Mechanism of Pyridoxal Phosphate-dependent Enzymatic Amino-acid Racemization

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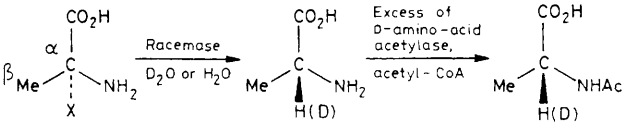
Conversion of L-[α-^{2}H]alanine in H_2O and unlabelled L-alanine in ^2H_2O into D-alanine, under nearly single-turnover conditions, with tyrosine phenol-lyase and with amino-acid racemase from *Pseudomonas striata* showed significant internal return of the α-hydrogen; the results support a single base mechanism for the racemization reactions catalysed by these two pyridoxal phosphate enzymes but with a third enzyme, alanine racemase from *E. coli*, the results were inconclusive, showing no detectable α-hydrogen return.

With only a few exceptions,[1,2] pyridoxal phosphate (PLP) enzymes catalyse group transfer reactions on one face only of a relatively planar PLP–substrate complex formed in their active site, while the other face of the complex is largely shielded.[3,4] However, PLP-dependent amino-acid racemases by definition have to operate on both sides of the complex since they have to remove a proton on one face and add one on the opposite face. This could involve the participation of two acid–base groups in the enzyme situated on opposite sides of the PLP–substrate complex. Such a two-base mechanism has been demonstrated for proline racemase,[5] an enzyme not dependent on PLP as cofactor. Alternatively, a single base could mediate both the abstraction of the α-hydrogen and the protonation of the resulting resonance-stabilized carbanion on the opposite face, requiring a re-orientation of the protonated base and the PLP–intermediate complex relative to each other during the catalytic process. A single base mechanism involving pivot rotation of the PLP Schiff's base, termed a 'swinging-door mechanism', has been proposed[6] for the alanine racemase from *Bacillus subtilis*, based on the pronounced asymmetry of the interaction of the enzyme with enantiomeric substrates and on the well documented[4] cofactor motions in other PLP enzymes.

In an attempt to distinguish between one and two base mechanisms for PLP-dependent amino-acid racemases, we probed for the internal return of the α-hydrogen in the conversion of L- into D-alanine under nearly single-turnover conditions, using three different enzymes. Internal α-hydrogen return would be expected for a single base mechanism, provided exchange of this hydrogen with solvent is slower than the forward transfer, but would not be expected for a two base mechanism.[5] Unlabelled L-alanine in ^2H_2O and L-[α-^{2}H]alanine in H_2O were incubated separately with the enzyme and the D-alanine formed was immediately trapped by acetylation with acetyl-coenzyme A (CoA) and acetyl-CoA:D-amino-acid acetyltransferase purified from yeast.[7] The resulting *N*-acetyl-D-alanine was isolated by passing the reaction mixture through a column of Dowex 50 H^+ and purified by paper chromatography (n-butanol–88% formic acid–water 2:1:1, R_f 0.83) and ion exchange chromatography (Dowex 1 acetate, gradient of 0.5 M to 2.0 M acetic acid). The product was then analysed by chemical ionisation mass spectrometry (Finnigan 4023, reagent gas isobutane), collecting at least four separate sets of spectral data for each point. The trapping efficiency was monitored by including a trace amount of D-[^{14}C]alanine in some of the incubations and measuring the partitioning of ^{14}C between *N*-acetyl-D-alanine and unchanged alanine; it was found to range from 79 to 97%, depending on the enzyme and reaction conditions. The results are summarized in Scheme 1.



Scheme 1. Internal return of α-hydrogen in the conversion of L- into D-alanine under nearly single-turnover conditions catalysed by several PLP-dependent amino-acid racemases.

Conditions	Internal return of α-hydrogen	Solvent-isotope effect (k_H/k_D)
Tyrosine phenol-lyase from *Escherichia intermedia*, H_2O, X = D	3.7%	
Tyrosine phenol-lyase from *Escherichia intermedia*, D_2O, X = H	>6.0%	
Amino-acid racemase from *Pseudomonas striata*, H_2O, X = D	0.75%	
Amino-acid racemase from *Pseudomonas striata*, D_2O, X = H	10.2 ± 0.9%	4.1 ± 0.6
Alanine racemase from *Escherichia coli*, H_2O, X = D	none	
Alanine racemase from *Escherichia coli*, D_2O, X = H	none	2.3

Tyrosine phenol-lyase (0.85 IU/mg), which normally catalyses the α,β-elimination of tyrosine to give phenol, pyruvate, and ammonia,[8] can also racemize various amino-acids, including alanine.[9] The conversion of L-alanine carrying 82% deuterium in the α-position into *N*-acetyl-D-alanine in H_2O with this enzyme proceeded with retention of 3% deuterium. In a control experiment it was established that the sample of L-[α-^{2}H]alanine used contained no more than 0.4% of the deuteriated D-isomer. Thus, the result indicates at least 3.7% (3.07/0.82) internal return of the deuteriated α-hydrogen. The experiment involving migration of an unlabelled α-hydrogen in a ^2H_2O environment is consistent with this conclusion, but the situation is complicated by the fact that the enzyme also catalyses exchange of the β-hydrogens. This is not surprising in view of the known ability of tyrosine phenol-lyase to catalyse the synthesis of tyrosine from pyruvate, ammonia, and phenol.[10] The presence of 6% of the undeuteriated species again indicates some internal return of the α-hydrogen. The similarity of the values for intramolecular transfer of α-^{2}H in H_2O and α-H in ^2H_2O suggests that the transfer of the hydrogen from the base to

the α-carbanion does not involve a large isotope effect, consistent with the implication of a monoprotic base, histidine, in the abstraction of α-H.[11]

With the broad spectrum amino-acid racemase from *Pseudomonas striata*[12] (120 IU/mg), the conversion of α-deuteriated L-alanine into *N*-acetyl-D-alanine in H_2O proceeded with only a very small degree (0.75%) of deuterium retention. In contrast, undeuteriated L-alanine in 98% ^2H_2O gave *N*-acetyl-D-alanine containing 18% normal hydrogen in the α-position. This result suggests substantial internal return of the α-hydrogen, but could also reflect a solvent-isotope effect. To explain the observed value of 18% ^1H entirely by an isotope effect, one would have to invoke a deuterium solvent-isotope effect of $k_H/k_D \cong 11$. To obtain a measure of the solvent-isotope effect, the experiment was repeated in ^2H_2O–H_2O mixtures containing 90% and 65% deuterium. The observed α-^2H/α-H ratios in the products, 60/40 and 19/81, respectively, were used to calculate the solvent-isotope effect.† In this way, it was determined that the reaction shows a solvent-isotope effect of 4.1 ± 0.6 and proceeds with 10.2 ± 0.9% internal return of the α-hydrogen (Scheme 1). The large difference in the degree of transfer of α-^2H in an H_2O environment *vs.* α-H in a ^2H_2O environment would suggest a large isotope effect in the protonation of the carbanion, implicating a polyprotic base, like an amino-group, in the deprotonation/protonation at C-α. On the other hand, it may merely reflect conformational differences of the enzyme in H_2O and in ^2H_2O.

The experiments with a third enzyme, the purified membrane-associated alanine racemase from *E. coli* B[13] (8 IU/mg), showed no detectable transfer of either α-^2H in H_2O or α-H in ^2H_2O. Repetition of the latter experiment in ^2H_2O–H_2O (50% ^2H) gave an α-^2H/α-H ratio in the product of 30/70, indicating a solvent-isotope effect of *ca.* 2.3.

In summary (Scheme 1), the results with tyrosine phenol-lyase and with the racemase from *P. striata* indicate significant internal return of the α-hydrogen in the conversion of an L- into a D-amino-acid. This supports a single base mechanism for the amino-acid racemization reactions catalysed by these two enzymes. The results with the alanine racemase from *E. coli* are inconclusive, as failure to observe internal return of the α-hydrogen is consistent either with a two base mechanism or with a single base mechanism in which equilibration of the proton abstracted from C-α with solvent is much faster than its forward transfer to the α-carbanion. However, the results with this latter enzyme serve as a negative control for the internal return observed with the other two enzymes.

We thank Professor Robert Weinkam, Purdue University, for a large number of careful mass spectral analyses. Financial support by the US Public Health Service (NIH grants to H.G.F. and C.W.), the National Science Foundation (Predoctoral Fellowship to S.A.W.), and the Purdue Research Foundation (David Ross Fellowship to S.-j.S.) is gratefully acknowledged.

Received, 2nd July 1982; Com. 765

† The calculation involves the assumption that the degree of internal α-hydrogen return is constant in the three experiments. The formula $A = I + (F \times k_H/k_D \times 100/(F \times k_H/k_D + 1 - F)$, where A = %α-^1H observed in *N*-acetyl-D-alanine, I = % internal return of α-^1H, and F = fraction of ^1H in deuteriated water, was applied to the three sets of data and gave three sets of values for k_H/k_D and I. The reasonably good agreement between the latter two values indicates that the assumption is not grossly in error.

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