Further Investigations about the Flavin in the L-Amino Acid Oxidase and a Possible Flavin in Photosystem II Complexes from the Cyanobacterium *Anacystis nidulans*

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Z. Naturforsch. **43c,** 545–553 (1988); received January 25, 1988

L-Amino Acid Oxidase, Flavoprotein, Photosystem II, Cyanobacteria, *Anacystis nidulans*

The absorption spectrum of the previously purified L-amino acid oxidase from the cyanobacterium *Anacystis nidulans* has shown considerable variation with each preparation and the spectrum in several preparations was quite different from the absorption spectrum of other simple flavoproteins (E. K. Pistorius and A. E. Gau, Biochim. Biophys. Acta **849**, 203, 1986). Here we show that the spectral complexity and variability of the L-amino acid oxidase can be largely explained by the presence of a modified flavin derivative of yet unknown structure besides oxidized FAD and FAD semiquinone. After removal from the enzyme this modified chromophore has absorption maxima at 260, 396 and in the 600 nm region. This derivative of FAD seems to be formed in variable amounts during the purification of the enzyme. On the other hand, extraction of Anacystis photosystem II complexes which contain the flavoprotein, almost exclusively yields modified flavin derivatives and practically no authentic oxidized FAD. The spectrum of the chromophores which have been extracted from photosystem II complexes at different purification stages, is either similar (although not identical) to the spectrum of the chromophore extracted from the isolated L-amino acid oxidase or similar to the spectrum of reduced flavin. All extracted chromophores show a fluorescence emission in the 420 to 560 nm region when excited with light of 390 nm. These results indicate that the flavin present in the L-amino acid oxidase protein as well as in photosystem II complexes from *A. nidulans* rapidly undergoes modification reactions of yet unknown nature to yield several closely related FAD derivatives. This might possibly be the reason why so far no flavin has been detected in photosystem II. The presence of such modified flavin derivatives in photosystem II complexes of *A. nidulans* as shown here is an additional support of our hypothesis that an unusual flavin is functional on the donor side of photosystem II.

Introduction

Previously we have isolated and partially characterized a flavoprotein which has an L-amino acid oxidase activity, from the cyanobacterium *Anacystis nidulans* [1]. The isolated L-amino acid oxidase contains FAD as a prosthetic group, but the amount of authentic oxidized FAD (always refers to the amount of FAD which was determined in the reconstitution assay with the apoprotein of the FAD specific kidney D-amino acid oxidase) present in various enzyme preparations was greatly variable. Moreover, the absorption spectra of the various purified enzyme samples have shown considerable variation. Although the enzyme has the general characteristics of flavin (absorption bands in the 375 and 465 nm region), it showed additional absorption bands in the 400 and 600 nm region. These results have indicated that part of the enzyme contains a modified flavin of yet unknown structure besides authentic oxidized FAD [1, 2].

Previous results have shown that the L-amino acid oxidase protein is partially associated with the thylakoid membrane in *A. nidulans*. When PS II complexes were isolated from these thylakoid membranes, the L-amino acid oxidase protein was found to be present in highly active, purified PS II complexes. Moreover, a number of results have indicated that this protein might be functional in photosynthetic water oxidation. Based on those experiments we have suggested that this enzyme may have a dual function: It has an L-amino acid oxidase activity in the absence of ions, but in the light and in the presence of Ca^{2+} und Cl^- this flavoprotein seems to become modified in such a way that it can now interact with Mn^{2+} and catalyze the water oxidizing reaction of PS II [2, 3]. Those results have implied that a

Abbreviations: PS, photosystem; Chl, chlorophyll.

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Verlag der Zeitschrift für Naturforschung, D-7400 Tübingen
0341–0382/88/0700–0545 $ 01.30/0

flavin could possibly be a functional component on the donor side of PS II. However, when we investigated whether purified PS II complexes from *A. nidulans* contain FAD, the results clearly showed that the amount of authentic oxidized FAD which we found, was much below the expected values [2]. Therefore, the functional flavin in PS II could only be a modified flavin or a flavin which rapidly undergoes modification reactions, if our hypothesis is correct. Here we present additional results about the modified flavin in the L-amino acid oxidase and in PS II complexes from *A. nidulans*.

Materials and Methods

Purification of the L-amino acid oxidase from A. nidulans

Purification A: This purification was basically the same as described in ref. [1] but the following modifications were introduced: After the ammonium sulfate precipitation step (30 to 55%) and after dialysis against 0.01 M potassium phosphate buffer, pH 7, the sample was heated in a water bath with gentle stirring to 60 °C and kept at this temperature for 10 min. After centrifugation at $20{,}000 \times g$ the supernatant was used for chromatography on a CM-Sephadex column, a DEAE-Sephadex column and a hydroxylapatite column as described previously [1]. The chromatography on the second DEAE-Sephadex column was omitted.

Purification B: This purification was basically the same as the purification A with the following changes: The sample obtained after the heating step at 60 °C was heated again to 67 °C in the presence of ammonium sulfate and at pH 3: To 1 ml sample 0.7 ml saturated ammonium sulfate solution and 0.8 ml 0.1 N HCl were added and this solution was heated with gentle stirring to 67 °C and kept at this temperature for 5 min. After centrifugation of this solution for 15 min at $27{,}500 \times g$, the pellet was extracted twice with 0.2 M potassium phosphate buffer, pH 7. The extract was recentrifuged at $27{,}500 \times g$ and the resulting supernatant was dialyzed against 0.01 M potassium phosphate buffer, pH 7, and used for chromatography on a DEAE-Sephadex column and a hydroxylapatite column [1]. In this purification the chromatography on the CM-Sephadex column could be omitted since the heating step at pH 3 resulted in an irreversible denaturation of a substantial amount of protein.

SDS polyacrylamide gel electrophoresis, activity and protein assays

SDS polyacrylamide gel electrophoresis was performed according to Laemmli [4]. Some gels were run with LiDS instead of SDS. Protein was determined with Coomassie Brilliant Blue G 250 (Bio-Rad) [5]. Bovine serum albumin (Serva) was used as standard. The L-amino acid oxidase activity was measured as O_2 uptake with a Clark type electrode. The solutions were saturated with air and the reaction temperature was 20 °C. The reaction mixture contained in a total volume of 3 ml: 160 μmol Hepes-NaOH buffer, pH 7, 97 μmol L-arginine, 32 μg beef liver catalase (Boehringer) and the enzyme. One unit of enzyme was defined as the amount which catalyzes the consumption of one μmol $O_2 \times min^{-1}$ (in the presence of catalase, L-arginine as substrate, in air and at 20 °C).

Isolation and purification of PS II complexes from A. nidulans

The PS IIa complex (complex after one sucrose gradient centrifugation) was isolated as described previously [2]. This complex was further purified by an additional sucrose gradient centrifugation. 5 ml PS IIa (0.05 to 0.15 mg Chl $\times$ ml^{-1}) were diluted with 10 ml 5% glycerol in H_2O. This solution was layered on a stepwise sucrose gradient consisting of 40%, 30% and 10% sucrose in 50 mM Hepes-NaOH buffer, pH 6.5, and 10% glycerol. After centrifugation for 90 min at $200{,}000 \times g$ (at r_{max}) a small pellet, a major and a minor green band on top of the 30 and 40% sucrose layer, respectively, were obtained. The green band on top of the 30% sucrose layer contained the PS II complex (PS IIb) with the highest specific activity (700 to 900 μmol O_2 evolved $\times$ mg^{-1} Chl $\times$ h^{-1}). The activity assay was the same as described previously [2].

Extraction of the "modified flavin" from PS II complexes

The PS II complex (total Chl 0.5 to 1.0 mg) was dialyzed against 10 mM potassium phosphate buffer, pH 7, to remove glycerol and sucrose. Then acetone (−20 °C) was added to give a final concentration of

90% acetone. The mixture was stirred for 30 min (at −20 °C) and then centrifuged for 30 min at 50,000 × *g*. The supernatant which contained chlorophyll, carotenoids and lipids was discarded. The pellet which contained the protein, cytochrome, blue pigments and the modified flavin, was once washed with acetone and then resuspended in 1 ml distilled water. After suspension 0.02 ml 10 N H_2SO_4 (to give a final concentration of 0.2 N H_2SO_4) was added and the sample was incubated for 30 min in ice. This acid extraction of the protein fraction removed the modified flavin from the protein but not the cytochrome or the blue pigments. Afterwards the sample was centrifuged for 20 min at 27,100 × *g* and the supernatant after filtration through a Sartorius filter (0.45 μm, type 113) was used for the characterization.

The absorption spectra were recorded with a Perkin Elmer spectrophotometer λ 3 and the fluorescence emission spectra were recorded with a Perkin Elmer fluorescence spectrophotometer MPF-44 A. The FAD content was determined either fluorometrically as described in ref. [1] or enzymatically as described in ref. [2]. The thin layer chromatography of the chromophore was done on Silica plates (Merck Si 60 F_{254}) with a solvent system consisting of 70% acetonitrile and 30% H_2O (v/v). The staining was done either by spraying with silver nitrate (0.33% in H_2O) or by spraying with 2 N H_2SO_4. After spraying the plates were heated to 150 °C for 5 min.

Results and Discussion

Purification and partial characterization of the L-amino acid oxidase from A. nidulans

The results of two different purifications of the L-amino acid oxidase from *A. nidulans* are given in Table I. We have modified the previous purification procedure [1], because we wanted to find conditions to obtain an L-amino acid oxidase which mainly contains oxidized FAD or mainly modified FAD. Purification A was basically similar to the previously described procedure [1]. Only a heating step to 60 °C at pH 7 (the enzyme is stable up to 70 °C – unpublished results) was included. In the purification B the solution obtained after heating to 60 °C at pH 7 was heated again to 67 °C at pH 3 in the presence of ammonium sulfate. A number of previous experiments have indicated that the flavin in the enzyme is tightly associated with the protein (but it is not covalently bound), since so far we have not been able to remove the flavin from the enzyme without denaturing the protein – as can be done for several other flavin enzymes [6, 7]. While doing these experiments, it became obvious that heating the enzyme to 67 °C at pH 3 in the presence of ammonium sulfate might be a good purification step, because most of the proteins (including the majority of the phycobiliproteins became irreversibly denatured and precipitated, while the L-amino acid oxidase protein which also precipitated, could be partly extracted from the

Table I. Purification of the L-amino acid oxidase from *A. nidulans*. Details are given under Materials and Methods. 1 unit of enzyme = 1 μmol O_2 consumed × min^{-1} (in the presence of catalase, L-arginine as substrate, in air and at 20 °C).

Purification step	Protein mg, total	L-Amino acid oxidase activity units, total	Specific activity units/mg protein
Purification A:			
French press extract	157,796	7,348	0.047
Ammoniumsulfate prec. 30–55%	29,652	1,907	0.064
Heating to 60 °C	26,925	1,803	0.067
CM-Sephadex column	254	863	3.4
DEAE-Sephadex column	6.6	611	93
Hydroxylapatite column	3.0	664	221
Purification B:			
French press extract	27,820	1,756	0.063
Ammoniumsulfate prec. 30–55%	4,464	801	0.179
Heating to 60 °C	2,530	515	0.204
Heating to 67 °C at pH 3 in presence of ammonium sulfate	95	234	2.46
DEAE-Sephadex column	5.8	56	9.7
Hydroxylapatite column	1.4	60	43

Table II. Specific activity and FAD content of the L-amino acid oxidase from purification A and B (fraction with highest specific activity). FAD (authentic oxidized FAD) was determined by reconstitution of the apoprotein from kidney D-amino acid oxidase which requires FAD for activity. The values in parenthesis were determined by fluorometric measurements.

Sample	Specific activity units/mg protein	FAD content mol FAD/mol enzyme	
L-amino acid oxidase from purification A	350	1.85	(1.94)
L-amino acid oxidase from purification B	50	0.30	(0.21)

pellet with only approximately 50% loss of the activity (see Table I). After both purifications the enzyme was homogeneous based on SDS PAGE, and the molecular weight of the two subunits in the enzyme obtained by purification procedure A as well as in the enzyme obtained by purification procedure B was 50 kDa. This value agrees well with the previous value of 49 kDa [1]. The flavin content and the specific activity of the best fraction from both purifications are given in Table II (in Table I the average specific activity of the top four fractions is given). The corresponding absorption spectra and the fluorescence spectra of the two enzyme forms are given in Fig. 1 and 2, respectively.

As can be seen in Fig. 1, the purification A gave an enzyme which mainly contained oxidized flavin (absorption bands with maxima at 391 and 469 nm). However, a shoulder in the 400 nm region and a small absorption band in the 600 nm region were also present. This enzyme had a high L-amino acid oxidase activity of 350 units $\times$ mg^{-1} protein (Table II) which corresponded to a turnover number of 70,000 (mol L-arginine deaminated $\times$ mol^{-1} enzyme $\times$ min^{-1}). This is the highest specific activity which we have so far obtained (previously reported turnover number was 57,000 [2]). The enzyme A shows a fluorescence emission in the 420 to 560 nm and 600 to 670 nm region (Fig. 2). The absorption spectrum and the fluorescence emission spectrum of enzyme A (both spectra are different from the corresponding spectra of oxidized FAD) could either be explained by the presence of FAD semiquinone which has *e.g.* been shown to be present in yeast methanol oxidase [8, 9] or to the presence of modified flavin of yet unknown

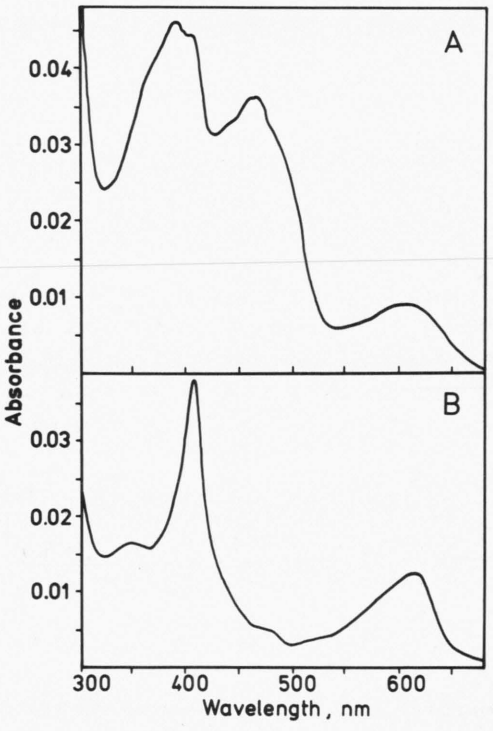

Fig. 1. Absorption spectrum of L-amino acid oxidase from purification A and B.
A: Absorption spectrum of L-amino acid oxidase from purification A. The sample was in 0.02 M potassium phosphate buffer, pH 7, and contained 0.20 mg protein/ml. The specific activity was 350 units/mg protein. The absorbance values were: $A_{275\,nm}$: 0.434, $A_{391\,nm}$: 0.046, $A_{469\,nm}$: 0.037 and $A_{613\,nm}$: 0.010.
B: Absorption spectrum of L-amino acid oxidase from purification B. The sample was in 0.02 M potassium phosphate buffer, pH 7, and contained 0.13 mg protein/ml. The specific activity was 50 units/mg protein. The absorbance values were: $A_{275\,nm}$: 0.126, $A_{408\,nm}$: 0.038 and $A_{610\,nm}$: 0.012.

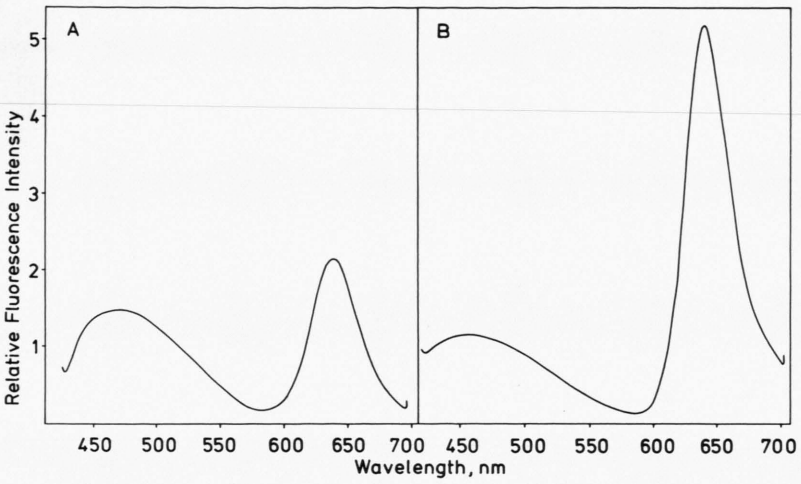

Fig. 2. Fluorescence emission spectra of L-amino acid oxidase from purification A and B. The enzyme samples were the same as in Fig. 1. The fluorescence emission spectra were recorded after excitation with light of 390 nm. A: L-amino acid oxidase from purification A and B: L-amino acid oxidase from purification B.

structure (as in enzyme B). After removal of the prosthetic group from the enzyme by acid treatment, the chromophore showed a typical FAD absorption spectrum (absorption bands with maxima at 265, 376 and 450 nm) and a typical FMN fluorescence emission (after hydrolysis of FAD to FMN) with a maximum at 530 nm (not shown). This indicates that the absorbance bands in the 400 and 600 nm region which are seen with the enzyme, are due to the presence of some enzyme stabilized FAD semiquinone [8–10] which is oxidized to FAD when removed from the enzyme. This conclusion that enzyme A partly contains a chromophore which can still be converted to FAD, is supported by the observation that we found almost the theoretically expected value of 2 mol FAD per mol enzyme in the reconstitution assay with the FAD specific kidney D-amino acid oxidase (Table II).

On the other hand, the enzyme from purification B mainly contained a modified flavin of yet unknown structure (absorption bands with maxima at 408 nm and in the 600 nm region) (Fig. 1B). In addition to the modified flavin a small amount of authentic oxidized FAD was also present (0.3 mol FAD per mol enzyme). As a consequence of the low FAD content this enzyme only had a low L-amino acid oxidase activity of 50 units per mg protein (Table II). We think that probably the L-amino acid oxidase activity is only associated with the enzyme containing oxidized FAD, while the enzyme containing the modified flavin is most likely inactive in this reaction. The absorption band with maxima at 408 nm is not due to a cytochrome, since dithionite greatly reduces this absorption (Fig. 3). The chromophore in enzyme B must have undergone a modification which could not any longer be reversed to oxidized FAD (as in enzyme A), since removal of the chromophore from enzyme B by acid treatment gave a chromophore which had absorption bands with maxima at 260, 396 and in the 600 nm region (Fig. 4). Although the absorption spectrum of enzyme B is quite different from that of enzyme A, the fluorescence emission spectra of the two enzymes were rather similar (Fig. 2). Only the emission in the 640 nm region was significantly higher in enzyme B than in enzyme A.

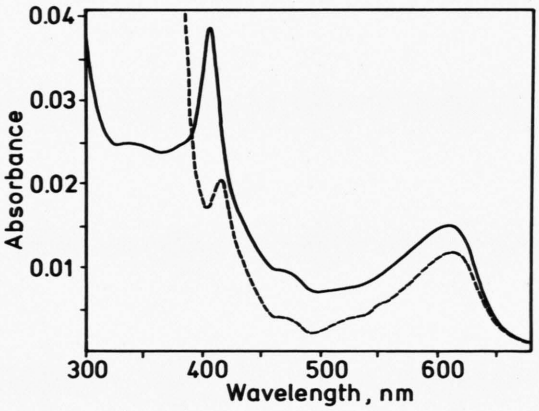

Fig. 3. Effect of sodium dithionite on the absorption spectrum of the L-amino acid oxidase from purification B. The enzyme was similar to the one in Fig. 1B; ——— without sodium dithionite, – – – with excess sodium dithionite.

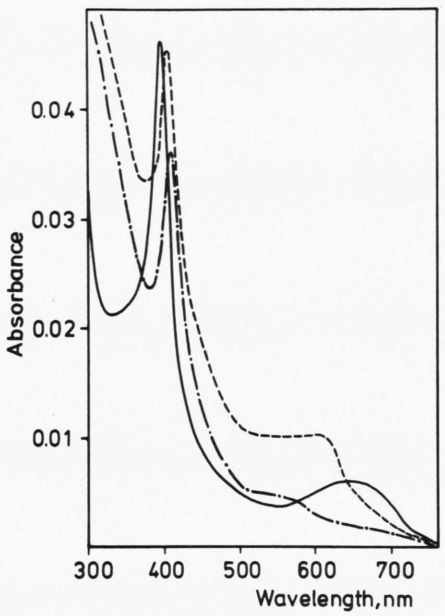

Fig. 4. Absorption spectrum of the chromophore extracted from the L-amino acid oxidase obtained in purification B. The chromophore was extracted from 0.066 mg protein (L-amino acid oxidase from purification B as in Fig. 1B) into 0.77 ml 0.2 N H_2SO_4. The absorbance values at pH < 2 were: $A_{260\,nm}$ (not shown): 0.145, $A_{396\,nm}$: 0.047 and $A_{650\,nm}$: 0.006. The ratio of A_{260}: A_{396}: A_{650} was 1 : 0.32 : 0.04. The curves show the effect of pH on the absorption spectrum: ——— pH < 2, – – – pH 6–7 and –.–.– pH > 10.

After removal of the chromophore from the enzyme B a broad fluorescence emission in the 470 nm region was present while the band in the 640 nm region was not any longer seen (not shown). It is not entirely clear at the present time whether the fluorescence emission band in the 640 nm region (seen with the enzyme but not with the extracted chromophore) is entirely due to the modified flavin or whether a small amount of phycocyanin remained associated with the enzyme throughout the entire purification.

The here shown results indicate that the L-amino acid oxidase can stabilize a semiquinone form of FAD under aerobic conditions and as a consequence a modification of FAD seems to occur fairly easily. This conclusion that a modification occurs, is supported by the fact that two different purifications (A and B) gave the two major types of the enzyme although the same starting material was used: Purification A gave an L-amino acid oxidase which contained oxidized FAD and FAD semiquinone which could be oxidized to FAD, while purification B gave an enzyme which mainly contained a modified FAD of yet unknown structure. Although variable amounts of the modified chromophore have been present in all previous enzyme preparations, the amount present in enzyme B was much higher than in all previous preparations. These results indicate that heating the crude extract at pH 3 in the presence of ammonium sulfate favored the conversion of FAD to the modified flavin of yet unknown structure(s) in the enzyme. Since the purified enzyme is very stable and only shows minor changes in its absorption spectrum upon storage, it seems that this modification reaction preferentially occurs at the early stages of the purification. So far we have not been able to find conditions to convert the modified flavin back to the oxidized FAD. We think that this modification is an irreversible alteration of the isoalloxazine ring – at least under the conditions here described (possibly not under *in vivo* conditions).

Flavin in photosystem II complexes from A. nidulans

Although we have previously shown that the L-amino acid oxidase protein is present in PS II complexes from *A. nidulans*, the amount of authentic oxidized FAD in these PS II complexes was much below the expected value [1]. However, the experiments in the previous section have shown that the flavin in the L-amino acid oxidase seems to undergo modification reactions fairly easily. Therefore, it seemed likely that these modification reactions could occur even more rapidly in PS II complexes. Here we have reexamined PS II complexes from *A. nidulans* for the presence of such modified flavin derivatives. For extraction of the chromophore we have first dialyzed the PS II complexes against 0.02 M potassium phosphate buffer, pH 7, to remove glycerol and sucrose and then acetone was added to give a final concentration of 90% (v/v). This treatment resulted in extraction of chlorophyll, carotenoids and lipids into the organic solvent phase, while the proteins which still contained the flavin, cytochrome and the residual blue pigments, were precipitated. After centrifugation the protein fraction was extracted with 0.2 N H_2SO_4. This procedure removed the flavin from protein but not the cytochrome or the blue pigments. The absorption spectra of the chromophores extracted from PS II complexes from *A. nidulans* after one or two sucrose gradient centrifugations

(PS IIa and PS IIb) are given in Fig. 5, and the effect of pH on the chromophore obtained from PS IIa is shown in Fig. 6. PS IIa was isolated as described previously [2] and still contained some phycobiliproteins and some L-amino acid oxidase which was not or only loosely associated with the complex. This PS IIa complex was further purified by an additional sucrose gradient centrifugation after the $CaCl_2$ concentration had been reduced. The reduction of ions in the medium caused an aggregation of the PS II complex and gave a better separation of the complex from solubilized proteins not associated with the complex (PS IIb). This complex was basically similar to the previously described PS II complex obtained after chromatography on a Sepharose 6B column [3]. The here described procedure gave a more concentrated PS II complex than the one in ref. [3].

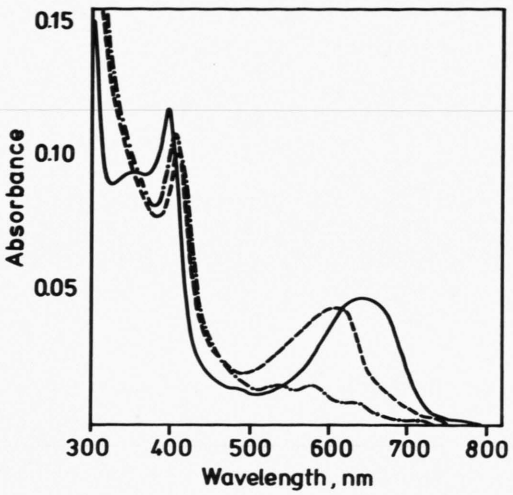

Fig. 6. The effect of pH on the absorption spectrum of the chromophore extracted from PS IIa. Absorption spectrum of the chromophore extracted from PS IIa at ——— pH < 2, – – – pH 6–7 and –.–.– pH > 10.

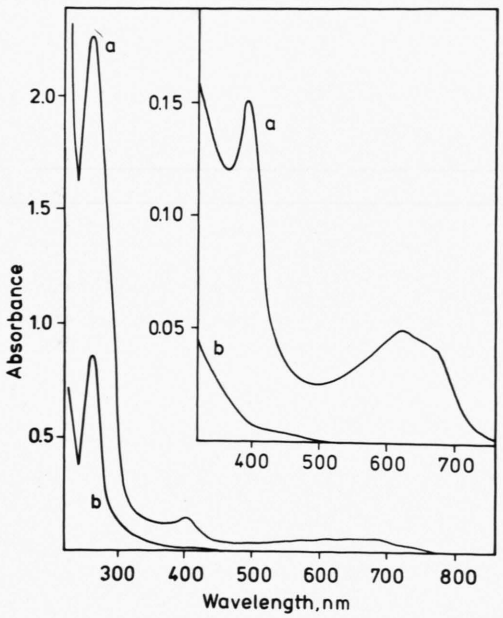

Fig. 5. Absorption spectra of chromophores extracted from PS II complexes of *A. nidulans*. The chromophores were extracted from the PS II complexes as described under Materials and Methods.
a: Chromophore extracted from PS IIa (total chlorophyll 0.5 mg) into 1.02 ml 0.2 N H_2SO_4. Absorbance values were: $A_{260\,nm}$:2.3, $A_{396\,nm}$:0.15 and $A_{626\,nm}$:0.049. The ratio of $A_{260}:A_{396}:A_{626}$ was 1:0.065:0.021.
b: Chromophore extracted from PS IIb (total chlorophyll 0.77 mg) into 1.02 ml 0.2 N H_2SO_4. Absorbance values were: $A_{260\,nm}$:0.853 and $A_{396\,nm}$:0.007. The ratio of $A_{260}:A_{396}$ was 1:0.008.

The chromophore obtained from the PS IIa complex had absorption bands with maxima at 260, 396 nm and in the 600 nm region and was similar to the chromophore extracted from the isolated L-amino acid oxidase with respect to the absorption maxima. However, the relative heights of the absorption bands to each other were different and were also variable in various preparations. When the ratios of $A_{260}:A_{396}:A_{600-650}$ were compared, then these ratios were approximately 1:0.32:0.04 for the chromophore extracted from the isolated L-amino acid oxidase (enzyme B – Fig. 4) and 1:0.065:0.021 for the chromophore extracted from the PS IIa complex (Fig. 5a). It seemed that the chromophore from the PS IIa complex was more bleached than the chromophore from the L-amino acid oxidase. This became even more obvious with the chromophore extracted from PS IIb (Fig. 5b). This chromophore showed a featureless spectrum which was somewhat similar to the spectrum of reduced flavin or to the spectrum of flavin sulfite complexes [11–13]. The chromophore from PS IIa and PS IIb were fluorescent with a fluorescence emission in the 420 to 560 nm region (not shown). It is obvious that more than one derivative of FAD is formed. However, the derivatives seem to be very similar since we have so far not been able to separate the derivatives from each other by thin layer chromatography, although

they could be separated from FAD (not shown). This aspect requires a much larger amount of the chromophore than is available at the present time. In general, it could be concluded that the various extracted chromophores were all identical with respect to the absorption band at 260 nm, but showed considerable variation in the absorption in the visible region of the spectrum. They were also quite similar with respect to the fluorescence emission in the 420 to 560 nm region.

As the results of Table III clearly show, the PS II complexes contained a substantially higher quantity of the chromophore than would be expected on the basis of the L-amino acid oxidase activity which we could detect in the PS II complexes. Moreover, if we try to correlate the amount of the extracted chromophore from PS II complexes with the expected amount of 2 chromophores per approximately 50 chlorophylls in these complexes, then we find 2.4 or 0.6 chromophores per 50 chlorophylls in PS IIa and PS IIb, respectively. Our calculation is based on the assumption that in the L-amino acid oxidase from purification B (Fig. 1B) two modified chromophores per mol enzyme are present (no corrections are made for the small amount of authentic oxidized FAD present). This would allow the calculation of a molar absorption coefficient as being $\varepsilon_{260\,nm} = 85{,}000\ M^{-1}cm^{-1}$. We have used the absorption at 260 nm for calculation, since this absorption seemed to show the least variation. Of course, these values can only be taken as a very rough estimation, since we are obviously dealing with a family of flavin derivatives which have to be further characterized.

Concluding Remarks

Here we show that the spectral complexity and variability of the L-amino acid oxidase can be largely explained by the presence of modified flavin derivatives of yet unknown structures besides authentic oxidized FAD and FAD semiquinone. These derivatives of FAD seem to be formed in variable amounts during the purification of the enzyme. On the other hand, extraction of the purified *Anacystis* PS II complexes which contain the flavoprotein, almost exclusively yields modified flavin derivatives and practically no authentic oxidized FAD. Our results have indicated that more than one derivative of FAD (although closely related derivatives) can be formed in these modification reactions. Which derivative eventually will be formed, seems to depend on the conditions under which the modification occurs. We think that the spectra of some of the modified flavin derivatives have a certain similarity to 10,10[a]-ring opened derivatives of flavin [14] and possibly also to 6-OH-FAD [15], while other chromophores show a spectrum similar to the spectrum of reduced flavin or flavin sulfite complexes [11–13]. If such modification reactions would occur as described by Mager and Addink [14], then this could explain the variability which we observe in the formed products – especially, when the known chemical reactivity of the isoalloxazine ring is taken into consideration. A chemical analysis of the flavin derivatives requires further work, but seems to be extremely difficult because of the great variability which we observe in the formed flavin derivatives.

Table III. Comparison between the L-amino acid oxidase activity and the amount of chromophore (given as absorbance values at 260 nm) extracted from the isolated L-amino acid oxidase and PS II complexes from *A. nidulans*. The absorption spectra of the extracted chromophores are given in Fig. 4 and 5.

Sample	L-amino acid oxidase activity total units	Extracted chromophore in 1 ml 0.2 N H_2SO_4 $A_{260\,nm}$
L-Amino acid oxidase (enzyme B)	3.29	0.11
PS IIa	0.25	2.35
PS IIb	0.04	0.87

Although there still remains a great deal of uncertainty about these here described chromophores, we think that these results further support our hypothesis about a functional flavin in PS II. However, if we suggest that flavin has a functional role in photosynthetic water oxidation, then, of course, the problem arises that normally flavins do not have a redox potential which is positive enough for this reaction and moreover FMN and FAD in the oxidized or reduced form have only a very poor affinity for metal ions [16, 17]. However, recently modified flavins which have an additional chelation site for metals, have been synthesized [18]. Such substances can form flavin → metal charge transfer complexes. In these complexes flavin has a more positiv redox potential and as a consequence this will lead to an activation of flavin as an oxidizing agent. These model experiments come very close to our hypothesis about the water oxidizing enzyme. We have suggested that the flavoprotein which has an L-amino acid oxidase activity under certain conditions, becomes modified in the light and in the presence of Ca^{2+} and Cl^- in such a way that the flavin in the enzyme could interact with Mn^{2+}. Such a flavin → Mn charge transfer complex might then be able to catalyze the water oxidation of PS II [2, 3]. In the enzyme possibly one of the amino acid residues could provide an additional chelation site to allow the formation of such a flavin → Mn charge transfer complex. Whether the suggested functional flavin on the donor side of PS II is "Z" [19] which presently is thought to be a tyrosine radical [20], or an additional redox component can not be decided at the present time.

Acknowledgements

The financial support of the Deutsche Forschungsgemeinschaft is gratefully acknowledged. We thank Prof. Georg H. Schmid for valuable discussions.

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