Isolation of a Photosystem II Associated 36 kDa Polypeptide and an Iron-Stress 34 kDa Polypeptide from Thylakoid Membranes of the Cyanobacterium *Synechococcus* PCC 6301 Grown under Mild Iron Deficiency

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Cyanobacteria, *Synechococcus* PCC 6301, Photosystem II, L-Amino Acid Oxidase, Iron Stress

A 36 kDa polypeptide which previously was shown to be present in purified photosystem II complexes from *Synechococcus* PCC 6301 and which crossreacts with the antiserum raised against the soluble L-amino acid oxidase of 50 kDa from *Synechococcus* PCC 6301 (A. E. Gau, G. Wälzlein, S. Gärtner, M. Kuhlmann, and E. K. Pistorius, Z. Naturforsch. **44c**, 971, 1989), was isolated from thylakoid membranes of the same cyanobacterium grown under mild iron deficiency. This peptide is present in about equal amounts in thylakoid membranes of *Synechococcus* PCC 6301 grown under regular or iron deficient conditions. The antiserum raised against this thylakoid membrane bound 36 kDa peptide crossreacts with the soluble L-amino acid oxidase of 50 kDa. These results further support our conclusion that the thylakoid membrane bound 36 kDa polypeptide is a modified form of the soluble 50 kDa L-amino acid oxidase. In addition, a 34 kDa polypeptide was isolated from iron stressed thylakoid membranes, and an antiserum was also raised against this protein. Immunoblot experiments with this antiserum show that the 34 kDa peptide is present in elevated amounts in thylakoid membranes from *Synechococcus* cells grown under iron deficiency and that it is almost absent in thylakoid membranes from cells grown under regular conditions.

Introduction

As described in the preceeding paper [1] our hypothesis of the water oxidizing enzyme suggests that this enzyme evolved from an L-amino acid dehydrogenase/oxidase with high specificity for basic L-amino acids, such as L-arginine. The antiserum raised against the isolated L-amino acid oxidase of 50 kDa present in the soluble fraction of French press extracts from *Synechococcus* PCC 6301 recognizes a 36 kDa polypeptide in isolated and purified PS II complexes of *Synechococcus* PCC 6301 and in the closely related *Synechococcus* PCC 7942 [1, 2]. These results indicate that the "soluble" L-AOX protein becomes modified in an unknown fashion (processed, covalently modified or associated with lipids) when incorporated into the thylakoid membrane, resulting in an altered migration velocity of this peptide in SDS polyacrylamide gels.

To confirm that the PS II associated 36 kDa polypeptide is a major protein in thylakoid membranes and that it actually is a modified form of the soluble L-AOX protein, we have isolated this 36 kDa peptide from thylakoid membranes of *Synechococcus* PCC 6301 for further characterization and for raising an antiserum. Sherman's group [3, 4] has shown that unter iron deficiency substantial changes in the ultrastructure of thylakoid membranes of *Synechococcus* PCC 7942 occur, such as *e.g.* a decrease of phycobilisomes as well as of the chlorophyll protein complexes of PS I and II, and a substantial increase in a chlorophyll protein complex, called CPVI-4, which copurifies with PS II and which consists of polypeptides of 36, 34 and 12 kDa. Therefore, we thought that thylakoid membranes of *Synechococcus* PCC 6301 grown under mild iron deficiency might possibly be a good starting material for isolation of the 36 kDa polypeptide being present in PS II complexes and showing a crossreactivity with the anti-L-AOX.

Abbreviations: L-AOX, L-amino acid oxidase; Chl, Chlorophyll; MSP, manganese stabilizing protein; PS, photosystem.

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Materials and Methods

Growth of Synechococcus PCC 6301, isolation of thylakoid membranes, and activity measurements

Synechococcus PCC 6301 was grown as previously described [5]. When the cells were grown

under Fe deficient conditions, $FeCl_3$ and Fe-EDTA were omitted. Thylakoid membranes for the experiments of Table I were prepared as described in [2], and photosynthetic O_2 evolution and L-AOX activity were determined as described in [1].

For the isolation of the peptides thylakoid membranes were isolated from *Synechococcus* PCC6301 cells grown under Fe deficient conditions as described above. After two days of growth in Fe deficient medium the cells were harvested by centrifugation, washed once with 50 mM Mes-NaOH, pH 6.5, containing 1 mM phenylmethylsulfonylfluoride and 1 mM ε-NH_2-caproic acid, and resuspended in the same buffer to give 100 µl cells/ml. The resuspended cells were passed twice through a French pressure cell at 137.9 MPa. The French press extract was centrifuged for 5 min at 3000 × *g*, and the supernatant was then centrifuged for 75 min at 95000 × *g* (F28-36 – Sorvall). After centrifugation the pellet was resuspended in the above buffer to give 0.5 mg Chl/ml.

Dodecyl-β-D-maltoside extraction and column chromatography

Thylakoid membranes which were isolated from *Synechococcus* PCC6301 grown under Fe deficient conditions as described above, were recentrifuged and suspended in 25 mM Tris-HCl, pH 8.2, containing 10 mM NaCl to give 0.5 mg Chl/ml. Dodecyl-β-D-maltoside was added to give a final concentration of 2% (wt/vol). After incubation for 30 min on ice under stirring, the extract was centrifuged for 1 h at 150000 × *g* (Ti60, Beckman) to remove the insoluble material and the supernatant was used for chromatography.

DEAE Sephacel column: The dodecyl-β-D-maltoside extract (15 mg Chl) was loaded on a DEAE Sephacel (Pharmacia) column (size 30 × 2 cm) equilibrated with 25 mM Tris-HCl, pH 8.2, containing 10 mM NaCl and 0.05% dodecyl-β-D-maltoside (buffer A). After washing the column with 50 ml buffer A, the proteins were eluated with a linear gradient of 10 to 300 mM NaCl in 300 ml buffer A. Three proteins in the 34 to 36 kDa region (called protein I, II, and III in the sequence of elution) were obtained before the major Chl proteins eluated.

Mono S column coupled to FPLC (Pharmacia): Protein II was further purified on a Mono S column which was equilibrated with 25 mM Mes-NaOH, pH 6.0, containing 10 mM NaCl and 0.05% dodecyl-β-D-maltoside (buffer B). After applying the sample (15 ml, 1.1 mg protein), washing the column with 20 ml buffer B, the protein was eluated with a linear gradient of 10 to 500 mM NaCl in 15 ml buffer B (two times repeated).

Mono Q column coupled to FPLC: Protein III was further purified on a Mono Q column which was equilibrated with buffer A. After applying the sample (15 ml, 2.1 mg protein), washing the column with 20 ml buffer A, the protein was eluated with a linear gradient of 10 to 300 mM NaCl in 15 ml buffer A (three times repeated).

SDS-PAGE (10% SDS polyacrylamide gels, 1 mm thick and denaturation of the sample for 2 h at 40 °C) and immunoblotting were performed as previously described [1, 2]. Denaturation of the thylakoid membrane samples used for the immunoblots with anti-protein III was performed with 2% docedyl-β-D-maltoside in the denaturation mixture. Protein was determined after trichloracetic acid precipitation according to Lowry, and the organic prosthetic group of the 36 kDa polypeptide was extracted as described in [6]. Ouchterlony double diffusion tests were performed on 5 × 5 cm plates coated with 1% agar in 0.06 M sodium phosphate buffer, pH 6.8, containing 1% Triton X-100.

Antisera against protein II and III were raised in rabbits according to standard procedures. Antisera raised against the soluble L-AOX of 50 kDa (from *Synechococcus* PCC6301) and against D1 and MSP from oat were the same as used in [1, 2]. The soluble L-AOX of 50 kDa used in this paper was isolated from *Synechococcus* PCC6301 according to the procedure described in [1].

Results

Chlorophyll content, photosynthetic O_2 evolution and L-amino acid oxidase activity in Synechococcus PCC6301 grown under regular and iron deficient conditions

Synechococcus PCC6301 was grown under regular and Fe deficient conditions. As the results in Table I show, growing the cells for two times two days on Fe deficient medium resulted in a significant reduction of growth and in a drastic decrease of the chlorophyll content of the cells. From such cells thylakoid membranes were prepared. When

Table I. Growth rate, chlorophyll content, photosynthetic O_2 evolution and L-AOX activity of *Synechococcus* PCC6301 grown under regular and iron deficient conditions.

A. Cell suspensions

	Growth rate µl cells/ml growth medium	Chlorophyll content mg Chl/100 µl cells
Cells grown under:		
Regular conditions	0.24 → 3.0*	0.74
2 Days Fe deficiency	0.24 → 2.6	0.34
2 × 2 Days Fe deficiency	0.48 → 1.3	0.21

B. Thylakoid membrane preparations

	Chlorophyll content mg Chl/mg protein	Photosynth. O_2 evolution µmol O_2/mg Chl × h	L-AOX activity (O_2 uptake) µmol O_2/mg Chl × h
Thylakoid membranes prepared from cells grown under:			
Regular conditions	0.066	84 (5.6)	25 (1.6)
2 Days Fe deficiency	0.042	124 (5.2)	60 (2.5)
2 × 2 Days Fe deficiency	0.041	140 (5.7)	69 (2.8)

Values in parenthesis were calculated on protein basis (µmol O_2/mg protein × h).
* Cell density at inoculation → after two days of growth.

the O_2 evolution was examined, the results of Table I show that the O_2 evolution increased on chlorophyll basis but not on protein basis in thylakoid membranes from Fe deficient cells. However, the L-AOX activity in thylakoid membranes from Fe deficient cells was elevated on chlorophyll as well as on protein basis. This could mean that either the amount of the L-AOX protein increased relative to the other proteins or that the enzyme was more accessible to the hydrophilic substrate L-arginine in thylakoid membranes from Fe stressed cells than in thylakoid membranes from cells grown under regular conditions (see later). SDS-PAGE of such thylakoid membrane preparations shows that a protein band in the 34 to 36 kDa region significantly increased under Fe deficiency (Fig. 1). The correspondent immunoblot with the anti-L-AOX indicated that one component in this band is the 36 kDa peptide which is immunologically related to the L-AOX (not shown).

Isolation of three polypeptides in the 34 to 36 kDa region from thylakoid membranes

Thylakoid membranes obtained from *Synechococcus* PCC6301 cells grown under iron deficiency for two days were extracted with the detergent dodecyl-β-D-maltoside, and the supernatant obtained after cenfrifugation was subjected to chromatography on a DEAE Sephacel column (Fig. 2). When the early fractions eluating from the column before the major chlorophyll proteins eluated, were ex-

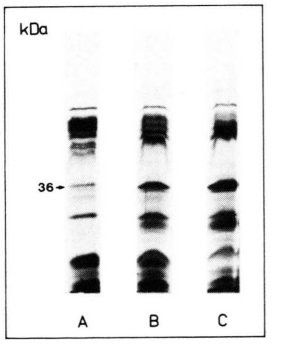

Fig. 1. Polypeptide composition of thylakoid membranes from *Synechococcus* PCC6301 grown under regular and Fe deficient conditions. Coomassie brilliant blue stained SDS polyacrylamide gel: A: thylakoid membranes from cells grown under regular conditions (7 µg Chl), B: from cells grown for 2 days under Fe deficient conditions (7 µg Chl), and C: from cells grown for 2 × 2 days under Fe deficient conditions (8 µg Chl).

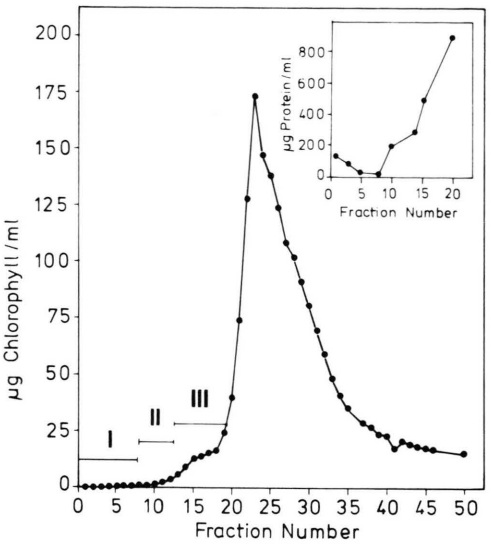

Fig. 2. Fractionation of the dodecyl-β-D-maltoside-solubilized thylakoid membranes from iron stressed *Synechococcus* PCC 6301 on a DEAE Sephacel column. The Chl (and partially the protein) elution profile is shown. The NaCl gradient started at fraction 1. Before the fractions with the main Chl proteins eluated, three proteins called I (fraction 1 to 3), II (fraction 9 to 11), and III (fraction 15 to 20) in the 34 to 36 kDa region could be collected. (The anti-L-AOX gave a positive signal with fractions 15 to 35, but only fractions 15 to 20 were used for further purification.)

amined by SDS-PAGE, three proteins with an apparent molecular weight of about 34 to 36 kDa were detected. The proteins were numbered protein I (35 kDa), protein II (34 kDa), and protein III (36 kDa) in the sequence in which they eluated from the column. At this stage of purification they all contained substantial amounts of carotenoids, but almost no chlorophyll. However, we think that these carotenoids are not specifically associated with these proteins, since the carotenoids could greatly (protein III) or almost completely (protein II) be removed during the subsequent purification step. Most likely these carotenoids were trapped in the protein detergent complexes.

As the corresponding immunoblot with the anti-L-AOX showed, protein III cross-reacted with the anti-L-AOX (Fig. 3), while the other two proteins did not. Protein I was not further investigated in this paper, while the other two proteins were puri- was raised. Protein II was subjected to chromatography on a Mono S column, and the protein raphy on a Mono S column, and the protein eluated in a sharp peak at 250 mM NaCl. Further purification of protein III was achieved by chromatography on a Mono Q column. Although the fractions 15 to 35 from the DEAE Sephacel column gave a cross-reaction with the anti-L-AOX, only those fractions (fractions 15 to 20) which contained minor contaminations, were used for further purification. The 36 kDa protein eluated in a relative broad band in the range of 125 mM NaCl from the Mono Q column indicating that either variable amounts of detergent or lipids were associated with this protein. Both proteins were homogenous based on SDS-PAGE. As expected, protein III still gave a cross-reaction with the anti-L-AOX (see later in Fig. 6), but had no detectable L-AOX activity. So far, we were not able to extract this protein in an active form from the thylakoid membranes.

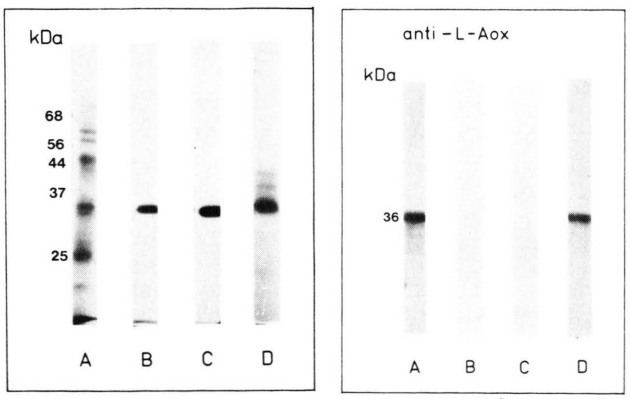

Fig. 3. SDS polyacrylamide gel of the dodecyl-β-D-maltoside extract of thylakoid membranes from Fe stressed *Synechococcus* PCC 6301 and of the three peptides in the 34 to 36 kDa region obtained after chromatography of the dodecyl-β-D-maltoside extract on a DEAE Sephacel column. A: dodecyl-β-D-maltoside extract of thylakoid membranes from Fe stressed *Synechococcus* PCC 6301, B: protein I (35 kDa), C: protein II (34 kDa), and D: protein III (36 kDa). Left: Coomassie brilliant blue stained gel; right: gel immunostained with anti-L-AOX (Dilution of the anti-L-AOX was 1 : 50).

Comparative immunoblots with the antisera raised against protein II and III

With the antisera raised against proteins II and III, we examined whether one of these two proteins was responsible for the increase of the 34 to 36 kDa protein band in thylakoid membranes of Fe deficient cells (see Fig. 1). The results of Fig. 4 clearly show that protein II significantly increased in thylakoid membranes isolated from cells grown under Fe deficiency as compared to thylakoid membranes from regularly grown cells. This peptide does not seem to be a general stress protein, since it does not increase when *Synechococcus* PCC 6301 was grown at elevated temperature (40 °C) (not shown). In contrast to protein II, the amount of the 36 kDa polypeptide (protein III) did not increase, but decreased on protein basis in thylakoid membranes from cells grown under Fe deficiency as compared to thylakoid membranes from regularly grown cells. However, the results clearly show that the 36 kDa polypeptide is present in substantial amounts in both cells types. As reported previously and in the accompanying paper [1, 2], this polypeptide is present in isolated PS II complexes from *Synechococcus* PCC 6301 and PCC 7942 and is suggested to be the water oxidizing enzyme. Therefore, we have also examined the D 1 and MSP content. A slight reduction in the quantity of these peptides on protein basis could also be observed when thylakoid membranes from cells grown under regular or Fe deficient conditions were examined (Fig. 4). Of course on chlorophyll basis all examined peptides increased due to reduction of chlorophyll in thylakoid membranes from Fe stressed cells, but on protein basis only the 34 kDa peptide (protein II) increased.

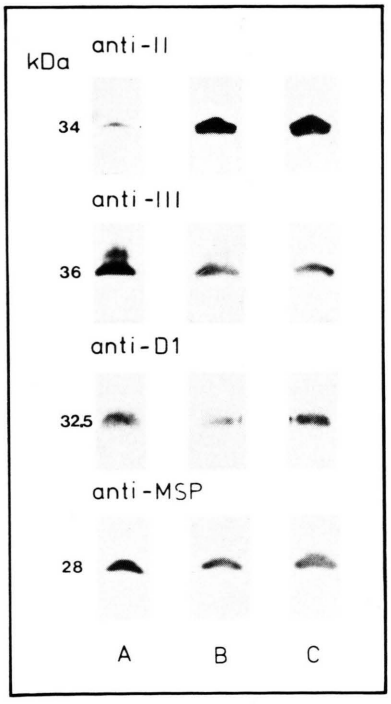

Fig. 4. SDS polyacrylamide gel of thylakoid membranes from *Synechococcus* PCC 6301 grown under regular or Fe deficient conditions immunostained with the antisera raised against protein II and protein III (this paper) as well as with the antisera raised against D 1 and MSP. A: thylakoid membranes from *Synechococcus* PCC 6301 grown under regular conditions (12 µg Chl, 119 µg protein), B: thylakoid membranes from cells grown for 2 days under Fe stress (5 µg Chl, 117 µg protein), and C: thylakoid membranes from cells grown for 2 × 2 days under Fe stress (5 µg Chl, 118 µg protein). (Antisera dilution: anti-protein II (34 kDa) 1 : 1000, anti-protein III (36 kDa) 1 : 2400, anti-D 1 1 : 100, anti-MSP 1 : 200.)

Immunological relation between the 50 kDa L-AOX protein present in the soluble fraction and 36 kDa polypeptide associated with thylakoid membranes

The antibody raised against the L-AOX protein which is present in the soluble fraction of French press extracts of *Synechococcus* PCC 6301 and which has an apparent molecular weight of 50 kDa, recognizes a 36 kDa peptide in isolated PS II complexes from *Synechococcus* PCC 6301 and PCC 7942 as shown in [1, 2]. That these two proteins are immunologically related is further supported by the results of Fig. 5. The antibody raised against the thylakoid membrane bound 36 kDa polypeptide (this paper) cross-reacts with the soluble L-AOX protein of 50 kDa [1, 6] in Quchterlony double diffusion tests. We have not succeeded to obtain a positive result in immunoblot experiments. Most likely SDS masks the antigene determinants of the protein which are recognized by the antiserum. In Fig. 6 SDS polyacrylamide gels of the purified 36 kDa peptide and of purified PS II complexes from *Synechococcus* PCC 6301 and PCC 7942 immunostained with the anti-L-AOX (50 kDa) and the anti-36 kDa peptide are shown. Both antisera recognize the isolated 36 kDa peptide and the same 36 kDa peptide in PS II complexes. As expected, the antiserum raised

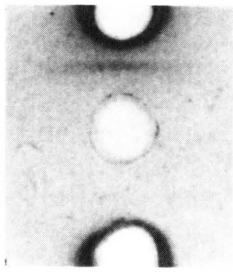

Fig. 5. Ouchterlony doubly diffusion test with purified L-AOX of 50 kDa present in the soluble fraction and the antiserum raised against 36 kDa peptide (protein III) associated with thylakoid membranes. Central well: 1.6 μg L-AOX from *Synechococcus* PCC6301 (50 kDa – isolated from the soluble fraction of French press extract), upper well: antiserum (undiluted) raised against 36 kDa peptide (thylakoid membrane bound), and lower well: control serum (undiluted).

against protein II does not recognize the L-AOX protein of 50 kDa or the 36 kDa peptide, and the antisera raised against the L-AOX (50 kDa) and the 36 kDa peptide do not recognize protein II (not shown).

Extraction of an organic prosthetic group from the 36 kDa polypeptide

Previously we have shown that the isolated L-AOX of 50 kDa from *Synechococcus* PCC6301 and PCC7942 contains variable amounts of FAD and a modified flavin of unknown structure(s) [1, 6]. An organic prosthetic group comparable to the modified flavin of unknown structure could also be extracted from isolated PS II complexes of *Synechococcus* PCC6301 [6]. When the 36 kDa polypeptide isolated from thylakoid membranes of *Synechococcus* PCC6301 was subjected to the same extraction procedure as previously described for PS II complexes, then a comparable compound could be extracted from this protein as the absorbance and fluorescence spectra indicate (Fig. 7). Whether this organic prosthetic group is a modified flavin of unknown structure [7] – possibly caused by an irreversible damage (due to highly oxidizing species in the membrane) or a modified flavin with better metal chelating properties [8] or even a methoxatin-like compound [9] can not be answered at the present time.

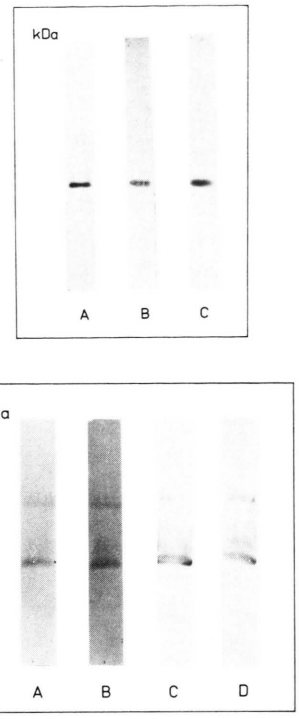

Fig. 6. Cross-reactivity of the anti-L-AOX (50 kDa) and anti-36 kDa peptide (protein III) with the purified 36 kDa peptide (protein III) isolated from thylakoid membranes and with the corresponding band in purified PS II complexes from *Synechococcus* PCC6301 and PCC7942. Top: SDS gel with the purified 36 kDa protein (after Mono Q – 2.8 μg protein). A: Coomassie brilliant blue stained, B: immunostained with anti-L-AOX and C: immunostained with anti-36 kDa peptide. Bottom: SDS gel of purified PS II complexes from *Synechococcus* PCC6301 (A and C – 1.5 μg Chl) and from PCC7942 (B and D – 1.5 μg Chl) immunostained with anti-L-AOX (A and B) and with anti-36 kDa (C and D). Antisera dilution: anti-36 kDa 1:2000, anti-L-AOX 1:100.

Discussion

The results presented support our previous conclusion [1, 2] that the membrane bound 36 kDa polypeptide and the soluble L-AOX of 50 kDa are immunologically related, since both antisera (the one raised against the soluble L-AOX of 50 kDa and the one raised against the membrane bound 36 kDa polypeptide) cross-react with the soluble L-AOX of 50 kDa as well as with the 36 kDa thylakoid membrane bound peptide and since both antisera recognize the same 36 kDa polypeptide in purified PS II complexes from *Synechococcus*

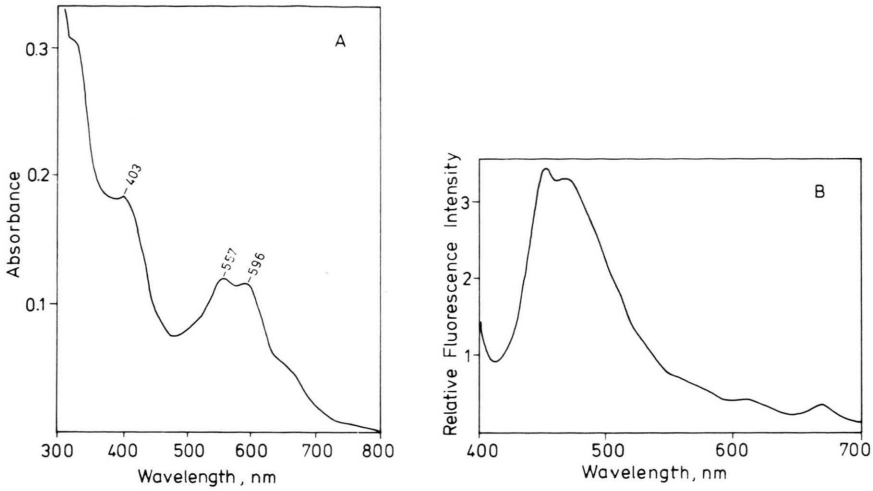

Fig. 7. Absorbance spectrum (A) and fluorescence emission spectrum (B) of the organic prosthetic group extracted from protein III (36 kDa). The prosthetic group was extracted from 0.7 mg protein as previously described [6]. The fluorescence spectrum was recorded after excitation with light of 390 nm.

PCC 6301 and PCC 7942. The 36 kDa polypeptide most likely represents a modified form of the soluble L-AOX of 50 kDa. However, the modification process which leads to the different mobility of these two immunologically related proteins on SDS polyacrylamide gels is still unknown.

As the results in [1, 6] have clearly shown, the 50 kDa polypeptide with L-AOX activity is present in very small amounts in the soluble fraction of French press extracts (isolation of 1 mg L-AOX required about 500 ml packed cells). Most of this protein (although in the 36 kDa form) seems to be associated with thylakoid membranes, since isolation of an equivalent amount of the 36 kDa polypeptide from thylakoid membranes only required about 1 ml packed cells (rough approximation). The isolated detergent solubilized 36 kDa peptide did not have detectable L-AOX activity – indicating that either the activity is lost due to the detergent treatment (possibly causing an irreversible damage of the prosthetic group) or that in the 36 kDa peptide this activity is totally suppressed due to an unknown modification.

In addition to the 36 kDa polypeptide, a 34 kDa polypeptide was also isolated from thylakoid membranes of *Synechococcus* PCC 6301. This polypeptide is not immunologically related to the L-AOX protein and strongly increases under iron stress in thylakoid membranes of *Synechococcus* PCC 6301. Most likely the 34 kDa polypeptide is related to the 34 kDa polypeptide which was shown to increase under iron stress in *Synechococcus* PCC 7942 and to be a component of the CPVI-4 complex isolated by Sherman's group [3, 10].

We hope eventually to answer the question of the function of the 34 kDa peptide (Fe stress protein) and of the 36 kDa peptide (possible water oxidizing enzyme – based on our hypothesis) [11, 12] in *Synechococcus* PCC 6301 and PCC 7942 by performing the corresponding gene inactivation experiments.

Acknowledgements

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