Affinity Labeling of Alanine Aminotransferase by 3-Chloro-L-alanine*

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The pyridoxal form of alanine aminotransferase from pig heart catalyzes the $\alpha\beta$ elimination reaction with 3-chloro-L-alanine as the substrate to form equimolar amounts of pyruvate, ammonia, and chloride. The maximum rate of the $\alpha\beta$ elimination reaction was 2.5 $\mu$mol/min/mg at pH 7.0 (25°C), approximately 0.5% that of the transamination reaction between L-alanine and 2-oxoglutarate. Time-dependent decrease in the rate of $\alpha\beta$ elimination was found to result from an irreversible inactivation of the enzyme. Michaelis constants for 3-chloro-L-alanine were identical in both the $\alpha\beta$ elimination and transamination reactions, indicating that the inactivation occurs via a key intermediate in the $\alpha\beta$ elimination reaction. Furthermore, the whole carbon moiety of 3-chloro-L-[U-14C]alanine was found to be covalently incorporated into the enzyme protein in a stoichiometric fashion. These observations clearly indicate that chloroalanine acts as an affinity label for alanine aminotransferase as was the case with aspartate aminotransferases (Morino, Y., Osman, A. M., and Okamoto, M. (1974) J. Biol. Chem. 249, 6684-6692). The 427 nm band of the native enzyme disappeared instantaneously upon the addition of chloroalanine to form a spectral species absorbing at 325 nm. The pseudo-first order rate constant for this fast process was 53 s$^{-1}$, which was much greater than that ($\sim$5 s$^{-1}$) for the overall $\alpha\beta$ elimination reaction. This fast spectral change was followed by the slow appearance of a new absorption band at 435 nm, which was accompanied by a negative circular dichroic band at 415 nm. The rate of formation of this spectral species (0.36 min$^{-1}$ at 25°C) coincided exactly with the rate of inactivation of the enzyme. When extrapolated to the infinite concentration of chloroalanine, the rates of inactivation as well as the $\alpha\beta$ elimination reaction was almost pH-independent. In contrast, the Michaelis constant for chloroalanine varied markedly with the change of pH. The analysis of this pH-dependent variation of Michaelis constants revealed the presence of two ionizable groups on the enzyme with $pK_a$ 6.3 and 7.9. Based on these kinetic and spectral data, a possible mechanism of the reaction of alanine aminotransferase with 3-chloro-L-alanine was discussed.

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Materials—3-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 (21). 3-Chloro-L-[U-14C]alanine was synthesized from L-[U-14C]serine (120 mCi/mmol, Daiichi Pure Chemicals Co., Ltd., Tokyo) as described previously (14). Its specific activity was 9.3 $\times$ 10$^7$ dpm/μmol. Alanine aminotransferase was isolated from pig heart by a modification of the procedure described by Saier and Jenkins (1). Details will appear elsewhere. The pure preparations employed in the present investigation showed an absorbance ratio ($A_{235}$ nm/$A_{435}$ nm) of 4.05 in 50 mM potassium acetate buffer (pH 5.5) containing 0.2 mM dithiothreitol and 2 mM EDTA. The specific activity range from 470 to 500 μmol/min/mg at 25°C. The enzyme concentrations were calculated using a value of 6.4. The molecular weight of the monomeric unit of this enzyme was assumed to be 58,000 (1, 2). Crystalline lactate dehydrogenase was obtained from pig heart as described (22). Tryptsin (type IX, hog pancreas) was obtained from Sigma.

Methods—The reaction mixture for transamination contained, in a total volume of 1.0 ml, 80 mM L-alanine, 4 mM 2-oxoglutarate, and 20 mM Tris/HCl buffer (pH 8.0). The reaction was initiated by adding alanine aminotransferase and performed at 25°C. The absorbance change at 340 nm was recorded in a Hitachi recording spectrophotometer model 200-10.

The reaction mixture for $\alpha\beta$ elimination reaction contained, in a total volume of 1.0 ml, varied concentrations of 3-chloro-L-alanine.

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†The subunit structure of this enzyme had not been determined. However, based on its molecular weight and the coenzyme content, it is assumed here that this enzyme is of a dimeric structure. Its precise nature is currently under investigation.
Adding alanine aminotransferase and the decrease in absorbance at 340 nm was recorded in a Hitachi model 200-10 recording spectrophotometer equipped with a digital print-out system. As described under “Results,” the rate of pyruvate formation slowed down with the progress of $\alpha,\beta$ elimination reaction with 3-chloro-L-alanine, which was ascribed to the inactivation of the enzyme. To obtain the apparent first order rate constant for the inactivation, the logarithm of average slopes read at every 5 to 10 s from the progress curve for the $\alpha,\beta$ elimination reaction were plotted against time. The pseudo-first order rate constant for the inactivation, $k$, was obtained from the plot using the equation, $\ln(1 - T/100) = k t$ (where $t$ (in minutes) denotes the time required for inactivating one-half the enzyme initially present.

Absorption spectra were obtained on a high sensitivity spectrophotometer model SM-401 (Union Giken, Osaka). Circular dichroic spectra were taken in a Jasco recording spectropolarimeter J-40AS (Japan Spectroscopic Co. Ltd.). Spectral measurements of a fast reaction were performed in a Hitachi RS-II rapid scan spectrophotometer.

Determination of pyridoxal phosphate was performed by the procedure described by Wada and Snell (23). Radioactivity was measured in a Tri-Carb liquid scintillation spectrometer model 3320 with Bray's solution (24) as scintillant.

One unit of the enzyme activity was defined as the amount of the enzyme which produces 1 pmol of pyruvate/min at 25°C in either the transamination or $\alpha,\beta$ elimination reaction under the assay conditions described above. The specific activity was expressed as units/mg of protein.

**RESULTS**

Alanine Aminotransferase-catalyzed $\alpha,\beta$ Elimination Reaction with 3-Chloro-L-alanine—When the pyridoxal form of alanine aminotransferase was incubated with 3-chloro-L-alanine, there were observed equimolar formations of pyruvate, ammonia, and chloride (Table I). It is thus evident that alanine aminotransferase catalyzes the $\alpha,\beta$ elimination reaction as previously reported for aspartate aminotransferase isoenzymes (14).

Fig. 1 shows the time course of the alanine aminotransferase-catalyzed formation of pyruvate from 3-chloro-L-alanine in a coupled system containing NADH and lactate dehydrogenase. The progress curve showed a marked decrease in the rate of pyruvate formation with time. This time-dependent decrease in the rate of pyruvate formation was assumed to result from the inactivation of the enzyme as was the case with the aspartate aminotransferase-catalyzed reaction (14). This assumption was verified by measuring the normal alanine aminotransferase activity on aliquots with drawn at various time intervals from the same reaction mixture (data not shown). One can obtain a pseudo-first order rate constant for the inactivation by plotting the logarithm of the magnitude of the slope of the progress curve (Fig. 1A) against time since the slope of the progress curve at a particular time represents the amount of the remaining active enzyme. Such a plot was shown in Fig. 1B, which yielded the pseudo-first order rate constant for inactivation of 0.36 min$^{-1}$.

That the inactivation represents an irreversible process was also indicated by the following experiment. A preparation of alanine aminotransferase (approximately 1 mg) was incubated with 20 pmol of 3-chloro-L-alanine in 1.0 ml of 20 mM potassium phosphate buffer (pH 7.0) containing 0.2 mM dithiothreitol. The resulting enzyme solution was dialyzed against the same buffer at 5°C. The resulting enzyme solution was examined for the enzyme activity in the absence or presence of 0.1 mM pyridoxal 5'-phosphate. No trace of transamination activity was detectable.

The Michaelis constant for 3-chloro-L-alanine in the $\alpha,\beta$ elimination reaction was 0.15 mM at pH 7.0 (in 20 mM potassium phosphate buffer). The corresponding constant in the inactivation reaction was also 0.15 mM as calculated from the double reciprocal plot of the rate constant for inactivation versus the substrate concentration (Fig. 2). Identity of the values for these two constants is consistent with a mechanism of inactivation in which a common intermediate is assumed for both the $\alpha,\beta$ elimination and the inactivation.

**Effect of pH on Some Kinetic Parameters in the $\alpha,\beta$ Elimination and Inactivation Reactions**—The maximum initial velocity of $\alpha,\beta$ elimination reaction increased slightly with the increase in pH while the maximum rate constant for inactivation remained unaltered over the pH range tested (Fig. 3). Comparison of the rate of $\alpha,\beta$ elimination reaction with the rate of inactivation yields the turnover number of $\alpha,\beta$ elimination reaction required for inactivating 1 molecule of the enzyme. Thus 1 molecule of the enzyme was inactivated during approximately 500 turnovers of $\alpha,\beta$ elimination reaction at pH 5.0 as compared with 1050 turnovers at pH 9.0, indicating that the inactivation occurs more efficiently at lower pH.

The Michaelis constant for 3-chloro-L-alanine in the $\alpha,\beta$ elimination reaction as well as the corresponding constant in the inactivation reaction showed a marked change with pH (Table II). Furthermore, these constants, $K_m$ and $K_{react}$, reflecting the affinity for the substrate in the $\alpha,\beta$ elimination
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FIG. 2. Double reciprocal plots of the initial rate of α,β elimination (○—○) or the rate constant for inactivation (■—■) versus the concentration of 3-chloro-L-alanine. The reaction mixtures contained, in a total volume of 1.0 ml: varied concentrations of 3-chloro-L-alanine as indicated, 11.6 μg of alanine aminotransferase in pyridoxal form, 0.15 mM NADH, 10 μg of lactate dehydrogenase, 0.1 mM EDTA, 0.1 mM dithiothreitol, and 20 mM potassium phosphate buffer (pH 7.0). The reaction was started by adding the aminotransferase and carried out at 25°C. The initial rate of α,β elimination reaction was obtained from a value for AAd , extrapolated to the zero time in a pseudo-first order plot as shown in Fig. 1B. The rate constant for inactivation (k) was obtained using the equation \( k = 0.693/t_{1/2} \), where \( t_{1/2} \) is the time (in minutes) required for inactivating one-half the enzyme initially present. The half-time was obtained from a first order plot as shown in Fig. 1B.

FIG. 3. Effect of pH on the maximal rate (Vmax) of α,β elimination reaction and the maximal rate constant (kmax) for inactivation of alanine aminotransferase. The reaction mixtures were varied as described for the experiments in Fig. 2, except that the buffers (20 mM each) were varied as follows: sodium acetate, pH 5.0 and 5.5 (●—●); potassium phosphate, pH 6.0, 6.5, 7.0, and 7.5 (○—○); imidazole/HCl, pH 6.0, 6.5, and 7.0 (□—□); Tris/acetate, pH 7.5, 8.0, 8.5, and 9.0 (■—■). Vmax and kmax were calculated from double reciprocal plots obtained at each pH and buffer condition as described for Fig. 2.

and the concomitant inactivation, respectively, were exactly identical at any single pH and buffer, indicating that the inactivation occurs via an intermediate in the α,β elimination reaction at any pH studied. Although a considerable degree of the buffer ion effect was observed, the affinity for 3-chloro-L-alanine increased markedly with increasing pH to reach a maximum at a pH range from 7.0 to 7.5, and then, decreased with the further increase in pH. Both of log \( (V_{max}/K_v) \) and log \( (k_{max}/K_{max}) \) versus pH plots (Fig. 4) revealed two ionizable groups on the free enzyme with \( pK_v \) 6.3 and \( pK_{max} \) 7.9, which would be involved in the interaction with the substrate.

Spectral Observations on the Interaction of Alanine Aminotransferase with 3-Chloro-L-alanine—The native pyridoxal form of alanine aminotransferase showed an intense absorption band at 427 nm and a small band at 327 nm, which are attributed to the bound pyridoxal 5’-phosphate. The yellow color of the enzyme solution bleached instantaneously upon the addition of 3-chloro-L-alanine. This apparently fast reaction was followed by use of the stopped flow technique.

TABLE II Variation of Michaelis constants for 3-chloro-L-alanine with pH

<table>
<thead>
<tr>
<th>Buffers</th>
<th>pH</th>
<th>( K_m )</th>
<th>( K_{max} )</th>
</tr>
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<tbody>
<tr>
<td>Sodium acetate</td>
<td>5.0</td>
<td>2.8</td>
<td>2.8</td>
</tr>
<tr>
<td>Sodium acetate</td>
<td>5.5</td>
<td>0.83</td>
<td>0.83</td>
</tr>
<tr>
<td>Potassium phosphate</td>
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<td>0.85</td>
<td>0.84</td>
</tr>
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<td>Potassium phosphate</td>
<td>6.0</td>
<td>0.45</td>
<td>0.45</td>
</tr>
<tr>
<td>Potassium phosphate</td>
<td>6.5</td>
<td>0.23</td>
<td>0.22</td>
</tr>
<tr>
<td>Potassium phosphate</td>
<td>7.0</td>
<td>0.15</td>
<td>0.15</td>
</tr>
<tr>
<td>Imidazole/HCl</td>
<td>7.0</td>
<td>0.13</td>
<td>0.12</td>
</tr>
<tr>
<td>Tris/HCl</td>
<td>7.0</td>
<td>0.05</td>
<td>0.06</td>
</tr>
<tr>
<td>Tris/acetate</td>
<td>7.0</td>
<td>0.06</td>
<td>0.07</td>
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<tr>
<td>Potassium phosphate</td>
<td>7.5</td>
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<td>0.17</td>
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<td>0.05</td>
</tr>
<tr>
<td>Tris/acetate</td>
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<td>0.06</td>
<td>0.06</td>
</tr>
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<td>Tris/HCl</td>
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<td>0.09</td>
<td>0.10</td>
</tr>
<tr>
<td>Hepes*</td>
<td>8.0</td>
<td>0.08</td>
<td>0.09</td>
</tr>
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<td>Tris/acetate</td>
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<td>0.29</td>
<td>0.29</td>
</tr>
<tr>
<td>Tris/acetate</td>
<td>9.0</td>
<td>0.53</td>
<td>0.53</td>
</tr>
</tbody>
</table>

* Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

FIG. 4. Effect of pH on the affinity of alanine aminotransferase for 3-chloro-L-alanine. The kinetic parameters in the α,β elimination reaction (---) and in the inactivation reaction (-----) were obtained from the experiments described for Fig. 3. Symbols used in this figure correspond to those in Fig. 3. Vmax is expressed here as a first order rate constant in minutes⁻¹. A value of 116,000 is assumed for the molecular weight of the enzyme.
Fig. 5 shows the spectral change of the reaction mixture in the visible region. Within a period of 1 s, an apparently single spectral species lacking visible region bands but absorbing at 325 nm was observed (Figs. 5A and 6A). The time course of the decrease in absorbance at 427 nm was also shown in Fig. 5B. The semilogarithmic plot showed that the predominant faster process appeared to be preceded by a short slower phase (Fig. 5B). The pseudo-first-order rate constant for the predominant faster phase was calculated to be 53 s\(^{-1}\) at 25°C, which was much greater than the overall rate of \(\alpha\beta\) elimination reaction (\(-5\) s\(^{-1}\)) under the present condition. Although the formation of a spectral band around 500 nm ascribable to a quinoid intermediate was reported for some pyridoxal enzymes (28, 29), no spectral band was detected at the 500 nm region during 20 ms to a few seconds after alanine aminotransferase was mixed with 3-chloro-L-alanine.

The enzyme solution which had become colorless upon mixing with 3-chloro-l-alanine was found to become yellow again over a period of a few minutes. Finally it showed a discrete band at 435 nm (Fig. 6A), which was obviously distinct from the band exhibited by the native enzyme at 427 nm. The absorbance at 435 nm increased with time (Fig. 6B) and reached a maximum value of 0.12 at 16 to 20 min after the addition of 3-chloro-l-alanine under the condition described for Fig. 5. Hence, the absorbance of the completely inactivated enzyme, \(A_x\), is assumed to be 0.12. Then, the value, \(\Delta A = A_x - A_t\), where \(A_t\) denotes the absorbance value at 435 nm at time \(t\) should represent a measure of the enzyme species which is still active. Thus, \(\log \left[\frac{A_x - A_t}{A_x}\right]\) was plotted against time. As can be seen in Fig. 6C, such a plot yielded a straight line. The pseudo-first-order rate constant for this spectral change was found to be 0.36 min\(^{-1}\), which coincided with the value (0.36 min\(^{-1}\)) previously described (Fig. 1) for the rate constant for inactivation. This finding clearly indicates that the species absorbing at 435 nm represents a direct product formed upon the inactivation of the enzyme.

The reaction of the enzyme with 3-chloro-l-alanine was also followed by measuring the circular dichroic spectra of the enzyme. The absorption band of the native enzyme at 427 nm was accompanied by an intense positive circular dichroic band at 430 nm (Fig. 7A). Upon the addition of 3-chloro-l-alanine, circular dichroism at this wavelength disappeared instantaneously and then started to change gradually in the negative direction (Fig. 7B) to form the species exhibiting an intense negative circular dichroism band centering at 415 nm (Fig. 7A). This time-dependent change in the circular dichroism at 415 nm was analyzed as described above for the change in absorbance at 435 nm exhibited upon the enzyme inactivation.
The result (Fig. 1C) showed that the change in circular dichroism at 415 nm followed pseudo-first order kinetics. The rate constant for this change was found to be 0.35 min⁻¹, which coincided with that for the spectral change at 435 nm as described above and, hence, with the rate of the enzyme inactivation. Thus, the spectral species exhibiting an intense negative circular dichroic band at 415 nm represents the inactivated enzyme. The striking difference in circular dichroic spectra between the two similarly absorbing species, the native enzyme at 417 nm and the inactivated enzyme at 435 nm, indicates that a drastic alteration in the state of the bound pyridoxal 5'-phosphate was induced upon the inactivation.

Spectral Change Occurring after the Inactivation of the Enzyme — A slow spectral change was found to occur when the inactivated enzyme was kept for a prolonged period after the enzyme was fully inactivated. Fig. 8 illustrates this secondary slow change in absorption and circular dichroism spectra of the fully inactivated enzyme. Over a period of several hours at 25°C, the 435 nm band decreased gradually with concomitant increase in the band at 335 nm. This spectral change appeared to follow pseudo-first order kinetics (data not shown) and the rate constant was approximately 0.4 h⁻¹ at 25°C. After 10 h at 25°C, the inactivated enzyme showed a predominant band at 335 nm with a small shoulder around 400 nm. The enzyme activity was not observed with this preparation in the absence or presence of added pyridoxal 5'-phosphate, indicating that this spectral species represents another form of the inactivated enzyme. To study a possible alteration in the chemical structure of the bound coenzyme, the content of pyridoxal 5'-phosphate was measured on the preparations which were kept for various periods after the inactivation. As can be seen from Table III, the bound pyridoxal 5'-phosphate was found to decrease with time and the decrease was closely correlated to that in absorbance at 435 nm of the preparations. This finding seems to indicate that the enzyme-bound pyridoxal 5'-phosphate has undergone presumably a slow conversion to a pyridoxamine (or ketimine) form via an intramolecular transamination reaction with the covalently incorporated label derived from 3-chloro-L-alanine (see Fig. 9).

Covalent Incorporation of Three-carbon Moiety of 3-Chloro-L-alanine into Enzyme — That the three-carbon moiety derived from 3-chloro-L-alanine was covalently bound to the active site during the inactivation was demonstrated by the following experiment. The pyridoxal form of alanine aminotransferase (1.73 mg, 30 nmol with respect to the bound coenzyme) was incubated with 20 pmol of 3-chloro-L-[U-14C]alanine in 1.0 ml of 50 mM Tris/HCl buffer (pH 7.5) at 25°C. After 10 min, when the preparation showed a residual activity of 3%, 0.2 mg of NaBH₄ was added with a small drop of 1-octanol. The resulting solution was then dialyzed against 1 liter of 20 mM Tris/HCl buffer (pH 7.5), containing 0.5 mM dithiothreitol, for 2 days with two changes of the outer solution. Radioactivity measurement on an aliquot revealed that 0.92 mol of the label derived from the radioactive chloroalanine was bound per 58,000 g of the enzyme protein. Subsequently, 1 g of guanidine HCl was added/ml of the dialyzed solution and the mixture was kept at 37°C for 1 h to ensure the unfolding of the protein. Then, the solution was dialyzed against distilled water for 30 h with two changes of the outer solution. The precipitated protein in the dialysis bag was collected by a brief centrifugation, suspended in 1.0 ml of 50

![Fig. 8. Slow spectral change occurring after the inactivation of the enzyme.](http://www.jbc.org/)

Table III

<table>
<thead>
<tr>
<th>Preparations</th>
<th>Time after reaction with chloroalanine (min)</th>
<th>Absorbance at 435 nm</th>
<th>Amount of bound pyridoxal phosphate (nmol/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native enzyme</td>
<td>0</td>
<td>0.180 (100)</td>
<td>21 (100)</td>
</tr>
<tr>
<td>Inactivated</td>
<td>20</td>
<td>0.137 (76)</td>
<td>16 (76)</td>
</tr>
<tr>
<td></td>
<td>70</td>
<td>0.105 (58)</td>
<td>11 (55)</td>
</tr>
<tr>
<td></td>
<td>120</td>
<td>0.065 (36)</td>
<td>8.2 (39)</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>0.034 (19)</td>
<td>4.2 (9)</td>
</tr>
<tr>
<td></td>
<td>300</td>
<td>0.020 (11)</td>
<td>1.8 (9)</td>
</tr>
<tr>
<td></td>
<td>420</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Corresponds to the data in Fig. 8. Values in parentheses are percentages.

Values in parentheses are percentages.
FIG. 9. Schematic representation of the reaction of alanine aminotransferase with 3-chloro-L-alanine. Pyridoxal 5'-phosphate is assumed to bind to the active site via an imine linkage with the ε-amino group of a lysyl residue. The direct product formed upon the affinity labeling of the enzyme is depicted as the complex VI, which may be in equilibrium with the protonated complex VII. Since the 435 nm species is predominant upon the inactivation of the enzyme, the equilibrium is assumed to be in favor of the formation of VII. The slow secondary conversion of the inactivated enzyme (see the data in Fig. 8 and Table III) is tentatively shown to proceed from VI via a hypothetical carbanion intermediate in the bracket to form a ketimine complex VIII or pyridoxamine form IX. X−, an amino acid residue within the enzyme active site.

mM NH₄HCO₃, and solubilized by digestion with 0.1 mg of trypsin for 3 h at 30°C. The resulting solution was analyzed for radioactivity on an aliquot and for the absorbance at 320 nm on another aliquot. The result indicated that 0.86 mol of the label was bound per mol of the bound pyridoxyl derivative in the trypsin digest. Incubation of a NaBH₄-reduced holoenzyme with the radioactive chloroalanine under the condition similar to that described above did not result in the covalent incorporation of any trace of radioactivity.

**DISCUSSION**

The present result demonstrates that the pyridoxal form of alanine aminotransferase from pig heart catalyzes the αβ elimination reaction with 3-chloro-L-alanine with concomitant inactivation of the enzyme. The inactivation was accompanied by a covalent incorporation of the three-carbon moiety derived from 3-chloro-L-alanine into the active site of the enzyme. Together with some kinetic observations, these findings indicate that 3-chloro-L-alanine acts as a potent affinity label (it may also be called a suicide substrate) for alanine aminotransferase as in the case of aspartate aminotransferase (13-15). Previously there was a brief description that rat liver alanine aminotransferase was efficiently inactivated by 3-chloro-D,L-alanine or 3-fluoro-D,L-alanine (31).

Considerable differences in various aspects of the reaction with 3-chloro-L-alanine were observed between alanine aminotransferase and aspartate aminotransferases. First, Vₘₐₓ/Kₘ, an index reflecting both the kinetic and binding specificities for 3-chloro-L-alanine in the αβ elimination reaction, was 1.1 and 7 mM⁻¹ min⁻¹ (14) for the cytosolic and mitochondrial aspartate aminotransferases, respectively. In contrast, this value for the alanine aminotransferase-catalyzed reaction was approximately 5,000 mM⁻¹ min⁻¹ at pH 7.0, indicating that chloroalanine is much better as a substrate for alanine aminotransferase than for aspartate aminotransferase. Second, although the maximal velocity for the αβ elimination reaction catalyzed by both aminotransferases was of a similar magnitude (2 to 4 μmol/min/mg at 25°C) in the absence of formate ion and is approximately 0.5 to 1% the rate of the transamination with the normal substrates, the effect of formate ion was quite different between these two aminotransferases. Namely, it was previously described that with aspartate aminotransferase the presence of formate ion resulted in a striking enhancement of the rates of both the αβ elimination and inactivation reactions (12, 14). In contrast, when the alanine aminotransferase-catalyzed reaction with 3-chloro-L-alanine was examined in sodium cacodylate buffer (pH 6.8), the presence of 0.2 mM potassium formate enhanced the αβ elimination only 1.4-fold and decreased slightly the rate of inactivation (data not shown). A similar stimulatory effect of formate ion was reported previously by Saier and Jenkins (3) on alanine aminotransferase-catalyzed reaction between the natural substrates. Third, spectral changes observed during the reaction with chloroalanine were considerably different between these two aminotransferases. A notable difference was that the inactivated alanine aminotransferase exhibited an absorption band at 435 nm, whereas the inactivated aspar-
tate alanine aminotransferase showed a predominant peak at 390 nm (14). These differences must reflect the difference in the structure of the active site between both aminotransferases, each of which shows a strict substrate specificity.

Spectral observations on the interaction of alanine aminotransferase with chloroalanine lead to some mechanistic consideration about the catalytic event occurring at the active site of the enzyme. The adsorption of chloroalanine to the enzyme active site may occur via its α-carboxyl group. The combination of the carboxyl group with a specific positively charged group in the active site would assist the deprotonation of the α-amino group of the substrate (32). This would facilitate the nucleophilic attack of the amino group to the coenzyme aldimine carbon atom to form the complex II (Fig. 9). Then, transamidination ensues to form the complex III. A rapid disappearance (k = 53 s⁻¹) of the 427 nm band of the native enzyme observed upon mixing with chloroalanine (Fig. 5) might be attributed to the formation of this complex III. The next step prerequisite to the α,β elimination reaction is the removal of the α-hydrogen atom of the bound substrate (33, 34) to form a quinoid complex IV. In the α,β elimination reaction catalyzed by Escherichia coli tryptophanase, a species (λmax ~ 500 nm) representing this deprotonated intermediate was demonstrated to accumulate during the reaction and this was attributed to the fact that the rate-determining step was in the release of the β substituent from the complex IV but not in the deprotonation step (29). In contrast, no spectral evidence for the formation and accumulation of such intermediate was obtained in the present investigation on alanine aminotransferase-catalyzed α,β elimination reaction with chloroalanine. Therefore, the deprotonation but not the elimination of the β substituent appears to be a rate-determining step in the alanine aminotransferase-catalyzed α,β elimination reaction. Removal of the β substituent from this deprotonated complex IV results in the formation of an α-aminoacyl-enzyme complex V, which then decomposes into free enzyme and α-aminoacylate. The latter hydrolyzes into free enzyme and a-aminoacrylate. The identification of a residue modified by 3-chloro-L-alanine, or by the analysis of the chemical process for the active site labeling with other affinity labels.

REFERENCES
Affinity labeling of alanine aminotransferase by 3-chloro-L-alanine.
Y Morino, H Kojima and S Tanase


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