Understanding Nature's Strategies for Enzyme-Catalyzed Racemization and Epimerization

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Received August 23, 2001

ABSTRACT
Epimerases and racemases are enzymes that catalyze the inversion of stereochemistry in biological molecules. In this article, three distinct examples are used to illustrate the wide range of chemical strategies employed during catalysis, and the diverse set of ancestors from which these enzymes have evolved. Glutamate racemase is an example of an enzyme that operates at an "activated" stereocenter (bearing a relatively acidic proton) and employs a nonstereospecific deprotonation/reprotonation mechanism. UDP−*N*-Acetylglucosamine 2-epimerase acts at an "unactivated" stereocenter and uses a mechanism involving a nonstereospecific elimination/addition of UDP. L-Ribulose phosphate 4-epimerase also acts at an unactivated stereocenter and uses a nonstereospecific retroaldol/aldol mechanism.

Racemases and Epimerases

A widely recognized property of enzymes is their ability to catalyze reactions with an extremely high degree of stereospecificity. The inherent chirality of enzymes and their ability to orient both substrates and catalytic residues permit these catalysts to preferentially stabilize one stereoisomeric transition state over another. This specificity is partnered with the general trend in nature to utilize only one enantiomer of a given molecule in essential biochemical pathways (e.g., L-amino acids and D-sugars). In many instances, however, an organism benefits from the ability to use amino acids and sugars of unusual stereochemistry as biosynthetic building blocks or metabolic precursors. An efficient way of generating or degrading such compounds is through the use of epimerases and racemases, enzymes that are capable of catalyzing inversions of stereochemistry.[1,2] The requirements imposed on this family of enzymes are somewhat different from those discussed above, since they must evolve to stabilize two stereoisomeric transition states and thereby exhibit a pronounced lack of stereospecificity.

To catalyze an inversion of stereochemistry, an epimerase or racemase must break and reform a bond in a nonstereospecific manner. In a typical biological molecule, this process could involve a carbon−hydrogen bond, a carbon−heteroatom (N or O) bond, or a carbon−carbon bond (Figure 1). This has interesting implications with regard to the evolution of these enzymes, since essentially any enzyme capable of catalyzing bond cleavage at a stereogenic carbon atom could potentially evolve into an epimerase. The catalytic machinery required to break (and hence, remake) the bond would already exist, and it would remain for evolutionary changes to engineer in the lapse of stereospecificty. In many cases, the evolutionary origins of these enzymes have become clear from studies on structure and mechanism.[3−9]

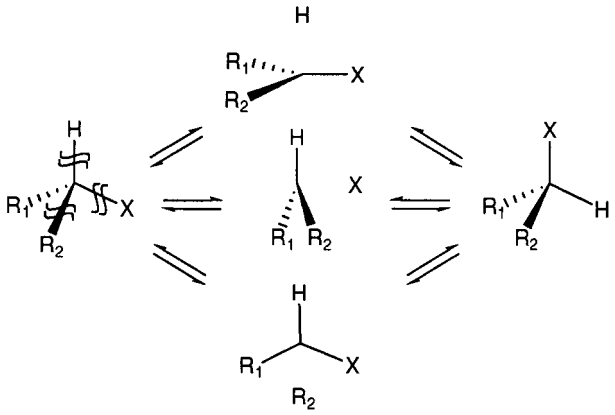

FIGURE 1. Three strategies for the inversion of stereochemistry (X = OH or NH_2).

The majority of racemases and epimerases act at a stereogenic center adjacent to a carbonyl functionality and reversibly cleave a C−H bond.[1] The pK_a of the hydrogen is lowered as a result of resonance stabilization of the resulting anion, allowing the enzymes to utilize a deprotonation/reprotonation mechanism. This is the case for the amino acid racemases as well as many sugar epimerases in which the center is adjacent to an aldehyde, ketone, or carboxylic acid functionality. In this article, such substrates will be referred to as "activated" and the reaction catalyzed by the enzyme glutamate racemase will serve as an example. A smaller subset of racemases and epimerases operate at stereogenic centers that lack acidic protons (pK_a > 30) and must employ different catalytic strategies. UDP−*N*-acetylglucosamine 2-epimerase and L-ribulose 5-phosphate 4-epimerase will serve as examples of enzymes that accept "unactivated" substrates.

Racemization at an Activated Center: Glutamate Racemase

The 19 optically active amino acids required to produce ribosomally derived polypeptides and proteins are all of the L configuration. The D-amino acids, however, also play very important roles in the biology of both prokaryotes and eukaryotes. Notable examples include D-alanine and D-glutamate that are components of the bacterial cell wall and D-serine that serves as an agonist of the NMDA receptor in the human brain. In the vast majority of cases, these D-amino acids are derived from the L-amino acids by the action of racemases and epimerases.[1]

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10.1021/ar000056y CCC: $22.00
Published on Web 03/23/2002

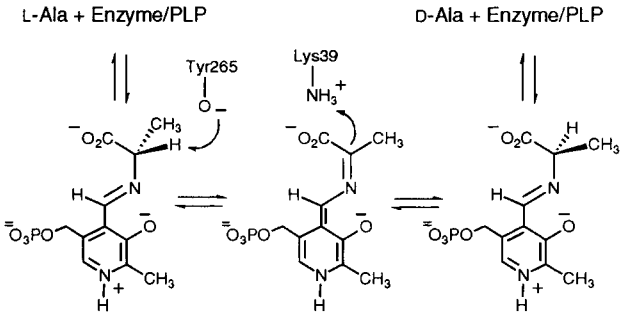

FIGURE 2. The mechanism employed by alanine racemase.

All known amino acid racemases operate via an initial deprotonation of the substrate's α-proton, followed by a reprotonation on the opposite face of the resulting planar anionic intermediate. Despite the increased acidity of this proton over those at unactivated centers, this is a difficult task. In the fully protonated form of the amino acid, the pK_a of the α-proton is ∼21; however, in solution at neutral pH, this value would be considerably higher because of the presence of the carboxylate anion. One way in which nature has managed to overcome this difficulty is through the use of the pyridoxal phosphate cofactor (PLP), as in the case of alanine racemase. The formation of an imine linkage between the cofactor and the substrate greatly acidifies the α-proton, since the anionic intermediate is stabilized by resonance (Figure 2).[10]

Despite the obvious logic of utilizing the PLP cofactor in this fashion, there exists a reasonably large subset of amino acid racemases/epimerases that operate in a cofactor-independent manner. The first of these enzymes to be studied in detail was proline racemase.[11,12] Subsequently, the enzymes glutamate racemase, aspartate racemase, and diaminopimelate epimerase have all been found to utilize a similar mechanism.[1,13]

Initial studies on the glutamate racemase reaction supported a deprotonation/reprotonation mechanism. The complete racemization of glutamate in a deuterated buffer led to the incorporation of deuterium at C-2.[14,15] This experiment supports the involvement of a nonstereospecific proton transfer and rules out a nonstereospecific hydride transfer mechanism. Two scenarios can explain how the proton transfer takes place: the one-base mechanism (Figure 3a), and the two-base mechanism (Figure 3b). In the former, a single enzymic base deprotonates the substrate and then reprotonates the resulting anionic intermediate on the opposite face. In the latter, one enzymic base deprotonates the substrate, and a second residue in its acidic form reprotonates the opposite face. The incorporation of solvent isotope into product must be observed in a two-base mechanism, but it can also be explained with a one-base mechanism if the substrate-derived proton can be exchanged for a deuteron during the lifetime of the anionic intermediate or if the base is polyprotic (e.g., lysine). To distinguish between these possibilities, the extent of solvent isotope incorporation was studied under initial velocity conditions.[15,16] In both reaction directions, all of the initially formed product molecules contained solvent-derived deuterium, whereas

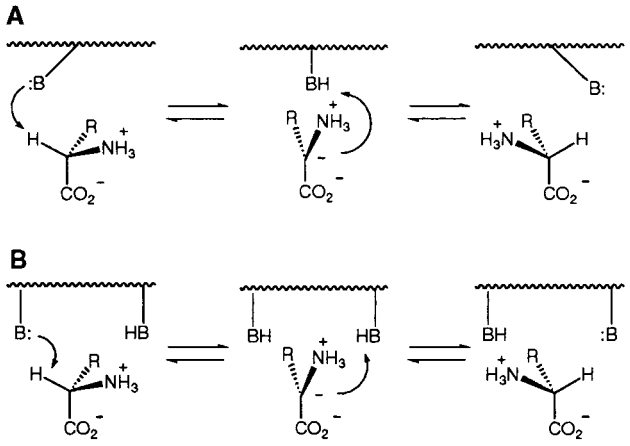

FIGURE 3. Mechanisms for amino acid racemization: (A) one-base mechanism and (B) two-base mechanism.

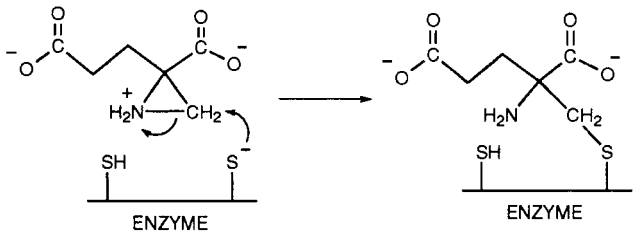

FIGURE 4. Irreversible inhibition of glutamate racemase by aziridino-glutamate.

the recovered starting material did not. These findings cannot be explained with a one-base mechanism, since the anionic intermediate would partition both forward and backward, resulting in solvent isotope incorporation into both the product and the starting material. Instead, the results are consistent with a two-base mechanism involving monoprotic residues.

In all cases, the cofactor-independent amino acid racemases are thought to employ cysteines as the catalytic acid/base residues.[1,13] In the *Lactobacillus fermenti* glutamate racemase, only two cysteine residues (Cys73 and Cys184) are present, and when these were individually mutated to alanine, the mutant enzymes were devoid of detectable racemase activity.[17] Although this demonstrates that the cysteines are critical for catalysis, it does not establish that they are proximal to the α-carbon of the bound substrate(s). This was done through the use of an irreversible inhibitor, aziridino-glutamate, that covalently modifies an active-site residue of the epimerase (Figure 4).[18] The covalent adduct was shown to be attached to a thiol, indicating that at least one of the cysteines is located in a position suitable to serve as an acid/base catalyst. Additional mutagenesis studies investigated the Cys73Ser and Cys184Ser mutants.[19] Surprisingly, the mutants retained a considerable fraction of their activity (∼1% of k_{cat}) despite the substitution of an alcohol (p$K_a \approx 16$) for a thiol (p$K_a \approx 10$). This could be explained by invoking a much higher reactivity for the alkoxide form of the enzyme despite there being much less of it in the correct protonation state. Alternatively, a catalytic diad could be responsible for substrate deprotonation in which another basic residue aids a neutral thiol/alcohol. This could

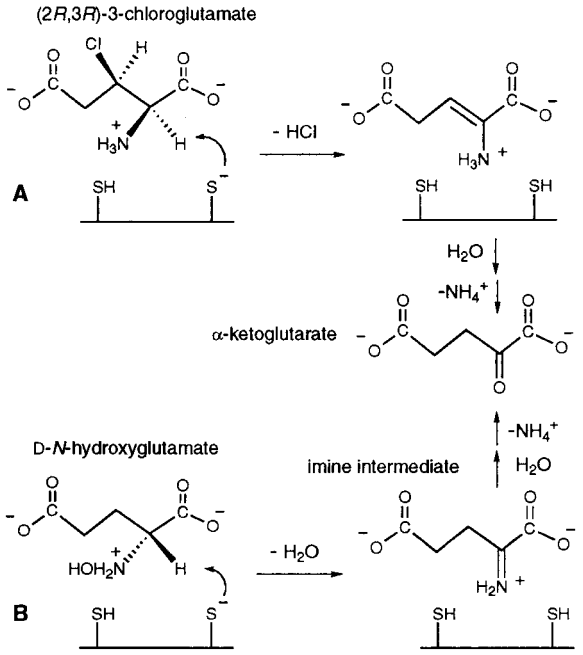

FIGURE 5. Alternative "one-base-requiring" substrates for glutamate racemase: (A) *threo*-3-chloroglutamate and (B) *N*-hydroxyglutamate.

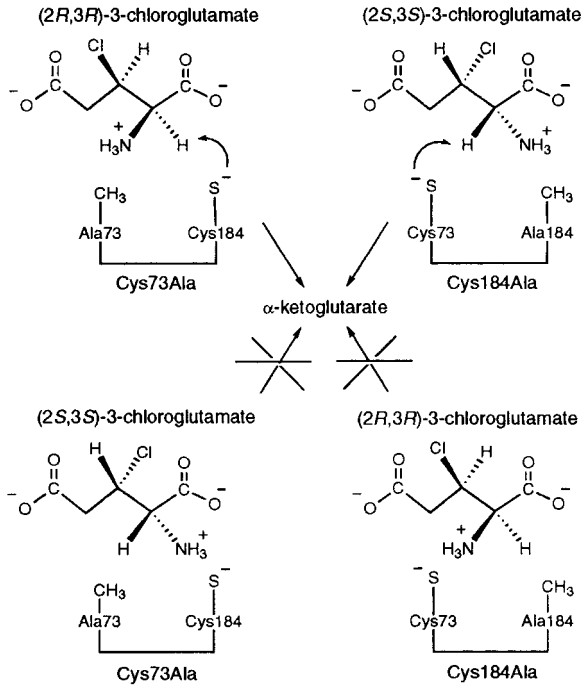

FIGURE 6. Reactivity of glutamate racemase mutants with *threo*-3-chloroglutamate enantiomers.

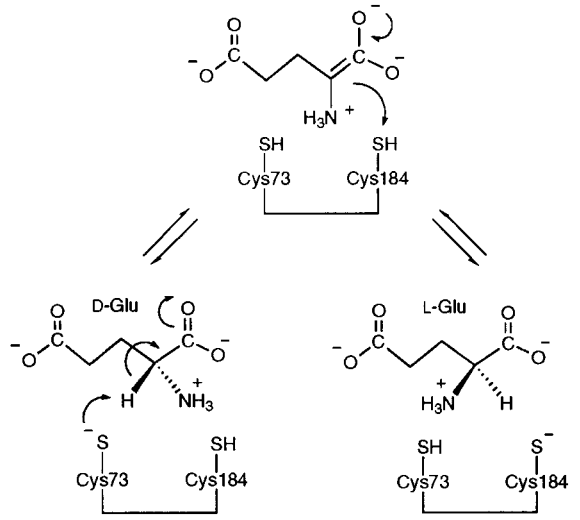

FIGURE 7. Mechanism employed by glutamate racemase.

explain how the Cys-to-Ser mutants could promote catalysis without first forming a discrete alkoxide to act as a catalytic base.

Further studies have focused on determining which cysteine deprotonates a given enantiomer. The assignments can be made with the use of alternative reactions that require only one base for catalysis. One such reaction is the elimination of HCl from *threo*-3-chloroglutamate (Figure 5a).[17] The wild-type enzyme will cleanly convert racemic *threo*-3-chloroglutamate into α-ketoglutarate, ammonia, and HCl. The mutant Cys73Ala, however, was found to convert (2*R*,3*R*)-3-chloroglutamate, but not (2*S*,3*S*)-3-chloroglutamate, into these products, and the opposite results were obtained with Cys184Ala (Figure 6).[20] Since the absolute configuration at C-2 of (2*S*,3*S*)-3-chloroglutamate corresponds to that of D-Glu, one can infer that Cys73 is responsible for the deprotonation of D-Glu and that Cys184 is responsible for the deprotonation of L-Glu (Figure 7). Another reaction that was used in this manner is the elimination of water from either enantiomer of *N*-hydroxyglutamate to give α-ketoglutarate and ammonia (Figure 5b).[19,21] *N*-Hydroxy-D-glutamate is a reasonably good substrate for wild-type glutamate racemase and is also a competitive inhibitor (K_i = 56 μM). It is reasonable to assume the inhibition is due to the binding of the imine intermediate that is formed immediately upon the elimination of water, since this species should mimic the planar anionic intermediate in the normal reaction. Interestingly, *N*-hydroxy-L-glutamate is a much poorer alternate substrate, pointing out that the pseudosymmetry that this enzyme displays toward two enantiomers is restricted to the natural substrate, glutamate. When Cys73Ser was treated with these alternate substrates, the specificity constant for *N*-hydroxy-D-glutamate dropped dramatically, whereas that for *N*-hydroxy-L-glutamate actually increased slightly. The opposite results were obtained with Cys184Ser, which leads one to conclude that Cys73 serves to deprotonate *N*-hydroxy-D-glutamate (and by analogy, D-Glu) and that Cys184 serves to deprotonate the L-enantiomers (Figure 7).

A much more subtle way of assigning the roles of the cysteines is to use kinetic isotope effect measurements to uncover the asymmetry introduced by mutations.[19] If the cleavage of the α-C−H bond is a rate-determining step, then the replacement of deuterium for hydrogen should slow the reaction considerably. In a two-base mechanism, this substitution will probe the deprotonation step only in a particular reaction direction. With the wild-type racemase, primary kinetic V_{max}/K_M isotope effects were observed with both L-[2-^{2}H]-Glu (K.I.E. = 2.5) and D-[2-^{2}H]-Glu (K.I.E. = 3.4). The fact that isotope effects are seen in both reaction directions indicates that both cleavage

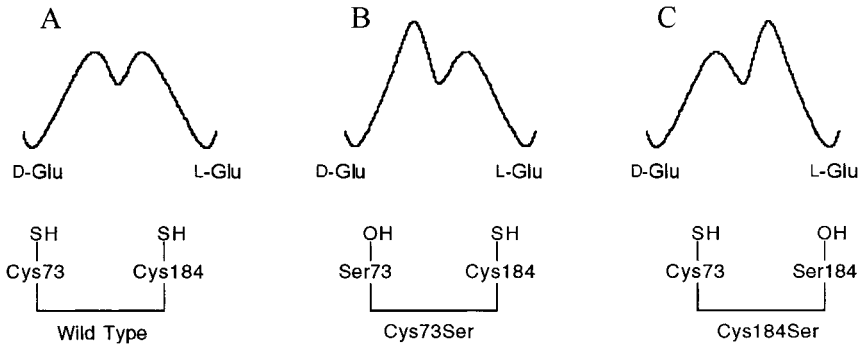

FIGURE 8. Conceptual free energy profiles for wild-type and mutant glutamate racemases (corresponding proteins shown below).

and reformation of the C−H bond are partially rate-determining steps of the reaction and that the reaction energy profile is somewhat symmetric (Figure 8a). With the Cys73Ser mutant, however, the isotope effect on the racemization of D-Glu (K.I.E. = 5.1) was found to increase, whereas the isotope effect on the racemization of L-Glu (K.I.E. = 1.85) decreased. This indicates that an asymmetry had been induced in the reaction energy profile and that the barrier toward the deprotonation of D-Glu had increased, making this step more cleanly rate-determining (Figure 8b). Thus, Cys73 is responsible for the deprotonation of D-glutamate. The opposite trend was observed with the Cys184Ser mutant (K.I.E. = 4.8 for L-Glu, and K.I.E. = 2.3 for D-Glu) indicating that Cys184 is responsible for the deprotonation of L-glutamate (Figure 8c).

More recently, the importance of other active-site residues was probed when four strictly conserved residues containing ionizable side chains were subjected to mutagenesis (Asp10Asn, Asp36Asn, Glu152Gln, and His186Asn).[22] The Asp10Asn and His186Asn mutants displayed 1000-fold reductions in the value of k_{cat}, indicating that these residues were essential for catalysis. X-ray structural data on a complex with the weak inhibitor glutamine confirmed that both of these residues are located in the active site of the enzyme (along with the two cysteines).[23] A detailed analysis of their positioning, however, is complicated, since the glutamine is bound in a reversed orientation and likely does not represent the Michaelis complex. One role for these residues could be to stabilize the anionic intermediate by hydrogen bonding to the carboxylate, as in the case of mandelate racemase.[3] Alternatively, they could act in conjunction with the catalytic thiols to help deprotonate the substrate. Evidence in support of the latter scenario was obtained using kinetic isotope effects that showed asymmetry had been introduced by the mutations and suggested that Asp10 aids Cys73 in the deprotonation of D-Glu, whereas His186 aids Cys184 in the deprotonation of L-Glu.[22]

Epimerization at Unactivated Centers

As discussed earlier, the majority of racemases and epimerases act at activated stereocenters and utilize deprotonation/reprotonation mechanisms.[1] A smaller group of enzymes operate at unactivated stereocenters and must use alternate approaches to catalysis. Most of these enzymes use an NAD^+ cofactor in a transient oxidation

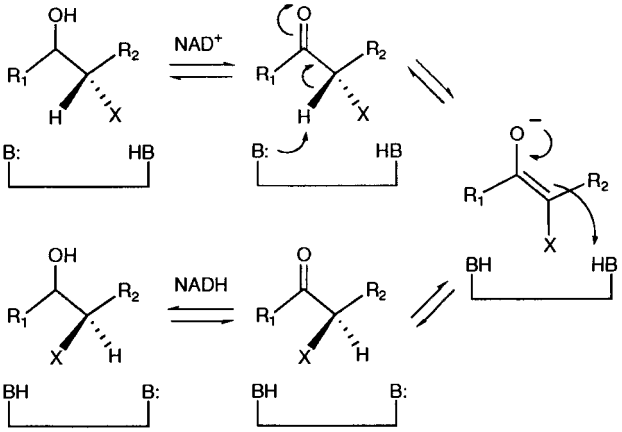

FIGURE 9. Deprotonation/reprotonation mechanism involving transient oxidation.

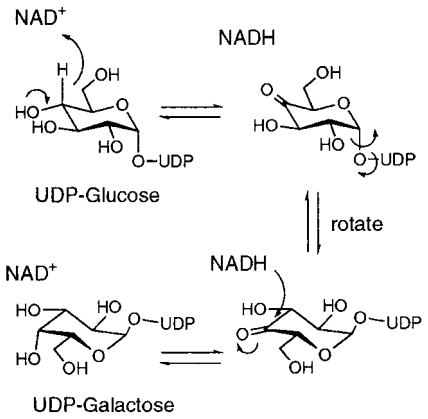

FIGURE 10. Mechanism employed by UDP−galactose 4-epimerase.

step.[2,6] One tactic is to simply activate the stereocenter by oxidizing an adjacent hydroxyl group and, thereby, to acidify the proton of interest (Figure 9). A deprotonation/reprotonation sequence inverts the stereochemistry and a final reduction of the keto-intermediate generates the epimeric product. An alternative approach used by UDP−galactose 4-epimerase is to oxidize a hydroxyl group located directly at the targeted stereocenter (Figure 10).[5] The abstracted hydride is then redelivered to the opposite face of the resulting keto-intermediate to give the epimeric product. Much rarer still are examples of cofactor-independent epimerases that operate at unactivated stereocenters. Two such enzymes will be described below.

(A) UDP−*N*-Acetylglucosamine 2-Epimerase: A Masked Transferase. The bacterial enzyme UDP−*N*-

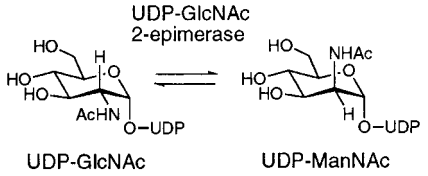

FIGURE 11. Reaction catalyzed by UDP−*N*-acetylglucosamine 2-epimerase.

acetylglucosamine 2-epimerase catalyzes the reversible interconversion of UDP−*N*-acetylglucosamine (UDP−GlcNAc) and UDP−*N*-acetylmannosamine (UDP−ManNAc) (Figure 11).[24] This enzyme serves to produce an activated form of ManNac residues (UDP−ManNAc) for use in the biosynthesis of a variety of cell surface polysaccharides. The bacterial enzyme is also of interest because it shows sequence homology to, and shares mechanistic similarities with, the mammalian form of the enzyme.[25] The mammalian UDP−GlcNAc "hydrolyzing" 2-epimerase catalyzes both the inversion of stereochemistry at C-2 and the hydrolysis of the UDP−sugar linkage to generate free ManNAc. ManNac is a precursor to *N*-acetylneuraminic acid and its derivatives, the sialic acids, that are found at the termini of cell surface oligosaccharides and are known to play key roles in cellular recognition events, such as viral infectivity.[26]

UDP−GlcNAc 2-epimerase was characterized as a homodimer in the late 70s and was shown not to require any exogenous cofactors (such as NAD^+) or metal ions for activity.[24] In addition, it was shown that the enzyme was tightly regulated by its own substrate UDP−GlcNAc. This is most evident when the enzyme is incubated with pure UDP−ManNAc, the thermodynamically less stable of the two epimers. No significant reaction takes place until a small amount of UDP−GlcNAc is added,and only then does the reaction proceed to equilibrium. The activation can also be seen in the sigmoidal nature of a plot of rate vs [UDP−GlcNAc].[24,27]

Early studies showed that epimerization was accompanied by solvent-derived tritium incorporation at C-2.[28] This is consistent with a mechanism that ultimately involves removal and replacement of a proton at the stereogenic center. In 1976, Salo proposed a mechanism in which the enzyme first oxidizes the C-3 hydroxyl of the substrate using NAD^+, then inverts the stereochemistry at C-2 via deprotonation/reprotonation, and finally, reduces the ketone at C-3 to give product (Figure 12).[28] The lack of any requirement for exogenous NAD^+ could be explained by a tightly bound cofactor that survives purification of the enzyme and is regenerated by each catalytic turnover. Although this mechanism is reasonable, an alternative possibility is that the enzyme catalyzes the *anti*-elimination of the C-2 proton and UDP from UDP−GlcNAc to give 2-acetamidoglucal and UDP as intermediates (Figure 13). A subsequent *syn*-addition, in which both the proton and UDP are delivered to the same face of the double bond, would generate UDP−ManNAc.

In more recent studies, the solvent isotope incorporation experiment was repeated in D_2O, and the resulting equilibrated epimers were found to contain deuterium

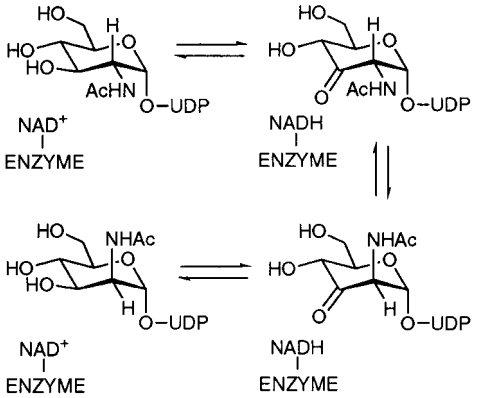

FIGURE 12. Salo's postulated mechanism of the reaction catalyzed by UDP−GlcNAc 2-epimerase.

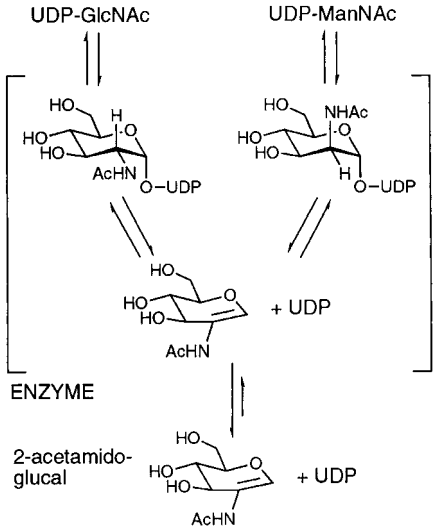

FIGURE 13. Mechanism employed by UDP−GlcNAc 2-epimerase.

exclusively at C-2.[27,29] This provided a source of [2-^{2}H]-UDP−GlcNAc that was used to measure a primary kinetic isotope effect of 1.8 on the value of k_{cat}. This experiment clearly indicates that the C−H bond at C-2 is cleaved during a rate-determining step of the reaction. A most informative result came from a positional isotope exchange (PIX) experiment that tests for transient C−O bond cleavage (Figure 14). The substrate UDP−GlcNAc with an ^{18}O-label specifically introduced at the anomeric center (bridging position) was synthesized and then incubated with the enzyme. Analysis of the resulting epimeric mixture by ^{31}P NMR spectroscopy showed that the label had scrambled into both bridging and nonbridging positions during catalysis. This scrambling process could only occur if C−O bond cleavage occurred to generate UDP as a transient intermediate and if the lifetime of the intermediate is comparable to the rate of P−O bond rotation in the terminal phosphate. The observation of isotopic scrambling clearly indicates that C−O bond cleavage occurs at C-1 during catalysis, and when this is combined with the evidence for C−H bond cleavage at C-2, a strong case for the glycal mechanism is made.

A final observation that strongly supports the glycal mechanism was made during extended incubations of the equilibrating UDP−sugar substrates with high concentra-

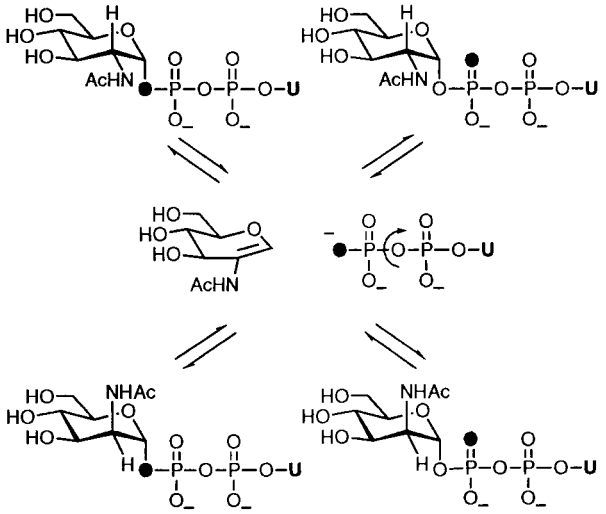

FIGURE 14. positional isotope exchange experiment. Darkened atoms represent ^{18}O labels.

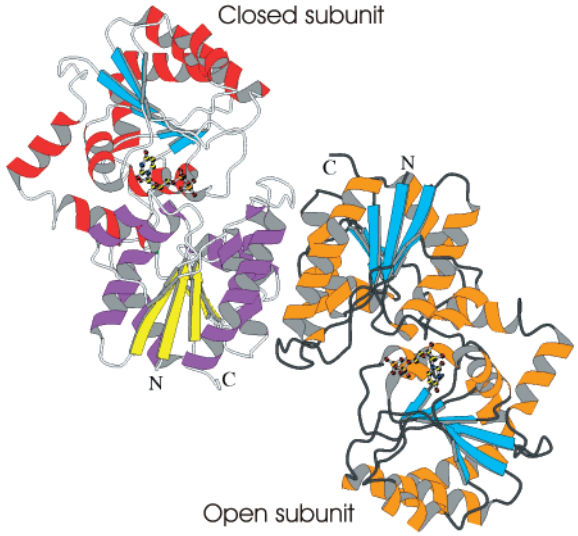

FIGURE 15. Structure of the UDP−GlcNAc 2-epimerase dimer. UDP is located in the active site of both subunits.

tions of enzyme.[27,29] It was found that the intermediates UDP and 2-acetamidoglucal are released into solution at a rate that is about 1/400 that of UDP−GlcNAc epimerization (Figure 13). Furthermore, these intermediates are significantly more thermodynamically stable than the sugar-nucleotides, and given sufficient incubation time, they are quantitatively generated. It appears that this enzyme is somewhat "sloppy" and occasionally releases its intermediates into solution in an essentially irreversible event. In contrast to many situations in which enzymes must protect high-energy intermediates from the aqueous environment, this enzyme must keep very stable intermediates kinetically trapped to avoid falling into this thermodynamic well.

Recently, the structure of the epimerase was solved using X-ray crystallographic methods, and the enzyme was shown to be a homodimer (Figure 15).[9] Each subunit is composed of two similar $\alpha/\beta/\alpha$ sandwich domains with the active site located in the deep cleft at the domain interface. The enzyme was crystallized in the presence of

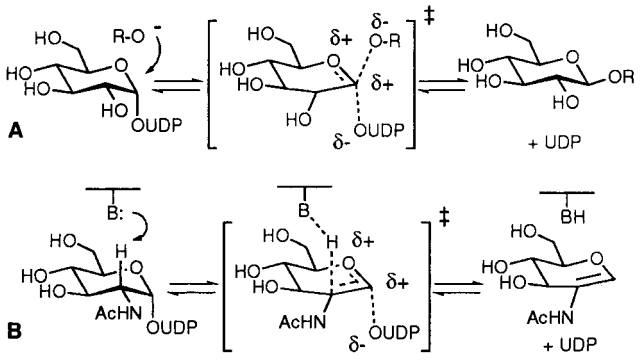

FIGURE 16. (A) Substitution reaction catalyzed by T4 phage β-glucosyltransferase and its postulated transition state (R = 5-hydroxymethylcytosine in DNA). (B) Anti elimination catalyzed by UDP−GlcNAc 2-epimerase and its postulated transition state.

UDP−GlcNAc, and electron density corresponding to UDP was observed in both subunits. It would appear that the reaction intermediates UDP and 2-acetamidoglucal were generated either during or following crystallization and that the 2-acetamidoglucal has either diffused out of the active sites or is disordered. A notable difference between the two subunits is that one is "open" and one is "closed" by an interdomain rotation that allows the enzyme to clamp down on the substrate. These conformations may help to explain the unusual regulation of activity by the substrate UDP−GlcNAc. The open subunit may play a regulatory role and allow only the closed subunit to be catalytically active when UDP−GlcNAc is bound. It is tempting to make this assignment, since the active-site residue His213 makes a hydrogen bond with the β-phosphate of UDP in the closed conformation, whereas this interaction is absent in the open conformation. This residue may serve as an acid catalyst during the elimination event.

Perhaps the most interesting aspect of the structural studies is the results of a search for proteins that share structural homology with the epimerase.[9] The enzymes T4 phage β-glucosyltransferase and MurG and the core of glycogen phosphorylase were all found to share a remarkably similar fold with UDP−GlcNAc 2-epimerase, and almost all secondary structural elements were conserved. Although this common architecture implies an evolutionary relationship between the enzymes, the link is quite distant, since none of these enzymes share any significant degree of sequence homology. The comparison becomes intriguing, however, when one considers the reaction catalyzed by each of the enzymes. All three are glycosyltransferases that catalyze a substitution reaction in which a nucleophile displaces either a uridine diphosphate or a phosphate from the anomeric center of a sugar (Figure 16a). These displacements are thought to follow S_N1-like S_N2 mechanisms with a significant amount of oxocarbenium ion character in the transition state.[30] A similar situation may help to explain how UDP−GlcNAc 2-epimerase is able to promote elimination reactions involving the nonacidic proton at C-2 (Figure 16b). The enzyme may employ E1-like E2 or E1 mechanisms that involve a significant amount of oxocarbenium ion char-

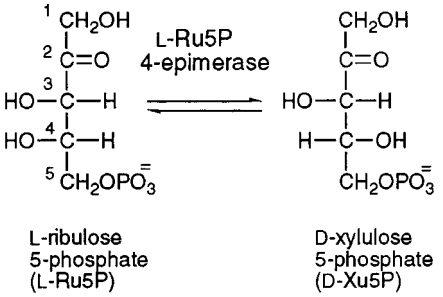

FIGURE 17. Reaction catalyzed by L-ribulose-5-phosphate 4-epimerase.

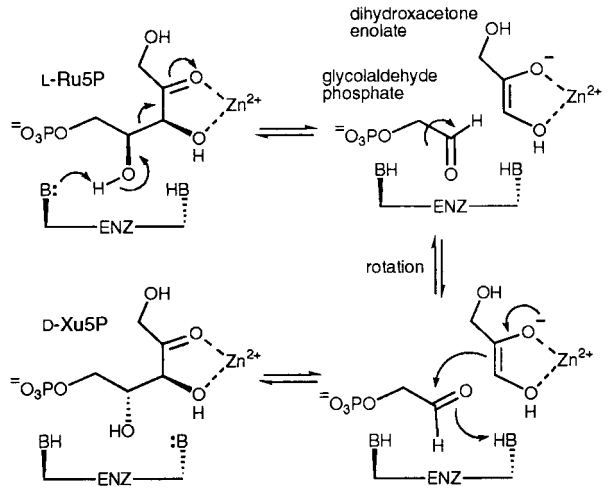

FIGURE 18. Retroaldol/aldol mechanism employed by L-Ru5P 4-epimerase.

acter in the transition state. The build-up of positive charge at C-1 would facilitate the removal of the proton at C-2. It is therefore reasonable to speculate that although the sequence and substrate specificity of these enzymes have diverged over time, they have retained the ability of their common ancestors to promote the cleavage of an anomeric sugar−phosphate bond via oxocarbenium ion-like transition states.

(B) L-Ribulose 5-Phosphate 4-Epimerase: A Masked Aldolase. The bacterial enzyme L-ribulose 5-phosphate 4-epimerase catalyzes the interconversion of L-ribulose 5-phosphate (L-Ru5P) and D-xylulose 5-phosphate (D-Xu5P) with the use of a divalent metal ion (Figure 17).[1] The enzyme is intriguing from a mechanistic point of view, because it operates at a stereogenic center that does not bear an acidic proton. Early studies demonstrated that NAD^+ was not utilized, ruling out oxidation/reduction mechanisms. Furthermore, no solvent isotope incorporation (^2H or ^{18}O) could be detected, indicating that any mechanism involving nonstereospecific deprotonation/reprotonation is highly unlikely. In the mid-70s, two mechanisms were forwarded that could explain these results.[31] One was a retroaldol/aldol mechanism in which a carbon−carbon bond cleavage occurs between C-3 and C-4 to generate glycolaldehyde phosphate and the enolate of dihydroxyacetone (Figure 18). The metal ion would serve as a Lewis acid catalyst and stabilize the enolate intermediate. A reorientation would then occur that would expose the opposite face of the aldehyde to the same face

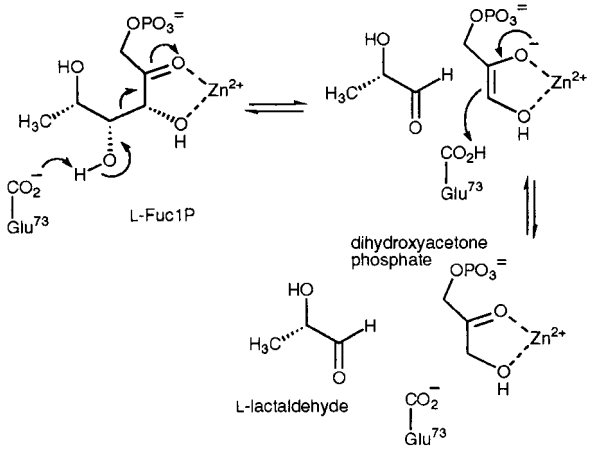

FIGURE 19. Mechanism employed by L-fuculose-1-phosphate aldolase.

of the enolate (perhaps only a simple bond rotation), and an aldol addition would reform the C−C bond. The second proposed mechanism involved a dehydration event between C-3 and C-4 to generate an enone, followed by a rehydration of opposite stereospecificity (not shown).

Indirect evidence supporting the retroaldol/aldol mechanism came from a report in 1993 that the epimerase shares 26% sequence identity with L-fuculose 1-phosphate aldolase, an enzyme that catalyzes the reversible aldol condensation between dihydroxyacetone phosphate and L-lactaldehyde to yield L-fuculose 1-phosphate (L-Fuc1P) (Figure 19).[32] This is a bacterial Class II aldolase that uses a divalent metal during catalysis. X-ray structures of both the free and inhibitor-bound enzyme show that the free aldolase uses three histidines and a glutamate (Glu73) as active site metal ligands and that the glutamate is displaced upon substrate binding.[33] The reaction proceeds via deprotonation of the hydroxyl group at C-4 with C−C bond cleavage to produce L-lactaldehyde and the metal bound enolate. Protonation of the enolate gives the second product, dihydroxyacetone phosphate. Recent mutagenesis studies indicate that the displaced Glu73 serves as both the key acidic and basic catalyst during both the formation and the protonation of the enolate intermediate.[34] The sequence similarity between the aldolase and the epimerase suggests they have evolved from a common ancestor and have retained the ability to form/cleave carbon−carbon bonds via metal-promoted aldol chemistry. The epimerase, however, has prevented the protonation of the enolate intermediate from occurring and instead has allowed a lapse in the stereospecificity of the aldol addition.

Initial studies probed the relationship between the enzymes through the use of site-directed mutagenesis.[35] Three histidines (His95, His97, and His171) and an aspartate (Asp76) of the epimerase align with the known metal ligands of the aldolase; therefore, the mutants His95Asn, His97Asn and Asp76Asn were prepared. The activity of the mutant enzymes was reduced 3−200-fold, as might be expected for catalysts in which the coordination sphere of the metal has been altered. Perhaps more importantly, the affinity of these mutants for Zn^{2+} was

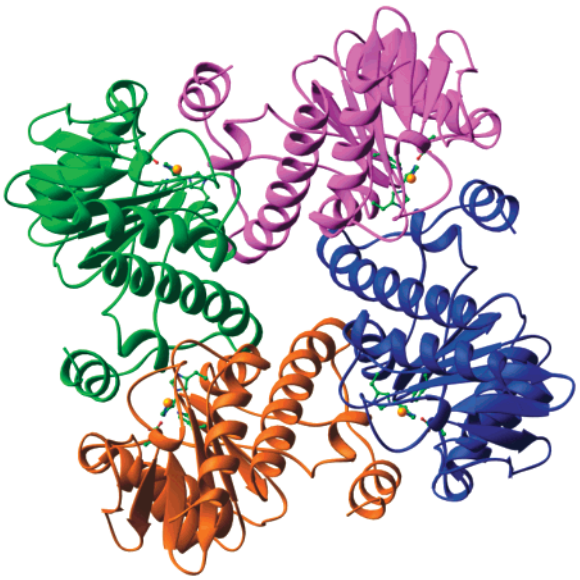

FIGURE 20. Structure of the L-Ru5P 4-epimerase homotetramer.

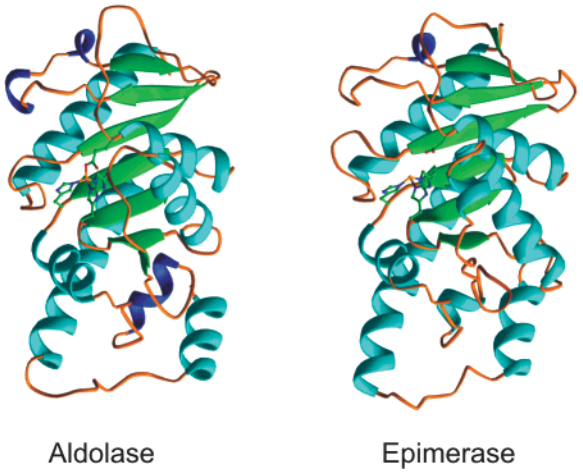

FIGURE 21. Structures of the L-Fuc1P aldolase monomer and the L-Ru5P 4-epimerase monomer.

reduced, and they required exogenous Zn^{2+} for full activity. This is unlike the wild-type enzyme that may be purified with one equivalent of metal bound per subunit and is fully active in the absence of exogenous metal ions. These results suggest that the architecture of the metal-binding site is common to the two enzymes and strongly supports the notion of a common evolutionary ancestry.

A common metal-binding motif does not necessarily indicate that a common mechanism is employed, since both postulated mechanisms could utilize a metal ion during catalysis. The first evidence pointing toward the retroaldol/aldol mechanism was the observation of a very low level of residual aldolase activity with the purified epimerase.[35] If the epimerase had evolved from an aldolase and retained the ability to cleave and reform carbon−carbon bonds, there would be a reasonable chance that it would occasionally protonate the enolate intermediate and release the retroaldol products dihydroxyacetone and glycolaldehyde phosphate. Indeed, when the epimerase was incubated with dihydroxyacetone and glycolaldehyde phosphate, the slow production of L-Ru5P and D-Xu5P could be observed.

Studies from the group of Cleland and co-workers provided the definitive evidence in support of the retroaldol/aldol mechanism.[36] ^{13}C kinetic isotope effects were observed on both the C-3 carbon (1.85%) and the C-4 carbon (1.5%) of L-Ru5P, which is consistent with a mechanism involving C−C bond cleavage. This also shows that the chemical steps of the reaction are rate-determining. They further determined that there were no primary deuterium isotope effects on the protons at either C-3 or C-4, an observation that rules out the dehydration mechanism.

Ultimately, the most telling comparison of the two enzymes was provided when the structure of the uncomplexed epimerase was solved by X-ray crystallography.[7] Like the aldolase, the epimerase is a homotetramer composed of four single domain subunits (Figure 20). The catalytic Zn^{2+} is located at the subunit interface, and therefore, residues from both adjacent subunits contribute to the active site cleft. In both enzymes, the eight C-terminal residues are disordered and could not be located in the structure. Nevertheless, they could easily be positioned to form an active-site cap, as has been previously suggested for the aldolase.[34] This is strongly supported in the case of the epimerase, since mutagenesis of Tyr229 in the C-terminal tail dramatically decreased activity (Tyr229Phe activity was down 1000-fold).[37]

A comparison of the epimerase secondary structure with that of the aldolase revealed a remarkable degree of homology (Figure 21). When the two structures are superimposed, 93% of α-carbons align with a root-mean-square deviation of only 1.5 Å. This agreement clearly indicates the two enzymes belong to a superfamily of aldolases/epimerases and have evolved from a common ancestor. An inspection of the epimerase active site reveals that the three histidines are metal ligands; however, the shortened side chain of Asp76 (as compared to Glu73 of the aldolase) does not permit it to directly bond to the metal. Instead, undetected water molecules must serve as the direct ligands. This had been anticipated by earlier EPR studies on the Mn^{2+}-substituted epimerase that showed that three waters served as metal ligands in the free enzyme.[37]

It is clear that the two enzymes share a great deal of mechanistic and structural features. However, notable differences must exist between them because of the position of the phosphates in the respective substrates. Although both enzymes must position a metal at the C-2 carbonyls and acid/base catalysts near the C-4 hydroxyls, the epimerase must position a phosphate-binding site at C-5, and the aldolase must position one at C-1. Since the residues lining the phosphate-binding pocket of the aldolase are all strictly conserved in the epimerase, it is reasonable to suggest that both enzymes utilize this region in the same manner. This would mean that the epimerase binds its substrate in a reversed or "flipped" orientation with respect to the aldolase, and therefore, it must recruit new acid/base residues to participate in catalysis. These notions were probed using site-directed mutagenesis.[8,37]

Two of the putative phosphate-binding pocket residues (Asn28 and Lys42) and all of the potential acid/base catalysts within the vicinity of the active site (including those on the disordered C-terminal tail) have been investigated. With Asn28Ala and Lys42Met, the values for K_M increased dramatically, reflecting a decreased binding affinity for the substrate. Since the phosphate is likely to be a key recognition element for binding, these results suggest that the two enzymes use a common phosphate-binding site and, therefore, bind the substrates in reversed orientations. When the aspartate (Asp76) corresponding to the key catalytic residue of the aldolase (Glu73) was mutated to asparagine, the mutant showed only a 100-fold decrease in activity, a value that is somewhat less than expected for the substitution of a critical residue. In contrast, the mutants Asp120Asn and Tyr229Phe showed activity decreases of 3000- and 1000-fold, respectively.[8,37] Thus, Asp120 and Tyr229 are reasonable candidates for the key acid/base catalysts in the epimerase, and it is interesting to note that both of these residues have been recruited from an adjacent subunit.

Concluding Remarks

This Account has outlined studies on three quite different enzymes in order to showcase the breadth of nature's strategy in catalyzing inversions of stereochemistry. In addition, the last two serve as excellent examples of how new enzymatic activities can evolve from preexisting ones on the basis of similarities in chemical mechanism.[4] Since epimerization requires only the cleavage and reformation of a single bond, epimerases can evolve from a wide variety of enzyme families (or perhaps vice versa) and thus exhibit diverse modes of catalysis.

I am grateful for the invaluable help of all of my co-workers and collaborators who have contributed to this work: K. A. Gallo; J. R. Knowles; S. Miao; A. E. Johnson; P. M. Morgan; R. F. Sala; S. Glavas; R. E. Campbell; J. Samuel; S. C. Mosimann; Y. Luo; and N. C. J. Strynadka.

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AR000056Y