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ASPARTATE AMINOTRANSFERASES FROM RIBBED MUSSEL GILL TISSUE: REACTIVITY WITH β-L-CYSTEINESULFINIC ACID AND OTHER PROPERTIES

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Abstract—1. The β-form of the cytosolic aspartate aminotransferase (cAAT) and the mitochondrial aspartate aminotransferase (mAAT) from ribbed mussel (*Modiolus demissus* = *Gukensia demissa*) gill tissue each exist as dimers of equal mol. wt (44,000).

2. Both the cAAT and mAAT are reactive with β-L-cysteine sulfinic acid (CSA) as an amino donor substrate.

3. The apparent K_ms for CSA are very high (19–250 mM) and increase with decreasing pH.

Aspartate aminotransferase (AAT) (L-aspartate; 2-oxoglutarate aminotransferase, EC 2.6.1.1) activity has been found in all tissues of all the molluscs investigated (see Bishop *et al.*, 1983). Studies on multiple molecular forms or isozymes of AAT in some bivalve tissues are suggestive of distinct mitochondrial (mAAT) and cytosolic (cAAT) variants which may or may not be polymorphic (see Paynter *et al.*, 1984a,b). The study of Paynter *et al.* (1984a) on partially purified preparations of the β-form of the cAAT and the single mAAT form from ribbed mussel gill tissue indicated some considerable differences between the two forms in terms of the substrate K_ms and V_{max} values at different pHs. For instance, the cAAT showed a change in the aspartate K_m from about 1 mM at pH 9.5 to 150 mM at pH 6.5 whereas the mAAT showed only a modest change in the aspartate K_m from 0.7 mM at pH 9.5 to 2.5 mM at pH 6.5. These differences in behavior with regard to the aspartate K_m may have a considerable regulatory effect on aspartate metabolism as aspartate levels fall and succinate plus alanine levels rise with the acidification of the cytosol during anaerobic stress in these bivalves (Ellington, 1983). However, in order to better understand how changes in behavior of the AATs with pH might affect asparate metabolism, one needs to know how the K_ms for glutamate and oxaloacetate change with changing pH, an aspect omitted in the study by Paynter *et al.* (1984a).

A second important aspect of the AAT activity is its possible reactivity with β-L-cysteinesulfinic acid (CSA) as an analog of L-aspartate (Singer and Kearney, 1955, 1956; Jenkins and D'Ari, 1966). The reactivity of purified AATs with CSA has not been investigated in molluscs and may be of importance in marine molluscs when one considers the high levels of tissue taurine and the possible role of CSA as an intermediate in the biosynthesis of taurine from cysteine (Allen and Awapara, 1960; Kochakian, 1976; Amende and Pierce, 1978; Bishop *et al.*, 1983).

Therefore, we have purified one of the cAAT's (β-form) and the mAAT from ribbed mussel gill tissue to homogeneity and have determined the reactivity with CSA plus the dimeric nature of these enzymes.

MATERIALS AND METHODS

Ribbed mussels (*Modiolus demissus*), purchased from Northeast Environment Laboratories (Monument Beach, MA) were maintained in artificial sea-water (Jungle Laboratories Inc., Standard, FL, USA) as described by Greenwalt and Bishop (1980). Except where noted all reagents and coupling enzymes were purchased from Sigma Chemical Co., St. Louis, MO, USA. Ammonium sulfate (enzyme grade) was obtained from Schwartz–Mann (Orangeburg, NY, USA).

Enzyme assay

During purification and standard assay, the AAT activity was determined spectrophotometrically by measuring oxaloacetate production as NADH oxidation (340 nm) in a reaction mixture containing 20 mM aspartate, 10 mM α-ketoglutarate, 70 μM NADH, 4 units malate dehydrogenase, 100 mM Tris–HCl (pH 8.3) and enzyme in a 2 ml cocktail. The reverse (aspartate forming) direction was measured by coupling the transaminase reaction to α-ketoglutarate dehydrogenase and measuring the reduction of NAD (340 nm) in a reaction mixture containing 20 mM glutamate, 5 mM oxaloacetate, 70 μM NAD, 0.1 mM CoA, 50 mM Tris HCl (pH 8.3), 1 unit α-ketoglutarate dehydrogenase and enzyme in a 1 ml cocktail.

Reactivity with CSA was determined by replacing either L-glutamate or L-aspartate with 25 mM CSA in combination with either oxaloacetate or α-ketoglutarate, respectively. Lactate dehydrogenase (LDH) was used in place of the other coupling enzymes to measure the formation of the pyruvate resulting from the spontaneous breakdown of the transamination product (β-sulfinylpyruvate) as NADH oxidation in the recording spectrophotometer.

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Incubations were at room temperature (23°C). Modifications of these assay mixtures for kinetic measurements are described below. One unit of activity synthesized 1 μmol of product/min under conditions specified. Kinetic constants were determined using computer-assisted analysis of initial rates using least-squares analysis to determine the best linear fit. Protein was estimated by the procedure of Miller (1959).

Isozyme distribution

Starch gel electrophoresis was performed as described previously using the Tris–borate buffer system at pH 7.0 rather than pH 6.5 (Paynter *et al.*, 1984a). Isozyme distribution and purity of electromorphic types was determined using non-denaturing polyacrylamide gel (PAG) electrophoresis in the following manner. A litre of stock electrophoresis buffer contained 14.4 g glycine and 3 g Tris (base). Samples of purified enzyme were mixed with a glycine solution (33.3% w/v), applied onto a 10% non-denaturing PAG and electrorophoresed at approximately 35 amp at 4°C. Gels were stained for activity in 200 mg L-aspartate, 100 mg α-ketoglutarate and 150 mg fast blue BB in 100 ml of 100 mM Tris pH 8.3. To test the gels for reactivity with CSA, CSA (200 mg) was added in place of L-aspartate and the reaction coupled with LDH to measure pyruvate formation as described for the alanine aminotransferase (Paynter *et al.*, 1984b). A Coomassie blue solution (1%) in isopropanol:acetic acid:water (25/10/65; v/v/v) was used to stain for protein. Gels were destained in this solution but without Coomassie blue.

Cytosolic AAT preparation

Initial purification procedures were performed as indicated in Paynter *et al.* (1984a), with modifications (as described below) beginning with the later column chromatography phases of separation. Starting with the HAP column step, a linear gradient (10 mM–400 mM) of degassed phosphate buffer (pH 6.8) was used to elute a single peak of AAT activity from the HAP column. Fractions (2 ml) were collected and all fractions showing activity were pooled (~60 ml) and ultrafiltered (10,000 mol. wt cut-off) at 30 psi to a vol of 5 ml. Pyridoxal phosphate (10 μM) was added and the preparation applied carefully to a leveled column bed of Sephadex G-150 column (45 × 2 cm) in 100 mM potassium phosphate buffer (pH 6.8) and eluted with this buffer at a flow rate of 52 ml/hr. Fractions (2 ml) were collected and all fractions showing activity were pooled (~40 ml) then concentrated (as described above) to a vol of 5 to 7 mls. The retentate was then applied to a Reactive Blue column (1 cm × 16 cm) in with 10 mM potassium phosphate buffer (pH 8.0) and eluted with this buffer. Fractions (2 ml) were collected and those containing activity were pooled (approx. 8–10 ml). This preparation was used for subsequent experiments.

Mitochondrial AAT preparation

Initial purification from lysed gill mitochondria was as indicated by Paynter *et al.* (1984a). The ammonium sulfate pellet containing the mAAT activity was resuspended in a minimum vol of 10 mM phosphate buffer, dialyzed, then applied to the HAP, Sephadex and the Reactive Blue columns as described above for the cytosolic AAT preparation.

Denaturing electrophoresis and gel-filtration for mol. wt measurements

Denaturing PAG electrophoresis was performed according to the Laemmli (1970) procedure using a slab with a stacking gel, a 10% "running" gel and the standard Tris glycine SDS running buffer at pH 8.3 at room temp (22°C) and 35 mM (100–150 V). Samples were prepared by boiling in the sample buffer (Tris–HCl pH 6.8) with 10% SDS, 1 mM DTT and 20% glycerol) then cooled. After electrophoresis, protein in the polyacrylamide gels was detected by Coomassie blue staining as described above. The standards used to calibrate the migration pattern were lysozyme (14,400 mol. wt), soybean trypsin inhibitor (21,500 mol. wt), carbonic anhydrase (31,000 mol. wt), ovalbumin (45,000 mol. wt), bovine serum albumin (66,200 mol. wt) and phosphorylase B (92,500 mol. wt), from BioRad Labs.

Gel-filtration for mol. wt estimation of the active enzymes was performed using the Sephadex G-150 described above by the procedure of Andrews (1964) with β-amylase (200,000), lactate dehydrogenase (120,000), ovalbumin (45,000) and cytochrome *c* (12,700) as mol. wt standards.

RESULTS AND DISCUSSION

The purification procedure for the cAAT (β) and the mAAT results in a preparation that showed single Coomassie blue staining bands that were coincident with AAT activity staining with non-denaturing PAG electrophoresis. The two activities showed very different electrophoretic mobilities which was in agreement with previous work (Paynter *et al.*, 1984a).

The specific activities of the purified enzymes varied somewhat from one preparation to another and neither were particularly stable in the pure form. Unlike the less pure forms described previously, more than 50% of the activity was lost with 1 week storage at 2°C or frozen at −20°C. The final specific activities for the cAAT and the mAAT were 11–23 units/mg protein and 35–65 units/mg protein, respectively.

With SDS–PAG electrophoresis, both the cAAT and the mAAT showed single Coomassie blue staining bands with identical or nearly identical mol. wts of 44,000. With gel-filtration using the calibrated Sephadex G-150 column, the average mol. wts of the native enzymes were about 90,000 for both the cAAT and the mAAT preparation. Therefore, it would appear that both the cAAT and mAAT activities showed a dimeric structure of two monomeric units of identical mol. wts. AAT from tissues of vertebrates have been shown to have a dimeric structure (see Paynter *et al.*, 1984a).

With non-denaturing PAG electrophoresis of both purified enzymes, the CSA reactive band migrated in the same position as the L-aspartate reactive band. Using starch gel-electrophoresis with crude cytosolic and mitochondrial preparations (see Paynter *et al.*, 1984a) all of the CSA reactive bands migrated in positions that were coincident with the L-aspartate reactive bands. It would appear that most if not all of the CSA transaminase activity was associated with the mAAT or cAAT activities and that there was little or no separate CSA transaminase activity associated with other proteins. A separate CSA transaminase has been reported in oak leaves (Perez-Milan *et al.*, 1959).

Both purified AAT's showed considerable reactivity with CSA as a substrate in place of L-aspartate or L-glutamate using the spectrophotometric assay. The relative CSA reactivities compared to L-aspartate reactivities at different pHs and at fixed substrate concentrations with both the mAAT and cAAT are described in Fig. 1. Under these conditions, the reactivity with CSA showed a sharp optimal pH around pH 8 with both enzymes, whereas the reactivity of L-aspartate with cAAT decreased almost an

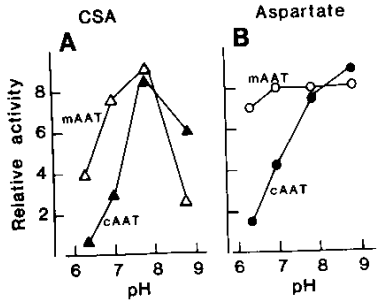

Fig. 1. Variation in relative activity of the mAAT with pH. The amount of enzyme added was adjusted so as to be of nearly equal reactivity (units) at pH 7.8. The reaction mixtures contained 25 mM CSA (β-L-cysteine sulfinic acid) and 20 mM L-aspartate in panels A and B, respectively. Concentrations of other substrates and reactants are described in the Materials and Methods.

order of magnitude between pH 8.6 and 6.3; with mAAT the reactivity with L-aspartate changed only slightly between pH 8.6 and 6.3. This result with L-aspartate confirmed the earlier experiments (Paynter *et al.*, 1984a) and indicated that the relative reactivity of CSA compared to L-aspartate was pH dependent.

Early studies at a single pH have indicated that the bovine heart cAAT was more reactive with CSA than L-aspartate (Singer and Kearney, 1956; Jenkins and D'Ari, 1966). To evaluate this aspect, the apparent K_ms for CSA were determined. The K_ms for CSA were considerably higher than the K_ms for L-glutamate or L-aspartate with both AATs (Table 1). With the cAAT at decreasing pH, the apparent K_m for L-aspartate and CSA increased more than an order of magnitude and the K_m for oxaloacetate fell whereas the apparent K_m for L-glutamate changed only slightly. With the mAAT, the apparent K_ms for L-glutamate at both pHs were much higher than those for L-aspartate and the apparent K_ms for L-aspartate and CSA increased only 2–5-fold between pH 8 and pH 6.3–6.5.

These results mean that at about pH 8, the V_{max} for both ATTs with CSA is considerably higher than the V_{max} with L-aspartate as a substrate. Secondly, it would appear that the cAAT would be more reactive in the L-aspartate forming direction at supposed cytosolic substrate concentrations and pH, whereas the mAAT would be more reactive in the L-glutamate forming direction at supposed mitochondrial substrate concentrations at all physiological pH's. Given these data and the high K_ms for CSA with both enzymes, it would appear that the mAAT would be more reactive with CSA than the cAAT and that turnover of CSA would require entry of CSA into the mitochondria.

Table 1. Cytosolic and mitochondrial aspartate aminotransferase (AATs) from ribbed mussel gill tissue: apparent K_ms for substrates and for β-L-cysteinesulfinate

Substrate*	Assay pH	Apparent K_m (mM)* (cAAT)	(mAAT)
L-aspartate	8.3	6.7	0.56
L-aspartate	6.5	160	2.6
α-ketoglutarate	8.3	0.043	0.63
α-ketoglutarate	6.5	0.03	0.36
L-glutamate	8.3	5.0	19.5
L-glutamate	6.3	6.0	39
oxaloacetate	7.8	0.014	0.05
oxaloacetate	6.5	0.010	0.035
β-L-cysteinesulfinate	8.7	76	—
β-L-cysteinesulfinate	7.8	19	28
β-L-cysteinesulfinate	6.3	250	62

*Buffers and the fixed concentrations of the amino acceptor (keto acid) or amino donor amino acid are described in the Materials and Methods. cAAT and mAAT refer to the cytosolic AAT and mitochondrial AAT, respectively.

In this regard then, the concentration of CSA in the cytosol and the mitochondrion and access of CSA to the mitochondrion becomes an important point. Kochakian (1976) reports between 0.09 and 0.32 μmol of CSA per g of tissue in various male reproductive organs of a squid. Livingstone *et al.* (1979) report three unknown ninhydrin positive compounds eluting between taurine and aspartate in the amino acid analysis chromatogram of extracts of *M. edulis*; one of these unknowns is probably hypotaurine (see Amende and Pierce, 1978) and at least one of the other two could be CSA and would be in the range of 0.31–0.64 μmol/g dry wt. Assuming Kochakian's (1976) weights were wet weights, then the average sea mussel CSA concentration could be in the range of 3 to 10 μM which is far below the apparent K_ms for CSA with the mAAT and the cAAT.

The tracer study by Allen and Awapara (1960) using [^{35}S]cysteine and [^{35}S]methionine injected into individual sea mussels (*M. edulis*) and brackish water clams (*Rangia cuneata*) indicated probable bivalve species differences in the shunting of carbon and sulfur from these amino acids to taurine. With the clam, cysteic acid, CSA and taurine were radiolabelled whereas cysteic acid, hypotaurine, and taurine but not CSA were labelled with the sea mussel. Because the sea mussel and the ribbed mussel have many common metabolic aspects such as high mAAT levels (Paynter *et al.*, 1984a,b) and a relatively non-specific L-amino acid oxidase that will deaminate both cysteine and methionine (Burcham *et al.*, 1980), one might predict that, if formed, CSA would not accumulate and may participate as an intermediate in taurine biosynthesis in mussels. These problems and other uncertainties concerning taurine biosynthesis in these molluscs (see Bishop *et al.*, 1985) are under investigation.

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