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Stereochemistry of Transamination Catalyzed by Pyridoxamine–Pyruvate Transaminase *

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ABSTRACT: In the reaction of pyridoxamine with pyruvate, giving pyridoxal and L-alanine, catalyzed by pyridoxamine–pyruvate transaminase, stereospecific transfer of a single deuterium atom to and from the 4-methylene position of pyridoxamine occurs. By conducting the reaction with unlabeled pyridoxamine in D_2O or with dideuteriopyridoxamine in H_2O, the two enantiomers of monodeuteriopyridoxamine are readily obtained. Direct comparison of products showed that the absolute stereospecificity of transfer in this reaction is the same as that observed earlier in the reaction of pyridoxamine with α-ketoglutarate, giving pyridoxal and L-glutamate, catalyzed by apoglutamate–oxaloacetate transaminase. Deuterium in either the active or inactive position of pyridoxamine causes an increase in the Michaelis constant for pyridoxamine for the transaminase reaction catalyzed by pyridoxamine–pyruvate transaminase. The Michaelis constant of the cosubstrate, pyruvate, is also increased slightly by the presence of deuterium in the active position of pyridoxamine. A relatively small kinetic isotope effect also is observed. By conducting the transaminase reaction in D_2O, α-deuterio-L-alanine was prepared. When this compound was used with pyridoxal as the substrate for the transaminase reaction in H_2O solution, significant amounts of deuterium were transferred to the pyridoxamine formed. The result shows that an internal prototropic shift occurs during enzymatic transamination that is consistent only with the *cis* removal and addition of a proton presumably *via* a single group of the apoenzyme which functions as a general base–acid catalyst.

Reaction 1, catalyzed by apoglutamate–oxaloacetate transaminase (Wada and Snell, 1962a), serves as a model for a half-reaction of the over-all transamination

pyridoxamine + α-ketoglutarate $\rightleftharpoons$ pyridoxal + L-glutamate (1)

reaction (reaction 2) catalyzed by the corresponding holoenzyme. In reaction 1, pyridoxal and pyridoxamine act as poorly bound analogs of pyridoxal phosphate and pyridoxamine phosphate; the reaction proceeds much more slowly than the over-all reaction 2. Dunathan *et al.* (1968a,b) showed that only one of the two protons present on the 4-methylene carbon atom of pyridoxamine was labilized during reaction 1, thus demonstrating the stereospecificity of proton transfer in this and, by implication, other transamination reactions.

L-glutamate + oxaloacetate $\rightleftharpoons$ α-ketoglutarate + L-aspartate (2)

We have now extended these observations to pyridoxamine–pyruvate transaminase, a pyridoxal phosphate independent enzyme that catalyzes reaction 3 (Wada and Snell, 1962b). This enzyme, which catalyzes an essential

pyridoxamine + pyruvate $\rightleftharpoons$ pyridoxal + L-alanine (3)

step in the degradation of pyridoxamine by a soil bacterium (Burg *et al.*, 1960), is exceptionally useful for this type of investigation since it shows relatively high affini-

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ties for pyridoxal and pyridoxamine and exhibits high catalytic efficiency. Kinetic studies demonstrate that the mechanism of its action is analogous to that of pyridoxal phosphate dependent transaminases (Ayling and Snell, 1968a,b). The results presented in this paper show that the proton transfer to and from pyridoxamine in reaction 3 also is stereospecific, and that the absolute stereochemistry of monodeuteriopyridoxamine formed in reactions 1 and 3 is the same. Appropriate experiments also show that when α-deuterio-L-alanine is used as substrate in reaction 3, deuterium is incorporated into pyridoxamine, thus demonstrating that an internal prototropic shift occurs in the intermediate azomethine, and that loss and addition of the proton must occur from the same side of the imine π system.

Materials and Methods

Pyridoxamine–pyruvate transaminase was purified and assayed as described by Ayling and Snell (1968a). For experiments in D_2O, the enzyme was crystallized, sedimented, and then resuspended in D_2O prior to dissolving the desired amount in D_2O. Preparation of dideuteriopyridoxamine (designated D_2-pyridoxamine)[1] has been described by Dunathan *et al.* (1968b). Pyridoxamine, pyridoxal, D_2O (>99.5%), and other chemicals were from commercial sources. Nuclear magnetic resonance spectra were determined with a Varian A-60 instrument; mass spectroscopy was carried out with an Atlas CH4 instrument with direct inlet system. Other methods and materials are specified in the individual experiments.

Results

Stereospecificity of Deprotonation of Pyridoxamine by Pyridoxamine–Pyruvate Transaminase in Reaction 3. REACTION WITH UNLABELED PYRIDOXAMINE IN D_2O. Pyridoxamine (40 mM), pyruvate (0.4 mM), L-alanine (40 mM), EDTA (0.3 mM), and varying concentrations of pyridoxamine–pyruvate transaminase in 10 ml of D_2O were adjusted with 2 N NaOD to pH 8.5 and incubated at 37° with shaking for 8 hr. During this time there was no diminution in the specific activity of the enzyme. Reactions were stopped by freezing, the mixtures were lyophilized, and the residues were dissolved in 0.7 ml of deuterioacetic acid. Insoluble protein was removed by centrifugation and the supernatant solution was analyzed by nuclear magnetic resonance spectroscopy. Figure 1 (curve 1) shows that only one of the two protons present in the 4-methylene group of pyridoxamine could be replaced by deuterium even at very high concentrations of enzyme. The monodeuteriopyridoxamine formed, which carries the deuterium in the "labile" or "active" position of the 4-methylene group, is referred to as DH-pyridoxamine (pyridoxamine–pyruvate transaminase).

REACTION WITH DIDEUTERIOPYRIDOXAMINE IN H_2O. The experiment described in the preceding paragraph was modified by replacing D_2O with H_2O and pyridoxamine with D_2-pyridoxamine containing 90% of deuterium in the 4-methylene group (Dunathan *et al.*, 1968b). In this case, half of the deuterium was removed from the pyridoxamine, but no more, even at high enzyme concentrations (Figure 1, curve 2). We may designate the remaining monodeuteriopyridoxamine, which contains no deuterium in the active position, as HD-pyridoxamine (pyridoxamine–pyruvate transaminase).

Kinetic Isotope Effect. Reaction mixtures containing the two stereoisomers, DH-pyridoxamine (pyridoxamine–pyruvate transaminase) and HD-pyridoxamine (pyridoxamine–pyruvate transaminase), prepared enzymatically as described in the preceding section, were lyophilized, and the residues were dissolved in a minimal volume of 1 N HCl. The resulting solutions were passed separately over a column of Dowex 50-X8 (200–400 mesh) in the H^+ form. The column effluent was monitored by ultraviolet absorption and by ninhydrin assay. All components, except pyridoxamine, were eluted from the column with 1 N HCl. Pyridoxamine was eluted between 2 and 3 N HCl. The fractions containing pyridoxamine were combined and lyophilized. The residue was dissolved in boiling methanol, and the deuterated pyridoxamine dihydrochloride was precipitated from the solution by cooling and then adding ether.

The two enantiomorphic monodeuteriopyridoxamines obtained in this way, DH-pyridoxamine (pyridoxamine–pyruvate transaminase) and HD-pyridoxamine (pyridoxamine–pyruvate transaminase), were compared with D_2-pyridoxamine and unlabeled pyridoxamine (H_2-pyridoxamine) in the kinetic assay described previously (Ayling and Snell, 1968a). With initial concentrations (checked spectrophotometrically) of pyridoxamine of 0.05 mM, the rates of reaction 3 in the presence of varying pyruvate concentrations were affected not only by the presence of deuterium in the active position, but also by the deuterium in the "inactive" position (Figure 2A). To eliminate the possibility that this effect was due to impurities in the pyridoxamine solutions, the experiments were repeated using pyridoxamine isomers purified by use of a basic resin column (Dunathan *et al.*, 1968b) in place of an acidic column; the results were identical. Similar effects are seen when the rates are compared at a constant concentration of pyruvate and varying pyridoxamine concentrations (Figure 2B). The effects of the introduced deuterium are more clearly seen in Figure 2C,D, which are secondary reciprocal plots equivalent to Figure 2A,B at different pyridoxamine and pyruvate concentrations. The maximal velocities for H_2-pyridoxamine and HD-pyridoxamine, both of which contain hydrogen in the active position, are the same. Values of V_M for D_2-pyridoxamine and DH-pyridoxamine, both of which contain deuterium in the active position, are also the same but are significantly lowered by the presence of the deuterium. It is clear that the apparent secondary isotope effect shown by HD-pyridoxamine and D_2-pyridoxamine, both of which contain one

[1] The convention for describing deuterated pyridoxamine samples is described in the preceding paper (Dunathan *et al.*, 1968b).

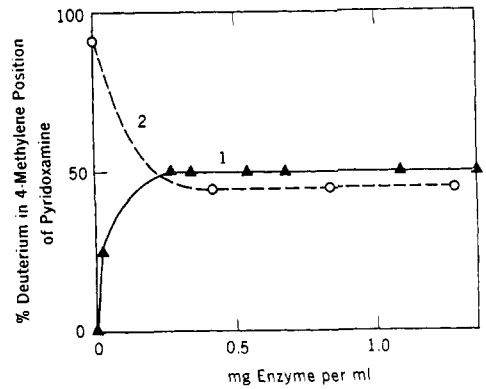

FIGURE 1: Incorporation of deuterium from D_2O into pyridoxamine and its removal from dideuteriopyridoxamine during transamination with pyruvate catalyzed by pyridoxamine–pyruvate transaminase. Curve 1: reaction of H_2-pyridoxamine in D_2O. Curve 2: reaction of D_2-pyridoxamine (90% deuterated) in H_2O. Reaction time was 8 hr in each case. The per cent of deuterium was determined by nuclear magnetic resonance spectroscopy from the ratio of the 4-aminomethyl to the 5-hydroxymethyl peak (Dunathan, 1968b). See text for other details.

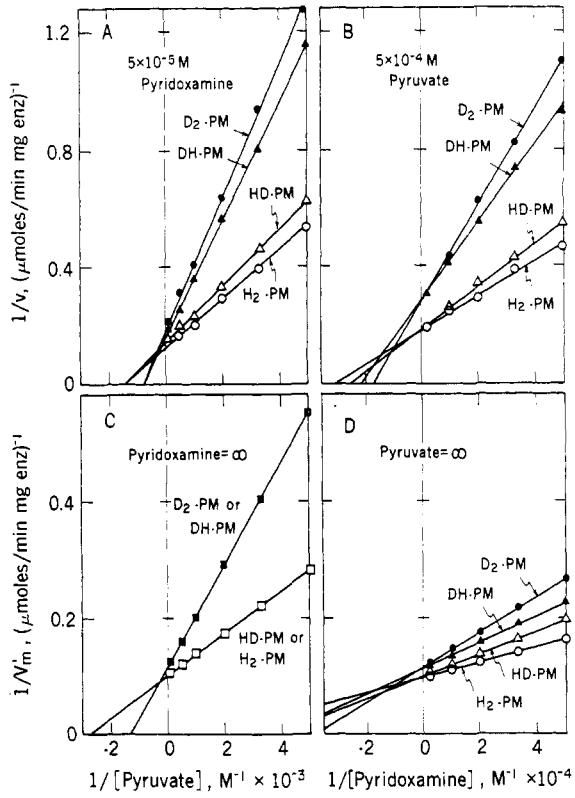

FIGURE 2: Kinetics of reaction of variously deuterated samples of pyridoxamine in reaction 3. (A) Pyridoxamine constant, as indicated, pyruvate varied; (B) pyruvate constant, as indicated, pyridoxamine varied; (C) secondary plots of $1/V'_m$ (obtained from a series of experiments such as that shown in B) *vs.* $[\text{pyruvate}]^{-1}$; (D) secondary plot of $1/V'_m$ (determined as in A) *vs.* $[\text{pyridoxamine}]^{-1}$. In all cases rates were measured in a cuvet with a 10-cm light path in a Cary spectrophotometer and recorded on a 10-fold-expanded recorder scale. All reactions were at 25° in 0.05 M sodium pyrophosphate buffer (pH 8.85) containing 0.16 μg of pyridoxamine–pyruvate transaminase/ml (Ayling and Snell, 1968a).

deuterium in the inactive position, results from a slight increase in the K_M value for pyridoxamine as a result of the deuterium substitution, and does not reflect an effect on the rate of catalysis. The presence of deuterium in the active position has a similar effect on K_M values, and the two effects are additive.

Comparison of the Absolute Stereospecificity of Pyridoxamine–Pyruvate Transaminase and Apoglutamate–Aspartate Transaminase in Catalyzing the Transamination of Pyridoxamine. The asymmetric monodeuteriopyridoxamine samples produced by pyridoxamine–pyruvate transaminase, HD-pyridoxamine (pyridoxamine–pyruvate transaminase) and DH-pyridoxamine (pyridoxamine–pyruvate transaminase), were used as substrates in transamination with α-ketoglutarate (reaction 1) catalyzed by the apoglutamate–aspartate transaminase in CO_2-free solutions under the assay conditions described by Dunathan *et al.* (1968b). Under these conditions, HD-pyridoxamine (pyridoxamine–pyruvate transaminase) gave a rate identical with that using pyridoxamine, and the rate with DH-pyridoxamine (pyridoxamine–pyruvate transaminase) was identical with that using D_2-pyridoxamine.

The rate ratios observed were k(HD-pyridoxamine (pyridoxamine–pyruvate transaminase))/k(H_2-pyridoxamine) = 1.03, k(DH-pyridoxamine (pyridoxamine–pyruvate transaminase))/k(D_2-pyridoxamine) = 1.00, and k(H_2-pyridoxamine)/k(D_2-pyridoxamine) = 1.90. These reaction rates show that apoglutamate–aspartate transaminase removes *hydrogen* from a sample of monodeuteriopyridoxamine containing *hydrogen* in the configuration activated by pyridoxamine–pyruvate transaminase; conversely, when the deuterium has the configuration activated by pyridoxamine–pyruvate transaminase, apoglutamate–aspartate transaminase also removes that deuterium. Thus, both enzymes show the same absolute stereospecificity in removing a proton from the 4-methylene group of pyridoxamine during transamination.

Determination of the Existence of an Intramolecular Prototropic Shift. To determine whether or not a direct transfer of a proton occurs between the two substrates, pyridoxamine and alanine, reaction 3 was studied in reverse, *i.e.*, α-deuterio-L-alanine was transaminated with pyridoxal, since relative values of the velocity constants for the reaction in the reverse direction (Ayling and Snell, 1968a) were more favorable for detecting a direct transfer.

α-Deuterio-L-alanine was prepared by extended incubation of L-alanine with pyridoxamine, pyruvate, and pyridoxamine–pyruvate transaminase in D_2O. The incubation mixture contained L-alanine (0.2 M), pyridoxamine (0.04 M), pyruvate (0.002 M), and enzyme (0.36 mg/ml of D_2O) at a pH (glass electrode) of 8.5. The reaction was run at 37° for 40 hr with shaking. Sufficient DCl to bring the solution to 0.5 N was then added, and the precipitated protein was removed by centrifugation. A nuclear magnetic resonance spectrum

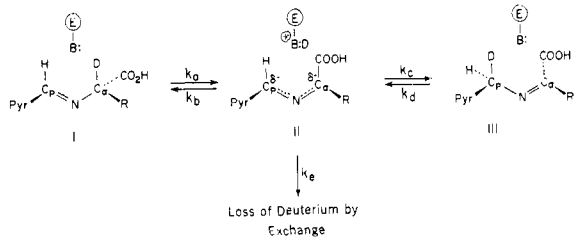

FIGURE 3: Transfer of the deuterium label in the prototropic shift of enzymatic transamination. C_P represents the carbon atom attached to the 4 position of pyridoxal, pyridoxamine, pyridoxal phosphate, or pyridoxamine phosphate; the remaining portion of these molecules is represented by Pyr. C_α represents the α-carbon atom of the amino acid or keto acid substrate.

of the supernatant solution showed that the peak representing the α-hydrogen atom of alanine was absent and the methyl peak was no longer a doublet but a single peak. To isolate the deuterated alanine the reaction mixture was evaporated to dryness, dissolved in H_2O, and applied to a column of Dowex 50-X8 (200–400 mesh) in the H^+ form. The column was then eluted with five column volumes of 0.01 N HCl (which removed all pyruvate), five column volumes of 0.1 N HCl, and then with 1 N HCl. The column effluent was monitored by its ultraviolet absorption and by its ninhydrin reaction. For the latter purpose, 0.2-ml samples were neutralized with $NaHCO_3$, spotted on filter paper, and then sprayed with 0.5% ninhydrin in acetone. Alanine was eluted with the 1 N HCl front. Pyridoxal was also eluted with 1 N HCl but requires several column volumes to displace it from the column. Pyridoxamine remained on the column. The fractions containing alanine were combined and lyophilized.

That α-deuterioalanine can be synthesized in this way shows that the proton removed from pyridoxamine in the ternary complex, EXY (eq 4), cannot be transferred directly to the α position of alanine with 100% efficiency. However, if the proton were first accepted by a basic group on the protein, such as an amino group, then transferred to the α-carbon atom, its chances of exchanging with protons of the medium would depend upon the closeness of the spatial relationships within the active site. In determining whether any such intramolecular proton transfer occurs, relatively dilute solutions of substrate and a short incubation time (compared with that required to reach equilibrium) were used, so that the reverse reaction, which could cause loss of any transferred deuterium, was minimized. A solution (200 ml) of α-deuterio-L-alanine (5 mM) and pyridoxal (5 mM), adjusted to pH 8.5 with NaOH, was incubated with pyridoxamine–pyruvate transaminase (0.88 μg/ml) for 18 min at 25°. At this time only 2% of the reactants had been converted into products, so that 20 μmoles of pyridoxamine was formed. The reaction was stopped by adding concentrated HCl to 0.5 N, the reaction mixture was then lyophilized, the residue was dissolved in H_2O, and the pyridoxamine was isolated from a Dowex 50 column by the procedure described for DH-pyridoxamine and HD-pyridoxamine. The residual alanine was also isolated and assayed by nuclear magnetic resonance spectroscopy; about 90% of the deuterium initially present in the α position still remained.

The isolated pyridoxamine was analyzed by mass spectroscopy using an Atlas CH4 instrument with direct inlet system,[2] and was 4.0 ± 0.2% monodeuterated. When the reaction was repeated using alanine that was approximately 95% deuterated in the α position and allowing the reaction to proceed to 5% conversion, the isolated pyridoxamine contained 2.0 ± 0.3% of the monodeuterio species.

Discussion

Since loss of only one deuterium atom from D_2-pyridoxamine is catalyzed by pyridoxamine–pyruvate transaminase when reaction 3 is carried out in H_2O, and only one deuterium atom is gained when the reaction with H_2-pyridoxamine is carried out in D_2O, transfer of a proton to and from pyridoxamine by this enzyme obviously is stereospecific. No detectable nonenzymatic transamination took place under the experimental conditions.

Stereospecificity in the reversible protonation of pyridoxamine during enzymatic transamination has also been observed in transamination of pyridoxamine by apoglutamate–aspartate transaminase (reaction 1; Dunathan *et al.*, 1968b). It should be emphasized that apoglutamate–aspartate transaminase and pyridoxamine–pyruvate transaminase show the same absolute stereospecificity in protonating both the 4-methylene group of pyridoxamine and the amino acid α-carbon, both being specific for the L-amino acid substrate. This reemphasizes the close relationship between the mechanism of action of pyridoxamine–pyruvate transaminase (which binds pyridoxal phosphate or pyridoxamine phosphate very poorly (Ayling and Snell, 1968b)) and that of the "normal" transaminases. It encourages the hope that a fixed relationship exists in all transaminases between the symmetry of the amino acid substrate and that of the pyridoxamine proton which is "activated."

The observed kinetic isotope effect in reaction 3 resulting from the presence of deuterium in the active position of pyridoxamine is relatively small. There is also a small apparent secondary isotope effect. However, when the pyridoxamine concentration was extrapolated to infinity, the rates of reaction with H_2-pyridoxamine and HD-pyridoxamine were equal, as were the rates with DH-pyridoxamine and D_2-pyridoxamine. Therefore, the apparent secondary kinetic isotope effect, given by the compounds with deuterium in the inactive position (Figure 2B), results from a slight increase in the K_M for pyridoxamine as a result of this substitution, and does not reflect an effect on the rate of catalysis. Deuterium in the active position also increased the K_M for pyridoxamine. This increase is predictable from the definition of this Michaelis constant, $K_M = k_5k_7/k_1(k_5 + k_7)$, in terms of the individual velocity constants of eq 4, which was

[2] We are most grateful to Dr. Thomas Kinstle of the Department of Chemistry, Iowa State University, Ames, Iowa, for supervising the mass spectral analyses.

$$E + PM \underset{k_2}{\overset{k_1}{\rightleftarrows}} E\cdot PM \underset{k_4}{\overset{(Pyr)k_3}{\rightleftarrows}} EXY \underset{(Ala)k_6}{\overset{k_5}{\rightleftarrows}} E\cdot PL \underset{k_8}{\overset{k_7}{\rightleftarrows}} E + PL \quad (4)$$

previously shown to define the kinetic mechanism of reaction 3 (Ayling and Snell, 1968a). The K_M value for pyruvate, which is equal to $k_7(k_4 + k_5)/k_3(k_5 + k_7)$, is increased twofold by deuterium in the active position of pyridoxamine (Figure 2C), indicating that a decrease in k_3 (or a major increase in k_4) must occur in addition to the calculated increase in k_5 as a result of this substitution. Since k_7 in this instance does not involve a deuterium-containing species one must assume that it is unchanged. The K_M value for pyruvate is not changed by the presence of deuterium in the inactive position of pyridoxamine.

The maximum velocity, $V_M = k_5k_7/(k_5 + k_7)$, is the same for DH-pyridoxamine and D_2-pyridoxamine, and since the K_M values for these two species are different, k_1 for D_2-pyridoxamine is lower than for DH-pyridoxamine. Similarly, since V_M is also the same for HD-pyridoxamine and H_2-pyridoxamine, k_1 with HD-pyridoxamine is lower than with H_2-pyridoxamine. Thus, k_1 is lower for all of the deuterated compounds.[3] The values for V_M are affected only when deuterium is in the active position. We previously reported values of $k_5 = 44$ sec^{-1} and $k_7 = 18$ sec^{-1} for reaction 3 with H_2-pyridoxamine as substrate (Ayling and Snell, 1968a). If one assumes that k_7 is unchanged by deuterium substitution in pyridoxamine, and substitutes the values for V_M (from Figure 2C,D) of 12.9 for H_2-pyridoxamine and 10.9 for DH-pyridoxamine into the expression for V_M given earlier, the value of k_5 is reduced from 44 sec^{-1} (for H_2-pyridoxamine) to 29 sec^{-1} (for DH-pyridoxamine); *i.e.*, the kinetic isotope effect is 1.5.

The observation that direct transfer of deuterium occurs from L-alanine to pyridoxal sets important limits on the detailed path for the 1,3-prototropic shift in this transamination reaction. The fact that the per cent of deuterium appearing in pyridoxamine is small does not detract from the importance of this result, since many paths exist whereby the per cent of deuterium transfer could be lowered. In the scheme of Figure 3, a basic group of the apoenzyme removes the C_α-deuterium of L-alanine in I leading to the ion pair II which can collapse to either I or III. The deuterium label in II may be diluted by exchange (k_e) with solvent or with groups on the apoenzyme, or by mixing with equivalent acidic protons on B:, as in the case where $BH^+ = NH_2D^+$. As a result of these processes deuteron transfer is competing with proton transfer in the step represented by k_c and is reduced by a primary isotope effect of indeterminate size. If one assumes that I and III are in rapid equilibrium with their hydrolysis products in solution, the per cent deuterium transfer in the initial stages of the reaction will depend upon the ratio of the rate constants, k_e and k_c, and upon the primary isotope effect in k_c.

In the transaminases, a histidine or lysine residue seems the most probable sources of group B:. If the ϵ-amino group of a lysyl residue is filling the role of B:, one would expect no more than about 10% deuterium transfer even in the absence of any exchange processes represented by k_e. This calculation assumes the equivalence of the two hydrogen and one deuterium atom in the NH_2D^+ of II and a primary isotope effect of 5 in k_c. If the imidazole group of a histidine residue acts as B:, k_e/k_c would have to be much larger to arrive at the observed per cent transfer.

The observed transfer of label, though small, effectively rules out a transamination mechanism involving two apoenzyme groups, one acting as general base, the other as a general acid catalyst. This "bimolecular" mechanism for prototropic shifts was first proposed by Ingold. There have been several suggestions that this mechanism could apply to enzymatic or model transamination systems (Metzler *et al.*, 1954; Bruice and Topping, 1963; Banks *et al.*, 1968).

Recently a number of prototropic rearrangements have been found to follow a different mechanistic path, essentially like that of Figure 3 (Ohlsson *et al.*, 1966; Guthrie *et al.*, 1967), and speculative schemes based upon this same idea (*e.g.*, Snell, 1962) also have been proposed for enzymatic transamination. The stereochemistry of these model reactions can be understood by assuming that the intermediate anion, corresponding to II in Figure 3, maintains its asymmetry with bond breaking and making both taking place on one side of the plane of the allylic anion. In these same systems a transfer of deuterium label almost always accompanies the stereospecific shift.[4] These rearrangements are very attractive as models for enzymatic transamination (Dunathan *et al.*, 1968a,b).

The observed transfer of label in pyridoxamine–pyruvate transaminase catalyzed transamination, the complete stereospecificity of the protonation of the pyridoxamine formed, and the existence of excellent model reactions all support the mechanism of Figure 3 for rearrangements that occur in the EXY complex during enzymatic transamination. This path involves participation of only one catalytic residue in the apoenzyme in the prototropic shift which proceeds in a *cis* manner through an asymmetric ion pair.

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[3] If the differences in K_M values for DH-pyridoxamine and H_2-pyridoxamine were due only to an altered k_5, the K_M value of DH-pyridoxamine would in fact be smaller than that of H_2-pyridoxamine, and the plots for these compounds in Figure 2B,D would be parallel. This behavior is not observed.

[4] Although the loss of asymmetry with retention of isotopic label, termed "isoracemization," has been observed (Cram and Gosser, 1964), we do not feel that this is a likely component of enzymatic transamination.

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