Selective Modification of the Mitochondrial Isozyme of Aspartate Aminotransferase by β-Bromopropionate. II. Chemical Structure of the Modified Site†

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ABSTRACT: The reaction of [1-^{14}C]β-bromopropionate with pig heart mitochondrial aspartate aminotransferase (L-aspartate:2-oxoglutarate aminotransferase, EC 2.6.1.1) irreversibly inactivates the enzyme with concomitant incorporation of radioactivity into several amino acid residues. A kinetic study of differential rates of incorporation of radioactivity into these amino acid residues indicated that carboxyethylation of a lysyl residue is directly correlated to the inactivation. A peptide containing N^{ϵ}-(carboxyethyl)lysine was isolated from a cyanogen bromide treated, carboxymethylated preparation of the ^{14}C-labeled inactive enzyme by chromatography over Sephadex G-50, followed by high-voltage paper electrophoresis. This peptide showed the following amino acid composition: *S*-carboxymethyl-$Cys_2Asx_5Ser_2Glx_4Gly_4Ala_5Val_2Ile_2Leu_2Tyr_2Phe_2$[^{14}C]$N^{\epsilon}$-(carboxyethyl)$Lys_1Lys_1His_1Arg_1$Hse (plus its lactone)$_1$. Digestion of this peptide with chymotrypsin yielded seven major ninhydrin-positive spots on a peptide map, one of which was radioactive. Dansylation and aminopeptidase digestion of this radioactive peptide indicated a sequence, Ala-(N^{ϵ}-carboxyethyl)Lys-Asn-Hse, which was identical with that of the phosphopyridoxyl tetrapeptide obtained by chymotryptic digestion of borohydride-reduced mitochondrial aspartate aminotransferase except that the phosphopyridoxyllysine residue is replaced by an N^{ϵ}-(carboxyethyl)lysine residue. Specific inactivation of the mitochondrial enzyme by β-bromopropionate results, therefore, from the carboxyethylation of the ε-amino group of the lysyl residue involved in aldimine formation with pyridoxal phosphate. Several structural features exhibited by the inactivated enzyme are discussed in the light of this fact.

In the preceding paper (Okamoto and Morino, 1972), the specific inactivation of pig heart mitochondrial aspartate aminotransferase by β-bromopropionate was shown to be caused by the carboxyethylation of a lysyl residue in the active site. To understand the molecular mechanism of the selective action of this alkylating reagent and to further clarify the structural basis for several properties of the inactivated enzyme, it seemed of primary importance to determine whether the same lysyl residue involved in binding pyridoxal phosphate was also modified in the inactivation process. The present paper describes the results of this investigation.

Experimental Section

Materials. Mitochondrial aspartate aminotransferase was purified from pig heart by a modification of the procedure described by Morino *et al.* (1963) for the beef liver enzyme. [1-^{14}C]β-Bromopropionic acid (1.59 Ci/mole) was purchased from the International Chemical and Nuclear Corp. α-Chymotrypsin was obtained from Worthington and aminopeptidase M from Rohm & Haas. Polyamide thin layer was obtained from Cheng Chin Trading Co., LTD., Taiwan. Other chemicals were of reagent grade or of spectrograde.

Measurement of Radioactivity in Effluents from the Amino Acid Analyzer. Radioactivity in colum effluents was monitored continuously (Becker, 1967) by connecting the outflow from the column of the amino acid analyzer (Yanaco LC 5S) directly with capillary tubings to a 2-ml Lucite flow-cell (Packard Instrument Co.) packed with anthracene (Packard, blue-violet fluorescent grade). The cell was set in the detector system of the scintillation spectrometer (Packard Model 3021) equipped with a recorder. The effluent, after being measured for radioactivity, was led again into the amino acid analyzer, mixed with ninhydrin reagent, and measured for optical density, according to the usual analytical procedure. The delay time of 7 min was observed from the radioactive peak to the ninhydrin peak of a [^{14}C]amino acid.

Isolation of the Peptide Containing N^{ϵ}-(Carboxyethyl)lysine. Mitochondrial aspartate aminotransferase (130 mg) was incubated, in a total volume of 8.0 ml, with 320 μmoles of [1-^{14}C]β-bromopropionate (1.02×10^5 dpm per μmole) at pH 7.2 in a pH-Stat at 37°. After 6 hr, the reaction mixture was turbid with the formation of insoluble protein.[1] After a brief centrifugation, the supernatant solution was passed through a Sephadex G-50 (fine) column (3.5 × 12 cm) which was equilibrated with 20 mM Tris-HCl buffer (pH 8.0). The resulting protein fractions (112 mg) were pooled, and the combined solution (14 ml) was made to 5 M in guanidine-HCl, 50 mM in Tris-HCl buffer (pH 8.0), 1 mM in dithiothreitol, and 10 mM in EDTA. After 30 min at 30°, 500 μmoles of iodoacetate was added and the mixture was incubated for 20 min at 30° with occasional adjustment of the pH to 8.0 by adding dilute NaOH solution. The mixture was then dialyzed against 100 volumes of 10 mM Tris-HCl buffer (pH 8.0) for 30 hr with two changes of buffer. The resulting precipitate was

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[1] The precipitate separated from the reaction mixture contained approximately 2.6 times as many counts as the inactivated enzyme in solution. Analysis of the acid hydrolysate of the precipitated proteins revealed an increased formation of the radioactive material emerging before the aspartic acid peak. This material is derived from the carboxyethyl ester of the dicarboxylic amino acids. Incorporation of the carboxyethyl group into a glutamic or aspartic acid residue seems, therefore, to decrease the solubility of the enzyme protein.

TABLE I: Incorporation of Radioactivity into Mitochondrial Aspartate Aminotransferase during Reaction with [1-^{14}C]β-Bromopropionate in the Absence and Presence of Succinate.[a]

Time of Reaction (min)	−Succinate (dpm/mg of Enzyme)			+Succinate (dpm/mg of Enzyme)		
		Treated with 0.05 N NaOH			Treated with 0.05 N NaOH	
	Total Count	Supernatant	Precipitate	Total Count	Supernatant	Precipitate
85	970	496	560	630	425	115
150	1920	870	950	926	755	190
210	2360	1100	1390	1530	1125	380
420	3310	1500	1630	1860	1580	450

[a] The composition of the reaction mixture was the same as that described for Figure 1. When succinate was present, the reaction mixture contained 35 mM sodium succinate (pH 6.9). Each reaction mixture was incubated at pH 7.2 in a pH-Stat at 37°. Aliquots (0.5 ml) withdrawn at indicated times were passed over a Sephadex G-50 column as described for Figure 1. Each of the resulting enzyme solutions was diluted to 1.5 ml. One portion (0.2 ml) was then used for the determination of radioactivity and enzyme activity. To the remaining portion (1.3 ml) there was added 0.2 ml of 0.4 N NaOH; the mixtures were then incubated at 30° for 15 hr. Proteins were precipitated by adding trichloroacetic acid to a final concentration of 5% (w/w). After a brief centrifugation, 0.5 ml of the supernatant solutions was transferred to test tubes and 0.1 ml of 1 N NaOH was added to bring the solutions to slightly alkaline pH (This avoids a low count rate which is caused by quenching in the presence of strong acid (Bray, 1960)). A 0.4-ml portion of each solution was then counted in the scintillation spectrometer. Precipitates were washed twice with 0.5 ml of distilled water, then dissolved in 1.0 ml of 0.05 N NaOH; 0.2-ml portions were then counted in the scintillation spectrometer, while a second portion (0.4 ml) was used for the protein determination.

collected by centrifugation, dissolved in 4 ml of 75% formic acid, then mixed with 100 mg of cyanogen bromide and allowed to stand for 14 hr at 20°. Then the mixture was diluted tenfold and lyophilized. The lyophilized material was dissolved in 1.5 ml of 5% acetic acid and placed on a Sephadex G-50 (fine) column (2.6 × 40 cm) previously equilibrated with 5% acetic acid. Peptide fragments were eluted by washing the column with the same solvent at a flow rate of 20 ml/hr at 20°. Each fraction was examined for the absorbance at 280 nm and aliquots (0.2 ml) from every third fraction were measured for radioactivity in a liquid scintillator.

Other Analytical Procedures. The enzyme assay was performed as described previously (Okamoto and Morino, 1972). Samples for amino acid analyses were hydrolyzed in 6 N HCl at 110° for 24 hr in evacuated sealed tubes.

Radioactivity of a solution was determined in Bray's solution (Bray, 1960) with the use of a Tri-Carb liquid scintillation spectrometer Model 3320. The detection and measurement of radioactivity on a filter paper were performed in a Tri-Carb radiochromatogram scanner Model 7201. Spectral measurements were done in a Hitachi spectrometer Model 124 or a Cary spectrophotometer Model 14.

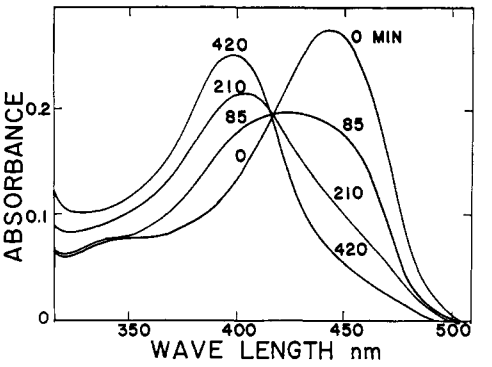

FIGURE 1: Spectral change of mitochondrial aspartate aminotransferase during reaction with [^{14}C]β-bromopropionate. The mitochrondrial enzyme (24 mg) was incubated, in a total volume of 3.0 ml, with 120 μmoles of [1-^{14}C]β-bromopropionate (7.52×10^4 dpm/μmole) at pH 7.2 in a pH-Stat at 37°. At indicated times, 0.5-ml aliquots were withdrawn and passed over Sephadex G-50 (fine) column (1 × 10 cm) previously equilibrated with 30 mM potassium succinate buffer (pH 6.0). The protein fractions were pooled. Curves were corrected for various concentrations of different enzyme samples and represent the spectra at an enzyme concentration of 2.0 mg/ml as calculated from a value of the extinction coefficient $E^{1\%}_{278\ nm}$ of 13.6 (Okamoto and Morino, 1972). The numbers in the figure refer to the reaction time in minutes. Under the present conditions, native enzyme showed an absorption maximum at 440 nm. Residual activity of the preparation at 420 min was 12%.

The amino-terminal amino acid was identified by the dansyl chloride procedure described by Gray (1967). The radioactive tetrapeptide (approximately 2 nmoles) was incubated with 5 μl of 0.25% dansyl chloride (in acetone) in a total volume of 10 μl of 0.05 M $NaHCO_3$ at 37° for 1 hr. The mixture was dried under vacuum, then 50 μl of 6 N HCl was added and the tube was evacuated, sealed, and heated at 105° for 24 hr. The hydrolysate was dried under vacuum and the dansyl amino acid was extracted twice with 0.1 ml of ethyl acetate (saturated with water). The organic layer was dried and the residue was redissolved in 5 μl of methanol. The dansylamino acid was identified by thin-layer chromatography on polyamide sheet as described by Woods and Wang (1967).

Results

Reaction of [1-^{14}C]β-Bromopropionate with Mitochondrial Aminotransferase. Upon incubation of the enzyme with [^{14}C]β-bromopropionate, the 430-nm band of the native enzyme decreased with a concomitant increase in absorbance at 395 nm (Figure 1), in accord with previous observations (Okamoto and Morino, 1972). Aliquots (0.5 ml) withdrawn at various time intervals were passed through a Sephadex G-50 column to remove unbound radioactive materials. The determination of radioactivity on these samples revealed a time-dependent incorporation of [^{14}C]β-bromopropionate into the enzyme protein (Table I). After incubation for 7.5 hr a sample contained radioactivity equivalent to 2.1 μmoles of [^{14}C]β-bromopropionate/μmole of the monomeric unit of the enzyme, corresponding to alkylation of more than two amino acid residues. In fact, a hydrolysate of the modified preparation showed on amino acid analysis three radioactive, ninhydrin-positive peaks and also a radioactive, ninhydrin-negative peak. The latter peak emerged earlier than the aspartic acid peak and its radioactivity was approximately one-half of the total radioactivity in the preparation. The alkylations of the hydroxyl group of tyrosine and also the carboxyl group of

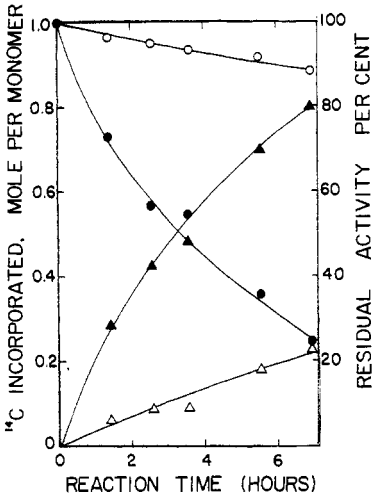

FIGURE 2: Correlation of the enzyme inactivation with the "alkali-stable" radioactivity incorporated into the enzyme protein and the prevention of both processes by the presence of succinate. The experimental conditions were the same as those described in Table I. Incorporation of ^{14}C was calculated on the basis of the molecular weight of 47,000 for the monomeric unit of enzyme (Okamoto and Morino, 1972). Residual activity of preparations incubated with β-bromopropionate in the presence (○) or absence (●) of succinate. ^{14}C incorporation into preparations incubated in the presence (△) or absence (▲) of succinate.

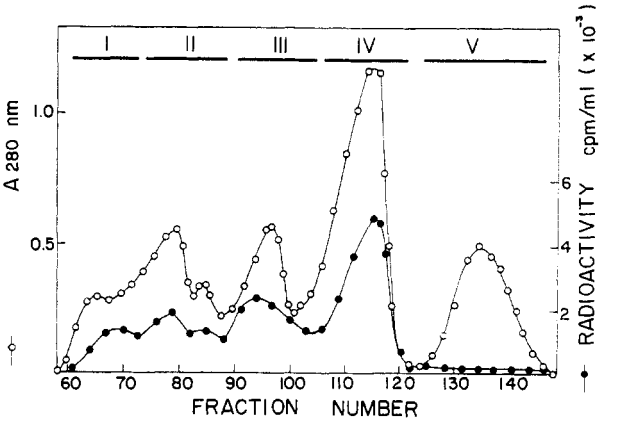

FIGURE 4: Elution profile of peptide fragments obtained by cyanogen bromide cleavage of [^{14}C]β-bromopropionate-inactivated enzyme. Procedures for carboxymethylation of the inactivated enzyme, cyanogen bromide treatment and chromatography on a Sephadex G-50 column are described in the Experimental Section. Fractions of 2 ml were collected. The bars indicate the fractions that were pooled and concentrated separately.

glutamic or aspartic acid residue are known to yield covalent bonds which are readily cleaved by acid hydrolysis (Greenstein and Winitz, 1961). Of these, the ester linkages formed by alkylation of the latter two amino acid residues are presumably unstable and easily hydrolyzed in a mild alkaline medium (Takahashi *et al.*, 1967). When a modified preparation was subjected to such alkaline treatment, a considerable portion of the radioactivity was released into the supernatant after the enzyme protein was precipitated by the addition of trichloroacetic acid (Table I). The rate of incorporation of the radioactivity into the alkali-labile form was essentially identical whether the reaction with β-bromopropionate was performed in the presence or absence of succinate, which is known to protect the enzyme from inactivation by β-bromopropionate (Okamoto and Morino, 1972). Thus the formation of such alkali-labile bonds cannot be the cause of the inactivation; instead, the modification of an amino acid in an alkali-stable form must be closely related to the inactivation of the enzyme. Figure 2 clearly illustrates such correlation between the incorporation of radioactivity into an alkali-stable form and the inactivation rate.

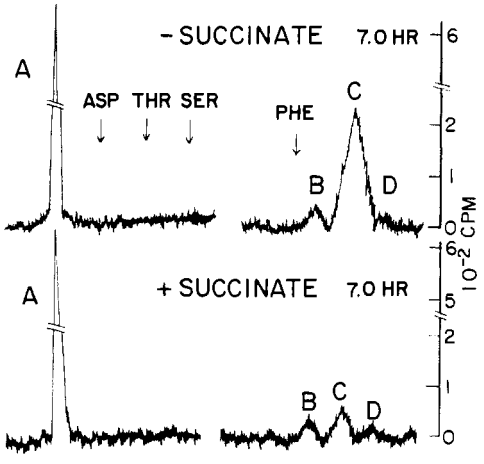

FIGURE 3: Elution patterns of radioactive materials in the acid hydrolysates of the ^{14}C-labeled enzyme on the amino acid analyzer. Samples were those withdrawn at 420 min from the reaction mixtures incubated in the absence or presence of succinate as described in Table I. Each sample (0.5 ml) was dialyzed against 500 volumes of distilled water for 40 hr with two changes of the outer solution. The dialyzed preparations were lyophilized and hydrolyzed *in vacuo* in 6 N HCl at 110° for 24 hr. Hydrolysates were analyzed for radioactive materials in the amino acid analyzer equipped with the scintillator flow cell as described in the Experimental Section. Peak A was ninhydrin negative and was assumed to be a degradation product of [^{14}C]carboxyethyl ester of a glutamyl or aspartyl residue. Peak B represents 1-(2-carboxyethyl)histidine, peak C, N^{ϵ}-(2-carboxyethyl)-lysine, and peak D, 3-(2-carboxyethyl)histidine (Okamoto and Morino, 1972). The residual enzymatic activities were 76% for the preparation inactivated in the presence of succinate and 12% for that inactivated in the absence of succinate.

Carboxethylation of a Lysyl Residue during the Inactivation. The supernatant solution after the alkaline treatment gave on amino acid analysis only one radioactive but ninhydrin-negative peak, emerging before the aspartic acid peak, and corresponding in position to the ninhydrin-negative radioactive peak observed with acid hydrolysates of a modified preparation. The acid hydrolysate of the precipitate after alkaline treatment gave on amino acid analysis three peaks emerging after the phenylalanine peak (Figure 3). A preparation in which inactivation by β-bromopropionate was retarded by the presence of succinate yielded on acid hydrolysis much less N^{ϵ}-(carboxyethyl)lysine (peak C) than one in which an efficient inactivation was achieved in the absence of succinate. Indeed, the amount of N^{ϵ}-(carboxyethyl)lysine paralleled the extent of the inactivation of these preparations whereas the other three components (peaks A, B, and D) were apparently unrelated to the extent of the inactivation. Thus the tentative conclusion (Okamoto and Morino, 1972) that inactivation of mitochondrial aspartate aminotransferase by β-bromopropionate results from alkylation of a lysine residue is strongly supported by the present results with [^{14}C]β-bromopropionate.

Position of the Alkylated Lysyl Residue in the Primary Structure. Mitochondrial aspartate aminotransferase was first

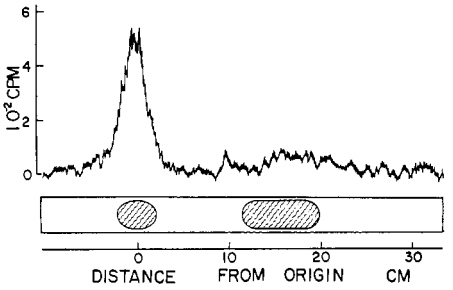

FIGURE 5: High-voltage electrophoresis of fraction IV from Figure 4. An aliquot (5200 dpm) from fraction IV was applied on Whatman No. 3MM paper. The run was for 90 min at 3000 V, pH 1.8 (99% formic acid–acetic acid–H_2O (1:8:91 v/v)) and 30°.

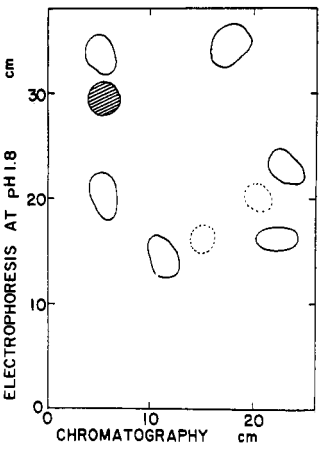

FIGURE 6: Peptide map of the chymotryptic digest of [^{14}C]peptide isolated by electrophoresis of fraction IV. Fifty nmoles of the [^{14}C]peptide, purified by high-voltage electrophoresis, was digested with 10 μg of α-chymotrypsin in 0.1 ml of 0.1 M NH_4HCO_3 at 30° for 5 hr. The lyophilized digest was applied to Whatman No. 3MM paper. Descending chromatography with 1-butanol–acetic acid–H_2O (3:1:1, v/v) was followed by electrophoresis for 90 min at 4000 V, pH 1.8 and 30°. The paper was sprayed with 0.3% ninhydrin in acetone. Radioactivity was then detected by applying 5-cm wide strips to a radiochromatogram scanner. The hatched area indicates radioactive spot.

inactivated by incubating with [1-^{14}C]β-bromopropionate, then carboxymethylated and treated with cyanogen bromide. The elution profile of the cyanogen bromide peptides on a Sephadex G-50 column (Figure 4) is essentially identical with that described by Watanabe and Wada (1971). Several fractions contained ^{14}C-labeled peptide fragments. Each of the five pooled fractions was concentrated and their acid hydrolysates were analyzed for radioactive amino acid derivatives by the use of an automatic amino acid analyzer equipped with a radioactivity detector system as described in the Experimental Section. [^{14}C]Carboxyethyllysine was enriched predominantly in the hydrolysate of fraction IV (Table II).

On high-voltage electrophoresis at pH 1.8, fraction IV gave a ninhydrin-positive spot at the origin and one or two diffuse spots with a mobility of aspartic acid; radioactivity was observed only in the spot remaining at the origin (Figure 5). This spot was cut out and eluted with 50% acetic acid. Its amino acid composition is shown in Table III. Another sample of the peptide was digested with chymotrypsin. An aliquot portion (2570 dpm, 25 nmoles) was applied to a Whatman No. 3MM paper and subjected to descending chromatography, followed by high-voltage electrophoresis at pH 1.8. The peptide map thus obtained showed 7 major ninhydrin-positive spots, one of which was radioactive (Figure 6). On the basis

TABLE II: Distribution of Radioactivity in Fractions Obtained by Chromatography of Cyanogen Bromide Fragments over Sephadex G-50.[a]

Fractions	Radioactivity Found (dpm × 10^{-3})				
	Total[b]	Peak A[c]	Peak B	Peak C	Peak D
I	29	17	3	3	6
II	44	29	6	9	*d*
III	48	39	4	5	*d*
IV	110	27	*d*	84	*d*

[a] Five per cent of each pooled fraction (I, II, III, and IV in Figure 4) was hydrolyzed in 6 N HCl as described under the Experimental Section. Acid hydrolysates were analyzed for radioactive materials in the amino acid analyzer equipped with the radioactivity detector system. Peaks A, B, C, and D refer to those defined in Figure 3. [b] This represents the total radioactivity in each pooled fraction as determined by scintillation counting of a portion of the acid hydrolysate. [c] Values were calculated from the relative magnitude of the area under each peak. [d] No discrete peak was observed.

TABLE III: Amino Acid Composition of the [^{14}C]Carboxyethyl Peptide Purified from Cyanogen Bromide Fragments of Inactivated Enzyme.[a]

Amino Acids	Found (nmoles)	Integral No. of Residues
S-Carboxymethylcysteine	18.6	2
Aspartic acid	46.0	5
Threonine	0.0	0
Serine	18.0	2
Glutamic Acid	41.2	4
Proline	0.0	0
Glycine	40.9	4
Alanine	49.0	5
Valine	20.6	2
Isoleucine	20.4	2
Leucine	13.3	2
Tyrosine	19.4	2
Phenylalanine	20.2	2
N^{ϵ}-Carboxyethyllysine[c]	8.7	1[b]
Lysine	9.0	1
Histidine	8.6	1
Arginine	8.6	1
Homoserine plus its lactone	9.4	1
Total		37

[a] [^{14}C]Carboxyethyl peptide which was isolated by high-voltage paper electrophoresis of fraction IV was hydrolyzed under vacuum for 20 hr in 6 N HCl at 110°. Data were not corrected for the loss of amino acids during hydrolysis or for incomplete hydrolysis. A single radioactive peak was observed at the position of N^{ϵ}-(carboxyethyl)lysine. [b] Radioactive (900 dpm). [c] The ninhydrin color value for this derivative was assumed to be equivalent to that for leucine.

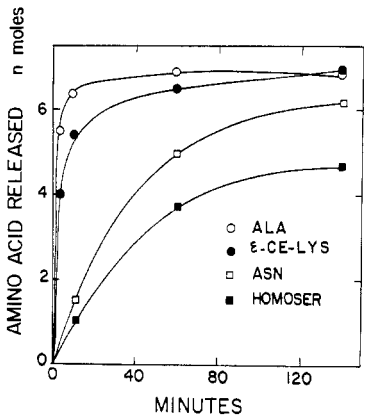

FIGURE 7: Time course of liberation of free amino acids from [^{14}C]-carboxyethyl tetrapeptide during digestion with aminopeptidase M. The reaction mixture contained, in a total volume of 0.25 ml, 31.4 nmoles of [^{14}C]carboxyethyl tetrapeptide (3200 dpm), 10 munits of aminopeptidase M, and 50 mM potassium phosphate buffer (pH 7.0). At indicated times, 0.06-ml sample was withdrawn, heated in a boiling-water bath for 1 min to stop the reaction and kept frozen at $-10°$ until analysis; 0.5 ml of 0.02 N HCl was added to each sample just before applying to the amino acid analyzer. Low recovery of homoserine may result from its partial conversion to its lactone.

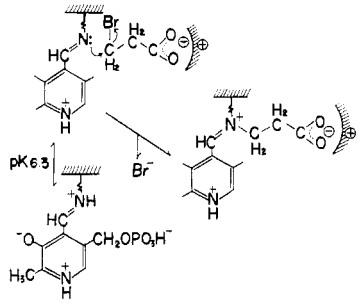

FIGURE 8: Proposed scheme for the reaction of β-bromopropionate with the active site of mitochondrial aspartate aminotransferase.

of this result, a larger portion of the same digest (11,000 dpm, 0.11 μmole) was applied to a Whatman No. 3MM paper and subjected to high-voltage electrophoresis at pH 1.8 for 90 min at 3000 V. The radioactive material after elution with 20% acetic acid and lyophilization contained 8100 dpm (74% yield). The amino acid composition of the radioactive peptide (Table IV) was identical with that of the coenzyme-combining site peptide (Morino and Watanabe, 1969), except for the replacement of the phosphopyridoxyllysine residue by an N^{ϵ}-(carboxyethyl)lysine residue.

To determine whether this peptide was derived from the same site as the phosphopyridoxyl peptide, it was subjected to dansylation and to digestion with aminopeptidase M. The dansylated peptide on acid hydrolysis gave only dansylalanine, as identified by thin-layer chromatography. Digestion with aminopeptidase M released alanine and N^{ϵ}-(carboxyethyl)-lysine first, the former at a slightly faster rate than the latter Figure 7). Asparagine and homoserine were released at a much slower rate. That homoserine is the carboxyl-terminal residue was readily inferred from the fact that the original large peptide fragment was obtained by cyanogen bromide treatment. These findings conform to the sequence: Ala-N^{ϵ}-(carboxyethyl)Lys-Asn-Hse, and clearly indicate that the carboxyethyl tetrapeptide was derived from the pyridoxal phosphate binding site of the mitochondrial enzyme. The selective inactivation of this enzyme by β-bromopropionate thus results from carboxyethylation of the ε-amino group of the lysine residue involved in the formation of aldimine linkage with pyridoxal phosphate.

TABLE IV: Amino Acid Composition of the Chymotryptic [^{14}C]Tetrapeptide.[a]

Amino Acids	Found (nmoles)	Radioactivity (dpm)
Aspartic acid	10.3	0
Alanine	9.6	0
N^{ϵ}-Carboxyethyllysine	9.8	930
Homoserine plus its lactone	9.2	0

[a] [^{14}C]Peptide purified from chymotryptic digest of the large cyanogen bromide fragment was hydrolyzed *in vacuo* for 24 hr in 6 N HCl at 110°. An aliquot (1050 dpm) of the acid hydrolysate was applied to the long column of the amino acid analyzer and another aliquot to the short column. The radioactivity in the column eluate was calculated by comparing the area under the [2-^{14}C]phenylalanine peak (an internal standard) with that under the radioactive peak derived from the hydrolysate of the peptide.

Discussion

The reaction of the mitochondrial aspartate aminotransferase with β-bromopropionate results in the modification of several amino acid residues (lysyl, glutamyl, or aspartyl and histidyl). A kinetic study on the rate of incorporation of [^{14}C]β-bromopropionate into these residues has clearly indicated that the carboxyethylation of a lysyl residue is directly correlated with the inactivation of the enzyme. Analyses of the [^{14}C]β-bromopropionate-inactivated enzyme revealed that the modified lysyl residue is identical with that involved in the formation of an aldimine bond with 4′-formyl group of the coenzyme, pyridoxal phosphate. A schematic representation (Figure 8) depicts the event occurring in the enzyme active site upon its reaction with β-bromopropionate. The proposed mechanism is consistent with several observed features of the inactivation process and the inactivation product. (1) The pH–rate profile in the inactivation indicated that an ionizing group with a pK of 6.2 is involved (Okamoto and Morino, 1972), whereas a pK of 6.3 has been assigned to the protonation of the nitrogen atom of the aldimine between the ε-amino group of lysine and pyridoxal phosphate of the extramitochondrial aspartate aminotransferase (Sizer and Jenkins, 1963) as well as of the mitochondrial enzyme (Wada and Morino, 1964). (2) The observation of saturation kinetics with respect to β-bromopropionate as an inactivating reagent, together with the demonstration that this reagent is a competitive inhibitor in the transamination reaction (Okamoto and Morino, 1972) make it plausible that the carboxyl group of this reagent binds to a site which normally binds one of the carboxyl groups of dicarboxylate substrates. Other halo acids inhibit the transamination reaction competitively, indicating that their carboxyl groups can also bind to the site (unpublished data). However, none of them inactivated the enzyme effectively, indicating that an effective inactivating reagent

must have an appropriate carbon chain length and a halogen substituent at a position properly distanced from the carboxyl group. In the present case, β-bromopropionate satisfies this structural requirement. (3) The inactivation product absorbs at 395 nm with a molar extinction coefficient considerably smaller than that observed at 355 nm in the native enzyme (Okamoto and Morino, 1972). The former spectral species showed a negative circular dichroism band at 410 nm. It is the incorporation of the carboxyethyl group into the aldimine nitrogen atom which produces the observed drastic change in spectral characteristics of the chromophore. The resistance of this spectral species to reduction with borohydride (Okamoto and Morino, 1972) is of interest. This phenomenon may possibly be explained by assuming that the bulky carboxyethyl substituent on the nitrogen atom of the aldimine linkage prevents the access of borohydride anion to it or, alternatively, such modification of the aldimine bond renders its environment more hydrophobic and makes the action of borohydride less effective. The latter situation was recently suggested for the case of glycogen phosphorylase (Shaltiel and Cortijo, 1970).

While there have been reports on the successful isolation of reduced coenzyme–substrate complexes following borohydride reduction of intermediary enzyme–substrate complexes (Fasella, 1967), an experimental difficulty in isolating such adduct was reported in some case (see, for example, Morino and Snell, 1967). This difficulty might result from the same factor as that described above, *i.e.*, the microenvironment of the aldimine linkage in such enzyme–substrate complexes may not allow access to the borohydride anion due either to hydrophobicity or steric hindrance, whereas in the active site of the holoenzyme itself the reducing anion can reach freely to the aldimine linkage. This speculation assumes that the microenvironment of the aldimine linkage in the holoenzyme is quite different from that of the analogous linkage in enzyme–substrate complexes. Under ordinary experimental conditions intermediary enzyme–substrate complexes exist in equilibrium with the holoenzyme itself, and addition of borohydride anion to such equilibrium mixture reduces only the aldimine bond in the holoenzyme and not that in an enzyme–substrate complex.

The large [^{14}C]peptide obtained after cyanogen bromide treatment of the β-bromopropionate-inactivated enzyme contains 37 amino acid residues, and is clearly derived from the coenzyme binding site of the enzyme. We previously isolated a pyridoxyl peptide containing 25 residues from tryptic digests of borohydride-reduced mitochondrial aspartate aminotransferase (Morino *et al.*, 1968). These two large peptides exhibit several features in common, namely, both contain two cysteinyl residues and one histidyl residue, neither contains threonine or proline, and a methionyl residue in the tryptic peptide is replaced by a homoseryl residue in the cyanogen bromide peptide. It therefore appears that some portion of the COOH-terminal part of the cyanogen bromide peptide overlaps with most of the NH_2-terminal part of the tryptic peptide.

The inactivating effect of halo acids has so far been tested solely on the pyridoxal form of each isozyme of aspartate aminotransferase. It would be interesting to examine their effect on the pyridoxamine forms of these isozymes, in which the microenvironment around the bound coenzyme is probably quite different from that in the pyridoxal form. Experimental results so far obtained on this point indicate that β-bromopropionate does not inactivate the pyridoxamine form of the mitochondrial isozyme and iodoacetate inactivates efficiently the pyridoxamine form of the supernatant enzyme but not its pyridoxal form (M. Okamoto and Y. Morino, unpublished observation). Thus, the ε-amino group of lysine which undergoes azomethine formation in the pyridoxal form of the enzyme no longer occupies the same relative position in the pyridoxamine form. Comparative studies of this general nature which include the pyridoxamine form of aspartate aminotransferase and its apoenzyme may provide important information concerning the topography of the active site in these isozymes. A systematic study along this line is now in progress in this laboratory.

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