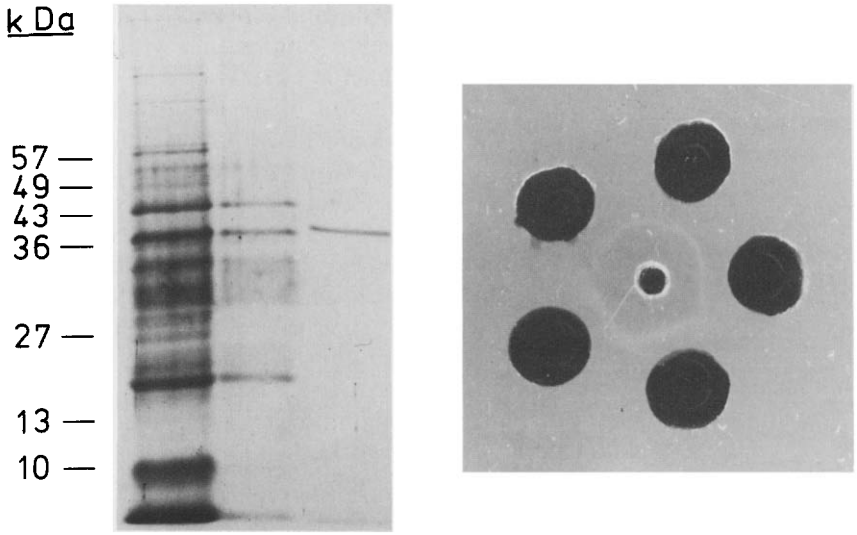

Fig.1. SDS-polyacrylamide gel electrophoresis and Ouchterlony double-diffusion test. (Left) SDS-polyacrylamide gel electrophoresis of PS II complex after sucrose gradient, 2.3 μg chl (A), PS II complex after Sepharose 6B column, 1 μg chl (B), and purified L-amino acid oxidase, 4 μg protein (C). (Right) Reaction of the PS II complex after Sepharose 6B column, 1.1 μg chl (upper three surrounding wells) and of the purified L-amino acid oxidase, 2.4 μg protein (lower two surrounding wells) with an antibody raised against the purified L-amino acid oxidase (center well) in an Ouchterlony double-diffusion test.

Table 2

Inhibition of photosynthetic O_2 evolution and L-amino acid oxidase activity

	Photosynthetic O_2 evolution (50% inhibition at (μM))	L-Amino acid oxidase activity (O_2 uptake) (50% inhibition at (μM))
2,2′-Bipyridine	no inhibition[a]	no inhibition[a]
DCMU	0.12	no inhibition[a]
Ioxynil	0.35	no inhibition[a]
Chlorpromazine	340	350
Diphenylamine DP 15	4.4	34
o-Phenanthroline	400	870

[a] Tested at 5 mM 2,2′-bipyridine, 10 μM DCMU, and 500 μM ioxynil

Photosynthetic O_2 evolution and L-amino acid oxidase activity were measured as described in section 2. In the absence of added inhibitors the O_2 evolution was 800–1200 μmol $O_2 \cdot$ mg chl$^{-1} \cdot$ h^{-1} (PS II complex after sucrose gradient) and the L-amino acid oxidase activity was 55 μmol O_2 taken up $\cdot$ mg protein$^{-1} \cdot$ min^{-1} (purified enzyme and a substrate concentration of 1.6 mM L-arginine was used)

2,2′-bipyridine does not inhibit [18]. Therefore, a structural element obviously contributes to the inhibition.

In fig.3 we give a hypothetical model for the prosthetic group of the water-splitting complex. We believe that during the light-dependent activation of the water-splitting enzyme, the flavin in the enzyme might be modified to a form which is identical or similar to the structure of the 10,10[a]-ring-opened derivative of flavin [25] and that this ring-opened derivative might be able to chelate Mn^{2+}. The suggested chelation property of this compound is based on the observation that two of the N atoms of the flavin derivative are in similar positions to those in *o*-phenanthroline. This, of course, is only a tentative model and does not exclude interaction of Mn^{2+} with additional ligand, such as Cl^- or amino acid residues.

Recently, a 3,5-di(*tert*-butyl)quinone has been described which can form a complex with metals. Lynch et al. [26] have suggested that such a quinone-Mn complex might have properties like those which would be expected of the water-splitting enzyme. In our model we also suggest that

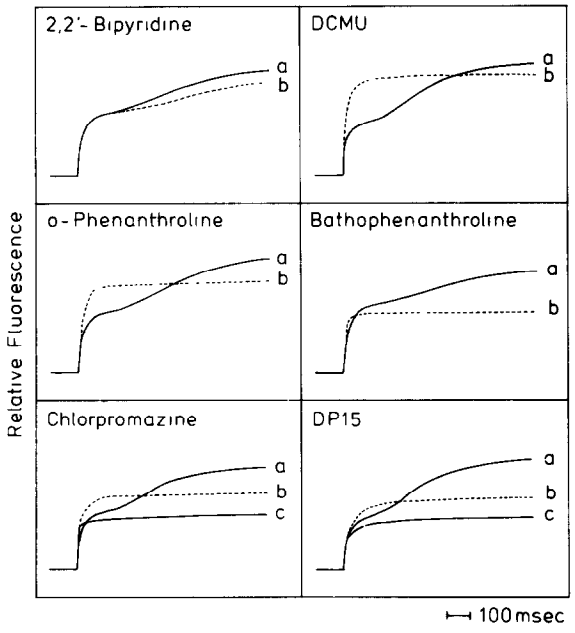

Fig.2. Effect of inhibitors on chl *a* fluorescence. The chl *a* fluorescence of the PS II complex was determined as described in section 2. Curve a was always the control without added inhibitor and curves b and c were obtained after addition of the inhibitors: 2,2'-bipyridine, 2 mM; DCMU, 5 μM; *o*-phenanthroline, 2 mM; bathophenanthroline, 50 μM; chlorpromazine, 1 mM (curve b) and 4 mM (curve c); DP 15, 1 μM (curve b) and 5 μM (curve c). No corrections were made for dilution by adding the inhibitor. Maximal dilution was 10% (2,2'-bipyridine and *o*-phenanthroline). In all other samples the dilution was less than 10%.

Mn^{2+} forms a complex with the organic prosthetic group of the water-splitting enzyme, but we think that a flavin-derived compound as suggested above is a more likely candidate. This would then imply that the flavoprotein which has an L-amino acid oxidase activity (in the absence of Mn^{2+}, Ca^{2+} and Cl^-), is modified in the light and in the presence of Ca^{2+} and Cl^- in such a way that it can now interact with Mn^{2+} (possibly mediated by the extrinsic Mn-stabilizing protein [27]) and catalyze the water-splitting reaction of PS II. We hope eventually to prove our hypothesis by a chemical analysis of Z.

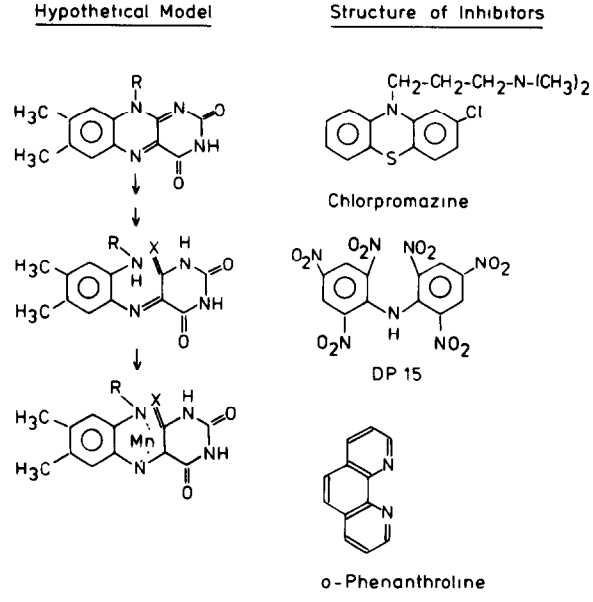

Fig.3. Hypothetical structural model of the prosthetic group of the water-splitting enzyme and structure of three types of inhibitors which inhibit photosynthetic O_2 evolution as well as L-amino acid oxidase activity. R = ribitol-phosphate-phosphate-D-ribose-adenine; X = O, $\overset{+}{O}\text{-}\bar{O}$ [25].

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