LABELING OF THE ACTIVE SITE OF CYTOPLASMIC ASPARTATE AMINOTRANSFERASE BY β-CHLORO-L-ALANINE

Yoshimasa Morino and Mitsuhiro Okamoto

Department of Biochemistry
Osaka University Medical School
33 Joancho, Kitaku, Osaka, Japan.

Received January 3, 1973

SUMMARY

Syncatalytic inactivation of pig heart cytoplasmic aspartate aminotransferase by β-chloro-[U-^{14}C]L-alanine resulted in the incorporation of radioactivity corresponding to one mole of the label per mole of the monomeric unit of the enzyme. A borohydride-reduced and then carboxymethylated preparation of the labeled enzyme was digested by trypsin. A radioactive peptide was isolated and found to contain a covalently linked pyridoxyl derivative which absorbed at 325 nm. The amino acid sequence of this peptide was Tyr-Phe-Val-Ser-Glu-Gly-Phe-Glu-Leu-Phe-Cys-Ala-Gln-Ser-Phe-Ser-Lys*-Asn-Phe-Gly-Leu-Tyr-Asn-Glu-Arg. In the peptide the phosphopyridoxyl group seems to be covalently bound via alanyl moiety derived from β-chloro-L-alanine, the β-carbon atom of which is covalently linked to the ε-nitrogen atom of the lysyl residue(Lys*). From a comparison with the amino acid composition of the phosphopyridoxyl peptide isolated from the tryptic digest of a borohydride-reduced holoenzyme, it was concluded that the modified lysyl residue was identical to that involved in binding pyridoxal phosphate to the apoenzyme.

The previous report (1) from this laboratory described the aspartate aminotransferase-catalyzed α,β-elimination of β-chloro-L-alanine with concomitant inactivation of the enzyme. A striking enhancement of the rate of the inactivation by the presence of formate ion greatly facilitated the labeling of the enzyme active site by β-chloro-L-alanine. The present communication describes the result of the chemical analysis on the modified site.

MATERIALS AND METHODS

The cytoplasmic isozyme of aspartate aminotransferase was purified from pig heart by a modification of the procedure described for the beef liver enzyme (2). β-Chloro-[U-^{14}C]L-alanine was synthesized according to the method described by Fischer and Raske (3). [U-^{14}C]L-Serine methyl ester was prepared by bubbling dry HCl gas through a solution containing 0.5 g L-serine and 0.2 mCi [U-^{14}C]L-serine (120 mCi per mmole, Daiichi Pure Chemicals CO.LTD. Tokyo) in 16 ml dry methanol. The over-all yield of the radioactive β-chloro-L-alanine was 42 % and the specific activity was 9.3 X 10^4 dpm per μmole. Assays for normal transamination and α,β-elimination reaction were performed as previously described (1,4). The concentration of the enzyme was calculated from a value of the molecular weight of 47,000 for the monomeric unit of the enzyme and a value

of $A^{1\%}_{278\ nm}$ of 12.5. The molar absorption coefficient of the phosphopyridoxyl derivative was assumed to be 7,500 $M^{-1}cm^{-1}$. Radioactivity was determined in Bray's solution (5) with the use of a liquid scintillation spectrometer, Packard model 3320. Spectral measurement was performed in a Hitachi spectrophotometer model 124 and circular dichroism measurement was done in a Jouan dichrograph II. Paper electrophoresis of peptides was carried out in a buffer system of pyridine-acetic acid-water (1:10:289), pH 3.6, at 4000 V for 1.5 hour on a Whatman No.3MM paper and descending paper chromatography was done in a solvent system of n-butanol-pyridine-acetic acid-water (15:10:3:12). The amino acid sequence was determined by the Edman degradation (6). The subtractive procedure was employed in some cases. Amino acid analysis was performed on an automatic amino acid analyzer (Hitachi Perkin-Elmer KLA 3B) according to the procedure of Spackman et al (7). Peptides were hydrolyzed in 5.7 N HCl for 24 hours at 110° in sealed evacuated tubes.

RESULTS

Labeling of the Enzyme: Four milliliters of 0.5 M β-chloro-[U-^{14}C]L-alanine (9.3 X 10^4 dpm per μmole) were added to 16 ml of the solution containing 180 mg (3.8 μmoles with respect to the monomeric unit) of the pyridoxal form of the cytoplasmic aspartate aminotransferase in 3 M potassium formate containing 0.1 M sodium pyrophosphate buffer (pH 7.8) and the mixture was incubated for 2 minutes at 37°. The inactivation was 98 % complete. Then, as quickly as possible, 10 mg of KBH_4 and one drop of n-octanol were added to the reaction mixture. After standing for 30 minutes at 37°, the mixture was dialyzed overnight against 500 volumes of distilled water at 5°. The dialyzed solution was further dialyzed against 100 ml of 7 M guanidine HCl containing 0.1 M Tris-HCl buffer (pH 8.0), 1 mM dithiothreitol and 10 mM EDTA[1] for 10 hours at 20°. To the resulting solution was added 0.3 ml of 1 M sodium iodoacetate and the mixture was incubated for 20 minutes at 30°. The carboxymethylated preparation was then dialyzed overnight against 1000 volumes of distilled water at 5°. The heavy precipitate was collected by centrifugation, suspended in 20 ml of 1 % NH_4HCO_3, and digested with 1 % (w/w) TPCK-trypsin for 3 hours at 30°. The resulting preparation contained radioactivity corresponding to 8.0 X 10^4 dpm per μmole of the monomeric unit of the enzyme, indicating that 0.86 mole of the three-carbon moiety derived from β-chloro-L-alanine was covalently bound per mole of the monomeric unit.

Isolation of a Radioactive Peptide: The tryptic digest thus obtained was lyophilyzed, dissolved in 7 ml of 1 % NH_4HCO_3, and passed over a Sephadex G-50 column

1. Abbreviations used are: EDTA, ethylenediaminetetraacetate; TPCK-, L-(1-tosylamido-2-phenyl)ethyl chloromethyl ketone-treated.

(3.5 X 120 cm) which was previously equilibrated with 1 % NH_4HCO_3. Peptides were eluted with the same solvent at a flow rate of 40 ml per hour. Fractions of 6 ml were collected. The elution pattern was shown in Fig. 1,A. Earlier fractions contained radioactivity derived from β-chloro-[U-^{14}C]L-alanine, and, in addition, absorbed at 325 nm, suggesting the presence of a pyridoxyl derivative in these fractions. Radioactive fractions were combined and lyophilized. The lyophilized peptides were dissolved in 2 ml of 20 mM Tris-HCl buffer (pH 7.8) containing 1 mM EDTA, and adsorbed onto a DE-32 column (0.8 X 12 cm) previously equilibrated with the same buffer. Elution was performed by applying first a linear gradient from 0 to 0.1 M NaCl, and then from 0.1 to 0.5 M NaCl. Radioactive fractions appeared around 0.4 M NaCl of the gradient, which exactly coincided with those exhibiting absorbance at 325 nm (Fig. 1,B). These fractions were combined, lyophilized and desalted on a Sephadex G-25 column. The resulting preparation showed on high voltage electrophoresis and paper chromatography a single radioactive, fluorescent and ninhydrin-positive spot which remained at the origin. The amount of the peptide thus obtained was 1.6 μmole (42 % yield) as determined from the value of absorbance at 325 nm and radioactivity. The peptide showed absorption bands at 325, 282 and 276 nm with a shoulder around 250 nm (Fig. 2). Circular dichroic spectra exhibited positive bands at 325 and 255 nm, suggesting that the phosphopyridoxyl moiety in this peptide exists in a dissymmetric environment.

Primary Structure of the Peptide from the Labeled Site: The amino acid composition of the radioactive peptide was shown in Table I. It can be seen that the composition was the same as that of the pyridoxyl peptide (8) isolated from tryptic digests of the borohydride-reduced cytoplasmic aspartate holoaminotransferase except for the absence of pyridoxyllysine residue in the radioactive peptide. This indicates that the lysyl residue involved in binding pyridoxal phosphate was modified during reaction with β-chloro-L-alanine.

The amino acid sequence of the peptide was determined as follows. The sequential Edman degradation was performed on the peptide up to the 13 th residue. The remaining part of the sequence was determined from the analysis on the peptide fragments obtained by digestion with chymotrypsin. Chymotryptic peptides were separated by chromatography on a Dowex 1-X2 column. Isolated fragments and their partial sequences were listed in Table II. A major radioactive peptide, C-3, exhibited an absorption band at 325 nm. The second cycle of Edman degradation on this peptide released a major portion of radioactivity bound to this peptide. All these results, taken together, indicated the primary structure of the labeled-site peptide shown in Fig. 3. Further confirmation of this tentative structure of the derivatized lysyl residue is now under way.

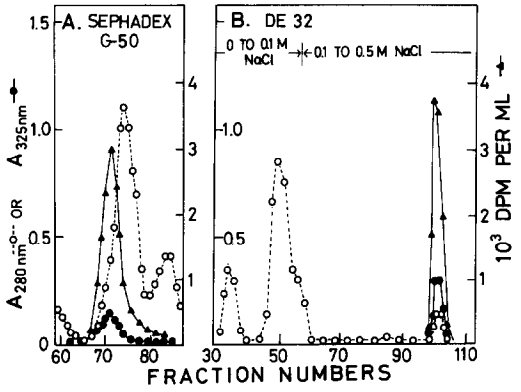

Fig. 1.

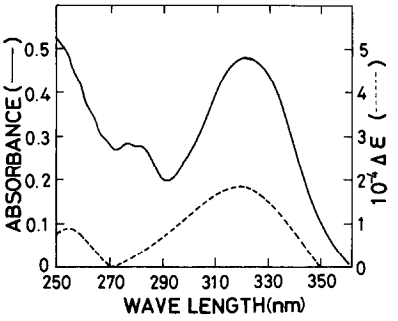

Fig. 2.

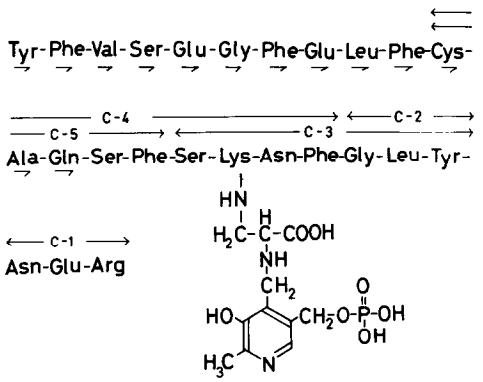

Fig. 3.

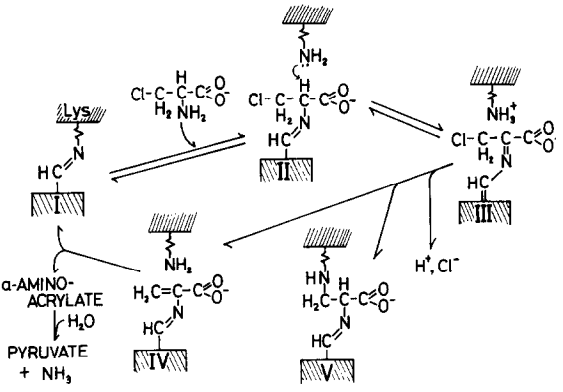

Fig. 4.

Fig. 1. Elution diagram of the radioactive peptide. A: On Sephadex G-50, see the text for the details. B: On DE-32; the first linear gradient was made between 50 ml of 20 mM Tris-acetate buffer (pH 7.8) and 50 ml of the same buffer containing 0.1 M NaCl. The second gradient was made between 50 ml of the same buffer containing 0.1 M NaCl and 50 ml of the same buffer containing 0.5 M NaCl. The flow rate was 20 ml per hour at 20°. Fractions of 1.7 ml were collected.

Fig. 2. Absorption and circular dichroic spectra of the radioactive peptide. The radioactive peptide (0.1 μmole) was dissolved in 1.5 ml of 0.04 M potassium phosphate buffer (pH 7.0). Solid curve, absorption spectrum; dashed curve, circular dichroic spectra. A quartz cuvette of 1-cm light path was used.

Fig. 3. Schematic summary of amino acid sequence data on the radioactive peptide from the labeled site of the cytoplasmic aspartate aminotransferase. Residues established by Edman degradation on the original tryptic peptide were underlined by horizontal arrows. Chymotryptic peptides derived from the original peptide were shown above the parent peptide and their nomenclature corresponds to that described in Table II.

Fig. 4. Schematic representation of the reaction of aspartate aminotransferase with β-chloro-L-alanine.

Table I. Amino acid composition of the radioactive peptide and its comparison with that of the phosphopyridoxyl peptide isolated from the borohydride-reduced holoenzyme[a].

Residues	Phosphopyridoxyl peptide		Radioactive peptide	
	nmoles		nmoles	
N^{ϵ}-Pyridoxyllysine	10.1	(1)[b]	-	(0)[c]
Arginine	12.1	(1)	4.9	(1)
S-Carboxymethylcysteine	11.1	(1)	4.6	(1)
Aspartic Acid	22.2	(2)	9.5	(2)
Serine	24.2	(3)	12.1	(3)
Glutamic Acid	44.3	(4)	19.1	(4)
Glycine	21.4	(2)	9.1	(2)
Alanine	10.7	(1)	5.0	(1)
Valine	10.1	(1)	4.6	(1)
Leucine	21.5	(2)	9.1	(2)
Tyrosine	21.7	(2)	9.3	(2)
Phenylalanine	56.3	(5)	24.4	(5)

a. The phosphopyridoxyl peptide was isolated from the tryptic digest of a borohydride-reduced preparation of the cytoplasmic aspartate aminotransferase by the procedure identical to that described for the peptide from the modified site. The purified peptide exhibited an absorption spectrum indistinguishable from that of the radioactive peptide, but did not show any ellipticity in the region where the phosphopyridoxyl moiety absorbs.
b. Numbers in parenthesis represent the integral numbers of residues.
c. The radioactive, derivatized lysyl residue was eluted by washing the short column of the automatic amino acid analyzer with 0.1 N NaOH.

DISCUSSION

The present investigation has clearly shown that the labeling of the active site of the cytoplasmic aspartate aminotransferase by β-chloro-L-alanine occurs at the ε-amino group of the lysyl residue involved in binding the coenzyme, pyridoxal phosphate. The schematic representation (Fig. 4) will depict the chemical events occurring at the active site of the enzyme during α,β-elimination of β-chloro-L-alanine. Upon the reaction of the enzyme with β-chloro-L-alanine, the internal coenzyme-aldimine bond (in I in Fig. 4) must be split to form the substrate-coenzyme aldimine (II), the step designated as transaldimination. The subsequent removal of the α-H atom of the enzyme-bound substrate is a prerequisite step in the α,β-elimination reaction as well as in transamination (10, 11). The structure of the deprotonated enzyme-substrate intermediary complex (III) thus formed facilitates elimination of the electronegative β-substituent on the substrate. Removal of Cl^- from this complex yields an α-aminoacrylate-enzyme complex (IV), which decomposes into free enzyme and α-aminoacrylate. The latter hydrolyzes spontaneously to pyruvate and ammonia. During this α,β-elimination reaction, the β-C atom is attacked by the juxtaposed nucleophilic ε-N atom of the lysyl residue (V) as has been demonstrated in the present study. If so, the same ε-amino group

Table II. Isolation and structure of the peptide fragments obtained by digestion of the tryptic radioactive peptide with chymotrypsin.[a]

Peptides	Amino Acid Sequence	Determination[b] Procedure	Yield
			μmole
C-1[c]	Asn-Glu-Arg	PTH	0.3
C-2	Gly-Leu-Tyr	PTH	0.07
C-3[d]	Ser-Lys*-Asn-Phe-Gly-Leu-Tyr	Subtractive	0.15
C-4[d]	Cys(cm)-Ala-Gln-Ser-Phe-Ser-Lys*-Asn-Phe	PTH & Subtractive	0.07
C-5	Cys(cm)-Ala-Gln-Ser-Phe	PTH	0.2

a. The radioactive peptide (0.4 μmole) was further digested by α-chymotrypsin (2 μg) in 0.5 ml of 1 % NH_4HCO_3 for 3 hours at 30°. Lyophilized digest was dissolved in 0.5 ml of pyridine(1 %)-collidine(1 %)-acetate(1 % acetic acid) buffer (pH 8.6) and applied on a Dowex 1-X2 (acetate form) column (0.8 X 12 cm) previously equilibrated with the same buffer. The chromatography was carried out at a flow rate of 10 ml per hour at 20°. Fractions of 1.5 ml were collected. The following buffer system was used: 1) 30 ml of equilibration buffer; 2) a linear gradient between 50-ml equilibration buffer and 50-ml 0.1 N acetic acid; 3) a gradient between 50-ml 0.1 N acetic acid and 50-ml 2 N acetic acid; 4) 20 ml of 2 N acetic acid. An aliquot (0.1 ml) from each fraction, after alkaline hydrolysis, was tested for the reaction with ninhydrin. Chymotryptic peptides were numbered in the order of their appearance from the column.

b. PTH, determined by identification of the phenylthiohydantoin derivative of an amino acid; Subtractive, determined from the amino acid composition of a peptide remaining after each cycle of the Edman degradation.

c. Peptides C-1 and C-2 were eluted in one peak and separated by paper chromatography.

d. The sequence " Ser-Lys*-Asn-Phe " in these peptides is identical to that of the phosphopyridoxyl tetrapeptide isolated from the chymotryptic digest of a borohydride-reduced holoenzyme (9). Lys* refers to a derivatized lysyl residue.

may be juxtaposed also to the α-H atom of the substrate. Thus it is tempting to conceive that the lysyl ε-amino group acts as an efficient base to abstract the α-H of the substrate, a key intermediary step in the pyridoxal catalysis requiring the removal of the α-H atom of a substrate as a step prerequisite to the subsequent reactions. This catalytic role of the lysyl residue was suggested earlier by Snell (12) and Ayling et al (13), and, later, by Ivanov and Karpeisky (14) without any really experimental evidence. The present result seems to supply a substantial support to this concept and leads to a general mechanistic consideration that an essential lysyl residue not only participates in the formation of an aldimine bond with pyridoxal phosphate but also functions as a base to abstract the α-H atom of the bound substrate.

The tryptic peptide from the labeled site was shown to exhibit positive cir-

cular dichroic bands at 325 and 255 nm whereas the phosphopyridoxyl chromophore in the tryptic peptide isolated from a borohydride-reduced holoenzyme did not show any ellipticity. This difference resulted probably from the fact that in the former peptide the alanyl moiety derived from the label is intercalated between the lysyl ϵ-N atom and the phosphopyridoxyl group, while in the latter the phosphopyridoxyl moiety is directly bound to the lysyl residue. It seems likely that the phosphopyridoxyl moiety of the peptide isolated from the labeled site interacts in a unique fashion with some parts of the peptide chain. Further physical and chemical analyses on the peptide should be expected to supply a structural basis of the dissymmetric nature of the environment where the chromophore is embedded.

Similar analysis on the mitochondrial isozyme is now under way and a preliminary result so far obtained is essentially identical to that obtained with the cytoplasmic isozyme.

It is also of interest to note that the syncatalytic inactivation of aspartate aminotransferase by bromopyruvate results from the formation of a covalent bond between a nucleophilic cysteinyl residue and the β-C atom of the label bound to the phosphopyridoxamine form of the enzyme (15) whereas the β-chloro-L-alanine-mediated inactivation results from the nucleophilic attack of the lysyl residue to the β-C atom of the label bound to the pyridoxal form. Indeed, these two findings clearly indicate a subtle but distinct difference in steric positions of these two important nucleophiles relative to the β-C atom in each of these two different labels having a specific affinity for either of the pyridoxal or pyridoxamine form of the active site.

REFERENCES

1. Morino,Y., and Okamoto,M., Biochem. Biophys. Res. Commun. 47, 498 (1972)
2. Morino,Y., Itoh,H., and Wada,H., Biochem. Biophys. Res. Commun. 13, 348 (1963)
3. Fischer,E., and Raske,E., Ber. 40, 3717 (1907)
4. Okamoto,M., and Morino,Y., Biochemistry 11, 3188 (1972)
5. Bray,G.A., Anal. Biochem. 1, 279 (1960)
6. Edman,P., in Protein Sequence Determination (Needleman,S.B. ed.) p.211 Springer-Verlag, Berlin,Heidelberg,New York (1970)
7. Spackman,D.H., Stein,W.H., and Moore,S., Anal. Chem. 30, 1190 (1958)
8. Morino,Y., Watanabe,T., and Okamoto,M., Proc. 19th Symp. Structure Proteins, p.97 (1968)
9. Morino,Y., and Watanabe,T., Biochemistry 8, 3412 (1969)
10. Braunstein,A.E., and Shemyakin,M.M., Biokhimiya 18, 393 (1953)
11. Metzler,D.E., Ikawa,M., and Snell,E.E., J. Amer. Chem. Soc. 26, 648 (1954)
12. Snell,E.E., Brookhaven Symp. Biol. 15, 32 (1962)
13. Ayling,J.E., Dunathan,H.C., and Snell,E.E., Biochemistry 7, 4537 (1968)
14. Ivanov,V.I., and Karpeisky,M.Ya., Advan. Enzymol. 32, 21 (1969)
15. Okamoto,M., and Morino,Y., J. Biol. Chem. 247, in press (1972)