The Primary Structure of Leucine Aminopeptidase from Bovine Eye Lens*


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The amino acid sequence of bovine eye lens leucine aminopeptidase has been determined. Cyanogen bromide fragments, the COOH-terminal hydroxylamine fragment, and a large fragment obtained by digestion with Staphylococcus aureus protease were isolated from reduced and S-alkylated leucine aminopeptidase. The amino acid sequences of these fragments were determined by automated sequence analysis, by manual direct Edman degradation, and by the dansyl-Edman technique. Overlapping peptides were obtained by tryptic digestion of the S-alkylated protein or the citraconylated S-alkylated protein. The polypeptide chain of leucine aminopeptidase comprises 478 residues, corresponding to a molecular weight of 51,891. No significant sequence homology with any other published protein primary structure could be detected. This is the first report of a complete amino acid sequence of an enzyme belonging to the class of two-metal peptidases.

Leucine aminopeptidase is an exopeptidase catalyzing the hydrolysis of amino acids from the NH$_2$ terminus of polypeptide chains, and belongs to the class of aminopeptidases which are widely distributed in nature. Aminopeptidases with similar or identical properties have been found in many tissues, including lens, kidney, pancreas, muscle, and liver. Among these, leucine aminopeptidase from bovine lens has been studied most extensively (see review by Hanson and Frohne (2)). Leucine aminopeptidase from bovine lens has a molecular weight of 326,000 (3, 4) and an isoelectric point of 4.9 ± 0.2 (3). It consists of six identical subunits of $M_r = 54,000$ (4, 5). The crystalline enzyme contains two zinc ions per subunit, one of which can easily be replaced by a manganese or magnesium ion, resulting in an increase in peptidase activity (6, 7).

Electron microscopic investigations of leucine aminopeptidase in solution showed that the subunits are arranged at the vertices of a distorted triangular prism (8), and electron microscopic investigations of crystals of leucine aminopeptidase showed six asymmetric bilobal subunits arranged in such a way that the principal lobes are eclipsed and the minor lobes are staggered (9). X-ray studies revealed that leucine aminopeptidase crystallizes in the hexagonal space group $P6_122$ with unit cell dimensions $a = b = 132$ Å and $c = 122$ Å (10). Limited tryptic digestion of leucine aminopeptidase resulted in specific splitting of a single bond, while the leucine aminopeptidase aggregate remained intact (11, 12).

Whereas the knowledge of the so-called one-metal peptidases (for instance, carboxypeptidases and thermolysin) has increased rapidly, the structure and reaction mechanism of the two-metal peptidases, to which class leucine aminopeptidase belongs, are still poorly understood (13). No x-ray structural analysis of any of the two-metal peptidases is available as yet, and no primary structure of these enzymes has been published.

In the present paper, the complete amino acid sequence of the polypeptide chain of leucine aminopeptidase is presented. Part of the sequence, namely residues 1-171 comprising the NH$_2$-terminal cyanogen bromide fragment, has been reported in a preliminary communication (12). The accompanying paper (14) describes the determination of the total number of sulfhydryl groups and their reactivity in the zinc metalloenzyme, in the enzyme activated by Mg$^{2+}$, Mn$^{2+}$, and Co$^{2+}$, and the metal-free enzyme.

EXPERIMENTAL PROCEDURES

Most of the experimental evidence for the purification of fragments and for the determination of amino acid sequences is presented as a miniprint supplement immediately following the references.

Essentially pure leucine aminopeptidase was isolated from calf lenses by the method described. About 150 mg of enzyme was purified routinely from 800 lenses. Some preparations showed a minor band of $M_r = 20,000$ when analyzed by polyacrylamide gel electrophoresis.

2 Portions of this paper (including "Experimental Procedures," part of the "Results," and Figs. 5 to 35) are presented in the miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Further supplementary data to this article (Tables II to XLI) are deposited with the miniprint supplement and can be obtained with the full size photocopies, which are available from the Journal of Biological Chemistry, 9550 Rockville Pike, Bethesda, MD 20814. Request Document No. 81M-2264A for the miniprint and 81M-2264B for the tables. Please specify the authors, document number, number of copies, and (in the case of the tables) whether microfiche or full sized photocopies. Orders must be accompanied by a check to the order of the Journal in the amount of $17.60 for photocopies of 81M-2264A and $16.40 for 81M-2264B, $33.00 for both, or $2.50 for a microfiche of 81M-2264B only. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.

3 The abbreviations used are: CNBr, cyanogen bromide; HA, hydroxylamine; SDS, sodium dodecyl sulfate.
polyacrylamide gel electrophoresis in the presence of SDS. This protein, believed to be a γ-crystallin fraction on the basis of amino acid analysis, two-dimensional gel electrophoresis, and determination of the NH₂-terminal sequence (results not shown), could be removed only with extreme difficulty once the enzyme solution had been frozen and lyophilized. Purification could, however, very simply be achieved by chromatography of the native leucine aminopeptidase solution on Ultrogel AcA 34 in 0.1 M Tris-HCl buffer, pH 8.0. Purified enzyme revealed a single homogeneous peak in the ultracentrifuge (Fig. 1) and gave a single band both on SDS-containing polyacrylamide gels and on acidic urea gels (Fig. 2). The molecular weight of the subunit was estimated to be 54,000. After activation by manganese ions, the hydrolytic activity of the leucine aminopeptidase solution was determined. Kinetic parameters were determined using a series of six substrate concentrations ranging from 20 to 100 mM. The Michaelis constant for the Mn²⁺-activated leucine aminopeptidase was determined to be 29.4 mM and \( V_{\text{max}} \) was 1.35 mmol/min/mg of enzyme.

The amino acid composition of leucine aminopeptidase is presented in Table I. For comparison, the values reported by Carpenter and Vahl (6) and by Kettmann et al. (15) are included. The enzyme contains a high percentage of hydrophobic amino acids. Values found for tryptophan vary considerably. We used three different methods for the tryptophan determination, but were not able to obtain reproducible results. However, none of the values determined exceeded 4.0. The number of tryptophan residues deduced from the sequence results is 6. The NH₂-terminal residue of native and of S-carboxymethylated leucine aminopeptidase was determined to be threonine. By automated Edman degradation, the NH₂-terminal amino acid sequence of leucine aminopeptidase was determined up to Thr-21 (Table II).

The general strategy for the sequence determination of leucine aminopeptidase is outlined in Fig. 3. Cleavage by CNBr at the 12 methionine residues of reduced and S-alkylated enzyme yielded 13 fragments, of which 11 fragments could be purified partially or completely. We were not able to purify fragments CB 7 and CB 13. It is conceivable that these fragments precipitate during gel filtration or ion exchange chromatography. Fragments CB 8 and CB 11 were isolated as a mixture, but fragment CB 11 could be obtained in pure form after CNBr cleavage of hydroxylamine fragment HA 2. A large part of the sequence of fragment CB 8 was deduced from fragment HA 2-CB 8b purified from a CNBr cleavage mixture of fragment HA 2. Treatment of leucine aminopeptidase with hydroxylamine resulted in cleavage of the only Asn-Gly bond in the chain (Fig. 3B). CNBr cleavage of the COOH-terminal fragment HA 2 yielded five fragments in pure form (CB 8b, CB 9, CB 10, CB 11, and CB 12). Overlaps between these fragments were obtained from trypsin, chymotryptic, thermolysin, and staphylococcal protease peptides of HA 2. Tryptic digestion of citraconylated HA 2 yielded two peptides, TC 5 and TC 6. They originated from the COOH-terminal part of HA 2 and contain sequences which were never found in a

![Fig. 1. Sedimentation velocity analysis of leucine aminopeptidase.](image)
CNBr fragment. Peptide HA 2-Tc 6 was identified as the COOH-terminal peptide of HA 2 and hence of the complete leucine aminopeptidase chain. The COOH-terminal alanine residue could not be identified by digestion with carboxypeptidases A, B, C, or Y, even when the digestions were performed under denaturing conditions, such as in the presence of SDS or urea, and by heat denaturation. Probably the COOH-terminal residues of leucine aminopeptidase are shielded in such a way that they are not accessible to the carboxypeptidases. All overlaps between the CNBr-fragments have been rigidly established. Tryptic digestion of S-carboxymethylated leucine aminopeptidase and purification of methionine-containing peptides T 25, T 30, and T 31 together with the purification of the tryptic peptide Tc 170-205, obtained from a digest of citraconylated S-carboxymethylated leucine aminopeptidase, established the remaining overlaps. The sequence of residues 275 to 304 was derived from analysis of peptide SP 232-304 obtained from a staphylococcal protease digestion of S-aminoethylated leucine aminopeptidase (Fig. 3A). The sequence of SP 232-304 also confirmed the order of fragments CB 4, CB 5, and CB 6.

The proposed amino acid sequence of leucine aminopeptidase is presented in Fig. 4.

DISCUSSION

In contrast to earlier reports (16) proposing that bovine lens leucine aminopeptidase is made up of 10 subunits with molecular weights of 32,000, it is now firmly established that the enzyme consists of six identical subunits of $M_r = 54,000$ (4, 5). We have never found any indication of a component of leucine aminopeptidase smaller than the $M_r = 54,000$ subunit; not even on prolonged incubation of the carboxymethylated subunit at 37 °C or in SDS and $\beta$-mercaptoethanol. This contradicts the claims that prolonged incubation of the subunits in 1% SDS and 1% $\beta$-mercaptoethanol results in the appearance of smaller components, or even that polypeptide chains of $M_r = 10,000$ are present in the aggregate (17). The results of cross-linking studies by Carpenter and Harrington (5), which indicated that leucine aminopeptidase is composed of identical subunits, are confirmed by our findings that the subunits are identical both in molecular weight and in charge, and by the fact that we never found any evidence for heterogeneities in the primary structure during our sequence studies.

Comparison of the total amino acid composition, derived from the sequence results, with the amino acid analysis of leucine aminopeptidase reveals very good agreement. The results of our amino acid analysis correspond reasonably well with the values reported by Kettmann et al. (15) and Carpenter and Vahl (6) (Table I). Only for tryptophan is a significant discrepancy observed. Although we determined the tryptophan content of leucine aminopeptidase by three different methods, we were not able to obtain reproducible results. A total of 6 tryptophan residues was deduced from the sequence results.

Initial attempts to isolate CNBr fragments were hampered by incomplete cleavage, insoluble core material, and considerable aggregation between fragments. These problems were solved by extensive reduction and alkylation of leucine aminopeptidase prior to cleavage by CNBr. Purification of CNBr fragments could be achieved only by ensuring complete solubilization of these polypeptides during chromatographic manipulations. All separations were therefore carried out in solvents containing 6 M urea. Despite these precautions, we were still unable to purify CNBr fragments CB 7 and CB 13. All amino acid residues in the sequence of leucine aminopeptidase were identified more than once in peptides obtained by different methods. All overlaps between larger fragments could be established conclusively, apart from the overlap between residues 472 and 473, which was confirmed by an indirect method, based on the normal specificity of trypsin and the lack of lysine or arginine in Tc 6.

The polypeptide chain of leucine aminopeptidase contains a total of 478 amino acid residues. The calculated molecular weight is 51,691. However, polyacrylamide gel electrophoresis in the presence of SDS resulted in an estimated molecular weight of 54,000. The discrepancy of 2300 between the two values is of the same magnitude as that found for CNBr fragment CB 1: a value of 18,837 was calculated from the sequence results, while a value of 21,000 was estimated from polyacrylamide gel electrophoresis in the presence of SDS (12). It may well be that the NH$_2$-terminal part of leucine aminopeptidase binds relatively less SDS than the remaining part of the polypeptide chain (18).

The amino acid sequence of bovine lens leucine aminopeptidase proposed here represents the first primary structure
**Sequence of Leucine Aminopeptidase**

![Proposed amino acid sequence of bovine lens leucine aminopeptidase. Determination of the sequence of the complete polypeptide chain was performed as outlined in the schematic representation (Fig. 3).](image)

determination of a member of the class of two-metal peptidases. To detect sequence relationships between leucine aminopeptidase and other proteins, six pieces of 25 residues from the total sequence of 478 residues were compared with all 25-residue segments of each sequence in the data base of the Atlas of Protein Sequence and Structure, using the computer program SEARCH (19). No statistically significant relationship was found with any sequence in the data base, indicating that leucine aminopeptidase represents a separate protein superfamily.

The sequence of leucine aminopeptidase can provide a basis for the interpretation of x-ray crystallographic studies, of which a preliminary account has been presented (10). It has also been useful in interpreting experiments involving the affinity labeling of cysteine residues in metallo-activated leucine aminopeptidase, as presented in the following paper (14). These results may provide a better understanding of the mechanism of action on a molecular basis of this class of enzyme.

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REFERENCES
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