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 m μ (Marino *et al.* 1966). The enzyme concentration was determined spectrophotometrically by using $E_{1\text{cm}}^{1\%}$ 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 Labora-

tories 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 m μ , 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 conc. HCl to 0.2–0.3 ml. portions of the enzyme solution (5.2–10.5 mg./ml.) for a final concentration of 5.7N. The tubes, thoroughly frozen in an acetone–solid CO₂ 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 4.5 ml. of 0.2M-citrate buffer, pH 2.2. Samples (2 ml.) of the resulting clear solution were applied on the 150 cm. and 15 cm. columns of the Spenco 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

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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 NaHCO_3 (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 *N*-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 transferred to a Pyrex hydrolysis tube. After vacuum hydrolysis in 6*N*-HCl for 20 hr. at 110°, the 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, Muntwyler, 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 mM with respect to arginine acid (2-hydroxy-5-guanidinovaleric acid) to inhibit traces of carboxypeptidase B activity.

The carboxypeptidase B digestion (enzyme sample/carboxypeptidase B 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 mM 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 acetanilide (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) HClO_4 .

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 $E_{1\text{cm}}^{1\%}$ 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 holoenzyme 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 70 hr. Values for ammonia, threonine, serine and methionine were extrapolated to zero time; values for arginine, isoleucine, leucine and valine were taken at 70 hr. All other values were calculated from the average of 22, 40 and 70 hr. 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.

Constituent	Composition (g./100 g. of enzyme)	No. of residues/mol. (to nearest integer)	Minimum mol.wt.	N content (g./100 g. of enzyme)	$\bar{v}^*$	$\bar{v} \times \%$
Lys	5.60	21	2290	1.34	0.82	4.59
His	2.28	8	6025	0.69	0.67	1.55
NH ₄ ⁺		32		0.60		
Arg	7.81	24	2000	2.75	0.70	5.47
Asp	10.55	44	1095	1.28	0.60	6.33
Thr	5.26	25	1920	0.73	0.70	3.68
Ser	5.99	33	1455	0.96	0.63	3.72
Glu	13.45	50	960	1.46	0.66	8.87
Pro	6.47	32	1490	0.93	0.76	4.92
Gly	3.80	32	1490	0.93	0.64	2.43
Ala	5.84	40	1180	1.17	0.74	4.33
CySH	1.11	5	9130	0.15	0.61	0.67
Val	3.72	18	2600	0.52	0.86	3.20
Met	1.64	6	7860	0.17	0.75	1.23
Ile	4.24	18	2620	0.52	0.90	3.82
Leu	8.49	36	1335	1.05	0.90	7.65
Tyr	3.74	11	4240	0.32	0.71	2.65
Phe	6.74	22	2200	0.64	0.77	5.19
Trp	3.40	9	5480	0.52	0.74	2.52
				16.73†		72.82

* From Cohn & Edsall (1943).

† By nitrogen analysis, 16.64.

corresponding to 0.224 μ mole for 15 mg. of enzyme (Fig. 1). The hydrolysis recovery of the DNP-amino acid is about 80% as checked with pure DNP-alanine. After correction, a single mole of *N*-terminal alanine can be assumed/48000 g. of pure enzyme. The same result was obtained by Turano, Vecchini & Giartosio (1962) for pig-heart aspartate aminotransferase, thus indicating that the same enzyme from two different species has the same *N*-terminal residue.

The *C*-terminal residue analysis, by hydrolysis with carboxypeptidase A, gave only leucine, the other amino acids being present in very small traces, except for moderate amount of glutamine deriving from self-digestion of carboxypeptidase A (Bargetzi, Thompson, Sampath Kumar, Walsh & Neurath, 1964). The amount of leucine released under the conditions described above was 55% of the expected amount calculated by assuming 1 mole of *C*-terminal leucine/48000 g. of enzyme. Preliminary experiments with carboxypeptidase B followed by chromatography on the 15 cm. column failed to show the release of lysine or arginine.

No comparative conclusions can be drawn on this structural feature of aspartate aminotransferase

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.

because of the absence of similar data for the enzyme from pig heart or any other animal species.

The results of the analyses reported in the present paper permit the calculation of the partial specific volume, $\bar{v}$, of the enzyme protein. The value of this parameter, calculated by the procedure of Cohn & Edsall (1943) from the weight percentage of amino acid residues and their specific volume (see Table 1), was 0.728, whereas for the measurement of the

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

- Bargetzi, J. P., Thompson, E. O. P., Sampath Kumar, K. S. V., Walsh, K. A. & Neurath, H. (1964). *J. biol. Chem.* **239**, 3767.
- Boyer, P. D. (1954). *J. Amer. chem. Soc.* **76**, 4331.
- Cohn, E. J. & Edsall, J. T. (1943). *Proteins, Amino Acids and Peptides as Ions and Dipolar Ions*, p. 375. New York: Reinhold Publishing Corp.
- Crestfield, A. M., Moore, S. & Stein, W. H. (1963). *J. biol. Chem.* **238**, 622.
- Harrington, W. F. & Schellman, J. A. (1956). *C.R. Trav. Lab. Carlsberg, Sér. chim.* **30**, 21.
- Jenkins, W. T., Yphantis, D. A. & Sizer, I. W. (1959). *J. biol. Chem.* **234**, 51.
- Kesner, L., Muntwyler, E., Griffin, G. E. & Abrams, J. (1963). *Analyt. Chem.* **35**, 83.
- Marino, G., Greco, A. M., Scardi, V. & Zito, R. (1966). *Biochem. J.* **99**, 589.
- Marino, G., Zito, R. & Scardi, V. (1964). *Boll. Soc. ital. Biol. sper.* **40**, 720.
- Moore, S., Spackman, D. H. & Stein, W. H. (1958). *Analyt. Chem.* **30**, 1185.
- Patchornik, A., Lawson, W. B. & Witkop, B. (1958). *J. Amer. chem. Soc.* **80**, 4747.
- Schreiber, G., Eckstein, M., Maass, G. & Holzer, H. (1964). *Biochem. Z.* **340**, 21.
- Turano, C., Giartosio, A., Riva, F. & Fasella, P. (1964). *Biochem. biophys. Res. Commun.* **16**, 221.
- Turano, C., Vecchini, P. & Giartosio, A. (1962). *Experientia*, **18**, 544.
- Weil-Malherbe, H. & Green, R. H. (1951). *Biochem. J.* **49**, 282.