Chapter 18

TRIOSEPHOSPHATE ISOMERASE AND PROLINE RACEMASE: REVELATION OF REACTION ENERGETICS

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I. INTRODUCTION

Explaining the origins of enzymatic catalysis in physical organic chemistry terms requires knowing the chemical nature of the reaction intermediates and the relative Gibbs free energies of these intermediates and their flanking transition states. This information can often be obtained through the use of isotopes as labels and kinetic probes. Two enzymes whose catalysis has been most thoroughly investigated by isotopic experiments are triosephosphate isomerase and proline racemase. This chapter describes the isotopic work that has revealed the complete reaction energetics for catalysis by these enzymes.

II. TRIOSEPHOSPHATE ISOMERASE

A large number of enzyme-catalyzed reactions rely on acid-base catalysis for the movement of protons to or from the substrate. In many of these reactions, substrate protons become partially or fully equilibrated with solvent protons at an intermediate stage of the reaction. The ability to feed tritium or deuterium from solvent directly into the middle of the reaction pathway affords the opportunity to use isotope tracer experiments as well as kinetic isotope effects (KIEs) to explore otherwise hidden aspects of enzymatic catalysis. Such an opportunity is present in the reaction catalyzed by the glycolytic enzyme triosephosphate isomerase (E.C. 5.3.1.1).

Triosephosphate isomerase catalyzes the interconversion of the two triose phosphates, dihydroxyacetone phosphate and (*R*)-glyceraldehyde 3-phosphate (or D-glyceraldehyde 3-phosphate).[1] The enzyme from chicken muscle is a dimer of identical subunits, each of molecular weight 26,500, and has no cofactor or specific metal ion requirement.[2] As an isomerase, with a single substrate and a single product, it is particularly amenable to mechanistic study. Further, since the equilibrium constant for the reaction is close to unity (an equilibrium mixture of triose phosphates contains 96% dihydroxyacetone phosphate and 4% glyceraldehyde phosphate[3], the enzyme-catalyzed reaction can be followed in either direction, most readily by use of a coupling enzyme, α-glycerophosphate dehydrogenase to remove dihydroxyacetone phosphate or glyceraldehyde-3-phosphate dehydrogenase to remove glyceraldehyde phosphate, as shown in Figure 1. In the presence of the appropriate dehydrogenase, the isomerase can be studied under conditions where its reaction is rate limiting and effectively irreversible.[4]

A. ISOTOPIC LABELING TO REVEAL MECHANISM

The work of Rieder and Rose[5-7] and of Bloom and Topper[8,9] established the mechanism of the reaction catalyzed by triosephosphate isomerase. For example, when dihydroxyacetone phosphate was incubated with the enzyme in tritiated water, the specific radioactivity of the reisolated dihydroxyacetone phosphate was found to be almost identical to that of the solvent. Insignificant labeling occurred in the absence of enzyme. Conversely, when stereospecifically-labeled [1(*R*)-^{3}H]dihydroxyacetone 3-phosphate was incubated with triosephosphate isomerase under irreversible conditions (where the immediate product glyceraldehyde phosphate was rapidly removed by the coupling dehydrogenase), less than 6% of the radioactivity was found in the product 3-phosphoglycerate (see Figure 1). These results indicate that, although neither substrate nor product exchanges carbon-bound protons with the solvent, exchange evidently does occur from an enzyme-bound intermediate. Further, when the enantiomeric [1(*S*)-^{3}H]dihydroxyacetone 3-phosphate was incubated with triosephosphate isomerase under irreversible conditions, all of the radioactivity was retained in the product. The

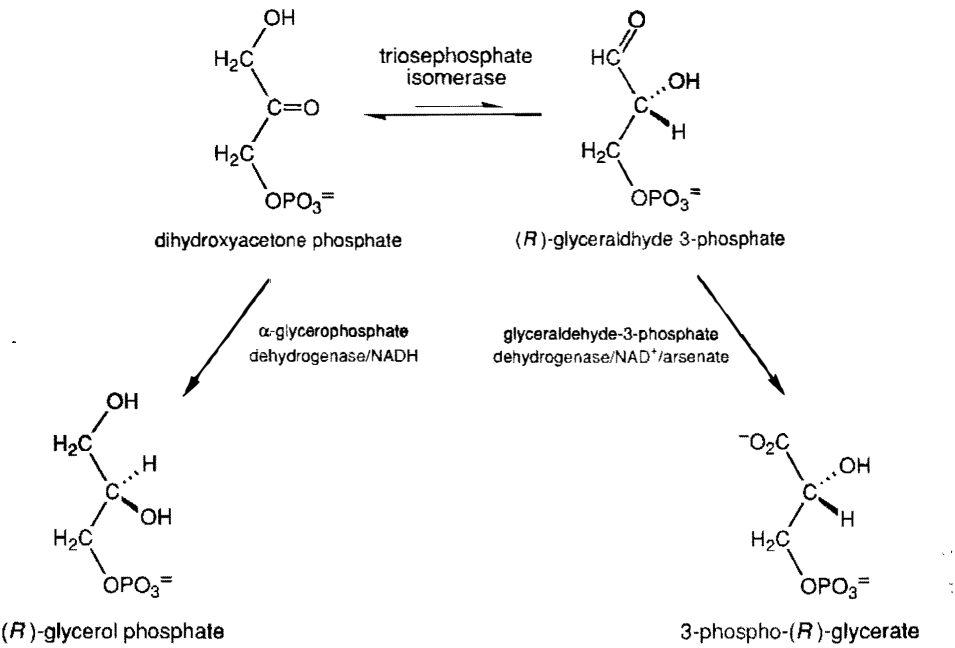

FIGURE 1. Reactions catalyzed by triosephosphate isomerase and by the coupling dehydrogenases.

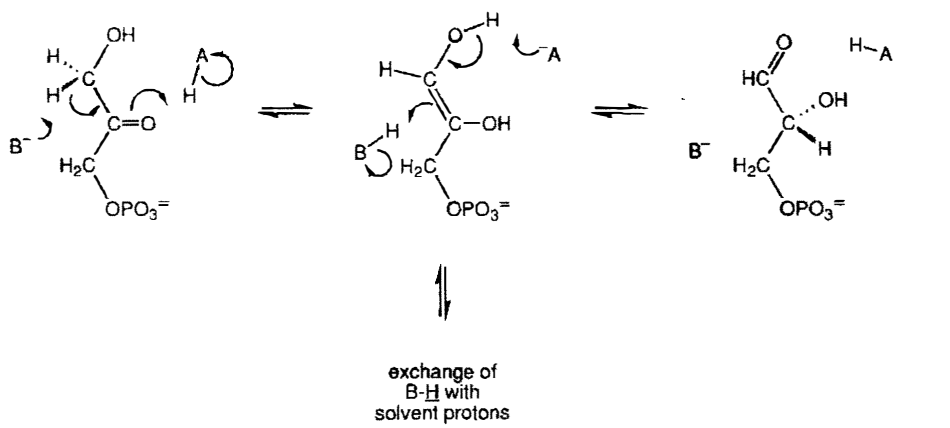

FIGURE 2. The mechanism of the reaction catalyzed by triosephosphate isomerase. B^- is the enzymic base (Glu-165) and HA is an enzymic acid.

most attractive mechanism for the triosephosphate isomerase reaction therefore involves the abstraction of either the 1-*pro-R* proton of dihydroxyacetone 3-phosphate or the C-2 proton of glyceraldehyde phosphate by an enzymic base (B^-) to give an enediol (or enediolate) intermediate, as shown in Figure 2.

B. ISOTOPE EFFECTS TO REVEAL REACTION ENERGETICS

In 1976, Albery and Knowles described a method for extracting the energetic details of enzyme catalysis in which a reaction intermediate exchanges isotope with solvent, using, in particular, data from experiments in which the isotopic content of the substrate or product is measured as a function of the extent of reaction.[10] The results of

these and other experiments can be used to derive the rate constants for the individual steps of the enzyme-catalyzed reaction and, thereby, the relative Gibbs free energies of each intermediate and transition state in the catalytic pathway.

The work of Rieder and Rose[7] and of Bloom and Topper[8] had previously demonstrated isotopic exchange between an enzyme-bound intermediate and the solvent in the reaction catalyzed by triosephosphate isomerase. Albery, Knowles, and colleagues proceeded to apply their method to triosephosphate isomerase, employing four types of experiments: (1) incubation of stereospecifically tritiated substrate in ^1H_2O, the specific radioactivity of product (transfer) and of remaining substrate (enrichment or depletion) being measured as a function of the extent of reaction;[11] (2) incubation of unlabeled substrate in tritiated water, the specific radioactivity of product (discrimination) being measured as a function of the extent of reaction;[12,13] (3) incubation of unlabeled substrate in tritiated water, the specific radioactivity of remaining substrate (exchange) being measured as a function of the extent of reaction;[12,13] and (4) comparison of the initial steady-state rates of unlabeled and stereospecifically deuterated substrates in ^1H_2O.[14] Together, these experiments can be used to deduce the energetics of any enzyme that exchanges a proton from an intermediate. (The method has recently been applied to the reaction catalyzed by alanine racemase.[15]) The results of the experiments on triosephosphate isomerase are discussed here (and elsewhere[16]) nonalgebraically in order to provide a qualitative view of the way in which the kinetic information is obtained.

1. Transfer Experiments

The extent to which a tritium label is transferred from the substrate to the product (a 'transfer' experiment) measures the efficiency of the exchange of isotope with solvent at the stage of the enediol intermediate. With triosephosphate isomerase, the existence of any direct transfer of isotopic label between carbon centers in the triosephosphates also has implications as to the number of enzymic groups involved in the proton transfer and to the nature of these groups. When [1(*R*)-^{3}H]dihydroxyacetone 3-phosphate was incubated with isomerase, 3 to 6% of the tritium was transferred to the product glyceraldehyde phosphate.[11] (The extent of tritium transfer increases at greater extents of reaction due to the accumulation of tritiated substrate, see below.) If two active-site bases are involved in the reaction, a negligible amount of transfer from substrate to product would have been expected. The small but significant amount of observed proton transfer from C-1 to C-2 suggests that a single active-site base abstracts the proton from each substrate. The participation of only one enzymic base, together with the stereochemistry of the overall reaction, suggests that proton transfer is to the *re-re* face of a *cis*-enediol intermediate.[17] Affinity labeling studies with glycidol phosphate[18] and bromohydroxyacetone phosphate[19,20] implicated a glutamic acid residue, Glu-165, as this active-site base. This conclusion was confirmed when the crystal structures of isomerase[21] and an isomerase-substrate[22] complex were solved, and Glu-165 was found on one side of the active site of the enzyme. The role of Glu-165 in the mechanism of the enzyme is now secure.

The accumulation of tritium in the remaining substrate during a transfer experiment using tritiated substrate is a measure of the extent to which the proton-transfer step is rate limiting in the reaction *up to* the exchanging intermediate, at which point the label is largely lost to solvent. With [1(*R*)-^{3}H]dihydroxyacetone 3-phosphate as the substrate for isomerase, tritium accumulated dramatically in the starting material. The preferential consumption of unlabeled substrate by the enzyme indicates that abstraction of the 1-*R* proton limits the rate of the isomerase-catalyzed conversion of dihydroxyacetone phosphate to the enediol intermediate.

2. Discrimination Experiments

The discrimination against the appearance of solvent tritium in the product (a 'discrimination' experiment) measures the extent to which the proton-transfer step is rate limiting in the steps *after* the exchanging intermediate. For example, when dihydroxyacetone phosphate was incubated with isomerase in tritiated water, the specific radioactivity of the product was found to be about 80% that of the solvent.[12] This 1.3-fold discrimination against tritium is indicative of almost complete equilibration in the protonation of the enediol intermediate at C-2. The bound enediol and the bound glyceraldehyde phosphate are therefore essentially at equilibrium. In the reverse direction (when unlabeled glyceraldehyde phosphate was the substrate in tritiated water), the specific radioactivity of the product was found to be only about 11% that of the solvent.[13] This nine-fold discrimination against tritium suggests that the protonation of the enediol intermediate to form enzyme-bound dihydroxyacetone phosphate limits the rate of the conversion of the enediol intermediate to this product.

The results of the isotope transfer and discrimination experiments discussed above reveal a qualitative picture of the relative free energies of several of the transition states in the isomerase reaction. As shown in Figure 3, the free energy of transition state 1 must be less than that of transition state 2, and the free energy of transition state 3 must be less than that of transition state 4. This ordinal analysis would be complete if the relative free energies of transition states 2 and 4 were known.

3. Exchange vs. Conversion Experiments

The appearance of solvent tritium in the remaining starting material (an 'exchange-conversion' experiment) measures the partitioning of the exchanging intermediate between the reaction forward to product and the reaction back to substrate. In an exchange-conversion experiment, the reaction is run under irreversible conditions with initially unlabeled substrate in tritiated water, and is then quenched at a known extent of substrate to product conversion. The isomerase-catalyzed reaction of unlabeled dihydroxyacetone phosphate in tritiated water was used to determine how the enediol intermediate partitions between 'exchange' back to substrate (to give tritiated dihydroxyacetone phosphate) and conversion to product glyceraldehyde phosphate, which is swept on by the coupling dehydrogenase.[12] This experiment produced the exchange-

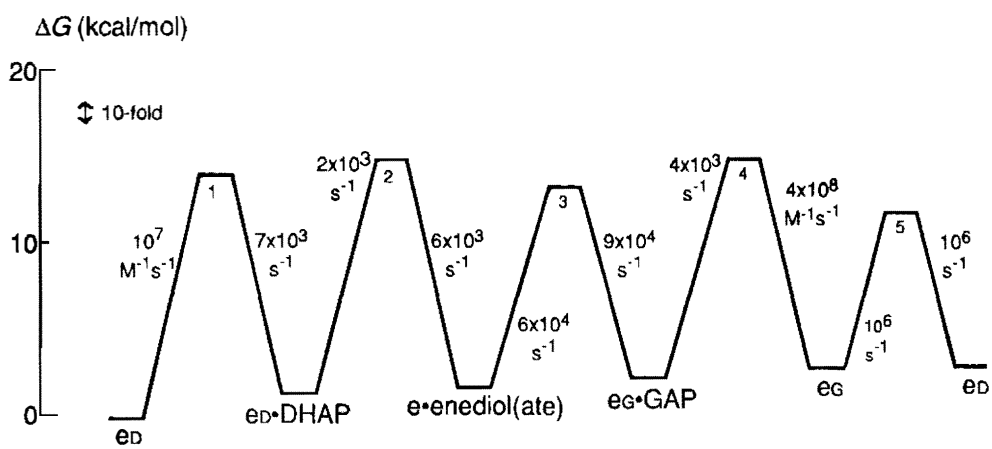

FIGURE 3. Free energy profile for the reaction catalyzed by triosephosphate isomerase. The standard state for the triose phosphates is 40 μM. DHAP is dihydroxyacetone phosphate and GAP is (*R*)-glyceraldehyde 3-phosphate.

conversion plot shown in Figure 4. Early on in the reaction, the slope of the line is about 0.3, indicating that the enediol intermediate undergoes conversion to product about three times for every time it suffers exchange, having picked up tritium in the rate-limiting transition state (transition state 2) on its return to dihydroxyacetone phosphate. At later times, the specific radioactivity of the remaining starting material increases because the kinetic isotope effect (KIE) in the processing of dihydroxyacetone phosphate leads to the preferential consumption of ^1H substrate, leaving the labeled ^3H substrate behind. When the exchange-conversion experiment was performed in the opposite direction, with glyceraldehyde phosphate as substrate, the plot shown in Figure 5 was obtained.[13] The initial slope of the line is again ca. 0.3, indicating that the intermediate partitions forward to product dihydroxyacetone phosphate about three times for every time it returns to glyceraldehyde phosphate having picked up tritium in a transition state that is *not* rate limiting (transition state 3). Thus, the results of the exchange-conversion experiment indicate that the free energy of transition state 2 (the higher of transition states 1 and 2) is less than that of transition state 4 (the higher of transition states 3 and 4). Indeed, the observed 3:1 partitioning of the enediol intermediate between transition state 4 and transition state 2 (in which a tritium is transferred), and the observed 3:1 partitioning between transition states 2 and 4 (in which a hydrogen is transferred), are in gratifying agreement with the tritium kinetic isotope effect on step 2 of about 9 ($= 3 \times 3$), determined from the results of the discrimination experiment using glyceraldehyde phosphate as substrate.

4. Deuterium Kinetic Isotope Effects

Isotopic studies using deuterium can complement those using tritium. In experiments using trace levels of tritium, the labeled molecules compete with the unlabeled ones in proton-transfer steps, thus providing information on the relative rates of steps that may not limit the rate of the overall reaction. In contrast, experiments using substrates that are labeled with stoichiometric levels of deuterium are not competitive, and probe the kinetic importance of particular proton transfer steps in the overall reaction.

The results of experiments with stereospecifically deuterated triosephosphates support the conclusions from the tritium experiments.[14] For example, when [1(*R*)-^2H]dihydroxyacetone 3-phosphate was the substrate, a kinetic isotope effect of $k_{cat}^H/k_{cat}^D = 2.9$ was observed. According to Figure 3, relatively slow abstraction of the 1(*R*)-^2H atom by the enzyme gives the enediol intermediate, at which point essentially all the deuterium is lost irreversibly by equilibration with solvent ^1H_2O. Since the isotopic content of the enediol intermediate depends only on that of the solvent, the partitioning of this intermediate will be the same regardless of the isotope initially in the 1-*R* position of dihydroxyacetone 3-phosphate. The relative rate of reaction of labeled and unlabeled dihydroxyacetone phosphate molecules is therefore governed only by the flux of molecules into the enediol intermediate. Thus, measuring the steady-state rate difference between labeled and unlabeled dihydroxyacetone phosphate measures the *full* primary kinetic isotope effect on a step (step 2) that does not necessarily limit the rate of the overall reaction.

When [2-^2H]glyceraldehyde phosphate was the substrate for isomerase, no isotope effect on either k_{cat} or k_{cat}/K_m was observed. According to Figure 3, the 2-^2H atom is removed to give the enediol intermediate, at which point deuterium is irreversibly washed out into the solvent before the rate-limiting collapse of the enediol by transfer of a solvent-derived hydrogen to C-1. Since the deuterated substrate loses its label in a step (step 3) that does not limit the rate of formation of the enediol intermediate, no isotope effect is expected.

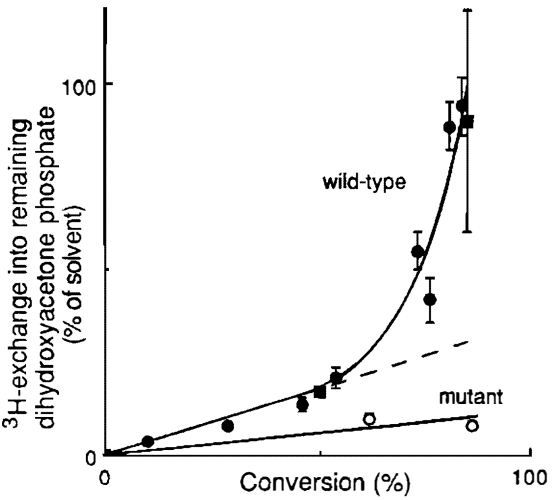

FIGURE 4. Exchange-conversion plot for chicken (●) and yeast wild-type (■), and for chicken Asp-165 mutant (○) triosephosphate isomerases with dihydroxyacetone phosphate as substrate.

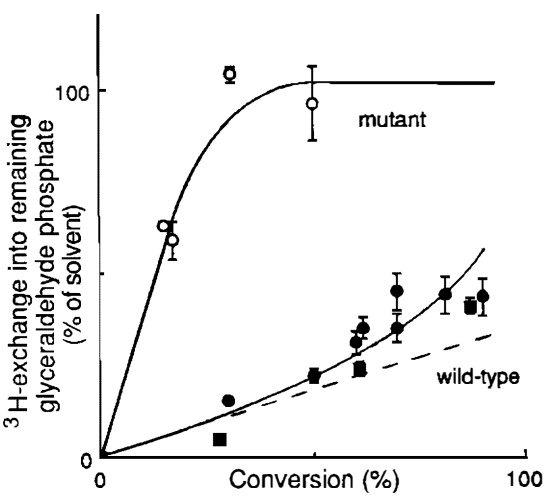

FIGURE 5. Exchange-conversion plot for chicken (●) and yeast wild-type (■), and chicken Asp-165 mutant (○) triosephosphate isomerases with glyceraldehyde phosphate as substrate.

5. Free Energy Profile for the Catalyzed Reaction

The results of the transfer, discrimination, exchange-conversion, and deuterium isotope effect experiments, together with the steady-state kinetic parameters, were used to calculate the individual rate constants of the isomerase-catalyzed reaction.[23] No assumptions were made about the kinetic importance of any step. (For example, the rate at which a proton on Glu-165 exchanges with solvent was demonstrated, not assumed, to be fast.[24]) The results of the analysis are shown as a free energy profile in

Figure 3, which is drawn for a standard state of 40 μM, the approximate concentration of triose phosphates in muscle.[25]

The identities of the particular ground state and the transition state that define the largest free energy difference in the profile of Figure 3 illustrate the efficiency of triosephosphate isomerase catalysis. At the *in vivo* substrate concentrations in which the isomerase operates, the lowest ground state in the free energy profile relates to the unliganded enzyme plus the more stable substrate dihydroxyacetone phosphate. The highest transition state in the free energy profile involves the dissociation of the less stable substrate, glyceraldehyde phosphate. (Viscosity variation experiments have demonstrated that this dissociation step is indeed diffusive, as distinct from involving a conformational change in the liganded complex prior to diffusive substrate release.)[26] The relative free energies of the highest transition state and the lowest ground state in the free energy profile are therefore independent of the nature of the enzyme. Such an enzyme is a 'perfect' catalyst, in the sense that any further acceleration of the chemical steps will not affect the rate of the overall reaction.[27-29]

C. DETECTION AND KINETIC CHARACTERIZATION OF UNLIGANDED ENZYMIC FORMS

Catalysis is cyclic in that, after facilitating the conversion of substrate to product, the catalyst must return to the form that accepts substrate so the next cycle can begin. The regeneration of the substrate-accepting form of a catalyst may not be a trivial process, especially in enzymatic reactions where the transformation of substrate to product can involve a variety of molecular events.[30] This regeneration can, in principle, occur during or after the release of product. If regeneration occurs during product release, then the catalytic pathway need contain only one form of unliganded enzyme that binds either the substrate or the product. In contrast, if enzyme regeneration occurs after the release of product, then two forms of unliganded enzyme are required, one of which binds the substrate and the other of which binds the product.[31]

One method that can detect the intermediacy of two enzyme forms is the tracer perturbation approach of Britton,[32,33] which was applied to catalysis by triosephosphate isomerase as follows.[34] A small quantity of ^{14}C-labeled dihydroxyacetone phosphate and glyceraldehyde phosphate, preequilibrated in the presence of isomerase, was perturbed by the addition of a relatively large amount of unlabeled glyceraldehyde phosphate. As the concentration of unlabeled glyceraldehyde phosphate fell to its equilibrium value, the concentration of *labeled* glyceraldehyde phosphate was observed to increase transiently, as shown in Figure 6. In other words, a transient flux of labeled material occurred in the direction *opposite to* that of the (much larger) flux of unlabeled material. This 'countertransport' of labeled material demonstrates that triosephosphate isomerase exists in two unliganded forms: (1) e_D, which binds and isomerizes dihydroxyacetone phosphate, and (2) e_G, which binds and isomerizes glyceraldehyde phosphate. During the tracer perturbation experiment, e_D and e_G are initially at equilibrium. This equilibrium is perturbed when the large amount of glyceraldehyde phosphate drives e_G over transition states 4, 3, 2, and 1 (see Figure 3) to e_D. The increased ratio of e_D to e_G leads to an increase in the relative rate of the bimolecular reaction of e_D with radiolabeled dihydroxyacetone phosphate (which, immediately after the perturbation, has a high specific radioactivity) and a commensurate decrease in that of e_G with glyceraldehyde phosphate (which has a low specific radioactivity). A flux of label from dihydroxyacetone phosphate to glyceraldehyde phosphate therefore occurs.

As shown in Figure 6, the magnitude of the observed perturbation gives a rate constant of about 10^6 s^{-1} for the interconversion of e_D and e_G. Although the results of

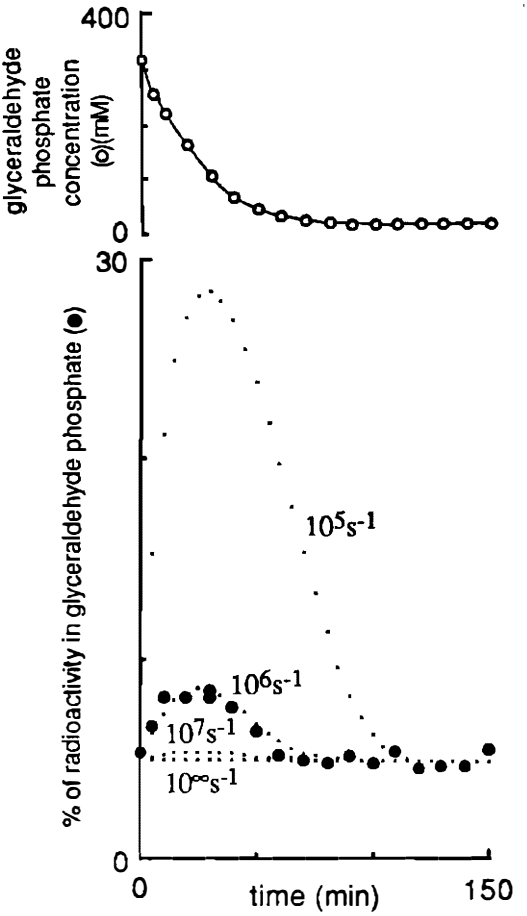

FIGURE 6. Tracer perturbation experiment for the reaction catalyzed by triosephosphate isomerase: time course after perturbation of an equilibrated mixture of [^{14}C]triose phosphates by addition of unlabeled glyceraldehyde phosphate. Dotted lines are data calculated using the indicated rate constants for the interconversion of unliganded forms of isomerase. Data are corrected for an observed change in the overall equilibrium constant upon perturbation.

the tracer perturbation experiment demand the existence of two unliganded forms of isomerase, the molecular nature of these two forms is not addressed by this experiment. Nevertheless, the results of the tracer perturbation experiment, combined with the rate constants for the substrate-handling steps of catalysis, complete the kinetic characterization of the catalytic cycle for triosephosphate isomerase. The reaction energetics for the enzyme are depicted in Figure 3.

D. REACTION ENERGETICS OF ACTIVE-SITE MUTANTS

Changing active-site residues using site-directed mutagenesis and observing the functional consequences can be an informative way to reveal the contribution of particular amino acid side chains to the reaction energetics.[35] With triosephosphate isomerase, the opportunity exists for a thorough evaluation of the consequences of subtle changes in critical amino acid residues, using the isotopic experiments described above. The stud-

ies described below illustrate how the use of isotopes as labels and as kinetic probes has delineated the energetic and mechanistic consequences of two mutations at the isomerase active site.

1. Replacing the Active-Site Glutamate with Aspartate: A Change in Reaction Energetics

To take a step towards defining the relationship between intermolecular distance and reaction rate, site-directed mutagenesis was used to replace Glu-165 of triosephosphate isomerase with an aspartate, thereby retaining the active-site carboxyl group but (presumably) moving it away from the substrate (see Figure 7).[36] The specific catalytic activity of this Asp-165 isomerase was found to be less than that of wild-type enzyme by a factor of about 1000.

Two experiments were performed to determine whether the removal of a methylene group from the enzyme allows the carboxylate of Asp-165 to act as a general base through an intervening water molecule.[37] First, solvent deuterium isotope effects were used to probe for an extra proton in flight in the transition state for substrate enolization in the mutant isomerase. Although the absolute magnitude of a solvent isotope effect is difficult to interpret because of unpredictable medium effects, a comparison of $k_{cat}^{H_2O}/k_{cat}^{D_2O}$ for a mutant and wild-type enzyme is more meaningful. The two isomerases displayed identical deuterium solvent isotope effects of $k_{cat}^{H_2O}/k_{cat}^{D_2O} = 1.5$, suggesting that the mutant enzyme does not recruit a water molecule but abstracts a proton directly from the substrate, as does the wild-type isomerase. Second, if the mutant isomerase acts through an intervening water molecule, then bromohydroxyacetone phosphate (a titrant for Glu-165 of the wild-type enzyme) will not inactivate the mutant isomerase as efficiently. Indeed, if Asp-165 acts as a general base, the mutant enzyme will catalyze the hydrolysis of the bromo compound. Yet, bromohydroxyacetone phosphate was found to inactivate the wild-type and mutant isomerases with the same efficiency. The mutant enzyme evidently does not act on the bromo compound through an intervening water molecule.

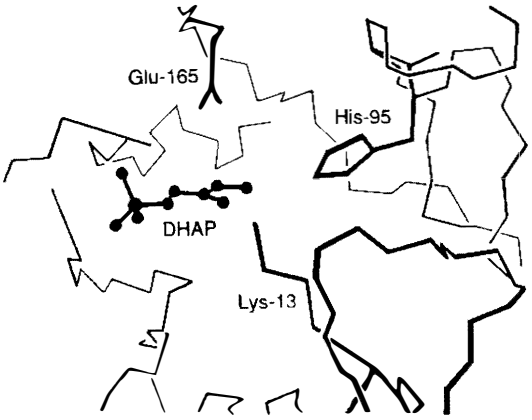

FIGURE 7. Active site of wild-type triosephosphate isomerase. The coordinates are those of the native chicken enzyme. Dihydroxyacetone phosphate has been positioned to allow Glu-165 to abstract its 1-*R* proton, His-95 to form a hydrogen bond with its hydroxyl group, and Lys-13 to polarize its carbonyl group.

The energetic details of catalysis by the Asp-165 isomerase were evaluated by the methods described above.[38,39] The results of the exchange-conversion experiments (shown in Figures 4 and 5) highlight the differences in the reaction energetics of the mutant and wild-type isomerases. When unlabeled dihydroxyacetone phosphate was the substrate for the mutant isomerase in tritiated water, essentially no radioactivity was found in the remaining dihydroxyacetone phosphate (see Figure 4). The enediol intermediate now partitions forward (crossing transition states 3 and 4) more than 30 times for every time it returns to give tritiated dihydroxyacetone phosphate. When unlabeled glyceraldehyde phosphate was the substrate for the mutant isomerase in tritiated water, the exchange-conversion plot (see Figure 5) shows that the intermediate partitions back to substrate (to give tritiated glyceraldehyde phosphate) about six times for every time it goes forward (crossing transition states 2 and 1). These exchange-conversion data indicate how much higher transition state 2 is than transition state 3 and, coupled with steady-state parameters and data from experiments that define the transfer of tritium from substrate to product and the discrimination against the appearance of solvent tritium in the product, provide the results needed to construct the free energy profile for the reaction catalyzed by the mutant isomerase. This profile is shown in Figure 8.

The mutation of Glu-165 to aspartate has made triosephosphate isomerase catalysis imperfect. The two enolization steps in the reaction have each been slowed by about three orders of magnitude, and the overall reaction is now limited by a transition state that involves covalency changes rather than one that involves substrate diffusion.

2. Replacing the Active-Site Histidine with Glutamine: A Change in Reaction Mechanism

Simple enolization reactions are known to be catalyzed by general acids as well as general bases.[40] Since the rate of substrate interconversion mediated by triosephosphate isomerase is more than 10^9 times faster than that from nonenzymatic general base catalysis,[41,42] an electrophilic component to isomerase catalysis is not unexpected.[43]

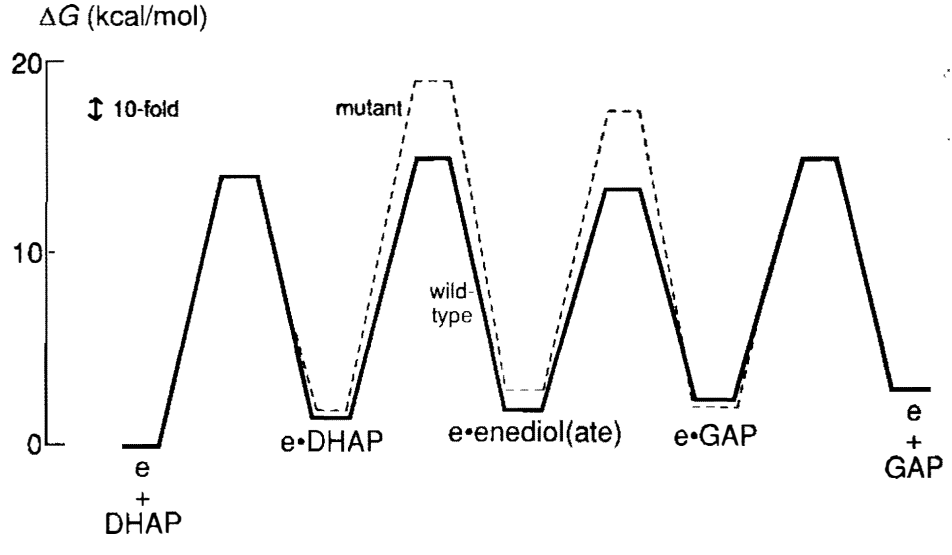

FIGURE 8. Free energy profiles for the reactions catalyzed by wild-type and Asp-165 mutant triose phosphate isomerases. The standard state for the triose phosphates is 40 μM. DHAP is dihydroxyacetone phosphate and GAP is (*R*)-glyceraldehyde 3-phosphate.

Polarization of the carbonyl group of dihydroxyacetone phosphate by the enzyme has indeed been detected by two types of experiments. First, the rate of reduction of the carbonyl group of dihydroxyacetone phosphate by borohydride is enhanced when the substrate is bound to the enzyme.[44,45] Second, the carbonyl stretching frequency of dihydroxyacetone phosphate is decreased upon binding to the enzyme.[46] Although the available crystal structures of isomerase do not allow the unambiguous identification of the putative electrophilic residue(s), Lys-13 and His-95 are good candidates (see Figure 7).

The role of His-95 in catalysis by triosephosphate isomerase was investigated by use of a mutant in which His-95 has been changed to glutamine.[47] The specific catalytic activity of this Gln-95 isomerase was found to be less than that of wild-type enzyme by a factor of nearly 400. Since analogues of the enediol(ate) intermediate of the mutant enzyme, the Gln-95 enzyme is apparently less able to stabilize this intermediate.

The evaluation of the reaction energetics of the Gln-95 isomerase proceeded as follows. In the transfer experiment with [1(*R*)-^{3}H]dihydroxyacetone 3-phosphate as the substrate, even less tritium was transferred to product in the reaction catalyzed by the mutant isomerase[47] than in that catalyzed by the wild-type enzyme.[48] With [2-^{3}H]glyceraldehyde phosphate as substrate, no significant transfer of tritium to the product was observed. In both cases, the remaining substrate became enriched in tritium in the course of the mutant enzyme-catalyzed conversion.

In the exchange-conversion experiment with unlabeled dihydroxyacetone phosphate as substrate for the mutant isomerase, little solvent tritium was incorporated back into the remaining substrate. Since the results of the transfer experiments indicate that the intermediate is in rapid isotopic exchange with solvent, the absence of solvent tritium incorporation into the remaining substrate dihydroxyacetone phosphate could suggest that the intermediate partitions forward to product glyceraldehyde phosphate. If the intermediate partitions to glyceraldehyde phosphate, then when the exchange-conversion experiment is performed with glyceraldehyde phosphate as the substrate, the C-2 proton of glyceraldehyde phosphate will preequilibrate completely with the labeled solvent protons, and the specific radioactivity of tritium in the remaining substrate will rapidly rise to a value approaching that of the solvent. Yet, when this experiment was performed, little solvent tritium was incorporated into the remaining substrate glyceraldehyde phosphate.

The observed lack of incorporation of solvent tritium into the remaining substrate regardless of the direction in which the reaction is run, as well as the enrichment of tritium in each remaining substrate when labeled substrates are used, could be explained by large tritium kinetic isotope effects on each of the enolization processes. The size of these isotope effects is set by the results of the 'discrimination' and deuterium kinetic isotope effect experiments. With dihydroxyacetone phosphate as substrate, the discrimination against tritium incorporation into the product was only 4.2-fold (while $k_{cat}^H/k_{cat}^D = 2.4$); when glyceraldehyde phosphate was the substrate, the discrimination against tritium in the product was only threefold (while $k_{cat}^H/k_{cat}^D = 2.2$). These isotope effects are too small to account for the anomalously slow entry of solvent tritium into the remaining substrate in each direction.

The results for the Gln-95 mutant seem to be best accomodated by a changed mechanism, in which Glu-165 plays the two roles depicted in Figure 9. First, Glu-165 abstracts the 1-*R* proton of dihydroxyacetone phosphate (now without the catalytic assistance provided by histidine) to give enediolate A. The enediolate is then rapidly reprotonated by Glu-165 on O-2 to form enzyme-bound enediol B. Glu-165 then proceeds to remove the O-1 proton of the enediol to give enediolate C, which is rapidly reprotonated by Glu-165 on C-2 to form the product glyceraldehyde phosphate. In the

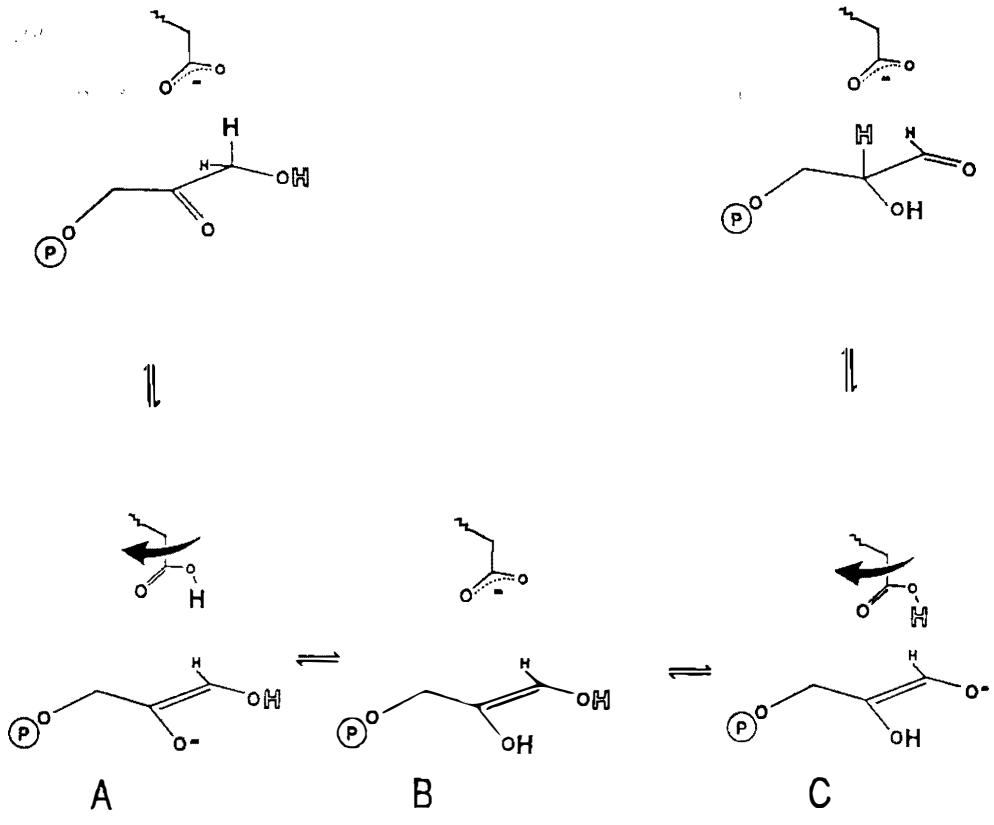

FIGURE 9. Proposed mechanism for the reaction catalyzed by Gln-95 mutant triosephosphate isomerase. Glu-165 abstracts the 1-*R* proton of dihydroxyacetone 3-phosphate to give the enediolate A, which collapses to the enediol B. Glu-165 then abstracts the other oxygen-bound proton to yield the enediolate C, which collapses to (*R*)-glyceraldehyde 3-phosphate.

reaction catalyzed by the mutant isomerase, Glu-165 apparently shuttles protons between C-1 and O-2, and between O-1 and C-2.

The mechanism of Figure 9 accounts for the isotopic labeling patterns observed for the mutant isomerase. First, tritium is not transferred from substrate to product because the new carbon-bound proton of the product derives from the substrate hydroxyl group. Second, the appearance of solvent tritium in either product is minimal, since the specific radioactivity of product will reflect that of the solvent, modulated only by the tritium isotope effect in the proton-transfer step (via A to dihydroxyacetone phosphate, or via C to glyceraldehyde phosphate). Third, solvent tritium does not exchange into the remaining substrate in either direction because the conjugate acid of Glu-165 (A or C in Figure 9) does not exchange its proton with solvent faster than it is deprotonated by the neighboring enolate.

As with the movement of the active-site base that occurred on changing Glu-165 to aspartate, removal of an active-site electrophile by changing His-95 to glutamine dramatically slows the enolization steps of the triosephosphate isomerase reaction. In addition, removing His-95 has forced a subtle change in mechanism that becomes evident from the results of the isotopic experiments described.

III. PROLINE RACEMASE

Most kinetic investigations of enzyme-catalyzed reactions are performed *in vitro* under irreversible conditions: either the reaction is followed to a few percent completion in the exergonic direction or the concentration of product is kept at low levels by the use of coupling enzymes. The results of experiments performed under these conditions may not be relevant *in vivo* where, for most enzymes, the concentration of substrates and products is maintained close to the equilibrium concentrations.[49] (In the glycolytic pathway, for instance, the substrate and product concentrations of only 3 of the 12 enzymes are substantially displaced from the equilibrium levels.) Indeed, kinetic events that are undetected by experiments performed under irreversible conditions may dominate the behavior of a system. Such kinetic events are most readily characterized for an enzymatic reaction in which the approach to equilibrium can be eaily monitored. Proline racemase (E. C. 5.1.1.4) is such an enzyme.

Proline racemase catalyzes the interconversion of (*R*)- and (*S*)-proline (or D- and L-proline, respectively)(Figure 10).[50] Anaerobic bacteria use this enzyme along with (*R*)-proline reductase to convert (*S*)-proline into δ-aminovaleric acid, a process that is coupled to the oxidation of other amino acids. Although amino acid racemases usually require the cofactor pyridoxal 5′-phosphate, which labilizes the α-proton by forming an aldimine with the α-amino group, such a pathway is not followed for imino acids such as proline. The enzyme proline racemase from the bacterium *Clostridium stricklandii* is a dimer of identical subunits, each of molecular weight 38,000; it has no cofactor requirement and it contains a single binding site per dimeric unit.[51,52] The racemization of either of the proline enantiomers can be followed precisely and continuously by the change in optical rotation.

A. ISOTOPIC LABELING TO REVEAL MECHANISM

Preliminary work by Cardinale and Abeles[51] and Rudnick and Abeles[52] illuminated the mechanism of proline racemase catalysis. First, incubating proline with the enzyme in deuterated or tritiated water resulted in the incorporation of solvent isotope at C-2 of the substrate. The racemase can therefore be presumed to catalyze the exchange of protons, and not of hydrogen atoms or hydride ions. Second, when the enzyme-catalyzed racemization of one proline enantiomer was quenched after a small extent of reaction, solvent isotope was found in the product enantiomer but not in the substrate. Hence, unlike the reaction catalyzed by triosephosphate isomerase (see Section II), substrate-derived protons do not equilibrate with solvent protons at an intermediate stage of the reaction catalyzed by proline racemase. The observation that no solvent isotope appears in remaining substrate in either direction (that is, when either *S*- or *R*-proline is the substrate) eliminates a mechanism in which a single enzymic base abstracts the C-2 proton from the substrate and (after exchange with solvent) delivers a

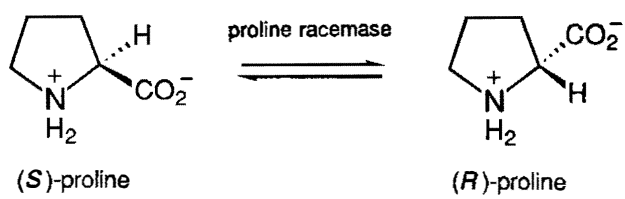

FIGURE 10. The reaction catalyzed by proline racemase.

solvent-derived proton to the putative carbanion to form the product. This finding is consistent, however, with a two-base mechanism in which an enzymic base abstracts the substrate's C-2 proton from one face of the proline ring, and the conjugate acid of another enzymic base delivers a solvent-derived proton to the other face. (Similar studies indicate the importance of two enzymic bases in catalysis by hydroxyproline epimerase,[53] methylmalonyl-CoA epimerase,[54,55] diaminopimelic acid epimerase,[56] and, probably, mandelate racemase.[57]) Third, although the rate at which tritium was released into the solvent from [2(*S*)-^{3}H]proline was not reduced by increasing the concentration of (*S*)-proline, the rate at which tritium was released from [2(*R,S*)-^{3}H]proline did decrease at high concentrations on (*R,S*)-proline. In other words, to capture the enzyme-bound proton derived from one proline enantiomer, the other enantiomer must be present. Thus, the form of the enzyme produced after the release of one proline enantiomer cannot bind the other enantiomer. As shown in Figure 11, the catalytic cycle for proline racemase must include two unliganded forms of the enzyme: (1) e_S, which binds and inverts the configuration of (*S*)-proline, and (2) e_R, which binds and inverts (*R*)-proline. Finally, the ability of the product enantiomer to capture a substrate-derived proton indicates that the interconversion of the two proline isomers must be faster than the release of the substrate-derived proton into the solvent; and the inability of the substrate enantiomer to capture a substrate-derived proton indicates that the release of the substrate-derived proton into the solvent must be at least as fast as the interconversion of the two unliganded forms of the enzyme.

In 1986, Albery and Knowles built on the early work of Abeles and colleagues and performed a series of isotopic experiments on proline racemase to explore the mechanism and energetics of catalysis under reversible conditions, and to illuminate the phenomenon of enzyme interconversion.[58] The results of these experiments are qualitatively presented here (and elsewhere[59]). The general equations that quantitatively describe enzymatic catalysis under reversible conditions have been published.[60]

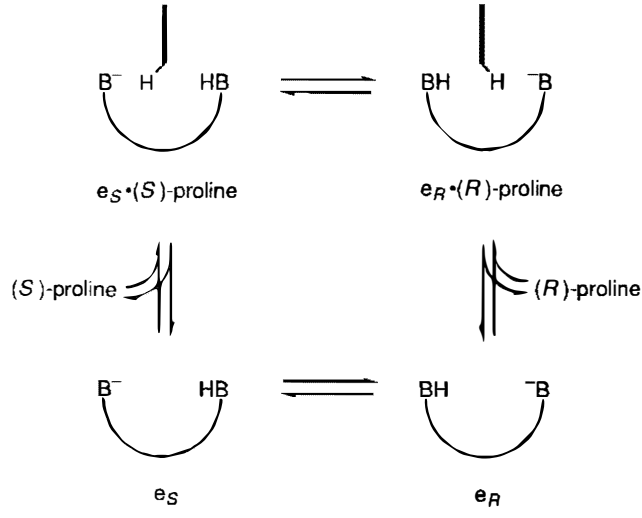

FIGURE 11. Minimal scheme for catalysis by proline racemase. The proline ring is represented edge-on as a heavy bar.

B. DEUTERIUM KINETIC ISOTOPE EFFECTS

The values of k_{cat} and K_m for proline racemase are essentially the same for (*R*)- and (*S*)-proline.[58] This identity is not prescribed, of course, since the complexes of the enzyme with (*R*)-proline and with (*S*)-proline are diastereomeric. Nevertheless, this observation suggests that the free energy profile for proline racemization is rather symmetrical. Since the second-order rate constant, k_{cat}/K_m for proline racemase is below the diffusion limit, the rate-limiting transition state is expected to be that for proton transfer (if the concentration of product is low, see below). Observable kinetic isotope effects (KIEs) are therefore expected for the reaction catalyzed by proline racemase.

The existence of a KIE in the reaction catalyzed by proline racemase was graphically demonstrated by Cardinale and Abeles,[51] who followed the racemization of (*S*)-proline in D_2O. As shown in Figure 12, the optical rotation is initially negative, becomes zero, is then positive, and finally approaches zero again. This transient 'overshoot' is the manifestation of a KIE. (An overshoot was not observed in H_2O, see Figure 12.) Initially, unlabeled (*S*)-proline substrate is converted into deuterated (*R*)-proline product, the conversion of which back to deuterated (*S*)-proline is slowed by a deuterium KIE. The rate constant for the reaction of *un*labeled (*S*) enantiomer exceeds that for the reaction of labeled (*R*) enantiomer and the observed optical rotation changes sign. Eventually, isotopic exchange between proline and the solvent becomes complete, the two (now fully labeled) enantiomers achieve equilibrium, and the optical rotation becomes zero.

The most direct method of measuring the magnitude of a KIE on an enzyme-catalyzed reaction is to compare the velocity of the reaction of labeled substrate with

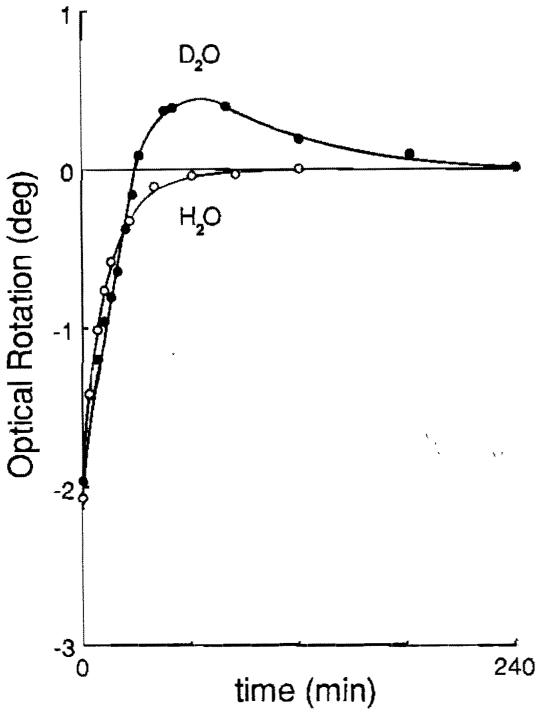

FIGURE 12. Proline racemase-catalyzed reaction of (*S*)-proline in D_2O and in H_2O. The concentration of racemase is 1.2-fold larger in the D_2O reaction than in H_2O.

that of unlabeled substrate. The absence of coupling enzymes for proline racemase means, however, that the measurement is more rapidly made under reversible conditions. To determine the deuterium KIE, the rate of loss of deuterium from one (labeled) substrate is compared with the rate of turnover of the other (unlabeled) substrate in a 'competitive deuterium washout' experiment.[61] In this experiment on proline racemase, enzyme is added to a solution containing equal concentrations of the proline enantiomers, only one of which is deuterated at C-2. Since the reaction of the deuterated substrate to its enantiomer proceeds more slowly than that of the unlabeled substrate to its enantiomer, an imbalance in the concentrations of the two enantiomers is generated, and the optical rotation diverges from zero, as shown in Figure 13. Ultimately, all the deuterium label is lost to solvent and the rotation returns to zero as the unlabeled enantiomers reach both chemical and isotopic equilibrium. The size of the perturbation in the optical rotation that results from a particular concentration of proline gives a deuterium KIE of 3.1 for (*S*)-proline and 2.7 for (*R*)-proline. (The deuterium KIEs are calculated from transition-state fractionation factors of 0.375 and 0.44 for the reactions of (*S*)- and (*R*)-proline, respectively, and a ground-state fractionation factor of 1.17 for each enantiomer. The deuterium fractionation factor of a molecular site is defined as the affinity of deuterium for that site relative to a site in bulk water, the arbitrary standard for comparison. The deuterium KIE is therefore given by the ratio of the fractionation factor of the ground state to that of the transition state: $k_H/k_D = \phi_{g.s.}/\phi_{t.s.}$).

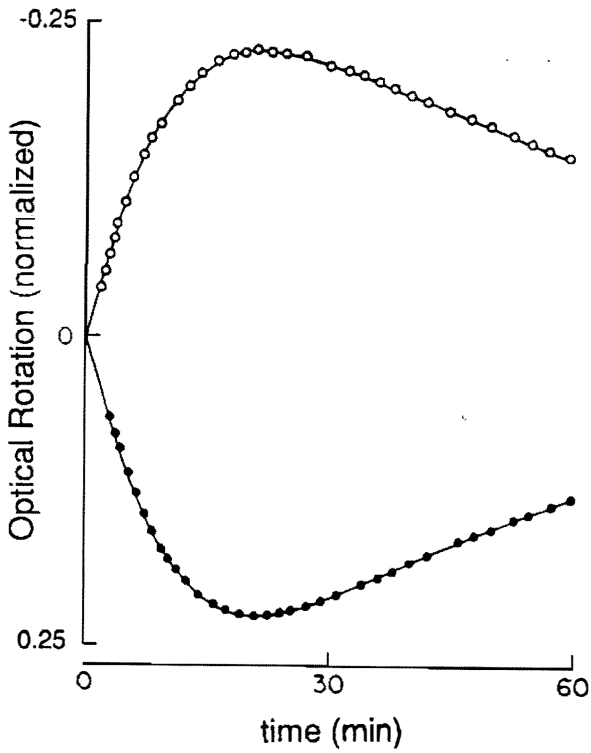

FIGURE 13. Competitive deuterium washout experiment for the reaction catalyzed by proline racemase: time course after addition of enzyme to (*S*)- (●) or (*R*)-[2-^{2}H]proline (○) in the presence of an equimolar amount of the unlabeled proline enantiomer.

Although $^{D}K_{eq}$ necessarily equals unity for a racemase reaction, the observed deuterium KIEs differ for the *R* and the *S* substrates. This difference does not contravene the Haldane relationship, however, because these isotope effects are on k_{cat}, not k_{cat}/K_m. The difference in the deuterium KIEs simply indicates that the e_R·[2(*R*)-^{2}H]proline complex reacts about 17% faster than does the diastereomeric e_S·[2(*S*)-^{2}H]proline complex.

The magnitude of the deuterium KIE for the interconversion of unliganded e_S and e_R was also measured. A competitive deuterium washout experiment was performed in which the total concentration of proline was so high that the transition state for the interconversion of e_S and e_R [rather than that for the interconversion of e_S·(*S*)-proline and e_R·(*R*)-proline, see Figure 14] was rate limiting. Again, a transient perturbation in the optical rotation was observed. The size of the perturbation that resulted from a particular concentration of proline demands a deuterium KIE of 2.4 for the reaction of e_S and 2.0 for that of e_R (as calculated from transition-state fractionation factors of 0.42 and 0.49, respectively).

C. KINETIC CHARACTERIZATION OF UNLIGANDED ENZYMIC FORMS

A two-base mechanism requires that proline racemase exist in two unliganded forms that differ at least in the position of a proton. Nevertheless, the interconversion of these two forms need not be kinetically insignificant. To investigate this question, the tracer perturbation method of Britton[32,33] was used to probe the kinetic importance of the two forms of proline racemase.[62] An equilibrated mixture of (*R,S*)-[^{14}C]proline in the presence of proline racemase was perturbed by the addition of a relatively large amount of unlabeled (*S*)-proline. A transient flux of ^{14}C meterial from (*R*)- to (*S*)-proline was observed as the enzyme catalyzed the equilibration of the added unlabeled (*S*)-proline. The existence of 'countertransport' established that the interconversion of the two forms of unliganded racemase can be kinetically significant. From the maximum in the tracer perturbation plot, a rate constant for this interconversion of 10^5 s^{-1} was obtained.

When the tracer perturbation experiment was repeated in ammonium carbonate buffer (instead of the Tris buffer used previously), no perturbation in the distribution

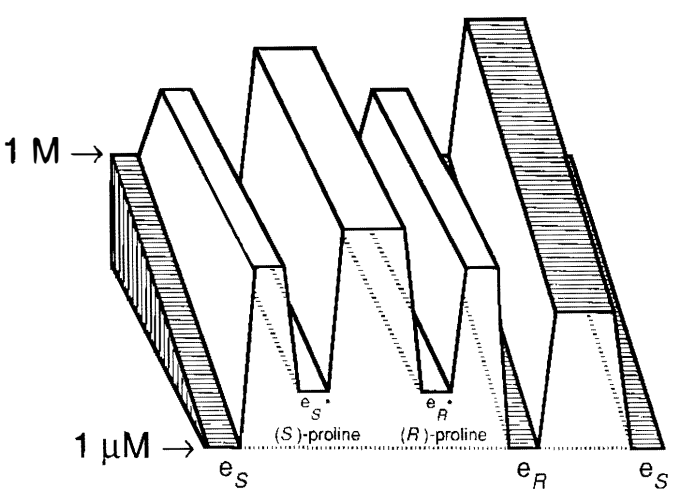

FIGURE 14. Free energy profile for the reaction catalyzed by proline racemase. The standard state for proline is as indicated.

of the ^{14}C material was detected. A change in buffer ion is unlikely to accelerate a conformational change in the protein. On the other hand, ammonium ion can, in principle, catalyze the interconversion of e_S and e_R, and a simple model where the two forms of the protein differ only in protonation state is therefore attractive. This model is consistent with the results of kinetic studies that demonstrate catalysis of the interconversion of e_S and e_R by other small buffer species with pK_a values close to 7.[63]

D. NATURE OF THE INTERCONVERSION OF UNLIGANDED ENZYMIC FORMS

The tracer perturbation experiment described above shows that the two unliganded forms of proline racemase, e_S and e_R, interconvert with a rate constant of about $10^5\ s^{-1}$. Additional isotopic experiments were performed to address the nature of this interconversion. Two questions are apparent: (1) can the two forms of unliganded enzyme exchange protons directly (that is, can labeled e_R be converted into labeled e_S in unlabeled water); and (2) when a proton is released from one form of the enzyme, is the initial product the same form or the other form of the enzyme (that is, is labeled e_R converted into unlabeled e_S when the label washes out)?

The issue of direct transfer of a proton between the enzymic bases was addressed by determining how much tritium is transferred to (*R*)-proline when (*S*)-[2-^{3}H]proline is racemized in ^1H_2O.[61] If label is indeed transferred between the enzymic bases, then the tritium in e_R that is formed from (*S*)-[2-^{3}H]proline and e_S will migrate across the active site to give tritiated e_S which, on reaction with a new molecule of (*S*)-proline, will yield (*R*)-[2-^{3}H]proline. When this possibility was investigated experimentally, less than 0.1% of the tritium initially in (*S*)-[2-^{3}H]proline was found to be transferred to (*R*)-proline. The direct transfer of protons between the two enzyme bases is evidently insignificant.

The initial enzymic product formed upon the release of a proton from one enzymic form was identified by determining whether labeled e_S results in unlabeled e_R or unlabeled e_S.[63] As before,[61] a competitive deuterium washout experiment was performed in which the concentration of proline was so high that the transition state for the interconversion of e_S and e_R was rate limiting (see Figure 14). Here, racemase was added to an equimolar mixture of deuterated (*R*)-proline and unlabeled (*S*)-proline, such that an imbalance was generated in the concentrations of the two proline enantiomers [towards (*R*)-proline] and (presumably) in the concentration of the two enzyme forms (towards e_R). The fate of labeled e_S was determined by comparing the rate of substrate racemization with that of deuterium release into solvent. Qualitatively, if the release of deuterium from labeled e_S produces unlabeled e_S, which must then cross the rate-limiting transition state of enzyme interconversion for the equilibration of (*R*)- and (*S*)-proline to proceed, the rate of deuterium release will exceed that of substrate racemization. On the other hand, if the release of deuterium from e_S produces unlabeled e_R, then the rate of deuterium release and of substrate racemization will be similar, because both processes require crossing the rate-limiting transition state. The latter result was found experimentally in both this and the converse experiment with deuterated (*S*)-proline and unlabeled (*R*)-proline. Thus, the deprotonation of one unliganded form of proline racemase evidently involves a commitment to crossover to the other unliganded form.

The isotopic work described above reveals the similar nature of the interconversion of e_S and e_R as mediated by proline [via e_S·(*S*)-proline and e_R·(*R*)-proline], or small buffer species. First, each of these reactions involves a commitment to crossover to the other enzymic form, in that deuterated e_S reacts with proline, water, or appropriate buffer ions to yield *only* unlabeled e_R. Second, the transition-state fractionation factors for the proline-mediated and water-mediated reactions of e_S are similar, as are those for

the proline-mediated and water-mediated reactions of e_R. Third, the transition state fractionation factor for e_S is 17% greater than that for e_R for each kind of reaction. Thus, the racemization of substrate occurs with the switch of the enzyme-bound protons illustrated in Figure 11, and the interconversion of the unliganded enzyme forms occurs by an analogous water-mediated switch of the same enzyme-bound protons. This parallelism is illustrated by the scheme shown in Figure 15.

E. CONCERTEDNESS OF THE CATALYZED REACTION

The work of Cardinale and Abeles established a two-base mechanism for the reaction catalyzed by proline racemase.[51] Their results cannot, however, be used to distinguish a concerted mechanism involving synchronous proton transfers from a stepwise mechanism involving sequential proton transfers and a carbanion intermediate. To make this more subtle distinction, concert in catalysis was probed by performing a 'double fractionation experiment' in which the racemase reaction was run in each direction in mixed H_2O-D_2O, and the effect of substrate deuteration on discrimination against the appearance of solvent deuterium in the product was ascertained.[64,65] (A discussion of the theoretical basis for this effect, and of the inauspicious propensity for coupling between the protonic motions, is presented in Chapter 4.) In either a concerted or stepwise reaction, the incorporation of solvent deuterium into the product is discriminated against. If the reaction is concerted, no change will occur in the magnitude of this discrimination because the abstraction of a hydrogen or a deuterium by one enzymic base coincides with the delivery of a hydrogen or deuterium (whichever hap-

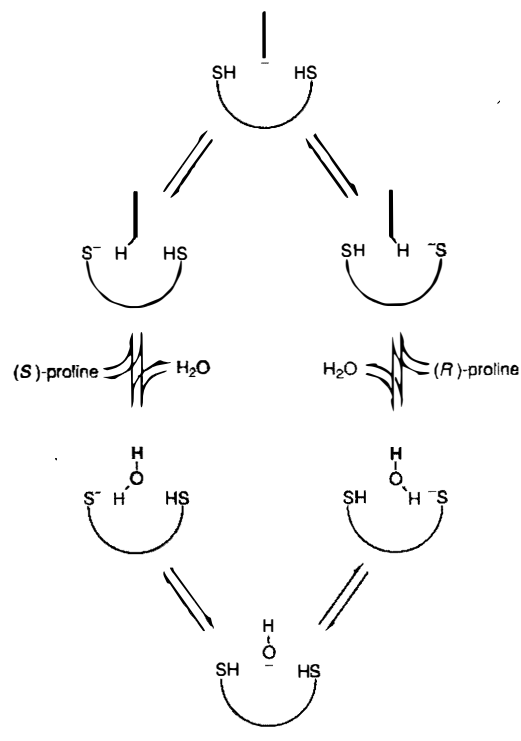

FIGURE 15. The mechanism of the reaction catalyzed by proline racemase. The two catalytic bases are cysteine residues. The proline ring is represented edge-on as a heavy bar.

pens to be bound) from the other enzymic base. The deuterium content of the product will therefore be the same, whether or not the substrate is deuterated. In contrast, if the reaction is stepwise, the abstraction of a deuterium from the substrate (which is slower than the abstraction of a hydrogen because of the deuterium KIE) makes the subsequent protonation step less rate limiting. The deuterium content of the product therefore increases because of the increased commitment to accept whatever (either a deuterium or a hydrogen) is bound to the enzymic base. When the experiment was performed with proline racemase, the reactions of (*R*)-[2-^{1}H]-and (*R*)-[2-^{2}H]-proline both showed solvent isotope effects of 3.2. Deuterium substitution in the substrate evidently does not affect the discrimination against solvent deuterium incorporation into the product.

The equivalence of the two solvent isotope effects is consistent with a concerted reaction. The actual magnitudes of these isotope effects are, however, also consistent with a stepwise reaction in which the enzymic bases are the sulfhydryl groups of cysteine residues. Compared to proline, a sulfur base has an inherent discrimination against deuterium of about twofold,[66,67] which is similar to the observed deuterium KIEs. If the two bases at the active site of proline racemase are indeed cysteines, then the fractionation factor for the solvent-derived proton, when it is in flight, will be similar to that when it is bound to the enzyme. Thus, as in a concerted mechanism, substrate deuteration will slow the rate of the reaction but will not alter the discrimination against the appearance of solvent deuterium in the product. To distinguish between a concerted reaction and a stepwise reaction catalyzed by sulfhydryl bases, the inherent discrimination against deuterium of the enzymic bases was determined directly.

One way to measure the ground-state fractionation factors of the enzymic bases is to compare the effect of D_2O on reaction velocity with the discrimination against the incorporation of solvent deuterium into the product. The KIE is given by the ratio of the fractionation factor of the ground state to that of the transition state, and the discrimination against the incorporation of solvent deuterium depends only on the transition-state fractionation factor. These data therefore allow calculation of the ground-state fractionation factor, which for proline racemase was found to be 0.44.[68] Since the interpretation of measurements of reaction velocities in D_2O can be severely compromised by medium effects of unknown magnitudes, the inherent discrimination against deuterium of the enzymic bases was also measured by a different method.

A more subtle way to introduce deuterium to an enzymic base is by a molecule of deuterated substrate in ^1H_2O. The time courses of two competitive deuterium washout experiments were therefore compared to determine the fractionation factors of the two essential active-site bases of proline racemase.[68] The rate of achievement of the maximum perturbation in the optical rotation was measured under two conditions: (1) with an equimolar mixture of deuterated substrate and unlabeled product, and (2) with an equimolar mixture of substrate and product in which only 20% of the substrate was deuterated. Both experiments were performed under conditions where the proline concentration was so high that catalysis required reaction from an equilibrated pool of liganded enzyme [e_S·(*S*)-proline and e_R·(*R*)-proline] over the transition state for the interconversion of the two free forms of the enzyme (e_S and e_R, See Figure 14). The size of the maximal perturbation of the optical rotation depends only on the initial concentration of labeled substrate. On the other hand, different concentrations of deuterated substrate produce different levels of deuteration of the enzymic bases, and the *time* taken for the perturbation to achieve its maximum depends on the fractionation factors of the equilibrated pool of liganded enzyme. The value observed for the fractionation factors of each enzymic base was 0.55, which is consistent with these groups being

thiols. This conclusion is supported by the value of the deuterium solvent isotope effect, by kinetic studies not employing isotopes,[63] and by chemical modification work.[52]

The results of the double fractionation experiment, coupled with those of the appropriate competitive deuterium washout experiment, are best described by a mechanism in which proline racemase catalyzes a stepwise reaction using two active-site cysteine residues, as shown in Figure 15. The e_S thiolate first abstracts a proton either from the (*S*)-proline enantiomer to give an intermediate in which a prolyl carbanion is bound to the diprotonated enzyme, or from water to give an intermediate in which hydroxide ion is bound to the diprotonated enzyme. The intermediate species then collapses to generate the (*R*)-proline enantiomer (from the enzyme-prolyl carbanion complex) or e_R (from the enzyme-hydroxide ion complex).

F. FREE ENERGY PROFILE FOR THE CATALYZED REACTION

The isotopic work described above suggests that the reaction catalyzed by proline racemase proceeds by the mechanism shown in Figure 15. By building upon this framework with kinetic measurements of the interconversion of (*S*)- and (*R*)-proline and of e_S and e_R, the free energy profile in Figure 14 can be constructed.[69] The third dimension of Figure 14, is used to illustrate the consequence of varying the *total* concentration of proline [that is, the sum of the proline concentrations in an equilibrium mixture of (*S*)- and (*R*)-proline]. This representation shows that enzyme interconversion becomes rate limiting at high proline concentrations under reversible conditions, a phenomenon often neglected in other enzymatic systems. Indeed, the results of isotopic experiments performed at high concentrations of proline have led to: (1) the detection and kinetic characterization of two forms of unliganded enzyme, (2) the definition of the nature of the interconversion of these two forms, and (3) the demonstration of the participation of thiol groups as the two active-site bases, which is consistent with an enzyme-bound prolyl carbanion and an enzyme-bound hydroxide ion as intermediates in the two pathways for the interconversion of e_S and e_R.

IV. ENVOI

Enzymologists can often exploit avenues other than substrate labeling to introduce isotope into the course of an enzyme-catalyzed reaction. For example, triosephosphate isomerase transfers a proton via an intermediate that exchanges its proton with the solvent, thereby allowing isotope from the solvent to be fed into the middle of the reaction pathway. Proline racemase delivers either a solvent- or product-derived proton to the substrate, enabling isotope to enter the reaction pathway from the solvent or from the product (in a reaction performed under reversible conditions). Analogous mechanistic avenues are open in a large number of other enzyme-catalyzed reactions. As described in this chapter, the use of deuterium or tritium as labels and as kinetic probes has led to the revelation of the complete reaction energetics for catalysis by triosephosphate isomerase and proline racemase. Similar isotopic experiments can be used to illuminate mechanistic and energetic details of catalysis by other enzymes as well.

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