The amino acid sequence of the aspartate aminotransferase from baker's yeast (Saccharomyces cerevisiae)

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INTRODUCTION

Structural studies of the aspartate aminotransferases (EC 2.6.1.1) from animal and microbial sources have attracted considerable interest in recent years, in the context both of mechanism of action and of evolution of the isoenzymes.

The cytosolic and mitochondrial isoenzymes from a particular animal source generally share about 48% identity of amino acid sequence, indicative of evolution from a common ancestral form [1,2]. Furthermore, quantitative comparisons of the sequences of animal isoenzymes known to date (those of human, horse, pig, rat, mouse and chicken) have shown that the cytosolic isoenzyme has evolved at about 1.3 times the rate of the mitochondrial form [3]. This difference may be attributed to extra constraints on the structure of the mitochondrial isoenzyme concerned with its uptake from the site of synthesis in the cell cytosol into mitochondria [4,5].

The earlier history of the aspartate aminotransferases is as yet unclear. The sequence of the enzyme from Escherichia coli has been determined by both protein [6] and DNA [7] methods and appears to be equally related to those of the eukaryotic cytosolic and mitochondrial forms (about 40% identity). This suggests that the event that gave rise to the isoenzymes of aspartate aminotransferase occurred after the emergence of eukaryotic cells. Consistently the eukaryotic isoenzymes are roughly equally, but very distantly, related to the aspartate aminotransferase from the extreme thermophilic archaebacterium Sulfolobus solfataricus [8]. This is in distinct contrast with the situation with malate dehydrogenase, for example, where the mitochondrial isoenzyme is closely related to the enzyme from E. coli and the cytosolic isoenzyme is closely related to that from Thermus flavus; these interesting differences have recently been reviewed [9].

To date no amino acid sequences have been reported for aspartate aminotransferases from plants or from eukaryotic micro-organisms; this represents a considerable gap in knowledge of structural relationships in this family of enzymes. The cytosolic aspartate aminotransferase from baker's yeast (Saccharomyces cerevisiae) has previously been isolated and partially characterized [10-13], and hence we chose that enzyme for further study. The (nearly complete) amino acid sequence is reported here. A point of particular interest concerning yeasts (at least those of the genus Saccharomyces) is that they lack a mitochondrial aspartate aminotransferase [13,14]. Recently it has been shown that this is not true of all yeasts, and a mitochondrial aspartate aminotransferase has now been purified from Rhodotorula marina [15]. This difference may reflect the fact that R. marina is a non-fermentative yeast in distinction from Saccharomyces.

A preliminary account of some of this work has already been published [9].

MATERIALS AND METHODS

Materials

CNBr, fluoren-9-yloxycarbonyl chloride and iodoacetic acid were from Aldrich Chemical Co., dansyl-aminoc acids were from Mann Research Laboratories, phenyl isothiocyanate used for manual sequence analysis was from Pierce Chemical Co., whereas all reagents required for automated sequence analysis were from Applied Biosystems. Pyridine, trifluoroacetic acid and h.p.l.c-grade solvents were from Rathburn Chemical Co. All proteolytic enzymes and most other materials were from Sigma Chemical Co.

Enzyme purification

Aspartate aminotransferase was isolated from fresh commercial samples of compressed baker's yeast as follows.

Step 1. Compressed yeast (1.5 kg wet wt.; approx. 500 g dry wt. of cells) was mixed with 387 ml of water and 48 ml of conc. aq. NH₄OH (d = 0.88) and the mixture left for 16-18 h at room temperature. Subsequently water (1760 ml) and 0.5 M-EDTA (pH 8.0, 60 ml) were added, followed by adjustment of the pH to around 7 with 1.0 M-acetic acid. Then (NH₄)₂SO₄ was added to 47% saturation, followed by removal of debris by centrifugation. 

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at 3500 g for 30 min. Active enzyme was precipitated by increasing the (NH₄)₂SO₄ concentration to 60% saturation and was recovered by centrifugation at 3500 g for 45 min.

**Step 2.** The precipitate from step 1 was suspended in sodium acetate buffer (10 mM, pH 5.8, 250 ml) containing phenylmethanesulphonyl fluoride (1.0 mM), pepstatin (0.4 μM) and protamine sulphate (3.7 g) and dialysed against running tap water for 4 h. After this the solution was dialysed against three lots of 5 litres of acetate buffer (10 mM, pH 5.8) containing 10 μM-phenylmethanesulphonyl fluoride and 10 μM-pyridoxal 5'-phosphate.

**Step 3.** The pH of the dialysed protein solution was adjusted to 5.8 with acetic acid, and precipitated protein was removed by centrifugation at 3500 g for 45 min. The clarified solution was then applied to a column of CM-cellulose CM23 (7 cm × 15 cm) equilibrated with the acetate buffer used in step 2. The column was washed with 2 litres of this buffer at a flow rate of 2 litres h⁻¹, after which the enzyme was eluted by using a linear gradient from 0 to 0.1 M-NaCl in a total volume of 4 litres.

**Step 4.** Active fractions from step 3 were pooled, diluted 1.5-fold with water, and applied directly to a column (25 cm × 20 cm) of CM-Sephadex C50 equilibrated in sodium acetate buffer (10 mM, pH 5.8) containing 0.05 M-NaCl. After washing the column with starting buffer (100 ml), the enzyme was eluted by using a linear gradient from 0.05 M- to 0.14 M-NaCl in a total volume of 400 ml.

**Step 5.** Active fractions from step 4 were pooled and applied directly to a column (25 cm × 30 cm) of Cibacron Blue-Sepharose equilibrated in sodium acetate buffer (10 mM, pH 5.8) containing 0.12 M-NaCl. After washing with starting buffer, the enzyme was eluted with the same acetate buffer containing 10 mM-2-oxoglutarate. Active fractions were pooled and concentrated to about 50 ml by ultrafiltration through an Amicon PM-30 membrane.

**Step 6.** If necessary, final purification was achieved by column chromatography using CM-cellulose CM52 (column size 2.5 cm × 18 cm; equilibrated with 10 mM-acetate buffer, pH 5.8). Active enzyme was eluted by using a linear gradient of 0 to 0.15 M-NaCl in a total volume of 400 ml.

A typical procedure yielded 40 mg of enzyme of specific activity 525 units/mg, representing a recovery of 42% of the activity in the initial lysate.

**Enzyme assays and protein determination**

Up to step 3 in the purification procedure enzyme activity was measured using the direct method of Cammarata & Cohen [16], whereas for later steps the linked-assay method of Karmen [17] was used under conditions previously described [13]. Protein concentrations were estimated by measurement of A₂₈₀ using a specific absorption coefficient (A₂₈₀) of 9.5 [12].

**Structure analysis**

Experimental details of the following procedures are described in Supplementary Publication SUP 50164: carboxymethylation of the enzyme; cleavage of carboxymethylated protein with trypsin and with CNBr; peptide fractionation by gel filtration and by reverse-phase h.p.l.c.; subdigestion of purified peptides with Staphylococcus aureus V8 proteinase and with pepsin; amino acid analysis; N-terminal analysis by dansyl-Edman method and by automated gas-phase methods.

**C-Terminal analysis**

Peptide (5–10 nmol) was dissolved in 50 μl of pyridine acetate buffer (0.1 M, pH 5.6) and 5 μl of a solution of carboxypeptidase Y (0.01 nmol/μl in water) was added. Aliquots (6 μl) were withdrawn at suitable time intervals from 0 to 20 min and transferred to vials containing 2 μl of acetic acid. Samples were dried under vacuum and then derivatized with fluoren-9-ylmethyloxycarbonyl chloride for analysis by reverse-phase h.p.l.c. (see Supplementary Publication SUP 50164).

**Identification of the N-terminal blocking group**

A blocked N-terminal nonapeptide (2.5 nmol) was introduced onto the probe of a Kratos MS50 mass spectrometer in a glycerol-thioglycerol matrix. Positive and negative ion mass spectra were obtained as previously described [18].

**RESULTS AND DISCUSSION**

**Purification of aspartate aminotransferase from baker's yeast**

Results of a typical purification starting from 1.5 kg of compressed yeast are given in Table 1 of Supplementary Publication SUP 50164. Cells were broken using the ammonia-lysis method of Morena et al. [19], and the subsequent steps in the procedure were based on those developed by Sheehan [20].

The product was pure as judged by SDS/PAGE, and the subunit molecular mass was estimated to be 45 kDa (results not shown); the native enzyme is a dimer of identical subunits [12,13]. Attempts to confirm purity by N-terminal analysis failed; it is now known that this is because the N-terminal residue is acetylated (see below).

**Peptides from digestion with trypsin**

Carboxymethylated protein (50 mg) was digested with trypsin. Product peptides were fractionated by gel filtration through Sephadex G-25 (superfine grade) and fractions pooled on the basis of t.l.c. on cellulose plates with the solvent system butanol-1/ol/acetic acid/water/pyridine (15:3:12:10, by vol.). Eight pooled fractions were taken, and these were further fractionated by h.p.l.c. on C₁₈ reverse-phase columns.

In total 49 peptides were isolated in pure form as judged by N-terminal analysis. Peptides were sequenced by using either the dansyl-Edman method or automated gas-phase sequencing or both. Peptides were also subjected to amino acid analysis. Experimental details and results are given in Supplementary Publication SUP 50164.

The positions of most of the peptides obtained are shown in Fig. 1 with peptides numbered in order from the N-terminus. Peptides covering the entire sequence were obtained, with the exception of residues 21–22 and probably 235–236 (see below).

Some peptides originated from internal chymotryptic-like cleavages; these peptides are not shown in Fig. 1 if the parent peptide was also obtained. Peptides T34s and T35s originated from cleavage of a Tyr-Leu bond; the parent peptide was not isolated. Two peptides (T5e and T23e) arose from failure to cleave at Lys-Pro bonds.

Peptide T1 was resistant to sequence analysis. Further investigation of its structure is described below.

**Peptides from digestion with CNBr**

Carboxymethylated protein (30 mg) was fragmented with CNBr and the product was fractionated by gel filtration through Sephadex G-50 (fine grade). Five pooled fractions were taken on the basis of analytical t.l.c. Fraction F1 contained a single peptide (M1). The components of fractions F2 and F3 were further purified by h.p.l.c. using a macroporous C₄ column, whereas fractions F4 and F5 were purified by h.p.l.c. using a C₁₈ reverse-phase column.
Yeast aspartate aminotransferase

1. Amino acid sequence of the aspartate aminotransferase from yeast

Underlinings show the peptides on which the sequence was based; broken underlinings show peptides or parts of peptides for which only amino acid compositions were obtained. Lack of a final arrowhead implies that direct evidence for the point of termination of a peptide was not obtained. For details of sequence analysis of peptide VT1-1 and identification of the N-terminal acetyl group, see the text. T and M signify tryptic and CNBr peptides respectively. VT, VM and PM denote peptides produced by subdigestion of parent peptides with S. aureus V8 proteinase (V) or with pepsin (P). In numbering the protein the first digit is placed over the residue specified by that number. The sequence probably contains two missing residues at positions 235 and 236.

The positions of the fragments obtained in the sequence of the protein are shown in Fig. 1. Peptide M1 was resistant to sequence analysis. Partial sequences were obtained for peptides M2, M3 and M8, and complete sequences were obtained for peptides M6 and M7 using automated gas-phase methods. Peptide M4e arose from failure to cleave at a Met–Ser bond. Peptides M4 and M5 arising from cleavage of the Met–Ser bond were also isolated and sequenced, but are not shown in Fig. 1. Details of sequence analysis and amino acid analysis for all these peptides are given in Supplementary Publication SUP 50164.

Subdigestion of peptides

Peptides T1, T16, M1, M2, M3 and M8 were subjected to subdigestion with S. aureus V8 proteinase, and peptide M1 was also subdigested with pepsin. Product peptides were purified by h.p.l.c. using C18 reverse-phase columns and subjected to sequence analysis and/or amino acid analysis as appropriate. The peptides isolated are shown in Fig. 1. Detailed analytical results are given in Supplementary Publication SUP 50164.

The N-terminus of the protein

As stated above, the intact protein was resistant to N-terminal analysis as were peptides T1 and M1, suggestive of a blocked N-terminal residue.

Peptide T1, thought to contain residues 1 to 20 of the protein, was digested with S. aureus V8 proteinase and the two product peptides purified by h.p.l.c. Peptide VT1-2 had an amino acid composition entirely consistent with its arising from residues 10–20. Peptide VT1-1 was resistant to dansylation and had the amino acid composition: Ser1, Asp2, Glu1, Thr1, Ala1, Phe1, Ile1, Leu1.

The peptide was digested with carboxypeptidase Y and aliquots were taken for amino acid analysis at appropriate time intervals. The results are shown in Fig. 2 and are consistent with the amino acid sequence:

Xaa-Ala-Thr-Leu-Phe-Asn-Asn-Ile-Glu

The definite appearance of phenylalanine before leucine in the analysis was sufficient to establish the order of these two residues.

By comparison of the above sequence with the amino acid composition of peptide VT1-1 it is clear that Xaa is a modified serine residue.

The nature of the blocking group was established by using...
fast-atom-bombardment m.s. Peptide VM1-1 (identical with TM1-1) was used for this study.

The most abundant species in the negative-ion spectrum (spectra not shown) had a mass of 1049, corresponding to a peptide mass of 1050. This would correspond to a mass of 43 for the blocking group, the likely identity of which is therefore CH$_3$CO$^-$. In the positive-ion spectrum the most abundant species was at mass 1072. Assuming that this represented the peptide + Na$^+$ (as seemed likely, given that substantial peaks were observed for bradykin + Na$^+$, bradykin having been added as an internal standard) then again a mass of 43 could be assigned to the blocking group. A peak was also observed that could be attributed to the loss of acetylserine from the cationized parent, but no other convincing sequence ions could be identified.

It seems therefore, that the yeast enzyme shares the property of N-terminal acetylation with the cytosolic isoenzymes from horse [2] and from chicken [21]. The N-termini of the cytosolic isoenzymes from pig [22] and human [3] are not blocked. The situation with the isoenzymes from mouse [23] and rat [24] is unknown, since these structures were inferred from cDNA sequences.

Amino acid sequence of cytosolic aspartate aminotransferase from baker's yeast

The (nearly complete) amino acid sequence of the enzyme, based on the results summarized above, is given in Fig. 1.

In most cases where direct evidence for junction of two tryptic peptides was not strong (T6/T7, T16/T17, T17/T18, T19/T20, T29/T30), recourse was had to considerations of sequence identity with other known aspartate aminotransferases to confirm the overlaps (see Fig. 3).

Identification of the C-terminus of the protein rests on the isolation of VM8-5 as the only peptide lacking C-terminal glutamic acid from the digest of CNBr fragment M8 with S. aureus V8 proteinase.

Attempts to confirm this by carboxypeptidase digestion of the native aspartate aminotransferase were unsuccessful, since the protein appeared to be resistant to attack.

The remaining doubt concerns the region of the structure between peptides T18 and T19. On the basis of comparison with the sequences of all other known aspartate aminotransferases, two further amino acid residues would be expected to occur between these two peptides. In all other cytosolic isoenzymes this is a relatively highly conserved region of the structure, and the dipeptide Tyr-Phe occurs at the position in question [3]. In mitochondrial isoenzymes the residues are Tyr/His-Phe [2] and the second residue is also phenylalanine in the enzyme from E. coli (see Fig. 3). Examination of the three-dimensional structure of the cytosolic isoenzyme from chicken [23] shows that this part of the sequence forms a constrained a-helix (helix 9), making it highly improbable that a two-residue deletion could have occurred in the yeast enzyme at this point. The structure of the chicken cytosolic isoenzyme was examined by using co-ordinates (entry 1AAT; communicated by E.G. Harutyunyan & V.A. Malashkevich) obtained from the Protein Data Bank at Brookhaven National Laboratory.

Hence it seems most likely that the amino acid sequence in Fig. 1 is lacking two residues between peptides T18 and T19. It may be that the second of these residues is phenylalanine and that an N-terminal dipeptide was lost from T19 by chymotryptic-like cleavage. Attempts to isolate a peptide from the subdigest of M2 with S. aureus V8 proteinase covering the region of interest failed (the expected peptide would have contained 54 residues), and hence it did not prove possible to resolve this point. In the absence of evidence to the contrary, it is assumed that two residues, 235 and 236, are missing from the sequence shown in Fig. 1 and the C-terminal portion of the sequence has been numbered accordingly. The sequence then contains 414 residues. All other amino acid residues were directly identified, including tryptophan residues and the state of amidation of aspartic acid/asparagine and glutamic acid/glutamine (see details in Supplementary Publication SUP 50164), and in the majority of cases each peptide was sequenced at least twice. Hence, with the exception of the presumed residues 235 and 236, the structure presented is securely based.

Comparison of the sequence with those of other aspartate aminotransferases

The results reported here add a new dimension to comparative studies of aspartate aminotransferases by providing a sequence from a member of a phylum not previously represented. Comparison of the sequence of the yeast enzyme with representative vertebrate cytosolic and mitochondrial forms (those from pig heart) and with the enzyme from E. coli is shown in Fig. 3, with particular emphasis on those amino acid residues that are common to all four proteins.

Various insertions/deletions in the sequences must be assumed in order to align the structures. Two of these are of particular note. Firstly, the yeast enzyme contains four residues more than does any of the other three proteins in the region following residue 280. This part of the structure of the yeast enzyme is firmly based, and the existence of these extra residues seems certain. In the cytosolic isoenzyme from chicken [25], the amino acids preceding the proposed point of the insertion form a surface loop and those following form a surface a helix (helix 10). Hence the insertion of four extra residues in the yeast enzyme could be accommodated without major disruption of the structure, assuming that the overall folding of the two proteins is similar.

The other point of particular interest concerns the region around residue 130. As has previously been noted, vertebrate mitochondrial aspartate aminotransferase and the E. coli enzyme share the common feature of a deletion of four or five residues at this point compared with vertebrate cytosolic forms; this is the main feature which argues for a closer relationship between the E. coli and the mitochondrial isoenzymes [23]. It is therefore remarkable that this feature is shared also by the yeast cytosolic
isoenzyme, which is five residues shorter in this region than is the cytosolic isoenzyme from pig heart.

The comparisons in Fig. 3 also help in defining amino acid residues that are important in the formation of active aspartate aminotransferases. Known active-site residues [26] are, of course, conserved, and the other regions of strong sequence identity presumably represent features essential for correct folding (the three-dimensional structures of vertebrate cytosolic, vertebrate mitochondrial and the E. coli enzyme are very similar [27]). The structures shown in Fig. 3 contain approx. 25% of identical residues. This value decreases somewhat (to 23%) if other known vertebrate sequences are included in the comparison.

Recently Christen and his co-workers have reported sequence identities between the aspartate aminotransferases, the tyrosine aminotransferases of rat and E. coli and the histidinol-phosphate aminotransferases of E. coli and S. cerevisiae [28]. These proteins contain only 12 invariant amino acid residues (and one of these, Arg-386, seems to be missing in the aspartate aminotransferase from Sulfolobus solfataricus [8]). There is as yet, however, no information on the three-dimensional structures of these other aminotransferases, and it is safer to assume that a set of residues similar to those shown as conserved in Fig. 3 is required to produce eukaryotic and eubacterial-like aspartate aminotransferase folds.

The m.s. of peptide VM1-1 was carried out by Dr. Peter Farmer, MRC Toxicology Unit, Carshalton, Surrey, U.K. to whom we are grateful. Part of the work reported in this paper will be submitted by B.M. in partial fulfilment of the requirements for the Ph.D. degree of the Università di Roma 'La Sapienza'.

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