Adenosine Triphosphate-Activated Adenylate Deaminase from Marine Invertebrate Animals

PROPERTIES OF THE ENZYME FROM LUGWORM (*ARENICOLA CRISTATA*) BODY-WALL MUSCLE

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Adenylate deaminase (AMP aminohydrolase, EC 3.5.4.6) from lugworm (*Arenicola cristata*) body-wall muscle was partially purified by extraction in KCl solutions and chromatography on phosphocellulose. Enzyme activity was eluted from the column at two salt concentrations. Both forms show co-operative binding of AMP (Hill coefficient, h , 2.85) with $s_{0.5}$ values of 20 mM and 15.6 mM. ATP and ADP act as positive effectors, lowering h to 1.07 and $s_{0.5}$ to 2 mM. The apparent K_a (activation) for ATP was 1.5 mM. GTP is an inhibitor with an apparent K_i of 0.12 mM. *In vivo* the ATP-activated adenylate deaminase is in the active form and may be regulated by changes in GTP concentrations. Adenylate deaminase may act as a primary ammonia-forming enzyme in ammonotelic marine invertebrates with the purine nucleotide cycle.

The primary ammonia-forming mechanisms among ammonotelic marine invertebrate animals are poorly understood (Campbell, 1973; Bishop, 1976). Physiologically significant mechanisms are generally coupled systems linking ammonia formation to the metabolic rate. Among mammals, the commonly suggested schemes are the amino acid transaminase systems linking a series of transaminases to glutamate formation for deamination by glutamate dehydrogenase, or to aspartate formation for deamination by the purine nucleotide cycle (Braunstein, 1957; Lowenstein & Tornheim, 1971; Lowenstein, 1972).

The importance of glutamate dehydrogenase as the primary ammonia-forming enzyme in rat liver has been questioned (McGivan & Chappell, 1975). The mechanism appears to be more efficient in the glutamate-forming direction. Glutamate dehydrogenase from crab muscle and land-snail mitochondria also functions poorly in the ammonia-forming direction (Florkin & Schoffeniels, 1965; Sollock, 1975). Among other invertebrate animals, low activity of glutamate dehydrogenase has been detected only when the enzyme is assayed in the glutamate-forming direction (Chaplin *et al.*, 1965, 1970; McDonnell & Tillinghast, 1973; Gilles, 1974; Zebe, 1975; Sugden & Newsholme, 1975; Wright, 1975; Addink & Veenhof, 1975; Wickes & Morgan, 1976). Therefore glutamate dehydrogenase may play a role in amino acid biosynthesis rather than in ammonia production.

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The purine nucleotide cycle (Scheme 1) may be the primary ammonia-forming mechanism in both hepatic and extrahepatic mammalian tissues (Lowenstein, 1972; Moss & McGivan, 1975; Schultz & Lowenstein, 1976). Data on the role of the purine nucleotide cycle in invertebrate animals are lacking. Adenylate deaminase, the ammonia-forming enzyme in the cycle, has been detected in a number of invertebrate animals (Dingle *et al.*, 1968; Bishop & Barnes, 1971; Flick & Lovell, 1972; McDonnell & Tillinghast, 1973; Dingle & Hines, 1974; Gibbs *et al.*, 1976). A proper evaluation of the purine nucleotide cycle as a primary ammonia-forming mechanism in ammonotelic invertebrate animals requires some understanding of the regulatory properties of this

Scheme 1. Purine nucleotide cycle

enzyme. The present paper describes the activation by ATP and ADP and the allosteric nature of adenylate deaminase from an ammonotelic marine annelid.

Methods and Materials

Animals were obtained from the Supply Department at The Marine Biological Laboratory, Woods Hole, MA, U.S.A., and NEMSCO, Bourne, MA, U.S.A., and were collected south of Cape Cod in the vicinity of Woods Hole.

Phosphocellulose, GSH (reduced glutathione), Tris, AMP, ADP and ATP were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. Nessler's reagent was obtained from Thomas Scientific Co., Philadelphia, PA, U.S.A. Cacodylic acid and its sodium salt were purchased from Matheson, Coleman and Bell, Cincinnati, OH, U.S.A. Other reagents were reagent grade from Fisher Scientific Co., Houston, TX, U.S.A. and Baker Chemical Co., Philipsburg, NJ, U.S.A.

Adenylate deaminase activity was assayed by measuring the ammonia formed from AMP in the presence of the enzyme (Bishop & Barnes, 1971). The standard assay mixture contained Tris/cacodylate (80 μ mol), ATP (disodium salt; 5 μ mol), AMP (sodium salt; 10 μ mol) and 600 μ g of enzyme at pH 7.1 in 1.5 ml. All substrates were adjusted to the proper pH with Tris base and all incubation were at 32°C. The reaction was initiated by addition of the enzyme and terminated by addition of 0.5 ml of 10% (w/v) trichloroacetic acid. Ammonia was determined by the diffusion/nesslerization procedure (Seligson & Seligson, 1951). Absorptions were determined with a Coleman spectrophotometer. A unit of enzyme activity was the amount of enzyme that converted 1 μ mol of AMP into ammonia/min at 32°C under the conditions of assay. Protein was determined by the method of Christian and Warburg (Muirhead & Bishop, 1974). Specific activity was expressed as units of activity/mg of protein.

Enzyme preparation

All procedures were performed at 0–4°C. Centrifugations were at 20000g for 30 min in a Sorvall RC2-B refrigerated centrifuge. All buffers contained 1 mM-GSH.

A summary of the purification procedure is described in Table 1. Cell-free extracts were prepared by homogenizing 480 g of washed blotted tissue in 1700 ml of 50 mM-potassium phosphate (pH 7.3) in a Waring blender at high speed for 1 min. After centrifugation, the pellet was rehomogenized in 1700 ml of the same buffer and left for 12 h. The preparation was centrifuged and the pellet resuspended in 1700 ml of the same buffer containing 0.6 M-KCl and homogenized. The preparation was centrifuged after standing for 12 h, and the supernatant dialysed

Table 1. Purification of adenylate deaminase from lugworm body-wall muscle

Initial activity was 3 μ mol/min per g wet wt. of tissue. Activity in gills and gut were 6 and 5 μ mol/min per g respectively, measured by the standard assay procedure.

Purification step	Specific activity (μ mol/min per mg)
0.6 M-KCl extract of pellet	0.006
Supernatant fluid after dialysis	0.046
Phosphocellulose chromatography	4.1

Fig. 1. Separation of lugworm adenylate deaminase by phosphocellulose chromatography

Conditions and column preparation are described in the text. $\circ$, Adenylate deaminase activity; $\bullet$, protein. Elution was with 0.7 M-KCl to 390 ml (arrow), then with 500 ml of 1.2 M-KCl.

against two changes of 10 litres of 50 mM-phosphate buffer (pH 7.3) for 140 min. The contents of the dialysis bags were centrifuged to remove the gelatinous precipitate and the supernatant was mixed with 120 g of wet phosphocellulose, which had been equilibrated with the solution from the second dialysis. After 17 h, the phosphocellulose was collected by centrifugation, resuspended in 50 mM-phosphate buffer and poured into a chromatographic column. The column was washed with the phosphate buffer until no material absorbing at 280 nm was eluted. The enzyme was then eluted from the column with 390 ml of 0.7 M-KCl followed by 500 ml of 1.2 M-KCl, both in the phosphate buffer, and collected in test tubes in 10 ml portions (Fig. 1). Adenylate deaminase activity was found in both KCl solution washes. The tubes with the greatest activity in each wash were combined separately. For convenience, the adenylate deaminase activities eluted at 0.7 M-KCl and at 1.2 M-KCl were designated preparations A and B respectively. The specific activities of the two preparations were essentially identical (4.1 units/mg).

All kinetic studies were performed on these preparations. No activity was lost on storage for 6 months at 4°C, but all activity was lost on dialysis for 1 h at 4°C.

Subsequently, enzyme prepared in 50mM-Tris/HCl (pH7.3) rather than the phosphate buffer had a specific activity of 26.6 units/mg. These preparations showed the same kinetic behaviour as enzyme prepared in phosphate buffer. The chromatograms (Fig. 1) indicated that the AMP deaminase preparations were not pure. Each of the preparations exhibited between five and ten Coomassie Brilliant Blue-staining bands after electrophoretic separation on 6% polyacrylamide gels in Tris/glycine buffer (pH8.3) at 4°C (Davis, 1964). In each case, any single component comprised no more than 10–20% of the 100 µg of protein added to the gels. No enzymic activity was evident when 0.5 cm cross-sectional pieces of unstained gels were incubated in enzymic reaction mixtures for 3 h. This loss of activity with electrophoresis has been observed for the AMP deaminase from mammalian tissues (Ogasawara *et al.*, 1975b). Adenylate kinase, adenosine triphosphatase and 5'-nucleotidase activities were below detectable limits of 0.1% of the adenylate deaminase activity. Assay procedures for these enzyme activities have been described (Bishop & Barnes, 1971).

Results

Adenylate deaminase A

Ammonia formation from AMP in the presence and absence of ATP increased linearly with time of incubation and with the amount of enzyme in the reaction mixture. No ammonia was produced in the absence of AMP. The deaminase activity was greatest between pH6.8 and 7.3 (Fig. 2) in the presence of ATP or ADP. In the absence of ATP and ADP, ammonia production from AMP was optimal at pH7.6. Tris/cacodylate did not inhibit the enzyme activity and was used as a buffer for all subsequent experiments (Zielke & Seultzer, 1971b).

The activating effect of increasing concentrations of ATP or ADP at various AMP concentrations is shown in Figs. 3(a) and 3(b) respectively. The observed velocity of the reaction increased in a sigmoidal fashion with increasing AMP concentrations in the absence of ATP and ADP. Addition of ADP or ATP to the reaction mixture shifted the kinetics from sigmoidal to hyperbolic, thereby lowering the apparent $s_{0.5}$ for AMP. From the Hill equation

$$\log \left(\frac{v}{V_{\max} - v} \right) = h \log [\text{AMP}] - \log K$$

the h value (Hill coefficient) for plots with AMP at low AMP was 2.85 (Hill, 1910). With the addition

Fig. 2. Effect of pH on lugworm adenylate deaminase activity

All reaction mixtures contained enzyme A (0.57 mg), 53 mM-Tris/cacodylate buffer at the appropriate pH, and substrates, final volume 1.5 ml. ATP or ADP (3.3 mM), and AMP (6.67 mM) were added to the assay mixture to evaluate the ATP- (○) or ADP- (□) activated activity. Incubations without added ATP or ADP contained 10 mM-AMP (▽).

Fig. 3. Effect of ATP (a) and ADP (b) on lugworm adenylate deaminase activity

The reaction mixtures contained 53 mM-Tris/cacodylate, enzyme A (0.57 mg) and the indicated concentrations (mM) of ATP or ADP, and AMP, at pH7.1, final volume 1.5 ml.

Fig. 4. (a) Effect of ATP on the apparent $s_{0.5}$ for AMP, and (b) determination of the apparent K_a (activation) for ATP

Apparent K_s values for AMP were obtained from Lineweaver & Burk (1934) plots. Data for these replots are taken from Fig. 3(a) and other similar Lineweaver & Burk (1934) plots. The slope is calculated as the value without ATP minus the value with ATP. α is defined as: αK_s (without ATP). The equation for the line was solved for $y=0$. The intercept on the abscissa, $6.68 = -1/\alpha K_a$.

of ATP and ADP at 6.67mM, the h value for the same plots was 1.07. The apparent $s_{0.5}$ for AMP changed from 20mM to 2.2mM with the addition of 6.67mM-ATP or -ADP. Although ADP and ATP had identical effects on the slopes of the Hill plots and on the shift of the apparent $s_{0.5}$ for AMP, the $V_{\max}$ with ADP was 25% lower than the $V_{\max}$ obtained in the presence and absence of ATP.

Double-reciprocal plots (Lineweaver & Burk, 1934) were used to evaluate the binding of ATP. A replot (Fig. 4b) of $1/\Delta\text{slope}$ of the double-reciprocal plots ($V_{\max}/s_{0.5}$ for AMP) against $1/ATP$ (Segel, 1975) yielded an apparent K_a (activation) for ATP of 1.5mM. This apparent K_a for ATP was approximately the concentration of ATP (1.14mM) that would cause a doubling of the minimal $s_{0.5}$ for AMP (Fig. 4a). The minimal apparent $s_{0.5}$ for AMP at maximal ATP concentrations was 2mM.

Other adenine nucleoside monophosphates and nucleosides at 20mM were not deaminated in the absence of ATP. ADP, dAMP and 2'-AMP were deaminated at 5–7% the rate of AMP in the presence of 3.3mM-ATP, whereas adenosine, deoxyadenosine, 3'-AMP and GMP were not deaminated. dAMP (10mM) inhibited deamination of 3.3mM-AMP about 65% in the presence of 3.3mM-ATP. Phosphate (17mM) inhibited deamination of 15mM-AMP completely in the absence of ATP, but addition of 3.3mM-ATP abolished this phosphate inhibition. No inhibition was observed with 20mM-acetohydroxamate, a urease inhibitor.

GTP (10mM) and GDP (6.7mM) showed complete and 90% inhibition respectively, of deamination of 3.3mM-AMP in the presence of 3.3mM-ATP. GTP inhibition was evaluated at various concentrations of AMP in the presence of 6.67mM-ATP. GTP was a non-competitive inhibitor of AMP and had an apparent K_i of 0.12mM.

KCl concentrations up to 0.2M increased the deaminase activity when assayed at 10mM-AMP in the absence of ATP. Maximal activation was observed at 0.2M-KCl, but higher KCl concentrations were inhibitory, activation being half-maximal at 0.6M-KCl. The activity at 0.2M-KCl was 50% of that with no added KCl in the presence of 6.67mM-ATP. When 6.67mM-ATP was included in the assay mixture, no activation by KCl was observed and KCl concentrations above 0.6M were inhibitory, the activity being decreased by one-half in the presence of 1.2M-KCl.

Adenylate deaminase B

The kinetic behaviour of the B form was very similar to that of the A form. The apparent $s_{0.5}$ for AMP in the presence of ATP, activation by ATP and ADP, inhibition by phosphate and GTP, and the activation by KCl in the absence of ATP were the same for both forms of the enzyme. The apparent $s_{0.5}$ for AMP in the absence of ATP was 15.6mM, which was slightly lower than the $s_{0.5}$ for AMP of the A form of the enzyme.

Discussion

Adenylate deaminase from the body wall of the lugworm (*Arenicola cristata*) resembles the enzyme from vertebrate tissues. All are allosteric enzymes, are strongly activated by ATP and ADP and have similar substrate specificity (Zielke & Sealter, 1971a).

ATP and ADP bind tightly (K_a for ATP = 1.5mM) and act as positive modulators which lower the apparent $s_{0.5}$ for AMP from 20mM to 2mM. The h value derived from the Hill plot decreases from 2.85 to 1.07 on addition of ATP. The enzyme changes from a form which exhibits co-operative binding of AMP to a form which conforms to Michaelis-Menten

kinetics. This shift in the h value of the Hill plots has been observed with the enzyme from calf brain (Setlow & Lowenstein, 1967) and rabbit skeletal muscle (Smiley & Seulter, 1967). The h value of 2.85 may indicate a minimum of three co-operative binding sites for AMP and is consistent with the proposed tetrameric structure of the vertebrate skeletal-muscle enzyme (Coffee & Kofke, 1975; Boosman & Chilson, 1976).

To understand the physiological significance of the low K_a for ATP and the high $s_{0.5}$ for AMP in the presence of ATP, one must examine the intracellular adenine nucleotide concentrations. Among non-insect invertebrate animals, the ATP concentrations vary between 2 and 7 mM and AMP concentrations vary between 0.1 and 0.9 mM, depending on the tissue, the organism and the physiological state of the organism (Rey, 1956; Nilsson, 1957; Dingle *et al.*, 1968; Hiltz & Dyer, 1970; McLaughlin, 1971; Flick & Lovell, 1972; Barrett & Beis, 1973; Hiltz & Bishop, 1975; Beis & Newsholme, 1975; Wijsman, 1976). The low binding constant for ATP means that the adenylate deaminase in these organisms is always in the active form. The high apparent $s_{0.5}$ for AMP under activating conditions indicates that the enzyme activity is substrate-limited; that is, the rate of ammonia production is a direct function of the availability of AMP. As AMP concentrations increase with metabolic work or for some other reason, ammonia production increases.

The inhibition by GTP is also of physiological significance. The intracellular concentrations of GTP are in the 0.1 mM range in most animals (Barrett & Beis, 1973). The apparent K_i of 0.12 mM for GTP was determined in the presence of high physiologically significant ATP concentrations. In the lugworm, therefore, changes in GTP concentrations would tend to modulate the activity of the ATP-activated adenylate deaminase and may regulate ammonia production from AMP. GTP also has a regulatory effect on adenylate deaminase from mammalian brain and skeletal muscle, in that it inhibits these ATP-activated enzymes (Setlow & Lowenstein, 1968; Tomozawa & Wolfenden, 1970; Smiley & Suelter, 1967).

K^+ concentrations in the physiological range activate the lugworm adenylate deaminase only in the absence of ATP. Therefore the physiological significance of this activating effect is uncertain. In contrast, the adenylate deaminase from some vertebrate tissues is activated by physiological K^+ concentrations in the presence of ATP (Setlow & Lowenstein, 1967; Ronca-Testoni *et al.*, 1970).

In the purification of the lugworm adenylate deaminase, advantage was taken of the apparent association of the enzyme with actomyosin. Similar observations have been reported for mammalian and shark muscle preparations (Currie & Webster,

1962; Makarewicz, 1969). Dialysis against phosphate buffer is used to enhance separation of these proteins. Subsequent preparations of the lugworm adenylate deaminase which were done in Tris buffers did not result in distinct separation from actomyosin but did improve the purification on the phosphocellulose column.

The lugworm enzyme was eluted from the phosphocellulose column as two distinct peaks (Fig. 1). Although the kinetic behaviour of the enzyme from these two peaks is qualitatively very similar, the $s_{0.5}$ values for AMP of enzymes A and B are 20 and 15.6 mM respectively, suggesting that distinct isoenzymes of adenylate deaminase may occur in the lugworm body-wall muscle. Isoenzymes of adenylate deaminase have been detected in most mammalian tissues (Ogasawara *et al.*, 1974a,b; 1975a,b). The isoenzymes from rat kidney, skeletal muscle and heart are also separated by chromatography on phosphocellulose and show quantitatively different kinetic properties.

The ATP-activated adenylate deaminase may have a wide distribution among invertebrate animals. The enzyme may have been overlooked in many previous investigations because it was assayed spectrophotometrically at low AMP concentrations in the absence of ATP (Stone, 1970; Bishop & Barnes, 1971; Senft *et al.*, 1972; Barrett, 1973). The apparent $s_{0.5}$ for AMP with ATP-activated enzyme from earthworm gut homogenates (McDonnell & Tillinghast, 1973) is similar to that reported here for the lugworm enzyme. In a survey of a variety of marine invertebrate animals, the ATP-activated AMP deaminase was detected in all species examined (Gibbs *et al.*, 1976; Bishop & Barnes, 1971).

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