Functional and Stereochemical Specificity at the $\beta$ Carbon Atom of Substrates in Threonine Dehydratase-catalyzed $\alpha,\beta$ Elimination Reactions*

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1-Threonine dehydratase catalyzes the nonoxidative conversion of $\beta$-substituted amino acids to $\alpha$-keto acids. In this manuscript the nature of the nucleophiles which are eliminated is established, new substrates are reported, and inhibition by $\beta$-substituted alanines is investigated. It is now shown that threonine dehydratase catalyzes the $\alpha,\beta$ elimination of $-\text{OH}$, $-\text{Cl}$, and $-\text{F}$ as $\text{H}_2\text{O}$, and $\text{HX}$ from a large number of $\beta$-substituted $\alpha$-l-amino acids. Threonine dehydratase is found to be nonspecific for the erythro and threo pairs of threonine, 3-chloro-$\alpha$-aminobutyrate, 3-fluoro-$\alpha$-aminobutyrate, 3-hydroxyleucine, and 3-hydroxynorvaline. There is, however, a catalytic preference shown for the threo isomers by threonine dehydratase in all cases.

Stereochromy at the $\beta$ carbon atom in substrates for threonine dehydratase influences the stereochromical course of the reaction as well as the catalytic efficiency. Substitution of the pro-$R$ or pro-$S$ proton of serine by alky and aryl groups has been shown to affect the binding and catalytic ability of threonine dehydratase for a number of substrates. By increasing the size of the alkyl groups at the $\beta$ carbon atom of serine we can demonstrate a peak in binding efficiency for the methyl group, i.e. threonine.

Threonine dehydratase from sheep liver undergoes inactivation when catalyzing the $\alpha,\beta$ elimination reaction of $\beta$-substituted alanines. Previously it was observed that serine caused a time-dependent inactivation of threonine dehydratase, it is now established that 3-bromo-, 3-chloro-, and 3-fluoro-L-alanine will also catalyze the $\alpha,\beta$ tautomerization of vinylglycine and L-2-amino-4-methoxy-3-butenoate (8) as well as the stereospecific $\alpha$-proton exchange of L-alanine and L-cysteine (5).

While previous studies have been directed toward an analysis of the stereochromy of the elimination reaction (5), current studies reported in this communication deal with specificity at the $\beta$ carbon atom. Functional specificity as well as conformational preferences on the effectiveness of the catalysis has been determined for a number of substrates for the enzyme. New substrates for the dehydratase reported herewith include: erythro- and threo-3-chloro-2-aminobutyrate, 3-fluoro-$\alpha$-aminobutyrate, 3-hydroxyleucine, and 3-hydroxynorvaline.

While dehydration of 4-carbon atom containing substrates proceeds with normal kinetics, inactivation of threonine dehydratase results from its action on $\beta$-substituted alanines. This inactivation results in an enzyme which will no longer catalyze the dehydration of either threonine or serine. Several explanations of the nature of this phenomenon have been advanced. Evidence was presented that the enzyme becomes resolved of its cofactor (6), or that oxazolidine ring conjugate between serine and pyridoxal phosphate occurs to account for loss of dehydratase activity (9). Finally it was proposed that the aminoacrylate formed as a result of enzymatic dehydration of serine becomes bound to some nucleophilic group which is essential for catalysis (10).

Neither the kinetics of this activation nor the ability of other amino acids capable of generating aminoacrylate to inactivate threonine dehydratase has been previously reported. This communication presents evidence supporting the idea that alkylation of an essential group by aminoacrylate is responsible for pseudo-first order inactivation of threonine dehydratase when acting on $\beta$ substituted alanines as substrates.

EXPERIMENTAL PROCEDURES

Materials—Sheep liver serine threonine dehydratase was prepared by the method of Kapko and Davis (1). A sample of L-allothreonine was obtained several years ago from Calbiochem. 3-Hydroxyleucine was purchased from United States Biochemical Corp. L-Threonine, L-cysteine, L-3-chloroalanine, and DL-$\beta$-hydroxynorvaline were obtained from Sigma. 3-Fluoro-L-alanine, 3-fluoro-D-alanine, and erythro- and threo-3-fluoro-$\alpha$-aminobutyrates were given to us by Merck.

Synthesis—dl-Vinylglycine was synthesized by the method of Rando1 from vinylglycolate (11). Cyanoglycine was synthesized according to the method of Ressler et al. (12).

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1 Personal communication helped in this synthesis.
The two diastereomers of 2-amino-3-chlorobutrate were prepared from L-threonine and D,L-allatohomine as reported by Walsh et al. (19). The methyl esters were employed for each synthesis in the chlorination step. The identity and purity of the compounds were established by paper chromatography (14). Each was free from the other diastereomer.

3-Hydroxyameline was purchased from United States Biochemical Corp. and separated into its diastereomers on preparative thick plates with a solvent system of 50% butanol, 6.25% acetone, 6.25% NH4OH, and 37% H2O.

Erythro- and threo-phenylserine were prepared and separated by the method of Shaw and Fox (14). Each was shown to be free from the other diastereomer by paper and thin layer chromatography.

DL-3-Bromoalanine was prepared from DL-3-chloroalanine and anhydrous HBr and acetic acid by the method reported by Dang et al. (15).

Methods—The assays have previously been described (16). One dehydratase unit is the amount of enzyme that will produce 1 μmol of keto acid from L-threonine/min at 37°C and at pH 7.2. K m and k cat values were determined with a least squares computer program. 4 Inactivated threonine dehydratase was prepared from 3.3 ml of active enzyme containing 30 units/ml which had been incubated with 1.1 ml of 0.1 M phosphate buffer, pH 7.2, and 0.1 M buffer L-serine for at least 30 min at 37°C. This treatment results in a 95% loss of original activity, and the excess serine is separated from the enzyme by adding the reaction mixture to a column (2.5 x 60 cm) of Sephadex G-25 (coarse) and eluting with 0.1 M phosphate buffer, pH 7.2. Completeness of separation was determined by testing the fractions containing protein with ninhydrin.

RESULTS

The Ionic Form of the Amino Acids Bound to the L-Threonine Dehydratase—In order to determine the ionic form of the amino acids bound by the dehydratase, K m values were determined for three cases as a function of pH. K m values were determined as a function of pH for L-threonine, L-serine, and 3-chloro-L-alanine. Over a pH range of 6.5 to 9 each K m versus pH graph showed an inflection around a pH corresponding to the basic pK a of each of the three amino acids. The slope changed from 1 to 0 for each amino acid tested, thus establishing preference for the anionic form of these amino acids by the dehydratase. Therefore, all of the studies reported in this correspondence were conducted at or above pH 9 where all the substrates tested were predominantly in their anionic form.

Preparation of L-Threonine Dehydratase with Unsaturated Amino Acids—With terminal dehydratase DL-vinylglycline is a good substrate. Fifty percent of a DL mixture of vinylglycline was selectively converted to α-ketoacids and an isomer was recovered. The reaction has a K m of 3.1 mM and a k cat of 1 s⁻¹ at pH 9. The enzyme showed no activity toward allylglycine and propargylglycine. The enzyme reacts with vinylglycine without any detectable loss of activity during a 24-h incubation.

Reaction of L-Threonine Dehydratase with Cyanoglycine—In experiments to compliment our vinylglycine results the dehydratase was treated with cyanoglycine. Cyanoglycine was observed not to function as a substrate or a k cat inhibitor for the dehydratase. Cyanoglycine was observed, however, to be a competitive inhibitor with a K I of 2.6 mM at pH 9.

L-Threonine Dehydratase Catalyzed Proton Exchange Reaction with Vinylglycine—Proton NMR was used to determine whether the α-proton of vinylglycine would exchange with solvent in the presence of l-threonine dehydratase. The proton (H α) of vinylglycine was used as a reporter for determining proton exchange at the α-position. The H α proton has a complex splitting pattern as a result of being split by three different protons with different coupling constants. An octet is observed as predicted at 6 ppm downfield with corresponding coupling constants.

Upon addition of dehydratase, which had been suspended in and lyophilized three times from 100% D2O, and incubated at ambient temperature with vinylglycine, four additional peaks appeared in the splitting pattern of the H α proton. This is exactly what is predicted when a deuterium replaces the H α proton of vinylglycine. Also, since the vinylglycine is a racemic mixture and only one isomer is active, one observes only a reduction in the peak height but not a complete disappearance. One would expect to see over a long period a 50% reduction.

TABLE I

<table>
<thead>
<tr>
<th>Substrates</th>
<th>K m</th>
<th>k cat</th>
<th>k cat/K m</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-Serine</td>
<td>1.8</td>
<td>65</td>
<td>36</td>
</tr>
<tr>
<td>L-Threonine</td>
<td>0.27</td>
<td>45</td>
<td>167</td>
</tr>
<tr>
<td>L-Allothreonine</td>
<td>0.30</td>
<td>1.5</td>
<td>5</td>
</tr>
<tr>
<td>L-β-Hydroxyamylene (threo)</td>
<td>0.27</td>
<td>0.75</td>
<td>2.8</td>
</tr>
<tr>
<td>L-β-Hydroxyamylene (erythro)</td>
<td>4.16</td>
<td>0.75</td>
<td>0.18</td>
</tr>
<tr>
<td>L-β-Phenylserine (threo)</td>
<td>100</td>
<td>35</td>
<td>0.35</td>
</tr>
<tr>
<td>L-β-Phenylserine (erythro)</td>
<td>0.18</td>
<td>1.5</td>
<td>8.3</td>
</tr>
<tr>
<td>L-β-Hydroxynorvaline</td>
<td>2.0</td>
<td>3.4</td>
<td>1.7</td>
</tr>
<tr>
<td>L-β-Fluoro-α-aminoacetate (threo)</td>
<td>0.4</td>
<td>0.1</td>
<td>0.25</td>
</tr>
<tr>
<td>L-β-Chloro-α-aminoacetate (threo)</td>
<td>0.77</td>
<td>7</td>
<td>9</td>
</tr>
<tr>
<td>L-β-Chloro-α-aminoacetate (erythro)</td>
<td>16.6</td>
<td>6</td>
<td>0.36</td>
</tr>
<tr>
<td>L-β-Vinylglycine</td>
<td>3.12</td>
<td>1.0</td>
<td>0.32</td>
</tr>
</tbody>
</table>

a All measurements were made at pH 9 in Tris-HCl buffer.

b Racemic mixture.

c Below level of detection.

TABLE II

<table>
<thead>
<tr>
<th>Substrates</th>
<th>K m</th>
<th>k cat</th>
<th>k cat/K m</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-β-Fluoroalanine</td>
<td>0.08</td>
<td>2.0</td>
<td>25</td>
</tr>
<tr>
<td>L-β-Chloroalanine</td>
<td>0.22</td>
<td>60</td>
<td>270</td>
</tr>
<tr>
<td>D,L-β-Bromomaline</td>
<td>3.12</td>
<td>146</td>
<td>46.7</td>
</tr>
</tbody>
</table>

a All measurements were made at pH 9 in Tris-HCl buffer.

b Racemic mixture.
yserine. The dehydratase was active on all of the substrates tested as shown in Table I. However, amino acids with two alkyl groups in the β-position (3-hydroxyvaline) are not substrates for the dehydratase.

Relative Reaction of erythro and threo Forms of Substrates for l-Threonine Dehydratase—In order to determine the ability of l-threonine dehydratase to distinguish between erythro and threo forms of substrates, the $K_m$ and $k_{cat}$ values were determined for the following pairs of diastereomers: threonine-allothreonine, erythro-threo 3-chloro-2-aminobutyrate, erythro-threo-3-fluoro-2-aminobutyrate, and erythro-threo-3-hydroxyleucine. The results are presented in Table III.

The Effect of Various Groups at Position 3 of l-Alanine on Inhibition of the l-Threonine Dehydratase—In order to assess the ability of close structural analogs of 3-hydroxyamino acids to inhibit L-serine-threonine dehydratase, the compounds in Table IV were tested as inhibitors of the enzyme. Also shown in Table IV is the inhibition caused by the poor substrates, erythro and threo 3-fluoro 2-aminobutyrate on the dehydratase. All $K_i$ values were determined at pH 9 with L-threonine as the substrate.

β-Haloalanines and Serine as Substrates and Inhibitors for Threonine Dehydratase—Threonine is the normal substrate for threonine dehydratase. In order to determine its specificity about the β-position, its ability to catalyze α,β elimination reactions of β-substituted alanines was investigated. When threonine dehydratase catalyzes β elimination of 3-carbon atom substrates the E/S complex partitions between product formation and inactivation. This behavior is demonstrated for L-serine, L-fluoroalanine, L-chloroalanine, and DL-bromoalanine. The kinetic parameters have been determined for these substrates in Table I and II. When threonine dehydratase is incubated with L-serine or any of the three halo alanines an initial rapid rate of pyruvate production occurs. The rate slows with time and after approximately 10 min, approaches zero for serine and chloroalanine. The time dependency of the rate changes with pH for serine is shown in Fig. 1. At pH 6.5 to 7.0 the rate of pyruvate production is slow and appears to be linear for 20 to 30 min. At pH values greater than 7.5 curvature appears in the pyruvate versus time plot in less than 10 min for threonine dehydratase acting on serine. These results indicate a pH dependence in the inactivation of threonine dehydratase by serine. If the dehydrated product, aminoaacrylate, is the putative inhibitor then perhaps the nucleophile to which it adds does not react when protonated or is not in a favorable position to react at acidic pH values.

**Table III**

<table>
<thead>
<tr>
<th>Substrates</th>
<th>$K_m$ (10$^{-3}$ M)</th>
<th>$k_{cat}$ (s$^{-1}$)</th>
<th>$k_{cat}/K_m$</th>
<th>($k_{cat}/K_m$)$<em>{threo}/(k</em>{cat}/K_m)_{erythro}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-Threonine</td>
<td>0.3</td>
<td>45</td>
<td>167</td>
<td>33</td>
</tr>
<tr>
<td>L-Allothreonine</td>
<td>0.3</td>
<td>1.5</td>
<td>5</td>
<td>30</td>
</tr>
<tr>
<td>L-β-Fluoro-AMB* (threo)</td>
<td>0.2</td>
<td>1.5</td>
<td>7.5</td>
<td>25</td>
</tr>
<tr>
<td>L-β-Fluoro-AMB (erythro)</td>
<td>0.4</td>
<td>0.1</td>
<td>0.25</td>
<td></td>
</tr>
<tr>
<td>L-β-Chloro-AMB (threo)</td>
<td>0.8</td>
<td>7</td>
<td>8.75</td>
<td>25</td>
</tr>
<tr>
<td>L-β-Chloro-AMB (erythro)</td>
<td>17</td>
<td>6</td>
<td>0.35</td>
<td>13</td>
</tr>
<tr>
<td>L-β-Hydroxyleucine (threo)</td>
<td>0.3</td>
<td>0.8</td>
<td>2.66</td>
<td></td>
</tr>
<tr>
<td>L-β-Hydroxyleucine (erythro)</td>
<td>4</td>
<td>0.8</td>
<td>0.20</td>
<td></td>
</tr>
</tbody>
</table>

* α-aminobutyric acid.

**Table IV**

**Inhibitors of L-threonine dehydratase**

All measurements were made in Tris-HCl buffer at pH 9 with L-threonine as substrate.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>$K_i$ (M)</th>
<th>$1/K_i$</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-Alanine</td>
<td>0.03</td>
<td>33</td>
</tr>
<tr>
<td>L-Cysteine</td>
<td>0.005</td>
<td>200</td>
</tr>
<tr>
<td>L-Diaminopropionate</td>
<td>4.8</td>
<td>0.2</td>
</tr>
<tr>
<td>L-α-Aminobutyrate</td>
<td>0.024</td>
<td>42</td>
</tr>
<tr>
<td>L-β-Fluoro-α-aminoalbuturate (threo)</td>
<td>0.18</td>
<td>5.6</td>
</tr>
<tr>
<td>L-β-Fluoro-α-aminoalbuturate (erythro)</td>
<td>0.108</td>
<td>9.2</td>
</tr>
<tr>
<td>Cyanoglycine</td>
<td>0.26</td>
<td>3.7</td>
</tr>
<tr>
<td>Glycine</td>
<td>0.22</td>
<td>4.5</td>
</tr>
</tbody>
</table>

Fig. 1. The effect of pH on the $k_m$ inhibition of threonine dehydratase when acting on L-serine.

Similar results are obtained with 3-fluoroalanine, 3-chloroalanine, and 3-bromoalanine. Only the L isomers are substrates for the dehydratase. No pyruvate is formed nor is any inhibition observed in the presence of d-3-chloroalanine, d-serine, or d-3-fluoroalanine. For the following reasons the inhibition is not due to accumulated product: added pyruvate does not inhibit threonine dehydratase activity toward threonine, there is no lag before the onset of inhibition, and upon the addition of new enzyme to the reaction mixture the rate of loss of enzyme activity is identical with the first case.

Relationship between Inactivation and Turnover of Threonine Dehydratase when Acting on Serine—A sample of threonine dehydratase was incubated with serine at pH 8.9. At various times pyruvate formed was determined in a sample denatured by trichloroacetic acid. At the same time a second sample was transferred to a L-threonine containing reaction mixture at pH 7.2. Residual activity was determined from the excess keto acid formed in a 10-min period. Fig. 2 shows the relationship between pyruvate production and remaining enzyme activity. Both pyruvate production and dehydratase activity falls off in parallel as the enzyme is inactivated.

With both serine and L-3-chloroalanine greater than 99% inactivation occurred after approximately 10,000 catalytic cycles have occurred at pH 7.2. At pH 9 inactivation at the 95% level occurs at many fewer catalytic cycles and 5% of estimated initial rate is never lost. The residual activity may represent reversal of the inhibition or intrinsic activity associated with a population of modified (alkylated) dehydratase molecules.

Time Dependence of Serine Inactivation of Threonine Dehydratase—Incubation of crude or purified threonine dehydratase from sheep liver with serine results in a time-dependent loss of activity (Fig. 1). This loss has been shown to follow pseudo-first order kinetics. Fig. 3 shows a reciprocal plot of pseudo-first order rate constants, determined by the Guggenheim method (17), as a function of various concentra-
tions of serine. From an analysis of the reciprocal graph as described by Kitz and Wilson (18) it appears that the enzyme can be saturated by serine both with respect to turnover and inactivation. The kinetic behavior shown in Fig. 3 consistent with inactivation proceeding only from a preformed $E$-$S$ complex. From the double reciprocal plot an apparent dissociation constant $K_s$ of $2 \text{mM}$ and a limiting rate constant for inactivation of $k_i = 2.4 \text{min}^{-1}$ are determined.

$$E + S \overset{k_1}{\underset{k_2}{\rightleftharpoons}} E$-$S \overset{k_3}{\rightarrow} E$-$I$$

There is partitioning of the $E$-$S$ complex to inactivate enzyme $E$-$I$, which may show residual reduced activity, and active enzyme plus products.

**Reactivation of Serine-treated Threonine Dehydratase—**

Threonine dehydratase from sheep liver is the only enzyme undergoing $k_{cat}$ inhibition that can be reactivated. When threonine dehydratase is inactivated by serine at pH 7.2 and the serine removed by Sephadex G-25, reactivation can be effected. Reactivation of the altered enzyme can be achieved by incubating it at pH 7 in phosphate or at pH 8.9 in Tris/carbonate buffer. The reactivation proceeds much more rapidly at pH 8.9 than 7 as shown in Fig. 4. Greater than 90% of the original activity can be regained by incubating the altered enzyme at pH 8.9 in 3 h, only 25% reactivation occurs at pH 7.2 over the same time period.

Reactivation of threonine is accelerated at pH 8.9 by pyridoxal 5'-phosphate. A 3-fold enhancement in rate of reactivation is observed by adding 1 mM pyridoxal 5'-phosphate to the reaction mixture. This reactivation in the presence of pyridoxal 5'-phosphate was taken for evidence that pyridoxal 5'-phosphate was a cofactor for threonine dehydratase from sheep liver (6). However, it is now shown that pyridoxal 5'-phosphate exerts its effect on the rate of reactivation and not on the final degree of reactivation. The same degree of activation is observed in the absence as in the presence of pyridoxal 5'-phosphate. In fact no enhancement in the rate of reactivation by pyridoxal 5'-phosphate occurs when conducted at pH 7.2 in phosphate buffer. This report is in disagreement with literature reports on the pH dependence of pyridoxal 5'-phosphate enhancement of reactivation (9).

**The Effect of Nucleophiles on Threonine Dehydratase—**

The effect of nucleophiles on enzymes undergoing $k_{cat}$ inhibition has been subjected to various interpretations. To assess the effect of nucleophiles on the inhibition of threonine dehydratase the following experiments were conducted. If alkylation of some functional group in threonine accounts for the $k_{cat}$ inhibition when acting on $\beta$-substituted alanines, then direct reaction with good alkylating agents might derivatize this group. To test this theory, threonine dehydratase from sheep liver was treated with N-ethylmaleimide and iodoacetate, and at $10^{-4}$ M neither produced any inhibition when preincubated with the dehydratase. These results suggest that there are no essential -SH groups present in this enzyme, it would also suggest that there are no essential $\epsilon$-$\text{NH}_2$ groups either, since Brewer and Riehm (19) demonstrated that N-ethylmaleimide will slowly attack the $\epsilon$-$\text{NH}_2$ groups of lysyl residues when sulphhydryl is not present and high concentrations of N-ethylmaleimide are used.

When the sulfur nucleophile, 2-mercaptoethanol is added to a reaction mixture actively catalyzing $\beta$ elimination of serine, production of pyruvate does not stop. Rather, 2-mercaptoethanol allows for a greater number of turnovers of the dehydratase before inactivation occurs. 2-Mercaptoethanol

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**Fig. 2.** Relationship between the loss of enzyme activity and production of pyruvic acid by threonine dehydratase with L-serine.

**Fig. 3.** Time-dependent loss of threonine dehydratase activity upon incubation with L-serine.

**Fig. 4.** Reactivation of $k_{cat}$ inhibited threonine dehydratase as a function of time. The pH effect of the ability of pyridoxal 5'-phosphate to catalyze the reactivation is also illustrated at pH 7.2 and 8.9.
Fig. 5. A scheme for $\beta$ elimination, $k_{cat}$ inhibition, and reactivation of threonine dehydratase with the substrates fluoroalanine, bromoalanine, chloroalanine, and serine.

While not preventing eventual inactivation of threonine dehydratase when acting on serine, does delay this process. If protection by mercaptoethanol and the greater number of turnovers result from addition of 2-mercaptoethanol to the putative $\alpha$-aminoacrylate then $S-(\beta$-hydroxyethyl)cysteine should be formed. However, in experiments with L-$^{[13]}$C-serine no evidence could be obtained from formation of this product.3

DISCUSSION

From results presented in this manuscript and previous work, the stereochemistry requirements and mechanistic aspects of the action of sheep liver threonine dehydratase are becoming clearer. The Michaelis complex is generated from threonine dehydratase and amionic forms of its substrates (7). Schiff base formation is a prerequisite step to further reaction (20), and the sheep liver threonine dehydratase is specific for $\alpha$-amino acids as substrates and inhibitors (6, 7).

After Schiff base formation the enzyme has been shown to catalyze three reactions: $\alpha$-proton exchange (5), $\alpha$,$\beta$ eliminations (6, 7), and $\beta$,$\gamma$ tautomerizations (8). The $\alpha$ proton exchange has been examined with L-alanine and L-cysteine and shown to be stereospecific with retention of configuration (5). Myles observed $k_{cat}$ inhibition of both the $\beta\gamma$ subunit and the $\alpha\beta\gamma\delta$ complex of tryptophan synthase by cyanoglycine (21). Since these units catalyze $\alpha$,$\beta$ eliminations it was decided to see if threonine dehydratase was similarly affected by cyanoglycine. If threonine dehydratase could remove the $\alpha$ proton of cyanoglycine then rearrangement might occur resulting in nitrogen labilization of a ketene. This intermediate could then react with some nearby nucleophile to inactivate the dehydratase. However, no evidence for a tautomerization of this nature was observed. Cyanoglycine was observed to be a weak competitive inhibitor for the dehydratase.

Threonine dehydratase plus two other enzymes (22, 23) have been reported to abstract the $\alpha$-hydrogen of vinylglycine after which a 1,3 proton exchange shifts evidently occurs resulting in turnover to form $\alpha$-ketobutyric acid and ammonia.

During the course of our work with threonine dehydratase we examined the ability of the enzyme to promote labilization of the $\alpha$ proton of vinylglycine. Previously we had been unable to observe a $\alpha$ proton exchange with the substrates L-serine, L-threonine, and L-allithreonine. Vinylglycine, however, undergoes both a proton exchange and a 1,3 prototropic shift to form $\alpha$-ketobutyrate in the presence of threonine dehydratase. The observation of incorporation of deuterium into vinylglycine by NMR spectroscopy implies a fast exchange relative to tautomerization on the NMR time scale.

The inability to observe exchange with the substrates undergoing $\alpha$,$\beta$ elimination would suggest that the $\beta$ elimination is fast once a proton removal occurs. This $\alpha$ proton labilization observed with vinylglycine is the only case of a substrate for the dehydratase undergoing both a proton exchange and eneamine formation.

Threonine dehydratase will catalyze the $\alpha$,$\beta$ elimination of H$_2$O from L-serine (6, 7). It is now shown to be able to catalyze the elimination of HX from 3-haloalanine. In all cases where an aminoacylate can be formed on the enzyme we note $k_{cat}$ inhibition. The aminoacylate should be less stable and more reactive than the aminocrotonate since the stability of a double bond may be significantly increased by alkyl substitution. For this reason one would expect the aminoacylate to be more susceptible to Michael-type additions than the aminocrotonate. The nucleophile-X lost from the $\beta$-carbon atom can be $\mathrm{OH}$ or halogen. No eliminations occur when the nucleophile is $\mathrm{OH}$, $\mathrm{NH}_2$, or $\mathrm{OR}$.

The activity of threonine is completely abolished at $\mathrm{pH}$ 7 by serine and the three haloalanines of Table II. The inactivation with serine follows pseudo-first order kinetics and the apparent dissociation constant is 2 mM for serine with a limiting rate constant for activation of 2.4 min$^{-1}$ (Fig. 3). A proposed scheme for $\beta$ elimination and inhibition is shown in Fig. 5. Once Schiff base formation takes place the $\alpha$-proton is removed. The rate of proton removal should be about the same for serine and the three haloalanines and there is no internal transfer of the $\alpha$-proton (5). $\alpha$-Proton removal is followed by $\beta$ elimination. The $\beta$ substituents of the three haloalanines appear to be lost at rates which reflect their leaving group tendencies. Thus with the substituents bromide chloride and fluoride (relative leaving group tendencies: 1, 0.02, and 0.001, respectively (22)), the fastest rate is observed with bromoalanine ($k_{cat}$ 146 s$^{-1}$) and the next fastest with chloroalanine (65 s$^{-1}$) and the slowest at 2 s$^{-1}$ for fluoroalanine.

In Fig. 5 the steady state concentration of the aminoacylate should also be in proportion to the relative leaving group tendency of each $\beta$ substituent, with bromoalanine generating the highest steady state concentration of IV and fluoroalanine the smallest amount. If inactivation proceeds by Reaction 5 in Fig. 5, then the rate of inactivation from the aminoacylate should also reflect its steady state concentration. The rate of inactivation should be slower with fluoroalanine and fastest with bromoalanine. This is what is observed, indeed, inactivation by bromoalanine is too rapid to obtain the initial branch of the product versus time curve using the stop-time assay. The earliest accurate stop line assay requires 30 s and by this time the enzyme is completely inactivated.

Sheep liver threonine dehydratase is the only enzyme investigated to date undergoing a $k_{cat}$ (aminoacylate) inactivation that can be reactivated. Reactivation can be effected by removal of excess serine or haloalanines plus pH adjustment in both the presence and absence of pyridoxal 5'-phosphate. A possible scheme for reactivation is also shown in Fig. 5.

Substitution of either the $\beta$-hydrogens of serine with an alkyl group has a pronounced effect on the rate of the reaction.

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and also eliminates the $k_{cat}$ inhibition. When a methyl group replaces the pro-R proton of L-serine to form threonine a smaller $K_{m}$ is observed. The $K_{m}$ for threonine is 10 times smaller than the $K_{m}$ for serine at pH 9. At the same time the overall catalytic ability drops somewhat from a $k_{cat}$ of 65 for serine to 45 for threonine. When the pro-S proton is replaced with a methyl group to form allothreonine the same binding enhancement shown by threonine over serine is observed. However, pro-S substitution by a methyl group results in a drastic reduction in $k_{cat}$.

Similar results are obtained when the β-protons of 3-fluoro-L-alanine and 3-chloro-L-alanine are replaced by methyl groups (Table II). The fluoro amino acids bind much more tightly to the threonine dehydratase than the −OH and −Cl containing analogs. This increased binding by the fluoro amino acids is not due to hydrogen bonding since a fluorine atom bonded to a carbon atom cannot act as hydrogen acceptor (25). It may be due to a size effect, since the fluorine atom is smaller than the other nucleophiles which are eliminated by the dehydratase.

Substitution of both β-hydrogens of serine with alkyl groups as in 3-hydroxyvaline renders the compounds inactive as substrates. It was previously shown that threonine dehydratase would act on both L-threonine and L-allo-threonine (6, 7). Now we can add to the list the erythro-threo pairs: 3-hydroxyvaline, 3-fluoro-2aminobutyrate, and 3-chloro-2-aminobutyrate. In all cases preference is shown in $k_{cat}$ and $k_{cat}/K_{m}$ for the threo configuration. Little difference is shown in $K_{m}$; however, when present, it too favors the threo configuration. A less efficient turnover is also observed as the bulk of the threonine is also observed as the bulk of the alkyl or aryl group is increased.

This preference for the threo form as well as the pronounced effect on the rate of elimination by pro-R proton substitution may be related to the mechanism and stereochemistry of elimination. We previously reported that the same chiral 2-keto[2H]butyrate is formed from L-threonine and L-allo-threonine by the dehydratase (5). This has also been shown for the action of β-amino oxidase catalyzing the α,β elimination reaction of 3-chloro-2-aminobutyrate (erythro and threo) (24). These observations could indicate that the C-4 methyl group of the diastereomeric 2-amino-3-hydroxybutyrate controls the stereochemical path of the elimination. In one case it would dictate a trans elimination and in the other case a cis elimination.

To compliment our understanding of the nature of the binding of amino acids by the dehydratase a series of substituted L-alanines were investigated as inhibitors. Alanine is a competitive inhibitor for L-threonine dehydratase with $K_{i}$ of 0.03 μM. Substitution of the nucleophiles shown in Tables I, II, and IV has various effects on the binding. Substitution of an −OH as in serine results in destabilization binding when $K_{i}$ and $K_{m}$ values are compared. Serine has a $K_{m}$ of 1.8 mM which is 60 times larger than the $K_{i}$ for L-alanine. The introduction of an amino function, as in diaminopropionic acid, results in a 160-fold decrease in binding when compared to alanine. β Substitution with either fluorine or chlorine has little effect on the binding over that of alanine except that fluoroalanine is bound more tightly than chloroalanine, again indicating that the enhanced fluorine binding is related to size.

Substitution with a −SH nucleophile results in an order magnitude better binding of cysteine over L-alanine by the threonine dehydratase. In addition to an increased binding capacity for L-cysteine, an increased rate of α-proton exchange is also observed (2). The tight binding by cysteine may be a result of thiazolidine ring formation.

No consistent picture of inhibition emerges which correlates with size of functional groups at the β-position, or hydrogen bonding capacity among the inhibitors in Table IV, or both. The only consistent feature which holds for these studies is an enhanced binding for substrates containing the 4-carbon structure, e.g. threonine as a substrate and aminobutyrate as an inhibitor. A preference is shown for the threo 4-carbon conformations. Therefore, these results would suggest a methyl binding site at the active site of sheep liver threonine dehydratase which also greatly influences the stereochemical course of its reactions.

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Functional and stereochemical specificity at the beta carbon atom of substrates in threonine dehydratase-catalyzed alpha,beta elimination reactions.

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