D_2O-ALANINE EXCHANGE REACTIONS CATALYZED BY ALANINE RACEMASE AND GLUTAMIC PYRUVIC TRANSAMINASE

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SUMMARY: NMR studies in D_2O (>90%) reveal that Alanine Racemase (5.1.1.1.) from B. subtilis catalyzes the exchange of the α hydrogen of D- and L-alanine with D_2O. Glutamic Pyruvic Transaminase (2.6.1.2.) and Glutamic Oxaloacetic Transaminase (2.6.1.1.) catalyze the exchange of α and β hydrogens of L-alanine. The rates of exchange of α and β hydrogens appear to be of the same order of magnitude. The transaminase catalyzed exchange is enhanced by catalytic amounts of pyruvate. The side chain of L-alanine is held more rigidly at the active site of transaminase so that the planar conjugated system can be extended to include the α and β carbons. A generalized mechanism is proposed for the action of pyridoxal phosphate dependent transaminases which extends Braunstein and Snell mechanism to include the structures which contribute to the labilization of β hydrogens of amino acids by the transaminases that have been studied.

Previous work in this laboratory has been carried out on purification and characterization of Alanine Racemase from B. subtilis (1, 2). Recent NMR studies of substrate-D_2O exchange reactions catalyzed by Alanine Racemase has prompted us to examine the substrate-D_2O exchange reactions catalyzed by other closely related pyridoxal phosphate-requiring enzymes. Racemization as catalyzed by pyridoxal phosphate-requiring enzymes and transamination are considered to proceed by a similar mechanism. This general mechanism independently proposed by Braunstein and Snell depicts the mechanism of action of pyridoxal phosphate catalyzed reactions (3, 4). Dunathan has extended this general mechanism to include the detailed conformation and reaction specificity in these pyridoxal phosphate-requiring enzymes (5 - 7).

The NMR studies reported here reveal a fundamental difference between amino acid-D_2O exchange reactions catalyzed by Alanine Racemase from B.

subtilis and those reactions catalyzed by transaminases obtained from pig heart. The exchange reaction catalyzed by Alanine Racemase occurs only at the α hydrogen of the amino acid while these transaminases catalyze the exchanges of both α and β hydrogens of the amino acid. IR studies of Oshima and Tamiya indicated that Glutamic Pyruvic Transaminase can catalyze the exchange of both α and β hydrogens of L-alanine with D_2O and suggested that the β hydrogen exchange may be important in the catalytic mechanism of transaminase (8, 9). The significance of their results has been criticized by the suggestion that the β hydrogen exchange might result due to a tautomerization reaction (10) Our results confirm by an independent method the conclusions of Oshima and Tamiya and establish that the observed catalytic exchange of β hydrogens of L-alanine cannot be due to a simple enolization of pyruvate and that the β hydrogen exchange is peculiar to the transaminase and not the racemase. Since the general mechanism for the transamination does not implicate the β hydrogens, an extension of the proposed mechanism is required to explain the properties of transaminases that our studies have revealed.

METHOD: Alanine Racemase was obtained from B. subtilis after ammonium sulfate precipitation, followed by CM-Sephadex ion exchange chromatography and Sephadex G-100 gel chromatography. The details of purification will be reported elsewhere. The racemase preparation of specific activity 150 (units/mg. protein) has no observable transaminase activity. Glutamic Pyruvic Transaminase (GPT) of specific activity 93 and Glutamic Oxaloacetic Transaminase (GOT) of specific activity 230 were purchased as ammonium sulfate suspensions from Sigma Chemical Company. The D_2O (99.8%) was obtained from NMR Specialties and the other materials were the same as previously described (1, 2).

The exchange reaction with racemase was conducted in a NMR tube containing a total volume of 0.46 ml. The solution contained 74 nmoles of pyridoxal phosphate, 1.5 μmoles of glutathione, 120 μmoles of alanine, 25 μmoles of potassium phosphate buffer pD 8.1 in 0.45 ml of D_2O and Alanine Racemase (2 units) in 10 μliters of water. The alanine solution was added to initiate

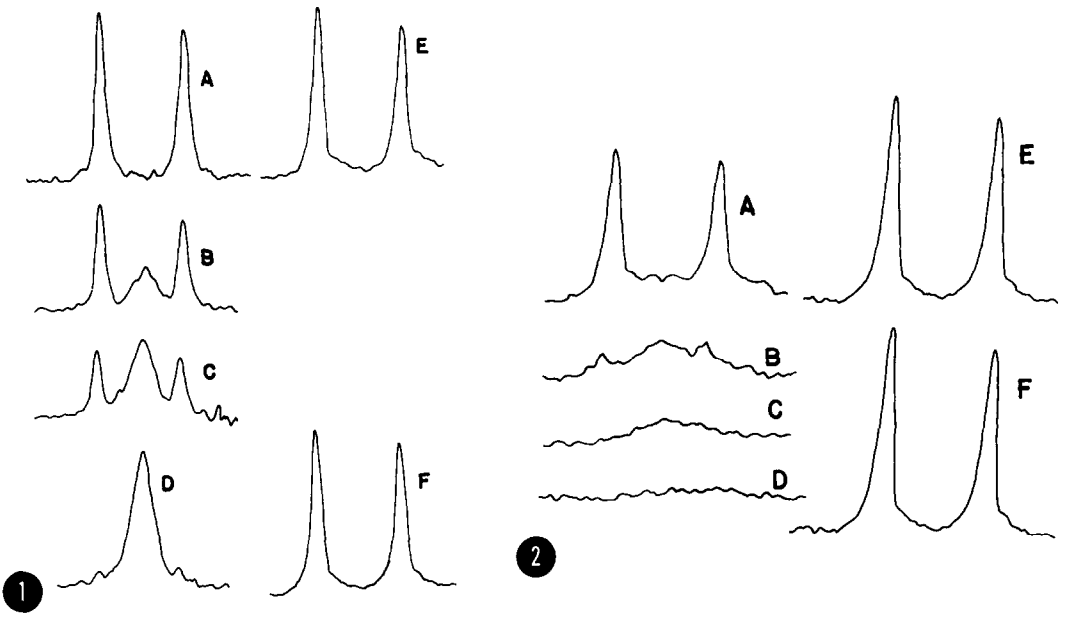

FIGURE 1. NMR spectra of D_2O-L alanine exchange as catalyzed by Alanine Racemase from B. subtilis. Spectra of methyl peak of alanine at the following instrument settings on a Varian A-60 NMR spectrometer. Filter band width 0.4, R. F. field 0.3, Sweeptime 250, Sweep width 100, and spectrum amplitude 12.5. A shows spectrum at 0 time, B at 2 minutes, C at 10 minutes, and D at 120 minutes of incubation with the enzyme under conditions described in the text. E and F show the spectra of alanine blank containing everything but the enzyme at 0 and 120 minutes respectively. The doublet centered at 1.48 ppm represents $-\underset{'}{CH}-CH_3$ and the singlet at 1.48 ppm represents $-\underset{'}{CD}-CH_3$.

FIGURE 2. NMR spectra of D_2O-L-alanine exchange as catalyzed by Glutamic Pyruvic Transaminase from pig heart. Instrument settings are the same as in Figure 1. A shows spectrum at 0 time, B at 15 minutes, C at 60 minutes and D at 120 minutes of incubation with the enzyme. E and F represent the spectra of alanine blank containing everything but the enzyme at 0 and 120 minutes respectively.

the reaction which was carried out at 37°C. At appropriate time intervals, the NMR spectra were taken on a Varian A-60 NMR spectrometer.

Studies on transaminase were conducted in a similar manner. The reaction mixture contained 74 nmoles of pyridoxal phosphate, 2 μmoles of sodium pyruvate, 120 μmoles of L-alanine, 25 μmoles of potassium phosphate buffer pD 8.1 in 0.45 ml of D_2O and transaminase (9.3 units of GPT or 18 units of GOT) in 10 μliters of water.

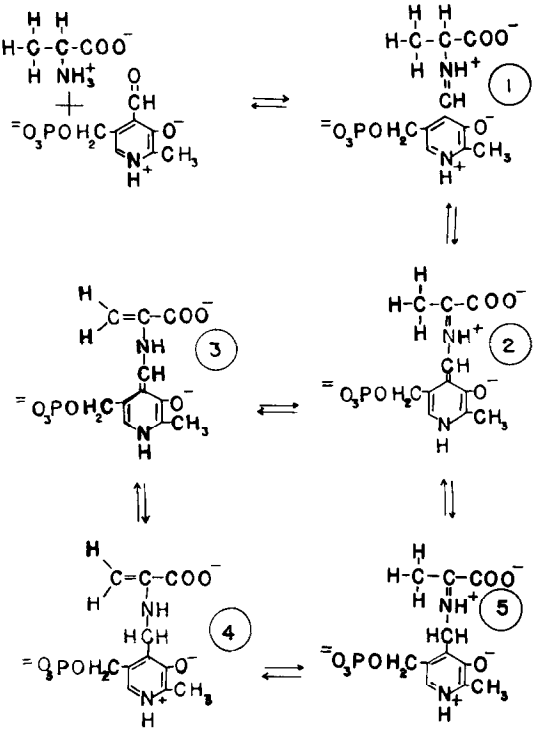

FIGURE 3. Proposed mechanism for transaminase reactions which exchange the β hydrogens.

RESULTS: The results of studies with purified Alanine Racemase from B. subtilis demonstrate that the enzyme catalyzes the exchange with D_2O of the α hydrogen of both D- and L-alanine. The NMR Spectra given in Figure 1 shows the progress of the exchange under the conditions of the experiment with L-alanine. With D-alanine the exchange proceeds at a slightly faster rate. The area of the single peak in the spectrum after two hours indicates that all of the exchange is limited to the α hydrogen (Figure 1D). The exchange is dependent upon presence of the enzyme, pyridoxal phosphate and glutathione. Pyruvate has no effect upon the rate of exchange. Similar experiments in which glycine was used in place of alanine have demonstrated that both the α hydrogens of glycine are exchanged with D_2O as a result of enzymic action.

The transaminase studies were undertaken in order to compare the exchange

properties catalyzed by racemase with those catalyzed by closely related pyridoxal phosphate-requiring enzymic reactions. The NMR spectra of a typical experiment with GPT are shown in Figure 2. Under the experimental conditions at the end of a two-hour period, no NMR peaks corresponding to the β hydrogens of L-alanine are observed. Thus, GPT had catalyzed the exchange of both α and β hydrogens of L-alanine with D_2O. D-alanine under these conditions exhibited no exchange with the medium. The rates of the exchange of the α hydrogen and the β hydrogens are of the same order of magnitude as indicated by the absence of the single peak corresponding to $-\underset{'}{CD} - CH_3$ of alanine as observed in the racemase reactions.

The exchange observed in the experiments conducted with the transaminase in which no pyruvate was added was extremely slow. The exchange was not complete even after 20 hours. The addition of catalytic amounts of pyruvate greatly accelerated the rate of exchange of α and β hydrogens of L-alanine with D_2O.

The transaminase also catalyzes the exchange of methyl hydrogens of pyruvate with D_2O, at pyruvate concentrations identical to that of alanine employed in the previously described experiment and catalytic amounts of L-alanine and pyridoxamine phosphate. The addition of catalytic amounts of L-alanine accelerated the rate of exchange of methyl hydrogens of pyruvate with D_2O. However, this exchange requires a longer period (80% exchange in 5 hours) than with L-alanine. After very long intervals of time (80% exchange in 20 hours) a non-enzymatic exchange of methyl hydrogens of pyruvate was observed.

Our results with glycine and GPT confirm the observation of Besmer and Arigoni that only one α hydrogen is exchanged (11).

GOT catalyzes similar exchanges of α and β hydrogens of L-alanine but at a much slower rate than GPT.

DISCUSSION: The exchange reaction catalyzed by Alanine Racemase differs from that observed with GPT in that it labilizes only the α hydrogen of the amino acid. The labilization of the α hydrogen by the enzymes can be readily ex-

plained by the proposed mechanism of Braunstein and Snell (3, 4). Our results would exclude any mechanism of racemization which implies any labilization of the β hydrogens.

The labilization of β hydrogens by the transaminase requires a specific interaction of the enzyme with the β carbon of the amino acid. This implies an extension of the conjugated system proposed for transaminase reaction to include the α and β carbon. Figure 3 shows the mechanism which is proposed to explain these results.

Structures 3 and 4 account for the exchange of the β hydrogens with D_2O. Presumably a basic group in the active site of the transaminase but not in the racemase would participate in the labilization of the β hydrogens. The transaminase reaction differs from the racemase reaction in (1) specificity, (2) the ability to be protonated at the formyl carbon of the coenzyme, and (3) the dissociation of the complex to form pyruvate. The contribution of structure 3 should be to facilitate the entry of the proton to the coenzyme by diminishing the charge on the adjacent nitrogen. Structures 3 and 4 which explain the β hydrogen exchange may be analogous to pyridoxal phosphate dependent reactions involving the metabolism of homocysteine, threonine and homoserine (12). The significance of the contributions by the various structures indicated in the mechanism to the catalytic process of transamination cannot be ascertained by our experiments. However, the nature of the GPT-L-alanine interaction is more accurately depicted by the inclusion of structures 3 and 4. The interaction of the methyl group of L-alanine with the transaminase would imply a more rigid binding to the surface of the enzyme with this enzyme than with the racemase. However, the methyl group is not required for the specificity of transaminase since glycine exchanges with the media in a stereospecific manner (11).

The exchange of α and β hydrogens of alanine depends upon transaminase concentration and is facilitated by catalytic amounts of pyruvate. The enzyme also catalyzes the incorporation of deuterium into pyruvate at a rate dependent

upon the amounts of L-alanine added. The rate of incorporation with pyruvate is slower than with L-alanine. No nonenzymatic exchange can be detected with L-alanine but pyruvate exchanges with the media nonenzymatically. The rate of nonenzymatic exchange of pyruvate is too slow to account for the rapid enzymatic exchange of the β hydrogens of L-alanine. The results reported here demonstrate that a fundamental difference exists in the D_2O exchange properties of the enzyme amino acid-pyridoxal phosphate complex which forms with the racemase and that which is formed in the case of the transaminases which have been studied.

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