The Leloir pathway: a mechanistic imperative for three enzymes to change the stereochemical configuration of a single carbon in galactose

PERRY A. FREY[1]

Institute for Enzyme Research, The Graduate School, and Department of Biochemistry, College of Agricultural and Life Sciences, University of Wisconsin-Madison, Madison, Wisconsin 53705, USA

ABSTRACT: The biological interconversion of galactose and glucose takes place only by way of the Leloir pathway and requires the three enzymes galactokinase, galactose-1-P uridylyltransferase, and UDP-galactose 4-epimerase. The only biological importance of these enzymes appears to be to provide for the interconversion of galactosyl and glucosyl groups. Galactose mutarotase also participates by producing the galactokinase substrate α-D-galactose from its β-anomer. The galacto/gluco configurational change takes place at the level of the nucleotide sugar by an oxidation/reduction mechanism in the active site of the epimerase·NAD^+ complex. The nucleotide portion of UDP-galactose and UDP-glucose participates in the epimerization process in two ways: 1) by serving as a binding anchor that allows epimerization to take place at glycosyl-C-4 through weak binding of the sugar, and 2) by inducing a conformational change in the epimerase that destabilizes NAD^+ and increases its reactivity toward substrates. Reversible hydride transfer is thereby facilitated between NAD^+ and carbon-4 of the weakly bound sugars. The structure of the enzyme reveals many details of the binding of NAD^+ and inhibitors at the active site. The essential roles of the kinase and transferase are to attach the UDP group to galactose, allowing for its participation in catalysis by the epimerase. The transferase is a Zn/Fe metalloprotein, in which the metal ions stabilize the structure rather than participating in catalysis. The structure is interesting in that it consists of a single β-sheet with 13 antiparallel strands and 1 parallel strand connected by 6 helices. The mechanism of UMP attachment at the active site of the transferase is a double displacement, with the participation of a covalent UMP-His^{166}-enzyme intermediate in the *Escherichia coli* enzyme. The evolution of this mechanism appears to have been guided by the principle of economy in the evolution of binding sites.—Frey, P. A. The Lenoir pathway: a mechanistic imperative for three enzymes to change the stereochemical configuration of a single carbon in galactose. *FASEB J.* 10, 461–470 (1996)

Key words: galactokinase · enzymatic catalysis · uridine nucleotide binding

THE METABOLISM OF GALACTOSE WAS delineated in 1948 to 1951, mainly by L. F. Leloir and his associates. The process was shown to take place through the initial phosphorylation of galactose to galactose-1-P by the action of galactokinase (galactose:ATP phosphotransferase, EC 2.7.1.6)[2] (1, 2). Galactose-1-P was shown to be transformed into glucose-1-P in the presence of an enzyme and a cofactor (3). The enzyme became known as galactose-1-P uridylyltransferase (hexose-1-P uridylyltransferase, EC 2.7.7.12), and the cofactor was identified as UDP-glucose (4, 5). The transformation of UDP-glucose into a galactose derivative (UDP-galactose) in extracts of *Streptomyces fragilis* was described by Leloir in 1951 (6). Leloir referred to this enzyme as a "Waldenase" (6) and to the system of enzymes for converting galactose into glucose-1-P as "galactowaldenase" in recognition of the apparent inversion of carbon-4 in the transformation of galactose into glucose. At the time, chemists referred to displacements at carbon that proceeded with inversion of stereochemical configuration as "Walden inversions." Mechanistic studies later showed that the process at carbon-4 of UDP-galactose is a reversible, nonstereospecific oxidation-reduction rather than a displacement. Therefore, Waldenase is now known as UDP-galactose 4-epimerase (UDP-glucose 4-epimerase, EC 5.1.3.2).

The transformation of galactose into glucose-1-P by these enzymes is illustrated in **Fig. 1**, which shows how UDP-glucose is cycled through UDP-galactose and thereby acts as a cofactor for the transformation of galactose-1-P into glucose-1-P. As an energy source, galactose is the equal of glucose. One molecule of ATP is required to convert galactose into glucose-1-P, and further transformation into glucose-6-P by the action of phosphoglucomutase allows its metabolism as an energy source through glycolysis. The metabolism of glucose through glycolysis also requires one molecule of ATP to phosphorylate it to glucose-6-P.

The discovery and characterization of UDP-glucose in the Leloir pathway for galactose metabolism (4, 5) consti-

[1]Send correspondence and reprint requests to Dr. Frey, at: Institute for Enzyme Research, University of Wisconsin-Madison, 1710 University Ave., Madison, WI 53705-4098, USA.

[2]Abbreviations: galactokinase, glalactose:ATP phosphotransferase.

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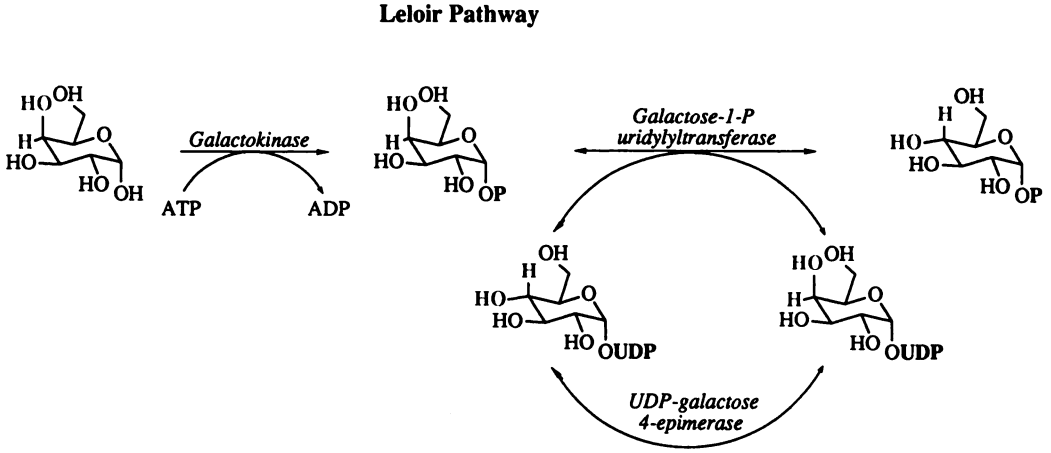


Figure 1. The Leloir pathway.

tuted its original discovery as the first nucleotide sugar. Its general role in the metabolism of complex carbohydrates was soon recognized. Leloir described galactose as a source of UDP-glucose for glycogen biosynthesis by coupling the Leloir Pathway (Eqs. 1–3) with the action of UDP-glucose pyrophosphorylase (Eq. 4) to give Eq. 5 (7).

$$\text{Galactose} + \text{ATP} \rightleftharpoons \text{Galactose 1-P} + \text{ADP} \quad [1]$$

$$\text{Galactose-1-P} + \text{UDP-glucose} \rightleftharpoons \text{UDP-galactose} + \text{Glucose-1-P} \quad [2]$$

$$\text{UDP-galactose} \rightleftharpoons \text{UDP-glucose} \quad [3]$$

$$\text{UTP} + \text{glucose-1-P} \rightleftharpoons \text{UDP-glucose} + PP_i \quad [4]$$

$$\text{Galactose} + \text{ATP} + \text{UTP} \rightleftharpoons \text{UDP-glucose} + \text{ADP} + PP_i \quad [5]$$

In this way, galactose is as efficient a source of glucosyl units for glycogen biosynthesis as glucose itself.

The mechanisms of action of the epimerase and transferase exemplify principles of enzymatic catalysis that have broad implications in biochemistry. This article focuses on these principles and their operation in the metabolism of galactose. It also seeks to answer the questions posed in the title by explaining why three enzymes are required to change the configuration at carbon-4 of glucose and galactose.

The evolution of three separate enzymes that function together solely in altering a single configuration appears to be unique to galactose metabolism in biochemistry. Other three-enzyme sequences leading to a single configurational change require the participation of enzymes that carry out additional metabolic functions. For example the transformation of glucose into mannose-6-P also requires three enzymes: hexokinase, phosphohexoisomerase, and phosphomannoisomerase. However, hexokinase and phosphohexoisomerase are required for glucose metabolism independently of the production of mannose. Therefore, only phosphomannoisomerase functions exclusively in mannose utilization or production. In contrast, the enzymes of galactose metabolism in Fig. 1 function solely in the biosynthesis and breakdown of galactose. No other enzymatic system for accomplishing this has evolved.

It may seem strange that catalysis of a single configurational change should require three enzymes. The gluco/galacto transformation takes place at the active site of the epimerase, so only that enzyme actually carries out the stereochemical change. The kinase and transferase assemble the uridine-5′-diphosphoryl moiety on the epimerase substrate UDP-galactose. One might imagine that the UDP-moiety is required for substrate recognition by the epimerase. This is true, but it is an oversimplification, because the UDP-substituent participates directly in essential mechanistic functions and plays at least two critical roles in the action of the epimerase. There is reason to believe that any enzyme catalyzing this epimerization would require either a nucleotidyl moiety or some analogous, alternative glycosyl substituent to participate in catalysis. The functions of the nucleotide in catalysis will occupy a large part of the discussion of the epimerase in this article. The mechanism of the attachment of the uridylyl-group to the phosphate group of galactose-1-P to produce UDP-galactose will be discussed as the main function of the transferase.

The *gal* operon of *Escherichia coli* encodes the three enzymes in Fig. 1 and also galactose mutarotase, which catalyzes the interconversion of the α- and β-anomers of either galactose (Eq. 6) or glucose (8). The mutarotase is important for lactose utilization in *E. coli* because β-galactosidase produces β-D-galactose from lactose, whereas galactokinase requires α-D-galactose as its sub-

[6]

strate. Although the mutarotation of sugars takes place

spontaneously, the rate is too slow to allow β-D-galactose to be metabolized efficiently. Galactose mutarotase catalyzes the transformation of β-D-galactose into α-D-galactose for use by galactokinase. The yeast enzymes corresponding to the enzymes in Fig. 1 are specified by three of the *GAL* genes in yeast.

The uridylyltransferase and epimerase reactions are readily reversible and nearly balanced at equilibrium, so that they also serve to produce UDP-galactose and galactose-1-P from UDP-glucose, which is available through the action of UDP-glucose pyrophosphorylase (Eq. 7).

$$\text{UTP} + \text{Glucose-1-P} \underset{\textit{UDP-glucose pyroposphorylase}}{\rightleftharpoons} \text{UDP-glucose} + PP_i \quad [7]$$

These enzymes are found in animals, plants, and bacteria, and are required for both galactose utilization and to produce galactosyl units for the biosynthesis of complex carbohydrates in glycoproteins, glycolipids, and cell walls.

The importance of galactose metabolism is accentuated by the consequences of inherited molecular defects in the human galactose-1-P uridylyltransferase (9, 10). The presence of a defective human transferase gene results in very low enzyme activity that causes galactosemia, a metabolic disease of galactose metabolism in which the transformation of galactose into glucose-1-P is acutely slowed. The resulting galactose toxicity has a number of clinical consequences, including enlargements of the liver and spleen, proteinuria, amino aciduria, elevated blood galactose derivatives, disorders of the central nervous system, and the early development of cataracts. The effects of this disease are alleviated by withholding milk and other lactose-containing foods from the diet. A number of variant human galactosemic genes have been described (11, 12).

UDP-GALACTOSE 4-EPIMERASE

When purified from *E. coli* or yeast, this enzyme contains tightly bound NAD^+ (13, 14). It does not require added NAD^+, but NAD^+ bound at the active site functions as an essential coenzyme in catalyzing the epimerization reaction. It does not dissociate from the enzyme, either in the course of catalysis or between catalytic cycles. The purified mammalian enzyme requires NAD^+ and appears to bind it very tightly (15). Both the yeast and *E. coli* enzymes are dimeric (125K and 79K, respectively) and composed of identical subunits. For many years they were thought to contain one molecule of NAD^+ per dimer (13, 14), but the *E. coli* epimerase is now known to contain one NAD^+ per subunit, that is, two molecules per enzyme dimer. The revised NAD^+ stoichiometry for the *E. coli* enzyme is based on the X-ray crystal structure (16) and a newly determined extinction coefficient at 280 nm ($E_{286}^{1\%} = 1.84$) (Y. Liu and P. A. Frey, unpublished results), which is more compatible with the amino acid composition than the earlier value. The original extinction coefficient led to high estimates of protein concentration that, when used with analytical data on the NAD^+ content, gave low values for the stoichiometry. The new data on the *E. coli* enzyme reopens the question of the NAD^+-content of the yeast epimerase.

The 3-dimensional structure of *E. coli* epimerase is depicted in **Fig. 2** by a ribbon diagram, showing the locations at which NAD^+ and the inhibitor UDP-phenol are bound. NAD^+ is bound to a typical Rossmann fold in the lower half of Fig. 2 that appears similar to the NAD^+ binding domains of pyridine nucleotide dependent oxidoreductases. UDP-phenol binds to a smaller domain in the upper half of Fig. 2 that forms a cleft with the larger NAD^+ domain. The uridine-5′-diphosphoryl portion of the inhibitor does not contact NAD^+, a fact that becomes significant in connection with chemical and spectroscopic

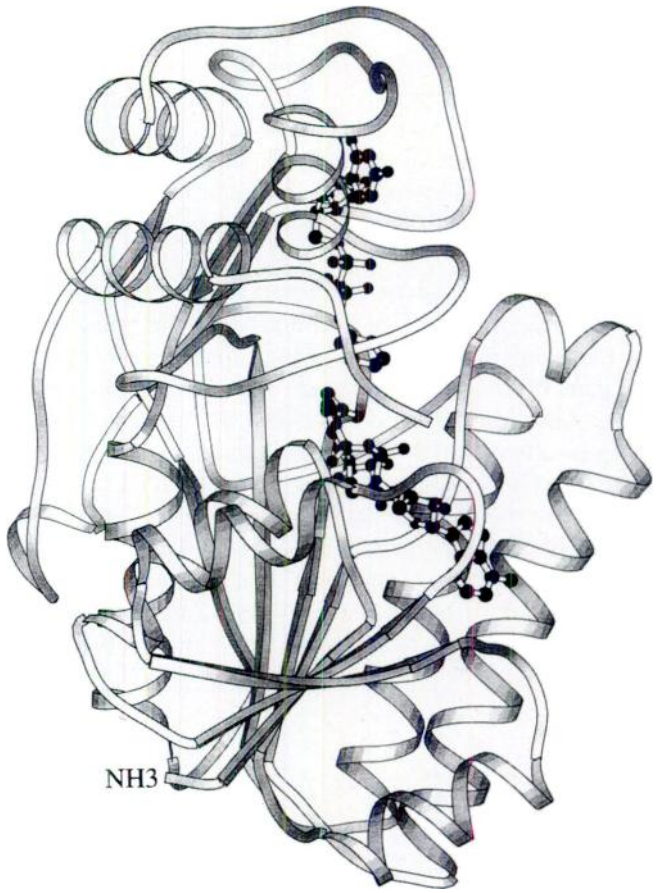


Figure 2. A ribbon diagram of the polypeptide chain of UDP-galactose 4-epimerase. The locations of NAD^+ and the inhibitor UDP-phenol are illustrated within the diagram of the polypeptide chain in one subunit of the dimeric epimerase from *Escherichia coli*. The larger domain at the base of the diagram is a dinucleotide binding domain with secondary structural elements typical of a Rossmann fold. NAD^+ is bound within this domain. The smaller domain in the upper part of the diagram forms a substrate binding cleft with the larger domain. A model of the inhibitor UDP-phenol is shown bound to this site. Courtesy of James Thoden and Hazel M. Holden.

observations that will be discussed in the following sections.

The close contacts between NAD^+ and enzymatic groups in the crystal structure indicate that the exceptionally tight binding of NAD^+ is brought about through hydrogen bonding between the adenosyl moiety of NAD^+ and side chain and main chain hydrogen bonding groups of the protein. There appear to be hydrogen-bonded interactions between nearly all of the heteroatoms of the adenosyl group and the protein (17). These stand in contrast to the interactions of NAD^+ with lactate dehydrogenase and other pyridine nucleotide-dependent oxidoreductases that bind NAD^+ reversibly, in which fewer adenosyl-protein hydrogen bonds appear to be formed.

The chemical mechanism by which UDP-galactose 4-epimerase catalyzes the interconversion of UDP-galactose and UDP-glucose was worked out in the 1970s and has been reviewed (18). This mechanism is illustrated in **Fig. 3.** NAD^+ participates as a redox cofactor by reversibly and *nonstereospecifically* dehydrogenating carbon-4 in the pyranosyl rings of UDP-galactose and UDP-glucose. The NAD^+/NADH transformation is itself stereospecific, with hydrogen transfer exclusively to and from the B-side (pro-S). However, the substrates are nonstereospecifically oxidized and reduced by way of the UDP-4-ketohexopyranose intermediate. The question of how this or any enzyme can function nonstereospecifically is interesting. Moreover, the answer in this case unmasks important biological information by explaining the functions of the UDP moiety in catalysis.

One essential catalytic function of the UDP-group is that it serves as the binding anchor for the galactosyl, glucosyl, and 4-ketohexopyranosyl groups of the substrates and the intermediate. Binding studies of the *E. coli* enzyme show that most of the binding free energy between the enzyme and these molecules is directed toward binding the nucleotide portion (19, 20). The standard free energy for binding the uridine nucleotide moieties of the substrates to the free enzyme (E·NAD+) is about -5 kcal/mol, whereas that for binding the glycosyl groups is near zero. The standard free energy for binding the UDP-moiety of the 4-keto intermediate to the reduced enzyme (E*·NADH) is about -7 kcal/mol, and that for binding the 4-ketohexopyranosyl group of the intermediate is about -2 kcal/mol.

Strong binding of the UDP group and weak binding of the hexopyranosyl group allows hydride transfer between the pyridine nucleotide and glycosyl C-4 to take place nonstereospecifically. Nonstereospecificity requires weak binding of either the nicotinamide or the 4-ketosugar, so that hydride transfer to either face of the sugar can take place. The following physicochemical observations indicate that the nicotinamide ring is firmly embedded in the active site (20–23): The fluorescence lifetime of NADH in **E** · NADH is very short and the emission is highly polarized. Chemical reduction of E·NAD^+ by NaB^3H_4 is stereospecific for the pro-*S* side (B side), and there is a large positive Cotton effect at 345 nm in the circular dichroism spectrum of the *E. coli* E·NADH. The weak binding of the 4-ketohexopyranosyl moiety indicates that it may be conformationally mobile within the active site. These facts led to the proposed mechanistic scheme in Fig. 3, in which the 4-ketohexopyranosyl group undergoes rotation about the bond linking P_β of UDP and the glycosyl oxygen of the 4-ketopyranosyl group. Additional rotational modes in the phosphoanhydride portion of the molecule can also participate in the positioning of the two faces of the hexopyranosyl moiety. This places the 4-keto group in positions relative to NADH that allow it to accept hydride transfer from either face to form either UDP-galactose or UDP-glucose. Space-filling models show that this rotation can allow virtually equivalent positioning of the hydroxyl groups at glycosyl C-3 and C-4 and the hydrogen at C-4 in the substrate epimers. This recognition is illustrated in **Fig. 4.** The absence of other binding interactions between the enzyme and the sugars would allow this recognition while permitting free rotation. Binding interactions to the 6-hydroxymethyl groups are clearly unimportant from the fact that the epimers UDP-D-xylose and UDP-L-arabinose are readily interconverted by the enzyme (24). Additional lines of evidence implicate weak binding interactions between sugars and the active site.

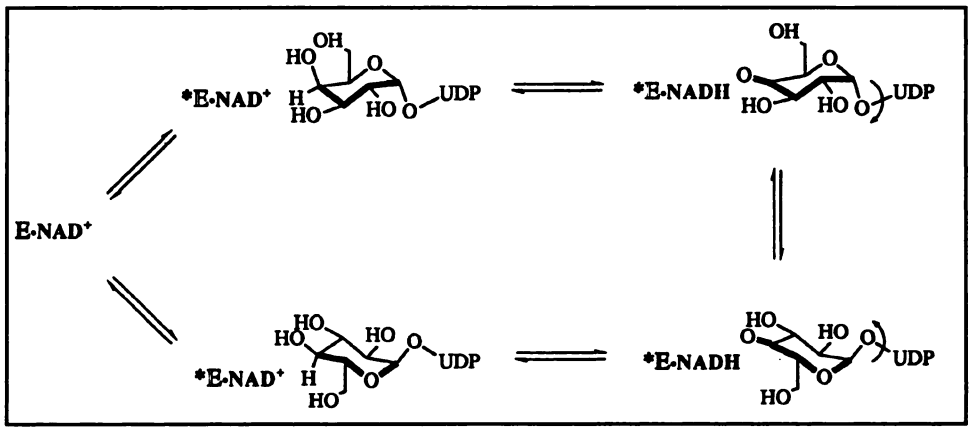


Figure 3. The mechanism of the interconversion of UDP-galactose and UDP-glucose by UDP-galactose 4-epimerase.

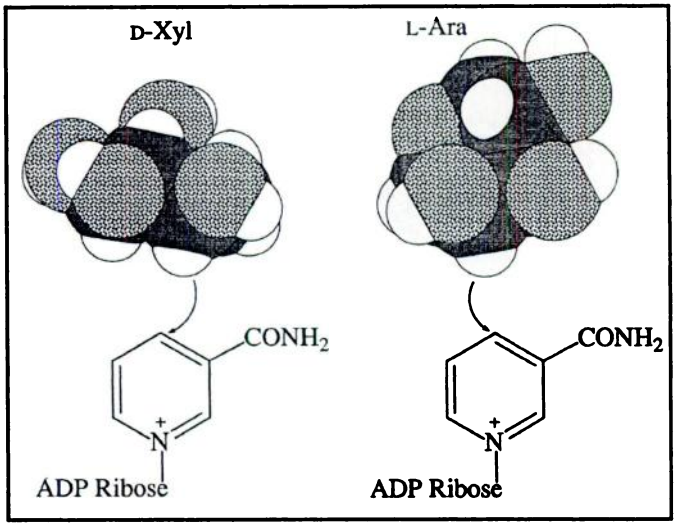


Figure 4. A stereochemical model for nonstereospecific hydride transfer by UDP-galactose 4-epimerase. The epimeric substrates UDP-D-xylose and UDP-L-arabinose are chosen to illustrate the stereochemical relationships among their C-4-OH, C-4-H, and C-3-OH in two conformations in which the α- and β-glycosyl faces interact with NAD^+. The conformational differences leading to these relationships are described in the text.

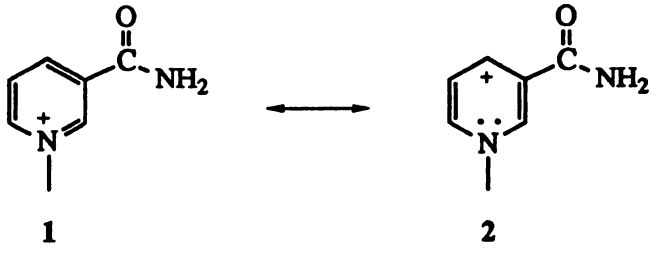
The anchoring function of the UDP group is critically important. There are five asymmetric carbons in α-D-galactose and α-D-glucose, and it is difficult to imagine how an enzyme could bind the α- and β-faces of both sugars with high affinity. For this reason, it would seem that weak binding is required for nonstereospecific hydride transfer at carbon-4 to be possible. If no binding anchor were available, and the enzyme were to facilitate dehydrogenation of the sugars themselves, a weakly bound dehydrogenated 4-ketosugar intermediate would be likely to diffuse away from the active site, leaving the enzyme in an inactive state with NADH instead of NAD^+. The reduced enzyme would be unlikely to bind the 4-ketosugar again to complete the catalytic cycle owing to the low affinity that is the essence of nonstereospecific action. The intermediate can be held at the active site while undergoing the reorientation in Fig. 3 and Fig. 4 because of the anchoring effect of the nucleotide portion in UDP-sugars.

Another important fact about this enzyme is that uridine nucleotides induce a conformational change in both the *E. coli* and yeast epimerases. The altered conformation is signified by the asterisk associated with enzyme forms in Fig. 3 to which uridine nucleotides are bound. The conformational change is a very important part of the catalytic process, and the conformationally altered form carries out hydride transfer and epimerization. A key property of this form of the enzyme is that the reactivity of E-NAD^+ uridine nucleotide toward reducing agents is higher than that of free NAD^+. This effect is observed with UMP, UDP, and UDP sugars (19, 20, 22, 25, 26). With UMP as the activating nucleotide, enzyme-bound NAD^+ is quickly reduced by glucose, arabinose, galactose, other reducing sugars, and $NaBH_3CN$; it is reduced less rapidly by Tris buffer and glycerol; and UMP itself at high concentrations will reduce the enzyme very slowly (27). The most reactive reducing agents will also reduce E·NAD^+ very slowly in the absence of a uridine nucleotide.

Uridine nucleotides also stabilize E·NADH against oxidation in the air (28–30). Complexes of the type E*·NADH·uridine nucleotide can be isolated and characterized aerobically, but E·NADH undergoes oxidation to E·NAD^+ very quickly in the air. The structural basis for uridine nucleotide-induced stabilization of NADH and destabilization of NAD^+ is important for understanding the mechanism of catalysis. The structure of the enzyme shows that the UDP group of a bound nucleotide is not in direct contact with NAD^+ (Fig. 2), so the effects must be mediated through the protein structure by way of the conformational change from E to E*.

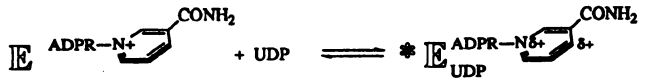
Information obtained from five independent lines of investigation can be integrated into a coherent model of the structural basis of the action of uridine nucleotides in destabilizing NAD^+ and stabilizing NADH at the active site. This evidence includes information from kinetic experiments, from the global structure of the epimerase in its active conformation, from ^{15}N and ^{13}C-NMR spectroscopic experiments, from physical organic chemical data, and from site-directed mutagenesis. The enzymatic kinetic work shows that UMP increases the rate at which glucose reduces E·NAD^+ by a factor of 3×10^3 and that UMP stabilizes the reduced enzyme in the form of E*·NADH·UMP against oxidation in the air. The global structure shows the close contacts between NAD^+ and the enzyme, which must explain the molecular/atomic basis for the effects of uridine nucleotides on the reactivity of NAD^+.

^{13}C- and ^{15}N-NMR data show that uridine nucleotides polarize the π-electron system of the nicotinamide ring in E·NAD^+ (31). UDP induces a 3.4 ppm downfield perturbation in the ^{13}C chemical shift for E·[nicotinamide-4-^{13}C]NAD^+ and a 3.0 ppm upfield perturbation in the ^{15}N chemical shift for E·[nicotinamide-1-^{15}N]NAD^+. Smaller perturbations occur at positions 2 and 6 of the nicotinamide ring, and none on the ^{15}N chemical shift of the carboxamide group. P^2-methyl-UDP induces slightly smaller perturbations in the same direction as UDP. The global structure of the complex E*·NAD^+·UDP rules out the possibility of local magnetic fields such as ring currents to explain the NMR data. Therefore the uridine nucleotide induced conformational change leads to some interaction between an enzymatic group and the nicotinamide ring that polarizes the π-electron system by destabilizing the nicotinamide resonance form **1**. This has



1 **2**

the effect of increasing the contribution of form **2** in the destabilized state. The increased importance of **2** accounts for the enhanced reactivity of NAD^+ as an oxidizing agent. The UDP-induced enzyme conformational change and polarization of the π-electrons in NAD^+ may be conceptualized as illustrated below.



Spectroscopic and kinetic studies of model compounds show that a chemical shift perturbation of 3.4 ppm downfield at carbon-4 of an *N*-alkylnicotinamide is correlated with a 3000 to 15,000-fold increase in reduction reactivity, depending on the polarity of the solvent, and a 125 to 150 mV increase in reduction potential (31). This accounts for the uridine nucleotide-dependent reductive inactivation of UDP-galactose 4-epimerase from *E. coli.*

The structure of the enzyme in its active conformation reveals an interaction that potentially explains the effect of uridine nucleotides on the reactivity of NAD^+. In the active conformation of the enzyme, the 6-ammonium group of Lys^{153} is hydrogen bonded to both the 2′- and 3′-hydroxyl groups of the nicotinamide riboside in NAD^+. The 6-ammonium group is much closer to nicotinamide-N-1 (5.3 Å) than to nicotinamide-C-4 (>7 Å). The spatial relationships are illustrated in **Fig. 5.** The positive electrostatic field between Lys^{153} and nicotinamide-N-1 in the active conformation should polarize the π-electrons in the nicotinamide ring, decreasing the positive charge on N-1 and increasing it on C-4. This would explain the uridine nucleotide effects. The properties of the specific mutant proteins K153M and K153A support this interpretation (32). The enzymatic activities of both mutants are about one ten-thousandth that of the wild-type enzyme, the effect being expressed mainly on k_{cat}. UMP binds to the mutants at least as tightly as to the wild-type enzyme. NAD^+ bound to these mutant forms cannot be reduced by glucose either in the presence or absence of UMP. NAD^+ in both mutants can be reduced at about the same rate with $NaBH_3CN$, but UMP has little or no effect on these reductions. Therefore, Lys^{153} is important in catalysis and essential for the UMP-dependent reduction of NAD^+.

GROUND STATE DESTABILIZATION IN ENZYMATIC CATALYSIS

The imposition of a positive electrostatic field on NAD^+ through enzymatic binding of nonreacting groups in the substrate is a particularly clear example of the importance of ground state destabilization in enzymatic catalysis. The reactivity of NAD^+ toward reducing agents is dramatically increased in epimerase-substrate complexes by the altered conformation induced by uridine nucleotide binding. This conformational change destabilizes the ground state of NAD^+ and forces it toward the transition state. The effect is brought about through electrostatic repulsion between the nicotinamide ring and a residue of the enzyme, Lys^{153}, and not through enzymatic binding of the transition state. This model for activation of NAD^+ implies that the positive electrostatic field is attenuated in the resting enzyme and strengthened in the enzyme-substrate complexes. The structure of the resting enzyme will provide information on the mechanism by which the effect is switched on and off.

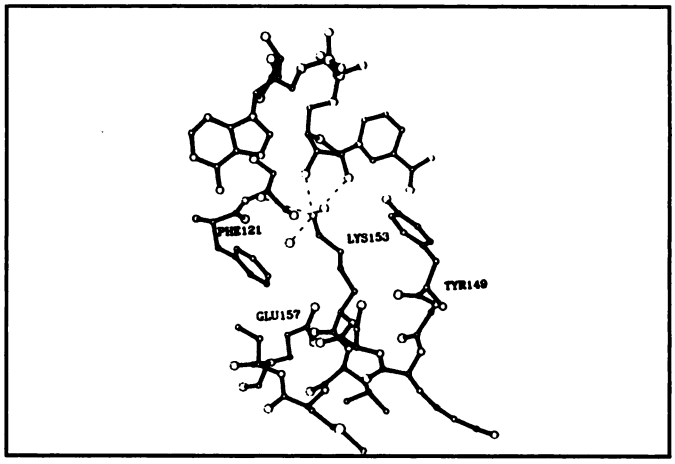


Figure 5. The interaction of Lys^{153} with NAD+ in the active conformation of UDP-galactose 4-epimerase from *E. coli.* The 6-ammonium group of Lys^{153} assists in binding NAD+ through hydrogen bonding to the 2′-OH and 3′-OH of the nicotinamide riboside portion. This creates a positive electrostatic field between Lys^{153} and N-1 of the nicotinamide ring. The electrostatic field is postulated to polarize the π-electrons of the ring by drawing electron density toward N-1 at the expense of C-4, increasing the reactivity of C-4 toward reducing agents. Note the presence of three fixed solvent molecules associated with the 6-ammonium group on the side opposite the ribose ring. Courtesy of Hazel M. Holden.

GALACTOSE-1-P URIDYLYLTRANSFERASE

The essential function of the transferase in the metabolism of galactose is to incorporate the uridine nucleotide moiety into the substrate by producing UDP-galactose and glucose-1-P from UDP-glucose and galactose-1-P. Glucose-1-P enters glucose metabolism and UDP-galactose undergoes epimerization to UDP-glucose. In this way, each turnover of the transferase produces phosphorylated glucose for metabolism and introduces the catalytically essential uridylyl moiety in UDP-galactose for epimerization into UDP-glucose, which in turn provides the uridylyl moiety for another cycle of uridylyltransfer and epimerization (Fig. 1).

The kinetic and chemical mechanism of action of the transferase has been reviewed (33). The reaction follows Ping-Pong kinetics, and the uridylyl (UMP) group of UDP-glucose is transferred to galactose-1-P in a double-displacement mechanism through a covalent uridylyl-enzyme intermediate. Equations 8 and 9 describe the kinetic and chemical mechanism for the transferase from *E. coli.* Transferases from other species act by the same mechanism.

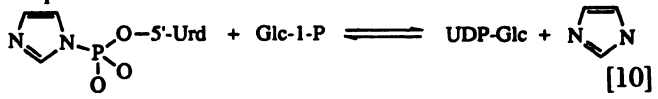
$$\text{E-His}^{166} + \text{UDP-Glc} \rightleftharpoons \text{E-His}^{166} \cdot \text{UDP-GLC} \rightleftharpoons \text{E-His}^{166}\text{-UMP} + \text{Glc-1-P} \quad [8]$$

$$\text{E-His}^{166}\text{-UMP} + \text{Gal-1-P} \rightleftharpoons \text{E-His}^{166}\text{-UMP} \cdot \text{Gal-1-P} \rightleftharpoons \text{E-His}^{166} + \text{UDP-Gal} \quad [9]$$

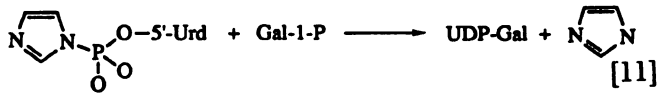
The Ping-Pong mechanism was established by a steady-state kinetic analysis of the overall reaction and of the appropriate exchange reactions, by the isolation and chemical characterization of the covalent intermediate, by the observation of retention of configuration at P_α in the overall reaction of (R_p)-UDP$_\alpha$S-glucose, and by the observation of inversion of configuration at P_α in the first step of the reaction of (R_p)-UDP$_\alpha$S-glucose to produce the covalent UMPS-enzyme intermediate (34). Retention of configuration in the overall reaction is accounted for by the double-displacement mechanism, in which inversion of configuration takes place twice, once in each of the two steps.

The characterization of the covalent intermediate as E-His^{166}-UMP is based on evidence obtained from chemical and specific mutagenic experiments (35–38). The intermediate can be isolated by gel permeation chromatography from glucose-1-P in reaction mixtures containing the transferase and [2-^{14}C]UDP-glucose or [α-^{32}P]UDP-glucose, and the radiolabeled intermediate reacts with glucose-1-P or galactose-1-P to produce the corresponding radiolabeled UDP sugar. The bond linking the UMP group to the enzyme is very labile to hydrolysis in slightly acidic solutions but stable in strongly basic solutions. These properties are characteristic of phosphoramides. The free enzyme is rapidly inactivated by diethylpyrocarbonate in phosphate buffer at pH 6.0 in a process that is readily reversed by hydroxylamine. The free enzyme is fully protected against diethylpyrocarbonate by UDP-glucose, but glucose-1-P or galactose-1-P do not protect the enzyme. Moreover, the UMP-enzyme is very resistant to inactivation by diethylpyrocarbonate. These properties are characteristic of an essential histidine in the enzyme that is protected both by the UMP-group in the substrate and by the UMP moiety in the covalent intermediate. Chemical degradation of the covalent [2-^{14}C]UM^{32}P-enzyme by treatment with periodate followed by mild alkaline elimination of the cleaved nucleoside gives the [^{32}P]phospho-enzyme, which upon hydrolysis in KOH yields [^{32}P]phosphohistidine as the only radiolabeled phosphoamino acid. These properties identify the active site nucleophilic catalyst as a histidine residue.

Specific mutagenic experiments, interpreted in the light of the chemical identification of the nucleophilic catalyst as a histidine residue, showed that His^{166} in the enzyme from *E. coli* provides the nucleophilic imidazole ring that catalyzes UMP transfer. Specific mutation of each of the 15 histidine residues in turn to asparagine, followed by enzymatic analysis of extracts in which these mutant proteins had been expressed, proved that His^{164} and His^{166} are the only essential histidine residues in the enzyme. To determine which of these two residues provides the catalytic nucleophile, two more specific mutants were prepared, H164G and H166G. These mutants lacked the β-carbon and imidazole ring of histidine in positions 164 and 166, respectively. The absence of the histidine-imidazole rings in these mutants generated cavities that might be filled by exogenous imidazole rings. To determine whether function could be restored to the cavity in one of the mutants by added imidazole rings, the proteins were examined for activity in each the reactions of Eqs. 10 and 11.



[10]



[11]

In this experiment, UMP-5′-imidazolate served as a surrogate to the covalent UMP-imidazole moiety of the uridylyl-enzyme intermediate; that is, the catalytic imidazole ring of the active site histidine residue was incorporated within the test substrate. The mutant H166G was found to catalyze reactions shown in both Eqs. 10 and 11 whereas the mutant H164G did not catalyze either reaction. The reaction depicted by Eq. 10 was observed in both forward and reverse directions. The reaction in Eq. 11 was examined in only the forward direction because of the lack of a convenient assay in reverse; however, there is no reason to expect it not to be as reversible as Eq. 10.

The simplest interpretation of the results is that either imidazole or the imidazole ring of uridine-5′-phosphoimidazolate can occupy the cavity created by the absence of the β-carbon and imidazole ring of His^{166} in the mutant H166G, and these exogenous imidazole rings can function in essentially the same way that His^{166} functions in the wild-type enzyme. This is illustrated in **Fig. 6.** However, the essential function of His^{164} cannot be replaced by added imidazole. Therefore, His^{166} provides the nucleophilic imidazole ring in the mechanism.

The *E. coli* sequence His^{164}Pro^{165}His^{166} is conserved in a number of species of transferase. This cluster of conserved amino acids points to the possible importance of all three of them for enzymatic activity. His^{166} is the active site nucleophile. The role of His^{164} will be discussed further in connection with the structure of the enzyme. There is presently no information about the role of Pro^{165}.

The wild-type transferase will not catalyze the reactions of Eqs. 10 and 11, and the mutant H166G will not catalyze the normal transferase reaction (Eqs. 8 and 9). Therefore, H166G is more than simply an inactive mutant of the transferase. It is an enzyme in its own right, a new enzyme that catalyzes reactions not catalyzed by any other enzyme. Moreover, it is a remarkably active enzyme. The k_{cat} value for H166G in reaction 10 in the forward direction is about one-fiftieth of that for the transferase in its specific reaction, and the value of

k_{cat}/K_m for the H166G-catalyzed reaction of UMP-Im is about one-thirtieth of that for the reaction of UDP-glucose with the transferase. The name UDP-hexose synthase has been suggested for H166G (38).

A mechanistic role that has been suggested for covalent intermediates in enzymatic reactions is that a covalent bond can immobilize a substrate and lead to a large decrease in its rotational and translational entropy at the active site. Losses of rotational and translational entropy can account for rate enhancements of up to 10^8. The high activity of UDP-hexose synthase (H166G) shows that the covalent bond is not essential and does not function in the wild-type transferase intermediate primarily to immobilize the substrate. Its most important function appears to be to maintain the uridylyl (UMP) group in a chemically activated state during the interchange of glucose- and galactose-1-phosphates at the binding subsite they share.

Examination of the amino acid sequence in the vicinity of $His^{164}Pro^{165}His^{166}$ of the transferase from *E. coli* and other species suggested the possibility that this enzyme might be a metalloprotein. Cys^{160} is a conserved residue, as is Glu^{152}. The presence of these metal ion-chelating residues suggested the possibility of the presence of tightly bound divalent metal ions such as Zn^{2+}. Metal analysis of the transferase from *E. coli* revealed the presence of both zinc and iron in amounts corresponding to about 1.2 Zn and 0.8 Fe per subunit (39). Experiments to remove the metal ions with chelating agents show that at least the zinc ion is required for activity. Both metal ions can be removed from the denatured enzyme, and renaturation experiments in the presence of divalent metal ions show that active enzyme can be reconstituted from the inactive, metal-free enzyme. Reconstitution with Zn^{2+}, Fe^{2+}, Co^{2+}, Mn^{2+}, or Cd^{2+} leads to active enzyme, with specific activities ranging from 20% to 50% that of the wild-type transferase. The fact that such a wide variety of divalent metal ions can reconstitute activity indicates that neither metal ion participates directly in catalysis and that both serve to establish and maintain the correct tertiary structure of the enzyme.

The structure of the *E. coli* transferase was recently solved by X-ray diffraction crystallography (40). The structure of the α-carbon backbone of one subunit is illustrated in **Fig. 7** by a ribbon diagram, which also shows the locations of the two divalent metal ions and UDP bound at the active site. The subunit structure includes an extended β-sheet with transitional helixes. The metal ions are well separated in the structure and do not share ligands. The ligands for Zn^{2+} are provided by His^{115}, His^{164}, Cys^{53}, and Cys^{55} in a tetrahedral array. The location of the imidazole ring of His^{164} is not near enough to the active site to constitute a part of it, so that His^{164} serves a structural function by providing a ligand for Zn^{2+} at its binding site. This interaction participates in maintaining the active conformation of the enzyme. The mutant H164G tends to undergo degradation during purification, and this may be due to a structural weakness that arises from the absence of the side chain of His^{164} as a Zn^{2+} ligand. The iron is bound through ligands provided by His^{281}, His^{296}, His^{298}, and Glu^{182}. Iron is also well outside the active site and cannot participate directly in chemical catalysis. The γ-carboxyl group of Glu^{182} provides a bidentate ligand to iron, which is held by five ligands in a distorted square pyramid.

BIOCHEMICAL FUNCTION OF A COVALENT INTERMEDIATE

The double displacement, Ping-Pong mechanism for galactose-1-P uridylyltransferase has presumably arisen because of the structural similarities of glucose-1-P and galactose-1-P. These molecules have the potential to occupy a single subsite when acting as uridylyl acceptors. In their absence, the corresponding glycosyl phosphate

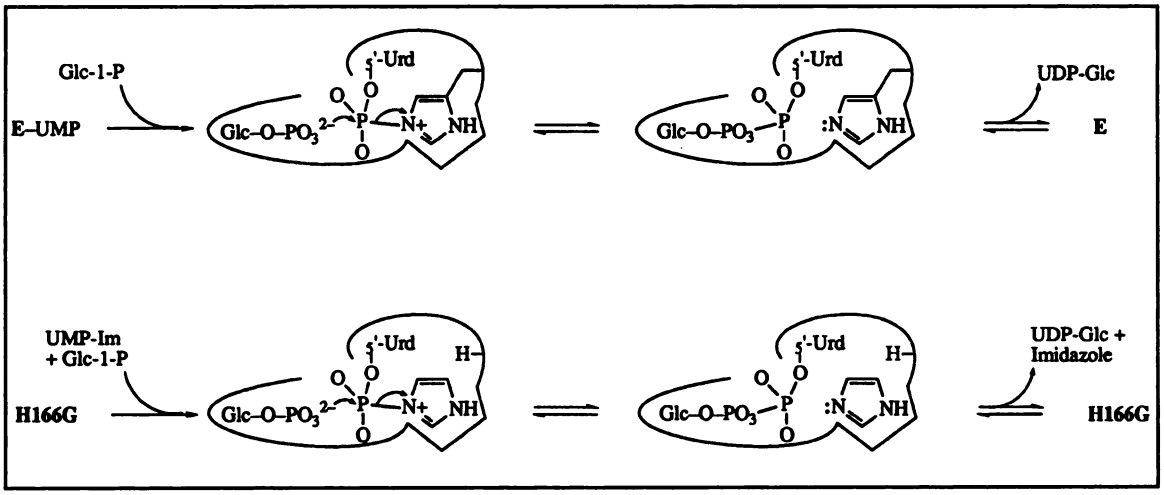


Figure 6. An illustration of the mechanism of UMP-transfer catalyzed by galactose-1-P uridylyltransferase from *E. coli* and its mutant H166G (UDP-hexose synthase).

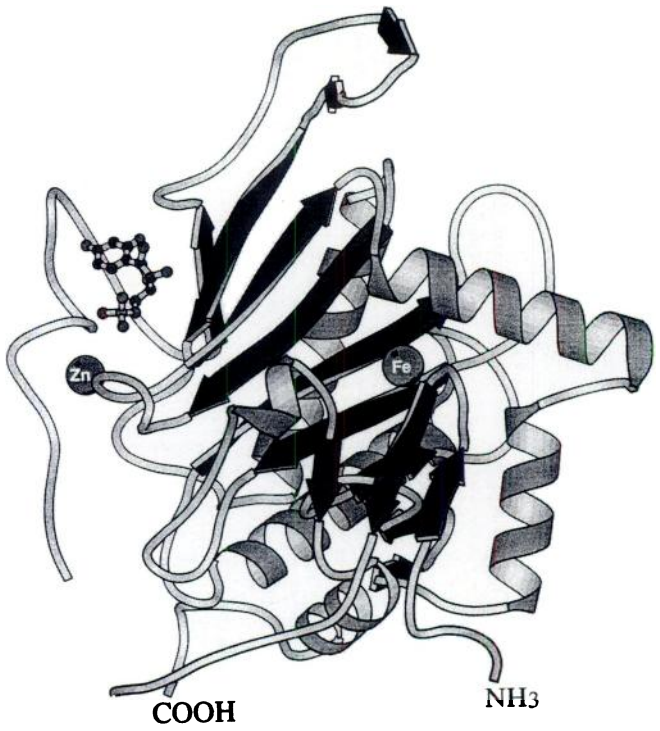


Figure 7. A ribbon diagram of the polypeptide chain in galactose-1-P uridylyltransferase from *E. coli*. Shown is a ribbon diagram of one subunit in the dimeric unit of galactose-1-P uridylyltransferase from *E. coli*. UDP binds to the active site, and its location is illustrated in the diagram. The two metal ions Zn^{2+} and Fe^{2+} are shown in their preferential binding sites. Neither metal ion appears to be located within the active site. At least one of the metal ions is required for enzymatic activity, and both may be required. Enzymatic activity is supported by either metal at both sites or by Co^{2+}, Mn^{2+}, or Cd^{2+} at both sites. The metal ions most likely participate in maintaining the active conformation of the enzyme. Courtesy of Joseph E. Wedekind and Ivan Rayment.

portions of UDP-glucose and UDP-galactose could also occupy the same site. The evolution of the enzyme has allowed a single subsite to serve as the binding site for both molecules. They cannot bind simultaneously, so that some provision for maintaining chemical activation of the uridylyl-group during the changeover from glucose-1-P to galactose-1-P is required. This is accomplished through the presence of His^{166} in position to accept the uridylyl-group in the form of the UMP-enzyme. The evolution of the active site and its mechanism of action appears to have been guided by the principle of economy in the evolution of binding sites. According to this principle, an enzyme evolves with the minimum number of binding sites that will allow it to function efficiently. In the case of the uridylyltransferase, this principle leads to a single site for binding UDP-galactose and UDP-glucose.

The mechanism of action of galactose-1-P uridylyltransferase may be contrasted with that of UDP-glucose pyrophosphorylase (Eq. 7). The action of UDP-glucose pyrophosphorylase does not entail a double-displacement on P_α of substrates—it proceeds with inversion of configuration—nor is there a covalent UMP-enzyme intermediate. The steady-state kinetics indicate ordered binding of substrates to form ternary complexes, and uridylyl transfer takes place directly between acceptor substrates within the ternary complexes. The transferase and pyrophosphorylase reactions are chemically similar, however, consisting of the transfer of a uridylyl (UMP) moiety from a phophoanhydride to a phosphate acceptor. UDP-glucose is a common phosphoanhydride substrate and uridylyl donor for the two enzymes, and both can use glucose-1-P as an acceptor. The differences are that UDP-glucose pyrophosphorylase uses UTP as a donor and PP_i as an acceptor in place of UDP-galactose and galactose-1-P, respectively.

The mechanism followed by galactose-1-P uridylyltransferase is evidently not practical for UDP-glucose pyrophosphorylase, presumably because of the structural differences between $MgPP_i$ and glucose-1-P, which could not easily bind to a single subsite. Therefore, the latter enzyme evolved with separate subsites for these molecules, obviating the need for a covalent intermediate by allowing direct transfer of the uridylyl-group from MgUTP to glucose-1-P.

URIDINE NUCLEOTIDE BINDING BY THE EPIMERASE AND TRANSFERASE

Considerations of biological unity might lead to the expectation of similarities in the binding for uridine nucleotides in the epimerase and transferase. The structures at the current state of resolution and refinement tell a different story. The binding sites for uridine nucleotides in these enzymes do not appear similar and may even be described as a study in contrasts. The binding of UDP-phenol to the epimerase active site is characterized by very strong ionic and hydrogen bonding to the diphosphate portion through Arg^{232}, Arg^{291}, Asn^{179}, and Asn^{199}. In the structure at 2.5 Å resolution, the uridine nucleoside appears to interact with Phe^{218} through its donation of a main-chain NH-hydrogen bond to the uracil-2-oxo group and a side-chain hydrophobic contact with the uracil ring. A higher resolution structure may reveal more detail. The transferase binds UDP with extensive hydrophobic and hydrogen bonding to the uridine nucleoside and ionic binding of the diphosphate. In the structure at 1.8 Å resolution, the uridine nucleoside forms hydrophobic and hydrogen bonded contacts with Leu^{54}, Phe^{75}, Asn^{77}, Asp^{78}, Phe^{79}, and Val^{61}. Residues in the human enzyme corresponding to Leu^{54} and Val^{61} have been implicated in human galactosemia (40). There are also ionic and H-bonded contacts of the diphosphate with Arg^{31} and Asn^{162}.

From a mechanistic standpoint, differences between the binding sites are understandable on the basis of the functions of the enzymes. In the epimerase, the uridine nucleotide is a binding anchor and an inducer of a conformational change but does not itself undergo a chemical reaction. In the transferase, the diphosphate portion of the uridine nucleotide is the substrate reaction center. These different functions may explain the need for different enzyme-nucleotide interactions.

GALACTOKINASE

As illustrated in Fig. 1, galactokinase initiates the process of nucleotide attachment to galactose by catalyzing its phosphorylation by ATP to galactose-1-P. The only mechanistic information is the observation that the steady state kinetics for the reaction requires the formation of a ternary Michaelis complex consisting of enzyme, ATP, and galactose before phosphoryl transfer. There is no evidence for or reason to expect a phosphoenzyme intermediate, although direct chemical tests for this have not been reported. Galactokinase appears to be unique in catalyzing the phosphorylation of an hemiacetal oxygen by ATP. Other phosphotransferases catalyze phosphorylations of alcohols, thiols, amines, amidines, and carboxylic acids. FJ

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