Isolation and Partial Characterization of a Manganese and Chloride Binding Protein Present in Highly Purified Photosystem II Complexes of the Thermophilic Cyanobacterium *Synechococcus* sp.: The Protein Being Detected by Its L-Arginine Metabolizing Activity

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This paper is dedicated to Professor Birgit Vennesland on the occasion of her 80th birthday

L-Arginine Metabolizing Enzyme, Water Oxidizing Enzyme, Photosystem II, Thermophilic Cyanobacteria, *Synechococcus* sp.

Photosystem II complexes were solubilized with the detergent sulfobetaine 12 from thylakoid membranes of the thermophilic cyanobacterium *Synechococcus* sp. and purified by two sucrose gradient centrifugations and by chromatography on a Mono Q column. In such photosystem II complexes having a photosynthetic O_2 evolving activity of 2938 μmol O_2 evolved/mg chlorophyll × h, an L-arginine metabolizing activity leading to ornithine and urea as major products, could be shown to be present. Besides ornithine and urea, a product (or products) of yet unknown structure is formed in addition – especially under aerobic conditions. This activity remained associated with photosystem II complexes even after substantial additional treatments to remove loosely bound proteins. On chlorophyll basis the maximal activity obtained under optimal assay conditions corresponded to 94 μmol ornithine formed/mg chlorophyll × h. This PS II associated, L-arginine metabolizing enzyme was isolated (utilizing a manganese charged chelating Sepharose 6 B column) and partially characterized. It could be shown that this enzyme requires manganese and chloride for its L-arginine metabolizing activity and that manganese becomes totally lost during purification indicating that manganese is bound to a fairly exposed site on the protein. Since it is rather unlikely that two different manganese and chloride binding proteins are present in such highly purified photosystem II complexes, the possibility of this protein being the water oxidizing enzyme will be discussed. Whether the manganese and chloride requiring L-arginine metabolizing activity of this protein which provided a suitable assay for its isolation from photosystem II complexes, has any physiological significance, can not be answered at the present time.

Introduction

One of the unanswered questions related to photosystem II (PS II) is the identity of the water oxidizing enzyme (WOE). The majority of groups working in this area favors the reaction center peptide D1 in connection with D2 (and/or other known PS II polypeptides) as the site of water oxidation. This model implies that the reaction center peptides D1/D2 can catalyze charge separation as well as water oxidation [1, 2]. In contrast, our model predicts that the WOE is a separate polypeptide – not being recognized by other groups as possible candidate for the WOE and being present in addition to the so far recognized seven polypeptides (CP47, CP43, D1, D2, MSP, and two cytochrome binding peptides of 9 and 4.3 kDa) in minimal O_2 evolving PS II complexes [3]. Our model is mainly based on results with the cyanobacteria *Synechococcus* PCC6301 and *Synechococcus* PCC7942 where we could show that a 36 kDa peptide (being distinct from D1, D2, and MSP) is present in highly purified PS II complexes [4–6]. This PS II associated 36 kDa peptide is immunologically related to a 100 kDa L-amino acid oxidase (L-AOX) consisting of two subunits of

Abbreviations: L-AME, L-arginine metabolizing enzyme; L-AOX, L-amino acid oxidase; CBB, Coomassie Brilliant Blue G250; Chl, chlorophyll; DM, dodecyl-β-D-maltoside; MSP, manganese stabilizing protein; PMSF, phenylmethanesulfonyl fluoride; PS II, photosystem II; SB 12, sulfobetaine 12 (N-dodecyl-N,N-dimethylammonio-3-propane-sulfonate); WOE, water oxidizing enzyme.

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50 kDa, catalyzing a regular oxidative deamination of L-amino acids, and having a high specificity for basic L-amino acids (such as L-arginine). The latter enzyme is detectable in the soluble fraction and in thylakoid membranes of French press extracts of *Synechococcus* PCC 6301 and PCC 7942. Interestingly, the L-AOX was shown to have affinities for Mn, Ca^{2+} and Cl^- (cations as well as anions inhibit the L-AOX activity of this protein) which are quite similar to the affinities which the WOE has for these ions. These and additional results were interpreted to suggest that the WOE could have evolved from an L-amino acid dehydrogenase/oxidase which originally mediated electron flow from L-arginine to the plastoquinone pool of the electron transport chain in these cyanobacteria [3]. It is, however, unclear whether the PS II associated 36 kDa peptide in *Synechococcus* PCC 6301 and PCC 7942 is a modified form (possibly processed) or an immunologically related isoenzyme of the 100 kDa L-AOX (two subunits of 50 kDa). Presently, the latter interpretation seems to be the more likely one. So far, we have not succeeded to isolate the PS II associated 36 kDa peptide with detectable L-AOX activity from PCC 6301 [6]. Therefore, it is uncertain whether this peptide after being incorporated into PS II has a reduced or no L-amino acid dehydrogenase/oxidase activity or possibly a low L-arginine consuming activity leading to different products than those obtained in the L-AOX reaction.

However, if our hypothesis should prove correct, then a comparable polypeptide should be present in all PS II complexes and such a protein should bind manganese, chloride and calcium – presuming that all three inorganic cofactors required for water oxidation [1, 2] are bound to one protein. We thought that successful isolation of such a protein with activity from PS II complexes could possibly be best achieved from the thermophilic cyanobacterium *Synechococcus* sp. which is known to have very stable PS II complexes still having O_2 evolving activity after several purification steps – indicating that the WOE is very stable [7]. Preliminary experiments with the thermophilic *Synechococcus* sp. have already shown that an L-arginine metabolizing enzyme activity (in this paper referred to as L-AME activity – to distinguish this activity from L-AOX activity) is associated with PS II complexes [8, 9]. Moreover, a manganese dependence of the PS II associated L-AME activity could be demonstrated [10].

In this paper we clearly show that this L-AME activity (measured as ornithine production from L-arginie) is tightly associated with highly purified PS II complexes from *Synechococcus* sp. Moreover, it was possible to isolate this PS II associated protein retaining its L-AME activity and to show that the L-AME activity of the isolated protein has a requirement for manganese and chloride – thus proving that this PS II associated protein can indeed bind manganese and chloride (and of course L-arginine).

Materials and Methods

Growth and isolation of thylakoid membranes from Synechococcus sp.

The thermophilic cyanobacterium *Synechococcus* sp. was grown as described in [8]. Thylakoid membranes from *Synechococcus* sp. were obtained according to [11] with slight modifications. 8 l cell suspension (about 25–30 ml packed cells) were harvested by centrifugation (20 min at 3000 × *g*), once washed with 20 mM Hepes-NaOH, pH 7.5, containing 10 mM NaCl, and the resulting pellet was resuspended in 100 ml HM buffer containing 1 mM PMSF (HM: 50 mM Hepes-NaOH, pH 7.5, containing 500 mM mannitol). The cell suspension was treated with lysozyme (1 mg lysozyme/ml cell suspension, 53000 units/mg lysozyme from egg white, Sigma) for 1 h at 35–40 °C in the dark. After centrifugation (12 min at 16000 × *g*) the pellet was resuspended in 75 ml MN buffer containing 1 mM PMSF (MN: 50 mM Mes-NaOH, pH 6.5, containing 10 mM NaCl) and passed twice through a French press at 6000 psi. After addition of 20 µg DNase/ml (2000 units/mg DNase, bovine pancreas, Boehringer) and 5 mM $MgCl_2$, the suspension was centrifuged for 10 min at 12000 × *g* and at 30 °C to remove whole cells. The supernatant was then centrifuged for 45 min at 250000 × *g* (50.2 Ti rotor, Beckman). The resulting pellet containing the thylakoid membranes was suspended in 20 mM Mes-NaOH, pH 6.5, containing 500 mM mannitol, 10 mM NaCl, 1 mM PMSF and 0.04% SB 12 (Serva). The detergent caused removal of substantial amounts of phycobiliproteins from the thylakoid membranes. After centrifugation

(45 min at 250000 × *g*) the membranes were resuspended in the same medium without SB 12 to give a final concentration of 1 mg Chl/ml and frozen at −50 °C for further use.

Extraction and purification of PS II complexes

Extraction and purification of PS II complexes were performed according to the procedure described by Dekker *et al.* [7] with slight modifications. PS II complexes were extracted from thylakoid membranes by addition of 0.35% SB 12 and incubation for 20 min in the dark at room temperature. All subsequent purification steps (except when indicated) were performed either at 4 °C or in ice. After centrifugation (40 min at 140000 × *g*) the resulting supernatant contained the extracted PS II complexes. 2 to 4 ml of this SB 12 extract were applied to a linear sucrose gradient (24 ml) of 10 to 40% sucrose in MCM buffer (MCM: 20 mM Mes-NaOH, pH 6.5, containing 10 mM $CaCl_2$ and 10 mM $MgCl_2$) and centrifuged for 16 h at 100000 × *g* (SW 27 rotor, Beckman). Under those conditions the PS II complexes aggregated in one band in the lower part of the sucrose gradient. In most centrifugations 0.04% SB 12 was added to the gradient. This resulted in additional removal of phycobiliproteins from PS II complexes. Aggregated PS II complexes obtained from the first sucrose gradient centrifugation were dialyzed against MMCM medium (MMCM: 20 mM Mes-NaOH, pH 6.5, 10 mM $MgCl_2$, 10 mM $CaCl_2$, and 500 mM mannitol). After addition of 0.045% DM (Sigma), they were submitted to a second sucrose gradient (18 ml, gradient consisting of 20 to 40% sucrose in MMCM containing 0.03% DM). The sample volume usually was 3 ml. After centrifugation for 20 h at 245000 × *g* (50.2 Ti rotor, Beckman) two bands containing PS II activity were obtained. The PS II complexes from the upper band (Fig. 1) were submitted to chromatography on a Mono Q HR 5/5 column (connected to a FPLC system, Pharmacia), equilibrated with MMCM medium containing 0.03% DM. After applying the sample, the column was washed with MMCM containing 0.03% DM, and PS II complexes were eluted with a linear gradient (30 ml) of 0 to 200 mM $MgSO_4$ in MMCM containing 0.03% DM. This resulted in two fractions (A and B) containing PS II complexes.

Various treatments of PS II complexes (after Mono Q column – fraction B) to remove loosely bound proteins (Experiments of Table II)

Tris treatment: Two parts of PS II complexes were mixed with one part 2.4 M Tris-HCl, pH 8.3. After incubation for 15 min at room temperature, this mixture was diluted 1 to 10 with bidest. water and centrifuged for 5 h at 250000 × *g* (50. 2 Ti rotor, Beckman). The resulting pellet was suspended in MMCM containing 0.045% DM. Sonification: PS II complexes were sonified three times for 10 sec at 40 W with intermissions of 60 sec (Labsonic 1510, Braun). DM treatment: PS II complexes were incubated with 0.1% DM at 50 °C for 10 min. After these treatments intact PS II complexes (although inactive for O_2 evolution) were separated from dissociated proteins by chromatography on a Mono Q column (under conditions as described for purification of PS II complexes). PS II complexes bound to this column at pH 6.5, while the L-AME (when removed from the PS II complex) did not bind. After elution of PS II complexes from the Mono Q column, they were dialyzed against 20 mM Hepes-NaOH, pH 7.5, containing 10 mM NaCl and 200 mM mannitol, and L-AME activity in those PS II complexes was determined.

Activity measurements

Photosynthetic O_2 evolving activity was measured at 40 °C with an O_2 electrode (Clark type electrode, Yellow Springs Instruments). The air-saturated reaction mixture contained in a total volume of 3 ml: 50 mM Mes-NaOH, pH 6.5, 1 M sucrose, 10 mM NaCl, 5 mM $MgCl_2$, 0.5% digitonin, 3 mM potassium ferricyanide, 0.5 mM phenyl-*p*-benzoquinone and the sample containing 30–80 μg chlorophyll (thylakoid membranes) or 2–20 μg chlorophyll (PS II complexes). Illumination was achieved with a halogen lamp (24 V, 250 W, from Spindler and Hoyer, Göttingen). The light was filtered through a red plexiglass filter and a 2% $CuSO_4$ solution (in a 2 cm cuvette).

L-AME activity was determined by incubation of the various samples at 60 °C for 5 h. The reaction mixture contained in a total volume of 3 ml: 33 mM Tricine-NaOH, pH 8.5, 6.7 mM L-arginine-HCl adjusted to pH 8.5, 0.5 mM $MnCl_2$, and the sample (further additions if made are listed in the

legends of the Tables and the Figures). After stopping the reaction by adding 0.1 ml 6 N H_2SO_4 and centrifugation (20 min at 20000 × *g*) the products ornithine and urea (or NH_4^+ after urease addition) were determined in the resulting supernatant.

Ornithine and urea were determined with colour tests according to Ratner [12] and Coulombe and Favreau [13], respectively. In some cases the formed urea was converted to NH_4^+ by addition of urease, and NH_4^+ was determined enzymatically as previously described [8]. For the conversion of the formed urea to NH_4^+, the reaction mixture was cooled to room temperature after the regular incubation for 5 h at 60 °C, and 50 µl urease solution (1 mg urease S from *Canavalia ensiformis*/ml, 45 units/mg, Boehringer) was added. After an incubation for 30 min at room temperature, 0.1 ml 6 N H_2SO_4 was added, and after centrifugation (20 min, 20000 × *g*) NH_4^+ was determined in the resulting supernatant (after adjusting the pH to 7 with 1 N NaOH). Arginine was determined using the method described by Ceriotti and Spandrio [14].

Treatments for "activation" of the L-AME: For some of the enzyme samples (especially when stored frozen for a longer period), an activation procedure was used before the activity test. Two parts of the enzyme sample were mixed with one part 2.4 M Tris-HCl, pH 8.3, containing 30 mM NaCl and 0.09% DM. For some samples preincubation with 0.5 to 1% SB 12 or 0.06 to 0.3% DM was more effective than the Tris treatment. After incubation for 20 min at room temperature, the enzyme was dialyzed against 20 mM Hepes-NaOH, pH 7.5, containing 10 mM or 100 mM NaCl.

Protein was determined either with CBB according to [15] or with bicinchoninic acid according to [16]. Manganese was determined with a plasma mass-spectrometer (Plasmaquad 2, Fisons Instruments). Mn^{3+} was used after solubilization of 53 mg Mn^{3+} acetate in 2 ml 150 mM sodium pyrophosphate. The concentration of Mn^{3+} in the filtered solution was determined according to [17].

SDS PAGE and immunoblots

SDS PAGE was performed according to Laemmli [18] – 10 or 12% SDS polyacrylamide gels were used. The gels were stained with CBB or with silver [19]. Immunoblot experiments were performed as previously described [9]. Protein transfer from the gels to the nitrocellulose matrix was achieved by pressure or by electroblotting [20]. The antisera raised against CP47, MSP, D1 as well as D2 were the same as in [5], and the antiserum raised against the soluble L-AME from *Synechococcus* sp. was the same as in [9]. All antisera were used with a dilution of 1 to 100.

Results

Purification and characterization of PS II complexes from the cyanobacterium Synechococcus sp.

Purification and O_2 evolving activities of PS II complexes

Thylakoid membranes from *Synechococcus* sp. were isolated according to Satoh [11]. From these thylakoid membranes PS II complexes were solubilized by the detergent SB 12 and further purified using basically the procedure described by Dekker *et al.* [7] (details are given in Materials and Methods). This purification procedure included two sucrose gradient centrifugations and chromatography on a Mono Q column. For the first sucrose gradient centrifugation PS II complexes were aggregated by reducing the SB 12 concentration during centrifugation. This resulted in one green band having PS II activity (Fig. 1). These PS II complexes were then dialyzed to remove sucrose and after addition of the detergent DM, the PS II complexes were layered on a second sucrose gradient. The subsequent centrifugation resulted in two green bands having PS II activity (Fig. 1). According to Dekker *et al.* [7], these could be classified as PS II monomers (upper band) and PS II dimers (lower band). The PS II monomers were further purified by chromatography on a Mono Q column. Again the results were similar as in [7]. We obtained one PS II fraction eluting at about 46 mM $MgSO_4$ and having no O_2 evolving activity (containing 1 Mn per 101 Chl – MSP being absent), and a second PS II fraction eluting at 62 mM $MgSO_4$ and having O_2 evolving activity (containing 1 Mn per 20 Chl – MSP being present) (Fig. 1). The O_2 evolving activities determined at the various purification steps are given in Table I. The value of 2938 µmol O_2 evolved/mg Chl × h obtained for purified PS II complexes corresponds quite well to the one obtained in [21] indicating that PS II com-

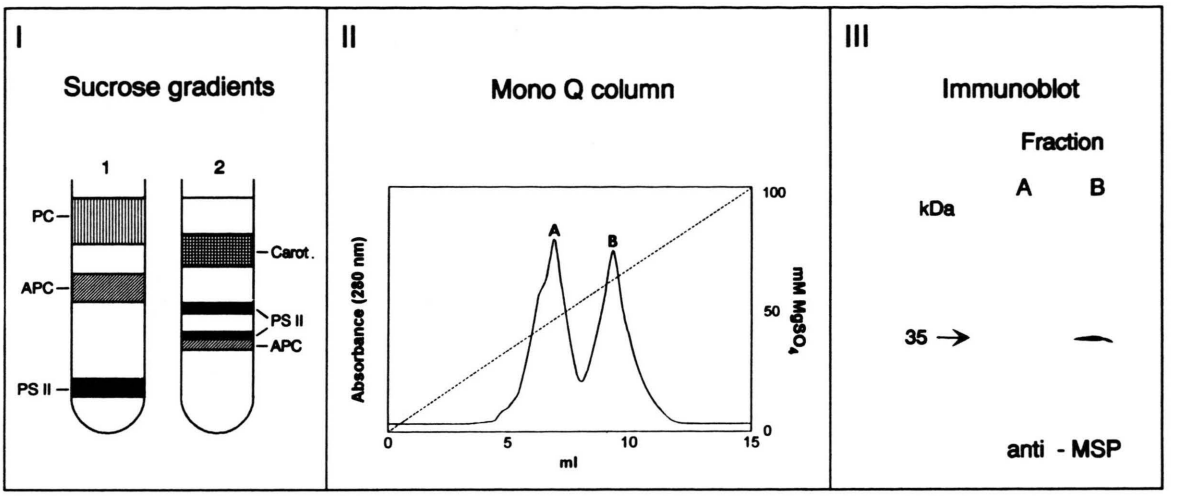

Fig. 1. Protein and pigment pattern obtained after the first and second sucrose gradient centrifugation, the elution profile of PS II complexes from the Mono Q column, and immunoblot with the two PS II fractions obtained after chromatography on Mono Q column. I: Sucrose gradients: The pattern obtained after the sucrose gradients was similar as described in [7]. APC: allophycocyanin, Carot.: carotenoids, PC: phycocyanin. II: Mono Q column: The elution profile was obtained after submitting PS II monomers from the second sucrose gradient centrifugation (upper band) to chromatography on a Mono Q column. Details are given under Materials and Methods. III: The two fractions obtained after Mono Q column chromatography were immunostained with an antiserum raised against MSP. Fraction A (having no oxygen evolving activity) contained no detectable MSP, while fraction B (having oxygen evolving activity) contained MSP.

Table I. Photosynthetic O_2 evolving activities and L-AME activities of PS II complexes from *Synechococcus* sp. at various purification stages. Details of the purification procedure and the activity assays are given under Materials and Methods. The L-AME activities were assayed after dialyzing the samples against 20 mM Hepes-NaOH, pH 7.5, containing 10 mM NaCl and 200 mM mannitol.

Purification steps	Chlorophyll total [mg]	Photosynthetic O_2 evolution [μmol O_2 evolved/ mg Chl × h]	L-AME activity [μmol ornithine formed/ mg Chl × h]
Thylakoid membranes	106	355	0.04
SB 12 extract	24	1245	0.20
1. Sucrose gradient	7.1	1666	0.27
2. Sucrose gradient			
Upper band	0.91	2232	2.73
Lower band	0.40	1798	2.10
Mono Q column:*			
Fraction A	0.45	n.d.**	3.97
Fraktion B	0.27	2938	2.70

* PS II monomers obtained after second sucrose gradient centrifugation (upper band) were applied to a Mono Q column. Chromatography of PS II monomers on a Mono Q column resulted in fraction A: Eluating at about 46 mM $MgSO_4$ and containing 1 Mn per 101 Chl – MSP being absent, and fraction B: Eluating at about 62 mM $MgSO_4$ and containing 1 Mn per 20 Chl – MSP being present.

** n.d. = not detectable.

plexes isolated by us could be considered of high purity.

L-AME activities of PS II complexes

In addition to measuring O_2 evolving activity, L-AME activity (determined as ornithine production from L-arginine in the presence of added $MnCl_2$) was measured (Table I). To standardize our assay conditions, L-AME activity values given in Table I were all obtained after dialyzing samples overnight against 20 mM Hepes-NaOH, pH 7.5, containing 10 mM NaCl and 200 mM mannitol. Under such assay conditions, an approximately 100-fold increase in the L-AME activity on chlorophyll basis was observed, when the activity of purified PS II complexes was compared to that in thylakoid membranes (Table I). However, these L-AME activities can only be taken as an approximation because of substantial difficulties in determining maximum enzymatic activity with a hydrophilic substrate, such as L-arginine, in thylakoid membranes or pigment-protein complexes. When trying to optimize assay conditions, we realized that the L-AME activity greatly varied depending on the aggregation state of PS II complexes as well as depending on sucrose, anion, cation and detergent concentration in the samples. This indicated that the activity is masked in PS II complexes to various extends which is supported by the finding that the detectable L-AME activity on chlorophyll basis is significantly higher in PS II complexes lacking MSP than in PS II complexes still retaining MSP (Table I and Fig. 1).

To obtain further prove that the L-AME really is a component of highly purified PS II complexes and not an impurity copurifiying with PS II, we submitted active PS II complexes (after Mono Q column – fraction B) additional treatments with the goal to remove proteins only loosely bound to the isolated complex (Table II). These treatments included washing of PS II complexes with 0.8 M Tris-HCl, pH 8.3 (a treatment known to remove several PS II polypeptides which are only peripherically bound to PS II complexes – such as *e.g.* MSP), sonification of PS II complexes or addition of dodecyl-β-D-maltoside (DM) in a concentration three times higher than the DM concentration used during purification of PS II complexes. After these treatments, PS II complexes (although inactive for O_2 evolution) were separated from dissociated proteins by chromatography on a Mono Q column at pH 6.5 (for details see Materials and Methods). At pH 6.5, PS II complexes bind to this column, while the L-AME (when dissociated from the complex) does not bind. After elution of PS II complexes from the column and after dialysis under standard conditions, L-AME activity was determined. As results in Table II show, the L-AME is clearly detectable in PS II complexes after these treatments. On chlorophyll basis the L-AME activity was higher in treated than in untreated PS II complexes (especially after DM treatment – L-AME activity approached values of 100 μmol ornithine formed/mg chlorophyll × h). This increase in activity is partly due to the fact that the L-AME became more accessible and partly due to an activation of the L-AME (see later).

Table II. L-AME activity detectable in PS II complexes submitted to various treatments to remove loosely bound proteins from PS II complexes. Purified PS II complexes (after Mono Q column, fraction B) were treated as indicated in the Table (for details see Materials and Methods). After the various treatments PS II complexes were separated from dissociated proteins by chromatography on a Mono Q column. L-AME activity was determined in intact PS II complexes after separation from dissociated proteins.

Treatment of isolated PS II complexes	L-AME activity [μmol ornithine formed/ mg Chl × h]
Untreated	4.0
Washing with 0.8 M Tris-HCl, pH 8.3	12.9
Sonification	40.4
Addition of 0.1% DM	94.3

It might be relevant to mention that PS II complexes after the two gradient centrifugations (Table I) still contained the α-subunit of CF_1-ATPase. This protein was almost removed after Mono Q column chromatography and totally removed after Tris washing of PS II complexes (detection with an antiserum raised against the α-subunit of CF_1 from spinach chloroplasts – results not shown). The α-subunit of CF_1-ATPase has recently been shown to share two highly conserved structural elements with chaperonins [22] – an observation which might explain why a fairly extended purification of PS II complexes (at least in case of cyanobacterial PS II complexes) is required, be-

fore this protein is totally removed. However, successful removal of such a protein from isolated PS II complexes without removing the L-AME is strongly in favor of our conclusion that the L-AME is an integral part of PS II and not just an impurity coincidently purifying with PS II.

Polypeptide composition of PS II complexes

After CBB staining of a SDS polyacrylamide gel the isolated PS II complexes show bands at 52, 50, 45, 42, 35, 31 and 28 kDa. The peptides in the 10 kDa region and below were not examined. In immunoblot experiments (Fig. 2) the 52 and 45 kDa bands crossreacted with the anti-CP47 (probably representing two forms of CP47 associated with different amounts of lipids and/or detergent or one band being CP43). The 35, 31 and 28 kDa bands corresponded to MSP, D2 and D1, respectively. The band pattern in the 40 to 50 kDa region was slightly variable depending on gel and denaturating conditions. L-AME was only poorly stained by CBB, as results with the purified L-AME showed (see later). Therefore, the L-AME protein could not be detected on a CBB stained gel, but identification was possible with an antiserum raised against a previously isolated "soluble L-AME" from the thermophilic *Synechococcus* sp. [9] which also recognizes this PS II associated L-AME (results see Fig. 3).

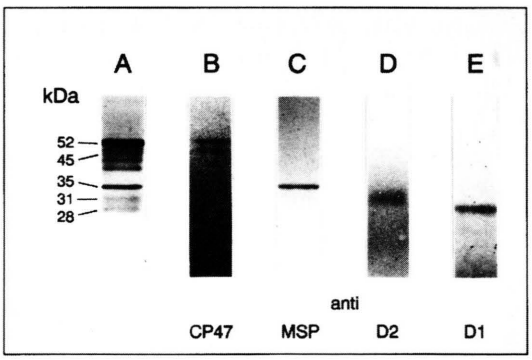

Fig. 2. Identification of polypeptides in PS II complexes from the thermophilic *Synechococcus* sp. A: CBB stained SDS polyacrylamide gel (12% polyacrylamide) of *Synechococcus* sp. PS II complexes obtained after Mono Q column (fraction B containing MSP). B to E represent the corresponding immunoblots which were immunostained with B: anti-CP47, C: anti-MSP, D: anti-D2 and E: anti-D1.

Purification of the L-AME from thylakoid membranes and from PS II complexes of Synechococcus sp.

Isolation of the L-AME from thylakoid membranes

To thylakoid membranes (1 mg Chl/ml – 150 ml total) 3% SB 12 (w/v) were added (thylakoid membranes were isolated as described under Materials and Methods – except that instead of Mes-NaOH, pH 6.5, Hepes-NaOH, pH 7.5, was used). The mixture was stirred for 20 min at room temperature and then centrifuged (40 min at 140000 × *g*). The resulting supernatant was dialyzed against 20 mM Hepes-NaOH, pH 7.5, containing 10 mM NaCl and then 0.05% DM and NaCl to give a final concentration of 0.7 M was added. This mixture was sonified (40 W) three times for 10 sec with intermission periods of 1 min – a procedure which diminished aggregation of the L-AME, thus improving binding of the L-AME to the column. Subsequently, the extract was chromatographed on a metal chelating Sepharose 6B column (Pharmacia, column volume 50 ml). Charging of the column was achieved by passing a solution of 1 mg $MnCl_2$/ml bidest. water through the column (100 ml). After equilibrating the column with 20 mM Hepes-NaOH, pH 7.5, containing 0.7 M NaCl and 0.03% DM (buffer A), the sample was applied. Subsequently the column was washed with buffer A, until all green material was removed from the column (about 5 column volumes). Approximately 50% of the L-AME (on activity basis) was bound to the column. The bound L-AME was eluted from the column with 100 ml buffer A containing 50 mM EDTA, and the fractions containing the L-AME were pooled.

To improve the yield, an increased concentration of DM (0.1% DM) was added to the L-AME which did not bind under the above conditions (in presence of 0.05% DM), and the mixture was incubated for 10 min at 50 °C. Then this mixture was reapplied to a recharged chelating column under the above conditions. This procedure increased the yield substantially. The eluted enzyme showed an approximately 100-fold increase in specific activity (Table III). The total activity regained after the chelating Sepharose column was higher than the total activity in the SB 12 extract. This increase in total activity is due to the presence of DM and

Table III. Purification of the L-AME from thylakoid membranes and from PS II complexes. The L-AME activities were determined after dialysis against 20 mM Hepes-NaOH, pH 7.5, containing 10 mM NaCl. The samples from purification A were in addition incubated for 20 min with 0.8 M Tris-HCl, pH 8.3, containing 10 mM NaCl and 0.03% DM, before dialysis (Details are given under Materials and Methods). 1 unit of enzyme corresponds to 1 μmol ornithine formed/h (at 60 °C).

Purification step	Protein total [mg]	L-AME activity total [units]	L-AME activity specific activity [units/mg protein]
A Purification of the L-AME from thylakoid membranes			
Thylakoid membranes	1600	35.2	0.022
SB 12 extract	496	64.5	0.13
Manganese charged chelating Sepharose column	9.5	104.5	11
Hydroxylapatite: batchwise treatment	0.71	13.5	19
B Purification of the L-AME from PS II complexes			
L-AME solubilized from PS II complexes*	1.31	3.6	2.75
Mono S column	0.017	0.52	30.6

* L-AME solubilized from PS II complexes after the first sucrose gradient centrifugation by addition of the detergent DM.

high concentrations of NaCl in the buffer optimizing the L-AME activity (see below).

A minor additional purification could be achieved by a batchwise hydroxylapatite treatment of the L-AME. The combined active fractions (total volume of 130 ml) from the Mn^{2+} chelating Sepharose column were dialyzed against 5 mM sodium phosphate buffer, pH 7.5 (buffer B). After addition of 0.03% DM, 10 ml hydroxylapatite suspension (equilibrated with buffer B containing 0.03% DM) was added. After centrifugation, hydroxylapatite was washed once with 200 ml buffer B containing 0.03% DM. The bound L-AME was eluted with 20 ml buffer B containing 0.03% DM and 1.2 M NaCl. Only a very small fraction of the L-AME actually bound to hydroxylapatite, and only a minor increase in specific activity was achieved (Table III).

Isolation of the L-AME from PS II complexes

When aggregated PS II complexes (see Table I and Fig. 1) were treated with DM and submitted to the second sucrose gradient centrifugation, part of the L-AME dissociated from PS II complexes and was present in the fraction above the carotenoid band of the second sucrose gradient. This fraction was collected and dialyzed against 20 mM MES-NaOH, pH 6.3, containing 0.2 M mannitol (buffer C). After addition of 0.05% DM, the enzyme solution was submitted to chromatography on a Mono S HR 5/5 column (FPLC system, Pharmacia) equilibrated with buffer C containing 0.05% DM. After washing the column with buffer C containing 0.05% DM, the enzyme was eluted with a linear NaCl gradient (10 ml, 0 to 1 M NaCl in buffer C containing 0.05% DM). Only 20% of the enzyme bound to the column (on activity basis). An approximately 10-fold increase in specific activity was obtained for the enzyme which bound resulting in a specific activity of 31 μmol ornithine formed/mg protein × min (Table III).

Comments on purification of the L-AME

The best purification of the PS II associated L-AME from *Synechococcus* sp. was achieved with a Mn^{2+} charged chelating Sepharose 6B column resulting in an approximately 100-fold increase in specific activity in one step. In a comparative experiment with partially purified L-AME (specific activity of 3) it could be shown that the L-AME bound best when the chelating Sepharose column was charged with Mn^{2+} (being a cofactor of the enzyme – 83% of the L-AME bound to the column), followed by Zn^{2+} (being a strong inhibitor of the

enzyme – 69% of the L-AME bound), while Cu^{2+} as a ligand did only poorly work (24% of the L-AME bound). These results indicate that the L-AME possesses a specific Mn^{2+} (and Zn^{2+}) binding site in a fairly exposed location.

A severe problem throughout the purification was the aggregation of the L-AME with itself and with other proteins. Therefore, addition of detergent at variable concentrations was required. Optimization of the detergent concentration was necessary for every purification step – optimal concentrations were in the range of 1 to 3% for SB 12 and in the range of 0.03 to 0.05% and in extreme cases up to 0.1% for DM. Among the buffers (pH 7.5) used for dialysis before determination of L-AME activity, buffers like Hepes (not binding cations) gave higher activity values than buffers like Tris (binding cations).

Partial characterization of the L-AME

Molecular weight

On a silver stained SDS polyacrylamide gel (L-AME stains poorly with CBB), the purified and highly active L-AME (specific activity of 20 to 30) gave either two bands of 58 and 70 kDa (and sometimes in addition a band of 25 kDa) or only one band of 25 kDa (Fig. 3, A–D). This problem of obtaining changing band patterns with purified L-AME (pattern depended on enzyme sample and previous treatment) was most likely due to aggregation of the L-AME with itself (not prevented by SDS and/or urea) and/or due to an extended interaction of the L-AME with anions or cations as well as detergents (probably interfering with proper SDS binding). We could show that centrifugation of purified L-AME (dialyzed against 20 mM Hepes-NaOH, pH 7.5, containing 10 mM NaCl, to reduce the detergent concentration) for 5 min at $3000 \times g$ resulted in a pellet and supernatant fraction (both having L-AME activity). When applied to a SDS gel, the pellet fraction gave mostly bands of 58 and 70 kDa, while the supernatent fraction gave mostly a band of 25 kDa. These results suggest that the L-AME monomer has an apparent molecular mass of 25 kDa and that the bands in the higher molecular weight region are aggregates. However, it cannot be ruled out that the 25 kDa species contains more lipids and/or detergent than the 58 and 70 kDa species resulting in a greater mobility during SDS PAGE as shown for several membrane proteins [23]. Thus, the molecular weight of the L-AME remains uncertain. All three species were detected by an antiserum raised against a previously isolated L-AME present in the soluble fraction of *Synechococcus* sp. [9] indicating that the PS II associated L-AME (here isolated) and the soluble L-AME [9] are immunologically related. Moreover, the here isolated and PS II associated L-AME is also recognized by an antiserum raised against the 100 kDa L-amino acid oxidase from *Synechococcus* PCC 6301 [4–6]. Since this antiserum recognizes the PS II associated 36 kDa polypeptide of *Synechococcus* PCC 6301 and PCC 7942, it can be concluded that the here isolated L-AME and the 36 kDa polypeptide of PCC 6301 and PCC 7942 are immunologically related.

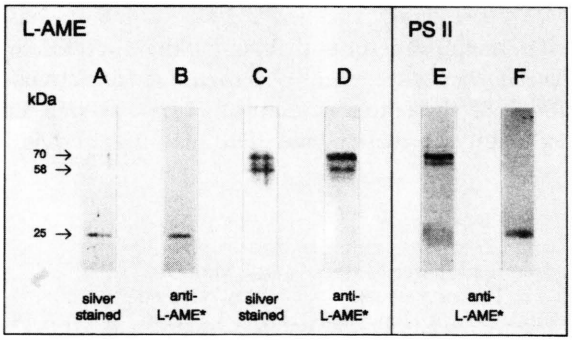

Fig. 3. Silver stained SDS polyacrylamide gel and immunoblot of purified L-AME and purified PS II complexes. A and C: Siver stained SDS polyacrylamide gels with purified L-AME (after Mono S column) form two different purifications. B and D: Corresponding immunoblots with anti-L-AME* [9]. E and F: SDS polyacrylamide gel with purified PS II complexes immunostained with the anti-L-AME* [9]. E: PS II complexes after Mono Q column (fraction B). F: PS II complexes as in E but in addition treated with 0.8 M Tris-HCl, pH 8.3 (as in Table II).

When PS II complexes (after Mono Q column) were investigated with the above mentioned antiserum [9], bands in the 58 to 70 kDa region were detected. Prior treatment of PS II complexes with 0.8 M Tris-HCl, pH 8.3, caused a substantial increase in mobility of the protein during SDS PAGE. Under such conditions one band having an apparent molecular mass of 25 kDa was detected (Fig. 3, E and F). Thus, the L-AME in PS II complexes gives a comparable band pattern as the purified L-AME. We think that this variable mo-

bility of the L-AME in PS II complexes has the same reasons as discussed above for the purified L-AME (variable aggregation, variable binding of anions and cations as well as lipids or detergents in response to different treatments).

Possible organic prosthetic group of the L-AME

The purified L-AME had a broad absorbance band in the visible region of the spectrum with an absorbance maximum at 400 nm (not shown) indicating that the enzyme might contain an organic prosthetic group comparable to the organic prosthetic group being present (besides authentic oxidized flavin) in the L-AOX from *Synechococcus* PCC6301 and PCC7942. The identity of this organic prosthetic group is unknown – it might be a modified flavin. The problems related to the unknown identity of this compound have been discussed previously and will not be repeated here [5, 24]. The purified enzyme did not contain significant amounts of manganese.

Substrate specificity and product formation

The L-AME has a temperature optimum at about 60 °C and is practically inactive at 20 or 80 °C. The pH optimum is in the range of pH 8.5 to 9.0 (not shown). The enzyme metabolizes L-arginine (Km value: 5.6 mM L-arginine) but no other L-amino acid or other guanidino compounds which we tested (such as L-homoarginine, L-argininic acid, L-canavanine or guanidino butyric acid). It is stereospecific for L-arginine, D-arginine is not a substrate. The major products formed by the L-AME are ornithine and urea with a stoichiometry of 1 to 1.5–2.0 indicating that besides ornithine another product (or products) of yet unknown structure(s) was formed from L-arginine. This is supported by the results given in Table IV showing that L-arginine consumption is almost twice as high as ornithine production. Under anaerobic conditions the stoichiometry of ornithine to urea production was 1 to 1.15 indicating that the formation of the additional product(s) besides ornithine and urea was higher under aerobic conditions than under anaerobic conditions. Although the major products formed from L-arginine by this enzyme (ornithine and urea – ratio 1 to 1.5–2.0) are identical to the products formed by arginase (ornithine and urea – ratio 1 to exactly 1), the formation of additional products and the presence of an organic prosthetic group strongly favors a complex reaction mechanism of yet unknown nature.

As shown in Fig. 3, the PS II associated L-AME is immunologically related to a previously isolated soluble L-AME [9] which metabolizes L-arginine to ornithine and NH_4^+ (but not urea) with a somewhat variable stoichiometry (indicating that an additional product(s) is also formed in this reaction). Whether the PS II associated L-AME is a modified form or an immunologically related isoenzyme of the soluble L-AME [9] is presently uncertain.

Manganese and chloride dependence of the L-AME

The membrane-bound form of the L-AME in *Synechococcus* sp. requires manganese for activity (added to the reaction mixture). Mn^{2+} as well as Mn^{3+} activate the enzyme. The maximal activity

Table IV. L-Arginine utilization and product formation by L-AME. L-Arginine utilization as well as ornithine, urea and ammonia production were determined as described under Materials and Methods. The incubation time of the reaction mixture was 16 h instead of 5 h. The enzyme used was highly purified (obtained after hydroxylapatite treatment) and was dialized against 20 mM Hepes-NaOH, pH 7.5, containing 10 mM NaCl, before determining enzyme activity.

Substrate/Product	L-arginine utilization and product formation [μmol]	Ratios of L-arginine utilization to product formation (L-arginine utilization = 1)
L-Arginine utilized	3.64	1.0
Ornithine formed	2.20	0.6
Urea formed	3.35	0.9
NH_4^+ (after addition of urease) formed	6.81	1.9

reached with Mn^{3+} (added as Mn^{3+} pyrophosphate) represents about 80% of that reached with $MnCl_2$. Maximal activities are obtained with about 10 µM $MnCl_2$ or 100 µM Mn^{3+}-pyrophosphate (Table V, A). Besides manganese only $CoCl_2$ caused a minor activation of the L-AME (11% of that reached with $MnCl_2$). Either Co^{2+} could be a substitute for Mn, or Co^{2+} might potentiate the effect of small amounts of manganese still bound to the isolated enzyme.

In addition to manganese, the L-AME requires chloride for activity (Table V, B). Maximal activity of the L-AME was reached with chloride concentrations between 100 and 300 mM chloride. Among the halides, Br^- is as effective or slightly more effective than Cl^-, and I^- is less effective than Cl^-, while F^- does not at all activate the enzyme (Table V, B). Best activation was achieved when the L-AME was dialyzed overnight against 20 mM Hepes-NaOH, pH 7.5, containing the various anions as sodium salts. Among other anions tested, bicarbonate and sulfate sometimes caused activation of the L-AME (although less than Cl^-/Br^-) – depending on the enzyme sample and previous treatment. Phosphate, nitrate and acetate did not increase the L-AME activity (not shown).

Table V. Stimulation of the L-AME activity by manganese and various anions.

A: Stimulation of the L-AME by manganese. The enzyme assays were performed as described under Materials and Methods (ornithine production was determined) with the corresponding changes in manganese addition to the reaction mixtures as indicated in the Figure. Before determining the enzyme activity all samples were dialized against 20 mM Hepes-NaOH, pH 7.5, containing 10 mM NaCl. Mn^{2+} was added as $MnCl_2$. Mn^{3+} was added as Mn^{3+} pyrophosphate (see Materials and Methods).

Manganese concentration present in assay [µM]	Addition of Mn^{2+}	Addition of Mn^{3+}
	Relative L-AME activity (%)*	
0	0	6
0.1	11	9
1	49	11
10	93	42
100	87	80
500	93	79
1000	100	79

* The L-AME activity obtained with 1 mM $MnCl_2$ corresponded to 0.9 µmol ornithine formed/mg protein of partially purified L-AME × h (= 100% activity).

B: Stimulation of the L-AME by various anions. Assays were performed as described under Materials and Methods (ornithine production was determined). Before measuring the L-AME activity the enzyme (in 5 mM Na-phosphate buffer, pH 7.5) was incubated with 1% SB 12. After incubation for 20 min at room temperature, the L-AME was dialyzed over night against 5 mM Hepes-NaOH, pH 7.5, containing 100 mM NaCl, NaBr, NaI or NaF.

Anions added during dialysis of L-AME	Relative L-AME activity (%)
None	6
Cl^-	86
Br^-	100
I^-	44
F^-	6

Inhibition of the L-AME

Among the transient metals tested, the L-AME is mainly inhibited by Zn^{2+}. 50% inhibition was reached with 0.06 mM $ZnCl_2$ (under regular assay conditions). When the $MnCl_2$ concentration in the reaction mixture was reduced from 0.5 mM to 0.05 mM $MnCl_2$, a 50% inhibition was reached at 0.004 mM $ZnCl_2$. Zn^{2+} most likely replaces manganese. Although Zn^{2+} is a strong inhibitor, other transient metals, like *e.g.* Cu^{2+} or Ni^{2+}, only poorly inhibit (1 mM $CuCl_2$ cause 48% inhibition, while 1 mM $NiCl_2$ did not inhibit). L-AME activity is inhibited by the product ornithine (50% inhibition at 4.7 mM L-ornithine), but not by urea or by ammonia (up to 5 mM). Moreover, hydroxylamine (50% inhibition at 7.0 mM hydroxylamine) and hydrogenperoxide (50% inhibition at 0.3 mM H_2O_2) inhibit the L-AME activity.

Discussion

The here presented results show that in highly purified PS II complexes from the cyanobacterium *Synechococcus* sp. an L-AME is present which converts L-arginine to ornithine and urea as major products with a ratio of about 1 to 1.5–2.0. An additional product (or products) of unknown structure(s) is also formed from L-arginine in this reaction. This activity was still associated with PS II complexes even after extensive efforts to remove this protein. On chlorophyll basis the activity in-

creased approximately 100-fold, when L-AME activity in thylakoid membranes was compared to that in purified PS II complexes. The maximal activity which we obtained in purified PS II complexes was 94 μmol ornithine formed/mg Chl × h (after DM treatment of purified PS II complexes) showing that this activity is a significant and not just a minor activity in PS II complexes.

Because of a fairly irregular behaviour of the L-AME during SDS PAGE which is not unusual for integral membrane proteins [23], an exact value for the molecular mass can not be given, but a value of about 25 kDa for the monomer seems to be more likely than a value in the 60 kDa region. The protein stains poorly with CBB. Therefore, detection of this protein as a distinct band on a SDS polyacrylamide gel pattern of PS II complexes was only possible with an antiserum raised against a previously isolated "soluble L-AME" of *Synechococcus* sp. [9] indicating that the membranebound form is either a modified form or an immunologically related isoenzyme of the "soluble L-AME".

The PS II associated L-AME of *Synechococcus* sp. could be isolated retaining its L-AME activity, and it could be shown that the L-AME requires added manganese (Mn^{2+} or Mn^{3+} are effective) for activity. Interestingly, the Mn binding site of this protein (at least one site) must be located in a largely exposed position, since the L-AME could effectively be purified on a manganese charged chelating Sepharose 6B column. The location of Mn at the surface of this protein probably explains the rapid loss of Mn during purification. In addition to Mn, the enzyme requires Cl^- for activity. When halides were compared, the results show that Br^- was as effective (or sometimes slightly more effective) as Cl^-, while I^- was less effective than Cl^-, and F^- was ineffective. Among other anions tested, bicarbonate and sulfate sometimes led to an increase of the L-AME activity, while phosphate, nitrate and acetate were totally ineffective.

The results of this paper show that we were able to isolate a PS II associated protein which can bind manganese and chloride. Since the WOE also requires Mn and Cl^- for activity [1, 2] and since it seems rather unlikely that two Mn and Cl^- binding proteins are present in such highly purified PS II complexes, we would like to suggest that this PS II associated protein which can be detected by its L-AME activity, might be the long searched for WOE – at least it seems likely that this protein contributes ligands to the Mn cluster which is possibly located at the interface of two or more polypeptides of PS II (D1, MSP and the described L-AME). The WOE also requires Ca^{2+} as a cofactor. Unfortunately, no statement can be made about Ca^{2+} binding of the L-AME, since Ca^{2+} does not influence the L-AME activity (up to a concentration of 5 mM $CaCl_2$). This does not necessarily mean that Ca^{2+} does not bind to the protein. However, another striking similarity could be shown to exist between the WOE and L-AME. The dependence of the L-AME activity on halides (Table V, B) is very similar to the effect which these anions have on the WOE activity (Cl^-, $Br^- > I^-$ activate, while F^- does not) [25, 26]. The influence of bicarbonate, sulfate, or nitrate on the WOE activity is somewhat contradictory [25–29]. Phosphate and acetate do not activate the WOE [25, 26] nor the L-AME. The L-AME is inhibited by Zn^{2+}, and Zn^{2+} inhibition has also been reported for the WOE [30]. This is not unusual, since manganese requiring enzymes are commonly inhibited by Zn^{2+}.

The PS II associated L-AME has a rather low turn-over number of about 20 for the here described activity (mol L-arginine metabolized/mol L-AME (25 kDa monomer) × min – approximation due to the uncertainty related to the molecular weight of the L-AME). Nevertheless this L-AME activity provides an efficient assay for detection of this manganese and chloride binding protein in PS II complexes. Whether the measurable L-AME activity of this PS II associated protein has any physiological significance or is just a cryptic activity based on an L-arginine binding capacity of this protein and being related to the suggested ancient L-arginine dehydrogenase/oxidase activity, can not be answered presently. However, having now optimized the assay conditions for this PS II associated protein, we should be able to detect such a protein in PS II complexes from other cyanobacteria as well as from plants – presumed our hypothesis suggesting that the protein is the manganese binding protein in PS II, is correct. Preliminary results have already indicated that such an activity can indeed be detected in thylakoid membranes or PS II complexes isolated from *Synecho-*

coccus PCC6301 and PCC7942 and from *Synechocystis* PCC6803 as well as from plants (A. Engels, A. E. Gau, and K.-P. Michel: unpublished results).

In conclusion it can be said that highly purified PS II complexes from the thermophilic cyanobacterium *Synechococcus* sp. contain a polypeptide in addition to the seven so far recognized major polypeptides in minimal O_2 evolving PS II complexes. This protein can be efficiently detected by its L-AME activity. Based on the extended efforts which we made to remove this protein from PS II complexes, it can be concluded that it is an integral part of PS II and not a copurifying impurity. Since this polypeptide binds manganese and chloride, the possibility should be taken into consideration that this protein might be the long searched for WOE and that the L-AME activity is due to an ancient activity of this protein.

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