Arginine deiminase from *Halobacterium salinarium*

Purification and properties

Gudrun M. MONSTADT and A. W. HOLLDORF*

Institut für Physiologische Chemie, Ruhr-Universität Bochum, Bochum, Germany

Arginine deiminase from the extreme halophilic archaeobacterium *Halobacterium salinarium* was purified to homogeneity in a four-step procedure with a 310-fold enrichment. The enzyme consists of two identical subunits of 55 kDa; its native molecular mass is 105 kDa. The pI of 4.7 indicates the acidic nature of the protein, which is evidenced by its amino acid composition, which shows an excess of more than 15% of acidic amino acids. The N-terminal amino acid of the enzyme is lysine. Arginine deiminase from *Halobacterium salinarium* exhibits its highest catalytic activity in the presence of 3.5 M-NaCl, pH 7.6, and at 40 °C. The half-activity constant, K_s , for arginine is 3.1 mM. The enzyme is inhibited by ornithine.

INTRODUCTION

Halobacteria are extreme halophilic organisms which inhabit brines containing concentrations of NaCl up to 5.2 M [1,2]. Phylogenetically they belong to the Archaeobacterial Kingdom, whose members live in extreme environments and show unusual properties [3]. The nutrition of halobacteria varies somewhat between different strains. *Halobacterium salinarium*, *H. cutirubrum* and *H. halobium* require amino acids as their carbon source, whereas *H. vallismortis* and *H. mediterranei* are able to grow on carbohydrates. To provide metabolic energy, halobacteria possess three energy-transducing pathways: oxidative phosphorylation, photophosphorylation, which has been well studied [4], and the fermentative arginine deiminase (ADI) pathway. Although the light-energy-converting chromoprotein bacteriorhodopsin of the photophosphorylation system is synthesized only under conditions of limited oxygen supply, Hartmann *et al.* [5] demonstrated that a continued growth of halobacteria by photophosphorylation in complete absence of oxygen is not possible. Those authors proved instead an anaerobic growth in complete darkness in the presence of the amino acid arginine. Dundas & Halvorson [6] have shown that decomposition of arginine in halobacteria occurs exclusively via the ADI pathway. The energy-providing pathway consists of three enzyme-catalysed reactions. ADI (EC 3.5.36) degrades arginine to citrulline; in the second step, citrulline is cleaved by ornithine transcarbamoylase (OTCase, EC 2.1.3.3) to ornithine and carbamoyl phosphate. Carbamate kinase (EC 2.7.2.2) catalyses the formation of ATP from carbamoyl phosphate and ADP. The ADI pathway is distributed widely among eukaryotes, eubacteria and with halobacteria in the Archaeobacterial Kingdom too [7,8]. In several organisms only ADI was described [7]. A peptidyl-ADI cleaving exclusively peptide-bound arginine to citrulline was found in mammalian tissues [9–11]. Of the archaeobacterial ADI pathway, only OTCase from *Halobacterium salinarium* has been isolated and characterized [12,13]. Here the purification and characterization of the first enzyme of this energy-providing pathway, ADI, is reported.

MATERIALS AND METHODS

Materials

All chemicals used were of analytical grade. L-Arginine, L-

citrulline, BSA and dansylated amino acid standards were from Sigma Chemical Co., hydrogen-bonding material HB1 from Whatman; other chromatographic materials were from Pharmacia. Marker proteins were purchased from Boehringer Mannheim. Bicine choninic acid (BCA) Protein Assay Reagent was from Pierce.

Bacterial strains

Halobacterium cutirubrum was kindly provided by Dr. A. T. Matheson, National Council of Canada, Division of Biological Sciences, Ottawa, Canada. Other strains used were purchased from the American Type Culture Collection: *H. salinarium* (A.T.C.C. 19700), *H. halobium* (A.T.C.C. 29841), *H. vallismortis* (A.T.C.C. 29715) and *H. mediterranei* (A.T.C.C. 33500).

Media and growth conditions

To purify ADI, *H. salinarium* was grown aerobically in 8 litres complex medium [14] at 43 °C for 40 h in a Biostat fermenter (Braun). Cells were harvested by centrifugation; the yield was 50 g wet weight. For quantitative determination of utilization of amino acids during the growth of *H. salinarium*, a chemically defined medium [15], without CMP, containing 5 g of L-glutamine/litre, was used. Cell growth was measured by absorbance at 580–640 nm.

For quantitative determination of ADI activity in crude extracts of other halobacterial strains, cells were grown in complex media, as described previously [16–19], and harvested by centrifugation.

Purification of the arginine deiminase

All steps of the purification of the ADI were carried out at room temperature unless otherwise stated. Ultrafiltration of enzyme fractions was done with a stirred cell (Amicon Corp.) fitted with a PM 30 membrane filter and operated under N_2 pressure. *H. salinarium* cells (80 g wet weight) were suspended in 300 ml of 50 mM-Tris buffer, pH 7.6, containing 3 M-NaCl (buffer 1). The suspension was twice sonicated in an ice bath (5 min at 70 W) and centrifuged at 40000 g for 20 min at 4 °C. A saturated solution of $(NH_4)_2SO_4$ was added dropwise to the crude extract, resulting in salt concentrations of 1.9 M-sulphate and 2 M-NaCl. This suspension was applied to a column (6.6 cm × 17.0 cm) of Sepharose CL-6B, previously equilib-

Abbreviations used: ADI, arginine deiminase; OTCase, ornithine transcarbamoylase; CK, carbamate kinase; BCA, bicinechonic acid; CTAB, cetyltrimethylammonium bromide.

* To whom correspondence and reprint requests should be addressed.

rated with buffer 2 [1.9 M-(NH₄)₂SO₄/2.0 M-NaCl/50 mM-Tris, pH 7.6]. The column was washed with 800 ml of buffer 2, followed by two antiparallel gradients of 1.9–1.0 M-(NH₄)₂SO₄ and 2.0–2.8 M-NaCl in 2000 ml of 50 mM-Tris, pH 7.6, at a flow rate of 3 ml/min. ADI was eluted at approx. 2.4 M-NaCl and 1.4 M-(NH₄)₂SO₄. Enzyme-containing fractions were pooled, and solid (NH₄)₂SO₄ was added until its concentration was 1.8 M. The solution was then applied to a HBI column (2.6 cm × 40.0 cm) equilibrated with buffer 3 [1.5 M-(NH₄)₂SO₄/2.4 M-NaCl/50 mM-Tris, pH 7.6]. The column was washed with 600 ml of buffer 3, followed by two antiparallel gradients of 1.8–1.0 M-(NH₄)₂SO₄ and 2.4–2.8 M-NaCl in 1200 ml of 50 mM-Tris buffer, pH 7.6, at a flow rate of 2 ml/min. ADI was eluted at approx. 1.2 M-(NH₄)₂SO₄ and 2.6 M-NaCl. The pooled enzyme fractions were concentrated 5-fold by ultrafiltration, washed twice with 30 ml of buffer 1 and applied to a column (1.8 cm × 30.0 cm) of hydroxyapatite (prepared as described by Levin [20]) equilibrated with the same buffer. ADI was eluted in a phosphate gradient from 0 to 500 mM-Na₂HPO₄ in 400 ml of buffer 1 with a constant pH of 7.6 at a flow rate of 0.5 ml/min at approx. 300 mM-phosphate. Pooled enzyme fractions were concentrated 3-fold by ultrafiltration, washed twice with buffer 1, twice with buffer 4 [buffer 1 containing 1.5 M-(NH₄)₂SO₄ and 0.13 M-Na₂HPO₄] and applied to a propyl-agarose column (1.0 cm × 30.0 cm). After washing with 30 ml of buffer 4, the enzyme was eluted with two antiparallel gradients of 0.13–0.50 M-Na₂HPO₄ and 1.5–0 M-(NH₄)₂SO₄ in 400 ml of buffer 1 at a flow rate of 0.5 ml/min. ADI was eluted at 0.4 M-phosphate and 0.5 M-sulphate. Pooled enzyme fractions were concentrated 10-fold by ultrafiltration, washed with buffer 5 (buffer 1 containing 0.5 M-Na₂HPO₄), and stored at room temperature.

Enzyme assay and protein determination

ADI activity was measured as the amount of citrulline formed as described by Oginsky [21] by the method of Archibald [22]. All enzyme assays were done in 3.5 M-NaCl, 14 mM-L-arginine, 50 mM-Tris, pH 7.6, at 37 °C, unless otherwise stated. Enzyme activity in column fractions was assayed directly in the eluate. Enzyme activities are expressed in katal (kat). Protein concentrations were determined by the method of Smith *et al.* [23], with the BCA Protein Assay Reagent (Pierce). Protein samples were precipitated with trichloroacetic acid and centrifuged. The supernatant was discarded and the protein dissolved in 0.5 M-NaOH. Solutions of BSA in water were used as standard.

Gel electrophoresis

PAGE under denaturing conditions was performed in the presence of anionic (SDS) and cationic (cetyltrimethylammonium bromide, CTAB) detergents. SDS/PAGE was performed in Tris/glycine buffer, pH 8.8, on 15% (g/ml) slab gels as described by Laemmli [24]. CTAB/PAGE was performed in Na₂HPO₄/NaH₂PO₄ buffer, pH 7.0, on 7.5 and 10% (w/v) slab gels as described by Eley *et al.* [25]. During the electrophoresis at 400 V and 60 mA for 8 h, the buffer of the anode was changed three times. Protein samples for PAGE were desalted by precipitation with trichloroacetic acid. After washing the protein sediment with acetone and re-centrifugation, the sediment was dissolved in a buffer solution. For SDS/PAGE, the Tris/glycine buffer, pH 8.8, contained 1% (w/v) SDS, 1% (w/v) 2-mercaptoethanol and Bromophenol Blue and was heated in a boiling-water bath for 5 min. For CTAB/PAGE the protein sediment was dissolved in 0.01 M-NaHPO₄/NaH₂PO₄ buffer, pH 7.0, containing 1% (w/v) CTAB and 1% (w/v) 2-mercaptoethanol and Malachite Green, and treated as indicated above. After electrophoresis the CTAB gels were fixed in boiling 10% (w/v) trichloroacetic acid

and stained with a solution of Coomassie Brilliant Blue R-250 (Serva) in 45% (v/v) ethanol/10% (v/v) acetic acid at 40 °C for 30 min and destained with 7.5% (v/v) acetic acid/30% (v/v) ethanol overnight. Molecular-mass determinations were performed by SDS/ and CTAB/PAGE. Proteins used as molecular-mass standards were fumarase (49 kDa), glutamate dehydrogenase (53 kDa), pyruvate kinase (59 kDa), BSA (68 kDa), transferrin (80 kDa), and phosphorylase *b* (94 kDa).

Gel filtration

The molecular mass of the native enzyme was determined by gel filtration on Sephacryl S-300 (1.6 × 90.0 cm) in two buffers of 50 mM-Tris (pH 7.6), containing 3 M-sodium chloride, 0.5 M-sodium hydrogen phosphate or 3.5 M-potassium chloride respectively. The flow rate was 1 ml/3 min. The elution of the enzyme was identified by measuring the catalytic activity. Marker proteins used were thyroglobulin (669 kDa), β -amylase (200 kDa), aldolase (160 kDa), BSA (68 kDa) and ovalbumin (43 kDa).

Amino acid analysis

Samples of 0.5 nmol of enzyme were desalted by dialysis, followed by freeze-drying and hydrolysis under N₂ with 6 M-HCl at 110 °C for 15, 24, 37 and 45 h. The vacuum-dried hydrolysates were chromatographed in a Biotronic amino acid analyser. Tryptophan was identified after hydrolysis of 2.5 nmol of enzyme with mercaptoethanesulphonic acid, and cysteine was identified as cysteic acid after oxidation with performic acid.

Isoelectric focusing

Isoelectric focusing was performed by the method of Gulian *et al.* [26]. The pH range was 3–6. Enzyme samples were dialysed for 1 h in a 1000-fold volume of 30% (w/v) glycerol at pH 7.0. Before electrofocusing, 4% (v/v) of a solution of ampholytes [Ampholine 3–10 (30%) and 3–5 (70%)] was added to the samples.

N-Terminal amino acid analysis

Determination of the N-terminal amino acid was performed as described in [27,28]. The dansylation was carried out at 37 °C for 45 min. For t.l.c., 5 μ l of the hydrolysate in acetone/acetic acid (3:2, v/v) were applied.

RESULTS AND DISCUSSION

Examinations during growth of *H. salinarum*

Consumption of arginine during growth of *H. salinarum* in a chemically defined medium was observed by analysing the amino acid composition of the medium. Without arginine the bacteria did not grow. However, a substitution of arginine by citrulline or ornithine is possible and results in identical growth curves. This contrasts with the findings of Dundas & Halvorson [6], who observed a reduced growth in presence of ornithine. In accordance with results of Ducharme *et al.* [29] for *H. cutirubrum*, we demonstrated for *H. salinarum* the metabolism of variable concentrations of arginine during the lag phase of growth, leaving the growth curves unchanged. Arginine concentrations from 0.3 to 1.0 g/litre did not lengthen the lag phase. At the beginning of growth, the importance of this pathway in halobacteria lies in the production of ATP.

Ducharme *et al.* [29] showed, for *H. cutirubrum*, that arginine is the only amino acid which is consumed quantitatively during the lag phase of growth. Our experiments reveal that the ADI pathway is constitutive and that the ADI is not inducible in the exponential phase. As Fig. 1 shows, arginine is metabolized to citrulline and ornithine, which are excreted in the medium. Both

Fig. 1. Consumption of arginine during growth of *H. salinarum*

During the growth of *H. salinarum* on a chemically defined medium, samples of the medium were analysed for their contents of the amino acids arginine (●), citrulline (▲) and ornithine (○). Cell growth (—) was measured as the absorbance at 580–640 nm.

amino acids are taken up again during the exponential phase. Whereas most organisms which possess the ADI pathway excrete ornithine [7], only *Mycoplasma hominis* [30] and *H. cutirubrum* [29] excrete citrulline. The ornithine uptake indicates that ornithine is consumed, as is the case with *Clostridium* and *Treponema* [8]. The excretion of citrulline and ornithine hints at the existence of arginine/citrulline or arginine/ornithine antiporters. An arginine/ornithine antiporter was demonstrated for *Streptococcus lactis* by Konings *et al.* [31]. This transport mechanism has not been described for halobacteria.

We determined the activities of ADI in crude extracts from late-exponential-phase cells of five halobacterial strains: *H. salinarum*, 0.33 nkat/mg; *H. mediterranei*, 0.11 nkat/mg; *H. halobium*, 0.35 kat/mg; *H. cutirubrum*, 0.34 nkat/mg; *H. vallismortis*, 0.44 nkat/mg. Comparable enzyme activities indicate that the ADI pathway is equally important among the different strains.

Results of the purification of ADI

The halophilic ADI was purified to electrophoretic homogeneity, as shown in Fig. 2 in a four-step procedure resulting in a 311-fold enrichment. The purification scheme is presented in Table 1. All steps were performed in buffers of high salt concentration. The first step was sulphate-mediated hydrophobic chromatography on Sepharose CL-6B. In presence of high concentrations of (NH₄)₂SO₄ the rate of hydration of proteins is lowered. The decrease in hydration induces an increase in net

Fig. 2. SDS/PAGE illustrating the purification of the ADI

Gel slots showing the results of the successful purification procedure: 1, crude extract; 2, enzyme eluate of the CL-6B column; 3, enzyme eluate of the HB1 column; 4, enzyme eluate of the hydroxyapatite column; 5, purified ADI after propyl-agarose column chromatography; 6, standard proteins (see under ‘Gel electrophoresis’ in the Materials and methods section).

entropy which enhances the formation of hydrophobic bonds [32]. Leicht & Pundak [33] demonstrated the feasibility of this method for large-scale purifications of halophilic proteins. The reduced hydration shell allows further purification of the enzyme by hydrogen-bonding chromatography on hydrogen-bonding material HB1. This method is also performed in the presence of high concentrations of (NH₄)₂SO₄. An 80-fold enrichment with a yield of 72% in the second step (Fig. 3) demonstrates the efficiency of the HB1 gel for purification procedures with buffers of high ionic strength. The third step was hydroxyapatite chromatography. Elution of the ADI with a gradient of increasing sodium phosphate concentration enhances the stability of the halophilic protein, being maximal in presence of 0.5 M-phosphate. Purification to homogeneity was finally achieved by hydrophobic chromatography on propyl-agarose using (NH₄)₂SO₄ for enhancing hydrophobic bonding with the gel matrix.

Purification of the ADI was seriously hampered by the fact that the enzyme is irreversibly denatured by NaCl concentrations lower than 2 M. Therefore chromatographic methods practicable in buffers of high ionic strength were required to retain catalytic activity. Previous attempts to purify the ADI by affinity chromatography on agaroses with arginine as ligands had been unsuccessful. Other methods tested in vain were ion-exchange chromatography on DEAE-cellulose and hydrophobic chromatography on phenyl- and octyl-Sepharose.

Table 1. Purification scheme for the ADI

Purification step	Protein (mg)	Activity		Enrichment (fold)	Yield (%)
		Total (nkat)	Specific (nkat/mg)		
Crude extract	3752.0	1076.9	0.33	1	100
Sepharose CL-6B eluate	910.8	905.2	1.00	4	84
HB 1 eluate	34.4	771.8	22.50	79	72
Hydroxyapatite eluate	18.6	611.8	33.34	115	57
Propyl-agarose eluate	2.9	258.4	90.02	311	24

Fig. 3. Elution profile of the HB1 column chromatography

The elution profile of the second step of ADI purification from *H. salinarium*. The elution of proteins (—) is shown as A_{280} . Enzyme activity (●) is expressed in nkat/fraction. Two anti-parallel gradients (----) were used to elute the enzyme.

Stability of the ADI

The purified enzyme is stable for at least 8 months in buffer 5, which contains 0.5 M- Na_2HPO_4 . Phosphate does not have any effect on the kinetics of the ADI (results not shown); it enhances the stability by its salting-out effect, i.e. by strengthening hydrophobic bonds between non-polar amino acids, as discussed in detail by Lanyi [34].

It was not possible to stabilize the enzyme by replacing salt with compatible solutes as proposed by Kushner [35]. In the presence of 4.0 M- β -alanine, 50 % of the initial catalytic activity

was retained for few hours, but then the ADI rapidly lost its activity. The enzyme was not stable in glycerol, poly(ethylene glycol)s and glycine buffers. No re-activation of the enzyme after dialysis against a salt-free buffer, containing Tris and 2-mercaptoethanol, pH 7.6, was possible.

Effect of salts on the catalytic activity of the ADI

Activities in the presence of different salts in a 50 mM-Tris buffer, pH 7.6, are illustrated in Fig. 4. We measured the highest activity with 3.5 M-NaCl, followed by Na_2HPO_4 at 2.0 M and KCl at 4.0 M. Ionic radii were not related to the salt-dependence of the ADI. Although the ionic radii of K^+ , NH_4^+ and Rb^+ with 0.13–0.15 nm are similar, activities in presence of their chlorides differ markedly. Bivalent cations are not able to substitute for

Fig. 4. Activity of the ADI in the presence of different salts

The activity of the ADI in the presence of different salts is expressed as a percentage of maximal reaction velocity (3.5 M-NaCl = 100 %). Salts examined were NaCl (●), KCl (○), RbCl and CsCl (□), NaBr (△), Na_2HPO_4 (■) and Na_2SO_4 (▲).

Table 2. Activity of the ADI in presence of different salts

Salt	Max. concn. examined (M)	Maximal activity (%)	Optimal concn. (M)
NaCl	5.0	100	3.5
$\text{NaH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$	4.0	77	2.0
KCl	4.0	74	4.0
RbCl	5.0	53	5.0
CsCl	5.0	52	5.0
$(\text{NH}_4)_2\text{SO}_4$	4.0	48	2.0
Na_2SO_4	2.0	39	1.5
NaBr	5.0	24	3.0
LiCl	5.0	< 5†	—
$\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$	5.0	< 5†	—
$\text{MgCl}_2 \cdot 6\text{H}_2\text{O}^*$	3.0	< 5†	—
NH_4Cl	5.0	< 5†	—
$\text{KH}_2\text{PO}_4/\text{K}_2\text{HPO}_4$	2.5	< 5†	—
KBr	4.0	< 5†	—

* 0.1 M-Tris, pH 6.0.

† No activity detectable.

univalent ones, and the activity does not depend on traces of bivalent cations as verified by assays with Mg^{2+} , Ca^{2+} and EDTA (results not shown). Activities of several halophilic proteins show a dependence on the salting-out effect of cations and anions according to the Hofmeister series [36]. This is not the case with the ADI, as is illustrated in Table 2. The rank of anions which have the greatest effect on catalytic activity is:

Although many proteins have been purified and characterized from extreme halophilic bacteria, the mechanism of the high salt-dependence of these molecules remains obscure. Charge-shielding by cations to hinder destabilization by electrostatic repulsion between acidic amino acid residues at the surface of proteins and salting-out effects of anions on apolar amino acids as discussed by Lanyi [34] are not sufficient to explain the mechanism of salt-dependence of ADI. Determination of structures of halophilic proteins in future will help to elucidate the effects of salts on these molecules.

Amino acid composition and *N*-terminal amino acid of ADI

The amino acid composition of the ADI from *H. salinarum* is illustrated in Table 3. We determined an excess of acidic amino acids of 15.6 mol % over basic amino acids. The high acidity is a typical feature of halophilic proteins, which is important for the stability of the molecules in the presence of high salt concentrations [34]. The amino acids asparagine and aspartic acid, and glutamine and glutamic acid, could not be separated.

Comparison of the composition of the ADI from *H. salinarum* with those of ADIs from two *Pseudomonas* strains and a mycoplasma show a higher percentage of acidic amino acids and their amines, but their numbers are not as high as with the halobacterial enzyme. The amount of hydrophobic amino acids

Table 3. Amino acid composition of the ADI from *Halobacterium salinarum* in comparison with those of non-halophilic ADIs

Amino acid	(mol %)	Composition			
		(residues/subunit of ADI)			
		<i>H. sal.</i> *	<i>P. put.</i> †	<i>P. aer.</i> ‡	<i>M. art.</i> §
Aspartate	14.6	60	50	37	44
Glutamate	13.5	52	48	40	37
Lysine	3.1	12	28	18	25
Histidine	4.3	16	14	9	9
Arginine	5.1	20	27	16	16
Serine	4.5	20	20	25	22
Threonine	5.1	20	30	25	22
Cysteine	0.6	4	6	7	18
Glycine	6.3	24	48	36	23
Tyrosine	2.7	12	13	11	13
Isoleucine	4.1	16	26	20	24
Leucine	8.1	23	43	39	39
Alanine	7.3	28	32	27	25
Valine	8.5	32	39	29	27
Methionine	2.6	12	11	10	10
Phenylalanine	3.9	16	18	13	16
Proline	5.1	20	32	22	18
Tryptophan	0.6	4	6	7	5
Total ...		392	492	401	393
Molecular mass ...		55 kDa	54 kDa†	46 kDa‡	49 kDa§

* *H. salinarum*.

† *P. putida* [48].

‡ *P. aeruginosa* [49] (the data were taken from the sequence analysis).

§ *M. arthritidis* [50].

in the halophilic ADI corresponds with that of non-halophilic analogues. This argues against Lanyi's [34] statement that halophilic proteins are deficient in apolar amino acids and therefore need the presence of high concentrations of salting-out salts to enhance their ability for hydrophobic bonding.

We have determined the *N*-terminal amino acid of the halophilic ADI to be lysine.

Determination of the molecular mass of ADI

Electrophoresis of the purified enzyme with cationic (CTAB) and anionic (SDS) detergents revealed that the ADI migrated as a single protein band, indicating the homogeneity of the preparation (see Fig. 2). The mobility of the enzyme in SDS/PAGE corresponded to a molecular mass of 75 ± 2 kDa. However, the molecular mass as determined by CTAB/PAGE was 55 ± 2 kDa. This can be explained by the acidic nature of the enzyme. Difficulties in estimating molecular masses of acidic proteins with SDS/PAGE have been observed previously [37]. Eley *et al.* [25] discussed this effect with a decreased bonding of SDS caused by electrostatic repulsions between negative charged detergent and acidic amino acid residues, resulting in a reduced electrophoretic mobility of the protein and an overestimation of its mass. This problem is circumvented by using the cationic detergent CTAB. Izotova *et al.* [38] demonstrated the practicability of CTAB/PAGE for the molecular-mass determination of an acidic serine proteinase from a halophilic organism. The amino acid composition of the ADI from *H. salinarum* shows a high percentage of acidic amino acids. As is the case with the halophilic serine proteinase, the molecular mass of the halophilic ADI determined by SDS/PAGE is about 25% higher than that obtained by CTAB/PAGE. The molecular masses of non-halophilic ADI subunits range from 47 to 54 kDa [39–43]. We determined the molecular mass of the native halophilic enzyme under different buffer conditions as 105 ± 3 kDa. The molecular mass determined by CTAB/PAGE corresponds to gel-filtration data, and suggests a dimeric enzyme.

General properties

The amino acid analysis indicates that the ADI from *H. salinarum* is an acidic protein. This is confirmed by the pI of 4.7. ADI enzymes from non-halophilic organisms are less acidic and show pI values of 7.0 for *Mycoplasma arthritidis* [40], 6.13 for *Pseudomonas putida* [43] and 5.3 for the peptidyl-ADI from rabbit skeletal muscle [9]. The high acidity of the halophilic enzyme is not a ubiquitous property of ADIs. It is a typical feature of a great number of enzymes from extreme halophilic organisms.

The velocity of the catalytic reaction is maximal at 40 °C. Non-halophilic ADIs show temperature optima from 30 to 50 °C [9,10,41,43–45]. The thermal stability of the halophilic enzyme is optimal at 35 °C; above 60 °C catalytic activity is lost. In contrast with the observations of Keradjopoulos & Holldorf [46], the halophilic ADI shows no thermophilic properties.

The catalytic activity depends from the pH and appears as a bell-shaped curve with a maximum at pH 7.6. pH optima for non-halophilic ADIs range from 6.0 to 10.0 [9,10,41,43–45,47–50]. Whereas eubacterial enzymes show pH optima that are more acidic than that of the ADI from *H. salinarum*, the pH optima for eukaryotic ADIs correspond to those of the ADIs from archaeobacteria.

Kinetic experiments

Plots of the reaction rate of the ADI versus substrate concentration are sigmoid-shaped, as illustrated in Fig. 5. The sigmoidicity cannot be altered by variation of phosphate concentration or addition of histidine, as demonstrated by

Fig. 5. Rate of reaction of the ADI: determination of K_s .

The plot of reaction rate, v , expressed in nkat/ml versus substrate concentration ($[S]$) in mM, is sigmoid-shaped. Data were analysed by using Graph PAD version 2.0 (ISI Software, San Diego, CA, U.S.A.). Linear regression of a Hill plot with $V_{\max} = 2.48$ nkat/ml results in a Hill coefficient (h) of 2.04 and a K' of 9.5. The half-activity constant, K_s , is 3.1 mM.

Venugopal *et al.* [51] for the enzyme from *Clostridium sporogenes*. We determined the half-activity constant (K_s) of the halophilic enzyme by analysing reaction-rate data by the Hill equation [56]:

$$\log \frac{v}{V_{\max} - v} = h \log [S] - \log K'$$

With $V_{\max} = 2.48$ nkat/ml we obtain from Fig. 5 a Hill coefficient (h) of 2.04 and K' (substrate concn. at the inflexion point of the curve) = 9.5, resulting in a K_s of 3.1 mM. Constants for non-halophilic enzymes vary over a wide range from 0.0015 mM for *Streptococcus faecalis* [48] to 2.84 mM for *Tetrahymena pyriformis* [50], a value that resembles the K_s for the halophilic enzyme.

The activity of the halophilic enzyme was dependent on free thiol groups. The ADI was inhibited quantitatively by 1 mM-*N*-ethylmaleimide, $-\text{HgCl}_2$ and $-\text{AgNO}_3$, whereas CuSO_4 and AlCl_3 had no effect. The ADI from *Pseudomonas putida* is inhibited almost quantitatively by Hg^{2+} , Al^{3+} , Cu^{2+} and Ag^+ ions [43]. AMP at 30 mM caused an increase of the activity of the enzyme to 148 % (activity at 3.5 M-NaCl = 100 %). The presence of ADP or ATP in concentrations of 20 mM reduced the activity slightly. Hutson *et al.* [53] demonstrated a feedback inhibition of the ADI from *Lactobacillus leichmannii* mediated by the adenosine nucleotides AMP, ADP and ATP. This is not the case with the halophilic ADI, where AMP shows an activating behaviour. A slight inhibition of the ADI is caused by the products of the ADI pathway, carbamoyl phosphate and ornithine, giving 89 % and 80 % activity respectively. Lysine, however, with one methylene group more than ornithine, showed no effect. The influence of citrulline could not be studied because the measurement of citrulline production is the basis of the enzymic assay. Several non-halophilic ADIs are also inhibited by ornithine [41,50,53,54].

The addition of guanidine as a possible substrate had no effect on the activity of the halophilic enzyme. This demonstrates that, in contrast with the ADI from *Mycoplasma arthritidis* [53], the halophilic enzyme is not able to metabolize guanidine.

The ADI from the extreme halophilic archaeobacterium *H. salinarum* shows a high acidity and an absolute salt-dependence. Apart from this, no unusual properties in its structure and kinetic data could be determined. Some properties are comparable with those of eubacterial enzymes; others resemble features of eukaryotic ADIs.

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