Isolation and Partial Characterization of a Manganese Requiring L-Arginine Metabolizing Enzyme Being Present in Photosystem II Complexes of Spinach and Tobacco

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A low L-arginine metabolizing enzyme (L-AME) activity leading to ornithine, urea and additional products not identified so far could be detected in photosystem II (PS II) membranes of spinach and of the chlorophyll deficient tobacco mutant *Su/su*. The detectable L-AME activity was very low in untreated PS II membranes, but increased significantly (about 10 fold) when the extrinsic peptides (*psb*O, P and Q gene products) were removed – suggesting that the L-AME is exposed at the lumen side of PS II. It was possible to isolate the detergent-solubilized protein from $CaCl_2$-washed PS II membranes of spinach by a combination of anion and cation exchange columns. On the basis of SDS PAGE the protein was homogenous and had an apparent molecular mass of 7 kDa. N-terminal sequencing of the polypeptide gave a contiguous sequence of 20 amino acids showing no homologies to PS II polypeptides as yet sequenced. After chromatography of the L-AME on an anion exchange column at pH 9.5 (last purification step) a completely inactive enzyme was obtained. Maximal reactivation was achieved by dialyzing the protein against Hepes-NaOH buffer in the pH range of 6.5 to 7.5 containing 100 mM chloride or sulfate (being the most effective anions). The L-AME activity was totally dependent on manganese added to the reaction mixture. Moreover, there were indications of a second cation binding site being more sequestered and requiring bound Ca^{2+} or Mn^{2+} for activity (Sr^{2+} was less effective and Mg^{2+} was ineffective). There are indications that the protein contains a redox active group – possibly an amino-acid-derived quinonoid (based on a redox cycling assay with glycine and nitroblue tetrazolium). The capability of this PS II associated protein to bind the cofactors of water oxidation and having a redox active group (preliminary results) suggests that this protein might be functional in photosynthetic water oxidation. This is further supported by the fact that the isolated L-AME has a low catalase activity.

Introduction

The overall reaction catalyzed by the thylakoid embedded PS II complex is the oxidation of water and the reduction of plastoquinone with O_2 released as by-product. The process of photochemical charge separation is fairly well understood, while the process of water oxidation requiring Mn, Ca^{2+} and Cl^- has remained uncertain in several aspects. Although substantial efforts have been made, the exact site of Mn, Ca^{2+} and Cl^- binding in PS II is unknown (see recent reviews: Debus, 1992; Rutherford *et al.*, 1992; Boussac and Rutherford, 1994). This is mainly due to the fact that Mn (Ca^{2+} and Cl^-) is rapidly lost when PS II complexes are totally dissociated. Therefore, identification of the Mn binding site by isolating a Mn-retaining polypeptide proved impossible. Most groups working in this field favour the reaction center peptide D1 in connection with D2 and/or other known PS II peptides, such as CP47, as the site of water oxidation (see recent reviews: Pakrasi

Abbreviations: L-AME, L-arginine metabolizing enzyme; Ches, 2-(N-cyclohexylamine)-ethanesulfonic acid; Chl, chlorophyll; DM, dodecyl-β-D-maltoside; Hepes, N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid; LHCP, light harvesting chlorophyll *a/b* protein complex; MSP, manganese stabilizing protein; PQQ, pyrroloquinoline quinone; PS II, photosystem II; SB 12, sulfobetaine 12 (N-dodecyl-N,N-dimethyl-ammonio-3-propanesulfonate; TPQ, topaquinone; Tricine, N-tris(hydroxymethyl)methylglycine; Tris, tris(hydroxymethyl)aminomethan; TTQ, tryptophan-tryptophan quinone; WOE, water oxidizing enzyme.

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and Vermaas, 1992; Vermaas, 1993). Their model is mainly based on results obtained by site directed mutagenesis (Diner *et al.*, 1991). It implies that a separate Mn, Ca^{2+} and Cl^- binding protein(s) is not present in PS II and that Mn is bound at the interface of D1 and one or two other known PS II peptides. In contrast, our PS II model suggests that the major (although not necessarily only) manganese binding protein in PS II is indeed a separate and not yet recognized polypeptide in PS II. Our initial idea was that the water oxidizing enzyme (WOE) might have evolved from a substrate dehydrogenase type enzyme (Pistorius, 1993). If this were the case, then this protein might possibly have retained a cryptic Mn-requiring activity being measurable independently from its physiological water oxidizing activity in the light. If such an activity were detectable, it could be used to isolate the protein from PS II.

With this strategy we successfully detected and isolated an L-arginine metabolizing enzyme (L-AME) which is present in highly purified PS II complexes from the thermophilic cyanobacterium *Synechococcus* sp. and which catalyzes with a very low rate the conversion of L-arginine to ornithine, urea, and additional not yet identified products. Moreover, we could show that this L-AME required added Mn (Mn^{2+} or Mn^{3+}) and Cl^- for its activity implying that the protein can indeed bind Mn as well as Cl^- and thus might be a possible candidate for the WOE (Ruff and Pistorius, 1994).

To obtain further support for our hypothesis suggesting the presence of a separate major Mn binding protein in PS II, it is essential to also identify a corresponding L-AME activity in PS II complexes of higher plants, where photosynthetic and respiratory electron transport are present in separate compartments. Here we report on the identification of such an L-AME activity in PS II complexes from spinach and tobacco as well as on the isolation, partial characterization and partial sequencing of this protein isolated from PS II complexes of spinach.

Materials and Methods

Spinach was purchased from local markets and the tobacco mutant *Su/su* which has a reduced light harvesting chlorophyll *a/b* protein complex (LHCP), was grown in the green house and is the same as previously described (Specht *et al.*, 1987). Isolation of PS II membranes, chlorophyll determination, measurements of photosynthetic O_2 evolution, and washing of PS II membranes with NaCl or $CaCl_2$ were performed according to Specht *et al.* (1987 and 1990). Tris washing of PS II membranes was done by mixing two parts of PS II membranes with one part of 2.4 M Tris-HCl, pH 8.3, or 2.4 M Tris-acetate, pH 8.3. After incubation for 30 min at 4 °C, this mixture was centrifuged at 50 000 x g for 30 min, and the resulting pellet was resuspended in 20 mM Hepes-NaOH, pH 6.5, containing 15 mM NaCl, 5 mM $MgCl_2$ and 400 mM sucrose. PS II core complexes and reaction center complexes were isolated according to Haag *et al.* (1990) and McTavish *et al.* (1988), respectively.

L-AME activity measurements: L-AME activity was determined by incubation of the various samples at 60 °C for 5 h (or for shorter times when L-AME activity was high). The reaction mixture contained in a total volume of 3 ml: 33 mM Tricine-NaOH, pH 8.5, 10 mM L-arginine-HCl, 16.6 mM NaOH (for adjustment to pH 8.5), 0.5 mM $MnCl_2$, and the sample. After incubation for 5 h, the reaction was stopped by adding 0.1 ml 3 M H_2SO_4 and centrifuged for 10 min at 10 000×*g*. In the resulting supernatant the products ornithine (Ratner, 1962) and urea (Coulombe and Favreau, 1963) were determined by colour tests. Variations from this standard assay procedure are given in the legends to the Tables and Figures.

Mn^{3+} was used after solubilization of 53 mg $Mn(acetate)_3$ in 2 ml 150 mM sodium pyrophosphate. The concentration of Mn^{3+} in the filtered solution was determined according to Kenten and Mann (1955).

For determinating catalase activity, the L-AME was dialyzed overnight against 10 mM Hepes-NaOH, pH 7.5, containing 100 mM NaCl, 1 mM $MnCl_2$ and 0.02% DM. Subsequently the catalase activity of the L-AME was measured in a Clark-type electrode (Rank Brothers) at 37 °C. The reaction mixture contained in a total volume of 3 ml: 50 mM Hepes-NaOH, pH 6.5, 240 mM H_2O_2, 0.5 mM $MnCl_2$ (when indicated) and L-AME (4.2 µg protein).

Protein was determined with bicinchoninic acid according to Smith *et al.* (1985). The protein determinations of PS II preparations were performed

after trichloroacetic acid treatment. SDS PAGE was performed according to Schägger and von Jagow (1987) and silver staining according to Heukeshoven and Dernick (1988). For SDS PAGE the protein samples were denatured in buffer as described by Heukeshoven and Dernick (1988) at 100 °C for 5 min.

The redox cycling assay for detection of quinonoid groups (decarboxylation of glycine is coupled to reduction of nitroblue tetrazolium to its formazan) was basically performed as described by Paz *et al.* (1988) with one modification: Reduced BSA was omitted. The reaction mixture contained in a total volume of 1.1 ml: 2 M glycine-NaOH, pH 10.0, 0.24 mM *p*-nitroblue tetrazolium and the L-AME. After incubation for 1 h at 60 °C formazan formation was determined as absorbance at 530 nm. For detection of possible non-covalently bound PQQ the enzymatic assay according to Geiger and Görisch (1987) was used. 2,4-dinitrophenylhydrazine treatment of the L-AME was performed according to van der Meer *et al.* (1986 and 1987).

For amino acid sequencing, the L-AME was concentrated in a Centricon 3 tube (Amicon) at 4 °C (coated with 0.1% Tween 20 – Pierce, sequencing grade). Subsequently 0.2 nmoles of the protein were sequenced on a pulsed liquid phase sequenator Model 477 A with online HPLC system 120A (Applied Biosystems) as described in Bökenkamp *et al.* (1994).

Results

Detection of an L-AME activity in PS II membranes from spinach and from tobacco

PS II membranes (BBY complexes – Berthold *et al.*, 1981) from spinach and tobacco were isolated as described by Specht *et al.* (1987), and L-AME activity was quantified by measuring ornithine production from L-arginine in the presence of added $MnCl_2$ as described under Materials and Methods. In Table I the L-AME activity detectable in PS II membranes from spinach and from tobacco (the tobacco mutant *Su/su* having a reduced LHCP complex was used) is given. The L-AME activity in most PS II preparations (BBY complexes) is very low and sometimes hardly detectable but increases substantially when PS II membranes were washed with NaCl to remove the two smaller extrinsic peptides and further

Table I. Photosynthetic O_2 evolving and L-AME activities of PS II membranes from spinach and from the chlorophyll deficient tobacco mutant *Su/su*. L-AME activity was determined in PS II membranes before and after removing the extrinsic peptides by various wash procedures. For spinach two representative experiments (marked a and b) are given to document the variable activity in untreated PS II membranes.

Treatment	Spinach			Tobacco mutant *Su/su*	
	Photosynthetic O_2 evolution [μmol O_2 evolved/mg Chl×h]	L-AME activity [μmol ornithine formed/mg Chl×h]		Photosynthetic O_2 evolution [μmol O_2 evolved/mg Chl×h]	L-AME activity [μmol ornithine formed/mg Chl×h]
PS II membranes (BBY)	336	0.15[a]	0.69[b]	446	0.33
PS II membranes after washing with 1.5 M NaCl	252	0.95[a]	1.22[b]	413	1.46
PS II membranes after washing with 1.5 M $CaCl_2$	34	2.76[a]	1.51[b]	n.d.	3.04
PS II membranes after washing with 0.8 M Tris-HCl, pH 8.3				n.d.	4.33
PS II membranes after washing with 0.8 M Tris-acetate, pH 8.3				n.d.	2.94

n.d., not determined.

increased when all three extrinsic peptides (*psb*O, P and Q gene products) were removed by $CaCl_2$ washing. This increase in activity by removing the extrinsic peptides was most pronounced, when the activity in untreated PS II membranes was low due to tight binding of the extrinsic peptides (compare the two experiments given for spinach PS II membranes in Table I). To make sure that the increase in detectable L-AME activity is truely due to removal of the extrinsic peptides and not due to a chloride or calcium effect on the L-AME activity (see below) the extrinsic peptides were removed by washing with 0.8 M Tris-HCl, pH 8.3, or Tris-acetate, pH 8.3, instead by washing with $CaCl_2$. Both Tris treatments also gave the expected substantial increase in detectable L-AME activity. Therefore, it can tentatively be concluded that the L-AME becomes better accessible for the hydrophilic substrate L-arginine in the absence of the three extrinsic polypeptides – suggesting that the L-AME protein is exposed at the lumen side of PS II.

To obtain further proof that the L-AME truly is a constituent of PS II complexes, O_2 evolving PS II core complexes from spinach were prepared according to the procedure described by Haag *et al.* (1990). These core complexes (having an O_2 evolving activity of 1040 μmol O_2 evolved/mg Chl×h) had an L-AME activity of 3.6 μmol ornithine formed/mg Chl×h (or 0.38 μmol ornithine formed/mg protein×h). On chlorophyll basis this value is about twice as high as the corresponding average value obtained with spinach PS II membranes (BBY). Moreover, it was possible to show that PS II reaction center complexes from spinach isolated according to McTavish *et al.* (1989) also possessed L-AME activity (0.25 μmol ornithine formed/mg protein×h) – suggesting that the L-AME protein is tightly associated with the D1/D2 heterodimer.

Optimization of the assay conditions for detecting the L-AME activity in PS II membranes

Initially we had substantial difficulties to detect L-AME activity in PS II membranes from plants in a reproducible manner. Therefore, substantial efforts were made to optimize the assay conditions. These experiments were performed with $CaCl_2$ washed PS II membranes from the tobacco mutant *Su/su* having a reduced LHCP, and the results are presented in Figs 1 and 2. As the results in Fig. 1 clearly show, the L-AME activity is hardly detectable when the reaction proceeds at room temperature but becomes detectable, when the temperature was raised above 50 °C – suggesting that the L-AME activity in plants has no physiological relevance, since only being detectable at elevated temperatures. The temperature optimum

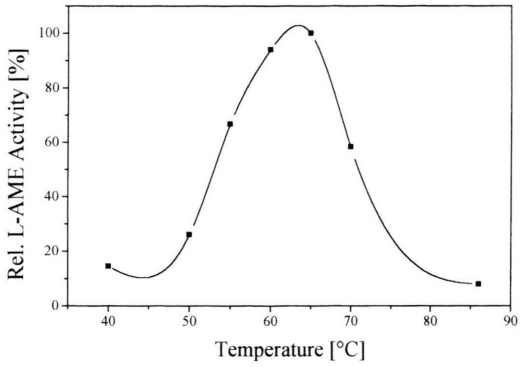

Fig. 1. L-AME activity of $CaCl_2$ washed PS II membranes (tobacco) as a function of temperature. The reaction proceeded for 5 h at the temperatures given in the figure. All other conditions were the same as given under Materials and Methods. At 60 °C the L-AME activity corresponded to 1.3 μmol ornithine formed/mg Chl×h ($CaCl_2$ washed tobacco PS II membranes were used: 126 μg Chl were present in the reaction mixture).

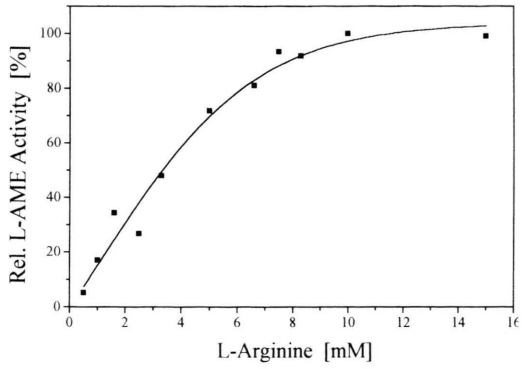

Fig. 2. L-AME activity of $CaCl_2$ PS II membranes (tobacco) as a function of L-arginine concentration. The reaction proceeded for 5 h at the L-arginine concentrations given in the figure. All other conditions were the same as given under Materials and Methods. In the presence of 10 mM L-arginine the L-AME activity corresponded to 1.2 μmol ornithine formed/mg Ch×h ($CaCl_2$ washed tobacco PS II membranes were used: 91 μg Chl were present in the reaction mixture).

for this reaction is in the range of 60 to 65 °C. The enzyme activity starts to decrease above 65 °C and was about zero at 80 °C. Because of this rather peculiar but very pronounced temperature effect we chose for regular assay conditions a temperature of 60 °C. The enzyme is very stable at this temperature, and the L-AME activity is linear with time for more than 20 h (not shown). The significant effect of elevated temperature on L-AME activity of this protein is most likely due to a conformational change improving the capability of this protein to bind the hydrophilic substrate L-arginine.

In Fig. 2 the dependence of the L-AME activity on L-arginine concentration is given. The enzyme shows a proper Michaelis-Menten behaviour giving a hyperbolic saturation curve with the substrate L-arginine (K_M value is 4 mM L-arginine) – supporting the conclusion that the activity which we measure is indeed an enzyme catalyzed reaction. D-arginine is not a substrate. The detectable L-AME activity of PS II membranes is very low without addition of manganese to the assay mixture. $MnCl_2$ as well as Mn^{3+} pyrophosphate (concentrations between 1 to 10^{-4} M are optimal – not shown but see Fig. 6). As the results of Fig. 1 show, significant L-AME activity can only be detected at temperatures between 50 and 75 °C. Under such conditions the PS II associated Mn would dissociate and become diluted into the reaction mixture. Thus, for L-AME activity measurements, Mn addition to the reaction mixture is required.

Purification of the L-AME from PS II membranes of spinach

For the purification of the L-AME, $CaCl_2$ washed spinach PS II membranes were used as starting material. The majority of the L-AME protein remains associated with PS II membranes after $CaCl_2$ washing, and only minor amounts are found in the wash solution. For solubilization of the PS II associated L-AME, $CaCl_2$ washed PS II membranes corresponding to 300 mg Chl were suspended in 20 mM Hepes-NaOH, pH 6.5, containing 15 mM NaCl, 5 mM $MgCl_2$, 400 mM sucrose, to give a Chl concentration of 1.0 mg Chl/ml. To this suspension 1% (w/v) of the ionic detergent sulfobetaine 12 (SB 12) was added. After incubation for 1 h with stirring at 4 °C, the suspension was dialyzed overnight against 10 mM potassium phosphate buffer, pH 7 (3 changes of buffer), to remove the ionic detergent which in great excess would disturb chromatography on ion exchange columns. After dialysis 0.02% of the nonionic detergent dodecyl-β-D-maltoside (DM) was added to keep the hydrophobic proteins in solution. For removal of the undissolved proteins, the solution was centrifuged for 15 min at 50,000×g.

Purification of the L-AME was achieved by a combination of three anion exchange columns (L-AME only binds at pH values above 9) and two cation ion exchange columns (L-AME binds well at pH 6.5) used in alternating order. After centrifugation, the above supernatent fraction was applied to a DEAE Sepharose A 50 column (size: 3×40 cm, equilibrated with 10 mM potassium phosphate buffer, pH 7, containing 0.02% DM). At pH 7 a substantial amount of the solubilized proteins bound to the column, while the L-AME did not bind or only poorly bound and was mainly in the initial eluate. To increase recovery, the column was washed with 10 mM potassium phosphate, pH 7, containing 300 mM NaCl and 0.02% DM. The initial eluate and this eluate were combined and then dialyzed against 10 mM Hepes-NaOH, pH 6.5. After adding 0.02% DM, the sample was submitted to chromatography on a SP-Sephadex C50 column (size: 3×30 cm, equilibrated with 10 mM Hepes-NaOH, pH 6.5, containing 0.02% DM). After applying the sample and washing the column with the same buffer, the L-AME was eluated with a linear NaCl gradient (0 to 500 mM NaCl in the above buffer, volume 300 ml). At a NaCl concentration of about 300 mM the main L-AME fractions were eluated in a sharp peak. The fractions with L-AME activity were combined and dialyzed against 20 mM potassium phosphate, pH 7.0.

After adding 0.02% DM, the above sample was applied to a second anion exchange column: DEAE Sepharose CL 6B column (size: 2×10 cm, equilibrated with 20 mM potassium phosphate buffer, pH 6.5, containing 0.02% DM). As above, since the L-AME did not bind to this column under the chosen conditions and was present in the initial eluate, the purpose of this column was removal of impurities which bound. Subsequently, the L-AME sample was dialyzed against 10 mM Hepes-NaOH, pH 6.5. After adding 0.02% DM,

the sample was applied to a second SP-Sephadex C50 column (size: 1×15 cm, equilibrated with 10 mM Hepes-NaOH, pH 6.5, containing 0.02% DM). After washing with 10 mM Hepes-NaOH, pH 6.5, containing 0.02% DM, the L-AME was eluated with 10 mM Hepes-NaOH, pH 6.5, containing 500 mM NaCl and 0.02% DM. The fractions containing L-AME activity were dialyzed against 10 mM Ches-NaOH, pH 9.5, containing 0.02% DM, and subsequently applied to a RESOURCE Q column of 1 ml coupled to a FPLC system (Pharmacia – several runs with about 100 µg protein) as the final purification step. The L-AME was eluated with two linear NaCl gradients (first gradient: 0 to 100 mM NaCl in the above buffer, volume 60 ml, and second gradient 100 to 1000 mM NaCl in the above buffer, volume 20 ml). At 20 mM NaCl an L-AME fraction was eluated containing no impurities detectable by SDS PAGE (see Fig. 3) and at 350 mM NaCl an L-AME fraction was eluated which was aggregated with additional proteins (not shown). For further use the L-AME fractions eluated at 20 mM NaCl were collected and concentrated by dialysis against 10 mM Ches-NaOH, pH 9.5, containing 0.02% DM and 40% polyethylene glycol 20,000. The results of such a purification are given in Table II.

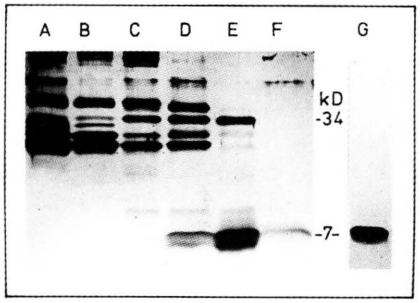

Fig. 3. SDS polyacrylamide gel pattern of protein samples after the various purification steps of the L-AME. Protein samples (A to F contained 2 µg protein and G contained 20 µg protein) from the various purification steps were submitted to SDS PAGE (14% SDS polyacrylamide gel in a Tris-Tricine buffer system according to Schägger and von Jagow, 1987) and subsequently silver stained. A: SB 12 extract, B: protein sample after DEAE Sephadex A50 column, C: after first SP-Sephadex column C50, D: after DEAE Sepharose CL 6B column, E: after second SP-Sephadex C50 column, F: purified L-AME protein after RESOURCE Q column, and G: same sample as F, but 10 times more protein. For the purified L-AME two runs are given with a 10 fold difference in protein content to demonstrate that the isolated protein is homogenous on the basis of SDS PAGE and to show which of the small peptides enriched during purification corresponds to the L-AME. Occasionally an aggregate or artefact band could be obtained in the upper part of a gel (see F: upper band).

Comments on purification

In the previous paper on the purification of the L-AME from PS II complexes of the thermophilic cyanobacterium *Synechococcus* sp. we reported on the use of a Mn^{2+} charged chelating Sepharose column (Ruff and Pistorius, 1994). For reasons unknown such a column could not be used successfully for purifying the L-AME from spinach PS II complexes. Therefore, it was necessary to develop a new purification scheme. Since the L-AME protein is hydrophobic, binds cations as well as anions (see below) and has a tendency to strongly aggregate with other proteins, purification proved to be extremely difficult. Another problem was that the L-AME became inactivated to various degrees during the chromatographic procedures. The most significant inactivation occurred when conditions were chosen in such a way that the L-AME would bind to an anion exchange column (pH 9.5) – as in the last purification step. Fortunately, this inacti-

Table II. Purification of the L-AME from $CaCl_2$ washed PS II membranes of spinach. L-AME activity in the samples obtained after the various purification steps was determined as described under Materials and Methods. For better comparison, all samples were dialyzed against 10 mM Tricine-NaOH, pH 8.5. Since the L-AME obtained after chromatography on a RESOURCE Q column at pH 9.5 was inactive, the L-AME was reactivated by dialysis against 10 mM Hepes-NaOH, pH 6.5, containing 100 mM NaCl and 0.02% DM. The L-AME activity value obtained after this treatment is given in parenthesis. 1 unit of enzyme corresponds to 1 µmol ornithine formed/h (at 60 °C) under standard assay conditions as given under Materials and Methods.

Purification step	Protein	L-AME activity	
	Total [mg]	Total [units]	Specific activity [units/mg protein]
$CaCl_2$ washed PS II membranes	536	150	0.28
SB 12 extract	759	167	0.22
DEAE-Sephadex A 50 column	312	659	2.11
SP-Sephadex C 50 column I	21	46	2.19
DEAE-Sepharose CL 6 B column	4.2	92	21.9
SP-Sephadex C 50 column II	2.5	21	8.4
RESOURCE Q column	0.42	0 (44)	0 (104)

vation could be reversed by dialyzing the inactive protein overnight against 10 mM Hepes-NaOH, in the pH range of 6.5 to 7.5, containing 100 mM NaCl and 0.02% DM (see below). Since the L-AME activity determined throughout the purification strongly depended on the treatment of the sample (other proteins, detergent, ions present and buffer used), the specific activities in Table II give only a rough approximation. Because of the small yield we have not tried to optimize the assay conditions for every purification step – except for the last purification step in which a totally inactive L-AME protein was obtained.

Apparent molecular weight

The L-AME protein was poorly stained by Coomassie Brilliant Blue, but could be well detected by silver staining (Heukeshoven and Dernick, 1988). The protein shows a rather problematic behaviour on SDS polyacrylamide gels. In a 10 to 12% SDS polyacrylamide gel the protein migrates faster than the tracking dye bromophenol blue, while it does not migrate into the gel when the polyacrylamide concentration was increased above 17% (using the Laemmli system – Laemmli, 1970). Best results were obtained using a 14% SDS polyacrylamide gel and Tris-Tricine buffer system according to the procedure described by Schägger and von Jagow (1987). Using this SDS PAGE system, it could be demonstrated that during purification a protein was enriched running in the lower part of such a gel (Fig. 3). On this basis the protein seems to be homogenous and has a molecular mass of approximately 7 kDa. When the protein (after RESOURCE Q column) was chromatographed on a Superose 12 column (Pharmacia), an apparent molecular mass of 20 to 35 kDa was obtained (see discussion).

N-terminal sequence of the L-AME

For further characterization the L-AME protein was submitted to N-terminal sequencing. It was possible to obtain a contiguous sequence of 20 amino acids which is given below: Ala–Glu–Ala–Gly–Thr–Ser–Asp–Asn–Arg–Gly–Leu–Ala–Leu–Leu–Leu–Pro–Ile–Ile–Pro–Ala–. Since no subsequence was obtained, it is highly likely that the purified L-AME was homogenous. This is in agreement with the results obtained by SDS-PAGE. Comparison of the N-terminal L-AME sequence to other so far sequenced PS II polypeptides revealed no homology. Thus, it can be concluded that this protein is an additional, so far unidentified PS II associated peptide (de Vitry *et al.*, 1991;Pakrasi and Vermaas, 1992). Moreover, since no significant homology was detectable to chloroplast DNA (Shinozaki *et al.*, 1986), it can further be concluded that the protein is nuclear encoded.

Absorbance spectrum of the L-AME

In Fig. 4 the absorbance spectrum of the purified L-AME is given. The L-AME exhibits an absorbance band in the ultraviolet, centered at 280 nm – either as a well defined peak (L-AME

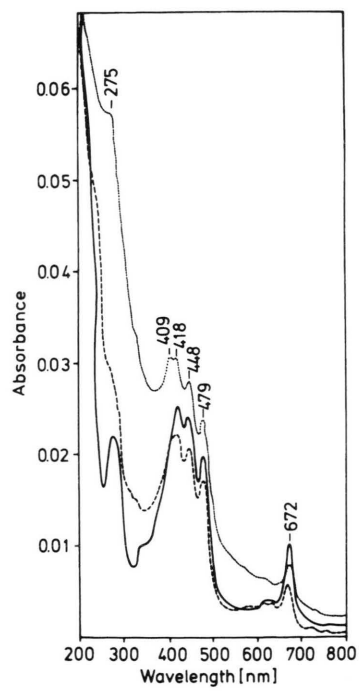

Fig. 4. Absorbance spectrum of the isolated L-AME. Before recording the absorbance spectrum of the isolated L-AME (after RESOURCE Q column – containing 7.8 µg protein), the enzyme was dialyzed against 10 mM Ches-NaOH, pH 9.5, containing 20 mM NaCl (——) or against 10 mM Ches-NaOH, pH 9.5, containing 100 mM NaCl (– – –) or against 10 mM Hepes-NaOH, pH 6.5, containing 100 mM NaCl (·····). All buffers contained 0.02% DM.

in 10 mM Ches-NaOH, pH 9.5, containing 0.02% DM) or as a shoulder (L-AME in 10 mM Hepes-NaOH, pH 6.5, containing 0.02% DM). In the visible region the L-AME shows a broad absorbance band in the 400 to 500 nm region with maxima at 409, 418, 448 and 479 nm and a small absorbance band centered at 672 nm. The absorbance is greatly variable depending on the buffer and the corresponding preparation resulting in quantitative as well as qualitative changes. The protein was fluorescent showing a fluorescence band in the range of 650 to 750 nm when excited at 405 nm (not shown).

Detection of a redox active group

While working with this protein, there were indications that the protein might contain a redox active component – possibly an amino-acid-derived quinonoid (see recent reviews: Duine, 1991; Klinman and Mu, 1994; McIntire, 1994). Support of such a possibility came from a redox cycling assay in which decarboxylation of glycine is coupled to reduction of nitroblue tetrazolium to its formazan (Paz *et al.*, 1988). Under the reaction conditions as given under Materials and Methods 2.1 μg or 4.2 μg purified L-AME gave an absorbance change at 530 nm (formazan formation) of 0.24 or 0.40, respectively – indicating that such an *o*-quinone group (PQQ, TPQ or TTQ) could be present in the here isolated protein. Since the bioassay for detection of noncovalently bound PQQ (Geiger and Görisch, 1987) was negative (not shown), the component in the protein giving a positive result in the redox cycling assay must be a covalently bound TPQ or TTQ or a similar group. The conclusion that the redox active group is covalently bound is also supported by the observation that prolonged incubation of the L-AME at 60 °C does not result in activity loss and thus not in loss of the organic cofactor. Although all purified L-AME samples gave a positive result in the redox cycling assay, formazane formation relative to protein concentration was variable – most likely due to different accessibility and to different redox states of the group. Thus, an exact quantification of the redox active group on the basis of this assay was not possible. This aspect requires further work when more homogenous L-AME is available.

The absorbance in the visible region of the L-AME could possibly be attributed to such an amino-acid-derived quinonoid group (see for comparison the absorbance spectrum of the TTQ containing methylamine dehydrogenase from bacterium W3A1 – Kenney and McIntire, 1983). Presence of such a group would also explain the variation in the absorbance spectrum observed in samples of different purifications – suggesting that the group can be present in different redox states. This is supported by the finding that prolonged incubation of the L-AME in an O_2 atmosphere alters the absorbance spectrum (Fig. 5). Treatment of the L-AME with 2,4-dinitrophenylhydrazine for 16 h in an O_2 atmosphere most likely leads to a cofactor adduct (Fig. 5). This result is also in favor of a quinonoid being associated with the L-AME, since 2,4-dinitrophenylhydrazine can

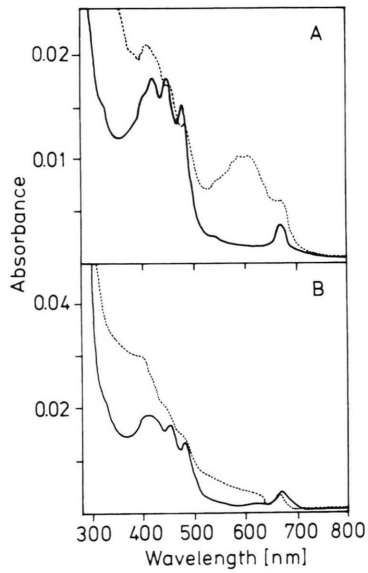

Fig. 5. Influence of O_2 and of phenylhydrazine on the absorbance of the L-AME. Before recording the absorbance spectrum, the L-AME was submitted to the following treatments: A: L-AME (3.1 μg protein) in 1 ml 50 mM sodium phosphate, pH 7.0, containing 0.02% DM was incubated for 16 h at 60 °C in a stream of oxygen. Absorbance spectrum before (——) and after treatment (·····). B: To L-AME (7.8 μg protein) in 1 ml 50 mM sodium phosphate, pH 7.0, containing 0.02 % DM, 50 μl of a saturated 2,4-dinitrophenyl-hydrazine solution was added. Subsequently, the mixture was incubated for 16 h at 60 °C in a stream of oxygen and then dialyzed against 50 mM sodium phosphate, pH 7.0, containing 0.02% DM to remove unbound 2,4-dinitrophenylhydrazine. Absorbance spectrum before (——) and after treatment (·····).

be used as a probe for identification of quinonoids (van der Meer *et al.*, 1986 and 1987).

Characteristics of the L-AME reaction

The isolated protein catalyzes the conversion of L-arginine (K_M value: 4.7 mM L-arginine) but not of D-arginine to ornithine and urea (ratio of ornithine to urea production was in the range of 1 to 1.24) and additional so far not identified products. Maximal L-AME activity of the purified protein was obtained at about 60 °C (not shown). Thus, the temperature dependence was about the same as that given in Fig. 1 for the L-AME activity detectable in PS II membranes. When the isolated enzyme was pretreated for 1 h at 60 °C, and activity measurements were subsequently performed at 40 °C, the pretreated L-AME had an about 2 to 3 fold higher activity than the enzyme without pretreatment at 60 °C – corresponding to about 30% of the activity obtained when the assay was performed at 60 °C. This finding suggests that the conformational change caused by the elevated temperature was only partly reversed by lowering the temperature (not shown).

The L-AME activity is totally dependent on manganese added to the reaction mixture (Fig. 6). The dependence of the isolated L-AME on Mn added to the reaction mixture is very similar to the requirement of the L-AME still incorporated into PS II membranes. The concentrations required for obtaining 50% of maximal activity was 0.05 mM $MnCl_2$ or 0.5 mM Mn^{3+} pyrophosphate for the isolated L-AME and 0.1 mM $MnCl_2$ or 0.2 mM Mn^{3+} pyrophosphat for the L-AME incorporated into PS II membranes. These results show that the isolated L-AME can bind di- as well as trivalent Mn. However, from these results it can not be concluded that the enzyme has a higher affinity for Mn^{2+} than Mn^{3+}, since Mn^{3+} was added in chelated form. Besides Mn we tested a number of other transient metals (such as Fe^{3+}, Co^{2+}, Ni^{2+}, Cu^{2+} and Zn^{2+}) for their influence on the L-AME activity (not shown). Among these only $CoCl_2$ had a minor stimulatory effect (giving 12% of the activity reached with $MnCl_2$). Whether this means that Co^{2+} could support a low L-AME activity of this protein or has just a potentiating effect (increasing a residual Mn concentration at the active center by displacing Mn from nonfunctional cation binding sites) can presently not be answered.

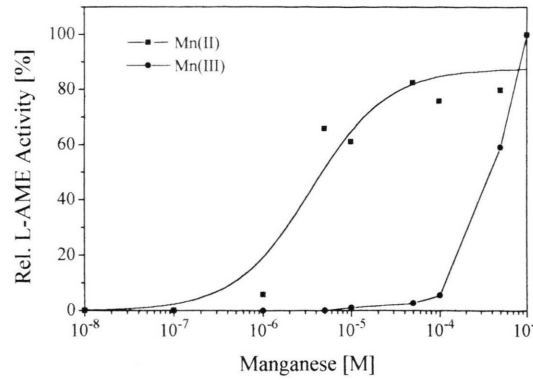

Fig. 6. Dependence of the isolated L-AME on manganese. The L-AME activity was determined as described under Materials and Methods with the corresponding changes in Mn addition to the reaction mixture as indicated in the Figure. $MnCl_2$ or Mn^{3+} pyrophosphate was used. In the presence of 0.5 mM $MnCl_2$ the L-AME activity corresponded to 27 μmol ornithine formed/mg protein×h. A partially purified L-AME preparation was used: 20 μg protein were present in the reaction mixture.

When in the presence of a suboptimal $MnCl_2$ concentration in the reaction mixture (0.05 mM instead of 0.5 mM $MnCl_2$) various alkali earth metals were added to the reaction mixture, then an inhibition of the L-AME activity could be observed. At 2 mM concentration an inhibition of 56%, 30%, 13% and 13% was obtained with $MgCl_2$, $CaCl_2$, $SrCl_2$ and $BaCl_2$, respectively. As expected, $MgCl_2$ was more inhibitory than $CaCl_2$, $SrCl_2$ or $BaCl_2$, because the chelate structure of Mg^{2+} is closest to that of Mn^{2+}.

An indication of a possible second cation binding site came from experiments in which the L-AME was submitted to elevated temperature (75 °C) in the presence of EDTA to remove tightly bound cations and subsequent dialysis to remove EDTA. After such a treatment addition of $MnCl_2$ to the reaction mixture was not sufficient to obtain maximal activity. Maximal activity was only obtained after incubating the L-AME in the presence of $CaCl_2$ at 50 °C for 30 min followed by a dialysis treatment, before L-AME activity was determined under standard assay conditions (presence of $MnCl_2$ in the reaction mixture). Mn^{2+} was about equally effective as Ca^{2+} under the chosen conditions, while Sr^{2+} was less effective and Mg^{2+} inef-

fective – suggesting that this more sequestered cation binding site has a broader specificity than the other cation binding site (Table III). These results indicate that the protein has two cation binding sites: To obtain maximal L-AME activity, one site (being easily accesssible) requires bound Mn (high specificity – only Co^{2+} gives a minor activity) and the other site (being more sequestered) requires bound Ca^{2+} or Mn^{2+} (Sr^{2+} being less effective and Mg^{2+} being ineffective). On the basis of these results it can be concluded that optimal conditions for L-AME activity are given when the protein contains either Mn–Mn or Mn–Ca.

Besides the cation requirement the enzyme showed a complex dependence on chloride for activity. This requirement became most obvious, when a totally inactive L-AME was obtained after chromatography on an anion exchange column at pH 9.5 – most likely due to partial or complete loss of chloride from the protein at alkaline pH. Partial reactivation could be achieved by dialyzing the enzyme against 10 mM Hepes-NaOH in the pH range of 6.5 to 7.5. However, maximal activity was only reached when NaCl (about 100 mM) was added to the dialysis buffer. Adding NaCl to the reaction mixture was not sufficient, a prolonged dialysis (overnight) in the presence of NaCl was necessary to reach maximal activation after chloride depletion – implying that a slow conformational change of the protein was required to convert the inactive to the active enzyme. Comparison of various halides (Fig. 7) showed that chloride and bromide were most effective in promoting this conformational change. However, chloride proved to be superior, because activation with chloride was possible over a broad concentration range, while activation with bromide was only successful in a narrow concentration range between 50 to 100 mM NaBr. Above this concentration a strong reduction of activity was observed. Chloride also became inhibitory at higher concentrations, but by far less than bromide. Iodine had only a minor stimulatory effect, and fluoride was inhibitory at all concentrations tested.

When testing other anions besides halides for their effectiveness in promoting the activation process of the enzyme, we observed that sulfate was as or occasionally even more effective than chloride in promoting the activating conformational change of the protein. This suggests that the slow conformational change required to convert the inactive to the active L-AME can be accelerated by a number of anions (also by acetate and phosphate – although to a lesser extent than by sulfate), but chloride and sulfate proved to be most effective. The effect of sulfate on the L-AME activity became most obvious when prior to the

Table III. Influence of various cations on the activity of an L-AME which was submitted to an EDTA-heat treatment prior to the activity measurements. The L-AME was incubated at 75 °C in the presence of 10 mM EDTA for 1 h and subsequently dialyzed against 10 mM Hepes-NaOH, pH 6.5, containing 0.02% DM, at 4 °C overnight. After dialysis the L-AME was incubated with the cations (1 mM) as indicated in the Table at 50 °C for 30 min. Thereafter the enzyme was dialyzed against 10 mM Hepes-NaOH, pH 6.5, containing 100 mM NaCl and 0.02% DM, at 4 °C overnight, and the L-AME activity (0.5 mM $MnCl_2$ added to the reaction mixture) was determined as described under Materials and Methods.

Additions made after the EDTA-heat treatment of the L-AME	L-AME activity [μmol ornithine formed/ml enzyme×h]
None	0.81
1 mM $CaCl_2$	3.45
1 mM $MnCl_2$	3.29
1 mM $SrCl_2$	2.15
1 mM $MgCl_2$	0.97

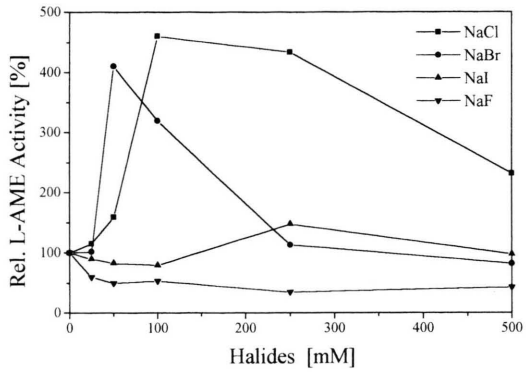

Fig. 7. Influence of halides on L-AME activity. Before determining activity, the L-AME was dialyzed overnight against 10 mM Ches-NaOH, pH 9.5, containing 0.02% DM. Thereafter, the L-AME was dialyzed against 10 mM Hepes-NaOH, pH 6.5, containing 0.02 % DM and the halides as indicated in the Fig. L-AME activity was determined (0.05 mM $MnCl_2$ added to the reaction mixture) as described under Materials and Methods. The L-AME dialyzed against buffer containing 100 mM NaCl had an activity of 421 μmol ornithine formed/mg protein×h. A partially purified L-AME preparation was used: 19 μg protein were present in the reaction mixture.

sulfate treatment the enzyme was treated with $CaCl_2$ to obtain maximal saturation of the more sequestered cation binding site. Under such conditions sulfate proved to be substantially more stimulatory than chloride. The reason most likely is that sulfate could remove Ca^{2+} from the easily accessible site (requiring Mn binding) more effectively than chloride by forming poorly soluble $CaSO_4$ (not shown).

Detection of catalase activity associated with the L-AME protein

A very low catalase activity is detectable when the L-AME is incubated with hydrogen peroxide. For these experiments the L-AME was dialyzed overnight against 10 mM Hepes-NaOH, pH 7.5, containing 100 mM NaCl, 1 mM $MnCl_2$ and 0.02% DM. At 37 °C the measurable catalase activity corresponded to 31 or 43 μmol O_2 evolved/mg protein×h in the absence or presence of $MnCl_2$ added to the reaction mixture, respectively. These results suggest that a low Mn mediated catalase activity (see for Mn catalase e.g. Shank *et al.*, 1994) is associated with the purified L-AME protein.

Discussion

As the results show, we were able to develop an effective purification procedure for the L-AME protein starting with $CaCl_2$ washed PS II membranes from spinach. One of the advantages working with plants instead of cyanobacteria is that the starting material (PS II membranes) could more easily be obtained in large quantities. However, since the protein strongly aggregates with other proteins, the yield of homogeous protein still remained rather low. On the basis of SDS PAGE the spinach L-AME has an apparent molecular mass of 7 kDa – thus superficially belonging to the family of low molecular weight proteins in PS II (Pakrasi and Vermaas, 1992). It is difficult to detect, since it is poorly stained by Coomassie Brilliant Blue. It is stained by silver, but the extent of staining is variable and depended greatly on conditions. We are uncertain whether the value of 7 kDa is the true molecular mass. Since the protein binds cations and anions, we think that the extensive ion binding might reduce SDS binding to the protein and as a consequence the apparent molecular weight obtained by SDS PAGE could be substantially lower than the correct value. Whether the molecular mass of 20 to 35 kDa obtained by chromatographing the detergent solubilized L-AME on a Superose 12 column represents the correct value for the monomer or represents a multimeric form of the protein is presently also uncertain. We feel that the correct molecular weight of this protein can only be obtained after identificating and sequencing the gene coding for this protein. It was possible to obtain a N-terminal sequence of 20 amino acids of this protein. Since there is no homology to any of the known PS II peptides and also no homology to DNA of plastides, it can be concluded that this peptide is an additional, nuclear encoded and so far unidentified PS II peptide.

The protein can exist in a form without detectable L-AME activity (obtained after chromatography on an anion exchange column at pH 9.5 (most likely due to partial or complete chloride loss) and a form having L-AME activity (maximal activity obtained after dialyzing the inactive protein against Hepes buffer in the pH range of 6.5 to 7.5, containing anions, such as chloride or sulfate). It is typical for many membrane bound enzymes when solubilized by detergent that they can exist in several possible conformations depending on detergent, lipids and ions present and also on buffer used (Gennis, 1989). Such a protein can be trapped in a metastable state under a certain set of conditions being unable to reach a stable and active configuration unless conditions are changed. Conversion from one state to another frequently is a rather slow process. In the case of the L-AME a prolonged dialysis treatment under adequate conditions is required.

Besides the effect of certain anions on the L-AME activity promoting the active conformation of the L-AME protein, specific cations are required for activity. Maximal L-AME activity is obtained when the protein contains Mn (Mn^{2+} or Mn^{3+}) bound to an easily accessible site and Ca^{2+} or Mn^{2+} bound to a more sequestered site. Addition of manganese to the reaction mixture is an absolute requirement for detection of L-AME activity (only Co^{2+} gives a minor activity) – clearly showing that this protein can effectively bind manganese.

Converting the inactive to the active L-AME (dialysis treatment in the presence of certain an-

ions) caused a significant change in the absorbance of the protein – mainly in the 300 nm region. Interestingly, an absorbance change in this region has also been described to occur during the S-state transition of the water oxidizing enzyme (Dekker, 1992). Since the L-AME has a low absorbance in the visible region (in the 350 to 500 nm region), the question arose whether the protein might contain an organic prosthetic group. On the basis of a redox cycling assay which was developed for detection of quinonoids (see recent reviews: Duine, 1991; Klinman and Mu, 1994; McIntire, 1994), it can be concluded that the isolated L-AME contains a redox active group – possibly such as TPQ or TTP (but not noncovalently bound PQQ, since the bioassay for PQQ gave negative results). This group is only detectable in the redox cycling assay when the reaction was performed at elevated temperatures (60 °C) – suggesting that the group in the isolated protein is not easily accessible for glycine and/or nitroblue tetrazolium binding. The presence of such an amino-acid-derived quinonoid might explain why the interaction of the protein with ions required for L-AME activity is rather complex – suggesting multiple interrelationships between the protein, the suggested *o*-quinone group, and the various ions.

Whether the low L-AME activity of this protein which we are able to measure, has any physiological significance, is related to an ancient activity of this protein or is just an artificial activity can presently not be answered. Since the L-AME activity is only detecable at elevated temperatures, it is more likely that the activity is nonphysiological. However, this activity provides an effective assay for detection and isolation of this PS II associated protein and enabled us to determine the affinities which this protein has for specific ions, via activity measurements. This is a great advantage, since ion binding studies with detergent solubilized membrane proteins are practically impossible.

When the ion requirement for the L-AME activity was compared to the ion requirement for the water oxidizing enzyme (WOE) activity (Debus, 1992; Boussac and Rutherford, 1994), then a significant similarity became obvious. Therefore, the question arises whether this agreement is coincidently or could imply that the PS II associated L-AME protein might provide ligands for the Mn cluster. The possibility of this protein being involved in water oxidation is supported by the observation that the protein most likely contains a quinonoid which could be a candidate for the organic radical involved in the S-state transition (Boussac and Rutherford, 1994). Moreover, detection of a low catalase activity associated with the L-AME protein also is a support for a functional role in water oxidation, since it is well documented that the WOE has a low catalase activity (Frash, 1992).

Our results suggest that a so far unrecognized, rather low molecular weight protein which most likely contains a redox active group of an *o*-quinone type and which has a capacity to bind ions, such as Mn, Ca^{2+} and Cl^-, is associated with the D1/D2/cytochrome *b*559 complex. We suggest that this combination represents the water plastoquinone oxidoreductase. In this respect it is relevant to mention that Zimmermann *et al.* (1993) suggested that results obtained by ESEEM of Cu containing amine oxidases (containing TPQ) seem to be similar to ESEEM results of the Mn S_2 signal. It might also be relevant to mention that in the TTQ containing methylamine dehydrogenase from *Methylobacterium extorquens* (Christoserdov *et al.*, 1990) TTQ is located on the small subunit of the multimeric enzyme (two subunits of 13 kDa and 2 subunits of 40 kDa) – comparable to our model of PS II which suggests that a small polypeptide with a redox active group and a capacity to bind Mn, Ca^{2+} and Cl^- is associated with D1/D2 heterodimer. If the here examined PS II associated L-AME should eventually turn out to be functional in water oxidation (a genetic approach is required for additional proof), then research on the interplay of the redox active organic cofactor (most likely an *o*-quinone having metal chelating properties – Schwederski et al., 1990) with the inorganic cofactors of water oxidation might help to obtain a final understanding of the mechanism of this reaction.

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