Amino Acid Composition and Terminal Residues of Aspartate Aminotransferase from Ox Heart

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1. The amino acid composition of highly purified aspartate aminotransferase from ox heart was determined. 2. Alanine is the only N-terminal residue. 3. Leucine was identified as the only C-terminal residue. 4. No disulphide bridges are present in the enzyme molecule. 5. The thiol groups are not equally accessible, the accessibility being comparatively easier in the apoenzyme molecule.

In the preceding paper (Marino, Greco, Scardi & Zito, 1966) a purification procedure for aspartate aminotransferase (l-aspartate-2-oxoglutarate aminotransferase, EC 2.6.1.1) from ox heart was reported. Some general properties of the purified enzyme were also investigated: These properties are essentially similar to the corresponding ones of the enzyme from pig heart and ox brain, except for the electrophoretic migration rate in stabilized electrolytes, which appears to depend on the animal species rather than on the organ used as enzyme source (Marino, Zito & Scardi, 1964). To investigate further along this line, some structural features of ox-heart aspartate aminotransferase were determined. The present paper deals mainly with the amino acid composition, the N- and C-terminal residues and the number of thiol groups of the purified enzyme.

EXPERIMENTAL

Materials. Deionized aspartate aminotransferase was prepared from ox heart as reported by Marino et al. (1966). Each stock enzyme preparation was tested for homogeneity by ultracentrifugal analysis, starch-gel electrophoresis and spectrophotometric measurements at 280 and 250 mμ (Marino et al. 1966). The enzyme concentration was determined spectrophotometrically by using E1%\textsubscript{1cm}. 14-40 (Marino et al. 1966).

The HCl used for hydrolysis was a product from Mallinckrodt Chemical Works, St Louis, Mo., U.S.A. N-Bromosuccinimide was obtained from Fluka A.-G., Basle, Switzerland. 1-Fluoro-2,4-dinitrobenzene was obtained from California Corp. for Biochemical Research, Los Angeles, Calif., U.S.A. DNP-l-alanine was from Mann Research Laboratories, New York, N.Y., U.S.A. Acetanilide (zone-refined) was from Koch–Light Laboratories Ltd., Colnbrook, Bucks. All other chemicals were analytical-grade products obtained from several commercial sources.

Carboxypeptidase A (EC 3.4.2.1) and carboxypeptidase B (EC 3.4.2.2) were purchased from Worthington Biochemical Corp., Freehold, N.J., U.S.A. (COA–DFP lot 6130 and COB–DFP lot 27 respectively).

Analytical methods

Pyridoxal 5'-phosphate content. The pyridoxal 5'-phosphate content of the purified enzyme was determined spectrophotometrically at 388 mμ, on the addition of 4N-KOH to the enzyme solution to give a final alkali concentration of 0-1N, by assuming the molecular extinction coefficient to be 6000.

Amino acid analysis. Amino acids, except tryptophan and cysteine, were determined according to the procedure of Moore, Spackman & Stein (1958). Acid hydrolysis was carried out in specially built Pyrex tubes by adding cone. HCl to 0-2-0-3ml. portions of the enzyme solution (5-2-10-5mg./ml.) for a final concentration of 5-7N. The tubes, thoroughly frozen in an acetone–solid CO\textsubscript{2} bath, were connected through a high-vacuum stopcock to a double-step oil pump (Edwards model SC150), reaching a final vacuum of about 10-12 μ Hg. The tubes were sealed on a air–gas burner, and after 5 min. at room temperature were placed in a air oven at 110° and left for the planned time. After removal from the oven, the tubes were kept at room temperature for 30 min., then opened and immediately placed in a high-capacity desiccator over NaOH pellets. The desiccator was evacuated for at least 1 hr. by an oil pump (Edwards model SC50). In the following hour the hydrolysates usually become dry. The dry residue was dissolved in 4-5ml. of 0-2N-citrate buffer, pH 2-2. Samples (2ml.) of the resulting clear solution were applied on the 15cm. and 15cm. columns of the Spincos model 120 automatic amino acid analyser and chromatographed in the usual way. The hydrolysis times used were 22, 40 and 70 hr.

Tryptophan determination. The spectrophotometric method of Patchornik, Lawson & Witkop (1958), which is
based on the decrease of the protein band on reaction of the tryptophan residues with N-bromosuccinimide, was used.

Cysteine determination. This amino acid was determined as cysteic acid after oxidation by performic acid (Harrington & Schellman, 1956), vacuum hydrolysis and automatic amino acid chromatography.

N-Terminal residues analysis. Solid NaHCO₃ (50 mg.) was added to 15-2 mg. of enzyme in 1 ml. of aqueous solution. To the mixture 50 μl. of fluorodinitrobenzene was added and allowed to react overnight in the dark at 25° under continuous magnetic stirring. The yellow precipitate formed was acidified with HCl and collected by centrifugation. It was then washed three times with deionized water and twice each with 95% (v/v) ethanol and peroxide-free ether. The residue (20 mg.), dried over silica gel overnight in the dark, was suspended in deionized water and allowed to react for 2 hr. at 110°, the N-DNP derivatives were recovered by extraction with peroxide-free ether. After evaporation of the ether, the sample was chromatographed on a Technicon automatic DNP-amino acid analyser, according to the procedure of Kesner, Muntywiler, Griffin & Abrams (1963).

C-Terminal residues analysis. Samples of the enzyme were incubated for 2 hr. at 25° with carboxypeptidase A in a final volume of 1 ml. (enzyme sample/carboxypeptidase A weight ratio, 20:1), the pH of the incubation mixture being adjusted to 8.0 with dilute NaOH. The solution was made 1 M with respect to arginine acid (2-hydroxy-5-guanidinovaleric acid) to inhibit traces of carboxypeptidase B activity.

The carboxypeptidase B digestion (enzyme sample/carboxypeptidase A weight ratio, 100:1) was performed essentially as described above for carboxypeptidase A. To inhibit traces of carboxypeptidase A activity the solution was made 2 M with respect to phenylpropionic acid (hydroxycinnamic acid). The reaction was stopped by the addition of 50% (w/v) trichloroacetic acid to give a final concentration of 5% (w/v). After centrifugation the supernatant together with the washings of the precipitate was extracted thrice with ether. After vacuum evaporation of the ether, 0.2 M-citrate buffer, pH 2.2, was added and the sample was quantitatively transferred to the columns of the Spinco model 120 automatic amino acid analyser.

Nitrogen analysis. This was performed in a Coleman model 29 automatic nitrogen analyser with two samples of deionized enzyme. The apparatus was calibrated with acetonilide (zone-refined).

Phosphorus analysis. This was performed by the method of Weil-Malherbe & Green (1951) on two samples of the deionized enzyme, after wet-ashing with 60% (v/v) HClO₄.

RESULTS AND DISCUSSION

Pyridoxal 5'-phosphate content. The pyridoxal 5'-phosphate content of the purified enzyme, determined spectrophotometrically, was found in two separate experiments to be 1.5 and 1.7 moles/96,000 g. of enzyme respectively. Total phosphorus analysis performed on two samples (16.8 mg.) of deionized enzyme gave a P content (10-9 μg., average value) very close to the theoretical P content (10-6 μg.) calculated by assuming a pyridoxal 5'-phosphate content of 2 moles/96,000 g. of enzyme. This suggests an average minimum molecular weight of 48,000.

Amino acid composition. Table 1 reports the amino acid composition of the purified aspartate aminotransferase from ox heart muscle. The composition given represents the values obtained from two distinct enzyme preparations, the difference being negligible. Ammonia, threonine, serine and methionine contents were extrapolated to zero hydrolysis time. For the slowly released amino acids (i.e. arginine, isoleucine, leucine and valine), the values at 70 hr. of hydrolysis were taken. In the acid hydrolysate cysteine was found only in traces. Part of the tryptophan was intact even after acid hydrolysis. The sum of the relative ultraviolet extinctions of tryptophan, tyrosine and phenylalanine gave ε½cm. 14-85, which is in excellent agreement with the extinction coefficient of the purified enzyme (i.e. 14-40). The absence of succinylcysteine, which is derived from a reaction between the thiol groups of the enzyme and the maleate added during the purification procedure (Turano, Giartosio, Riva & Fasella, 1964), seems to indicate that the reaction does not occur in the present case.

Cysteine residues, determined as cysteic acid, were found to be 4-9 moles/48,000 g. of enzyme. To ascertain whether all cysteic acid is derived from cysteine or cystine or both, direct titration with p-chloromercuribenzoate was carried out by the method of Boyer (1954). Only 2-2 moles of thiol groups/48,000 g. were detected in the deionized holoenzyme, even after treatment with 8 M-urea for 20 min. Reaction with native and urea-treated enzyme with iodoacetic acid, followed by dialysis and hydrolysis by the procedure of Crestfield, Moore & Stein (1963), showed 1.3 and 3.9 residues of S-carboxymethylcysteine respectively. Since ionic interaction might be responsible for the incomplete carboxymethylation, the apoenzyme and holo-enzyme treated with 8 M-urea were allowed to react with an equal mixture of iodoacetamide and iodoacetic acid. The S-carboxymethylcysteine from the apoenzyme was exactly 5.1 moles/48,000 g., whereas that from the holoenzyme was 2.0 moles/48,000 g.

The results indicate that (a) all cysteic acid is derived from cysteine residues, (b) two thiol groups are not accessible to p-chloromercuribenzoate, and (c) the apoenzyme can be more readily unfolded by urea treatment than the holoenzyme. Further, it should be pointed out that in the urea-treated apoenzyme some lysine residues were carboxymethylated and the dicarboxymethyl-lysine appeared in the chromatogram.

N- and C-Terminal residues. In the chromatogram recorded by the automatic DNP-amino acid analyser, besides the dinitrophenol and the dinitroaniline peaks, only the DNP-alanine peak was found,
**Table 1. Amino acid composition of ox-heart aspartate aminotransferase**

The hydrolysis times were 22, 40 and 70hr. Values for ammonia, threonine, serine and methionine were extrapolated to zero time; values for arginine, isoleucine, leucine and valine were taken at 70hr. All other values were calculated from the average of 22, 40 and 70hr. The hydrolysis were carried out on two distinct enzyme preparations. Cysteine was determined as cysteic acid and as S-carboxymethylcysteine (see the text). Tryptophan was determined spectrophotometrically (Patchornik et al. 1958). A minimum mol.wt. of 48000 was assumed.

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<th>Constituent</th>
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<th>N content (g./100g. of enzyme)</th>
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* From Cohn & Edsall (1943).
† By nitrogen analysis, 16-64.

**Fig. 1.** Record of the automatic analysis of ether-soluble DNP-amino acids of ox-heart aspartate aminotransferase. Peaks are: A, dinitrophenol; B, DNP-alanine; C, dinitroaniline. Experimental details are given in the text.
molecular weight of aspartate aminotransferase from pig heart and from yeast \( \bar{v} \) values of 0.745 and 0.749 respectively were assumed by Jenkins, Yphantis & Sizer (1959) and by Schreiber, Eckstein, Maass & Holzer (1964).

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REFERENCES


