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Selective Modification of the Mitochondrial Isozyme of Aspartate Aminotransferase by β-Bromopropionate. I. Inactivation Process and Properties of Inactivated Enzyme†

Mitsuhiro Okamoto and Yoshimasa Morino*

ABSTRACT: A comparative study on the inactivation of the phosphopyridoxal forms of the mitochondrial and supernatant isozymes of aspartate aminotransferase from pig heart muscles by various halo acids (iodoacetate, iodoacetamide, α-bromopropionate, β-bromopropionate, γ-bromobutyrate, and bromosuccinate) was conducted. Of these halo acids, β-bromopropionate was found to cause an irreversible inactivation of the mitochondrial enzyme. None of them inactivated the supernatant enzyme. The inactivation of the mitochondrial enzyme followed pseudo-first-order kinetics. The dependence of the inactivation rate on the concentration of β-bromopropionate showed typical saturation kinetics, indicating substrate-like structural features of this reagent. The maximum rate constant for inactivation was 0.4 hr^{-1} at 25° and the constant analogous to the Michaelis constant for β-bromopropionate was calculated to be 100 mM which was of a magnitude similar to that of the related constant found when the halo acid acted as competitive inhibitor in the transamination reaction. The presence of maleate or succinate protected the enzyme from the inactivation. The inactivation rate increased with increasing pH up to 7, above which the rate remained unchanged. The pK for the inactivation reaction was about 6.2. As inactivation progressed, a new peak appeared around 395 nm with a concomitant decrease in the 355-nm peak of the native enzyme. The new peak at 395 nm was not changed upon variation of pH or addition of substrates. Furthermore, the 395-nm band did not disappear upon the addition of $NaBH_4$ under the condition where the internal aldimine bond of the native enzyme was readily reduced. The 395-nm absorption band was accompanied by a negative circular dichroism band at 410 nm. The amount of bound pyridoxal phosphate did not change during the inactivation. The number of sulfhydryl groups of the enzyme was not affected by the modification. Amino acid analysis of the inactivated preparation demonstrated N^{ϵ}-(2-carboxyethyl)lysine as a major product of alkylation of the enzyme by β-bromopropionate, along with two minor products derived from histidine. From a quantitative correlation of the extent of inactivation with the amount of carboxyethylated amino acid residues, it was concluded that modification of one lysine residue per monomeric unit of the enzyme was responsible for the inactivation.

Two distinct forms of L-aspartate:2-oxoglutarate aminotransferase (EC 2.6.1.1) are present in mammalian tissues, one of which is localized in mitochondria and the other in the soluble cytoplasmic fraction (Boyd, 1961; Katsunuma *et al.*, 1962). A number of investigations (Morino *et al.*, 1963, 1964; Morino and Wada, 1963; Wada and Morino, 1964; Wada *et al.*, 1966; Jenkins and D'Ari, 1966; Martinez-Carrion and Tiemeier, 1967; Kagamiyama *et al.*, 1968; Michuda and Martinez-Carrion, 1969, 1970; Morino and Watanabe, 1969; Morino and Okamoto, 1970) have revealed that these two are differentiated from each other by kinetic, physicochemical, and immunochemical properties.

The similarities in the mode of action and the apparently clear structural distinction prompt a comparative study of the chemical nature of the active site in each isozyme. As an approach to this aim, we studied the primary structure of the tetrapeptides at the pyridoxal phosphate binding site obtained from chymotryptic digests of both enzymes. The two peptides are quite similar as indicated by the conservative substitution of amino acid residues at the corresponding positions with a sequence, Lys-Asn, common to both (Morino and Watanabe, 1969). Thus it has been anticipated that both isozymes should have essential common structural features at least in their active region.

To learn more about the chemical structure of the catalytic

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site, the reactivity with several halo acids of each isozyme from pig heart muscle was studied. Of the halo acids tested, β-bromopropionate was found to inactivate the mitochondrial enzyme but not the supernatant enzyme. Other halo acids did not inactivate either isozyme under similar conditions.

The present communication describes a specific inactivation of the mitochondrial aspartate aminotransferase by alkylation with β-bromopropionate and the consequence of such modification occurring in the structure of the active site. An accompanying paper (Morino and Okamoto, 1972) deals with the chemical analysis of the modified site.

Experimental Section

Materials. Iodoacetic acid was recrystallized from isooctane; iodoacetamide, from distilled water; β-bromopropionic acid, from carbon tetrachloride. γ-Bromobutyric acid was synthesized by the method described by Avison and Morrison (1950). Other chemicals were obtained from commercial sources and were used without further purification. Mitochondrial and extramitochondrial aspartate aminotransferases were purified from pig heart muscle by a modification of the procedures described by Morino *et al.* (1963) for the beef liver isozymes. Mitochondrial malic dehydrogenase was purified by the method described by Englard and Siegel (1969).

Assay for Transamination Activity. METHOD A. The reaction mixture contained, in 1.5 ml, 100 mM Tris-HCl buffer (pH 8.1), 10 mM L-aspartate, and 10 mM α-ketoglutarate. Incubations were at 25°. The increase in absorbance at 260 nm was recorded on a Hitachi Model 124 spectrophotometer with a recorder giving full-scale deflection for an absorbance change of 0.1.

METHOD B. The reaction mixture contained 0.1 mM NADH and 5 μg of malic dehydrogenase in addition to the components described for method A. The decrease in absorbance at 340 nm was followed at 25°.

Determination of Bound Pyridoxal Phosphate. One-tenth milliliter of 2% phenylhydrazine in 10 N sulfuric acid (Wada and Snell, 1961) was added to 1.0 ml of solutions containing 1–2 mg of the enzyme. After standing for a 0.5 hr at 37°, 0.4 ml of 10% trichloroacetic acid was added. After standing at 5° for 1 hr the precipitated protein was removed by centrifugation and the supernatant was measured for the absorbance at 410 nm. The calculation of the number of moles of bound pyridoxal phosphate per monomeric unit of the enzyme was based on a value of the molecular weight of 47,000 for the monomer of the mitochondrial enzyme, and a value of the extinction coefficient ($E^{1\%}_{278\ nm}$) of 13.6 as calculated from the dry weight of the enzyme.

Titration of Sulfhydryl Groups. The native and modified preparations were incubated in 5 M guanidine-HCl containing 0.2 M potassium phosphate buffer (pH 7.4) at 37° for 30 min to ensure the unfolding of the protein. The mixtures were diluted by the same guanidine-HCl buffer to give solutions containing approximately 0.5 mg of the protein/ml. To 1.45 ml of the resulting solution was added 0.05 ml of 20 mM DTNB[1] in 50 mM potassium phosphate buffer (pH 7.4). The reaction was followed at 412 nm in a cuvet of 1.0-cm path length. The reaction was complete within 3 min. The calculation of the number of sulfhydryl group was based on a molar absorbance of 13,600 for the reduction product of DTNB (Ellman, 1959). The absorbance of 0.1% solution of the enzyme in the same guanidine-HCl buffer was 1.3 at 280 nm. Cysteinyl residues in the enzyme were also determined as *S*-carboxymethylcysteine after hydrolysis of the preparation carboxymethylated by iodoacetate in the presence of 6 M guanidine-HCl according to the procedure described by Morino and Snell (1967).

Amino acid analysis was carried out by the use of an automatic amino acid analyzer (Hitachi Perkin-Elmer KLA-3B or Yanaco LC-5S), according to the method described by Spackman *et al.* (1958). Samples were hydrolyzed in 5.7 N HCl at 105° for 36 hr in sealed evacuated tubes.

For the purpose of identification of the modified amino acid residues in acid hydrolysates of alkylated preparations, carboxyethylated derivatives of cysteine, methionine, lysine, and histidine, candidates for such residues, were prepared by incubating 20 μmoles of the individual amino acid with 20 μmoles of β-bromopropionate in 1 ml of 20 mM sodium cacodylate buffer (pH 6.8) at 37° for 20 hr. The amino acid analysis of the reaction mixture for the alkylation of cysteine showed two peaks, one of which appeared at the same position as that of serine and the other was cystine. The earlier peak was assumed to be due to *S*-carboxyethylcysteine. The acid hydrolysate of the reaction mixture for methionine gave three major peaks, homoserine, homocysteine, and methionine, in the order of the appearance on the elution diagram. With histidine, two peaks appeared after the phenylalanine peak. The earlier peak was tentatively assigned to 1-carboxyethylhistidine and the later peak, to 3-carboxyethylhistidine. This assumption is based on the finding with carboxymethyl derivatives of this amino acid as described by Crestfield *et al.* (1963). The reaction mixture with lysine gave on amino acid analysis a peak just before the assumed 3-carboxyethylhistidine peak.

Synthesis of N^{ϵ}-(Carboxyethyl)lysine. N^{ϵ}-(Carboxyethyl)lysine was synthesized by carboxyethylation of lysine, the α-amino group of which was blocked by chelation with cupric ion. A solution containing 1.5 g of L-lysine hydrochloride and 3 g of cupric carbonate in 40 ml of distilled water was boiled for 2 hr under reflux. After insolubles were removed by centrifugation, the supernatant was concentrated to 10 ml. To this was added 3 ml of a neutralized solution containing 1.5 g of β-bromopropionate and the mixture was incubated at 65° with occasional addition of 4 N NaOH under stirring to keep the pH of the solution around 8. After 2 hr, termination of the reaction was indicated from the cessation of decrease in pH. Then the mixture was cooled to 20°, acidified to pH 2 with the addition of concentrated HCl, and bubbled with hydrogen sulfide gas. The resulting precipitate was removed by filtration. The filtrate was concentrated to 5 ml and to it were added 20 ml of acetone. Precipitates were washed by acetone and residual acetone was removed *in vacuo*. The dried material was dissolved in 10 ml of 0.01 N HCl and applied to a Dowex 50-X4 column (3 × 27 cm) which was equilibrated with 0.2 M pyridine–acetate buffer (pH 3.1). The buffer system employed was as described by Schroeder (1967). After washing the column with 450 ml of the starting buffer, a linear gradient between 1500 ml of the starting buffer and 1500 ml of 2 M pyridine–acetate buffer (pH 5.0) was applied. Five ninhydrin-positive peaks appeared at 120 ml (pH 3.1, 0.2 M in pyridine), 210 ml (pH 3.1, 0.2 M), 870 ml (pH 3.43, 0.43 M), 1160 ml (pH 3.65, 0.60 M), and 1360 ml (pH 3.80, 0.73 M), respectively (Figure 1). Of these peaks, earlier three peaks were minor components and were not further examined. The material in the fifth peak was identified to be lysine. The compound in the

[1] Abbreviation used is: DTNB, 5,5′-dithiobis(2-nitrobenzoic acid).

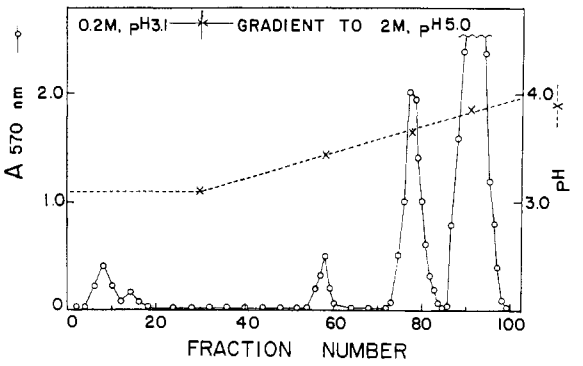

FIGURE 1: Elution profile of carboxyethylated derivatives of lysine. Chromatography was performed at 20°. The flow rate was 80 ml/hr. Fractions of 15 ml were collected. The reaction with ninhydrin (Rosen, 1957) was carried out on aliquots of 0.02 ml from each fraction. Details were described in the Experimental Section.

fourth peaks was eluted after the phenylalanine peak on the amino acid analyzer. The combined solution from the fourth peak was evaporated at 50° *in vacuo*. The oily material was dissolved in 2 ml of H_2O and lyophilized. The lyophilized material was dissolved in 2 ml of 70% ethanol. During concentration of the solution at 40° under reduced pressure, crystals appeared, and the evaporation was continued to dryness. The material thus obtained appeared highly hygroscopic and was further dried over phosphorus pentoxide at 60° *in vacuo* for 40 hr. The yield was 390 mg. The nuclear magnetic resonance spectrum (Figure 2) of this compound in D_2O showed chemical shifts and integral numbers of protons on each carbon atom all inconsistent with the proposed structure, N^{ϵ}-(2-carboxyethyl)lysine.

Circular Dichroism. The measurement was made in a Jouan dichrograph II at 25°. Silica cells with a light path 0.5 or 10 mm were used. Values of $\Delta\epsilon$ (differential circular dichroic absorption) were calculated from the following relation: $\Delta\epsilon = \Delta D/cl$, where ΔD is the observed value of circular dichroic optical density, l the length of the light path in centimeters, and c the molar concentration of the monomeric unit of the enzyme (Velluz *et al.*, 1965).

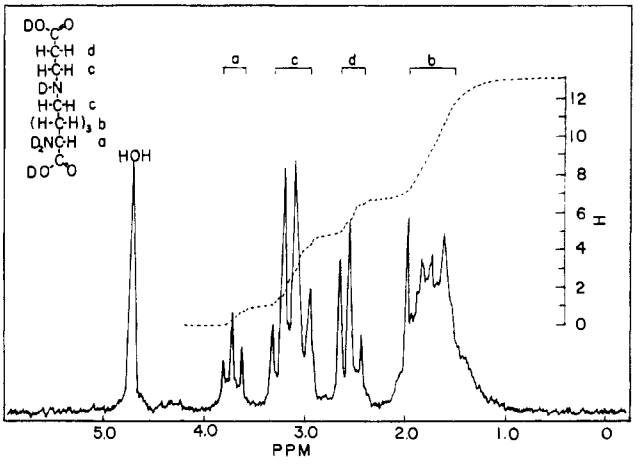

FIGURE 2: 60-MHz nuclear magnetic resonance spectrum of N^{ϵ}-(2-carboxyethyl)lysine in D_2O. Absorptions are assigned to protons as labeled in the structure. Dashed curve indicates the integral number of protons.

TABLE I: Effect of Halo Acids on the Activity of Mitochondrial and Supernatant Isozymes of Aspartate Aminotransferase.[a]

Enzyme	Reagents	pH	Time of Reaction (hr)	Inactivation (%)
Mitochondrial aspartate aminotransftrase	Iodoacetate	6.8	20	5
	Iodoacetamide	6.8	20	7
	α-Bromopropionate	6.8	20	5
	Bromosuccinate	6.8	20	5
	γ-Bromobutyrate	6.8	20	0
	β-Bromopropionate	5.4	4	8
		6.8	4	50
		8.8	4	60
Supernatant aspartate aminotransferase	Iodoacetate	6.8	20	5
		8.8	4	0
	Iodoacetamide	6.8	16	5
		8.8	4	0
	α-Bromopropionate	6.8	20	0
	Bromosuccinate	6.8	20	5
		8.8	4	0
	β-Bromopropionate	5.4	20	6
	γ-Bromobutyrate	6.8	20	0

[a] Each isozyme (1.2–1.8 mg/ml) was incubated, in a total volume of 1.0 ml, with 40 mM of each halo acid in 50 mM of a buffer (sodium acetate at pH 5.4, potassium phosphate at pH 6.8, Tris-HCl at pH 8.8) at 37° for the indicated time. The activity was measured by method B.

Ultracentrifugal Studies. Sedimentation velocity experiments were performed with a Hitachi analytical ultracentrifuge equipped with schlieren optics having a phase plate as the schlieren diaphragm. Single-sector cells with a 12-mm optical path, quartz window (plain and 2° wedged) and aluminum-filled centerpiece were used. The high-speed equilibrium experiment (Yphantis, 1964) was conducted with the ultracentrifuge equipped with interference optics. A six-channel Kel-F centerpiece and sapphire windows were used. Solutions of the native and inactivated preparations (0.02% each) in 50 mM sodium cacodylate buffer (pH 6.8) were centrifuged at 19,780 rpm at 20°. An inactivated enzyme sample with a residual activity of 26% was used. The liquid column height was 3 mm. The equilibrium was attained after 20 hr. Photographic plates were analyzed with the aid of a Nikon microcomparator. The calculation of the molecular weight of the native and modified preparations is based on a value of the partial specific volume of 0.736 which is obtained from the amino acid composition of the enzyme.

Results

Reaction of Alkylating Reagents with Aspartate Aminotransferase Isozymes. Table I summarizes the effect of various halogen compounds on the activity of the mitochondrial and supernatant isozymes of aspartate aminotransferase. Among these compounds, only β-bromopropionate inactivated mitochondrial enzyme; the others seemed to be almost without effect under the present conditions. The supernatant enzyme

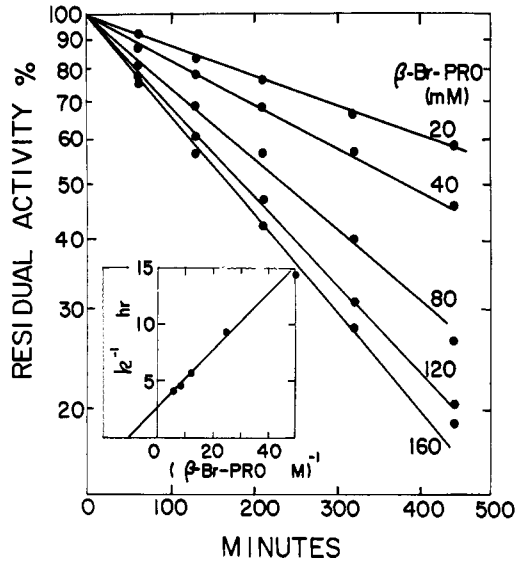

FIGURE 3: Inactivation of the mitochondrial enzyme by β-bromopropionate. The reaction mixture contained, in 1.0 ml, 1 mg of the mitochondrial enzyme, 50 mM sodium cacodylate buffer (pH 6.8), and β-bromopropionate as indicated. The concentrations of β-bromopropionate present were indicated on lines. Incubations were at 25°. At various times, aliquots (2 μl) were removed and assayed by method B. The inset is a double-reciprocal plot of the first-order rate constant (k) for the inactivation *vs.* the concentration of β-bromopropionate.

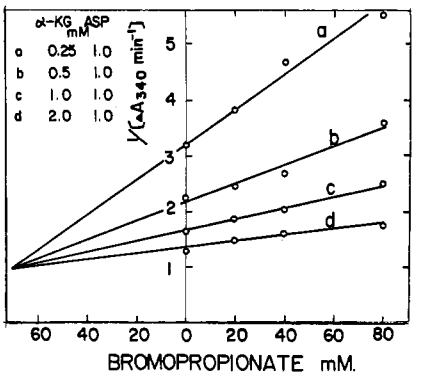

FIGURE 4: Inhibition of transamination reaction by β-bromopropionate. The reaction mixtures contained, in a total volume of 1.5 ml, α-ketoglutarate and L-aspartate as indicated, various concentrations of β-bromopropionate, 0.1 mM NADH, a few units of malic dehydrogenase, and 50 mM sodium cacodylate buffer (pH 6.8), and was maintained at 25°. The reaction was initiated by adding 3.2 μg of the mitochondrial enzyme and the change in absorbance at 340 nm was recorded.

was not inactivated by any of these compounds. Thus, it is clear that not the chemical reactivity but the structural feature of an alkylating reagent was important in causing the inactivation.

The rate of the inactivation of the mitochondrial enzyme followed pseudo-first-order kinetics (Figure 3). The rate constant (k) thus obtained was plotted in double-reciprocal fashion against varied concentrations of β-bromopropionate (the inset in Figure 3). This treatment provided a limiting rate of inactivation, 0.4 hr^{-1}, and a constant representing the extent of complex formation, 100 mM, for β-bromopropionate. This reagent was also found to act as a competitive inhibitor with respect to both α-ketoglutarate and L-aspartate in the transamination reaction. A typical experiment in which the concentration of α-ketoglutarate was varied is shown in Figure 4. The inhibition constant for β-bromopropionate was calculated to be 76 mM. This value was of a magnitude similar to that of the related constant observed in the inactivation process. These findings indicate that β-bromopropionate binds like a substrate to the active site to form a dissociable complex with the enzyme prior to inactivation. Figure 5 shows the variation of the rate of inactivation as a function of pH. The rate increases with increasing pH up to around 7 and remains almost the same over the range from pH 6.8 to 8. A half-maximum rate was attained around pH 6.2. Since no appreciable change in the affinity for β-bromopropionate was observed with the variation of pH, it is likely that a dissociating group with a pK value around this pH is concerned with the inactivation process *per se* but not with the formation of a dissociable complex prior to inactivation.

Spectral Properties of the Inactivated Enzyme. There was no appreciable change in the spectrum of the enzyme immediately after the addition of β-bromopropionate. With the progress of inactivation, a new peak appeared around 400 nm with concomitant decrease in the absorbance at 355 nm (Figure 6A). Figure 6B illustrates the close correlation of these spectral changes with the extent of inactivation. If we assume that the inactivated species comprised 68% of all species present in the preparation which was incubated with β-bromopropionate for 7 hr, an absorption spectrum for the completely inactive species could be obtained by subtracting the spectrum for the native enzyme corresponding to 32% of the total species present from that for the total species. The spectrum thus calculated was also shown in Figure 6A, and it clearly shows an absorption maximum at 395 nm.

The mitochondrial aspartate aminotransferase is known to show a pH-dependent spectral change (Morino and Wada, 1963) which is attributed to the dissociation of a protonated internal aldimine (Sizer and Jenkins, 1963). In contrast, the unique absorption band at 395 nm of the inactive enzyme did not show any spectral change with a variation of pH. At a pH higher than 9, the 395-nm peak shifted somewhat to shorter wavelength region. This is probably due to the dissociation of the coenzyme. The 395-nm peak due to the inactive species was also indifferent to the addition of L-cysteine-sulfinate which normally converts the enzyme to the pyridoxamine form absorbing at 334 nm (Figure 7). These spectral properties are quite different from those of the native enzyme

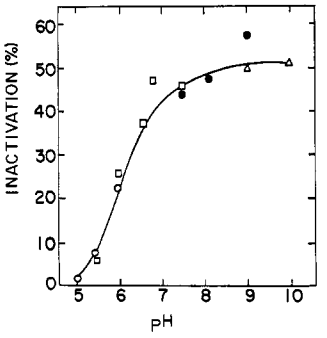

FIGURE 5: Effect of pH on the inactivation. The mitochondrial enzyme (2 mg) was incubated, in a total volume of 1.0 ml, 25 mM β-bromopropionate and 50 mM buffer. The used buffers were sodium citrate buffer (○), sodium cacodylate buffer (□), Tris-HCl buffer (●), and sodium carbonate bicarbonate buffer (Δ). After the incubation for 4 hr at 37°, the enzyme activity was measured by method A.

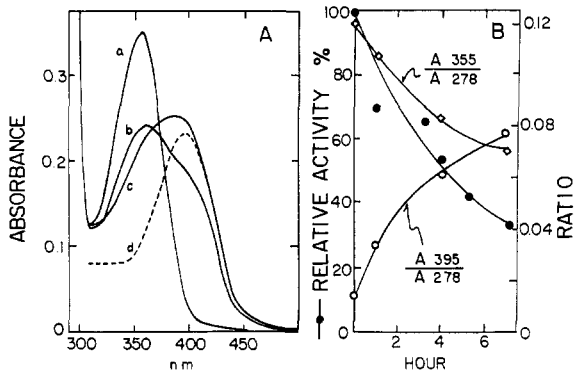

FIGURE 6: Spectral change of the enzyme upon incubation with β-bromopropionate (A) and its correlation with the extent of inactivation (B). The mitochondrial enzyme (15 mg) was incubated with 25 mM β-bromopropionate in 4.0 ml of 50 mM cacodylate buffer (pH 6.8) at 37°. Aliquots (1 ml) were withdrawn at the indicated times and dialyzed overnight against 10 mM Tris-HCl buffer (pH 8.1) at 5°. (A) Curve a, 0 time; curve b, after 4 hr; curve c, after 7 hr. Residual activities were 53% after 4 hr, and 32% after 7 hr. Curve d shows the spectrum for the completely inactivated species which is obtained from a calculation based on the assumption described in the text. Each spectrum was corrected for dilution of the preparation during dialysis and represents that of the solution containing 2.5 mg of enzyme protein. (B) The activity (●), the ratio of the absorbance at 355 nm to that at 278 nm (□), and the ratio of the absorbance at 395 nm to that at 278 nm (○) were plotted against the time of incubation.

and supply evidence that an important drastic change has occurred in the vicinity of the bound pyridoxal phosphate upon reaction with β-bromopropionate.

State of Pyridoxal Phosphate Bound to the Inactive Enzyme. The native mitochondrial aspartate aminotransferase, like other pyridoxal enzymes, binds pyridoxal phosphate through an aldimine linkage to a lysine residue of the apoprotein (Morino and Watanabe, 1969). Such bonds can be reduced by borohydride to give a derivative which absorbs around 330 nm. To test whether the coenzyme is bound to the inactivated enzyme in such a way that it can be transformed into reduced form, 0.2 mg of $NaBH_4$ was added to 1.0 ml of the solution containing 9 mg of the modified preparation with 50% residual activity in 50 mM sodium cacodylate buffer (pH 6.8). After standing for 30 min at 30°, excess borohydride was

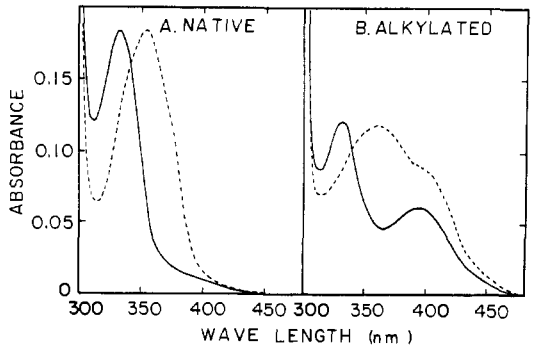

FIGURE 7: Effect of cysteinesulfinate on the spectrum of a modified preparation. (A) Native mitochondrial enzyme and (B) a modified preparation with 50% residual activity. Solid curves show the spectra after the addition of 10 μmoles of L-cysteinesulfinate; dashed curves, the spectra before the addition of the amino acid. All solutions contained 1.3 mg of the native enzyme or the modified preparation per ml of 50 mM cacodylate buffer (pH 6.8).

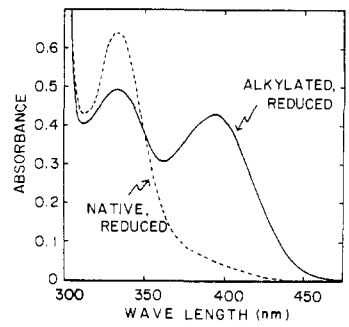

FIGURE 8: Spectral change of a modified preparation upon the addition of $NaBH_4$. Solid curve represents the spectrum of a modified preparation after borohydride reduction as described in the text. Dashed curve represents the spectrum of a similarly treated solution containing 5.1 mg of native enzyme/ml.

removed by dialysis against the same buffer for 6 hr. The resulting enzyme solution showed absorption maxima at 335 and 395 nm (Figure 8). Presumably the former peak results from the reduced form of the unmodified enzyme and the latter must be due to the modified species which remained unchanged during the treatment with $NaBH_4$. Thus the bond in the inactive enzyme seems to be resistant to the reducing action of borohydride.

Additional evidence regarding the state of pyridoxal phosphate bound to the inactive species can be provided by measuring the coenzyme level of inactivated preparations. Of the total bound pyridoxal phosphate in partially inactivated preparation, the fraction that is convertible to pyridoxamine by the addition of cysteinesulfinate is presumed to represent that in an active form. Table II shows that, of the total bound

TABLE II: Amount of Pyridoxal Phosphate Bound to the β-Bromopropionate-Treated Enzyme before and after the Addition of L-Cysteinesulfinate or KBH_4.[a]

Time of Reaction (hr)	Inactivation (%)	Amount of Bound PLP[b] (Moles/Monomer) Before CS (I)	After CS (II)	After BH_4 (III)	(II/I)·100 (%)
0	0	1.04	0.00	0.00	0
1	26	1.08	0.28	0.24	26
3	42	1.17	0.53	0.48	45
6	60	1.06	0.68	0.62	64

[a] The mitochondrial enzyme (31 mg/ml) was incubated with 25 mM β-bromopropionate in 5.0 ml of 50 mM sodium cacodylate buffer (pH 6.8) at 38°. At various time intervals, aliquots in triplicates (0.3 ml each) were withdrawn and mixed separately with 0.7 ml of the same buffer at 0°. To these solutions were separately added 0.1 ml each of distilled water (I), 50 mM L-cysteinesulfinate (II) or 0.2% KBH_4 solution (III). These mixtures were incubated at 37° for 20 min and then dialyzed overnight against 20 mM sodium acetate buffer (pH 5.4). Dialyzed solutions were determined for the absorbance at 278 nm and for the amount of pyridoxal phosphate by the reaction with phenylhydrazine as described in the Experimental Section. The enzyme activity was measured by method A. [b] PLP, pyridoxal phosphate; CS, L-cysteinesulfinate; BH_4, potassium borohydride.

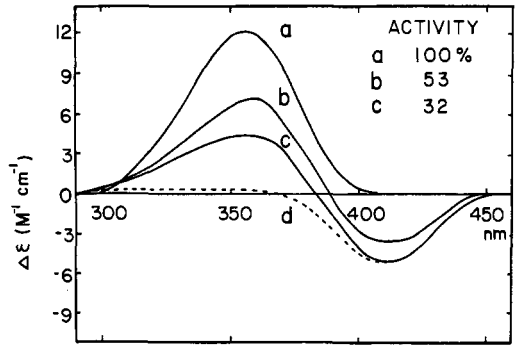

FIGURE 9: Circular dichroism spectra of modified preparations. The preparations used were the same as those described for Figure 6. Curves a, b, c, and d also correspond to those in Figure 6A.

pyridoxal phosphate, the portion which was not converted to the pyridoxamine form by cysteinesulfinate increased with the progress of inactivation. An excellent agreement is seen between the ratio of the pyridoxal phosphate bound in an inactive form to the total pyridoxal phosphate and the extent of inactivation. The fraction of pyridoxal phosphate in the inactivated preparation which was not reduced by the addition of KBH_4 corresponded quantitatively to the extent of inactivation.

Circular Dichroism of Modified Species. Figure 9 shows the variation of circular dichroism in the visible region of the mitochondrial enzyme during the reaction with β-bromopropionate. The native enzyme gave a positive circular dichroism band at 355 nm at pH 6.8. With the progress of the inactivation the dichroism band at 355 nm diminished and, instead, a new negative circular dichroism band appeared around 410 nm. This anomalous band remained unchanged upon the addition of either L-cysteinesulfinate or $NaBH_4$ which also did not exert any effect on the absorption band of the modified species at 395 nm.

Thus the appearance of a new peak in the inactive species which is accompanied by an anomalous circular dichroism would be attributed to a drastic change in ligand(s) involved in the formation of the chromophore. Such change in the mode of binding of the coenzyme must result from a modification of some amino acid residue(s) located in the vicinity of the bound coenzyme.

Identification of the Modified Amino Acid Residue Responsible for the Inactivation. One of the amino acid residues susceptible to alkylation by β-bromopropionate is cysteine. The native mitochondrial enzyme contains six sulfhydryl groups per monomeric unit of the enzyme. Preparations inactivated to various extents were titrated for the number of sulfhydryl groups. As shown in Table III, the number of sulfhydryls of these preparations was nearly the same irrespective of the extent of inactivation. A comparison between the number of half-cystines in the native enzyme and that in the inactivated enzyme preparations was also performed by the determination of *S*-carboxymethylcysteine in acid hydrolysates of carboxymethylated preparations. No significant variation in the carboxymethylcysteine content was observed in these preparations. These observations rule out the possibility that the cysteinyl residue has been modified.

To identify the modified amino acid residue(s), preparations inactivated to various extent were hydrolyzed and analyzed for anomalous amino acids. A portion of a typical analytical pattern is shown in Figure 10. One can see three unusual

TABLE III: Titration of Sulfhydryls by DTNB.[a]

Time of Reaction (hr)	Residual Act. (%)	No. of SH's/ Monomer
0	100	6.18
1	68	6.21
3.25	65	6.46
5.25	44	6.29
7.25	39	6.15

[a] The reaction mixture was the same as described in Table II. At the indicated time, an aliquot (0.4 ml) was mixed with 2.0 ml of 6 M guanidine-HCl containing 0.24 M phosphate buffer, pH 7.4, and incubated for 30 min at 37°. Titration of sulfhydryl groups was performed as described in the Experimental Section. The activity was measured by method A.

peaks emerging after the phenylalanine peak. One major peak, B, cochromatographed with N^{ϵ}-(carboxyethyl)lysine and two other peaks, A and C, were tentatively assigned to 1-carboxyethylhistidine and 3-carboxyethylhistidine, respectively, since these cochromatographed with carboxyethylated derivatives of histidine. The time course of the appearance of these three peaks indicated that N^{ϵ}-(carboxyethyl)lysine increased with

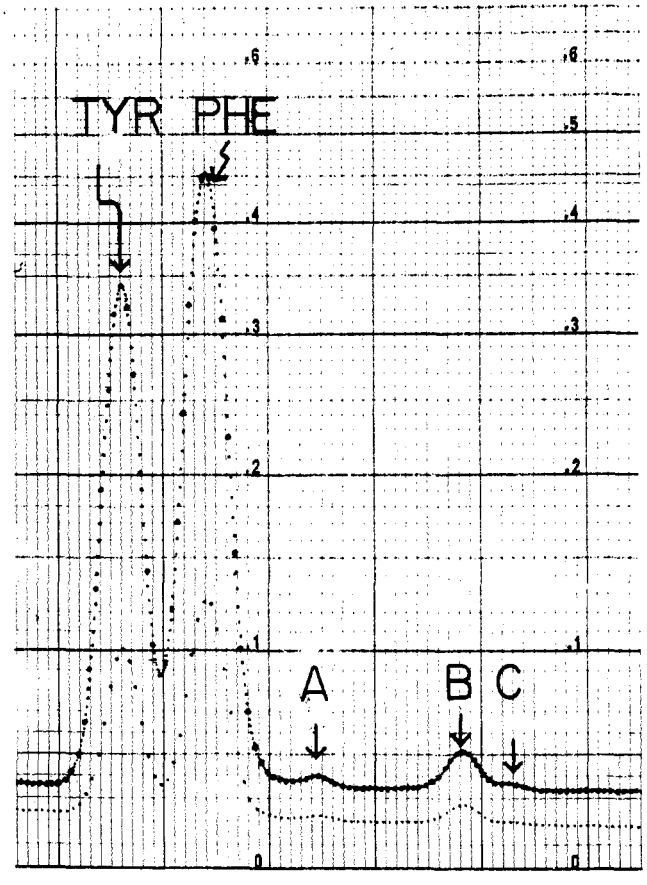

FIGURE 10: Elution pattern of alkylated amino acids in the acid hydrolysate of the modified preparation. Peaks A and C are assumed to be 1-carboxyethylhistidine and 3-carboxyethylhistidine, respectively. The peak B is N^{ϵ}-(carboxyethyl)lysine.

TABLE IV: Quantitative Correlation of Alkylated Amino Acid Residues with the Extent of Inactivation in the Presence or Absence of Substrate Analogs.[a]

Reaction Time (hr)	Substrate Analog[b]	Extent of Inactivation (%)	Mole/Monomeric Unit of Enzyme[d] 1-Carboxyethyl histidine[c]	N^{ϵ}-(Carboxyethyl)lysine	3-Carboxyethylhistidine
2	None	41	<0.1	0.65	<0.1
	Succinate	15	<0.1	0.10	0.10
	Maleate	3	<0.1	<0.1	<0.1
4	None	68	<0.1	0.74	<0.1
	Succinate	25	0.12	0.46	0.12
	Maleate	6	0.12	0.25	0.12

[a] The reaction mixture contained 4 mg of the mitochondrial enzyme and 50 mM β-bromopropionate in 2 ml of 100 mM cacodylate buffer (pH 6.8). Incubations were at 37°. At indicated times, an aliquot of 1 ml was withdrawn, assayed for the enzyme activity, and dialyzed overnight against distilled water, followed by lyophilization. The acid hydrolysis, amino acid analysis and identification of modified amino acids were performed as described in the Experimental Section. [b] Where indicated, 10 mM either of potassium succinate or potassium maleate was present in the reaction mixture described above. [c] 1-Carboxyethylhistidine, ε-carboxyethyllysine, and 3-carboxyethylhistidine correspond to peaks A, B, and C in Figure 10, respectively. [d] Color values of these modified amino acids were assumed to be equivalent to that of leucine.

the extent of inactivation. Other minor peaks due to modified histidyl residues also seemed to increase gradually with the progress of inactivation.

It has been known that dicarboxylic acids like succinic acid and maleic acid are potent competitive inhibitors for aspartate aminotransferase. Therefore it was expected that these dicarboxylic acids might protect the enzyme from inactivation by β-bromopropionate. The results shown in Table IV demonstrate that this was the case. Taking advantage of this fact, an unambiguous identification of the amino acid residue modified in the inactivation process was attempted by correlating the amounts of alkylated amino acid residues with the extent of inactivation when the enzyme was reacted with β-bromopropionate in the absence or presence of substrate analogs. A linear correlation of the formation of N^{ϵ}-(carboxyethyl)lysine with the extent of inactivation and lack of such correlation in the amount of the carboxyethylated histidines seem to indicate that the modification of a lysine residue is directly involved in the inactivation by β-bromopropionate while that of histidine residue(s) results from a side reaction.

Gross Structure of the Inactive Species. The modified species tended to form precipitates during incubation with β-bromopropionate at pH lower than 6.5. This fact seems to indicate that some change has occurred to a higher order structure of the enzyme molecule as a consequence of the modification leading to inactivation. However, a comparison of the circular dichroic spectrum of the native enzyme to that of the modified preparation at the far ultraviolet region did not reveal any difference between them.

No significant difference was observed in the sedimentation patterns of the native enzyme and inactivated preparations at a concentration of 5 mg/ml. The molecular weight (92,500) of the inactivated preparation at a protein concentration of 0.02% as determined by high-speed sedimentation equilibrium method was identical within experimental errors with that (94,100) of the native enzyme and no sign of dissociation to smaller molecules nor aggregate formation was detectable in the plot of the logarithm of fringe displacement *vs.* the distance from the rotor axis. Thus, the structural alteration of enzyme molecules accompanying the inactivation seems to be restricted to the active site of the enzyme.

Discussion

The results presented in this paper have shown that, of all the halo acids studied, β-bromopropionate inactivated specifically the mitochondrial aspartate aminotransferase and the inactivation was caused by alkylation of an essential lysine residue located in the active region. Since the supernatant enzyme was not inactivated by any of these halo acids, there must be distinct differences in the fine structure of the active site of each isozyme.

The apparent strict specificity of β-bromopropionate in inactivating the mitochondrial enzyme can be interpreted as showing that the molecular structure of this halo acid satisfies the structural requirements for binding apparently through interaction of a positively charged group in the active region with the carboxyl group of the halo acid, thus orienting its reactive group (β-carbon atom) properly to react with an accessible nucleophile (a lysine residue in the present case) in the active site. Analogous observations can be found in elaborate studies on the specificity of halo acids in alkylating certain amino acid residues of pancreatic ribonuclease (Stark *et al.*, 1961; Heinrikson *et al.*, 1965).

The pH dependency of the inactivation rate indicated that a group having a pK value around 6.2 is subject to the alkylation. A candidate for such group is the ε-amino group of lysine residue to which the 4′-formyl group of pyridoxal phosphate is linked, for a value of pK = 6.3 is assigned to the proton dissociation from the internal Schiff's base nitrogen from the study of the spectral change of the enzyme with a variation of pH (Sizer and Jenkins, 1963).

Loss of the pH-dependent spectral shift of the enzyme and lack of the interaction with substrates upon the inactivation resulted from one and the same cause, *i.e.*, the alkylation of an essential lysine residue. Thus, the carboxyethyl moiety seems to occupy the region of the active site which normally binds the substrates. This is plausible since the presence of substrate analogs like succinate or maleate was shown to protect the enzyme from inactivation by β-bromopropionate. It seems that the bulky carboxyethyl group, the carboxyl of which is held tightly by a positively charged group in the active region, perhaps gives rise to some distortion of the aldimine linkage, and the bond thus modified is responsible for the absorption band at 395 nm exhibited by the inactivated enzyme. This modification of the aldimine linkage is also reflected in the inverted circular dichroism exhibited by the chromophore of the inactivated enzyme.

The unique negative circular dichroism observed with the inactivated species is also reminiscent of the negative circular dichroism observed upon the reaction of the native enzyme with *erythro*-β-hydroxy-L-aspartate (Jenkins, 1961; Breusov *et al.*, 1964). Thus, the carboxyethylated active site simulates structurally an intermediary enzyme–substrate complex.

The inactivation product represented by a spectral species absorbing at 395 nm was shown to resist reduction by borohydride under the condition where the internal Schiff's base in the native enzyme is readily reduced. The difference in reducibility may be explained by assuming that the incorporation of a bulky carboxyethyl substituent into a lysyl residue located in juxtaposition to the internal aldimine linkage in the active site precluded the access and functioning of borohydride in the attempted reduction of the active site aldimine linkage.

The finding that an ionizing group with a value of $pK = 6.2$ is involved in the inactivation leads us to speculate that the alkylated lysyl residue might be the one that links to the formyl group of the coenzyme *via* an aldimine bond. The accompanying paper describes the experiment performed to examine the identity of the alkylated lysyl residue with that involved in binding pyridoxal phosphate.

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