SUMMARY

The action of partially purified L-threonine dehydratase of sheep liver on L-serine has been studied. This enzyme acts on L-serine as a substrate but is rapidly inactivated in the process, partially at pH 8.9 and more completely at pH 7.2. However, incubation at pH 8.9, with or without L-threonine, leads to a gradual restoration of up to 90% of the original activity. The addition of pyridoxal phosphate to the inactive enzyme solution has no effect on the activity or rate of reactivation at pH 8.9. However, at pH 7.2, the addition of pyridoxal phosphate leads to a tripling of the small activity toward L-threonine. These observations are discussed in terms of the proposed formation of an oxazolidine ring between serine and bound coenzyme.

The enzyme is inactive in an environment of low ionic strength. The immediate addition of potassium phosphate restores the activity to desalted enzyme. The amount of restoration by potassium phosphate depends on the time of its addition, for a slower, irreversible inactivation also occurs.

L-Threonine dehydratase (L-threonine hydro-lyase (deaminating), EC 4.2.1.16) catalyzes the conversion of L-threonine or L-allothreonine to α-keto acid and ammonia (1, 2). L-Serine is also deaminated to pyruvate; however, the enzyme, from sheep liver, is rapidly inactivated in the process. At pH 7.2 the inactivation is nearly complete, but at pH 8.9 a slow reaction with serine continues. L-Serine has a similar effect on the L-threonine dehydratase of Escherichia coli and of yeast (3, 4). Little is known about the mode of inactivation. Addition of L-threonine to the sheep liver enzyme before, after, or at the same time as the addition of L-serine did not affect the inactivation process.

(1, 2). Nishimura and Greenberg reported that dialysis of enzyme which had been inactivated at pH 7.2, followed by the addition of pyridoxal phosphate, restored up to 95% of the original activity at pH 7.2 (2). This suggested that serine facilitated the dissociation of the coenzyme from the apoenzyme.

We have studied the inactivation of partially purified L-threonine dehydratase by L-serine at pH 7.2 and its reactivation at pH 8.9. Our results, which agree with those of Moss et al. (5), show that there is no extensive loss of coenzyme during the inactivation. They are consistent with a mechanism involving the formation of an oxazolidine ring by intramolecular addition of a hydroxyl group in an aldime of L-serine and bound pyridoxal phosphate. The effects of various salts on the activity and stability of the enzyme have also been studied.

EXPERIMENTAL PROCEDURE

Materials

All amino acids and other reagents were commercial products. The assay has been described previously (1). A unit of enzyme was defined as that amount of enzyme producing 1 umole of α-keto acid in 1 hour at 37°, pH 8.9. During the purification, the absorbance of a diluted enzyme solution at 280 mμ was measured as an indication of the content of protein and other light-absorbing material. The purity index, defined as the units of enzyme activity per ml, divided by the optical density of the enzyme solution at 280 mμ, is approximately equal to the units of enzyme per mg of protein.

Purification Procedure

The method of Davis and Metzler (1) was modified as follows.

Second Ammonium Sulfate Fractionation—The solution from the second heat treatment was made 30% saturated with ammonium sulfate by adding 176 g of salt per liter of solution. The pH was adjusted to 7.2 with concentrated ammonia. After 5 hours, the precipitate was discarded, and the clear supernatant was brought up to 50% saturation with ammonium sulfate by adding 127 g of salt per liter of solution. After standing overnight, the precipitate was collected and dissolved in 10 to 25 ml of 0.1 m phosphate buffer, pH 7.2.

Chromatography on DEAE-Sephadex—Residual ammonium sulfate was removed, and the phosphate concentration was changed from 0.100 m to 0.025 m either by dialysis or by passage
I I I I I I I I I I I
10 20 30 40 50 60
TIME, MINUTES

\[ \text{a-Ketobutyrate production at pH 8.9 as a function of time for the altered enzyme.} \]

A solution containing 6.0 ml of altered enzyme (90 to 150 original units), 0.75 ml of 0.1 M phosphate buffer (pH 7.2), and 2.25 ml of 0.5 M Tris-carbonate buffer (pH 8.95) was incubated for 5 min at 37°C. Then 2.25 ml of 0.1 M buffered substrate, pH 8.9, at 37°C, was added. Aliquots of 1.2 ml were removed periodically and added to 0.4 ml of 25% trichloracetic acid. One milliliter of each of these solutions was used to determine the production of α-keto acid (1). When pyridoxal phosphate (PLP) was included in the reaction mixture, it replaced the 6.75 ml of phosphate buffer, and the altered enzyme was incubated with pyridoxal phosphate at pH 7.2 for 1 hour before the assay was started.

Through a column of Sephadex G-25 (coarse) in 0.025 M phosphate buffer, pH 7.2. A column (2.5 x 28 cm) of DEAE-Sephadex A-50 (medium) was prepared by adding the gel as a slurry. Samples containing 100 to 750 mg of protein were added to the column in a cold room at 2°C. A yellow band formed at the top of the gel.

A stepwise elution was carried out with phosphate buffers at 0.05 M, 0.10 M, and 0.50 M concentration, pH 7.2, with a flow rate of 45 ml per hour. Two fractions containing enzyme activity were obtained. Fraction I, eluted from the column by 0.10 M phosphate buffer, contained enzyme purified 5- to 10-fold over that added to the column. Fraction II, eluted by 0.50 M phosphate buffer, contained enzyme purified 2-fold. The final purity index was usually 300 to 600, but it was sometimes as high as 1600. As mentioned by other authors, the activity of the crude extracts varied greatly among livers, and this strongly affected the final activity of the enzyme preparation. Efforts to obtain a completely pure enzyme have not yet been successful.

Additional information concerning the purification procedure has been presented elsewhere (6).

RESULTS

Inactivation by L-Serine—When the enzyme reacts with L-serine at pH 8.9, there is an initial burst of activity for about 2 min, after which the activity decreases to a much lower level (1). At pH 7.2, the initial burst lasts for 4 min, and then the activity decreases to almost zero (1, 2). An increase in pH from 7.2 to 8.9 results in an increase in small residual activity (1). We will refer to the enzyme inactivated by serine as the "altered enzyme."

Isolation of Altered Enzyme—The altered enzyme was separated from other components of the reaction mixture by passage through a column of Sephadex G-25. In a typical experiment, 3.3 ml of enzyme solution, containing 200 to 1600 units per ml, were incubated with 1.1 ml of 0.1 M phosphate buffer, pH 7.2, and 1.1 ml of 0.1 M buffered L-serine, pH 7.2, for at least 15 min at 37°C. This resulted in a loss of about 95% of the original activity; longer incubations had little additional effect on the activity. The reaction mixture was added to a column (2.5 x 62 cm) of Sephadex G-25 (coarse). The altered enzyme was eluted with 0.1 M phosphate buffer, pH 7.2, within 15 min at room temperature or at 2°C. The ninhydrin test was used to establish the complete separation from serine (7). As a control, the same experiment was performed with L-threonine in place of L-serine. No loss of activity occurred. The altered enzyme was stable at 2°C for at least 5 days, but freshly prepared altered enzyme was used in the following experiments.

Activity of Altered Enzyme—Figs. 1 and 2 show the production, from L-threonine, of α-ketobutyrate as a function of time for the altered enzyme at pH 8.9 and 7.2, respectively. The rate of appearance of α-ketobutyrate was not linear, but increased with time. This suggested that the activity of the altered enzyme was being restored slowly to that of the original. Fig. 3 shows the production, from L-serine, of pyruvate as a function of time at pH 8.9 and 7.2. The curves resemble those obtained for the original enzyme, but the quantity of product formed by the altered enzyme was only 20% of that produced by the same amount of original enzyme under the same conditions.

The effect of pyridoxal phosphate on the activity of the altered enzyme is illustrated also in Figs. 1 to 3. At pH 8.9 the addition
of pyridoxal phosphate has little or no effect upon the activity or the rate of reactivation of the altered enzyme, but at pH 7.2 the addition of pyridoxal phosphate triples the small activity toward L-threonine or L-serine.

**Reactivation of Altered Enzyme**—Reactivation of the altered enzyme was achieved by incubation at pH 8.9 with Tris-carbonate buffer and L-threonine, in the same concentrations used for the assay. L-Threonine could be omitted from the incubation mixture and an equal volume of 0.5 M Tris-carbonate buffer, pH 8.9, substituted in its place. After incubation, the mixture was passed through a column of Sephadex G-25. In similar experiments, the altered enzyme was incubated in phosphate buffer at pH 7.2 with and without L-threonine. The results of the above experiments are shown in Table I. Incubation at pH 8.9 for longer than 2 hours caused almost complete reactivation. Enzyme, reactivated 90%, produced α-ketobutyrate from L-threonine at a constant rate for at least 1 hour.

**Fig. 3.** Pyruvate production at pH 8.9 and 7.2 as a function of time for the altered enzyme. The incubation mixture was the same as in Figs. 1 and 2. O-□, pH 8.9, without pyridoxal phosphate; △-△, pH 8.9, with pyridoxal phosphate; ●-●, pH 7.2, without pyridoxal phosphate; •-•, pH 7.2, with pyridoxal phosphate.

**Table I**

<table>
<thead>
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<th>Threonine</th>
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<th>Time</th>
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</tr>
<tr>
<td>+</td>
<td>7.2</td>
<td>300</td>
<td>25</td>
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* Purity index of original enzyme was 38.6 units per mg of protein.

**Fig. 4.** The effect of adding salts at different times on the activity of the desalted enzyme. Two experiments are illustrated. $t_1$: time required to elute the enzyme from the column; $t_2$: time of addition of the salt. O-□, desalted enzyme; ●-●, desalted enzyme in 5 × 10^{-3} M potassium phosphate; ■-■, desalted enzyme in 5 × 10^{-3} M potassium phosphate; △-△, desalted enzyme in 5 × 10^{-3} M potassium chloride; □-□, desalted enzyme in 5 × 10^{-4} M ammonium sulfate.

**Table II**

<table>
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<tr>
<th>Concentration of potassium phosphate after dilution</th>
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<th>Activity before addition of phosphate</th>
<th>Activity after addition of phosphate</th>
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<td>98</td>
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<tr>
<td>5 × 10^{-6}</td>
<td>120</td>
<td>77</td>
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</tr>
</tbody>
</table>

**K_m of Altered Enzyme**—In order to minimize reactions of the altered enzyme during the determination of its Michaelis constant in the presence of L-threonine, the reaction mixtures were incubated for only 10 min. A double reciprocal plot of the initial velocity with increasing concentrations of L-threonine was made. An approximate $K_m$ of 2.4 × 10^{-3} M at pH 8.9 was determined for the altered enzyme. This value agreed well with the $K_m$ of 2.5 × 10^{-3} M determined for the original enzyme, and is in approximate agreement with the value of $K_m$ of 8 × 10^{-4} M for the original enzyme at pH 9 previously reported (1).

**Effect of Ionic Environment**—Dialysis against distilled water resulted in a loss of enzyme activity. When the enzyme was desalted by passage through columns of either Sephadex G-25
or ion retardation resin, AG-11A8, a similar effect was observed (Fig. 4). After the desalting process, the enzyme solution still contained from 1 x 10^{-4} to 25 x 10^{-4} m potassium phosphate, as determined by the method of Sudo, Hamakawa, and Kubota (8). The inactivation was affected by the time required to pass the enzyme through the column; as the time increased, the extent of inactivation increased. A minimum inactivation of 34% was obtained when the enzyme was desalted within 15 min. The addition of ammonium sulfate, sodium chloride, mercaptoethanol, pyridoxal phosphate, or adenosine diphosphate prevented the enzyme from further inactivation but could not prevent the initial inactivation. The immediate addition of potassium phosphate to a concentration of 2 x 10^{-2} m restored 93% of its activity.

Similar results were obtained when the potassium phosphate concentration was reduced by dilution. The minimum salt concentration for maximum activity was 1 x 10^{-2} m potassium phosphate. As shown in Table II, at 5 x 10^{-4} m potassium phosphate 51 to 61% of the enzyme activity was lost. The immediate addition of potassium phosphate to a concentration of 0.1 m restored the activity. The addition of phosphate at later times could not restore all of the activity. Therefore, there appears to be a second, slower, irreversible inactivation.

**Discussion**

Our results with the altered enzyme, especially its reactivation at pH 8.9 with or without L-threonine, are not consistent with a mechanism involving the resolution of the enzyme. We suggest, instead, not only that L-serine is acted upon as a substrate, but that, in a competing reaction, it converts the enzyme to the inactive, altered form. The same conclusion has been reached independently by Moss et al. (5), who studied the reactivation of the "inert complex with serine" at pH 9. These authors also suggested that the enzyme in fresh sheep livers is often present largely in the inactive form, which can be activated by heated at pH 9.

We suggest that the inactivation occurs by the mechanism shown in Fig. 5. Serine is assumed to form an aldimine, the normal ES compound, by reaction with the carbonyl group of a tightly bound molecule of pyridoxal phosphate. Dehydration of this aldimine leads to production of pyruvate and ammonia, the normal dehydratase reaction (Reaction 1). A relatively very slow nucleophilic attack by the hydroxyl group of the serine side chain on the aldimine carbon results in the formation of an oxazolidine ring, inactivating the enzyme (Reaction 2). Incubation of the active enzyme with L-serine results in an initial burst of pyruvate production until Reaction 2 reaches equilibrium, with most of the enzyme in the form of the altered ES compound (oxazolidine). Removal of excess serine (at pH 7.2) by passage through Sephadex G-25 gives a solution containing a small but measurable fraction (10%) as the normal, free enzyme. The formation of free enzyme continues, but very slowly, presumably by breakdown to products of the very small amount of normal ES compound in equilibrium with the altered ES compound. The close agreement of the K_m for the original and altered enzymes supports our conclusion that the activity of the altered enzyme resides in the small fraction of free enzyme that is present.

At pH 8.9, the reactivation is relatively rapid. Evidently the equilibrium is shifted from the altered toward the normal ES compound, and the latter is formed by a slow interconversion of the two forms. The slow opening of an oxazolidine ring is completely compatible with this picture.

The reactivation of the inactive enzyme at pH 7.2 by pyridoxal phosphate has also been reported by Moss et al. (5). This cannot be represented as simply the addition of coenzyme to apoenzyme. Possibly, however, the pyridoxal phosphate displaces the bound, altered coenzyme. Moss et al. offered the alternative proposal that the added pyridoxal phosphate competes with the bound pyridoxal phosphate for the serine of the altered ES complex.

The idea that the inactivation by serine might involve formation of an oxazolidine ring was proposed earlier by Nishimura and Greenberg (2), but these authors evidently felt that this coenzyme derivative was then removed by dialysis. Our data are fully consistent with the formation of a tightly bound oxazolidine derivative, but no proof for this type of structure exists; other types of reversible alteration of the active site are not excluded.

Oxazolidine rings involving pyridoxal have been prepared en route to the synthesis of pyridoxal phosphate (9). An oxazolidine ring structure has also been proposed for the inactivation of asparagine decarboxylase by β-hydroxyaspartic acid (10, 11). In addition, the sulfur analogue cysteine is known to form a similar thiazolidine ring structure with pyridoxal phosphate (12-16), and related structures have been proposed for the structure of pyridoxal phosphate in phosphorylase and for the binding of pyridoxal phosphate to bovine plasma albumin (14, 17, 18).
The proposed oxazolidine structure is expected to absorb light at about 330 mp. Hence, with a highly purified enzyme, an easily observable change in the spectrum should accompany the inactivation. Unfortunately, even in preparations purified 420-fold over the crude extract, the spectrum of the bound coenzyme is not visible.

The stability and activity of L-threonine dehydratase is greatly affected by monovalent cations, especially potassium (1, 2). Potassium ions act by decreasing the Michaelis constant for L-threonine (1), and our results are consistent with this interpretation. However, the slow, irreversible inactivation suggests that K⁺ is also required to maintain the proper structural configuration.

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REFERENCES

The Reversible Inactivation of l-Threonine Dehydratase of Sheep Liver by l-Serine
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