MITOCHONDRIAL BOVINE ASPARTATE AMINOTRANSFERASE

Preliminary sequence and crystallographic data

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1. Introduction

Cytosolic aspartate aminotransferase (EC 2.6.1.1) from ox heart has been fully characterized [1,2]; the most significant results have been reviewed [3].

An investigation is now in progress to establish structural and evolutionary relationships between this enzyme [4] and the homologous one from pig heart, whose complete primary structure has been reported [5,6].

A more complete picture from an evolutionary point of view should also arise from a comparison of the mitochondrial enzymes. In fact the primary structure of the mitochondrial porcine enzyme has been reported [7,8]. Structural investigations on cytosolic and mitochondrial chicken aspartate aminotransferases are being pursued [9,10]. Therefore we have undertaken the purification and characterization of mitochondrial bovine aspartate aminotransferase. Preliminary sequence and crystallographic data are reported here.

2. Experimental

2.1. *Purification of the enzyme*

After removal of connective tissue and fat, about 2 kg fresh ox heart muscle were minced and homogenized in a waring blender (5 min, full speed) with 1.5 l 0.05 M Tris–HCl, 5 mM 2-oxoglutarate, 5 μM pyridoxamine-5′-phosphate, 0.1 mM dithiothreitol (pH 8.0). Insoluble material was collected by centrifugation (Sorvall RC 2B, 3000 × *g*, 30 min) and re-extracted with 1.0 l of the same buffer. The combined supernatants were heated for 10 min at 60°C. After centrifugation an ammonium sulphate precipitation at 50–75% saturation was performed. The precipitate was suspended in 0.1 M 2-oxoglutarate, 1 mM dithiothreitol (pH 5.4) and dialyzed against 0.05 M Tris–HCl, 1 mM dithiothreitol (pH 7.0). The dialysed solution was applied on the top of two 45 × 2.5 cm tandem columns [11] filled with CM–Sepharose and DEAE–Sepharose, respectively, equilibrated and eluted (~100 ml/h) with the above-mentioned buffer. The protein fraction not retained by the resins was used for the purification of the cytosolic enzyme. The mitochondrial enzyme was eluted from the anionic resin by using the same buffer with added 100 mM NaCl. The active fractions were collected and dialysed against 0.02 M glycine–NaOH, 1 mM dithiothreitol (pH 9.5). The enzyme solution was applied on an SP–Sephadex C-50 column (1.5 × 30 cm) equilibrated and eluted with the above buffer. After the elution of broad peak of impurities, the enzyme was eluted by using the same buffer with added 100 mM NaCl. The pooled active protein fractions (160 mg, spec. act. 228, $E^{1\%}_{1\,cm}$ at 280 nm 14.9) resulted to be homogeneous on polyacrylamide gel electrophoresis (pH 4.6).

2.2. *Chemical studies*

Quantitative amino acid analyses were performed on a Unichrom Bekman apparatus equipped with a

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mod.B calorimeter. Norleucine and α-amino-β-guanidopropionic acid were used as internal standards. A single dansyl-α-amino acid, identified as serine by thin-layer chromatography on polyamide sheets [12], was observed after dansylation and HCl hydrolysis of protein samples [13]. The pyridoxal-5′-phosphate content was 0.97 mol/46 500 g. The N-terminal sequence was determined on protein samples after denaturation and aminoethylation by using the dansyl-Edman procedure [13]. The phenylthiohydantoins were further identified by gas–liquid chromatography [14].

2.3. *Enzyme crystallization and X-ray studies*

Samples of the enzyme were crystallized by the vapor diffusion method adapted for microtechniques. Polyethylene glycol was used as 'salting out' agent [15]. A solution of the enzyme in deionized water was prepared (~5 mg/ml) and a 10μl droplet was mixed in the well of a siliconed microculture slide with an equal amount of a solution of polyethylene glycol (mol. wt 4000 ± 50, 18% w/v), buffered at the desired pH with 10 mM glycine–NaOH (pH 8.0–9.5). The slide was then placed in a closed glass box (20 ml), having a reservoir with ~5 ml of the buffered polyethylene glycol solution. Crystals invariably grew as platelets, diamond shaped, and reached 0.4 × 1.0 × 0.1 mm in ~1 week.

A crystal grown at pH 9.1, was mounted in a sealed capillary and X-ray photographs were recorded on a precession camera (diam. 60 mm), using Ni-filtered CuK_α radiation.

Table 1
Amino acid composition of mitochondrial aspartate aminotransferase from ox heart compared with the compositions of the homologous enzymes from pig and chicken heart

Amino acid	Bovine enzyme			porcine[a] enzyme	avian[b] enzyme
	mmol/100 g protein	mol/ 46 500 g protein	nearest integer	residue/ subunit	residue/ subunit
Arginine	47.5	20.4	20	20	25
Histidine	21.8	9.6	10	9	11
Lysine	71.4	30.7	31	28	36
Phenylalanine	41.2	17.6	18	13	16
Tyrosine	33.8	14.6	15	14	16
Leucine	63.3	27.1	27	29	31
Isoleucine	56.4	24.9	25	23	22
Methionine	22.6	9.9	10	11	9
Cysteine[c]	16.1	6.7	7	7	5[f]
Valine	66.8	29.4	29	27	23
Alanine	89.0	38.6	39	34	41
Glycine	48.3	31.0	31	33	33
Proline	44.4	19.2	19	20	19
Glutamic acid	89.7	39.3	39	39	36
Serine[d]	76.3	33.0	33	25	27
Threonine[d]	51.1	21.6	22	19	16
Aspartic acid	80.5	35.2	35	39	43
Tryptophan[e]	20.7	8.5	9	8	8

[a] Data from [7] calculated from the reported primary structure
[b] Data from [17]
[c] Calculated as cysteic acid according to [21]
[d] Extrapolated to 0 time hydrolysis (all the other values are the average of 24, 48, 72 h hydrolysis in 6 M HCl)
[e] Determined by the spectrophotometric method [22]
[f] Data from [23]

3. Results and discussion

The work in [10,16–18] on mitochondrial aspartate aminotransferases showed that these isozymes can be separated from the corresponding cytosolic ones by exploiting their different isoelectric points, the mitochondrial isozymes studied so far being strongly basic proteins. Our purification procedure combines some of above features with consecutive CM– and DEAE–Sepharose ion-exchange chromatography, currently employed in our laboratory for the purification of the cytosolic aspartate aminotransferase isozymes [11]. From 2 kg ox heart muscle tissue ~200 mg pure enzyme was obtained.

Mitochondrial aspartate aminotransferase has been purified and characterized [16] from beef kidney. However yields from this source cannot be considered fully satisfactory (~20 mg from 2 kg tissue).

SDS–gel electrophoresis [19] shows only a single band having the same mobility as the cytosolic bovine (subunit mol. wt 46 500) [4] and porcine (subunit mol. wt 46 500) [5,6] enzymes. Therefore a subunit mol wt 46 500 ± 2000 can be assumed.

Chemical studies were undertaken and preliminary results are summarized in table 1 and fig.1. The amino acid composition is quite similar to that of the corresponding porcine and avian isozymes, the only notable exception being the difference in cysteine content. However, we observed that sulphydryls of the native proteins have similar reactivity [20]. Also the N-terminal sequence except for the uncertainity of Glu in position 8, is identical with those already reported for the porcine [7,8] and avian isozymes [23]. The close homology among these proteins also arises from crystallographic studies. Precession photographs (fig.2) show that crystals are triclinic, space group P1, with: $a = 55.6$ Å; $b = 58.6$ Å; $c = 76.0$ Å; $\alpha = 85.5°$; $\beta = 109.1°$; $\gamma = 115.6°$. The unit cell parameters are, within the limits of experimental errors, identical with those reported for the chicken heart isozyme [10].

2	4	6	8	10	12
Ser-Ser-Trp-Trp-Ala-His-Val-Glx-Met-Gly-pro-Pro					

Fig.1. Amino terminal sequence of mitochondrial aspartate aminotransferase from ox heart.

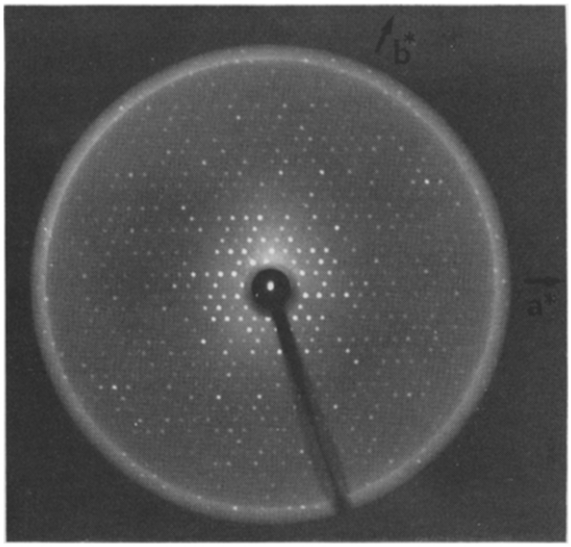

Fig.2. $\mu = 16°$ precession photograph, hKO zone, of mitochondrial bovine aspartate aminotransferase on a 60 mm Buerger precession camera (38 kV, 18 mA, Ni filtered CuK_α radiation).

Visual comparison of the hOl and hKO precession photographs of the ox enzyme with those published for the chicken heart enzyme shows that the intensities of corresponding spots closely follow a similar pattern. However, some inversion in the relative intensities can be seen.

The identity of the unit cell parameters of the two enzymes and the strict similarity of the intensity distribution strongly suggest that the tertiary structures of the two proteins are similar, whereas small variations in the intensities of the corresponding reflections indicate some minor changes in the amino acid sequence. Immunochemical studies indicated that a close interspecies homology exists between mitochondrial aspartate aminotransferases from pig and chicken heart [24]. Our results, obtained with the crystallographic technique, an independent experimental approach, indicate a similarity in tertiary structure between the two mitochondrial isozymes from chicken and ox heart, confirming the hypothesis of strong evolutionary constraints existing for the mitochondrial isozymes. On the contrary significant differences have been found for the cytosolic isozymes from ox [4], pig [5,6] and chicken heart [25]. As

mitochondrial isozymes are synthesized in the cytosol [26], of very much interest appear to be studies concerning the transport mechanism [27] which could be responsible for the observed evolutionary constraints concerning the structure of mitochondrial aspartate aminotransferases.

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