Biochimica et Biophysica Acta, 611 (1980) 227—240

BBA 68895

SOME PROPERTIES OF A BASIC L-AMINO-ACID OXIDASE FROM *ANACYSTIS NIDULANS*

ELFRIEDE K. PISTORIUS and HELGA VOSS

Forschungsstelle Vennesland der Max-Planck-Gesellschaft, Harnackstrasse 23, D-1000 Berlin 33 (Germany)

(Received June 19th, 1979)

Key words: L-Amino-acid oxidase, Basic amino acid; HCN; (A. nidulans)

Summary

An L-amino acid oxidase (L-amino-acid oxygen oxidoreductase (deaminating), EC 1.4.3.2) from the blue-green alga *Anacystis nidulans* has been purified to homogeneity with an overall yield of about 10%. Purification included ammonium sulfate fractionation and CM-Sephadex, DEAE-Sephadex, and hydroxyapatite chromatography. The purified enzyme has an absorption spectrum which is characteristic of a flavoprotein, and contains 1 mol FAD per mol enzyme. The native enzyme has a molecular weight of 98 000 as determined by gel exclusion chromatography. Electrophoresis in SDS-polyacrylamide gels gives a single protein band corresponding to a molecular weight of 49 000, which suggests that the native enzyme is composed of 2 subunits of equal molecular weight. As previously demonstrated, the enzyme catalyzes the oxidative deamination of the basic amino acids: L-arginine, L-lysine, L-ornithine and L-histidine. In the presence of catalase and of any of these amino acids, 0.5 mol O_2 is consumed, and 1 mol ammonia is formed for each mol amino acid oxidized. HCN is formed from L-histidine when the L-amino acid oxidase is supplemented with peroxidase. In addition to the unusual substrate specificity of this L-amino acid oxidase, it also has an unusual set of inhibitors including *o*-phenanthroline as well as divalent cations of which Cu^{2+}, Zn^{2+}, and Cd^{2+} are the most effective ones, but Mg^{2+} and Ca^{2+} also inhibit. This inhibition can be reversed by chelating agents such as EDTA. ATP and ADP, but not AMP, can also overcome the inhibition caused by Mg^{2+}, for example. The inhibitory effect of cations can be demonstrated in vivo.

Abbreviations: HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; SDS, sodium dodecyl sulfate; MES, 2-(*N*-morpholino)ethanesulfonic acid; Tricine, *N*-tris(hydroxymethyl)methylglycine.

Introduction

We have shown previously that the basic amino acids, L-arginine, L-lysine, L-ornithine and to a lesser extent L-histidine, strongly stimulate the O_2 uptake of cell suspensions of the blue-green alga or cyanobacterium *Anacystis nidulans* [1]. In the case of L-histidine, the extra O_2 consumption is associated with the formation in vivo of small amounts of HCN. The enzyme which is responsible for these activities has been identified as an L-amino acid oxidase specific for the basic amino acids. The present paper reports an improved purification procedure and a partial characterization of the enzyme.

Materials and Methods

Horseradish peroxidase (purity grade I), catalase (bovine liver crystal suspension, 20 mg/ml), DNAase I (purity grade II, 1000 units/mg), and RNAase (bovine pancreas, 40 units/mg) were purchased from Boehringer, Mannheim, as was FMN. FAD and riboflavin were purchased from Sigma, St. Louis, U.S.A.

Anacystis nidulans. L-1402-1, was obtained from the Sammlung von Algenkulturen, Universität Göttingen, Göttingen, F.R.G. The growth of cells and the preparation of extracts was the same as reported previously [1].

Enzyme assays

Oxidation of L-arginine. The assay was performed as described previously [1], except that the amount of added L-arginine was increased to 60 μmol. One unit of enzyme was defined as the amount which catalyzes the consumption of one μmol O_2 per min (in the presence of catalase, in air and at 20°C). Unless indicated otherwise, specific activities were determined with this assay.

HCN formation from L-histidine. The assay was performed as described previously [1], except that L-histidine was increased to 60 μmol per 3 ml reaction mixture (instead of 30 μmol) and the center trough of the Warburg vessel contained 0.2 ml 0.1 N NaOH. One unit of enzyme was defined as that amount which generates 1 μmol HCN per min (in O_2 and at 20°C).

Analytical methods

HCN determination. The HCN content of the base (from the center trough of the Warburg vessel) was determined by the method of Epstein [2] or Lambert [3].

Measurements of O_2 uptake. O_2 consumption was measured manometrically at 20°C. Some measurements were also made with a Gilson Oxygraph Model IC-OXY fitted with a Clark type electrode. Solutions were saturated with air and the reaction temperature was 20°C.

Protein determination. Protein was determined by a modification [4] of the method of Lowry et al. [23]. Bovine serum albumin (Serva, crystalline, purity grade reinst, >99.4%) was used as standard.

H_2O_2 determination. Hydrogen peroxide was determined without delay, with *o*-dianisidine and peroxidase as described by Bernt and Bergmeyer [5].

NH_4^+ determination. Ammonia was determined enzymically by measuring the

decrease of the absorbance of NADPH in the presence of excess α-ketoglutarate and glutamate dehydrogenase, as described by Da Fonseca-Wollheim [6].

Electrophoresis

Analytical disc gel electrophoresis of the native enzyme was performed at 4°C in 7% polyacrylamide gels in a Tris-barbital system [7]. No stacking or sample gels were used. Samples in 25% glycerol were applied directly to the resolving gels and overlayed with electrode buffer. Bromophenol blue was used as tracking dye. Gels were run at 1 mA/tube for about 15 min and then set at 4 mA/tube until the tracking dye front was close to the bottom of the gel.

Sodium dodecyl sulfate gel electrophoresis was performed as described by Weber and Osborn [8]. The subunit molecular weights of the standards were those given by Weber and Osborn [8]. The proteins which we used as molecular weight standards were: ribonuclease, 13 700; trypsin, 23 300; pepsin, 35 000; fumarase, 49 000; catalase, 60 000; bovine serum albumin, 68 000. The L-amino acid oxidase had a specific activity of 133 units/mg, and 16 μg protein were applied.

Molecular weight determination

The molecular weight of the native enzyme was determined with a Sephadex G-200 column (86.5 × 2.6 cm) equilibrated with 0.1 M potassium phosphate buffer, pH 7.0. The void volume was determined from the elution volume of Dextran blue 2000, run under identical conditions. The protein was applied in 0.5-ml sample and fractions of 4.6 ml were collected. The enzymes which we used as molecular weight standards, were: malate dehydrogenase, 70 000; lipoxygenase, 102 000; lactate dehydrogenase, 140 000; aldolase, 158 000. The L-amino acid oxidase had a specific activity of 133 units/mg and 33 μg were applied to the column.

Assay of proteins used for standards. The activities of malate dehydrogenase, lactate dehydrogenase and aldolase were estimated as described in the Boehringer Biochemica catalogue. Lipoxygenase activity was measured with an O_2 electrode as described by Christopher et al. [9].

Flavinanalysis

Identification of extracted flavin. Flavin was liberated by heating the enzyme in 0.01 M potassium phosphate buffer, pH 7.0, at 100°C for 12 min. Subsequently, the enzyme solution was cooled in ice and protein removed by centrifugation. The flavin was identified and estimated by titration of the apoprotein of the FAD-specific D-amino acid oxidase from mammalian kidney. The apoprotein was prepared according to Warburg and Christian [10]. A standard curve was produced by measuring the reactivation obtained with known amounts of FAD.

The total flavin content of the enzyme was measured fluorometrically after hydrolysis [11]. Aliquots of purified enzyme containing 0.2—0.6 nmol flavin were heated for 45 min at 100°C in 3.0 ml 0.1 N H_2SO_4. The pH was then adjusted to 4.6 by addition of 0.25 ml of 2.5 M sodium acetate. The volume was brought to 4.0 ml and the solution was clarified by centrifugation. Excitation of fluorescence was at 434 nm and emission was measured at 530 nm. A riboflavin

standard was run in parallel with enzyme solutions. The concentration of riboflavin in the standard was determined from absorbance measurements at 268, 374 and 448 nm.

To determine FAD fluorimetrically, flavin was first extracted for 15 min with 10% trichloroacetic acid at 0°C [11]. One aliquot of the extract was neutralized immediately with 0.25 vol. 4 M K_2HPO_4 while a second aliquot was neutralized after 48 h incubation in the dark at room temperature. Fluorescence was then measured on both samples. The fluorescence of the unhydrolyzed sample was 13% of the fluorescence of the hydrolyzed sample.

Metal analysis

The metal content (Fe, Cu and Mn) of the enzyme was determined with an atomic absorption spectrophotometer from Zeiss, Model FMD 4. $(NH_4)_2Fe(SO_4)_2 \cdot 6\ H_2O$, $CuSO_4$ and $MnSO_4 \cdot H_2O$ were used as standards.

Results

Purification of L-amino acid oxidase from A. nidulans

The enzyme was purified from extracts of *A. nidulans* broken by a Ribi Press. Treatment of the Ribi extract including DNAase and RNAase treatments and ammonium sulfate precipitation was the same as reported previously [1], but the protamine sulfate step was omitted. The pellet fraction which was obtained after the centrifugation following DNAase and RNAase treatment, and the material which precipitated between 0—20% saturation with $(NH_4)_2SO_4$, contained some green particles and retained approx. 20% of the original L-amino acid oxidase activity. These two fractions were refractionated with $(NH_4)_2SO_4$ which increased the recovery by another 10%.

Column chromatography. The combined protein fractions which precipitated between 30—55% $(NH_4)_2SO_4$ saturation were dialyzed against 20 mM sodium acetate buffer, pH 5.5. After being clarified by centrifugation, the dialyzed protein solution was divided into 3 portions and chromatographed on 3 separate CM-Sephadex columns (4 × 28 cm) which had been equilibrated with 20 mM sodium acetate buffer, pH 5.5. Elution was with a linear gradient from 0 to 0.3 M NaCl in 20 mM sodium acetate buffer, pH 5.5. The total volume of the gradient was 600 ml. The most active fractions from all 3 columns were combined and dialyzed against 20 mM potassium phosphate buffer, pH 7.0. The protein sample was then chromatographed on a DEAE-Sephadex column (3 × 28 cm) equilibrated with 20 mM potassium phosphate buffer, pH 7.0. Elution was with a linear phosphate gradient which ranged from 20 to 200 mM potassium phosphate buffer, pH 7.0. The total volume of the gradient was 600 ml. The most active fractions were combined, dialyzed against 5 mM potassium phosphate buffer, pH 7.0, and then subjected to chromatography on a hydroxyapatite column (2 × 10 cm) which had been equilibrated with 5 mM phosphate buffer, pH 7.0. Elution was again with a 200 ml linear phosphate gradient which ranged from 5 to 100 mM potassium phosphate buffer, pH 7.0. The most active fractions were combined, dialyzed against 20 mM potassium phosphate buffer, pH 7.0, and then subjected to chromatography on a second DEAE-Sephadex column (2 × 15 cm) which was equilibrated

with 20 mM potassium phosphate buffer, pH 7.0. Elution was carried out with a linear gradient of equal volumes of 20 and 100 mM potassium phosphate buffer, pH 7.0. The total volume of the gradient was 300 ml.

Comments on purification

The enzyme activity was measured in two different ways during the entire purification procedure: (1) O_2 uptake was measured with L-arginine as substrate in the presence of catalase; (2) HCN formation from L-histidine was determined with enzyme and added peroxidase. Typical results of the purification of the L-amino acid oxidase from *A. nidulans* are summarized in Table I. After the last DEAE-Sephadex column a specific activity of 120 units/mg was obtained in the oxidative deamination reaction with L-arginine as substrate and a specific activity of 1.4 units/mg in the HCN-generating test with L-histidine. The ratio of these two activities remained constant after the $(NH_4)_2SO_4$ step and was approx. 90. The overall yield was about 10%. Measurements of the enzyme activity in crude extracts were not very accurate, since crude extracts consumed small amounts of HCN and catalyzed a nonspecific O_2 uptake with various amino acids. However, after the $(NH_4)_2SO_4$ step, the two assays showed good reproducibility and linearity with enzyme concentration over a 20-fold range. The two fractions with highest specific activity from the last DEAE-Sephadex column were dialyzed against 1 l of 20 mM potassium phosphate buffer (pH 7.0) containing 1 mM EDTA, and three times against 1 l 10 mM potassium phosphate buffer, pH 7.0. The sample was then concentrated in an Amicon Model 12 ultrafiltration cell equippped with a PM 10 membrane.

Purity of enzyme

One major protein-staining band was detected after polyacrylamide gel electrophoresis of the purified enzyme at 4° C in a Tris-barbital buffer system (pH 7.0). Below the major band, there was a very light and diffuse minor band

TABLE I

PURIFICATION OF L-AMINO ACID OXIDASE FROM *ANACYSTIS NIDULANS*

	Protein (mg, total)	O_2 uptake (L-Arg)		HCN formation (L-His)		Ratio: O_2 uptake (L-Arg) to HCN formation (L-His)
		Total units	Spec. act. (units/mg)	Total units	Spec. act. (units/mg)	
French-press extract	208 760	2757	0.013	16.58	$7.9 \cdot 10^{-5}$	166
Ammonium sulphate prec. 30—55% saturation	34 207	1597	0.047	21.13	$6.2 \cdot 10^{-4}$	76
CM-Sephadex column	1 109	1169	1.05	12.41	$1.1 \cdot 10^{-2}$	94
DEAE-Sephadex column No. 1	60	563	9.38	5.69	0.095	99
Hydroxyapatite column	11.9	456	38.32	4.70	0.395	97
DEAE-Sephadex column No. 2	1.48	178	120.27	2.13	1.439	84

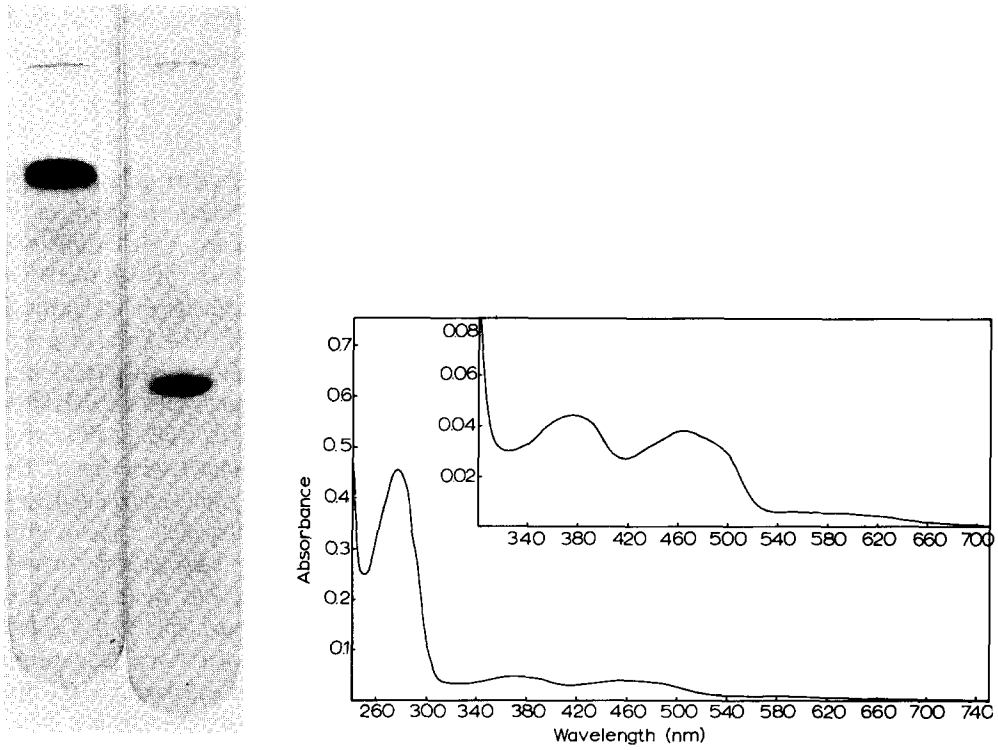

Fig. 1. Electrophoresis of purified L-amino acid oxidase. Left: Electrophoresis of 16 μg enzyme (specific activity 133 units/mg) on 7% polyacrylamide gel in Tris-barbital buffer system, pH 7.0 at 4° C [7]. Right: SDS gel electrophoresis of 16 μg enzyme (specific activity 133 units/mg) on 8% polyacrylamide gel according to the method of Weber and Osborn [8]. Details of the electrophoresis procedure are given in Materials and Methods.

Fig. 2. Absorption spectrum of purified L-amino acid oxidase. The enzyme was in 0.01 M potassium phosphate buffer, pH 7.0, the protein concentration was 0.31 mg/ml (specific activity 133 units/mg). The absorbance at 275 nm was 0.459, at 462 nm 0.042, and at 376—368 nm 0.045. The spectrum was recorded in a 1-cm cuvette (4 mm wide) with a Zeiss recording spectrophotometer Type DM 4.

which amounted to less than 5% of the major band (Fig. 1). One protein-staining band was also detected after SDS-polyacrylamide gel electrophoresis (Fig. 1) at a position corresponding to a subunit molecular weight of 49 000.

Spectral properties

The purified L-amino acid oxidase from *Anacystis* had a typical flavin absorption spectrum (Fig. 2), with maxima at 462—468, 368—376 and 275 nm. The ratio of absorbances at 275 and 462 nm was 10.9. For the crystalline L-amino acid oxidase from rat kidney mitochondria a ratio of 6.5 has been reported [12] and for the crystalline D-amino acid oxidase from pig kidney a ratio of 9.5—10 [13]. Anaerobic reduction of the enzyme by the substrate L-arginine led to rapid bleaching of the flavin absorbance bands (not shown).

Characterization of the prothetic group

The results of the prosthetic group analysis of the L-amino acid oxidase from 2 different preparations are presented in Table II. The flavin of the L-amino acid oxidase has been identified as FAD by titration with the FAD-specific apoprotein from kidney D-amino acid oxidase and by fluorescence measurements before and after hydrolysis. The latter measurements showed that the flavin was all FAD; no FMN was present. The results in Table II provide evidence that 1 mol enzyme contains 1 mol FAD.

Attempts to resolve the FAD and apoprotein reversibly have not been successful, either with acid $(NH_4)_2SO_4$ [10] or with $CaCl_2$ [14].

We could not detect iron in the purified enzyme. Nor did we find copper or manganese. To answer the question whether any other metal might be present, will require further experiments. So far all amino acid oxidases which have been characterized, contain only flavin and no metal [13].

Molecular weight determinations

The molecular weight of the native enzyme was determined by gel filtration on a Sephadex G-200 column and was calculated from the relationship between the logarithms of the molecular weights and elution volumes. Four proteins of known molecular weight are used as standards. This method resulted in a molecular weight of 98 000 for the L-amino acid oxidase from *A. nidulans* (average of 3 determinations). Electrophoresis of the purified enzyme on sodium dodecyl sulfate polyacrylamide gels gave one band which corresponded to a molecular weight of 49 000 (average of 3 determinations).

This indicates that the enzyme consists of 2 subunits of equal molecular weight.

Enzyme activities of Anacystis L-amino acid oxidase

Substrate specificity. The substrate specificity of the highly purified enzyme was identical to that previously reported [1]. The enzyme oxidized L-arginine, L-lysine, L-ornithine and to a lesser extent L-histidine. Other naturally occurring amino acids were not oxidized or only to a much lower extent

TABLE II

FLAVIN ANALYSIS

The flavin content of the purified L-amino acid oxidase was determined by three different methods. Expt. 1: FAD released by heat treatment and determined by reconstitution of activity with apoprotein from D-amino acid oxidase from kidney. Expt. 2: Fluorimetric determination after treatment of the enzyme with 0.1 N H_2SO_4; Expt. 3: Fluorimetric determination after treatment of the enzyme with trichloroacetic acid. Details of each method are given in the text. Enzyme from two separate purifications with specific activity of (a) 121 units/mg or (b) 133 units/mg was used.

Expt.	Protein (nmol)	FAD found (nmol)	mol FAD per mol enzyme
1 b	0.30	0.27	0.9
2 a	0.34	0.29	0.9
2 b	0.61	0.56	0.9
3 a	0.34	0.27	0.8
3 b	0.30	0.24	0.8

(below 0.1% of the activity observed with L-arginine).

Stoichiometry of O_2 uptake and NH_4^+ formation. The results in Table III show the stoichiometry of O_2 consumption and ammonia production in the presence and absence of catalase. These reactions were run to completion with small amounts of substrate. In each case 1 mol NH_3 was formed and 1 mol O_2 consumed for 1 mol amino acid oxidized in the absence of catalase. When catalase was added, the O_2 consumption was reduced to approx. 50% as expected. In the absence of added catalase some H_2O_2 accumulated. Normally, the H_2O_2 is utilized for the nonenzymic decarboxylation of the ketoacid. In the present case the formation of cyclic products may remove the ketoacid, as shown by Boulanger and Osteux [15] for the turkey liver L-amino acid oxidase which is also specific for basic L-amino acids.

Effect of peroxidase on O_2 uptake and NH_4^+ formation. With L-histidine as a substrate, in the absence of peroxidase, the reaction was also that of a simple oxidative deamination, with O_2 consumption equivalent to half the NH_4^+ production in the presence of catalase. Addition of peroxidase caused a marked increase in O_2 consumption, a decline in the ammonia production and substantial HCN formation (Table IV). HCN was formed only from L-histidine, but not from any of the other three amino acid substrates. This observation is in agreement with results obtained with other amino acid oxidases supplemented with peroxidase [16].

Reaction characteristics

Oxidative deamination. In HEPES buffer, O_2 consumption was highest at pH 7.0. In other buffers, the pH optimum was shifted slightly. The velocity of the reaction showed the expected dependence on O_2 concentration. Almost maximum activity was reached at about 50% O_2, with saturating concentration of L-arginine. At lower arginine concentrations, 20% O_2 was almost saturating (not shown). When the activity was measured as a function of L-arginine concentration, the usual hyperbolic saturation curve was obtained, with a K_m value

TABLE III

STOICHIOMETRY OF O_2 UPTAKE AND NH_4^+ FORMATION

The reactions were carried out in Warburg vessels, with 2 side arms and a center trough in an atmosphere of O_2, as described in the text. The center trough contained 0.2 ml 0.2 N NaOH. The reaction mixture contained in a total volume of 3 ml: 200 μmol Hepes buffer, pH 7.0, 3 μg (for arginine and lysine experiments) or 6 μg (for ornithine experiments) L-amino acid oxidase (specific activity 133 units/mg), 0.1 mg catalase and amino acids as indicated. The O_2 uptake was measured manometrically and the reaction was run to completion, after which 0.1 ml 4 N H_2SO_4 was tipped from a second side arm and NH_4^+ and H_2O_2 were determined.

Amino acid	Amount (μmol)	Catalase added	O_2 taken up (μmol)	NH_4^+ formed (μmol)	H_2O_2 found (μmol)
L-Arg	10	—	9.8	9.5	1.9
L-Arg	10	+	5.0	9.8	0
L-Lys	10	—	9.3	9.6	1.9
L-Lys	10	+	5.1	9.9	0
L-Orn	5	—	4.7	4.9	2.6
L-Orn	5	+	2.5	5.2	0

TABLE IV

EFFECT OF PEROXIDASE ON O_2 UPTAKE AND NH_4^+ FORMATION

The reactions were carried out in Warburg vessels, with 2 side arms and a center trough, in an atmosphere of 100% O_2, as described in Materials and Methods. The reaction mixtures contained in a total volume of 3 ml: 200 μmol potassium phosphate buffer, pH 6.5, 50 μmol KCl, 30 μmol L-histidine, 10 μg L-amino acid oxidase (specific activity) 82 units/mg), 100 μg peroxidase and 100 μg catalase as indicated. The reaction time was 4 h.

Additions	O_2 taken up (μmol	NH_4^+ formed (μmol)	HCN formed (μmol)
L-His	1.4	1.5	0.001
L-His + catalase	0.9	1.7	0.003
L-His + peroxidase	2.2	1.3	0.438
L-His + peroxidase + catalase	1.7	1.4	0.411

of about $5 \cdot 10^{-3}$ M. The requirement of high amino acid concentration and high O_2 concentration for maximal activity are properties typical of amino acid oxidases.

HCN forming reaction. The pH optimum for the HCN forming reaction with L-histidine as the amino acid substrate was between pH 6.0 and 6.5 in potassium phosphate. The initial reaction rate (30 min period) was faster in phosphate buffer than in HEPES, MES, Tricine or borate buffer. The K_m value for L-histidine was about $1 \cdot 10^{-2}$ M and the HCN yield was greater in 100% O_2 than in air.

Effect of inhibitors

Table V shows the effect of a variety of possible inhibitors on the O_2

TABLE V

EFFECT OF INHIBITORS

The O_2 uptake was measured with an O_2 electrode under standard conditions, as described under Materials and Methods.

Inhibitor	Concentration (M)	Inhibition (%)
NaF	$1 \cdot 10^{-1}$	0
NaN_3	$1 \cdot 10^{-1}$	77
o-Phenanthroline	$5 \cdot 10^{-3}$	72
m-Phenanthroline	$5 \cdot 10^{-3}$	9
Bathophenanthroline disulfonic acid	$5 \cdot 10^{-2}$	20
α,α-Bipyridyl	$1 \cdot 10^{-2}$	0
8-Hydroxyquinoline	$1 \cdot 10^{-3}$	0
4,5-Dihydroxy-*m*-benzene disulfonic acid	$1 \cdot 10^{-2}$	2
α-Naphthol	$1 \cdot 10^{-2}$	53
Nordihydroguaretic acid	$5 \cdot 10^{-4}$	63
Salicylhydroxamic acid	$1 \cdot 10^{-2}$	2
Sodium diethyldithiocarbamate	$1 \cdot 10^{-3}$	0
NaCN	$1 \cdot 10^{-2}$	0
EDTA	$1 \cdot 10^{-2}$	0
p-Chloromercuribenzoate	$1 \cdot 10^{-3}$	7
Hydroxylamine	$1 \cdot 10^{-3}$	3
C_6H_5COONa	$1 \cdot 10^{-2}$	0
p-Aminobenzoic acid	$1 \cdot 10^{-2}$	0

consumption with arginine as a substrate. The enzyme is not inhibited by sodium benzoate or aminobenzoic acid which are effective inhibitors of the better known amino acid oxidases. Nordihydroguaretic acid and α-naphthol are inhibitory, however, and so is *o*-phenanthroline as previously reported [1]. This compound caused 72% inhibition at $5 \cdot 10^{-3}$ M, compared to 9% inhibition by *m*-phenanthroline at the same concentration. Bathophenanthroline disulfonic acid gave 20% inhibition at $5 \cdot 10^{-2}$ M; α,α-bipyridyl and 8-hydroxyquinoline had no effect.

All divalent cations tested inhibited enzyme activity. Table VI lists these in the order of decreasing effectiveness in the oxidative deamination assay (with L-arginine as substrate) and shows the concentration at which 50% inhibition was observed. Monovalent cations such as Na^+, K^+ or NH_4^+ or the trivalent cation Al^{3+} had either no effect of inhibited only slightly (<10%) at concentrations up to 10^{-2} M. Except for Cu^{2+}, all the cations tested also inhibited HCN production from L-histidine in the presence of amino acid oxidase and peroxidase. The quantitative differences in inhibition of the two assay systems are probably due to differences in the complex formation between the cation and the two different substrates: histidine and arginine. The effect of Cu^{2+} was complex. At $6 \cdot 10^{-5}$ M it caused no or only slight inhibition of HCN production, but higher concentrations became stimulatory (up to 25%) and then above 10^{-3} M $CuSO_4$, an inhibition was again observed. The reasons for this behavior are not clear.

The kinetics of the inhibition of arginine oxidation by divalent cations was complex. Fig. 3A shows Lineweaver-Burk plots of the reaction with and

TABLE VI

EFFECT OF DIVALENT CATIONS

O_2 uptake was measured with an O_2 electrode under standard assay conditions as described in Materials and Methods. 4.5 μg L-amino acid oxidase (specific activity 22 units/mg) were used. O_2 uptake with L-arginine as substrate was 0.10 μmol O_2/min. The reactions leading to HCN formation were carried out in Warburg vessels with 2 side arms and a center trough, in an atmosphere at O_2, as described in Materials and Methods. The reaction mixture contained in a total volume of 3 ml: 200 μmol Hepes buffer, pH 7.0, 60 μmol L-histidine, 1.4 μg L-amino acid oxidase (specific acticity 84 units/mg), 100 μg peroxidase and the salts as indicated.

	Concentration (M)	Inhibition (%)	
		O_2 uptake (L-Arg)	HCN formation (L-His)
$CuSO_4$	$5.9 \cdot 10^{-5}$	50	0
$ZnSO_4$	$6.5 \cdot 10^{-5}$	50	41
$CdCl_2$	$1.5 \cdot 10^{-4}$	50	66
$MnCl_2$	$1.6 \cdot 10^{-4}$	50	93
$CoSO_4$	$3.8 \cdot 10^{-4}$	50	19
$MgCl_2$	$1.0 \cdot 10^{-3}$	50	80
$CaCl_2$	$1.6 \cdot 10^{-3}$	50	94
$BaCl_2$	$1.8 \cdot 10^{-3}$	50	87
$NiSO_4$	$3.4 \cdot 10^{-3}$	50	59
$SrCl_2$	$4.6 \cdot 10^{-3}$	50	93
$(NH_4)_2Fe(SO_4)_2$ *	$1 \cdot 10^{-3}$	—	94

* $(NH_4)_2Fe(SO_4)_2$ was not tested in the oxidative deamination assay, because Fe^{2+} is autooxidizable and gives an O_2 uptake by itself.

without different concentrations of $MgCl_2$. The results of a similar set of measurements with and without $ZnSO_4$ are shown in Fig. 3B. The inhibition clearly decreases with increasing substrate concentration, but is apparently partly noncompetitive.

As expected, a chelator which binds Mg^{2+} can relieve the inhibition of arginine oxidation by Mg^{2+}. EDTA can thus cause an apparent stimulation of arginine oxidation when divalent cations are present in the reaction mixture, as may be the case in crude extracts. It is of greater interest from a physiological point of view, that ATP and ADP can also relieve the Mg^{2+}-induced inhibition of arginine oxidation. Table VII shows these effects of EDTA, ATP and ADP, tested with partially purified enzyme plus Mg^{2+}. The table also shows that AMP did not have such an effect.

As reported previously [1], the O_2 uptake of intact *A. nidulans* cell suspensions can be strongly stimulated by added basic amino acids. These experiments were made with cell suspensions in dilute phosphate buffer. Use of the complete inorganic growth medium for preparation of the cell suspensions resulted in very diminished effects of added basic amino acids on O_2 consumption (Pistorius, E.K., Voss, H., Jetschmann, K. and Vennesland, B., unpublished).

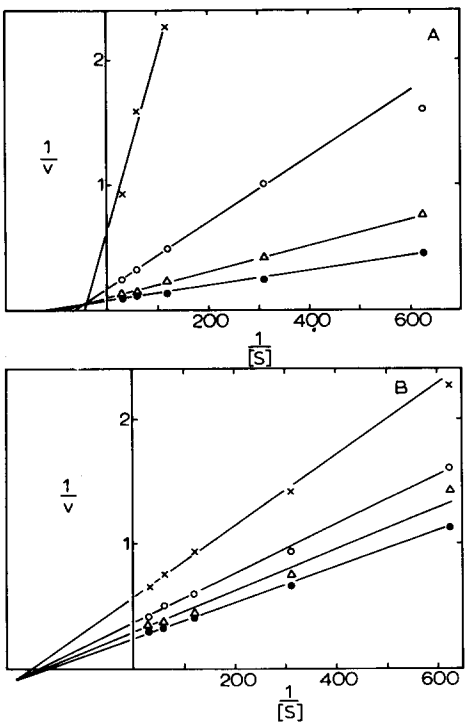

Fig. 3. Kinetics of inhibition by $MgCl_2$ or $ZnSO_4$. O_2 uptake was measured with an O_2 electrode under standard assay conditions as described in Materials and Methods. 2.6 or 4.5 μg L-amino acid oxidase were used (specific activity 25 or 22 units/mg). The L-arginine and metal content was varied. (A) ●———●, No $MgCl_2$; △———△, $5.4 \cdot 10^{-4}$ M $MgCl_2$; ○———○, $1.1 \cdot 10^{-3}$ M $MgCl_2$; X———X, $2.7 \cdot 10^{-3}$ M $MgCl_2$; (B) ●———●, No $ZnSO_4$; △———△, $2.7 \cdot 10^{-5}$ M $ZnSO_4$; ○———○, $5.4 \cdot 10^{-5}$ M $ZnSO_4$; X———X, $1.1 \cdot 10^{-4}$ M $ZnSO_4$.

TABLE VII

EFFECT OF EDTA AND ADENINE NUCLEOTIDES ON THE Mg^{2+}-INHIBITED OXIDATION OF ARGININE

O_2 uptake was measured with an O_2 electrode under standard assay conditions as described in Materials and Methods. 4.5 μg L-amino acid oxidase (specific activity 22 units/mg) were used. O_2 uptake with L-arginine and no other additions was 0.10 μmol O_2/min. Addition of 30 μmol EDTA, 20 μmol ATP or ADP or 40 μmol AMP alone had no effect on the O_2 uptake. Volume of reaction mixture was 1.85 ml.

Additions	Inhibition (%)
5 μmol $MgCl_2$; no addition	88
+3 μmol EDTA	56
+5 μmol EDTA	0
+5 μmol ATP	23
+10 μmol ATP	8
+10 μmol ADP	43
+20 μmol ADP	17
+40 μmol AMP	84

This is at least partly due to the divalent cations present in the complete growth medium. These results will be described elsewhere.

Discussion

The basic amino acid oxidase here described is in some respects a typical amino acid oxidase. Its molecular weight was estimated to be 98 000, and it contains two subunits of equal molecular weight (49 000). Since it contains only one molecule of FAD per molecule of enzyme, the results suggest that the FAD may be located between the two subunits, or that only one of the subunits is active catalytically. We can not exclude the possibility that some of the flavin was lost during purification, but the residual flavin seemed to be quite firmly bound, as indicated by the difficulty we experienced in removing flavin without denaturation of the apoenzyme. The possibility of the presence of a metal ion, which was suggested by the observed *o*-phenanthroline inhibition has not been excluded entirely and requires further study.

The turnover number of the *Anacystis* enzyme was calculated to be 26 000 mol L-arginine oxidized per mol enzyme per min, in air at 20°C. This value compares very favorably with the value of 2000 for kidney D-amino acid oxidase, 7000 for snake venom L-amino acid oxidase or 6 for L-amino acid oxidase from kidney mitochondria [12,17]. All of these three other amino acid oxidases have been shown to contain 2 molecules of flavin per molecule of enzyme [13], but the flavin in the L-amino acid oxidase from kidney mitochondria has been shown to be FMN instead of FAD, as in the others [12]. In connection with turnover number, it should be noted that the substrate specificity of the basic amino acid oxidase from *Anacystis* is a relative thing. If one uses massive amounts of enzyme, oxidation of nonbasic amino acids can also be detected. A complete study of the specificity of the purified *Anacystic* enzyme in the activity range of turnover numbers below 50 has not yet been made, but the enzyme can in fact oxidize L-leucine, for example, with a turnover number of about 11. Leucine is reported to be the best substrate for

the mitochondrial L-amino acid oxidase with a turnover number of 6 [12]. Thus, the *Anacystis* enzyme oxidizes L-leucine about as effectively as the mitochondrial enzyme. It is possible, but by no means certain, that the basic amino acid oxidase may be responsible for the low amino acid oxidase activity for L-phenylalanine and L-tyrosine, which has been detected in thylakoids of *A. nidulans* by Löffelhardt [18].

The most unusual characteristic of the *Anacystis* L-amino acid oxidase here described is its inhibition by divalent cations. This inhibition is probably related to the high preference of the enzyme for L-amino acids containing a basic R-group. The fact that the inhibition can be relieved by ADP and ATP, but not by AMP, and that both inhibition and relief of inhibition can be observed in a physiological concentration range, make the divalent cation effect particularly interesting. This problem has only been approached in the present paper and obviously requires far more detailed study. In our previous paper [1], we presented evidence with *o*-phenanthroline that the L-amino acid oxidase was functional in vivo. Similar evidence could be obtained with the divalent cations.

Scientific literature abounds with examples of divalent cation effects on respiration. Potent inhibitory effects of Zn^{2+} and other cations (Cd^{2+}, Ni^{2+}, Cu^{2+} and Co^{2+}) have been observed on mitochondrial respiratory systems [19—21], as well as on the respiration system of *Azotobacter* [22]. Though the site of inhibition seems to have been located with respect to the known components of the respiratory chain, no clear identification has been made of the inhibited reaction. One might consider the possibility of a basic amino acid oxidase of the type described here being involved.

Acknowledgements

We are deeply grateful to Professor Birgit Vennesland for her generous encouragement and help. We wish to thank Dr. G. Offer of the Institut für Gärungsgewerbe und Biotechnologie, Berlin, for the use of his atomic absorption spectrophotometer. This work was supported in part by a grant from the Deutsche Forschungsgemeinschaft.

References

1 Pistorius, E.K., Jetschmann, K., Voss, H. and Vennesland, B. (1979) Biochim. Biophys. Acta 585, 630—642
2 Epstein, J. (1947) Anal. Chem. 19, 272—274
3 Lambert, J.L., Ramasamy, J. and Paukstelis, J.V. (1975) Anal. Chem. 47, 916—918
4 Bailey, J.L. (1967) Techniques in Protein Chemistry, 2nd edn., p. 340, Elsevier, Amsterdam
5 Bernt, E. and Bergmeyer, H.U. (1970) Methoden der enzymatischen Analyse, 2nd edn. p. 2165, Bd. II, Verlag Chemie, Weinheim
6 Da Fonseca-Wollheim, F. (1973) Z. Klin. Chem. Klin. Biochem. 11, 421—426
7 Williams, D.E. and Reisfeld, R.A. (1964) Ann. N.Y. Acad. Sci. 121, 373—381
8 Weber, K. and Osborn, M. (1969) J. Biol. Chem. 244, 4406—4412
9 Christopher, J., Pistorius, E. and Axelrod, B. (1970) Biochim. Biophys. Acta 198, 12—19
10 Warburg, O. and Christian, W. (1938) Biochem. Z. 298, 150—165
11 Koziol, J. (1971) Methods Enzymol. 28, Part B, 253—285
12 Nakano, M. and Danowski, T.S. (1966) J. Biol. Chem. 241, 2075—2083
13 Bright, H.J. and Porter, D.J.T. (1975) in The Enzymes (Boyer, P.D., ed.), 3rd edn., Vol. XII, pp. 421—505, Academic Press, New York

14 Bookjans, G., San Pietro, A. and Böger, P. (1978) Biochem. Biophys. Res. Commun. 80, 759—765
15 Boulanger, P. and Osteux, R. (1956) Biochim. Biophys. Acta 21, 552—561
16 Pistorius, E.K., Gewitz, H.-S., Voss, H. and Vennesland, B. (1977) Biochim. Biophys. Acta 481, 384—394
17 Meister, A. (1965) Biochemistry of the Amino Acids, 2nd edn., Vol. I, pp. 294—319, Academic Press, New York
18 Löffelhardt, W. (1977) Z. Naturforsch. 32c, 345—350
19 Skulachev, V.P., Chistyakov, V.V., Jasaitis, A.A. and Smirnova, E.G. (1967) Biochem. Biophys. Res. Commun. 26, 1—6
20 Nicholls, P. and Malviya, A.N. (1968) Biochemistry 7, 305—310
21 Kleiner, D. (1974) Arch. Biochem. Biophys. 165, 121—125
22 Kleiner, D. (1978) FEBS Lett. 96, 364—366
23 Lowry, O.H., Rosebrough, H.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265—275