Formate-induced Labeling of the Active Site of Aspartate Aminotransferase by \( \beta \)-Chloro-L-alanine

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SUMMARY

The pyridoxal form of both the supernatant and mitochondrial isoenzymes of aspartate aminotransferase (EC 2.6.1.1) catalyzes \( \alpha,\beta \) elimination of pyruvate from \( \beta \)-chloro-L-alanine with concomitant inactivation. Rates of these reactions were greatly accelerated by formate ion. Acetate and propionate were much less effective and essentially ineffective with the mitochondrial isoenzyme. With both isoenzymes, dependency of rates of \( \alpha,\beta \) elimination as well as inactivation on formate concentration showed sigmoidal curves; the rate enhancement was especially marked at formate concentrations from 1 to 3 M. Michaelis constants for \( \beta \)-chloro-L-alanine were identical in both the \( \alpha,\beta \) elimination reaction and the inactivation. These results suggest that both reactions occur via a common intermediate. The presence of formate did not affect the Michaelis constants for \( \beta \)-chloro-L-alanine. Formate increased only the maximal velocities of these reactions. The pH optimum for the inactivation of both isoenzymes was approximately pH 7.5 whereas the rate of the \( \alpha,\beta \) elimination reaction was maximal at pH 7 and remained constant above this pH.

Formate induced a considerable change in the absorption and circular dichroism spectra of the pyridoxal form of both isoenzymes in visible regions. Upon reacting with \( \beta \)-chloro-L-alanine in the presence of 3 M formate, the absorption band at 345 nm, which is attributed to the bound pyridoxal phosphate, shifted to 337 nm, which was accompanied by a marked decrease in ellipticity. Prolonged incubation of the inactivated enzymes resulted in the formation of a new spectral species absorbing at 455 nm. Removal of formate produced a drastic change in the spectrum, giving rise to three absorption bands at 333, 375, and 420 nm. Addition of formate to this solution reversed these spectral changes to give a single 455-nm band again. All these observations are consistent with the proposal that formate binds to a discrete subsite within the active site which normally binds the distal carboxyl group of a natural dicarboxylic substrate.

Inactivation of both isoenzymes with \( \beta \)-chloro-L-[U-\( ^1\)C]-alanine and reduction with KBH\(_4\) resulted in a stoichiometric incorporation of the three-carbon moiety derived from chloroalanine as well as the pyridoxyl moiety into the enzyme protein via stable covalent linkages.

A comparative study on the effect of various halo acids on the mitochondrial and supernatant isoenzymes of aspartate aminotransferase has revealed that \( \beta \)-bromopropionate selectively inactivates the mitochondrial enzyme by alkylating the active site lysyl residue (1, 2). This finding indicated that this halo acid has been structurally well fit to the active site and thus reacted efficiently with the essential lysyl residue within the site. Therefore it was anticipated that an analogous halo acid having an additional substituent such as either amino or carbonyl group at the \( \alpha \) position might be a much better active site-directed reagent. In fact, bromopyruvate has been found to react as a keto acid substrate with the pyridoxamine form of both isoenzymes of aspartate aminotransferase and to inactivate them by alkylating an essential cysteinyl residue within the active site (3, 4). Similarly, another type of such analog, \( \beta \)-bromo-L-alanine, also was found to react with the pyridoxal form of these isoenzymes with formation of pyruvate via an \( \alpha,\beta \) elimination reaction (3), which is analogous to that observed with \( \beta \)-chloro-L-glutamate (5) or L-serine-O-sulfate (6). Both isoenzymes were inactivated slowly during this enzymatic reaction. During the study of the aspartate aminotransferase-catalyzed \( \alpha,\beta \) elimination reaction and concomitant inactivation of the enzymes, we have found a striking acceleration of these reactions by the presence of formate ion (7) which greatly facilitated a further investigation of the affinity labeling of the enzyme active site by 3-chloro-L-alanine. A brief account of a study on the structure of the modified site in the supernatant aspartate aminotransferase has appeared (8). It is the purpose of this communication to describe in detail the stimulatory effect of formate ion on the \( \alpha,\beta \) elimination reaction catalyzed by both isoenzymes and on the \( \alpha,\beta \) elimination reaction catalyzed by both isoenzymes and on the concomitant inactivation of these enzymes.
EXPERIMENTAL PROCEDURES

Materials—β-Chloro-l-alanine-HCl was synthesized from L-serine methyl ester (Protein Research Foundation, Osaka) and the HCl-free form was obtained as described by Fischer and Raske (5). β-Chloro-l-[U-14C]alanine was synthesized as follows. Dry HCl gas was bubbled for 30 min into a suspension containing 500 mg of L-serine and 0.2 mM of L-[U-14C]serine (120 mCi per mmole, Daiichi Pure Chemicals Co. Ltd., Tokyo) in 15 ml of dry methanol at 0°. The mixture then was heated under reflux for an additional 30 min. Methanol and excess HCl were removed under reduced pressure in a rotary evaporator. The radioactive L-serine methyl ester HCl was dissolved in hot dry methanol and crystallized by adding dry ethyl ether. Resulting crystals were dried in vacua over P2O5. Starting from this ester, radioactive β-chloro-l-alanine was obtained in a yield of 42% and the specific activity of potassium formate was prepared by a gradual addition of saturated KOH solution to 50% formic acid in an ice bath under stirring. The final pH was approximately 9. Sodium pyrophosphate buffer, which was used throughout the present study, was prepared by adding pyrophosphoric acid to a solution of sodium pyrophosphate. 

cysteine-β-Chloro-l-alanine-aminobutyric acid, and β-chloro-n-alanine were synthesized from L-threonine, L-α-threonine, and L-alanine, respectively, according to the procedure described for β-chloro-l-alanine. Mitochondrial and supernatant aspartate aminotransferases were purified from pig heart by a modification of the procedure described for beef liver enzymes (10). Lactate dehydrogenase was obtained in crystalline form from pig heart according to the procedure described by Reeves and Fimognari (11).

Methods—Aspartate aminotransferase activity was measured as described previously (4). Protein concentration was obtained from the values of E280 nm of 12.5 (supernatant enzyme) and 13.6 (mitochondrial enzyme). The molecular weight of each isoenzyme was assumed to be 94,000.

The rate of the α,β elimination reaction with β-chloro-l-alanine or β-chloro-l-α-aminobutyrate was determined from the rate of formation of pyruvate or α-ketobutyrate by following the decrease in absorbance at 340 nm in the presence of NADH and lactate dehydrogenase. Detailed conditions have been described in the legends to tables and figures. Absorption spectra were measured in a Hitachi model 124 spectrophotometer or a Cary model 14 spectrophotometer. Circular dichroism was measured in a Jasco Dichrograph II. Radioactivity was measured in a Tri-Carb liquid scintillation spectrometer model 3200. Bray's solution (12) was used as the scintillation medium.

RESULT

Stoichiometry of Aspartate Aminotransferase-catalyzed Decomposition of β-Chloro-l-Alanine—The data are shown in Table I. A similar result was obtained also with the mitochondrial isoenzyme. Thus it is evident that aspartate aminotransferases catalyze α,β elimination of pyruvate from β-chloro-l-alanine. Neither the pyridoxal phosphate form nor the borohydride-treated preparation of the pyridoxal form of both isoenzymes catalyzed the formation of pyruvate.

Inactivation of Aspartate Aminotransferases during α,β Elimination Reaction—As previously reported (3), an irreversible inactivation of both isoenzymes occurred during incubation with β-chloro-l-alanine. When the α,β elimination reaction was followed by recording the decrease in absorbance at 340 nm in the presence of NADH and lactate dehydrogenase, the rate of pyruvate formation was found to decrease with time (Fig. 1A). The extent of time-dependent decrease in the rate of pyruvate formation was exactly identical with the decrease in the rate of transamination between α-ketoglutarate and L-aspartate as determined with aliquots withdrawn from the same reaction mixture at various intervals during the α,β elimination reaction (Fig. 1B). Thus it is clear that the observed decrease in the rate of α,β elimination with time resulted from an irreversible inactivation of the enzyme rather than from the accumulation of an inhibitor in the reaction mixture. The inactivation followed first order kinetics. The pseudo-first order rate constant, k, for the inactivation was obtained from a semilogarithmic plot of the rate of NADH oxidation against time, using the following relation, k = 0.693/t1/2, where t1/2 denotes the time (in minutes) required for inactivating one-half the enzyme initially present. This procedure provides a convenient determination of both the initial velocity of the α,β elimination reaction and the rate of inactivation from a single experiment.

Effect of Monocarboxylate Anions—Inactivation of the supernatant isoenzyme was found to be accelerated when Tris-HCl...
buffer in the reaction mixture was replaced by Tris-acetate buffer (7). This finding led to the investigation of the effect of various monocarboxylate anions on the rates of $\alpha,\beta$ elimination and concomitant inactivation. Fig. 2 illustrates that formate was the most effective in the inactivation reaction while acetate and propionate, acids of longer carbon chain length, produced less stimulation. This was particularly true with the mitochondrial isoenzyme where acetate and propionate were virtually ineffective. Although data were not presented, similar results were obtained for the effect of these monocarboxylates on the $\alpha,\beta$ elimination reaction as well. Other anions such as chloride, phosphate, or sulfate did not replace the monocarboxylate anions in these reactions with both isoenzymes.

As shown previously (7), saturation curves for formate were not of a typical hyperbola but sigmoidal in the $\alpha,\beta$ elimination reaction as well as concomitant inactivation. In these experiments, reactions were performed using the substrate at a concentration far below saturation. Hence it was suggested that this anomaly might possibly arise from an increase in the affinity for the substrate in the presence of formate. Thus, Michaelis constants for the substrate were determined at various concentrations of formate. However, no variation was observed in these values over the range of formate concentrations tested. In addition, the data in Fig. 3 indicated that Michaelis constants for $\beta$-chloro-$L$-alanine were virtually identical in both of the $\alpha,\beta$ elimination reaction and the concomitant inactivation. This finding indicates that the inactivation occurs via an intermediate in the $\alpha,\beta$ elimination reaction. Similar results were obtained from experiments on the supernatant isoenzyme.

Plots of maximal velocities both of the $\alpha,\beta$ elimination reaction and inactivation versus formate concentration were hyperbolic from 0 to 0.6 M of formate while these rates increased in an anomalous fashion with a further increase in formate concentration, thus giving rise to sigmoidal curves (Fig. 4). The enhancing effect of formate was more striking on the reactions catalyzed by the supernatant enzyme. With either isoenzyme, the extent of rate acceleration was much greater in the inactivation process than in $\alpha,\beta$ elimination reaction.

Table II summarizes kinetic parameters for reactions of both isoenzymes with $\beta$-chloro-$L$-alanine. Maximal velocities of $\alpha,\beta$ elimination attainable at an infinite concentration of formate could not be adequately estimated since saturation curves for formate were anomalous (Fig. 4) and both isoenzymes tended to form precipitates at a concentration of formate greater than 4 M. However, it is clear that the maximal velocity would be

![Figure 2](http://www.jbc.org/)

**Fig. 2.** Effect of monocarboxylate anions on the rate of inactivation of aspartate aminotransferases by $\beta$-chloro-$L$-alanine. The reaction mixtures contained, in 1.0 ml: 40 mM $\beta$-chloro-$L$-alanine, 25 $\mu$g of supernatant aspartate aminotransferase (left), 10 $\mu$g of mitochondrial aspartate aminotransferase (right), 0.1 mM NADH, 2 $\mu$g of lactate dehydrogenase, monocarboxylate anions as indicated, and 100 mM sodium pyrophosphate buffer (pH 7.4). The reaction was started by the addition of aspartate aminotransferase and the absorbance change at 340 nm was followed at 20°. $H^+$, potassium formate; $CH_2CO_2^-$, potassium acetate; $CH_3CH_2CO_2^-$, potassium propionate. Values in parentheses indicate rates in the absence of these monocarboxylates.

![Figure 3](http://www.jbc.org/)

**Fig. 3.** Double reciprocal plots of the rate of $\alpha,\beta$ elimination (A) and the rate of inactivation (B) against concentrations of $\beta$-chloro-$L$-alanine. Reaction mixtures contained, in 3.0 ml, $\beta$-chloro-$L$-alanine as indicated, 22 $\mu$g of mitochondrial aspartate aminotransferase, potassium formate as indicated on each plot, 0.1 mM NADH, 5 $\mu$g of lactate dehydrogenase and 40 mM sodium pyrophosphate buffer (pH 7.4). The reaction was started by the addition of aspartate aminotransferase and followed by recording the absorbance change at 340 nm. Incubations were at 18°. Half time ($t_{1/2}$) is related to the rate of inactivation ($k$) by the equation $t_{1/2} = 0.693/k$.

![Figure 4](http://www.jbc.org/)

**Fig. 4.** Effect of formate on maximum velocities of $\alpha,\beta$ elimination of $\beta$-chloro-$L$-alanine and concomitant inactivation of the supernatant (A) and mitochondrial (B) isoenzymes of aspartate aminotransferase. A, reaction mixtures contained, in 3.0 ml: varied concentrations of $\beta$-chloro-$L$-alanine (8, 16, 32, and 64 mM), varied concentrations of potassium formate as indicated, 25 $\mu$g of supernatant aspartate aminotransferase, 0.1 mM NADH, 5 $\mu$g of lactate dehydrogenase, and 40 mM sodium pyrophosphate buffer (pH 7.4). The reactions were initiated by adding aspartate aminotransferase and the absorbance change at 340 nm was followed. Incubations were at 18°. Reciprocals of the initial velocity of $\alpha,\beta$ elimination and the rate of inactivation were plotted against reciprocals of concentration of $\beta$-chloro-$L$-alanine. Maximum velocities of $\alpha,\beta$ elimination and of concomitant inactivation were obtained from these plots at each concentration of formate. B, data from the experiments on the mitochondrial isoenzyme (Fig. 3) were plotted again.
Kineti... of 

<table>
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<th>Formate</th>
<th>( V_{\text{max}} ) (μmoles/min/mg)</th>
<th>( K_m ) (m)</th>
<th>( k_{\text{max}} ) (min⁻¹)</th>
<th>( K_m ) (m)</th>
<th>Transamination between L-aspartate and α-ketoglutarate (μmoles/min/mg)</th>
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\( a,\beta \) Elimination:

- \( V_{\text{max}} \) = 2.3 (μmoles/min/mg) at pH 7.4
- \( K_m \) = 0.2 (m) at pH 7.4

Inactivation:

- \( k_{\text{max}} \) = 0.08 (min⁻¹) at pH 7.4
- \( K_m \) = 20 (μmoles/min/mg) at pH 7.4

Transamination between L-aspartate and α-ketoglutarate:

- \( V_{\text{max}} \) = 100 (μmoles/min/mg) at pH 7.4
- \( K_m \) = 0 (μmoles/min/mg) at pH 7.4

* Values in parentheses are inactivation rate at a fixed concentration of substrates.
* n.d., not determined.

For the reaction with fl-chloro-L-alanine except that p-chloro-n-olate, or 10 mM erythro-fl-chloro-L-a-aminobutyrate in 1.0 ml of 0.1 M sodium pyrophosphate buffer (pH 7.4) in the presence or absence of formate and assayed for aspartate transaminase activity as described previously (4).

Effect of pH—With both isoenzymes, the pH rate profiles for the inactivation process showed a rather broad pH optimum centering around pH 7.5 in the presence of 3 M formate. In the absence of the activator, a sharp pH optimum was observed at pH 7.5 with the supernatant enzyme and at pH 7.0 with the mitochondrial enzyme. The rate of the \( \alpha,\beta \) elimination reaction also increased with the increase in pH, but the rate remained constant above pH 7.0 whether formate was present or not. No significant variations were observed in the values of Michaelis constant for \( \beta \)-chloro-L-alanine in either the \( \alpha,\beta \) elimination reaction or the inactivation process with each isozyme when the reactions were performed at pH 6.5, 7.0, and 7.5 in the presence or absence of 2 M formate.

Spectral Changes of Enzymes during Reaction with \( \beta \)-chloro-L-Alanine—In the absence of formate anion, absorption and CD spectra of the pyridoxal form of both isoenzymes changed slowly upon the addition of \( \beta \)-chloro-L-alanine (Fig. 5). The increase in absorbance at 330-nm region was mostly accounted for by the progressive accumulation of pyruvate produced during the \( \alpha,\beta \) elimination reaction. The extent of decrease in ellipticity at 350 nm was approximately parallel to the extent of inactivation of the enzyme.

Since formate has been known to exert a striking effect on the reaction of the enzyme with \( \beta \)-chloro-L-alanine, it was of interest to examine its effect on the spectral properties of the enzyme. As can be seen in Fig. 6, the 362-nm peak of the native pyridoxal form of the supernatant enzyme in the absence of formate was shifted to 345 nm in 3.5 M formate and, in addition, a small peak appeared around 430 nm which can be attributed to the presence of a protonated form (15). A value of pK 7.3 was found for the protonation of the enzyme in 3.2 M formate. This value was 1 pH unit more alkaline than the pK value for the protonation of the enzyme in the absence of formate. Corresponding shifts also were observed on the CD spectra (Fig. 6). Similarly the 355-nm band of the mitochondrial enzyme in the absence of formate was shifted to 342 nm in 3.2 M formate, although data were not presented. Other anions such as chloride, phosphate, and sulfate did not appreciably affect the absorption spectra of these isoenzymes.

Spectral changes occurring upon the addition of \( \beta \)-chloro-L-alanine were much more dramatic in the presence of formate than in its absence. The CD spectra of the pyridoxal form of both isoenzymes recorded between 330 and 440 nm at pH 7.4 in the absence of formate (Fig. 6) were characterized by a negative band centered around 345 nm with a smaller positive band at 360 nm. In the presence of formate, the 345-nm band of the pyridoxal form of the mitochondrial enzyme shifted to 330 nm, and a small negative peak appeared at 380 nm. The CD spectrum of the pyridoxal form of the supernatant enzyme showed a broad negative band from 330 to 350 nm with a peak at 350 nm and a smaller band at 380 nm. In the presence of formate, the 350-nm band of the pyridoxal form of the mitochondrial enzyme shifted to 330 nm, and a small negative peak appeared at 370 nm. The CD spectrum of the pyridoxal form of the supernatant enzyme showed a broad negative band from 330 to 350 nm with a peak at 345 nm and a smaller band at 370 nm.

Under the present reaction conditions, 98% inactivation of the enzyme was observed at 3 min after the enzyme was incubated with the radioactive substrate.
those observed in the absence of formate. Immediately after the addition of the substrate, the 345-nm band of the supernatant enzyme disappeared and a new peak appeared at 333 nm (Fig. 7). Under the present experimental conditions, more than 95% of inactivation of the enzyme was observed within 2 min after addition of β-chloro-L-alanine. Curve 1 in Fig. 7 (top) represents the spectra obtained within 4 min after the addition of the substrate. The increase in absorbance at the 330 nm region is partly attributed to the accumulation of pyruvate during the reaction. Thus the spectrum shown by Curve 1' in Fig. 7 (top) was obtained by subtracting the absorbance contributed by pyruvate and the resulting spectrum represents an enzyme species formed immediately after inactivation. Corresponding changes occurred in the CD spectra during reaction with β-chloro-L-alanine (Fig. 7, bottom). A positive band at 350 nm of the native supernatant enzyme was shifted to 340 nm with concomitant decrease in its ellipticity and the positive band at 430 nm in the native enzyme completely disappeared after addition of the substrate. Curve 1 in Fig. 7 (bottom) was obtained within 15 min after the substrate was added and, therefore, could represent the spectra of a species formed just after inactivation.

To determine whether these spectral changes arose from the inactivation of the enzyme or from the formation of an intermediary enzyme-substrate complex prior to inactivation, the reaction occurring upon the addition of the substrate was followed by recording the change in absorbance at 360 nm and also the change in ellipticity at 360 nm. The ellipticity at 360 nm and the absorbance at 360 nm decreased rapidly upon the addition of the substrate and remained constant within 15 s (Fig. 8). Under the present experimental conditions the half-time of inactivation was approximately 40 s. Therefore the rapid decrease in ellipticity at 360 nm seems to result from the interaction of the substrate with the enzyme, which must occur prior to the inacti-
tivation of the enzyme. Since no detectable change was observed in the absorption and CD spectra for several minutes further, it is likely that most of these spectral changes occurred before the inactivation proceeded and there might be no appreciable difference in spectral properties between the inactivated species and the intermediary species formed prior to the inactivation.

A slow spectral change appeared when the inactivated enzyme was kept for a prolonged period after the enzyme was completely inactivated. Over a period of a day at 18°, the absorption band at 455 nm increased and that at 340 nm decreased (Fig. 7). The rate of this slow spectral change was greater with the supernatant isoenzyme than with the mitochondrial enzyme. This broad 455-nm absorption peak was accompanied by a CD band at 440 nm. As will be shown later, this secondary slow spectral change seems to result from some chemical reaction occurring at the β-chloro-L-alanine-labeled active site.

The removal of formate ions completely changed these spectral characteristics, giving rise to three new absorption bands at 333, 375 and 420 nm which were accompanied by CD bands at the corresponding wave lengths (Fig. 9). These spectral characteristics were identical to those of the preparation which was inactivated by β-chloro-L-alanine in the absence of formate (see Fig. 5). These spectral changes were reversible and the addition of 0.1 M formate resulted in the restoration of the original spectra in a time dependent process (Fig. 9). No variation was observed in the spectrum of the inactivated species over the pH range from 6 to 9.5. These results described for the supernatant enzyme were essentially identical with those obtained with the mitochondrial enzyme.

Incorporation of Three-carbon Moiety of β-Chloro-L-alanine into Enzyme—The supernatant aspartate aminotransferase (57 mg, 1.2 μ mole monomer unit of the enzyme) was incubated at 30° with 300 μ moles of β-chloro-L-[U-14C]alanine in 5 ml of 0.1 M sodium pyrophosphate buffer (pH 7.8) containing 3 M potassium formate. At various time intervals, aliquots of 1.0 ml were withdrawn, mixed with 100 molar excess KBH₄ and 1 drop of 1-octanol, followed by dialysis. The spectra were taken before reduction with KBH₄ and after dialysis of the reduced preparation. The spectral properties of the inactivated enzyme were identical to those described earlier for Fig. 7. Upon reduction with borohydride the preparation showing a predominant absorption band at 455 nm gave a new species absorbing at 365 nm whereas the 340-nm species which was predominant immediately after inactivation yielded upon reduction a species absorbing at 325 nm (Fig. 10).

Determination of radioactivity incorporated from β-chloro-L-[U-14C]alanine revealed that all of these preparations contained radioactivity corresponding to 1 mole of the substrate per monomeric unit of the enzyme (Table III). To know whether the bound radioactivity is of a covalent nature or not, KBH₄-reduced preparations were denatured by incubating in 7 M guanidine HCl for 1 hour at 37°, followed by dialysis overnight against distilled water. After dialysis, heavy precipitates were collected, and the residue was suspended in 1% NH₄HCO₃ and digested by chymotrypsin. Radioactivity and absorbance at 325 nm of the resulting solutions were measured (Table III). The sample prepared by reducing with KBH₄ immediately after the inactivation contained per monomeric unit of the enzyme an amount of radioactivity corresponding to 0.9 mole of the 14C-substrate and an amount of pyridoxyl derivative corresponding to 0.9 mole of coenzyme. This finding indicates that the label derived from the substrate is covalently bound to some nucleophilic residue within the active site, and upon reduction with KBH₄, the coenzyme, pyridoxal phosphate, is fixed to some part of the label (presumably the α-amino group of the label). With the supernatant enzyme, this nucleophilic side chain was demonstrated to be the ε-amino group of the lysyl residue which originally binds the coenzyme (8).

As described earlier, a slow spectral change occurred in the inactivated enzyme upon standing for a long period, giving rise to a broad absorption band at 435 nm (see Fig. 7). The latter species exhibited upon reduction with KBH₄ an absorption band at 365 nm (Fig. 10). As shown in Table III, each preparation before denaturation contained the radioactivity corresponding to approximately 1 mole of the label per monomeric unit of the enzyme. However, after denaturation in 7 M guanidine HCl...
and digestion by chymotrypsin, the preparation predominant in the 365-nm species was found to retain both the radioactivity and the coenzyme derivative absorbing at 325-nm equivalent to only 20% of those of the preparation which was reduced immediately after inactivation. Table III also illustrates that both the radioactivity and pyridoxyl derivative retained in a denatured sample decrease in parallel with the extent of delay in the KBH₄ treatment.

After extensive dialysis of the 455-nm species against water neither recovery of enzymatic activity nor release of any radioactivity was observed. Thus the label derived from β-chloro-L-alanine was still tightly bound to the active site. Although the precise nature of the bond between the label and the enzyme is not known at this time, it might be a rather labile covalent bond which is decomposed upon the denaturation of the enzyme. Based on these observations we postulate the mechanism of α,β elimination reaction is the abstraction of the α-hydrogen atom of a substrate (16, 17). In this scheme the deprotonation is assumed to be preformed by the ε-amino group of the lysyl residue which originally binds pyridoxal phosphate. This idea comes from the recent finding (8) that the lysyl residue is covalently modified by β-chloro-L-alanine during the enzyme α,β elimination reaction. Namely, the ε-amino group must be in close proximity to the β-carbon atom of the bound substrate in an intermediary complex (III in Fig. 11). If so, one may suggest that the same ε-amino group is also in juxtaposition to the α-hydrogen atom of the bound substrate. This assumption is not too arbitrary since the ε-amino group is provided with a flexible arm characteristic of a lysyl residue. Such structural considerations on the relative positions of the ε-amino group of the lysyl residue and the α-hydrogen atom of the bound substrate further suggest that the ε-amino group might function as a base participating in the abstraction of the α-hydrogen atom of the substrate. The concept that an essential lysyl residue participates not only in binding the coenzyme but also acts as a catalytic residue within the active site was raised earlier by Snell (18) and was later supported by kinetic evidence on a bacterial pyridoxamine pyruvate transaminase (19). The striking acceleration of the rate of the α,β elimination reaction by formate ions as well as the concomitant inactivation of the enzyme is rather surprising. The maximal velocity of the α,β elimination reaction attained at an infinite concentration of β-chloro-L-alanine is of the same order of magnitude as that of the normal transamination between L-aspartate and α-keto-

### Table III

<table>
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DISCUSSION

Both mitochondrial and supernatant isoenzymes of aspartate aminotransferase have been shown to catalyze α,β elimination of β-chloro-L-alanine and to be irreversibly inactivated during the reaction. It has been demonstrated recently that a covalent labeling of the active site of the supernatant isozyme by β-chloro-L-alanine occurs at the ε-amino group of the lysyl residue involved in binding the coenzyme, pyridoxal phosphate (8). An identical conclusion has been drawn from a similar study on the mitochondrial isoenzyme and its detailed account will appear in a subsequent publication.

Based on these observations we postulate the mechanism of the reaction of both isoenzymes with β-chloro-L-alanine as shown in Fig. 11. A prerequisite step in the sequence of the reaction mixture contained, 7.5 ml: 32 mg of inactivated preparation of supernatant aspartate aminotransferase, 3.0 M potassium formate, and 50 mM sodium pyrophosphate buffer (pH 7.4). The reaction was started by adding 0.5 ml of 0.4 M β-chloro-L-[¹⁵N]alanine. Incubation was at 30°C. At indicated times aliquots of 1.5 ml were withdrawn and their absorption spectra were taken. Then 1 mg of KBH₄ and 1 drop of 1-octanol were added and spectra of the resulting solutions were recorded. Top, absorption spectra; bottom, CD spectra. Inset represents the absorption spectra of inactivated preparations before KBH₄ treatment.
imidazole ring of the histidyl residue. This produces a charge depicted as binding, for instance, to the γ-nitrogen atom of distal carboxyl group of a dicarboxylic substrate, formate can be active site of the supernatant aspartate aminotransferase had been suggested by Martinez-Carrion and his coworkers (21). On the assumption that this histidyl residue is a site binding the enzyme in the cw/β elimination reaction as well as the alanine transamination (16, 17). Therefore it is highly plausible that formate acts on this common step in the enzymatic catalysis, i.e. formate induces reactivity of a proton-abstracting base which has been assumed to be the ε-amino group of the essential lysyl residue. When this argument is carried over to the normal transamination reaction in which the distal carboxyl group of a dicarboxylic substrate binds to the imidazole group and participates in forming a charge relay system. This may provide a molecular mechanism of the substrate specificity of aspartate aminotransferase. Such a charge relay system mediated by a histidyl residue already has been proposed for the mechanism of action of chymotrypsin where an aspartyl residue provided by the enzyme protein is one of the components of the substrate (22). In contrast to this, a part of the substrate molecule is a participant in the relay system proposed here for aspartate aminotransferase.

Between the two isoenzymes some differences were observed in the Michaelis constants for substrates (Table II). In the inactivation reaction acetate and propionate were much less effective on the mitochondrial enzyme than on the supernatant enzyme (Fig. 3). With the supernatant isoenzyme, similar degree of stimulatory effect was observed on both the αβ elimination reaction and the inactivation. In contrast, the rate of αβ elimination reaction catalyzed by the mitochondrial isoenzyme was little affected at a range of formate concentration from 1 to 3 M where the inactivation was markedly stimulated (Fig. 4). All these observations seem to indicate that there is a subtle difference in the structure of the active site between these two isoenzymes.

The 365-nm species produced by KBR treatment of the 455-nm species cannot be of a simple aldamine structure since the latter should absorb normally at a shorter wavelength (325 to 330 nm). Thus the chemical nature of either the 455- or 365-nm species remains uncertain at present. However, it has been clearly demonstrated that the bond linking the label to the enzyme protein is rendered more labile during the slow process giving rise to the 455-nm species and KBR, should be added immediately after the inactivation of the enzyme to fix the label to the enzyme via a stable covalent bond. Based on this knowledge, a peptide was successfully isolated from the labeled site as a reaction product extending to the ε-amino group of the lysyl residue which has been assumed to act as a base to abstract the α-hydrogen atom of the substrate (L-alanine or β-chloro-L-alanine). Establishment of this relay system would result in the enhancement of the reactivity of the base. This may be extended to the normal transamination reaction in which the distal carboxyl group of a dicarboxylic substrate binds to the imidazole group and participates in forming a charge relay system. This may provide a molecular mechanism of the substrate specificity of aspartate aminotransferase. Such a charge relay system mediated by a histidyl residue already has been proposed for the mechanism of action of chymotrypsin where an aspartyl residue provided by the enzyme protein is one of the components of the substrate (22).

A possible mechanism of action of formate ion can be visualized as in Fig. 12. A common intermediary step in these reactions is the withdrawal of the α-hydrogen atom of a bound substrate (16, 17). Therefore it is highly plausible that formate acts on this common step in the enzymatic catalysis, i.e. formate induces reactivity of a proton-abstracting base which has been assumed to be the ε-amino group of the essential lysyl residue. When this argument is carried over to the normal transamination reaction, it is highly probable that the distal carboxylate ion not only assists in binding the substrate to the enzyme but also participates in the actual catalytic process by enhancing the reactivity of a catalytic residue within the active site.

A possible mechanism of action of formate ion can be visualized as in Fig. 12. The presence of a histidyl residue in or near the active site of the supernatant aspartate aminotransferase had been suggested by Martinez-Carrion and his coworkers (21). On the assumption that this histidyl residue is a site binding the distal carboxyl group of a dicarboxylic substrate, formate can be depicted as binding, for instance, to the γ-nitrogen atom of imidazole ring of the histidyl residue. This produces a charge relay system extending to the ε-amino group of the lysyl residue which has been assumed to act as a base to abstract the α-hydrogen atom of the substrate (L-alanine or β-chloro-L-alanine). Establishment of this relay system would result in the enhancement of the reactivity of the base. This argument may be extended to the normal transamination reaction in which the distal carboxyl group of a dicarboxylic substrate binds to the imidazole group and participates in forming a charge relay system. This may provide a molecular mechanism of the substrate specificity of aspartate aminotransferase. Such a charge relay system mediated by a histidyl residue already has been proposed for the mechanism of action of chymotrypsin where an aspartyl residue provided by the enzyme protein is one of the components of the substrate (22). In contrast to this, a part of the substrate molecule is a participant in the relay system proposed here for aspartate aminotransferase.

Between the two isoenzymes some differences were observed in the Michaelis constants for substrates (Table II). In the inactivation reaction acetate and propionate were much less effective on the mitochondrial enzyme than on the supernatant enzyme. In contrast, the rate of αβ elimination reaction catalyzed by the mitochondrial isoenzyme was little affected at a range of formate concentration from 1 to 3 M where the inactivation was markedly stimulated (Fig. 4). All these observations seem to indicate that there is a subtle difference in the structure of the active site between these two isoenzymes.

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REFERENCES


Fig. 11. Schematic representation of the reaction of aspartate aminotransferase with β-chloro-L-alanine. I, pyridoxal form; II, transaldimination; III, deprotonation; IV, inactivated enzyme; V, enzyme-α-aminoacrylate complex.

Fig. 12. A possible mechanism of action of formate anion.
Formate-induced Labeling of the Active Site of Aspartate Aminotransferase by \( \beta \)-Chloro-L-alanine

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