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A NON-SPECIFIC ADENINE NUCLEOTIDE DEAMINASE FROM *DESULFOVIBRIO DESULFURICANS*

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SUMMARY

1. A non-specific adenine nucleotide deaminase, which is equally active with AMP, ADP or ATP, was extracted from *Desulfovibrio desulfuricans*, strain Berre S, and purified approx. 800-fold.

2. Enzyme preparations contained 4% of nucleic acids, which stabilised the activity during storage.

3. The enzyme was competitively inhibited by various substances with the following values for K_i: urea (410 mM), *p*-mercuribenzoate (11.8 μM), $HgCl_2$ (5.3 μM), $CuSO_4$ (67 μM) and the anions, β,β-dimethyl glutarate (3.2 mM), citrate (6.0 mM), succinate (18 mM), maleate (22 mM), phosphate (83 mM), cacodylate (134 mM) and Cl^-.

4. Low concentrations of F^- and CN^- caused non-competitive inhibition.

INTRODUCTION

Enzymes that deaminate the adenine nucleus have been detected in many biological systems[1–6] and, excepting adenine deaminase from *Aspergillus oryzae*[7] and an ATP deaminase from *Microsporus audouini*[8,9], they are highly specific towards biologically active adenine compounds. The AMP deaminase from rabbit muscle[10–12], the non-specific deaminase from *A. oryzae*[13–16] and the specific adenosine deaminase from calf spleen[17,18] have been extensively purified and studied in detail. The present paper describes the isolation and properties of an enzyme from a nitrogen-fixing strain of *Desulfovibrio desulfuricans* which is unique in that it deaminates ATP, ADP and AMP equally. It will therefore be called adenine nucleotide deaminase. Evidence is presented that, despite this non-specificity, the enzyme binds to substrate phosphate groups and to sites antagonised by urea and mercurials.

MATERIALS AND METHODS

Adenine, nucleosides, nucleotides, horse heart cytochrome *c* (type III) and bovine serum albumin were purchased from Sigma (London) Chem., London, S.W.6.

Adenosine 5′-phosphosulphate was made from AMP and pyridine-SO_3[19]; the starting material, pyridine-SO_3, was prepared by reacting SO_3 with pyridine[20], and adenosine 5′-phosphosulphate was separated from AMP and SO_4^{2-} by thin-layer chromatography on a 1-mm layer of cellulose MN 300HR (purchased from Machery, Nagel and Co., Düren, Germany), using an isopropanol–ammonia–water solvent (6:3:1, by vol.). Gels for estimating the molecular weight of the enzyme were purchased from Bio-Rad Lab. (Richmond, California) and $(NH_4)_2SO_4$, especially low in heavy metals for enzyme work, came from British Drug Houses (Poole, England).

Growth of Desulfovibrio desulfuricans

The strains of bacteria used, followed by their National Collection of Industrial Bacteria numbers, were: *D. desulfuricans* var. *azotovorans*, strain Berre S, (8388); *D. desulfuricans*, strain Essex 6, (8307); *D. vulgaris*, strain Hildenborough, (8303); and *D. gigas*, (9332). They were grown in a lactate–sulphate–yeast extract medium C of POSTGATE[21] at 30° under anaerobic conditions. *D. desulfuricans*, strain Norway 4, (8301) was grown in medium C supplemented with 2.5% NaCl. For large-scale preparation of the enzyme, strain Berre S was grown in a nitrogen-free modification of medium C in semi-continuous culture under a flow of N_2 (200 ml/min) and CO_2 (30 ml/min). After 48 h, the cells (0.5 g wet wt./l) were harvested in a continuous flow centrifuge.

Enzyme assays

Adenine nucleotide deaminase activity. Enzyme activity is expressed throughout as μmoles of substrate hydrolysed per min. The assay chosen depended on the concentration of substrate necessary in the experiment: with substrate concentrations above 80 μM, the reaction rate was measured by the amount of NH_3 produced (Table IV indicates the stoichiometry between substrate hydrolysed and NH_3 produced); with 80 μM substrate and less, the reaction rate was measured by the decrease in light absorbance at 265 mμ caused by the hydrolysis of adenosine to inosine (deamination of 0.1 μmole of ATP, ADP or AMP caused a decrease of 0.290 *A* units at 265 mμ)[3]. Typical assays were conducted as follows: (1) *Using high substrate concentration:* 50 μl of enzyme were added to 1.95 ml of 50 mM sodium phosphate buffer (pH 5.8 or 6.0) containing 4 mM ATP, ADP or AMP. The reaction was stopped after 20 min by adding 2 ml of 1 M NaOH, and the NH_3 liberated was determined with Nessler's reagent at 425 mμ using an EEL colorimeter. (2) *Using low substrate concentration:* 100 μl of 1 mM substrate was added to the enzyme in 50 mM sodium phosphate buffer (pH 5.8) in a final vol. of 2.5 ml. The hydrolysis of adenosine phosphate to inosine phosphate was measured with a Unicam S.P. 500 spectrophotometer as the drop in absorbance at 265 mμ over 2 min, starting 30 sec after mixing the reactants. Low enzyme concentrations ensured a linear reaction rate during the assay period. These procedures will be cited in the text as Assay 1 and Assay 2, respectively.

ATPase activity. Purified deaminase fractions were assayed for ATPase activity in 50 mM sodium cacodylate buffer (pH 5.8) or in 50 mM Tris buffer (pH 8.0) containing 10 mM $MgCl_2$. The inorganic phosphate released was measured by the method of TAUSSKY AND SHOOR[22].

ATP-pyrophosphohydrolase activity. The activity of this enzyme was determined qualitatively by thin-layer chromatography of incubation mixtures on cellulose

powder, using *n*-butanol–acetone–glacial acetic acid–5% ammonia–water solvent (7:5:3:3:2, by vol.) containing 1% EDTA[23]. This mixture readily separated IMP or AMP from their corresponding triphosphates.

Enzyme purification

Washed bacteria (50 g wet wt.) were suspended in 25 mM Tris buffer (pH 7.4) (1:5, w/v) and passed through a French pressure cell from 10 000 lb/inch^2. The extrudate was centrifuged at 40 000 × *g* for 20 min and the supernatant fluid (150 ml, designated as Fraction 1) was further centrifuged at 200 000 × *g* for 1 h to yield a second supernatant fluid (Fraction 2) made slightly dark by suspended FeS. At this stage the enzyme was often stored at −20° for bulk purification, when it lost no activity over several weeks. Saturated $(NH_4)_2SO_4$ (pH 7.5) was added dropwise and with constant stirring to Fraction 2 at about 4°. At 50% saturation the suspension was allowed to stand for 20 min and was then centrifuged at 30 000 × *g* for 15 min. The supernatant fluid was made 60% saturated with $(NH_4)_2SO_4$ in the same manner and stood for 40 min with intermittent stirring before centrifuging. This precipitate (the 50–60% $(NH_4)_2SO_4$ fraction) was dissolved in 25 mM Tris buffer (pH 7.4, 20 ml) and dialysed overnight at 4° against the same buffer to remove traces of $(NH_4)_2SO_4$ and to yield Fraction 3. Fraction 3 was then applied to a 20 cm × 2 cm column of DEAE-cellulose suspended in 50 mM Tris buffer (pH 7.4) and the enzyme was eluted with a linear gradient of Tris buffer (pH 7.4) starting with 75 ml of 60 mM buffer in the closed mixing flask and 500 ml of 120 mM buffer in the reservoir. The flow rate was 0.2 ml/min and 5-ml fractions were collected. Fig. 1 shows the elution pattern

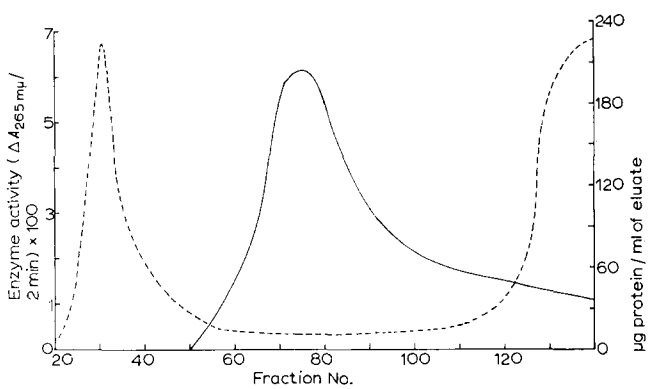

Fig. 1. DEAE-cellulose chromatography of Desulfovibrio adenine nucleotide deaminase (Fraction 3). Conditions: Assay 2 (see text) using 50 mM phosphate buffer (pH 5.8) ,40 μM ATP and 0.2 ml of each fraction eluted. Protein concentration was estimated from the absorbances at 260 and 280 mμ (ref. 26). ....., protein; ———, enzyme activity.

with such a column. Fractions of the eluate with the highest specific activities (approx. 20) were bulked and dialysed against 50 mM Tris (pH 7.4) and concentrated 10-fold by absorbing on a second DEAE-cellulose column (10 cm × 1 cm) similar to the first and eluting with 150 mM Tris buffer (pH 7.4). This preparation was dialysed again against the 50 mM Tris buffer to yield Fraction 4. Fraction 4 was dialysed against 50 mM succinate buffer (pH 5.0) and purified finally by passing through a 10 cm ×

1 cm column of CM-cellulose suspended in the succinate buffer, which was also used as the eluent. This last fraction (Fraction 5) had only 20% of the deaminase activity of Fraction 4, and on disc electrophoresis[24] it consisted largely of a single protein band with traces of two or three other proteins. Attempts to purify Fraction 5 by continuous disc electrophoresis resulted in complete loss of enzyme activity. Because so little enzyme was recovered in Fraction 5, most of the properties were tested using Fraction 4.

RESULTS

Physical properties of the adenine nucleotide deaminase

The enzyme preparation (Fraction 5) was colourless with a molecular weight of between 30 000 and 60 000 when determined by molecular sieve chromatography on Bio-Rad gel using horse heart cytochrome *c* and bovine serum albumin as standards[25]. Fraction 4 was stable indefinitely at −20°, for 3 days at room temperature and to freezing and thawing many times. Heating at 60° for 5 min destroyed all activity.

Effect of nucleic acids on the enzyme stability

Fraction 5 contained 4–5% nucleic acid when measured according to the method of Layne[26]. Cells disrupted in protamine sulphate solution (13 mg/ml at pH 7.5) contained 50% less nucleic acid in Fraction 5, but the stability of the enzyme was then decreased: the specific activity of ordinary Fraction 4 was 10 times that of a Fraction 4 derived from cells treated with protamine sulfate. Protamine sulfate treatment caused Fraction 2 to lose most of its activity when stored overnight at −20°.

Search for ATPase and ATP pyrophosphohydrolase

ATP or ADP activation of AMP deaminase is well documented[27–30]. AMP produced by ATP pyrophosphohydrolase (EC 3.6.1.8) and ADP produced by ATPase (EC 3.6.1.7) might, therefore, contribute to the apparent rate of deamination of ATP by Desulfovibrio deaminase. Both these enzymes were absent from Fraction 4. This evidence confirms the equal affinity of the deaminase for ATP, ADP or AMP.

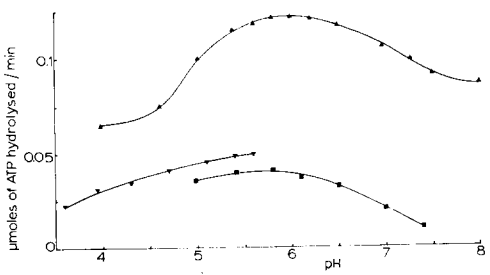

Fig. 2. pH optimum of Desulfovibrio adenine nucleotide deaminase . Conditions: top curve: Assay 1 (see text) using 50 mM succinate and phosphate buffers (▲) with 5 mM ATP and 40 µg of enzyme protein (Fraction 4); bottom curves: Assay 2 using 50 mM acetate (▼) and cacodylate (■) buffers.

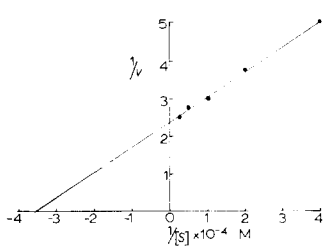

Fig. 3. Lineweaver–Burk[47] plot of ATP deamination by Desulfovibrio deaminase. Conditions: Assay 1; *v* µmoles of ATP hydrolysed per min using 30 µg of enzyme protein (Fraction 4).

pH optimum

With 5 mM substrate (Assay 1) the enzyme had a broad pH optimum and was active above pH 8.0; with low substrate concentration (80 μM) the pH optimum was narrower and the activity fell more sharply as the pH became alkaline (Fig. 2). The choice of acetate and cacodylate buffers to determine the pH optimum with low substrate was made because these buffers had low activities as competitive inhibitors of the enzyme, a problem which is discussed below.

Substrate specificity and affinity

The Michaelis constants and maximum velocities with ATP, ADP or AMP were almost identical and so were the initial velocities (Table II). The initial velocity with adenosine 5'-phosphosulphate was similar to that with ATP, but insufficient adenosine

TABLE I

PURIFICATION PROCEDURE FOR ADENINE NUCLEOTIDE DEAMINASE

Test conditions: Assay 1 (see text) run for 20 min in 50 mM phosphate buffer (pH 6.0). Protein concentrations were measured by biuret reagent[26] or by the method of LOWRY *et al.*[48].

Fraction	*Protein concn. (mg)*	*μmoles ATP hydrolysed per mg protein per min*	*Enzyme activity recovered (%)*
1. 40 000 × *g* supernatant	2450	0.035	100
2. 200 000 × *g* supernatant	450	0.15	65
3. 50–60% $(NH_4)_2SO_4$	105	0.37	43
DEAE-cellulose 1	2.4	7.8	24
4. DEAE-cellulose 2	1.2	12.5	16
5. CM-cellulose	0.16	26.4	4

5'-phosphosulphate was available to determine the K_m. Adenosine and the deoxyribonucleotides, dATP, dADP and dAMP were deaminated approx. one tenth as rapidly as ATP and had higher K_m values, suggesting that both ribose and phosphate groups of the adenine nucleotide were involved in enzyme–substrate binding. DALZIEL[31] showed that commercial samples of NAD contained 5% of a contaminant likely to be ADP-ribose. However, the approximately equal affinity of the adenine nucleotide deaminase for NAD and ADP-ribose suggests that a 5% contamination with ADP-ribose would contribute little to the apparent deamination of NAD. 3'-AMP, CMP, CDP, CTP, GMP, GDP or GTP were not deaminated by Desulfovibrio deaminase. 3'-AMP was deaminated as rapidly as adenosine by the nonspecific adenosine deaminase from *A. oryzae*[14] but was a poor substrate for the ATP-deaminase from *M. audouini*[8]. The Desulfovibrio enzyme deaminated old preparations of 3'-AMP but not freshly prepared solutions.

Inhibition by nucleotides

3'-AMP, 2'-AMP or adenosine inhibited the deamination of ATP according to Assay 1; adenine, GMP, GDP or GTP were ineffective.

TABLE II

SUBSTRATE SPECIFICITY OF DESULFOVIBRIO ADENINE NUCLEOTIDE DEAMINASE

K_m and v_{max} were determined by Assay 1 and calculated by a Lineweaver–Burk plot (Fig. 3). Initial velocity was determined by Assay 2 using substrate concn. of 40 μM.

Substrate	K_m *(mM)*	*Velocity (μmoles ATP hydrolysed per min per mg protein)*	
		Maximum	*Initial*
ATP	0.285	13.8	5.7
ADP	0.30	12.6	5.7
AMP	0.25	14.2	5.8
Adenosine 5′-phosphosulphate			5.5
dATP	1.0	1.25	0.6
dADP	0.93	1.3	0.5
dAMP	0.85	1.15	0.55
Adenosine	0.69	0.98	0.43
Deoxyadenosine	1.8	0.7	0.25
3′5′-Cyclic AMP			1.45
ADP-ribose	45	39	1.1
FAD			2.6
NAD^+	59	30	1.35
CoA			0.34
Adenine, 2′-AMP, 3′-AMP, 2′,3′-Cyclic AMP, $NADP^+$			0

Effect of anions

At low substrate concentrations (less than 80 μM) the enzyme was competitively inhibited by several buffers at concentrations above 10 mM (Fig. 4), whereas at high substrate concentration (5 mM AMP) most common anions, including these buffers, did not inhibit. A 10 mM buffer concentration was optimal: below this level the deaminase activity fell, due to a shift of pH away from the optimum. Sodium citrate and sodium β,β-dimethylglutarate were the most efficient inhibitors, being approx. 5 times as effective as succinate or maleate buffers on a molar basis, and 10 times as effective as phosphate ions. Table IIIA gives K_i values for these buffers, determined by plotting $1/v$ against $[I]$ (Fig. 5); this plot also showed that the inhibition was linear competitive rather than hyperbolic[32].

The inhibitions by citrate and phosphate ions were not additive: for instance, 20 mM sodium citrate inhibited the deamination of 80 μM ATP by 49% when compared with a control containing 10 mM sodium phosphate; 50 mM phosphate inhibited 25%. When added together 20 mM citrate and 50 mM phosphate only inhibited 50% of the deaminase activity. On the other hand, 10 mM sodium β,β-dimethylglutarate inhibited 60%, but when added with 50 mM sodium phosphate the inhibition was 83%. The simplest explanation for these observations is that citrate and phosphate ions bound the same site on the enzyme and that citrate excluded phosphate ions. β,β-Dimethylglutarate and phosphate ions would therefore not bind at the same site, but at similar sites, since they both inhibited competitively. Citrate and β,β-dimethylglutarate inhibitions were also additive, while sodium succinate inhibited at the same site as phosphate and citrate.

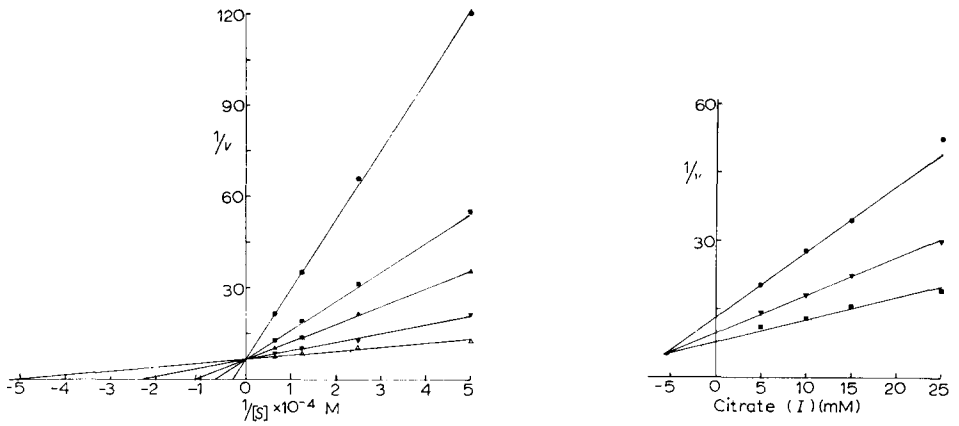

Fig. 4. Competitive inhibition of adenine nucleotide deaminase by buffer anions (sodium salts). Conditions: Assay 2; v = μmoles of ATP hydrolysed per min per 30 μg of enzyme protein (Fraction 4). ●—●, 20 mM β,β-dimethyl glutarate; ■—■, 25 mM citrate; ▲—▲, 36 mM succinate; ▼—▼, 50 mM phosphate; △—△, 10 mM phosphate.

Fig. 5. Inhibition of adenine nucleotide deaminase by sodium citrate; determination of inhibitor constant (K_i). Conditions: Assay 2; v = μmoles of ATP hydrolysed per min with 30 μg of enzyme protein (Fraction 4). ●—●, 20 μM ATP; ▼—▼, 40 μM ATP; ■—■, 80 μM ATP.

The nature of the cation was irrelevant to the inhibition: lithium, sodium or potassium citrates (10 mM) inhibited the enzyme equally.

As an example of a non-buffering anion, Cl^- was tested with the Desulfovibrio deaminase and was found to inhibit weakly: NaCl (40 mM) inhibited 20% of the enzyme activity in the presence of 10 mM sodium phosphate, while 10 mM β,β-dimethylglutarate inhibited 60%.

TABLE III

COMPETITIVE INHIBITION OF DESULFOVIBRIO ADENINE NUCLEOTIDE DEAMINASE BY BUFFERS AND SH GROUP BINDING AGENTS

Test conditions: Assay 2 (see text) using 10 mM sodium phosphate buffer (pH 5.8) as the control in B with substrate (ATP) concentrations ranging from 20 to 80 μM.

A. Buffers (sodium salt, pH 5.8)	K_i *(mM)*
Cacodylate	134
Phosphate	83
Maleate	22
Succinate	18
Citrate	6.0
β,β-Dimethylglutarate	3.2
B. SH group binding agents	K_i *(μM)*
p-Chloromercuribenzoate	11.8
$HgCl_2$	5.3
$CuSO_4$	67

Effect of F^- and CN^-

F^- and CN^- (below 4 mM) inhibit Desulfovibrio adenine nucleotide deaminase noncompetitively (Fig. 6). F^- also inhibited AMP deaminase from muscle[33] and amide transferase from *Pseudomonas aeruginosa*[34] non-competitively; in both cases increasing substrate concentrations increased the inhibition at a fixed F^- concn. With Desulfovibrio deaminase, this effect was pronounced: NaF (1–3 mM) caused 40–65% inhibition of the deaminase activity of Fraction 3 with 20–80 μM ATP and more than

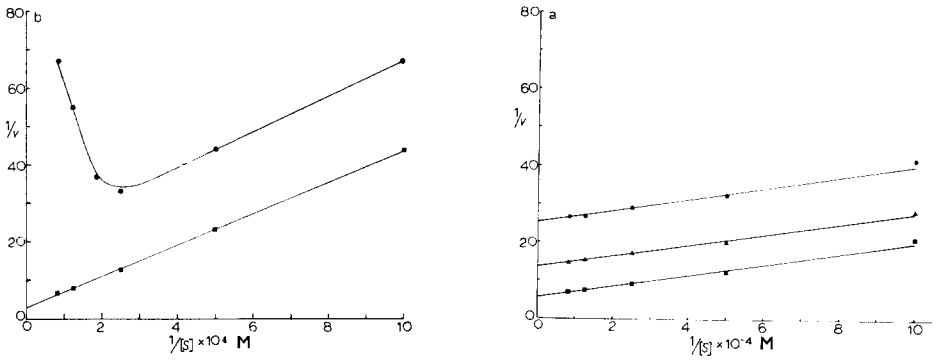

Fig. 6. a. Non-competitive inhibition of adenine nucleotide deaminase by F^- and CN^-. Conditions: Assay 2 using 50 mM phosphate buffer (pH 5.8). v = μmoles of ATP hydrolysed per min using 30 μg enzyme protein (Fraction 4). ■—■, control; ▲—▲, control + 4 mM KCN; ●—●, control + 120 μM NaF. b. Conditions: assay 2 using 400 μg of enzyme protein (Fraction 3). ■—■, control; ●—●, control + 120 μM NaF.

90% inhibition with 120 μM ATP (Fig. 3). Such kinetics are observed when an enzyme is inhibited by excess substrate. A more purified deaminase preparation (Fraction 4) did not exhibit this behaviour, therefore the inhibition is not due to a F^-–substrate complex as suggested for amide transferase[34] but must be due to the effect of an impurity. FMN forms a complex with adenine[35] and Fraction 3 contained flavin whereas Fraction 4 did not. However, FMN (100 μM) had no effect with F^-.

The effect of pH on the inhibition by F^- and CN^- was different (Fig. 7). Concentrations of CN^- above 5 mM competitively inhibited the deaminase, possibly by a mechanism similar to the inhibition with other anions.

Effect of SH-binding agents and reducing agents

p-Mercuribenzoate, Hg^{2+} and Cu^{2+} competitively inhibited adenine nucleotide deaminase (Table III). The inhibitions by these compounds were not additive when they were present together in the reaction, presumably because they all acted at SH groups involved in enzyme–substrate binding. On the other hand, Fe^{3+} and Mn^{2+}, which inhibited the enzyme activity as readily as did Cu^{2+}, did not compete with *p*-mercuribenzoate. Equimolar GSH or cysteine completely prevented the inhibition of deaminase activity by *p*-mercuribenzoate. GSH and cysteine did not themselves inhibit the enzyme activity.

Inhibition of adenine nucleotide deaminase by urea

Urea (0.2–1 M) competitively inhibited the Desulfovibrio deaminase with a K_i

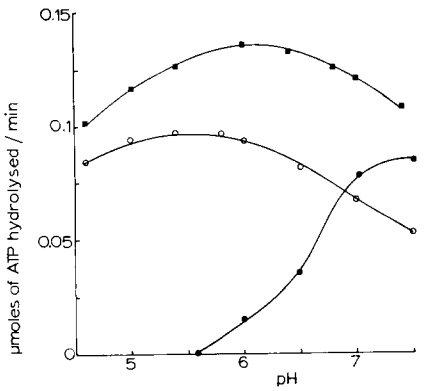

Fig. 7. Effect of pH on the inhibition of Desulfovibrio adenine nucleotide deaminase by F^- and CN^-. Conditions: Assay 1 (see text) using 50 mM succinate and phosphate buffers with 40 µg of enzyme protein (Fraction 4), ■—■, control; ○—○, control + KCN (5 mM); ●—●, control + NaF (0.5 mM).

of 410 mM. The inhibitions by phosphate and urea were additive, indicating two separate sites of enzyme–substrate binding. Urea and Hg^{2+} apparently competed as enzyme inhibitors but this may have been due to a urea–mercuric ion complex since the inhibition when both urea and Hg^{2+} were present was sometimes less than with Hg^{2+} alone.

Effect of metal ions

Desulfovibrio adenine nucleotide deaminase showed no evidence of a metal requirement, being inhibited by most common transition and heavy metals: 0.5 mM Fe^{3+}, Mn^{2+}, Cu^{2+}, Co^{2+}, Zn^{2+} ions all inhibited the enzyme activity approx. 50% using 40 µM ATP. 0.5 mM Ag^+ and Hg^{2+} inhibited 100%. The enzyme was not stimulated by $CaCl_2$ or $MgCl_2$ (10 mM) nor inhibited by metal-binding agents, EDTA, cysteine nor Na_2S; in fact, Na_2S stimulated the activity of Fraction 3 slightly, possibly by removing an inhibitory heavy metal bound to the active site.

Na^+ and K^+ activated AMP deaminase from rabbit muscle in the presence of

TABLE IV

STOICHIOMETRY OF THE ACTION OF DESULFOVIBRIO DEAMINASE ON ATP, ADP AND AMP

Conditions: As described for Assay 1 except that the amount of substrate hydrolysed (last two columns) was measured at each time interval by treating an 0.5 ml aliquot of the reaction with 2% $HClO_4$ (0.5 ml), diluting the resulting mixture 50 times with water and reading the absorbance at 265 mµ.

Substrate	*NH_3 produced (µmoles)*		*Substrate hydrolysed (µmoles)*	
	15 min	*30 min*	*15 min*	*30 min*
ATP	3.2	5.6	3.1	5.3
ADP	3.2	5.8	3.2	5.8
AMP	3.4	5.7	3.5	5.8

ATP[28,36]; at low AMP concentration (below 0.5 mM) activation occurred without ATP[37]. No comparable activation by Na^+ or K^+ on Desulfovibrio deaminase was detected with ATP, AMP or a mixture of both.

Stoichiometry of the reaction

Table IV indicates that NH_3 produced during the action of Desulfovibrio deaminase on ATP, ADP or AMP was proportional to the inosine nucleotide formed, calculated by the decrease in optical density at 265 mμ (ref. 3). Thin-layer chromatography in several solvents[23] showed that the Desulfovibrio deaminase converted ATP to ITP, ADP to IDP and AMP to IMP, although the resolution of these pairs of nucleotides was not adequate for quantitative estimation from the chromatograms.

Reversibility of Desulfovibrio adenine nucleotide deaminase

No evidence was obtained for enzyme reversibility using Fraction 4 either by direct reversal of deamination or by product inhibition. However, in a crude preparation of liquid-nitrogen-treated *D. desulfuricans*, strain Berre S, ITP or ATP stimulated the phosphoroclastic system, whereas only ATP stimulated the phosphoroclastic system of similarly treated *D. vulgaris*, strain Hildenborough, which does not possess an adenine nucleotide deaminase[38]; this might mean that the deaminase of strain Berre S is reversible *in vivo*. KALCKAR[3] quotes unpublished evidence that AMP deaminase from rabbit muscle is reversible *in vivo* although it is not so *in vitro*[3].

Adenosine nucleotide deaminase and nitrogen fixation

Berre S was the only strain of Desulfovibrio listed under MATERIALS AND METHODS found in this laboratory to fix nitrogen. It was also the only strain that possessed an adenine nucleotide deaminase. A relationship was possible between the ability to fix nitrogen and possession of the deaminase, but the specific activity of the enzyme remained the same after forty serial transfers of the organism in medium with fixed nitrogen.

DISCUSSION

The three non-specific deaminases that have been isolated and studied are quite dissimilar in their activities towards substrates. Desulfovibrio deaminase is equally active towards ATP, ADP or AMP and shows low activity with adenosine, in sharp contrast to the non-specific adenosine deaminase from *A. oryzae*, which deaminates ATP only one sixth as rapidly as AMP or adenosine[14]; it also differs from the ATP deaminase of *M. audouini*, which deaminates ATP faster than any other substrate[18].

The higher K_m for adenosine than for ATP, ADP or AMP with Desulfovibrio deaminase, and the competitive inhibition by phosphate, both suggest that substrate 5'-phosphate groups are involved in enzyme–substrate binding. However, the non-specificity of the enzyme towards ATP, ADP or AMP and the lack of specific inhibition by phosphate ions (citrate, β,β-dimethylglutarate, meleate and succinate were stronger competitive inhibitors than phosphate) indicate that no severe steric factors govern the enzyme 5'-phosphate group binding. The relatively high concentration of anions required for inhibition and the wide variation in anion structure precludes the possibility of inhibition by steric hindrance but suggests, instead, that a simple ion

pair is formed between enzyme and substrate during binding. 2'- and 3'-AMP must also bind to the enzyme, since they inhibited ATP deamination, although they did not act as substrates for the enzyme themselves. 3',5'-Cyclic AMP was deaminated less rapidly than AMP; possibly because of steric hindrance or lack of a secondary ionisable group on the cyclic phosphate group.

A second site of substrate binding is indicated by the competitive inhibition by SH group binding agents, *p*-mercuribenzoate Hg^{2+} and Cu^{2+}. HEMMERICH[39] has defined the reactions between Cu^{2+} and SH groups and stressed their reversibility. WOLFENDEN *et al.*[42] showed competitive inhibition of adenosine deaminase from *A. oryzae* by *p*-mercuribenzoate and mersalyl acid, and deduced that enzyme SH groups bound directly to the purine moiety of adenosine since adenine binding was just as readily inhibited by mercurials. Competitive inhibition of other adenosine deaminases by mercurials has been reported[40-42].

The additive nature of the competitive inhibitions by phosphate ions and urea suggests yet another site of substrate binding by adenine nucleotide deaminase, although evidence for absence of competition between urea and mercurials was not obtained. RONCA[43] suggested that urea prevented the purine moiety of adenosine from binding to adenosine deaminase from calf intestinal mucosa because 2,6-diaminopurine competetively inhibited the enzyme. However, 2,6-diaminopurine had no effect on the activity of Desulfovibrio adenine nucleotide deaminase towards ATP.

Since metal ions are not involved in adenine nucleotide deaminase activity, the non-competitive inhibition by F^- must affect other positively charged groups within the protein. According to PEARSON[44], the F^- is a very strong base and, as such, could readily combine with strong acids such as hydrogen-bonding groups. An increase of such positive charges with decreasing pH would account for the increased susceptibility of the enzyme to F^- as the pH dropped from 7.0 to 5.5. The CN^-, on the other hand, is a weak base which is more likely to bind to weak acids and would, therefore, not inhibit the enzyme in the same manner as F^-.

As explained in the text, the anomalous effect of F^- on the activity of Fraction 3 (Fig. 6b) could not be explained in terms of a simple association between inhibitor and substrate. An alternative explanation may be that F^- caused a conformational change in the active site which allowed the enzyme to be inhibited by high substrate concentrations. DIXON AND WEBB[45] have discussed the reasons for such behaviour.

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