The primary structure of ornithine aminotransferase

Identification of active-site sequence and site of post-translational proteolysis

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Tentative assignments of functional residues in rat liver mitochondrial ornithine aminotransferase have recently been made using the amino acid sequence deduced from a cDNA clone [(1985) J.Biol.Chem. 260, 12993-12997]. Partial sequences obtained using the pure mature protein demonstrate that one of these assignments, that of Lys 292 as the residue that binds the coenzyme pyridoxal phosphate, is correct. However, the identification of the Glu 34–Gln 35 bond as the site of post-translational proteolysis is in error. This cleavage occurs instead at Ala 25-Thr 26.

Ornithine aminotransferase *Active-site lysine* *Leader sequence* *Primary structure*

1. INTRODUCTION

Ornithine aminotransferase (EC 2.6.1.13) from rat liver is synthesised in the cytoplasm as a precursor of M_r 49000 [1] and transferred to its final location, the mitochondrial matrix, where it is found as a mature protein of M_r 43000–45000 [2–4]. Recently a cDNA clone covering the whole coding region for this enzyme was sequenced [5] and by combining this new evidence with that from earlier published work two significant deductions concerning the mature protein were made. Firstly, the lysine responsible for binding the coenzyme pyridoxal phosphate was identified on the basis of limited homology with aspartate aminotransferase. Secondly, the point at which the protein is cleaved during post-translational proteolysis was assigned assuming that a tentative suggestion [6] for the identity of the blocked amino terminus was correct.

As a prerequisite to studies aimed at a precise description of the site and mechanism of a class of 'suicide' inhibitors of this enzyme, we have undertaken the determination of the primary structure of ornithine aminotransferase and in particular of the sequence adjacent to the lysine that binds the coenzyme by classical protein sequencing methods.

Although our sequence data are not complete, by using the cDNA sequence [5] we are able to provide definite identification both of the active-site lysine and of the site of post-translational proteolysis.

2. MATERIALS AND METHODS

Ornithine aminotransferase was prepared from rat liver according to [7]. A sample of about 20 mg of holo-enzyme, dissolved in 3.0 ml of 0.01 M Hepes buffer, pH 8.0, was reduced by the addition of 60 μmol sodium cyanoborohydride. After 30 min at room temperature the solution was dialyzed against distilled water and the protein recovered by lyophilization. These and subsequent operations were performed in the dark to minimize photodestruction of the chromophore. The reduced enzyme was carboxymethylated with iodoacetate [8] and then suspended in 3.0 ml of 0.1 M ammonium bicarbonate and digested with

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0.5 mg trypsin for 3 h at 37°C. A second 20 mg portion of similarly treated enzyme was digested with 0.5 mg chymotrypsin in 0.1 M ammonium bicarbonate for 4 h at 37°C.

Preliminary fractionation of the peptide mixtures was performed by passing the digests through a Sephadex G-25 superfine column (25 × 120 cm) eluted with 10% acetic acid. Further purification of peptides was accomplished by high-performance liquid chromatography (HPLC) on a macroporous reverse-phase column (Brownlee Labs, RP-300, 10 μm) with gradients of acetonitrile in 0.2% (v/v) trifluoroacetic acid generated using a Beckman model 420 instrument at a flow rate of 1.0 ml/min. The absorbance of the effluent was monitored at both 220 and 325 nm using a Beckman model 165 variable-wavelength detector. Peptides were sequenced by the manual dansyl-Edman procedure and complemented by carboxypeptidase digestion. Details of these and other analytical procedures have been published [8]. Determination of the N-terminal residue of ornithine aminotransferase was performed by using a modified version of the procedure recommended by Gray [9]. 2 nmol carboxymethylated protein was dissolved in 30 μl of 1% SDS by heating the mixture in a boiling water bath for 2 min. 20 μl of 0.2 M sodium bicarbonate and 50 μl DNS-Cl (5 mg/ml acetone) were added; the mixture was thoroughly mixed and left overnight at room temperature. The labelled protein was precipitated by the addition of 0.5 ml acetone, collected and acetone-washed by centrifugation and hydrolysed with 6 N HCl for 5 h at 110°C. Dansyl-amino acids were identified on polyamide plates as described by Hartley [10].

3. RESULTS AND DISCUSSION

The elution pattern of the chymotryptic peptides of reduced ornithine aminotransferase from Sephadex G-25 showed a single peak absorbing at 325 nm. Further purification of this material by HPLC allowed the isolation of a phosphopyridoxyl peptide whose sequence proved to be Leu-Gly-Lys(Pxy)-Ala-Leu. Comparison of the sequence of chicken heart mitochondrial aspartate aminotransferase with that deduced for the precursor of ornithine aminotransferase from a cDNA clone showed moderately high-significance homology between the region around the active-site lysine of aspartate aminotransferase and the region around Lys 292 of ornithine aminotransferase, thus leading to the suggestion that Lys 292 serves to bind pyridoxal phosphate in ornithine aminotransferase [5]. Our results demonstrate the correctness of this proposal since the sequence of the phosphopyridoxyl pentapeptide corresponds uniquely to that adjacent to Lys 292. Moreover, this identification is in accord with the results from the tryptic digestion. The expected tryptic phosphopyridoxyl peptide should be 57 residues long, since it contains 1 Arg-Pro and 1 Lys-Pro sequence, both insensitive to tryptic cleavage, with Trp 275 as N-terminus. From the tryptic digest of reduced ornithine aminotransferase a high-M_r fragment absorbing at 325 nm was obtained with an amino acid composition well in accord with that calculated from the deduced sequence around Lys 292 and a mute N-terminus, as expected from the destruction of DNS-Trp during acid hydrolysis. Scarcity of material prevented further analysis of this fragment.

From the same tryptic and chymotryptic digests a number of additional peptides were obtained in pure form and sufficient quantity. The structural information obtained from the analysis of these peptides covered 63.8% of the entire sequence and was always in accord with the structure deduced from the cDNA sequence, with the latter being of great help in interpreting some ambiguous data, concerning the determination of a few amidation states, the position of tryptophan residues, etc. In particular, the peptide Thr-Ile-Leu-Ser-Phe was the only tryptic peptide found which terminates with a residue different from Lys or Arg thus suggesting its being the C-terminus of the protein, again in accord with previous carboxypeptidase experiments [6] and with the cDNA deduced sequence [5].

Most interesting is the problem of the identification of the N-terminus of the mature protein. It was postulated [5] that the amino terminus of the mature enzyme may correspond to glutamine in position 35 of the deduced sequence of ornithine aminotransferase precursor on the basis of the following evidence: (i) previous studies on the mature enzyme failed to reveal a free amino terminus and experiments employing acetyl-amino acid releasing enzyme and amino acylase suggested

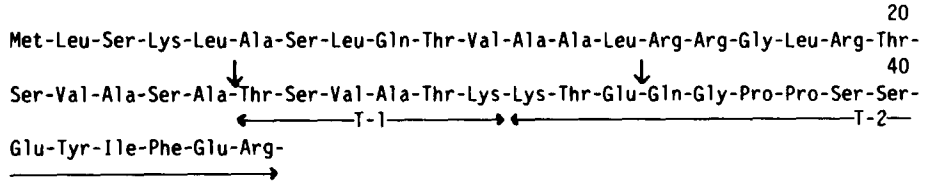

Fig.1. cDNA-derived sequence of the N-terminal portion of ornithine aminotransferase precursor [5]. The structure of tryptic peptides T-1 and T-2 is indicated by horizontal arrows under the sequence. The vertical arrows indicate the cleavage site between the precursor and mature enzyme as suggested by the present work (position 25–26) or as previously postulated (position 34–35) [5].

that the blocked amino-terminal residue is pyroglutamic acid [6]; (ii) Gln 35 is the only glutamine residue within the required sequence range to give a polypeptide chain of M_r corresponding to that found for the subunit of the mature enzyme. We have found and analysed two tryptic peptides (T-1 and T-2) whose sequence, indicated in fig.1 aligned along the pertinent region of the cDNA-deduced sequence, clearly identifies an amino terminus for the mature enzyme different from that postulated.

That Thr 26 is the real N-terminus of the mature enzyme is established on the following basis: (i) the N-terminus of the protein was determined after labelling with DNS-Cl and, since the expected DNS-Thr residue is acid-labile, a short hydrolysis time was adopted: a definite spot of DNS-Thr was visible on the polyamide plate; (ii) peptide T-1 unambiguously and uniquely corresponds to positions 26–34 of the precursor sequence, demonstrating that this section of the sequence remains with the enzyme after processing. The amino-terminal threonine of the mature protein is preceded by an alanyl residue and an -Ala-Thr- sequence should not be cleavable by trypsin. It is interesting to note that the cleavable sequence linking leader peptide to mature protein of ornithine aminotransferase -Ala-Thr- is similar to that of pig aspartate aminotransferase -Ala-Ser- [11] but different from that for ornithine transcarbamylase which is -Gln-Ser in rat [12–14] and -Gln-Asn- in human [15].

Positioning the N-terminus at Thr 26 gives an M_r of 45749 (414 residues), still quite compatible with the reported estimates [2] and leaves perfectly valid the observation on the homology of amino-terminal leader segments involved in targeting different enzymes to their common final destination in the mitochondrial matrix [5].

In conclusion, the reported identification of crucial sequences of ornithine aminotransferase appears to be a good example of how modern powerful techniques of protein sequence deduction from cDNA may be profitably complemented by the classical procedures of protein chemistry.

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