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# Partial characterization of an arginine-metabolizing enzyme from the thermophilic cyanobacterium *Synechococcus* sp.

Ralf Meyer and Elfriede K. Pistorius

*Universität Bielefeld, Fakultät für Biologie, Lehrstuhl Zellphysiologie, Bielefeld (F.R.G.)*

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**An L-arginine-metabolizing enzyme has been partially purified from the thermophilic cyanobacterium *Synechococcus* sp. and some of the properties of this enzyme are here described. The enzyme produces $NH_4^+$ and ornithine as the major products from L-arginine, and it does not require $O_2$ in the gas phase or any other added electron acceptor for the here described reaction. Superficially, the enzyme can be classified as an L-arginine dihydrolase catalyzing the combined reaction of an arginase-urease or deiminase-citrullinase. However, the purified protein does not have a separate arginase, urease, deiminase or citrullinase activity. Since the stoichiometry of $NH_4^+$ to ornithine production is somewhat variable, and since the enzyme still shows a cross-reaction with the antiserum raised against the L-amino acid oxidase from the cyanobacterium *Anacystis nidulans* and is also inhibited by cations as the L-amino acid oxidase, a more complex reaction as just an arginine dihydrolase reaction is highly likely. In *Synechococcus* sp. this enzyme is constitutive. However, when the *Synechococcus* sp. cells are transferred from nitrate to L-arginine as sole nitrogen source, growth only proceeds after an $Mn^{2+}$-requiring arginase is induced. Those results indicate that the enzyme does not play a major role in L-arginine catabolism but that it has an additional role – possibly of the type as previously suggested (Meyer, R. and Pistorius, E.K. (1987) Biochim. Biophys. Acta 893, 426–433).**

## Introduction

Recently, a flavoprotein which has an L-amino acid oxidase activity with specificity for basic L-amino acids was isolated from the cyanobacterium *Anacytis nidulans* [1,2]. This enzyme seems to be a functional component of Photosystem II, since its activity can be detected in highly purified Photosystem II complexes of *A. nidulans* and because it is affected by various reagents which also stimulate or inhibit photosynthetic water oxidation. Those results suggested that this flavoprotein could possibly become modified in the light and in the presence of $Ca^{2+}$ and $Cl^-$ in such a way, that it could interact with $Mn^{2+}$ and then catalyze the water-oxidizing reaction of Photosystem II [1,2].

To obtain more information about such enzymes in organisms other than *A. nidulans*, we have recently investigated the thermophilic cyanobacterium *Synechococcus* sp. [3]. This organism has very stable Photosystem II complexes which have been well characterized by several groups [4–7]. We could show that – based on $NH_4^+$ production – an L-amino acid metabolizing enzyme with a high specificity for L-arginine was also present in thylakoid membranes and purified Photosystem II complexes of the cyanobacterium *Synechococcus* sp. However, as in *A. nidulans*, the major amount of the detectable activity was found in the soluble fraction of a French press extract [3].

In this work we report on the partial purification and characterization of an enzyme which is responsible for one of the L-arginine metabolizing activities in *Synechococcus* sp.

## Materials and Methods

*Cyanobacterial strain and growth of the organism.* *Synechococcus* sp. was grown on nitrate in a medium as described in Ref. 3. Cells to be grown in the presence of L-arginine were harvested, washed twice with distilled water and then inoculated in a medium in which nitrate

Abbreviations: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; EDTA, ethylenediaminetetraacetic acid; EGTA, ethyleneglycol-bis(2-aminoethylethane)tetraacetic acid; TLC, thin layer chromatography.

Correspondence: E.K. Pistorius, Universität Bielefeld, Fakultät für Biologie, Lehrstuhl Zellphysiologie, Postfach 8640, D-4800 Bielefeld 1, F.R.G.

was replaced by 5 mM L-arginine. The L-arginine was added to the medium after sterilization by filtration.

*Preparation of cell extracts.* The preparation of French press extracts was carried out according to Ref. 3. The resulting extract after French press treatment was centrifuged at $20000 \times g$ for 20 min to remove the unbroken cells. The supernatant of this centrifugation was used for activity measurements with the L-arginine grown cells. For purification of the enzyme this extract was centrifuged again at $245000 \times g$ for 2 h. The supernatant obtained after this ultracentrifugation which was called 'supernatant of French press extract', was stored at $-20\,^{\circ}C$ before use.

*Determination of enzyme activity.* The enzyme reaction was carried out in 12 ml tubes in a water bath normally at $55\,^{\circ}C$ for 5 h with one exception: the activities for purification 1 (Table I) were determined at $50\,^{\circ}C$ and then converted to values at $55\,^{\circ}C$ according to the temperature curve of Fig. 2. The basal reaction mixture contained in a total volume of 3 ml: 6.7 mM L-arginine (Merck), 20 mM EDTA, 16.7 mM Hepes-NaOH buffer (pH 7.5) and the sample. The reaction was stopped by adding 600 mmol $H_2SO_4$. After centrifugation at $48000 \times g$ for 20 min the reaction products were determined. For thin-layer chromatography the incubation time was extended to 12–20 h and the above reaction mixture without EDTA was used. The reaction was then stopped by filtration of the reaction mixture through centricon 10 microconcentrators (Amicon). For experiments under anaerobic conditions the reaction was carried out in Warburg vessels equilibrated with argon and in the presence or absence of 3 mM glucose, 25 units of glucose oxidase (Boehringer) and 1300 units of catalase (Boehringer). Before the start of the reaction, this mixture was preincubated for 20 min at room temperature. All conditions gave the same results.

*Enzyme unit.* One unit of enzyme was defined as 1 nmol $NH_4^+$ produced per min. (L-Arginine as substrate, $55\,^{\circ}C$ reaction temperature).

*Determination of the reaction products.* $NH_4^+$ was determined enzymatically after neutralization of the sample with NaOH [3]. Arginine and ornithine were determined colorimetrically [8,9]. Thin-layer chromatography was performed on TLC aluminium sheets, cellulose $F_{254}$ (Merck) with a layer thickness of 0.1 mm in a solution of butanol/glacial acetic acid/$H_2O$ (12 : 3 : 5). Amino acids were stained by spraying with a solution of 0.1% ninhydrin and 0.1% collidin in chloroform and heating at $110\,^{\circ}C$. For specific determination of guanidino residues the FCNP-reagent according to Merck (no. 244) was used.

*Molecular-weight determination.* The molecular weight of the native enzyme was determined with a superose 12 column, coupled to an FPLC system (Pharmacia), equilibrated with 0.1 M sodium phosphate buffer pH 7.5 containing 0.1 M NaCl.

*Determination of protein concentration.* Protein concentration was determined using the Bradford protein assay [10] or in some cases using the Lowry protein assay [11]. In both cases bovine serum albumin (Sigma) was used as standard.

*SDS-PAGE and immunoblotting.* Polypeptide compositions were examined by SDS-PAGE [3]. The gels were stained with Coomassie Blue [3] or silver [12]. For immunoblotting experiments the protein bands were transferred by pressure from the unstained gel to nitrocellulose sheets for 70 h at $4\,^{\circ}C$. The nitrocellulose was then blocked with a solution of 5% milk powder in phosphate buffered saline. The first antibody, raised against the purified L-amino acid oxidase protein from *A. nidulans* [13], was usually diluted 50–500 fold. The second antibody, peroxidase-conjugated swine immunoglobulins to rabbit immunoglobulins (DAKO-PATTS), was diluted 100-fold. Specifically bound antibodies were stained by reaction of the peroxidase with $H_2O_2$ and 4-chloro-1-naphthol (Sigma).

*Metal analysis.* Metal analysis was performed by mass spectrometry with an inductively coupled plasma ion source (Plasma Quad, VG Instruments). The enzyme was dialyzed against 10 mM Hepes-NaOH (pH 7.5), containing 20 mM EDTA, and then with several changes against the same buffer without EDTA. The last dialysis buffer was used as a control and the values for the enzyme sample were corrected with the values obtained with the buffer alone.

*FAD determination.* FAD was determined enzymatically as in Ref. 1. Absorption and fluorescence spectra were performed as in Ref. 14. The excitation wavelength in the fluorescence measurements was 390 nm.

## Results

*Purification of the L-arginine metabolizing enzyme*

As we could show previously, the detectable activity of this enzyme, measured as $NH_4^+$ production from L-arginine, was mainly found in the soluble fraction of the French press extract from *Synechococcus* sp. (98% of the activity), while only 2% of the activity was associated with the thylakoid membranes. Therefore, we have only used the supernatant for the purification. However, in the supernatant of the French press extract the enzyme is not really a soluble protein, since dialysis of the supernatant against Hepes-NaOH buffer results in precipitation of the enzyme [3]. This could be prevented by addition of sucrose or phosphate ions to the buffer. Obviously, the protein aggregates with other proteins which might cause the precipitation, or the protein itself exists in differently soluble aggregation states which are influenced by the ions in the medium. This property of the enzyme most likely explains the difficulties which we have encountered during the purification procedures.

TABLE I

*Purification Procedures 1 and 2*

Activities were measured as described under Materials and Methods. 1 unit of enzyme was defined as 1 nmol $NH_4^+$ produced per min. (L-arginine as substrate; reaction temperature 55 °C)

| Step | Protein (mg, total) | Total activity ($NH_4^+$ production) (units, total) | Specific activity (units per mg protein) |
|---|---|---|---|
| Purification 1 | | | |
| 1 Supernatant of French-press extract | 5830 | 31208 | 5.4 |
| 2 30–60% $(NH_4)_2SO_4$ precipitation | 3900 | 30758 | 7.9 |
| 3 DEAE-Sephadex column No. 1 | 13.8 | 3214 | 233 |
| 4 DEAE-Sephadex column No. 2 | 8.1 | 1490 | 184 |
| 5 FPLC-Mono Q column | 0.75 | 653 | 871 |
| Purification 2 | | | |
| 1 Supernatant of French-press extract | 3402 | 14750 | 4.3 |
| 2 Pellet after Hepes dialysis | 494 | 9800 | 19.8 |
| 3 DEAE-Sepharose CL-6B column | 26.9 | 2100 | 78.1 |
| 4 Mono Q column | 4.8 | 1477 | 308 |
| 5 Pellet of Ultra-centrifugation | 0.66 | 549 | 833 |

We report here on two different purification procedures. All steps of the purification procedures were performed at 0–4 °C.

*Purification 1: ammonium sulfate precipitation and chromatography on ion-exchange columns (Table I)*

The supernatant (2.6 l) of French press extracts of *Synechococcus* sp. (see Materials and Methods), which corresponded to 40 ml packed cells, was fractionated by the addition of $(NH_4)_2SO_4$ and the protein obtained between 30 and 60% saturation was collected. After dialysis against 50 mM sodium phosphate buffer, pH 6.5 (buffer A), containing 0.2 M NaCl, and centrifugation at 27000 × *g* for 20 min to remove denatured proteins, the extract was chromatographed on a DEAE-Sephadex column (3 × 80 cm) which was equilibrated with the dialysis buffer. After washing the column with the same buffer, elution was performed with a gradient (1.3 l) of buffer A, containing 0.2–1.0 M NaCl. The fractions with the highest specific activity were dialyzed again and subjected to chromatography on a second DEAE-Sephadex column (50 × 2 cm). After washing the column as above, elution was performed with a gradient (500 ml) of buffer A, containing 0.1–0.8 M NaCl. The active fractions were dialyzed against buffer A and after centrifugation the solution was applied to a Mono Q column coupled to an FPLC system. The column was equilibrated with buffer A and the enzyme was eluted with a gradient (50 ml) of buffer A, containing 0–0.4 M NaCl.

*Purification 2: precipitation by dialysis against Hepes buffer, chromatography on anion-exchange columns and ultracentrifugation (Table I)*

The supernatant of the French press extract (1.1 l) obtained as above (corresponding to 12 ml packed cells) was dialyzed against 10 mM Hepes-NaOH buffer (pH 7.5), for 22 h. After centrifugation at 50000 × *g* for 20 min most of the phycobili proteins remained in the supernatant, while the L-arginine metabolizing activity was found in the pellet. After resuspending the pellet in 0.1 M sodium phosphate buffer (pH 6.5), and centrifugation at 50000 × *g* for 30 min, 95% of the activity was now recovered in the supernatant. This supernatant was then chromatographed on a DEAE-Sepharose CL-6B column (3 × 10 cm) equilibrated with buffer A. After applying the sample, the column was washed with buffer A, containing 0.1 M NaCl and the enzyme was eluted with a gradient (500 ml) of buffer A, containing 0.1–0.5 M NaCl. The fractions with the highest specific activity were dialyzed against buffer A and subjected to chromatography on a Mono Q column. After washing the column with buffer A, elution was performed with the same buffer, containing 0–0.5 M NaCl. The active fractions which eluted at 0.22 and 0.25 M NaCl were further purified by ultracentrifugation for 2 h at 245000 × *g*. Half of the activity was precipitated in an orange pellet which could be resuspended in buffer A and which now showed an increase in specific activity to 833 units/mg.

*Comments on the purification procedures.* Several observations which we made in the course of development of these purification procedures are worthy of comment. One of the major problems which we had throughout the purification was the great heterogeneity of the enzyme which most likely formed aggregates with other proteins and with itself. Therefore, the different active species showed a great variability in their binding capacities to columns. As a consequence, each column gave several protein peaks with activity. The heterogeneity was bigger in the second purification procedure – as would be expected, since the protein was separated from the bulk of the phycobili proteins by dialysis against Hepes buffer which resulted in precipitation of the protein. On the other hand, the advantage of this procedure is the separation of the enzyme from the majority of the blue proteins in the first purification step.

The enzyme obtained after the second purification still contained minor amounts of carotene while the

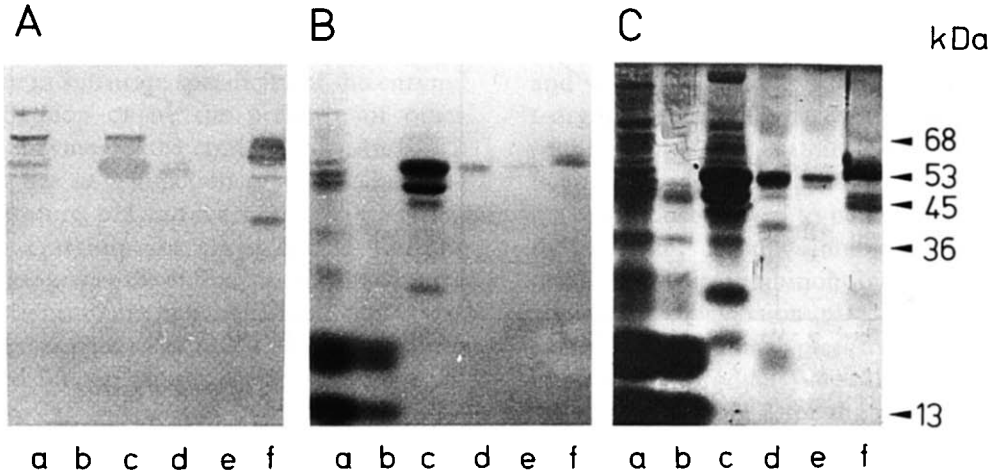


Fig. 1. SDS-PAGE and Western blot. SDS-PAGE and immunoblotting was performed as described under Materials and Methods. (A) Immuno-stained nitrocellulose; (B) Coomassie Blue stained gel, and (C) silver stained gel. The following samples were applied: a, supernatant of French press extract (containing the enzyme); b, supernatant of French press extract dialyzed against Hepes buffer (not containing the enzyme); c–f, four samples of the partially purified enzyme (c, 68 μg protein, 56 units/mg; d, 20 μg protein, 208 units/mg; e, 3.4 μg protein, 411 units/mg; and f, 17.7 μg protein, 500 units/mg). The protein concentration of lane e was too low to give a good band with the antiserum (band does not show on the photo but was weakly seen on the nitrocellulose).

enzyme obtained after the first purification procedure still contained minor amounts of phycobili proteins which could not be removed during the purification. Similar observations have been made for ATP-synthase purified from the thermophilic cyanobacterium *Mastigloca dus laminosus* [15].

The specific activity after both purifications was about 850 units/mg. So far we have not been able to purify the enzyme further, since purification on cation exchange columns was not possible because the enzyme binds to such columns only at pH values where it denatures. This indicates that the enzyme has a low isoelectric point. Moreover, no further increase in specific activity was achieved on hydroxylapatite or phenyl-sepharose columns.

Based on the specific activity obtained in the two purifications, a turn-over number of 170 (mol $NH_4^+$ produced from L-arginine per mol enzyme per min; molecular mass of the native enzyme: 200 kDa) can be calculated. This is a very low turn-over number even if we take into consideration that we might eventually be able to increase the specific activity slightly.

*Molecular weight, prosthetic group and cross-reaction of the enzyme with an antiserum raised against the L-amino acid oxidase from A. nidulans*

The molecular mass of the native enzyme is 200 kDa when determined by gel filtration on a Superose 12 column, and the molecular mass determined by SDS-PAGE gave a value of 50–60 kDa (Fig. 1). Possibly the enzyme consists of four subunits of equal molecular weight. After extensive dialysis of the enzyme against EDTA/Hepes-NaOH buffer it still contained substantial amounts of $Na^+$ which might influence the binding of SDS to the enzyme and this could explain some of the problems which we encounter with SDS-PAGE. We sometimes observe additional bands in the higher or lower molecular weight region in samples which previously did not have such bands.

The enzyme did not contain authentically oxidized FAD. However, after treatment of the enzyme with 0.2 M $H_2SO_4$ and after precipitation of the protein by centrifugation, an organic component which showed an absorption around 260 nm and a hardly detectable absorption in the yellow region of the spectrum was obtained. This component also had a fluorescence around 495 nm after excitation with light of 390 nm (not shown) and was in this respect somewhat similar to the modified flavin shown to be present in the L-amino acid oxidase from *Anacystis nidulans* [14].

The antibody raised against the L-amino acid oxidase from *A. nidulans* showed a cross reaction with the supernatant of French press extracts from *Synechococcus* sp., while the supernatant dialyzed against Hepes buffer from 'Purification 2' did not show a cross reaction (Fig. 1). This supernatant did not anymore contain the enzyme, since it was precipitated by the dialysis against Hepes buffer. The immunoblotting experiments also show that the protein which was enriched in the two purification procedures and which had a molecular mass of approx. 55 kDa still gave a cross reaction with the antiserum (Fig. 1). This would indicate that the enzyme is similar to the *A. nidulans* enzyme (see concluding remarks) or that the antibody recognizes L-arginine binding sites.

*Characterization of the enzyme activities of the purified protein*

*Substrates, reaction characteristics and inhibitors.* The purified enzyme produces $NH_4^+$ from L-arginine. All

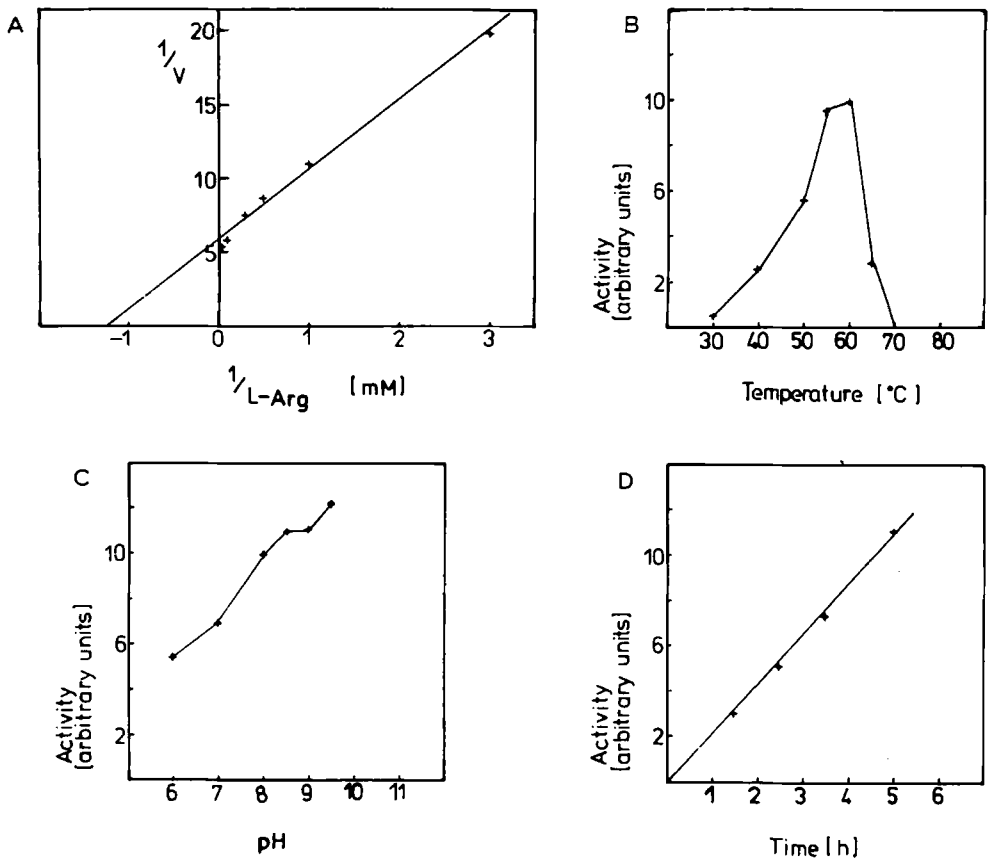


Fig. 2. Influence of L-arginine concentration, temperature, pH and incubation time on the enzyme activity. Enzyme activities were measured as described under Materials and Methods with the variations given in the diagrams. (A) Lineweaver-Burk plot; (B) temperature dependence; (C) pH dependence; and (D) dependence on incubation time.

TABLE II

*Inhibition of the enzyme*

Enzyme activity was determined as described under Materials and Methods (substrate: 6.7 mM L-arginine). The values represent the inhibition of $NH_4^+$ production, except the value for hydrazine where the inhibition of L-ornithine production was determined. Other substances which do not inhibit the enzyme (i.e., inhibition below 10%, up to 50 mM) were: L-serine, L-valine, γ-guanidino butyric acid, agmatine, L-argininic acid, L-canavanine, L-glutamic acid, salicylhydroxamic acid (up to 3 mM), urea and $NH_4^+$.

| Inhibitor | Concentration (mM) | Inhibition (%) |
|---|---|---|
| L-Ornithine | 17 | 95 |
| $H_2O_2$ | 0.15 | 50 |
| Hydrazine | 10 | 85 |
| Hydroxylamine | 10 | 71 |
| DL-2,3-Diamino-propionic acid | 17 | 60 |
| L-Cysteine | 20 | 50 |
| L-Alanine | 20 | 40 |
| Glycine | 20 | 17 |
| L-Lysine | 20 | 14 |
| D-Arginine | 17 | 0 |
| D-Ornithine | 17 | 0 |

other tested substances only led to $NH_4^+$ production below 1% of the L-arginine value. In the crude extract addition of L-ornithine, DL-2,3-diamino propionic acid and L-citrulline also resulted in $NH_4^+$ production [3].

TABLE III

*Influence of EDTA and cations on the enzyme activity*

Activity was measured as described under Materials and Methods. Dialysis of the enzyme was performed against 20 mM Hepes (pH 7.5).

| Sample treatment | Addition to assay | | Activity (%) |
|---|---|---|---|
| | EDTA or metal | Concn. (M) | |
| – | – | – | 35 |
| – | EDTA | $2 \cdot 10^{-2}$ | 100 |
| Dialysis | – | – | 96 |
| Dialysis | EDTA | $2 \cdot 10^{-2}$ | 94 |
| Dialysis | NaCl | $5 \cdot 10^{-1}$ | 85 |
| Dialysis | $CaCl_2$ | $5 \cdot 10^{-3}$ to $1 \cdot 10^{-2}$ | 85–50 [a] |
| Dialysis | $MnCl_2$ | $5 \cdot 10^{-5}$ to $1 \cdot 10^{-4}$ | 50 |
| Dialysis | $ZnCl_2$ | $5 \cdot 10^{-8}$ to $1 \cdot 10^{-7}$ | 50 |
| Dialysis | $LaCl_3$ | $1 \cdot 10^{-6}$ to $5 \cdot 10^{-6}$ | 50 |

[a] See text for explanation.

These activities were not associated with the purified enzyme. This discrepancy could possibly be explained either by a change in substrate specificity of the enzyme during the purification or by the presence of other $NH_4^+$-producing enzymes in the crude extract. In Fig. 2 the enzyme activities as a function of L-arginine concentration, temperature, pH and reaction time are given. A $K_m$ value for L-arginine of about 1 mM was obtained. Most striking was the influence of temperature on the reaction. An optimum between 55 and 60 °C was observed which resembles very much the temperature optimum obtained for the growth of *Synechococcus* sp. [16].

As shown for many L-arginine metabolizing enzymes, the here described enzyme is inhibited by L-ornithine (it is not inhibited by D-ornithine or D-arginine). Moreover, inhibition is also observed with $H_2O_2$, hydrazine and hydroxylamine (partly competitive and partly non-competitive inhibition – not shown) and with other small amino acids (Table II). Urea and $NH_4^+$ are not inhibitors of the enzyme.

*Effect of EDTA and cations.* Previously, we had shown that the addition of EDTA to the reaction mixture caused a stimulation of the $NH_4^+$ production from L-arginine [3]. This effect of EDTA could also be demonstrated with the purified enzyme (Table III). The majority of the purified enzyme samples showed a reduced activity (5–50% of the maximum activity) in the absence of EDTA (EGTA gave the same results). When the enzyme was extensively dialyzed against Hepes buffer, maximum activity could be observed without the addition of EDTA to the reaction mixture. This effect of EDTA is related to the inhibition of the enzyme by cations (Table III). Trivalent cations, as $La^{3+}$, are more effective than divalent cations, as $Ca^{2+}$, while monovalent cations, as $Na^+$, are ineffective. Among the group of divalent cations transition metals as $Zn^{2+}$ and $Mn^{2+}$ inhibited more strongly than alkali earth metals. Due to the heterogeneity of the enzyme, as mentioned above, the extent of the metal inhibition is somewhat variable. Therefore we have given a concentration range for each metal in Table III. This variability was more obvious with $Ca^{2+}$ than with the other metals. The purified enzyme (dialyzed against EDTA/Hepes) still contains $Zn^{2+}$, $Mg^{2+}$, $Cu^{2+}$ and $Al^{3+}$. This is possibly related to the high affinity which the enzyme has for some metals. Whether one of these metals has a functional role cannot be decided at the present time.

*Identification of the reaction products and investigations about the reaction type.* $NH_4^+$ production from L-arginine by the enzyme proceeded under aerobic as well as under anaerobic conditions. The enzyme did not require $O_2$ in the gas phase or any other added electron acceptor for the here described reaction. Identification of the products by TLC shows that besides $NH_4^+$ the major product obtained cochromatographed with L-ornithine (not shown). The ratio of $NH_4^+$ to ornithine production was 1.9–2.6 to 1 with L-arginine as substrate and with no further additions, and the amount of L-arginine metabolized corresponded to the amount of ornithine produced, within these deviations. The identification of additional products besides ornithine as the major product proved to be extremely difficult, because the reaction is strongly inhibited by the major product: ornithine. This inhibition by L-ornithine only allows about 30% conversion of L-arginine to product. We cannot exclude that traces of citrulline (and possibly other minor products) are formed. This could explain the deviation from 2 for the ratio of $NH_4^+$ to ornithine production. The here isolated enzyme does not have a separate arginase, urease, deiminase or citrullinase activity.

*Comparison of L-arginine metabolizing activities in Synechococcus sp. grown on nitrate or on L-arginine*

In bacteria a great variety of L-arginine metabolizing pathways which are either constitutive or inducible when grown on L-arginine, exists (see for a review Ref. 17).

Therefore we have examined whether *Synechococcus* sp. could grow on L-arginine as a sole nitrogen source.

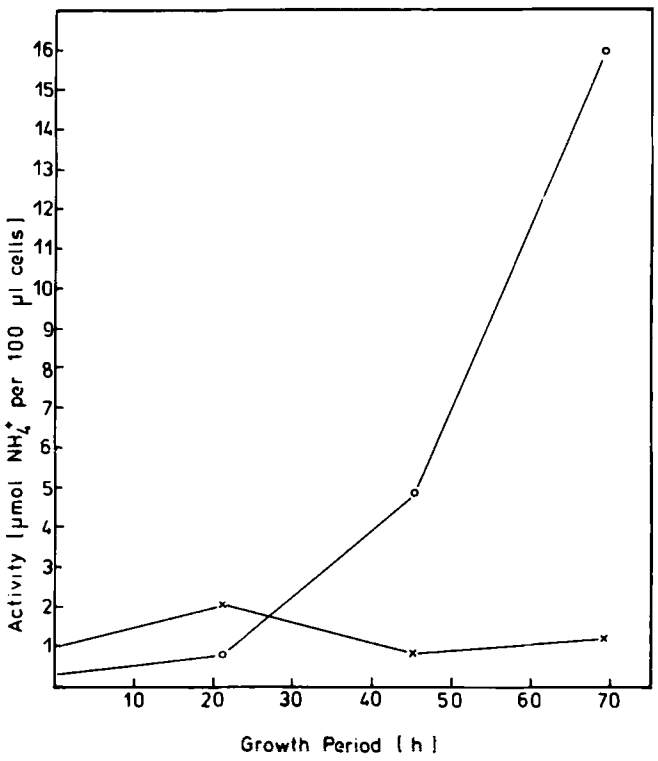


Fig. 3. $NH_4^+$ production from L-arginine in French press extracts from L-arginine-grown *Synechococcus* sp. cells. Activity was determined as described under Materials and Methods. ×——×, $NH_4^+$ production from L-arginine in the presence of EDTA (usual assay conditions). This assay mainly represents the here purified enzyme; o——o, $NH_4^+$ production from L-arginine in the presence of 1 mM $MnCl_2$ (urease present). This assay mainly detects the $Mn^{2+}$ requiring arginase activity.

Moreover, we have asked the question whether the here described enzyme plays a major role in L-arginine catabolism or whether other L-arginine utilizing enzymes, such as arginase, are induced, when the cells are grown on L-arginine. The results are given in Fig. 3. When *Synechococcus* sp. cells are transferred from nitrate containing medium to L-arginine-containing medium, a delay in growth of about 24 h was observed and the cells turned from a blue-green to a yellow-green colour. After that period the cells recovered and normal growth rates were obtained. The $NH_4^+$ producing activities (in the presence of EDTA) increased slightly during this period without growth and dropped to normal values after the first day. In addition to this activity, an $NH_4^+$ producing activity which was stimulated by $Mn^{2+}$ and inhibited by EDTA (urease present) was induced after the first day. Those results indicate that the cells could only grow on L-arginine after arginase had been induced. Therefore the L-arginine metabolizing enzyme which we have purified and described in this paper, does not seem to play a major role in L-arginine catabolism.

## Discussion

In this paper we describe an L-arginine metabolizing enzyme which produces $NH_4^+$ and ornithine as the major products. This reaction does not require oxygen in the gas phase or an additional cofactor as electron acceptor. The enzyme could superficially be classified as an L-arginine dihydrolase catalyzing the combined reaction of an arginase-urease [17] or of a deiminase-citrullinase [18]. A similar reaction has been proposed for the degradation of succinyl-arginine in *Pseudomonas cepacia* [19]. For several reasons we believe that such a straightforward concerted type of reaction does not explain all our experimental data. We observe a small variability in the ratio of $NH_4^+$ to ornithine production (1.9–2.6 to 1), and this indicates that minor amounts of additional products, such as citrulline and possibly 2-*keto*-arginine could also be formed. These minor products are extremely difficult to identify, since the reaction is strongly inhibited by the major product ornithine. Those side products could only be identified after we have isolated an L-ornithine metabolizing enzyme from *Synechococcus* sp.

Initially we had classified this enzyme as an 'L-amino acid oxidase type' enzyme [3], but the observation that the major products are $NH_4^+$ and ornithine and that no $O_2$ or other electron acceptor is required for the here described reaction, would speak against a typical L-amino acid oxidase reaction. However, the enzyme from *Synechococcus* sp. still shows a cross reaction with the antibody raised against the L-amino acid oxidase from *A. nidulans*. Moreover, it is inhibited by cations in a comparable fashion as the *Anacystis* enzyme [20], and it contains a yet unidentified organic prosthetic group which shows a certain similarity to the modified flavin which is present in the *Anacystis* enzyme besides oxidized FAD [14]. Therefore, it seems that the enzyme has a rather complex reaction mechanism of a yet unknown nature.

It is surprising that an antibody raised against the *A. nidulans* L-amino acid oxidase [13,20], which oxidizes L-arginine (and other basic L-amino acids) and which can utilize $O_2$ as one possible electron acceptor [21], shows a cross reaction with the here described L-arginine-metabolizing enzyme which does not seem to interact with $O_2$. However, a comparable cross-reactivity has recently been described for an antiserum which was raised against the hog kidney D-amino acid oxidase, which reacts with $O_2$. This antiserum cross-reacts with the membrane-associated oxidative L-alanine dehydrogenase from *E. coli* [22,23]. The latter enzyme is a component of the respiratory chain [24] and does not utilize $O_2$ but can use several artificial electron acceptors [23].

Since the L-arginine metabolizing enzyme which we have isolated from *Synechococcus* sp. shows a very low turn-over number of about 170, it could be possible that – although the here described reaction of the enzyme proceeds at very low rates without the addition of an electron acceptor – the rates might be significantly higher if we will eventually find the right combination of artificial electron acceptors or identify the physiological electron acceptor. Based on the observed cross-reactivity in the immunological experiments here reported, one possible explanation could be that the here described enzyme might initially form the imino acid from L-arginine, but that a number of consecutive internal reactions which we do not yet understand, might lead to the production of ornithine and $NH_4^+$ from L-arginine. This aspect requires further work.

As our results show, the enzyme does not seem to play a major role in L-arginine catabolism, since *Synechococcus* sp. can only grow on L-arginine after an $Mn^{2+}$-requiring arginase has been induced. We have recently hypothesized that this enzyme might have a role in photosynthetic water oxidation [3] and/or respiration as was shown for the *Anacystis* enzyme [21]. If this were the case, then, of course, it would not be surprising that the enzyme has a rather complex reaction mechanism (greatly depending on conditions), and it would also explain some of the difficulties which we encounter.

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