Purification and Properties of Diaminopimelic Acid Epimerase from *Escherichia coli* *

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Diaminopimelic acid epimerase was purified from *Escherichia coli*. The enzyme is a monomer of $M_s = 34,000$. Diaminopimelic acid epimerase is not a pyridoxal phosphate-dependent enzyme; there is no evidence for pyridoxal phosphate in the ultraviolet spectrum of the purified enzyme, and the epimerase is not inactivated by carbonyl reagents such as hydroxylamine and sodium borohydride. Exchange of the $alpha$-protons of the substrates, DL- and LL-diaminopimelic acid, with solvent accompanies epimerization; and exchange of $^3$H from solvent into diaminopimelic acid gives $^3$H primarily (80–90%) in the product isomer, regardless of whether the DL- or LL-isomer is substrate. From these results it is concluded that the epimerase utilizes a two-base mechanism for proton translocation. In these major aspects of its mechanism, diaminopimelic acid epimerase resembles proline racemase. It is argued that the relative values of the isotope fractionation factors for the two proton acceptor sites on the enzyme can be estimated from the isotope effects for the DL- and LL-isomers of diaminopimelic acid. The observed difference in the isotope effects predicts that one, but not both, of the proton acceptor sites is a thiol, and it is demonstrated that diaminopimelic acid epimerase has a single thiol which is necessary for activity and which reacts with iodoacetamide.

For DAP synthesis is utilized by some bacteria (Misono et al., 1979) to develop a rapid, convenient assay for DAP epimerase. In the alternative pathway $alpha$-amino-$k$-ketopimelic acid is transformed directly to DL-DAP by reductive amination by NH$_2$ and NADPH, catalyzed by DAP dehydrogenase. This reaction is reversible and allowed us to couple the epimerization of LL-DAP to DL-DAP with the DAP dehydrogenase-catalyzed oxidation of DL-DAP by NADP.

We present herein evidence that pyridoxal phosphate is not a cofactor for DAP epimerase and that DAP epimerase resembles proline racemase in the major features of its mechanism.

**Experimental Procedures**

**Materials**—[1,7-$^3$C]Iodoacetamide was obtained from New England Nuclear. [1,7-$^3$C]DAP, 114 Ci/mol, and [G-3H]DAP, 630 Ci/mol, were obtained from Amersham Corp., both as a mixture of stereoisomers in statistical proportion. Nonradioactive DAP, a mixture of stereoisomers, lyophilized *E. coli* (Strain W, ATCC 9637), reactive blue 2-agaroose, reactive red 120-agaroose, and Sephadex G-200 were from Sigma. DEAE-celluloses DE52 and DE55 were from Whatman. Ion exchange resins AG 1-X8 and AG 50W-X4, both 200–400 mesh, were from Bio-Rad.

DL-DAP specifically tritiated at the $d$ epimeric carbon, 2p,6$R$-[2-$^3$H]DAP, was synthesized using the reversible reaction catalyzed by DAP dehydrogenase. [3H]NADPH was generated from [1-3H]glucose (67 $mu$Ci, 0.5 mCi, from New England Nuclear) in 0.5 ml of pH 8.0 buffer containing 100 mM Tris-HCl, 10 mM MgCl$_2$, 0.5 mM ATP, 5 units/ml of yeast hexokinase, 0.135 mM NADP, and 0.068 mM DL-DAP. After incubating this mixture for 5 min at 25 °C, 0.02 ml of 45 units/ml of yeast hexokinase, 10 units/ml of glucose-6-phosphate dehydrogenase, 0.135 mM NADP, and 0.068 mM DL-DAP. After incubating this mixture for 5 min at 25 °C, 0.02 ml of 45 units/ml of DL-DAP dehydrogenase was added to give further NADPH formation due to the oxidation of DL-DAP. After 30 min the oxidation of DAP was reversed by the addition of 2 $mu$ 1 of 4 mM NH$_4$Cl. The reaction mixture was incubated for 16 h to allow isotopic equilibration and then acidified with 0.5 ml of 0.1 ml of 10% HCl. Final purification was accomplished by reverse phase HPLC with Cu$^{2+}$-dipropylalanine eluant as described below.

**Resolution of the Isomers of DAP**—Analytical separations of the stereoisomers of DAP were accomplished by HPLC on a radial-PAC C$_8$ column (0.5 $times$ 10 cm) (Waters Associates Inc., 5- $mu$ particle size) using 8 mM N,N-di-n-propyl-L-alanine and 4 mM cupric acetate in 5% acetonitrile as mobile phase (Weinstein et al., 1982). The isomers eluted in the order, DD-LL, LL-DD, Quantitation of radioactive DAP was accomplished by collecting fractions and counting. Quantitation of nonradioactive samples was accomplished by postcolumn derivatization using o-phthaldialdehyde (Benson and Hare, 1970). Preparative separation of the isomers of radioactive DAP was also accomplished by this procedure. Copper was removed after the chromatography in this case by passing the HPLC fractions over 1 ml of Chelex 100 resin (Na+ form) and washing the column with 2 ml of 10% pyridine.

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1 The abbreviations used are: DAP, diaminopimelic acid; $^{3}$H$/$K, the $^3$H isotope effect on $V_{max}/K_m$; HPLC, high pressure liquid chromatography.
The flowthrough and washes were combined and evaporated. No attempt was made to remove the N,N-di-n-propyl-l-alanine, which did not interfere with the activity of DAP epimerase.

Nonradioactive isomers of DAP were isolated from the mixture of isomers which is commercially available. DL-DAP was enriched to 91% purity by fractional crystallization of the mixture, in the ethanolic precipitate obtained by addition of ammonium sulfate to 45% of saturation. After centrifugation, the pellet was dissolved in 16 ml of buffer B (20 mM potassium phosphate, 1 mM EDTA, 10 mM diithiothreitol, pH 7.0) and after 1 h were lysed at 20,000 psi in a French pressure cell. The pellet from centrifugation at 22,000 × g for 40 min was washed with 300 ml of buffer A and the supernatants applied to a DEAE-cellulose column (8 × 13 cm), DE53, equilibrated in buffer A. A total of 173 g of cells were processed and applied to the column. Approximately 94% of the activity flowed through the column without binding and was discarded. The column was washed with 400 ml of buffer A, and the remaining activity was eluted with a linear 5-liter gradient of buffer B containing 0-0.22 M KCl. The pooled fractions from the DE53 column were concentrated and further purified by ammonium sulfate precipitation. Solid ammonium sulfate was added to 30% of saturation, and the precipitated protein removed by centrifugation at 20,000 × g. DAP epimerase was then precipitated by addition of ammonium sulfate to 45% of saturation. After centrifugation, the pellet was dissolved in 16 ml of buffer B (20 mM potassium phosphate, 1 mM EDTA, 10 mM diithiothreitol, pH 7.0) and dialyzed against the same buffer. The epimerase was applied to another DEAE-cellulose column (4 × 35 cm), DE52, equilibrated in buffer B. The column was washed with 1 liter of buffer B and eluted with a linear 3.8-liter gradient of buffer B containing 0-15 M KCl. The pooled fractions from the DE52 column were dialyzed against buffer C (100 mM potassium phosphate, 1 mM EDTA, 10 mM diithiothreitol, pH 8.0) and applied to a reactive blue 2-agarose column (4 × 8 cm) in buffer B containing 0.2 M KC1. Leading fractions of epimerase were contaminated by a high molecular weight impurity and were combined, concentrated, and eluted a second time over the G-200 column.

Isolation of DAP Dehydrogenase—Corynebacterium glutamicum (ATCC 13032) was grown at 31 °C in AZ broth, pH 7.2 (Ueda et al., 1978), with the modifications that thymine was omitted and Bacto-yeast extract was substituted for Adarnine Z yeast extract. The pH was adjusted back to 7.2 after 4 h and after 7 h of growth, and cells were harvested after 24 h of growth. The yield was 5 g of cells/liter of medium. DAP dehydrogenase was prepared from C. glutamicum (Oshima et al., 1984). Cells, 128 g, were washed in buffer D (10 mM potassium phosphate, 1 mM EDTA, pH 6.8), pelleted, frozen, and thawed in 200 ml of buffer D containing 10 mM spermidine hydrochloride and 0.02% phenylmethylsulfonyl fluoride. Lysis was accomplished in one pass at 40,000 psi in a French pressure cell. After centrifugation at 22,000 × g to remove cell debris, the supernatant was diluted to 500 ml with buffer D and applied to a reactive red 12-agarose column (5 × 40 cm), equilibrated in the same buffer. The column was washed with 400 ml of buffer D, and DAP dehydrogenase activity was eluted with 1.5 liters of buffer D containing 0.2 M KCl. A total of 177 units of dehydrogenase activity were recovered at a specific activity of 2.5 pmol/min. The enzyme was concentrated by precipitation with 0.5 g of ammonium sulfate/ml of enzyme. The concentrated sample was dialyzed against 0.5 M Tris-HCl, pH 7.8, and dialyzed to remove ammonium ion. DAP dehydrogenase can be stored at -78 °C for at least 2 years. Although the first report of the DAP dehydrogenase from C. glutamicum indicated that it co-purified with glutamate dehydrogenase, and presumably is the same enzyme (Oshima et al., 1964), we find that the two activities are separated by the above procedure. Before it is used in the assay of DAP epimerase, the dehydrogenase must be treated with iodoacetamide to remove a low DAP epimerase activity which is present in the preparation. Dithiothreitol (0.2 M) was added to allow the enzyme to be dialyzed in the presence of the DAP dehydrogenase and incubated for 1 h at 25 °C. This is followed by the addition of iodoacetamide to 15 mM and a further 15 min at 25 °C. The enzyme is isolated free of iodoacetamide by chromatography on Sephadex G-25.

Enzyme Assays—DAP dehydrogenase is assayed at 25 °C in 0.1 M Tris-HCl, 1 mM EDTA, 0.25 mM NADP, 0.33 mM DL-DAP, pH 7.8, following the increase in absorbance at 340 nm. The Km for DL-DAP under these conditions is 0.11 mM. The apparent equilibrium constant for the oxidation of DAP is approximately 1 mM at pH 7.8. A unit of activity is 1 μmol/min of NADPH formed under the conditions specified.

All reactions involving DAP epimerase were performed at 25 °C, pH 7.8, in 0.1 M Tris-HCl, 1 mM EDTA, 1 mM diithiothreitol. DAP epimerase is conveniently assayed by coupling the conversion of LL-DAP to DL-DAP with the oxidation of DL-DAP, which is catalyzed by DAP dehydrogenase. The assay solution contains 0.4 mM LL-DAP, 0.3 mM NADP, and 0.06 units of DAP dehydrogenase, in the standard pH 7.8 buffer. A unit of epimerase activity is 1 μmol/min of NADPH formed under these conditions. The assay is linear over time for a change of up to 0.2 absorbance units. This assay has limited applicability at low pH since the activity of the DAP dehydrogenase decreases with pH in this region. The assay may be used at higher pH subject to the limitation of increased rates of air oxidation of DAP epimerase at high pH. Pure DAP epimerase has a specific activity of 108 units/mg by this assay.

In order to determine the kinetic parameters for the enzyme in both reaction directions, the conversion of DL- to LL- and of LL- to DL-DAP was monitored by HPLC. The reaction mixture, 0.1 ml, was analyzed by injection directly onto the column, and HPLC conditions were as described above.

Since sodium borohydride interfered with the NADP-dependent assay of DAP epimerase, the effect of this reducing agent on epimerase activity was assayed by the epimerase-catalyzed release of 3H to water from [G-3H]DAP. Typically, 0.1 ml of reaction mixture was acidified with 0.5 ml of 10% trichloroacetic acid and applied to a column (0.5 × 2 cm) of AG 50W-X4 ion exchange resin (H+ form). The column was washed with 3 × 0.5 ml of water and the eluates combined and counted for radioactivity.

Protein was measured using bovine serum albumin (Schaffner and Weissman, 1973).

'H Exchange Kinetics—'H exchange studies were performed with epimerases of the highest purity available. The isotope effect for exchange of 'H from 20,61-[2-'H]DAP to solvent was monitored by HPLC. A 0.2-ml solution containing 0.09 μCi of 20,61-[2-'H]DAP, and 0.015 μCi of dl-[13C]DAP was incubated with approximately 0.01 units/ml of DAP epimerase and analyzed by injection directly onto the HPLC column (HPLC conditions as described above). Formation of 'H2O as well as the distribution of 'H and 13C between DL- and LL-DAP could be monitored directly by this method. No 'H was detected in the product Dl-DAP in these experiments. For a more sensitive determination of the amount of 'H transferred from DLL- to LL-DAP the amount of [3H]DAP was tripled and the epimerization reaction was quenched by addition of iodoacetamide to a final concentration of 2 mM. An aliquot was analyzed by HPLC to determine the extent of 'H2O formed and the remainder was treated with NADP (0.3 mM) and DAP dehydrogenase (0.16 units/ml) to oxidize any remaining DL-DAP. This sample was deproteinized by heating for 5 min at 90°C and centrifuging at 15,000 × g. The 'H content of the amount LL-DAP was then analyzed by HPLC.

The exchange of 'H from solvent into DAP was determined with 10 mM DAP (sum of both isomers) and 0.01 units/ml of DAP epimerase in 0.05 ml of buffered 0.1 M NH4HCO3, 1 Ci/g. Either DL- or LL-[13C]DAP were then removed by applying the residue to 0.5 ml of 1 M Tris-HCl, 0.1 mM EDTA, pH 7.8, and dialyzed to remove ammonium ion. DAP dehydrogenase can be stored at -78 °C for at least 2 years. Although the first report of the DAP dehydrogenase from C. glutamicum indicated that it co-purified with glutamate dehydrogenase, and presumably is the same enzyme (Oshima et al., 1964), we find that the two activities are separated by the above procedure. Before it is used in the assay of DAP epimerase, the dehydrogenase must be treated with iodoacetamide to remove a low DAP epimerase activity which is present in the preparation. Dithiothreitol (0.2 M) was added to allow the enzyme to be dialyzed in the presence of the DAP dehydrogenase and incubated for 1 h at 25 °C. This is followed by the addition of iodoacetamide to 15 mM and a further 15 min at 25 °C. The enzyme is isolated free of iodoacetamide by chromatography on Sephadex G-25.
Enzyme Purification and Molecular Properties—A summary of the purification of DAP epimerase is presented in Table I. A purification of 6000-fold was achieved in overall 3% yield. There is some indication that there are two forms of DAP epimerase in E. coli from the behavior of the enzyme in the early steps of the purification. Specifically, approximately 30% of the epimerase activity would not bind to DEAE-cellulose under a variety of conditions, and this fraction of the activity was precipitated at lower ammonium sulfate concentrations than the remaining activity. It is possible that one activity is a proteolytic fragment of the other although the activity was precipitated at lower ammonium sulfate concentrations than the remaining activity. It is possible that the protein is not adequately reactivated by thiols, how-

ever. This apparent air oxidation is slower at pH 7 than at pH 8. The enzyme is also inactivated by iodoacetamide. We conclude that the enzyme has a free thiol which is necessary for activity.

The criterion for purity of the DAP epimerase derives primarily from gel electrophoresis of the purified preparation. The DAP epimerase activity in fractions from the Sephadex G-200 column paralleled a protein band of M, = 34,000 (±6%) when these fractions were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. This is the molecular weight assumed for the monomeric enzyme in all subsequent calculations. For purified epimerase, this band accounted for greater than 95% of the protein detected by staining with Coomassie Blue and for 85% of the protein detected by silver staining. The molecular weight for the native epimerase was 45,406 (±5%) determined on Sephadex G-200, which is consistent with a monomeric enzyme. Enzymatic activity also co-migrated with the major protein species when purified epimerase was subjected to electrophoresis on cellulose acetate.

The inactivation of DAP epimerase by iodoacetamide was used to further assess the purity of the enzyme. DAP epimerase is inactivated by iodoacetamide with a stoichiometry of 1.2 mol of iodoacetamide/mol of enzyme. Sodium dodecyl sulfate-polyacrylamide electrophoresis of enzyme inactivated with [14C]iodoacetamide gave a major band which accounted for 96% of the radioactivity and which co-migrated with the major protein band. The rate of incorporation of radioactivity into protein was the same as the rate of inactivation. For example, at 0.25 mM iodoacetamide the half-life for inactivation was 9.6 min and the half-life for incorporation of radioactivity into protein was 11.3 min. In addition, substrate protected against both inactivation and incorporation of [14C]iodoacetamide into protein. In order to demonstrate this protection, DAP epimerase was incubated with 23 mM DAP (DL:LL ratio of 2:1) and 1 mM iodoacetamide. The reaction was quenched after 11 min by addition of 2-mercaptoethanol to a final concentration of 56 mM. Enzyme treated in duplicate but with DAP omitted was inactivated more than 99%. The epimerase was then recovered by chromatography on Sephadex G-25 and shown to have 65% of the original enzymatic specific activity. Subsequent incubation with [14C]iodoacetamide resulted in the loss of 86% of the remaining enzymatic specific activity. Subsequent incubation with [14C]iodoacetamide inactivated the DAP epimerase activity with a stoichiometry of 1.2 mol of iodoacetamide/mol of enzyme. Sodium dodecyl sulfate-polyacrylamide electrophoresis of enzyme inactivated with [14C]iodoacetamide gave a major band which accounted for 96% of the radioactivity and which co-migrated with the major protein band. The rate of incorporation of radioactivity into protein was the same as the rate of inactivation. For example, at 0.25 mM iodoacetamide the half-life for inactivation was 9.6 min and the half-life for incorporation of radioactivity into protein was 11.3 min. In addition, substrate protected against both inactivation and incorporation of [14C]iodoacetamide into protein.
If pyridoxal phosphate were present in 1:1 stoichiometry, the pyridoxamine form of the enzyme would have been expected to have an absorbance peak near 415 nm with an extinction coefficient of approximately 5,000 M⁻¹ cm⁻¹ (Johnson and Metzler, 1970). This peak is clearly absent. In agreement with an earlier report (White et al., 1969), the epimerase was not inhibited by 10 mM hydroxyamine or by 10 mM hydrazine, and we have observed no inhibition when 10 mM sodium borohydride was included in an epimerase assay. The specific activity of the enzyme was not affected by dialysis or by chromatography on Sephadex G-200, and the activity of purified enzyme was not increased by the addition of 0.01 mM pyridoxal phosphate to assay solutions. There seems to be no possibility, therefore, that DAP epimerase could be a pyridoxal phosphate-dependent enzyme.

In examining other possible cofactor requirements, no dependence on redox active cofactors could be demonstrated. There is no requirement for added NAD, NADP, or flavin. These cofactors, if tightly bound at the active site, might be expected to be reduced by 10 mM borohydride, but, as noted above, this reagent had no effect on activity. There is no indication of tightly bound flavin in the spectrum of the purified enzyme. The observation that tritium in [3H]DAP is lost to solvent in every turnover. For example, after exchange of 22% of the ³H from 2d,6l-[2-³H]DAP (230,000 dpm), no LL-[³H]DAP could be detected by HPLC. The limit of detection of [³H] in the LL-DAP was determined by the uncertainty in the background counts and was 160 dpm or 0.3% of the [³H]O produced.

Exchange of ³H from [³H]O into DAP was also examined (Table II). An obvious, and the most important, result from the hydrogen exchange data is that when DAP is epimerized in [³H]O, [³H] is incorporated preferentially into product, and this is true in both reaction directions. This is not an absolute preference, however. For example, with LL-DAP as substrate at very early reaction times [³H] is clearly incorporated directly into the substrate as well as the product. A total of six different exchange reactions are, therefore, possible, and these are listed in Table III. There is sufficient information available to evaluate the rate constants for each of these exchange reactions.

The evaluation of the rate constants of Table III is approached in the following manner. When LL-DAP is incubated in [³H]O, [³H] appears in LL-DAP approximately four times faster than in LL-DAP. This establishes the ratio of k₁/k₆. Conveniently, each reaction in which [³H] is exchanged between solvent and DAP shares the same equilibrium constant. This equilibrium constant is the fractionation factor for [³H] between amino acids and water and is here taken to be φDAP = 1.19 (Cleland, 1980). The pertinent equilibria are specified in Equation 1 and have been indicated in Table III. From the ratios in Equation 1, the ratio of k₆ to k₄ can be determined from the ratio of k₆ to k₄.

\[
\frac{k₆}{k₄} = \frac{k₆}{k₄} = \frac{k₆}{k₄} = \frac{k₆}{k₄} = \phiDAP
\]

The relative values of a second set of rate constants, k₃, k₄, k₅, and k₆, can be determined similarly from the data of Table II for exchange of [³H] into DAP with LL-DAP as substrate. In this case the observed relative amounts of [³H] appearing in substrate, DL-DAP, versus product, LL-DAP, is misleadingly large. This is due to the initial contamination of the DL-isomer by LL-DAP and the relatively long reaction times involved. Calculation of the actual ratio of k₃/k₄ indicates that

\[
\frac{k₃}{k₄} = \frac{k₃}{k₄} = \frac{k₃}{k₄} = \frac{k₃}{k₄} = \phiDAP
\]

In practice, the rate constants of Table III were determined by numerically integrating by computer the set of differential equations defined by the kinetic equations of Table III. Values of the rate constants were chosen by trial and error to fit the observed results. With one minor exception, however, the approximate values of the rate constants are obvious from inspection of the data, as described in the text. The details of the computer calculations have, therefore, been omitted in the interest of clarity and can be provided on request.

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**Table II**

<table>
<thead>
<tr>
<th>Initial isomer concentration</th>
<th>% Reaction</th>
<th>Relative incorporation</th>
</tr>
</thead>
<tbody>
<tr>
<td>DL</td>
<td>LL</td>
<td>DL-DAP</td>
</tr>
<tr>
<td>mM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>1.7</td>
<td>0.814</td>
</tr>
<tr>
<td>10</td>
<td>5.4</td>
<td>0.869</td>
</tr>
<tr>
<td>10</td>
<td>18.9</td>
<td>0.815</td>
</tr>
<tr>
<td>9.1</td>
<td>0.4</td>
<td>13</td>
</tr>
<tr>
<td>9.1</td>
<td>0.4</td>
<td>27</td>
</tr>
<tr>
<td>6.7</td>
<td>3.3</td>
<td>1.3</td>
</tr>
</tbody>
</table>

**FIG. 1. UV spectrum of purified DAP epimerase.** The absorbance of a 1 ng/ml of solution of epimerase is 0.736 at 277 nm, which corresponds to an extinction coefficient of 25,000 M⁻¹ cm⁻¹.
but have suggested the possibility that DAP epimerase might not be a pyridoxal phosphate-dependent enzyme. We have now re-examined this possibility with purified DAP epimerase, and can find no evidence that the epimerase requires this cofactor. The enzyme is clearly not inhibited at high concentrations of the carbonyl reagents sodium borohydride, hydroxylamine, and hydrazine. Unlike many pyridoxal phosphate-dependent enzymes, purified DAP epimerase is not stimulated by added pyridoxal phosphate; and there is no evidence for pyridoxal phosphate in the electronic spectrum of the purified enzyme. The spectral evidence that pyridoxal phosphate is not bound to DAP epimerase is particularly convincing, but only to the extent that the purity of the enzyme can be demonstrated. The purity of this preparation is 85–95%, depending on the staining procedure used, when examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

In addition, it is unlikely that DAP epimerase could function by an oxidation-reduction mechanism similar, for example, to that of UDP-D-glucose 4’-epimerase (Glaser, 1972). There is no requirement for added flavin or nicotinamide cofactor, and there is no evidence for tightly bound flavin in the spectrum of the enzyme. The observation that the labile hydrogen from DAP is exchanged with solvent further argues that this hydrogen is mobilized as a proton and not as hydride in a mechanism involving hydride transfer to a nicotinamide cofactor.

The possibility exists that DAP epimerase requires a metal ion for catalysis, and we do not have sufficient data at this time to address this possibility. It is noteworthy, however, that the epimerase is purified and stored in the presence of EDTA without any loss of activity attributable to the chelator, and we have been unable to demonstrate any inhibition of the enzyme by 1,10-phenanthroline at concentrations as high as 10 mM (data not shown).

DAP epimerase resembles proline racemase in lacking a requirement for pyridoxal phosphate (Cardinale and Abeles, 1968). Proline racemase is characterized by a two-base mechanism in which proline is protonated on one face as the proton on the other face is removed. Implicit in a two-base mechanism for proton transfer is the possibility that the formation of a high energy, unstabilized carbamion intermediate can be avoided by concerted proton transfers, which may result in a kinetic advantage. An alternative one-base mechanism is exemplified by the extraction of a proton by a single hydrogen acceptor site on the enzyme, which then transfers that proton to the opposite face of the substrate. Such a mechanism requires a carbamion intermediate of sufficient lifetime to allow translocation of a proton from one face to the other of the substrate. The mechanistic distinction between these two mechanisms for proton transfer was first made by Rose (1966). The two mechanisms are distinguishable by the patterns of hydrogen exchange which accompany epimerization (Cardinale and Abeles, 1968; Rudnick and Abeles, 1975).

A kinetic scheme for a one-base proton transfer mechanism is presented in Fig. 2. It is obvious that exchange of hydrogen between DAP and water can only occur if the intermediate carbamion (species I’ in Fig. 2) has a lifetime long enough to allow exchange of a proton between enzyme and solvent. In examining the possible patterns of hydrogen exchange, if exchange of 'H into D3L-DAP occurs via the route z-y-z, for example, then the intermediate (A) in the exchange pathway must partition most often to the right to give predominantly

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**Table III**

<table>
<thead>
<tr>
<th>Relative rate constants for 3H transfer reactions</th>
<th>Reaction</th>
<th>Relative rate$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$DL \overset{\kappa_a}{\rightleftharpoons} LL$</td>
<td>$k_a = 1$</td>
<td>$k_b = \frac{1}{2} \cdot \kappa_a = 0.49$</td>
</tr>
<tr>
<td>$DL + 3H_2O \overset{\kappa_a}{\rightleftharpoons} 3LL$</td>
<td>$k_a = \phi_{DAP} \cdot \kappa_a = 0.36'$</td>
<td>$k_b = \frac{k_a}{2} \cdot \phi_{DAP} = 0.30$</td>
</tr>
<tr>
<td>$DL \overset{\kappa_a}{\rightleftharpoons} LL + 3H_2O$</td>
<td>$k_a = k_a(2 \cdot \phi_{DAP} = 0.40'$</td>
<td>$k_b = k_b(2 \cdot \phi_{DAP} = 0.40'$</td>
</tr>
<tr>
<td>$D3L \overset{\kappa_a}{\rightleftharpoons} 3LL$</td>
<td>$k_a = k_a = 1$</td>
<td>$k_b = k_b = 1$</td>
</tr>
<tr>
<td>$DL + 3H_2O \overset{\kappa_a}{\rightleftharpoons} 3DL$</td>
<td>$k_a = \phi_{DAP} \cdot \kappa_a = 0.039'$</td>
<td>$k_b = k_b = 0.033$</td>
</tr>
<tr>
<td>$DL + 3H_2O \overset{\kappa_a}{\rightleftharpoons} 3DL$</td>
<td>$k_a = \phi_{DAP} \cdot \kappa_a = 0.039'$</td>
<td>$k_b = k_b = 0.033$</td>
</tr>
<tr>
<td>$3DL \overset{\kappa_a}{\rightleftharpoons} 3LL$</td>
<td>$k_a = k_b &lt; 0.0005$</td>
<td>$k_b = k_b = 0.033$</td>
</tr>
</tbody>
</table>

$^a$ DL is DL-DAP; D3L is 2D,6L-[2-3H]DAP; 3LL is DL-DAP; 3DL is 2D,6L-[2-3H]DAP; etc.

$^b$ Rate constants are V/K values relative to V/K for conversion of DL-DAP to LL-DAP; $k_a$ and $k_b$ are the isotope effects for the DL and LL-isomers of [3H]DAP; the isotope effect for the LL-isomer is statistically corrected. $\phi_{DAP}$ is the fractionation factor for DAP in $3H_2O$.

$^c$ The units chosen for the concentration of $3H_2O$ are immaterial, and the designated rate constants are arbitrarily normalized to the condition $3H_2O = 1$. 

$3H$ is incorporated into product a factor of 9.2 faster than into substrate. The relative values of $k_a$ and $k_b$ are again determined using Equation 1.

The two sets of rate constants which have been evaluated at this point are now linked by examining the incorporation of $3H$ into DAP starting with the equilibrium mixture of DAP isomers. The total rate of mass transfer in both reaction directions must be the same in this case, but $3H$ appears in DL-DAP at approximately half the rate that it appears in LL-DAP. This observation determines the ratio of rate constants $k_a$ and $k_b$. The relative values of all rate constants through $k_b$ are therefore determined by the data of Table II and Equation 1. The values of these rate constants normalized relative to $k_b$ can now be determined from the isotope effect which was observed for 2D,6L-[2-3H]DAP. The observed isotope effect represents the ratio $k_b/(k_a + k_b)$. 

The implications of the rate constants in Table III can be summarized as follows. There is a small (V/K) isotope effect for both isomers of [3H]DAP, defined as $k_a^{-1}$ and $k_b^{-1}$, and this isotope effect is approximately half as large for the LL-isomer as for the DL-isomer. Release of the substrate proton to solvent accompanies epimerization in every turnover, but the converse is not true since exchange of substrate protons with solvent can occur independent of epimerization (compare $k_a$ with $k_b$ and $k_b$ with $k_b$). Similarly, when $3H$ is exchanged into DAP from solvent, the $3H$ appears primarily in the product, but some $3H$ appears concurrently in substrate as well.
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\[
E + DLS \xrightarrow{\gamma} E \cdot DL \xrightarrow{\delta} E \cdot I \xrightarrow{\beta} EH \xrightarrow{\gamma} E \cdot LL \xrightarrow{\delta} E \cdot LL + LL
\]

\[
E + DL \xrightarrow{\gamma} E \cdot DL \xrightarrow{\delta} E \cdot I \xrightarrow{\beta} EH \xrightarrow{\gamma} E \cdot LL \xrightarrow{\delta} E \cdot LL + LL
\]

**Fig. 2. One-base mechanism for epimerization of DAP.** DL and LL denote DL- and LL-DAP. *DL* and *LL*, and *H₂O* denote *H*-labeled species. *I* is the carbocation formed by abstraction of *H* from the α-carbon of DAP.

**Fig. 3. Two-base mechanism for epimerization of DAP.** Symbolism is the same as for Fig. 2. Numbered reaction steps define rate constants used under "Appendix" in the derivation of rate expressions.

LL-[2-3H]DAP, in order to account for the product distribution that is observed. Exchange of *H* into DAP when LL-DAP is the substrate must also occur through the intermediate (A), however, which is common to the exchange pathways for both isomers of DAP. Since intermediate (A) must partition to give predominantly LL-[2-3H]DAP, the one-base mechanism would predict exchange primarily into substrate from LL-DAP, which is contrary to the observed result. Similar arguments apply for the other possible exchange pathways. The one-base mechanism is therefore inconsistent with the observed pattern of hydrogen exchange for DAP epimerase.

A kinetic scheme for a two-base proton transfer mechanism is presented in Fig. 3. The lower pathway of Fig. 3 represents exchange of *H* into DAP with the DL-isomer as substrate, and the upper pathway represents exchange with LL-DAP as substrate. Clearly the two pathways share no common intermediates. The scheme is consistent with the observed result that *H* is incorporated preferentially into product in both reaction directions. Likewise, the scheme predicts that when the substrate DAP is labeled with *H*, the *H* is lost to solvent in every turnover, as is observed. DAP epimerase is, therefore, similar to proline racemase in the essential features of its mechanism. There is no requirement for pyridoxal phosphate to activate the α-proton, and both enzymes catalyze proton transfer by a two-base mechanism.

We have observed (Table II) that *H* can be exchanged directly from solvent into substrate. If the base which abstracts the α-proton from the substrate is a monoprotic base, then this exchange reaction is a straightforward reversal of the catalytic step. If the base is monoprotic, then exchange between enzyme-bound and solvent protons must first occur, followed by reversal of the catalytic step. We would predict that at least one of the catalytic bases at the active site of DAP epimerase is a monoprotic base, the essential thiol which is alkylated by iodoacetamide. DAP epimerase differs from proline racemase in this detail of its kinetic mechanism since with proline racemase no incorporation of solvent protons into substrate is observed and no exchange of the substrate-derived proton with solvent is observed from the enzyme-proline complex.

It is also clear that dissociation of DAP from DAP epimerase cannot be very much faster than the catalytic step. If dissociation were much faster, then exchange of solvent protons into substrate could not occur. To the extent that dissociation of DAP is rate-limiting for DAP epimerase, we would expect to observe a small isotope effect for epimerization. Indeed, the observed isotope effects are only \( (V/K) = 5.9 \) for 2d,6l-[2-3H]DAP (\( k^{-1} \) from Table III) and \( (V/K) = 3.3 \) for LL-[2-3H]DAP (\( k^{-1} \) from Table III). The intrinsic isotope effects for this enzyme have not been determined. A small isotope effect, \( (V/K) = 2.5 \), has also been observed for proline racemase (Cardinale and Abeles, 1968). It was somewhat surprising to observe that the isotope effects for 2d,6l-[2-3H]DAP and LL-[2-3H]DAP were different. In contrast, the isotope effects on \( V/K \) for proline racemase are nearly equal for the two isomers (Cardinale and Abeles, 1968; Belasco et al., 1983). It is possible to isolate a single kinetic parameter that reflects the difference in isotope effects, and we will offer an explanation for why this kinetic parameter differs for the two isomers of DAP.

The kinetic expressions that define the observed isotope effects in terms of the kinetic scheme of Equation 3 have been derived under the “Appendix.” The derivation is simpler if one considers the total rates of release of *H* from [3H]DAP, which are represented by \( (k_1 + k_2) \) for DL-DAP and \( (k_3 + k_4) \) for LL-DAP (rate constants from Table III). These rates do not represent a true isotope effect but are more easily interpreted. The results are presented in Equations 2 and 3 in the form proposed by Northrup (1976).

\[
(k_1 + k_2)^{-1} = \frac{k_{DL} + C_i + C_r (\phi_{DAP/\phi_R}) \cdot \alpha_1}{1 + C_i + C_r}
\]

\[
(k_3 + k_4)^{-1} = \frac{k_{LL} + C_i + C_r (\phi_{DAP/\phi_R}) \cdot \alpha_1}{1 + C_i + C_r}
\]

The parameters in Equations 2 and 3 are defined under “Appendix.” The terms \( k_{DL} \) and \( k_{LL} \) are the intrinsic isotope effects for the DL- and LL-isomers; \( \phi_{DAP} \), \( \phi_{DL} \), and \( \phi_{LL} \) are ground state isotope fractionation factors, and their ratios define equilibrium isotope effects; \( \alpha_1 \) and \( \alpha_2 \) relate the rate of release of DAP to the rate of release of *H* from the enzyme-DAP complex; and \( C_i \) and \( C_r \) are termed the forward and reverse commitments to catalysis (Northrup, 1976). The following qualitative treatment of Equations 2 and 3 is justified quantitatively under “Appendix.” Since the equilibrium isotope effects should be small, and since \( \alpha_1 \) and \( \alpha_2 \) must have values between 0 and 1 (“Appendix”), the differences between the observed and the intrinsic isotope effects are determined primarily by the commitments to catalysis, \( C_i \) and \( C_r \). In addition, since the intrinsic isotope effects are at least as large as the observed effects, which are greater than 1, then the effects of \( C_i \) and \( C_r \) must be significantly greater in the denominators of Equations 2 and 3 than in the numerators. For example, if the observed isotope effect were only 2, then the sum \( (C_i + C_r) \) could be much greater than unity but, could not be greater than the intrinsic isotope effect. This allows us to neglect the terms containing \( C_i \) and \( C_r \) in the numerator. The validity of this approximation depends primarily on the size of the observed isotope effects and is acceptable even for isotope effects as low as those observed here. We now consider
the ratio of Equations 2 and 3. Since the denominators of Equations 2 and 3 are the same, then based on the above approximation, Equation 4 follows:

\[
\frac{(k_4 + k_1)}{(k_3 + k_4)} = \frac{\phi_{DL}}{\phi_{DAP}} = \frac{\phi_{DL}}{\phi_{DAP}}
\]

(4)

In other words, the ratio of the observed isotope effects is determined predominantly by the ratio of the intrinsic isotope effects. The intrinsic isotope effect in each direction is determined in turn by the ratio of the ground state fractionation factor, \(\phi_{DL\alpha}\), to the transition state fractionation factors \(\phi_{DL}\) and \(\phi_{DAP}\) as designated in Equation 5. In this case \(\phi_{DL}\) and \(\phi_{DAP}\) are the transition state fractionation factors for transfer of a proton from DAP to proton acceptor sites \(B_1\) and \(B_2\), respectively, at the active site (Fig. 3).

\[
\frac{(k_4 + k_1)}{(k_3 + k_4)} = \frac{\phi_{DL}}{\phi_{DAP}} = \frac{\phi_{DL}}{\phi_{DAP}}
\]

(5)

We conclude, therefore, that the difference in the observed isotope effects is determined predominantly by the difference in the transition state fractionation factors for the hydrogen transfer steps. The value for the ratio of the observed rate constants in Equation 5 is 0.60.

Classically, differences in transition state fractionation factors for abstraction of a proton from carbon acids have been correlated with differences in pK\(_a\) between the hydrogen donor and hydrogen acceptor (Bell, 1973). These deviations are not large enough to account for the results observed in this case, however. For a given carbon acid, a difference of a factor of 0.6 in the observed \(^3\)H isotope effect for proton abstraction by two different proton acceptors would require a difference on the order of 10\(^6\) in the basicity of the two acceptors. Based on the following argument, this would seem an unreasonably large difference in basicity for the two proton acceptor sites of DAP epimerase. Since DAP epimerase does not utilize pyridoxal phosphate to catalyze proton abstraction from DAP, we would predict that proton abstraction is an endothermic reaction. In this case, then, a difference in pK\(_a\) between the two proton acceptor sites on the enzyme will result in a correspondingly large difference in the rate of proton abstraction by the two bases (Eigen, 1964). At the same time, however, both \(k_{DL}\) and \(k_{DAP}/K_m\) in the two reaction directions are approximately equal, so that the proton abstraction step could not be rate-determining in both directions. The observation of an isotope effect in both reaction directions, which implies that the proton abstraction step is at least partially rate-determining in both directions, would, therefore, be inconsistent with a model in which there is a large difference between the pK\(_a\) values of the two proton acceptor sites.

We offer an alternative explanation for the difference in the isotope effects for DL- and LL-DAP that: we feel is more consistent with the known properties of DAP epimerase. It was argued above that it is likely that abstraction of the α-proton from DAP is a highly endothermic reaction. According to the Hammond postulate, therefore, the transition state for this reaction should resemble the products, with almost complete transfer of the proton from DAP to the proton acceptor site on the enzyme. We propose that Equation 6 should hold, where \(\phi_1\) and \(\phi_2\) are the ground state fractionation factors for proton acceptor sites \(B_1\) and \(B_2\) of Fig. 3. In other words, a difference between the transition state fractionation factors for proton abstraction by \(B_1\) and \(B_2\) could be the result of a difference between the ground state fractionation factors for the two bases.

\[
\frac{(k_4 + k_1)}{(k_3 + k_4)} = \frac{\phi_2}{\phi_1}
\]

(6)

Conveniently, among the functional groups which may be identified with \(B_1\) and \(B_2\), thiol s are unique in having a fractionation factor significantly different than unity (Cleland, 1980). Compared with the value of 0.60 estimated for the ratio \(\phi_2/\phi_1\), values of 0.42–0.55 have been determined for the fractionation factor for thiol s (Szawelski et al., 1982). We propose, therefore, that the thiol of DAP epimerase which is required for activity and which reacts with iodoacetamide can be identified with the active site base, \(B_1\) of Fig. 3 and that the second active site base is not a thiol.

Calculations of the expected kinetic isotope effects for transfer of a proton from carbon to a variety of types of proton acceptors (More O’Ferrall and Kouba, 1967) support the relation between Equations 5 and 6. In comparing mercaptides with amines and oxyanions as proton acceptors, these calculations predict that the full difference in ground state fractionation factors would be reflected in the ratio of the transition state factors for a wide range of relative acidities between the proton acceptor and the proton donor. In other words, the condition that the transition state for abstraction of a proton from DAP should resemble the products is not a very restrictive condition.

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APPENDIX

The expressions in Equations 2 and 3, which relate the observed and intrinsic isotope effects for DAP epimerase, were derived as follows. The kinetic schemes for epimerization of unlabeled and \(^3\)H-labeled DL-DAP are provided as Equations 7 and 8, which have been extracted from Fig. 3.

\[
\begin{align*}
E + DL & \rightleftharpoons E \rightleftharpoons E \rightleftharpoons LL \rightarrow E + LL \\
B_1H & \rightarrow B_1H \rightarrow B_1H \rightarrow B_1H \\
B_2H & \rightarrow B_2H \rightarrow B_2H \rightarrow B_2H \\
E + DL & \rightleftharpoons E \rightleftharpoons E \rightleftharpoons LL \rightarrow E + LL \\
B_1H & \rightarrow B_1H \rightarrow B_1H \rightarrow B_1H \\
E & \rightarrow E \rightarrow E \rightarrow E
\end{align*}
\]

(7) (8)

The epimerase reaction is unusual in that exchange of \(^1\)H with solvent may be the first irreversible step for isotopically labeled DAP, but this step does not appear in the scheme for unlabeled DAP, since the exchange in this case would be a virtual reaction. The rate equations for \(V/K\) for these two schemes have been derived by numerous authors, for example (Cleland, 1975), and are provided as Equations 9 and 10.

\[
(V/K)_{DAP} = \frac{k_4/k_3}{k_5/k_6} = \frac{k_4k_3}{k_5k_6}
\]

(9)

\[
(V/K)_{DL} = \frac{k_4/k_3}{k_5/k_6} = \frac{k_4k_3}{k_5k_6}
\]

(10)

The observed isotope effect \((\Delta K_{DL})_{DAP}\) is given by the ratio of Equations 9 and 10. In this case the "observed isotope effect" is defined as rate of release of \(^1\)H from \(^3\)H-DAP relative to the rate of epimerization of unlabeled DAP and is given by \((k_4 + k_1)^{-1}\) from Table III. As explained in the text, the
observed isotope effect is not purely an isotope effect since some release of $^3$H to solvent can occur without concomitant epimerization of DAP (see Table III). The result is presented in Equation 11 in the form proposed by Northrup (1976).

\[
(k_a + k_b)_{3}^{\text{N}} = \frac{k_a C_l + C_r (\varphi_{\text{DAP}}/\varphi_{b}) \cdot \alpha_2}{1 + C_l + C_r}
\]

(11)

\[
\frac{3\varphi_{\text{DAP}}}{{k}_{\text{DAP}}} = \frac{k_a k_4}{k_2 k_4} \cdot \frac{3\varphi_{b}}{k_k/k_4} = \frac{k_3}{k_2} C_l \frac{k_3}{k_4} C_r = \frac{k_4}{k_4} \frac{k_3}{k_2}
\]

In Equation 11, $3k_{\text{DAP}}$ is the intrinsic isotope effect, $\varphi_{b}$ is the ground state fractionation factor for the proton acceptor site $B_a$, and $\varphi_{\text{DAP}}$ is the ground state fractionation factor for DAP. The ratio $\varphi_{\text{DAP}}/\varphi_{b}$ is the equilibrium isotope effect for epimerization at the active site. We should point out that normally the equilibrium isotope effect at the active site is equated with the equilibrium isotope effect on the overall reaction in derivations of this kind (Northrup, 1976), but this is clearly not warranted in this case. It is also interesting that rate constant $k_4$, which represents exchange of $^3$H between the enzyme-DAP complex and solvent, only affects one term in Equation 11. If solvent exchange is slow relative to product dissociation, then $\alpha_2 = 1$, and the exchange reaction will have no effect on the observed isotope effect. On the other hand, if exchange is fast, the effect will be to decrease the effect of the reverse commitment to catalysis term, $C_r$, in the numerator of Equation 11. This will in turn strengthen the argument propounded in the text that the relative value of the observed and intrinsic isotope effects is determined largely by the denominator of Equation 11.

Due to the symmetry of the epimerization reaction, the corresponding equation for the observed isotope effect for LL-DAP, $(k_a + k_b)^{\text{N}}$, can be written by inspection and is given in Equation 12. Rate constants refer to Fig. 3.

\[
(k_a + k_b)^{\text{N}} = \frac{k_a C_r + C_l (\varphi_{\text{DAP}}/\varphi_{b}) \cdot \alpha_1}{1 + C_l + C_r}
\]

(12)

In addition to the observed isotope effects, it is useful to consider to partitioning between DL- and LL-DAP as products from the reaction of $[^3]H$DAP (see Table III). The kinetic expressions for this partitioning can be written in terms of the parameters of Equations 11 and 12 where $k_l/k_d$ determines the DL/LL product ratio with $2d,6l,-[2,-3]^3H$DAP as substrate and $k_a/k_d$ determines the LL/ DL ratio with LL-[3H]DAP as substrate.

\[
\frac{k_l}{k_d} = \frac{(1 - \alpha_2) \cdot C_l}{1 + C_l + \alpha_2 \cdot C_r}
\]

(13)

\[
\frac{k_a}{k_d} = \frac{(1 - \alpha_1) \cdot C_r}{1 + C_r + \alpha_1 \cdot C_l}
\]

(14)

Equations 11–14 represent four equations with eight unknowns: $k_a$, $k_{\text{DAP}}$, $\varphi_{b}$, $\varphi_{a}$, $\alpha_1$, $\alpha_2$, $C_l$, and $C_r$. Using these four equations it was possible to assess the validity of the approximations used in the text to relate the observed and intrinsic isotope effects. The fractionation factors $\varphi_{b}$ and $\varphi_{a}$ were assigned values of 1 and 0.5, to correspond to the approximate values for a nonthiol and a thiol, respectively. Values for $\alpha_1$ and $\alpha_2$ were then varied independently between 0 and 1, and the corresponding values of the commitment factors and intrinsic isotope effects were calculated. For reasonable values of the intrinsic isotope effects, i.e., $3k_{\text{DAP}} < 40$, it is straightforward to demonstrate that the ratio of $3k_{\text{DAP}}/3k_{\text{DAP}}$ must be within the limits 0.51–0.62. By comparison, the ratio of observed isotope effects was 0.60.

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