INTERACTION OF RAT LIVER ALANINE AMINOTRANSFERASE WITH L-PROLINE*

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A recent report from this laboratory (Segal, Abraham, and Schatz, 1968) described some in vitro stability studies with rat liver alanine aminotransferase in which it was found that L-proline protected the enzyme against heat denaturation better than any other amino acid tested. It was of interest to us to pursue this observation in order to decide whether the interaction was at the active site, and if so whether a complex was formed with the pyridoxal moiety. The results allow us to conclude that L-proline does interact with the prosthetic group and to speculate about the nature of the complex and its relationship to normal intermediates in the transamination reaction.

METHODS

For the stability and inhibition experiments rat liver alanine aminotransferase was purified through the first ammonium sulfate step (Gatehouse, Hopper, Schatz and Segal, 1967). The ammonium sulfate precipitate was dissolved in and dialyzed against 10 mM KPO_4 buffer, pH 7.3, containing 1 mM EDTA. Enzyme and protein assays were as described in the same paper.

For the spectrophotometric experiments crystalline enzyme was used.**

RESULTS

Effect of proline on enzyme inactivation - The effect of L-proline on the heat stability is shown in Table I and compared with that of certain other amino

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**The enzyme has recently been crystallized in this laboratory by Dr. T. Matsuzawa. Its spectral properties are indistinguishable from those reported previously for the homogeneous non-crystalline enzyme (Gatehouse et al., 1967).

TABLE I

The influence of added substances on alanine aminotransferase stability

Compound added	Percent protection
0.1 M L-proline	95 (6)
0.01 M L-proline	60 (2)
0.001 M L-proline	9 (1)
0.1 M hydroxy-L-proline	10 (3)
0.1 M D-proline	1 (1)
0.1 M L-alanine	43 (3)
0.1 M L-glutamate	70 (4)
0.01 M pyruvate	35 (4)
0.01 M α-ketoglutarate	23 (2)

The enzyme solution of specific activity about 12 was heated for 10 min. at 60°, then assayed. Protein concentration during heating was about 2.4 mg/ml in 5 mM KPO_4, pH 7.3, containing 0.5 mM EDTA. Inactivation with no additions averaged 61% with a tendency for greater inactivation as the preparation aged over a several week period. Figures in parentheses are the number of samples for which the average percent protection is recorded.

acids. L-proline also protects the enzyme against acetone inactivation. Treatment of the enzyme solution with 3 volumes of acetone at 0° led to a precipitate which, when redissolved, contained 10% of the original activity whether assayed with or without added pyridoxal phosphate. When the enzyme solution to which the acetone was added contained 50 mM L-proline, virtually full activity was recovered in the redissolved precipitate. Alanine also protected under these conditions.

Inhibition by proline - L-proline is an inhibitor of rat liver alanine aminotransferase, competitive with alanine (Fig. 1). In this experiment the apparent K_m of L-alanine was calculated to be about 50 mM and the K_i of L-proline about 10 mM. The relative affinities implied by these values are consistent with those which can be estimated from the protection data in Table 1.

Inhibition by proline was also observed with the alanine enzyme from rat

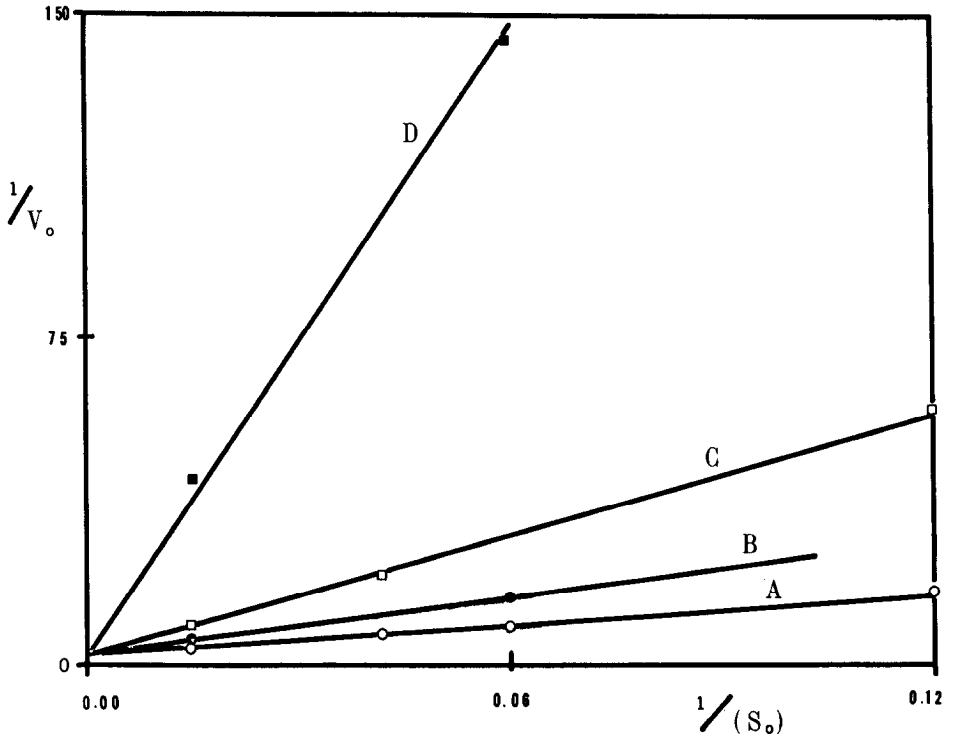

Fig. 1. Double reciprocal plot of velocity versus L-alanine concentration. In curves A, B, C and D there were present 0, 0.01 M, 0.03 M and 0.1 M L-proline, respectively. Alanine concentration was varied as shown.

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heart, while no inhibition of partially purified aspartic transaminase of liver or heart was obtained with 0.1 M L-proline.

Absorption spectrum of the complex with L-proline - The absorption spectra of the enzyme in the pyridoxal form, in the pyridoxamine form and complexed with proline are presented in Fig. 2.

DISCUSSION

The binding constant for L-proline estimated from the inhibition and protection experiments implies an affinity which compares favorably with the amino acid substrates (Hopper and Segal, 1962). The question of whether the binding is allosteric or isosteric is answered definitively by the absorption spectrum of the proline complex in which an interaction with the prosthetic group is apparent. The peak at 380 mμ suggests that the compound formed is a Schiff

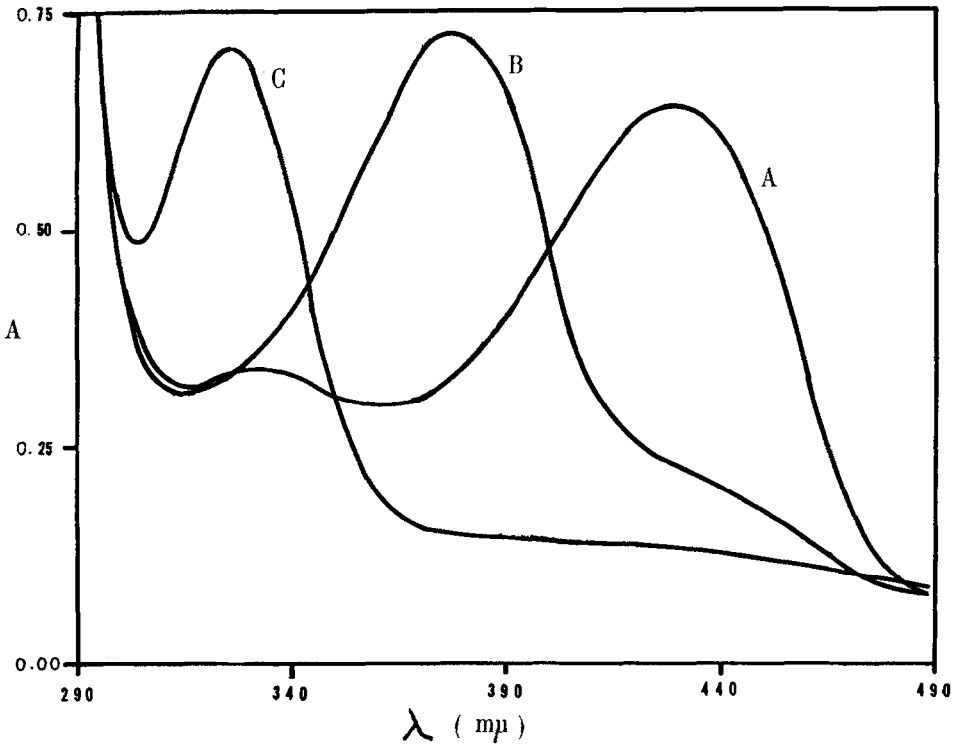
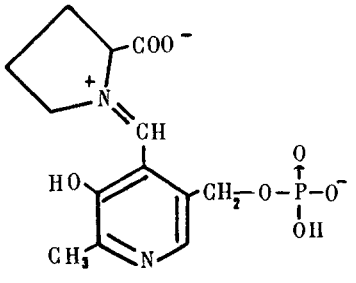


Fig. 2. Spectra of alanine aminotransferase. Enzyme concentration was 4.0 mg/ml of the crystalline preparation in 50 mM KPO_4 containing 0.5 mM dithiothreitol. The final pH was about 7. B contained in addition 0.1 M L-proline and C 0.1 M L-alanine.

Base, since tetrahedral carbon derivatives, such as the pyridoxamine forms (e.g., curve C of Fig. 2) and the secondary amines formed by the reduction of the Schiff base derived from enzyme-bound lysine (Hughes, Jenkins, and Fischer, 1962) absorb at much lower wavelengths.

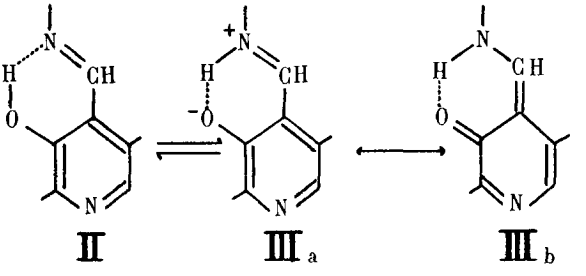
We therefore propose structure I for the pyridoxal-proline compound.



I

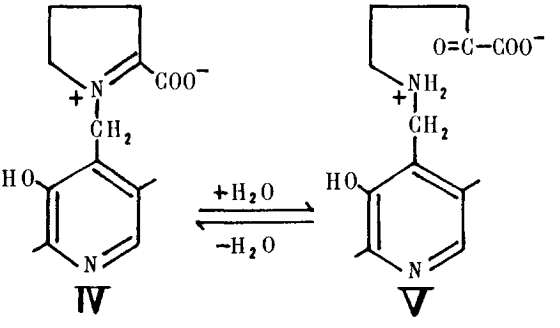
Evidence for the formation of quaternary Schiff bases with pyridoxal phosphate and cyclic imines has been described by Cordes and Jencks (1962).

If I is the correct structure it raises the question of why the absorbancy peak is shifted to 380 mμ from the 430 mμ peak characteristic of the pyridoxal form of the native enzyme, in which the pyridoxal group is presumably linked in a Schiff base to the ε-amino group of lysine. The answer may lie in the nucleophilicity of the Schiff base N in the lysine derivative, which is not a property of the Schiff base N of the proline derivative or of the hydroxylamine and hydrazine derivatives, which also show a blue shift (Jenkins, Orlowski, and Sizer, 1959; Gatehouse et al., 1967). Thus hydrogen bonding with and capture of the phenolic H (structures II and IIIa) promoting quinoid formation (structure IIIb) is possible in the former case but not in the latter cases.



II IIIa IIIb

Another question raised by the proposed structure is whether it rearranges to the ketamine form (IV) and thence to the hydrolysis product (V).



IV V

We would expect little or none of the open chain form (V) to exist at neutral pH by analogy with free Δ^1-pyrroline-2-carboxylic acid (Cabello, Leon, Prajoux,

and Plaza, 1964), although it is possible that binding to pyridoxal and the influence of the enzyme might alter this equilibrium.

The ketamine form (IV), with a tetrahedral carbon attached to the pyridine ring, would be expected to absorb in the 320-330 mμ region, however no peak was observed in this region (curve B of Fig. 2). Furthermore, the magnitude of the absorbancy of the proline complex at 380 mμ is equivalent to that of the pyridoxal form of the enzyme at its maximum (430 mμ). Therefore structure I would appear to be the predominant form.

As expected from the inability of D-proline to interact with the enzyme, we have found that L-proline is not racemized by the enzyme. These findings are consistent with but do not prove the non-formation of the ketamine. A definitive test would be to determine whether there is an enzyme-catalyzed incorporation of tritium into the α-carbon of L-proline.

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