Fluorescence and Nucleotide Binding Properties of *Escherichia coli* Uridine Diphosphate Galactose 4-Epimerase: Support for a Model for Nonstereospecific Action†

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ABSTRACT: The fluorescence emission spectrum for reduced diphosphopyridine nucleotide (DPNH) in *Escherichia coli* uridine diphosphate galactose 4-epimerase·DPNH complexes has a maximum at 435 nm, which is about twice as intense when the excitation is at 280 nm as at 340 nm. The fluorescence excitation spectrum monitored at 460 nm has two maxima, one at 340–345 nm and another about twice as intense at 280 nm. The polarization of DPNH fluorescence by these complexes is 0.43–0.44 compared with 0.46 for DPNH immobilized in propylene glycol at −20 °C. The small degree of fluorescence depolarization is due to rotational relaxation of the protein, relaxation time 205 ns. The excited-state lifetimes in epimerase·DPNH·nucleotide complexes are 3.5–4.2 ns. The fluorescence data show that the dihydropyridine ring in these complexes is highly immobilized and exhibits no detectable independent motion relative to rotational motions of the protein. The inhibition constants for uridine monophosphate (UMP) and 2,2,6,6-tetramethyl-4-piperidinyl-1-oxyl uridyl pyrophosphate acting as competitive reversible inhibitors of epimerase·DPN$^+$ are 1.2 and 0.2 mM, respectively, at 27 °C in 0.1 M sodium bicinate buffer at pH 8.5. A collection of K_i and K_m values for uridine nucleotide inhibitors and substrates indicates that the principal substrate binding interactions involve the nucleotide moieties of substrates. Dissociation constants for uridine nucleotides dissociating from epimerase·DPNH·nucleotide complexes, measured by ultraviolet absorption and fluorescence techniques, are 12 μM for UMP, 14 μM for UDP-hexopyranoses, 4 μM for UDP-pentopyranoses, 27 μM for *p*-bromoacetamidophenyl uridyl pyrophosphate, 0.14 μM for UDP-4-ketohexopyranose intermediate, and 0.36 μM for UDP-4-ketopentopyranose intermediate at 27 °C in 0.1 M sodium bicinate buffer at pH 8.5. Analysis of these data shows conclusively that the major part of the binding free energy for UDP-4-ketopyranose intermediates binding to epimerase·DPNH is attributable to the uridylpyrophosphoryl components and that the glycosyl-binding free energies are much smaller. The data show that the action of this enzyme does not require tight binding between the active site and glycosyl groups of either substrates or intermediates, although there is favorable binding of the uridylpyrophosphoryl components, particularly by epimerase·DPNH. It is postulated that nonstereospecific action results from and depends upon relatively weak, nonspecific active site binding of glycosyl groups in substrates and intermediates and that the uridylpyrophosphoryl groups serve as binding anchors in the epimerization process.

The mechanistic pathway for the action of UDPgalactose 4-epimerase has been studied in several laboratories in recent years. All evidence accumulated to date suggests that an enzyme·DPNH[1]·UDP-4-ketopyranose is a catalytic intermediate (Glaser and Ward, 1970; Nelsestuen and Kirkwood, 1971; Maitra and Ankel, 1971; Wee and Frey, 1973; Adair et al., 1973). An outstanding mechanistic question concerning this pathway is how the enzyme functions nonstereospecifically in converting both UDP-galactose and UDP-glucose reversibly to the common UDP-4-ketopyranose intermediate. Stereospecific action is a general property of enzymes. Those catalyzing hydrogen transfer between pyridine nucleotides and substrates are especially pertinent examples which function stereospecifically with respect to both pyridine nucleotides and cosubstrates (Vennesland and Westheimer, 1954; Popjak, 1970).[2] UDPgalactose 4-epimerase is exceptional in that, although it is B-side specific for hydrogen transfer to the nicotinamide ring of tightly bound DPN$^+$ (Nelsestuen and Kirkwood, 1971; Wee and Frey, 1973; Ketley and Schellenberg, 1973), it is nonstereospecific with respect to glycosyl C-4 of nucleotide sugar substrates. The essence of the epimerization mechanism is that by which the enzyme acts nonstereospecifically at this locus.

In a recent report from this laboratory, it was shown that the *E. coli* enzyme oxidizes C-1 of both α- and β-D-glucose in UMP-dependent reductive inactivation by glucose (Kang et al., 1975), a reaction described originally by Kalckar et al. (1970). It was proposed that nonstereospecificity results from the sugar-binding interactions at the active site, being sufficiently weak and nonspecific to permit the sugar to undergo binding with either face projecting into the site toward the nicotinamide ring of DPN$^+$. We suggested that this might be a model for nonstereospecific action by this enzyme if epimeric nucleotide sugar substrates can be bound at the active site in conformations which differ with respect to which face of the glycosyl ring is projected toward the nicotinamide ring of DPN$^+$. Inspections of molecular models revealed that the stereochemistry at glycosyl C-3 and C-4 of epimeric substrates is such that topographically similar arrays of C-3 and C-4

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[1] Abbreviations used are: TUP, 2,2,6,6-tetramethyl-4-piperidinyl-1-oxyl uridyl pyrophosphate; BUP, *p*-bromoacetamidophenyl uridyl pyrophosphate; NUP, *p*-nitrophenyl uridyl pyrophosphate; DPN, diphosphopyridine nucleotide; DPNH, reduced DPN; UMP, UDP, uridine mono- and diphosphates.

[2] An exception may be one of two enzymes involved in the formation of CDP-4-keto-3,6-dideoxyglucose, which is reported to lack A,B specificity for TPN$^+$ (Rubenstein and Strominger, 1974).

hydroxyl groups and C-4 hydrogen can be projected if the glycosyl moiety of one epimer relative to that of the other is rotated about the bond connecting the glycosyl oxygen and the β-phosphorus of the pyrophosphate linkage. This rotation was postulated to account for nonstereospecific action and epimerization by this enzyme (Kang et al., 1975).

Our model is based on two premises: (a) that the tightly bound DPN^+ occupies a single site and is essentially immobile, and (b) that the principal substrate binding interactions involve the uridylpyrophosphoryl moieties. It is postulated that the glycosyl binding interactions at the active site involve primarily the C-3 and C-4 hydroxyl groups and are not very strong, which would permit the glycosyl groups the mobilities and binding flexibility required for nonstereospecific action. This paper reports several lines of evidence in further support of the assumptions on which our model is based.

Materials and Methods

Materials. UDPgalactose 4-epimerase was purified by the procedure of Wilson and Hogness (1964), except that the hydroxylapatite chromatography was carried out at pH 7.0 instead of 6.5. In a recent paper, we mistakenly gave this pH modification to be from pH 6.5 to 7.3 (Kang et al., 1975) instead of from 6.5 to 7.0. We have found pH to be critical in this step and pH 7.0 to be near optimal.

Nucleotides, nucleotide sugars, $NaBH_4$, and D-glucose were obtained from commercial sources and used without further purification. 2,2,6,6-Tetramethyl-4-piperidinyl-1-oxyl uridyl pyrophosphate, TUP, was synthesized according to Wong (1974). Analytically pure *p*-bromoacetamidophenyl uridyl pyrophosphate, BUP, and *p*-nitrophenyl uridyl pyrophosphate, NUP, were synthesized by Y-H. Huang in this laboratory by the general procedure described by Winer (1972).

Fluorescence Measurements. Excitation and emission spectra were obtained at 25 °C on a laboratory-built spectrophotometer using two 0.25-m Jarrell-Ash monochrometers. Fluorescence emission was monitored with a 6256SA photomultiplier from EMI Electronics and displayed on a Bristol recorder. The excitation spectra were corrected. Quenching of epimerase·DPNH fluorescence by BUP and rates of epimerase·DPNH·nucleotide complex oxidation by TUP were measured on a Perkin-Elmer MPF-3 spectrofluorometer. Fluorescence polarization and lifetime measurements were carried out in Professor Gregorio Weber's laboratory at the University of Illinois using a photon counting polarization instrument and a phase-modulation cross-correlation fluorometer (Spencer and Weber, 1969).

Epimerase·DPNH·Nucleotide Complexes. Complexes of epimerase·DPNH·UMP or epimerase·DPNH·UDP-sugar were prepared for use in competitive exchange experiments and for measurements of the rate of oxidation by TUP. The UMP complex was prepared by incubating the enzyme with 0.1 mM UMP and 0.2 M glucose for 1 h in 0.1 M sodium bicinate buffer at pH 8.5 and then subjecting it to Sephadex G-25 chromatography on a 0.6 × 27 cm column equilibrated and eluted with 0.1 M sodium bicinate buffer containing 50 μM UMP. The UDP-sugar complexes, in which UDP-sugar was the epimeric mixture of UDP-D-glucose and UDP-D-galactose or UDP-D-xylose and UDP-L-arabinose, were prepared by incubating the enzyme with ≥5 mM UDP-D-glucose or UDP-D-xylose for longer than 2 h in 0.1 M sodium bicinate at pH 8.5 and then isolating the complex by Sephadex G-25 chromatography as above, substituting 28 μM UDP-glucose or UDP-xylose for UMP.

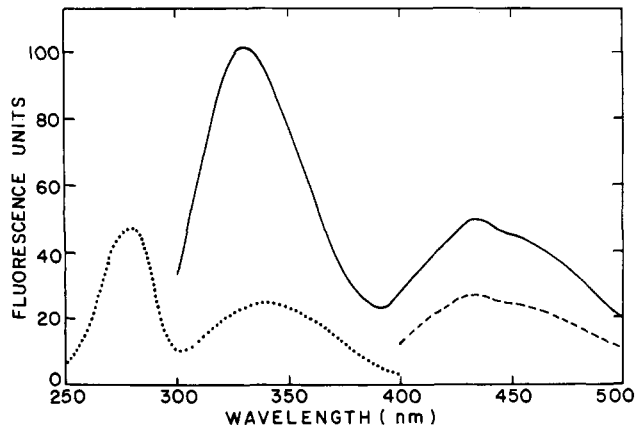


FIGURE 1: Fluorescence emission and excitation spectra of epimerase·DPNH·UMP complex. Sixty-eight micrograms of enzyme was reduced with 1 M glucose in the presence of 14.3 μM UMP. The dotted line is the excitation spectrum with fluorescence monitored at 460 nm, the solid line is the emission spectrum resulting from excitation at 280 nm, and the dashed line is the emission spectrum resulting from excitation at 340 nm.

Results

Fluorescence Properties of Epimerase·DPNH Complexes. We assumed in formulating our hypothesis for nonstereospecific action that the pyridine nucleotide associated with the enzyme is rigidly bound and essentially immobile (Kang et al., 1975). This assumption is consistent with the fact that the nucleotide is tightly bound throughout purification to homogeneity as well as that it is stereospecifically reduced to [4-B-^{3}H]DPNH by NaB^3H_4 (Nelsestuen and Kirkwood, 1971; Wee and Frey, 1973). To obtain further evidence bearing on this, we have investigated the fluorescence properties of epimerase·DPNH complexes.

Figure 1 shows the emission and excitation spectra of the epimerase·DPNH complex produced by UMP-dependent reduction of epimerase·DPN^+ by glucose. When excited at 340 nm, the complex exhibits an emission spectrum typical of enzyme-bound DPNH with the maximum shifted to 435 nm compared with the 460-nm maximum characteristic of free DPNH. When excited at 280 nm, there is also an emission maximum at 435 nm which is stronger than that resulting from excitation at 340 nm. The relative emission intensities upon excitation at these wavelengths are displayed in the excitation spectrum shown in Figure 1. The fluorescence properties of the epimerase·DPNH complexes obtained by reduction with $NaBH_4$ in the presence of UMP or UDP-glucose and by the equilibrium mixture of UDP-glucose and UDP-galactose to produce the epimerase·DPNH·UDP-hexose abortive complex (Wee and Frey, 1973) are similar to those in Figure 1. The fluorescence properties are similar to those reported by Bertland (1970) for the yeast enzyme, and the higher fluorescence emission observed at 435 nm upon exciting at 280 nm, as compared with 340 nm, is evidence of unusually efficient energy transfer from the protein fluorofors to DPNH. The energy transfer observed by Bertland (1970) with the yeast enzyme is even more efficient.

Fluorescence depolarization measurements on reduced enzyme complexes prepared by different methods are listed in Table I. The polarization values can be compared with that for DPNH in propylene glycol at −20 °C, which is 0.461. Under these conditions, the polarization of fluorescence is temperature independent, so that this value is taken to represent the theoretical maximum polarization for immobilized

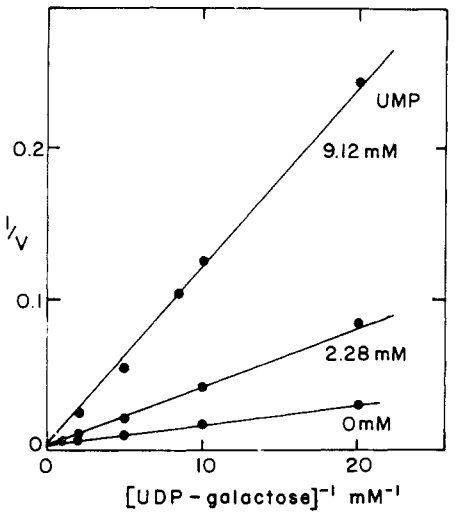


FIGURE 2: Competitive inhibition by UMP. The enzyme activity was measured as described by Wilson and Hogness (1964) with ionic strength adjusted to 0.2 M by addition of KCl. Each rate was measured in duplicate at [UDP-galactose] between 0.05 and 1.0 mM and duplicate rates differed by about 5% on the average and in no case by more than 10%. The mean values were plotted and fitted to the rate equation for competitive reversible inhibition of a one substrate–one product reaction. The K_m for UDP-galactose was found to be 0.28 mM, while the K_i for UMP was 1.19 mM at 9.12 mM UMP and 1.18 mM at 2.28 mM UMP.

DPNH. As shown in Table I, the emission from the epimerase·DPNH complexes is about 93–96% polarized when referred to 0.461 as the maximum. The residual small degree of depolarization disappears when Perrin plots as a function of temperature are made and extrapolated to infinite viscosity, as shown by the agreement of the P_0 values in Table I with 0.461. The linearities of these plots further support the interpretation that the small degree of depolarization is due to the Brownian motions of the protein.

The fluorescence lifetimes (τ), measured by two different methods in Table I, are in good agreement, which indicates no fluorescence heterogeneity in the solutions. The differences in τ between epimerase·DPNH·UMP complexes and epimerase·DPNH·UDP-hexose complexes appear to be significant and probably reflect minor differences in the binding interactions of UMP and UDP-hexoses with the active site.

The relaxation times in Table I were calculated from eq 1 (Perrin, 1926) assuming 0.461 for P_0 and the tabulated values for P and τ.

$$\left(\frac{1}{P}-\frac{1}{3}\right)=\left(\frac{1}{P_0}-\frac{1}{3}\right)\left(1+\frac{3\tau}{\rho}\right) \qquad (1)$$

Binding of Uridine Nucleotides by Epimerase·DPN⁺ Complex. Inasmuch as this enzyme reversibly converts both UDP-galactose and UDP-glucose to a common UDP-4-keto-pyranose intermediate concomitant with hydrogen transfer from glycosyl C-4 to DPN⁺, the epimeric substrates must bind in sterically different ways. The interactions of the active site with uridine nucleotides of various structures may shed some light on this.

We have measured the inhibition constants for some competitive reversible inhibitors, as well as the K_m for UDP-galactose. The data in Figure 2 were obtained for UMP inhibition of the initial rate of UDP-galactose epimerization. The parameters obtained by fitting these data to the equation for competitive reversible inhibition of a one substrate reaction produced values of 0.28 mM as the K_m for UDP-galactose, in reasonable agreement with the published value of 0.16 mM (Wilson and Hogness, 1964), and 1.2 mM as the K_i for UMP.

We have also studied the binding of TUP, a stable free-radical nitroxide derivative of UDP. This compound was found to be a competitive reversible inhibitor with a K_i of 0.2 mM, about the same as the K_m for UDP-galactose.

A collection of available data on the binding of uridine nucleotides by epimerase·DPN⁺ is given in Table II. Included are published data, as well as data from this work on UMP and TUP and additional data on BUP and NUP, an active site directed irreversible inhibitor and its precursor, respectively (Winer, 1972). The affinities of the enzyme for these compounds are all similar within a factor of about 5 or 6. The structural common denominator is that all are uridine nucleotide dianions and all but UMP are alkyl or aryl uridyl pyrophosphates. The similarities in their relative affinities, despite a wide variation in the nature of the alkyl and aryl moieties, from hydrophilic to hydrophobic groups and from the absence of any such group in UMP to the presence of the bulky 2,2,6,6-tetramethyl-4-piperidinyl-1-oxyl substituent, suggest that the major part of the binding interactions involve the

TABLE I: Fluorescence Depolarization and Lifetime of Reduced UDPgalactose 4-Epimerase.[a]

Complex	P	P_0	τ(ns)[b]	ρ(ns)[c]
Epimerase·DPNH·UDP hexose	0.433			
	0.433	0.458	4.10 ± 0.06	
			4.24 ± 0.16	164
Epimerase·DPNH·UMP	0.443	0.468	3.50 ± 0.03	231
			3.90 ± 0.04	
	0.443	0.459	3.50 ± 0.03	
			3.50 ± 0.03	219

[a] Epimerase·DPNH·UDP-hexose was prepared in one experiment by reducing the enzyme with 1.9 mM $NaBH_4$ in the presence of 8.75 μM UDP-galactose plus UDP-glucose at epimeric equilibrium and in the other experiment by permitting abortive complex to form in the presence of 2.5 mM UDP-hexopyranose substrates (Wee and Frey, 1973). Epimerase·DPNH·UMP was prepared by reducing epimerase with 2.5 mM $NaBH_4$ in the presence of 23 μM UMP in the first experiment and with 0.13 M glucose in the presence of 33 μM UMP in the second. [b] Fluorescence lifetime measured by phase decay and by degree of modulation. [c] Rotational relaxation times.

TABLE II: Uridine Nucleotide Binding by *E. coli* UDPgalactose 4-Epimerase·DPN⁺ Complex.

Nucleotide	Dissociation Parameter (mM)	Reference
UDP-D-glucose	1.0[a]	Imae et al., 1964
UDP-D-galactose	0.16[a]	Wilson and Hogness, 1964
	0.28[a]	This work
UDP-D-xylose	1.2[a]	Ankel and Maitra, 1968
UMP	1.86 ± 0.72[b]	Kang et al., 1975
	1.2[c]	This work
UDP-D-fucose	1.3[c]	Spencer et al., 1973
	0.76[b]	Blackburn et al., 1976
TUP	0.2[c]	This work
NUP	0.21[c]	Winer, 1972
BUP	0.21[b]	Winer, 1972

[a] Substrate K_m. [b] Dissociation constant measured kinetically in inactivation experiments. [c] Inhibition constant for competitive reversible inhibition.

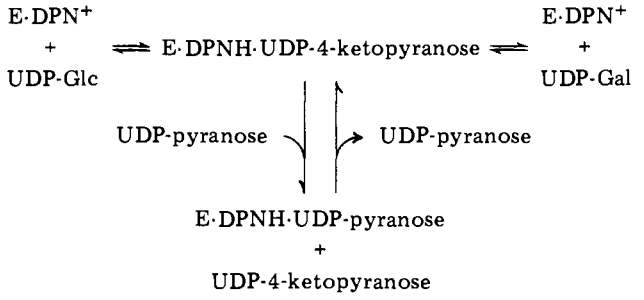
nucleotide components. An instructive case, which may be analogous to substrate binding, is the epimerase·DPN^+·UMP·glucose complex. From the dissociation constants for UMP, 1.86 mM, and D-glucose, 0.49 M (Kang et al., 1975), it can be calculated that the binding free energy for UMP is about -3.7 kcal mol^{-1}, while that for the binding of glucose to the epimerase·DPN^+·UMP complex is -0.4 kcal mol^{-1}, about a tenfold difference.

Binding of Uridine Nucleotides by Epimerase·DPNH Complex. The epimerase·DPNH complex is known to differ from the epimerase·DPN^+ complex in that it binds uridine nucleotides much more tightly (Bertland et al., 1971; Nelsestuen and Kirkwood, 1971; Wee and Frey, 1973). Inasmuch as the conformational transition leading to nonstereospecific action must occur in the epimerase·DPNH·UDP-4-ketopyranose complex, information on the binding of uridine nucleotides by epimerase·DPNH is at least as important as the foregoing information on epimerase·DPN^+ complex. Moreover, such data must be consistent with any acceptable hypothesis for nonstereospecific action.

Direct binding studies involving epimerase·DPNH complex have been complicated by the fact that it is unstable with respect to autoxidation in the absence of uridine nucleotides. We have, therefore, taken indirect approaches. We first measured equilibrium constants for competitive exchange reactions between pairs of uridine nucleotides competing for binding to epimerase·DPNH and then measured the dissociation constants for several of the same nucleotides by a kinetics technique. We collected sufficient data to calculate dissociation constants for the UDP-4-ketopyranose intermediates, UDP-pyranoses, UMP, and BUP.

The equilibrium constants for exchanges of UDP-4-ketopyranose intermediates with UDP-pyranose substrates were measured spectrophotometrically by observing the A_{345} changes accompanying (1) the additions of substrates to the enzyme, (2) the appearance of abortive complex (Wee and Frey, 1973), and (3) complete reduction of the enzyme to epimerase·DPNH complex. Earlier work from this laboratory showed that Scheme I describes the interconversion of UDP-

Scheme I



glucose and UDP-galactose, and the appearance of an equilibrium concentration of an abortive complex in which a molecule of UDP-pyranose has exchanged with UDP-4-ketopyranose in association with the epimerase·DPNH complex.

On the basis of this scheme, it can be shown that eq 2 gives the equilibrium constant, K_1, for the exchange of UDP-4-ketopyranose with UDP-pyranose substrates at epimeric equilibrium according to eq 3.

$$K_1 = \frac{6.2 \times 10^3 A^{\alpha}(A^{\gamma} - A^{\beta})(A^{\gamma} - A^{\alpha})[\text{UDP-pyranose}]}{[A^{\gamma}(A^{\beta} - A^{\alpha})]^2} \quad (2)$$

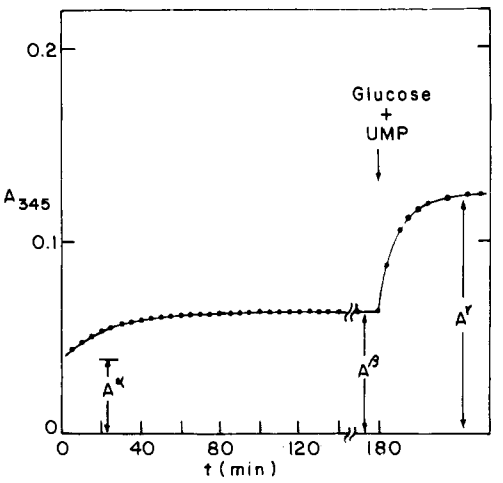


FIGURE 3: Abortive complex formation in the presence of substrates. About 1.3 mg of enzyme in 0.8 ml of 0.075 M sodium bicinate at pH 8.7 was placed in a spectrophotometric cuvet at 27 °C. A small aliquot of UDP-galactose was added to a final concentration of 0.94 mM and the A_{345} was monitored continuously. At the indicated time, UMP and glucose were added to 1 mM and 0.12 M, respectively. Plotted here are data points taken from the recorder chart record.

$$\text{E·DPNH·UDP-pyranose} + \text{UDP-4-ketopyranose} \underset{}{\overset{K_1}{\rightleftharpoons}} \text{E·DPNH·UDP-4-ketopyranose} + \text{UDP-pyranose} \quad (3)$$

In eq 2, 6.2×10^3 is the extinction coefficient of DPNH, [UDP-pyranose] refers to the total concentration of substrates of epimeric equilibrium, A^{α} refers to the A_{345} shortly after mixing and corresponds to [E·DPNH·UDP-4-ketopyranose] intermediate prior to the accumulation of abortive complex, A^{β} corresponds to the sum of the concentrations of abortive complex and intermediate complex when all of the processes in Scheme I are at equilibrium, and A^{γ} measures the total concentration of active enzyme in a reaction mixture. The experimental origins of these quantities are further defined in Figure 3, which presents sample data.

The measured K_1 values for six concentrations of the epimeric substrate pairs UDP-D-galactose and UDP-D-glucose between 1 and 5 mM were 99.3 ± 9.0. For the epimeric substrates UDP-D-xylose and UDP-L-arabinose, the values were 11 ± 6 in three determinations between 0.4 and 2 mM substrates. These values were less accurate than those for UDP-hexopyranoses because the A^{α} were smaller and less accurately measured. Thus, the equilibria favored the binding of UDP-4-ketopyranose intermediates by a factor of about 100 for the hexopyranoses and 5–20 for the pentopyranoses. The data indicated some binding interactions involving the C-6 hydroxymethyl groups which, however, are not an essential part of the epimerization mechanism.

We have also measured the equilibrium constants K_2 and K_3 for the exchanges of BUP with UDP-hexopyranoses and UMP bound to the epimerase·DPNH complex according to eq 4 and 5.

$$\text{E·DPNH·UDP-hexopyranose} + \text{BUP} \overset{K_2}{\rightleftharpoons} \text{E·DPNH·BUP} + \text{UDP-hexopyranose} \quad (4)$$

$$\text{E·DPNH·UMP} + \text{BUP} \overset{K_3}{\rightleftharpoons} \text{E·DPNH·BUP} + \text{UMP} \quad (5)$$

The UDP-hexopyranose referred to in eq 4 was the substrate

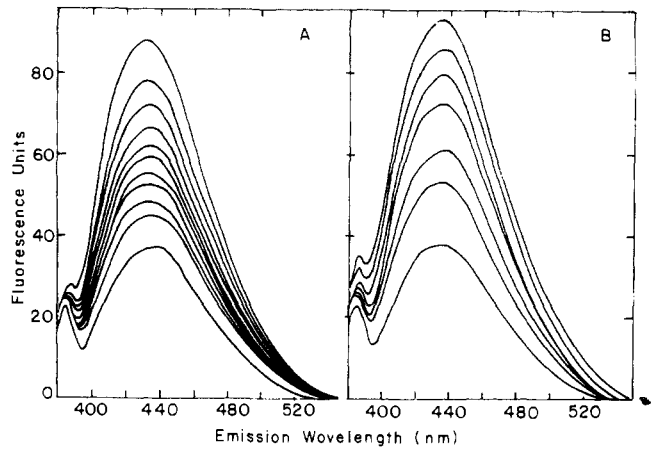


FIGURE 4: Reversible quenching of epimerase·DPNH fluorescence by BUP. In part A, a solution of epimerase·DPNH·UDP-hexopyranose in 0.1 M sodium bicinate buffer at pH 8.5 containing 28.5 μM UDP-hexopyranose was subjected to incremental additions of BUP. After each addition, the emission spectrum was measured with excitation at 340 nm. From the uppermost spectrum reading down, the BUP concentrations were: 0, 3.90, 7.78, 11.7, 15.5, 19.4, 23.3, 27.1, 34.8, 42.5, and 57.9 μM. In part B, the solution was back-titrated with UDP-hexopyranose. Reading up from the lowermost spectrum, the UDP-hexopyranose concentrations were: 28.2, 71.5, 157, 285, 412, 623, and 830 μM. UDP-hexopyranose refers to the mixture of UDP-glucose and UDP-galactose at epimeric equilibrium.

pair UDP-galactose and UDP-glucose at epimeric equilibrium. These measurements were made by observing the decrease in fluorescence of epimerase·DPNH·UMP and epimerase·DPNH·UDP-pyranose complexes upon adding BUP, which quenched the DPNH fluorescence when bound to the complex. The quenching was completely reversed when BUP was in turn displaced from the enzyme by high concentrations of UDP-pyranose or UMP, as shown in Figure 4.

It can be shown that in an experiment such as that in Figure 4 the equilibrium constant K_2 or K_3 is given by eq 6

$$\frac{[\text{BUP}]}{1-\theta} = \frac{[\text{nucleotide}]}{K\theta} - [\text{E}\cdot\text{DPNH nucleotide}](1/K - 1) \quad (6)$$

where θ is the ratio of fluorescence intensity in the presence and absence of BUP, [nucleotide] is the concentration of UDP-pyranose or UMP, and K is K_2 or K_3. Using this equation together with data such as that in Figure 4, we evaluated K_2 and K_3 from the slopes of plots of $[\text{BUP}]/1-\theta$ vs. $1/\theta$, which were straight lines. These values were 0.46 and 0.58 for K_2 in two determinations and 0.44 for K_3 at 27 °C and 0.1 M sodium bicinate buffer at pH 8.5. Using these experimental values, we also calculated the equilibrium constant K_4 for the exchange of UDP-hexopyranose with UMP according to eq 7.

$$\text{E}\cdot\text{DPNH}\cdot\text{UMP} + \text{UDP-pyranose} \overset{K_4}{\rightleftharpoons} \text{E}\cdot\text{DPNH}\cdot\text{UDP-pyranose} + \text{UMP} \quad (7)$$

The ratio K_3/K_2 gave K_4 values of 0.96 and 0.76 in two determinations, showing that the hexose moiety in UDP-hexopyranoses contributes little, if any, to the tight binding of these compounds by epimerase·DPNH.

The dissociation constants for UMP or the epimeric equilibrium mixtures of UDP-pyranoses dissociating from epimerase·DPNH·nucleotide complexes were measured kinetically. This was accomplished by studying the kinetics for a new reaction, recently discovered, in which TUP oxidizes the DPNH in these complexes (S. S. Wong and P. A. Frey, 1976).

In order for TUP free radical to react with an epimerase–DPNH nucleotide complex, it must first exchange with the nucleotide. The resulting epimerase·DPNH·TUP complex then undergoes an O_2-dependent decomposition to epimerase·DPN^+, reduced free radical, and superoxide. The kinetic measurement of the dissociation constant for the nucleotide depends upon the pathway for nucleotide exchange. The simplest exchange pathway which is consistent with our results is Scheme II.

Scheme II

$$\text{E}\cdot\text{DPNH} + \text{nucleotide} \overset{k_1}{\underset{k_2}{\rightleftharpoons}} \text{E}\cdot\text{DPNH}\cdot\text{nucleotide}$$

$$\text{E}\cdot\text{DPNH} + \text{TUP} \overset{k_3}{\underset{k_4}{\rightleftharpoons}} \text{E}\cdot\text{DPNH}\cdot\text{TUP} \xrightarrow{k_5} \text{E}\cdot\text{DPN}^+$$

This kinetic model does not state by what mechanism or at what step O_2 is involved, although we believe it to be involved in the k_5 step. All of our rate measurements were made in air-equilibrated solutions at constant O_2 pressure, so questions concerning O_2 involvement should not affect our analysis. According to Scheme II, the reaction starting with E·DPNH·nucleotide should follow the first-order rate law with respect to decay of this complex when [TUP] and [nucleotide] are much larger than the concentration of enzyme. The observed pseudo-first-order rate constant is given by eq 8

$$1/k_{\text{obsd}} = \phi_0 + \frac{\phi_1}{[\text{TUP}]} + \frac{\phi_2[\text{nucleotide}]}{[\text{TUP}]} \quad (8)$$

where ϕ_0 is k_5^{-1}, ϕ_1 is $(k_4 + k_5)/k_3k_5$, and ϕ_2 is $k_1(k_4 + k_5)/k_2k_3k_5$ when the steady state approximation is made. If the equilibrium binding approximation is made, ϕ_1 is k_4/k_3k_5 and ϕ_2 is $k_1k_4/k_2k_3k_5$. In either case, the ratio ϕ_1/ϕ_2 is k_2/k_1, the dissociation constant for nucleotide dissociating from the enzyme. All exchange pathways involving association of TUP with E·DPNH·nucleotide complex prior to dissociation of the nucleotide are inconsistent with eq 8.

We measured the rates of decay of the fluorescence of E·DPNH·nucleotide complexes as functions of [nucleotide] and [TUP]. All rates were first order, and plots of k_{obsd}^{-1} vs. $[\text{TUP}]^{-1}$ at several fixed [nucleotide] were consistent with eq 8, as shown in Figure 5. The replots of slope vs. [nucleotide] were straight lines, and the dissociation constants for UMP and UDP-pyranoses were calculated from ϕ_1 and ϕ_2, the intercepts and slopes of the replots respectively. The values obtained at 27 °C and pH 8.5 in 0.1 M sodium bicinate buffer were 12 μM for UMP, 14 μM for UDP-galactose and UDP-glucose at epimeric equilibrium, and 4 μM for UDP-xylose and UDP-arabinose. The ratio of the values for UMP and UDP-hexopyranoses, 0.86, was the same within error as the calculated value for K_4 in eq 7, which confirmed the consistency of the kinetically measured dissociation constants with the foregoing binding data. Using these data together with K_1, K_2, and K_3, we calculated dissociation constants for the nucleotides listed in Table III.

Table III shows that the affinities of UDP-hexopyranoses, UDP-pentopyranoses, UMP, and BUP for epimerase DPNH are remarkably similar. The UDP-4-ketopyranose intermediates are significantly more tightly bound, but, even in these cases, the dissociation constants are only 10- to 100-fold smaller than those of UDP-pyranoses. Comparing the dissociation constants for UMP and UDP-4-ketopyranoses, the binding free energy for UMP is -6.75 kcal mol^{-1}, while that

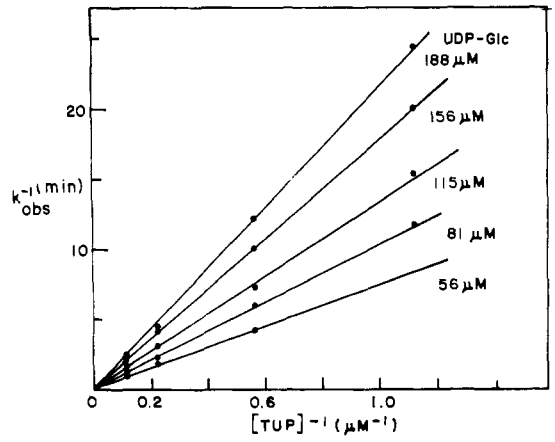


FIGURE 5: Kinetics for oxidation of epimerase·DPNH·nucleotide complexes by TUP. The observed pseudo-first-order rate constants were measured fluorimetrically in 0.1 M sodium bicinate buffer at pH 8.5 and 27 °C at the indicated concentrations of TUP and UDP-glucose. Under the experimental conditions, UDP-glucose was in epimeric equilibrium with UDP-galactose. The data were plotted according to eq 8; the lines do *not* intersect at zero on the ordinate. The intersection point is above zero but not easily seen.

for UDP-4-ketopentopyranose is −8.8 kcal mol^{-1} and for UDP-4-ketohexopyranose it is −9.4 kcal mol^{-1}. The differences in binding free energies between UMP and the catalytic intermediates are −2.0 and −2.6 kcal mol^{-1}, which are the maximum binding free energies attributable to sugar binding interactions, while −6.75 kcal mol^{-1} is the minimum attributable to the binding of the nucleotide moiety, minimum because UMP dianion probably does not bind quite as well as a uridylpyrophosphoryl dianion. Alternative values for keto sugar binding interactions can be obtained by comparing the dissociation constants for UDP-pyranoses with those for UDP-4-ketopyranoses. These differ by 11 and 99 for the UDP-pentose and UDP-hexose substrates, respectively, corresponding to binding free energies of −1.4 and −2.7 kcal mol^{-1} for the 4-ketopentopyranosyl and 4-ketohexopyranosyl groups. From these and the binding free energies for UDP-4-ketopyranoses, we estimate the uridylpyrophosphoryl binding free energies to be −7.4 and −6.7 kcal mol^{-1} for UDP-4-ketopentopyranose and UDP-4-ketohexopyranose. Both estimates show that of the −9 kcal mol^{-1} binding free energy for the intermediates about −7 kcal mol^{-1} is attributable to uridyl pyrophosphoryl binding, and about −2 kcal mol^{-1} to 4-ketopyranosyl binding. We conclude that the principal binding interactions involve the nucleotide components of intermediates.

UDP-Hexopyranose Dependent Reduction of Epimerase·DPN$^+$ by Glucose. The dissociation constants in Tables II and III show that, from the standpoint of binding energy, the major binding interactions between the active site and substrates involve the nucleotide portions of UDP-pyranoses. Sugar binding appears to be weak by comparison. This suggests that glucose at large concentrations might displace pyranosyl rings of bound UDP-pyranoses by binding at the sugar subsites without necessarily displacing the nucleotide components from their subsites; i.e., E·DPN$^+$·UDP-pyranose·glucose complexes might be possible. It would be difficult to detect such complexes by direct methods because of the low affinity of the enzyme for glucose (Kang et al., 1975); however, they could be detected indirectly if the nucleotide sugar were to activate reduction of epimerase·DPN$^+$ by glucose. This would occur if the nucleotide sugar could fulfill the structure-modifying role played by

TABLE III: Uridine Nucleotide Binding by *E. coli* UDPgalactose 4-Epimerase·DPNH Complex.[a]

Nucleotide	Dissociation Constant (μM)
UDP-hexopyranose[b]	14
UMP	12
UDP-pentopyranose[b]	4
BUP	27
UDP-4-ketohexopyranose	0.14
UDP-4-ketopentopyranose	0.36

[a] Data refer to 27 °C at pH 8.5 in 0.1 M sodium bicinate buffer. [b] UDP-hexopyranose refers to UDP-galactose and UDP-glucose at epimeric equilibrium and UDP-pentopyranose to UDP-D-xylose and UDP-L-arabinose at equilibrium.

UMP in UMP-dependent reductive inactivation by glucose (Kang et al., 1975).

Such a reaction has recently been described by Blackburn and Ferdinand (1976) who studied UDP-fucose-dependent reductive inactivation by D-fucose and L-arabinose. These workers did not detect UDP-glucose activation of this reaction and concluded that the glucosyl group is tightly bound; however, we have been able to detect UDP-glucose- and UDP-galactose-activated reduction by glucose under experimental conditions likely to favor the reaction, i.e., half-saturating nucleotide sugar and very large [glucose]. The experimental protocol and results were similar to those of Figure 3, except that UMP was omitted. Under conditions otherwise similar to Figure 3, the addition of glucose to 1 M final concentration to an enzyme solution containing 0.22 mM UDP-galactose and UDP-glucose at epimeric equilibrium subsequent to the appearance of the equilibrium concentration of abortive complex, i.e., A^β in Figure 3, led to an increase in A_{345} similar to that between A^β and A^γ in Figure 3 with a half-time of about 15 min. This would not have occurred in the absence of any nucleotide. Control experiments showed that it is very unlikely to have been caused by UMP contamination. If it had been, the rate of the A^β to A^γ change should have been dependent upon the degree of contamination; however, this rate was independent of whether the substrate was initially UDP-galactose or UDP-glucose, and the two samples were unlikely to have been contaminated by UMP to exactly the same extent. In addition, the presence of 0.1–0.7 IU of alkaline phosphatase prior to the addition of glucose did not affect the results.

On the basis of these experiments, we conclude that UDP-glucose- and UDP-galactose-dependent reduction of epimerase·DPN$^+$ by glucose occurs. Therefore, both glucose and UDP-hexopyranoses can simultaneously interact with the enzyme.

Discussion

The essential aim of this study has been to obtain information concerning the mechanism by which UDPgalactose 4-epimerase acts nonstereospecifically in transferring hydrogen between the B side of DPN$^+$ and glycosyl C-4 of epimeric substrates. For this process to be nonstereospecific with respect to glycosyl C-4, there must be a reversible isomerization of the epimerase·DPNH·UDP-4-ketopyranose intermediate involving at least two conformations, one for each of two reaction pathways leading to UDP-glucopyranose and UDP-galactopyranose. The isomerization could involve the conformation

of the enzyme itself, the conformations of enzyme bound substrates and intermediates, and the conformation of the pyridine nucleotide. Although there is good evidence that the binding of uridine nucleotides induces structural changes in epimerase·DPN^+ leading to increased reactivity of DPN^+ toward reducing agents (Davis et al., 1974; Kalckar et al., 1970; Wee and Frey, 1973) and that its conversion to epimerase·DPNH induces further conformational changes manifested by ORD–CD changes and increased binding affinity for uridine nucleotides (Bertland and Kalckar, 1968; Nelsestuen and Kirkwood, 1971; Bertland et al., 1971; Wee and Frey, 1973; Seyama and Kalckar, 1972), there is no information concerning whether these effects are related to nonstereospecific action by this enzyme. The present studies do not bear on this point; therefore, we shall not invoke enzyme conformational transitions in discussing nonstereospecificity. This is not to deny their possible involvement; such transitions could complement those of the UDP-ketopyranose intermediates postulated in the following, but they are not essential to our argument and will not be considered further at this time.

Available information on DPN^+ and DPNH binding does not offer any basis for proposing that nonstereospecific action by this enzyme results from mobility on the part of the nicotinamide ring of the coenzyme. Our findings of highly efficient energy transfer from aromatic residues in the enzyme to DPNH and 100- to 1000-fold tighter binding of uridine nucleotides by epimerase·DPNH than by epimerase·DPN^+ suggest that interactions between the enzyme and the dihydronicotinamide ring are strong. Our data on polarization of fluorescence also suggest that the nicotinamide ring of DPNH is rigidly bound. The small degree of fluorescence depolarization can be attributed to rotational relaxation of the protein itself.

Thus, our results show that any motion on the part of the dihydronicotinamide ring with a relaxation time comparable to or faster than the rotational relaxation time of the protein, ~200 ns, can be ruled out. Moreover, comparative experiments with lactate dehydrogenase show that the maximum polarization for DPNH bound to this enzyme is only 0.34 (D. Jameson and S. S. Wong, unpublished experiment), although the fluorescence lifetime is only 1.5 ns (Scott et al., 1970) and the molecular weight is larger than that of epimerase. This means that DPNH is substantially less immobilized when bound to lactate dehydrogenase than it is in the epimerase·DPNH complex.

Other indirect evidence is also most consistent with the interpretation that DPN^+ is rigidly bound at a highly specific site on the enzyme. It is essentially irreversibly bound (Wilson and Hogness, 1964). It is stereospecifically reduced to [4-B-^{3}H]DPNH by NaB^3H_4 (Nelsestuen and Kirkwood, 1971; Wee and Frey, 1973). It does not exchange with free DPN^+ at a detectable rate in the absence of denaturants (Pydeski, 1975). It readily exchanges with free DPN^+ (<1 h) in the presence of 5 mM *p*-hydroxymercuribenzoate (Davis and Glaser, 1971; Pydeski, 1975), but its exchange rate with $AcPyDPN^+$ and N^6-ethenoDPN$^+$ under the same conditions is much slower (>5 h) and only detectable with 100- to 500-fold excesses of the analogues, and the epimerase DPN^+ analogue complexes obtained in such experiments do not exhibit any detectable activity either in the epimerization of substrates or in the UMP-dependent reduction by glucose (Pydeski, 1975). On the basis of available information, the epimerases from yeast and mammalian sources exhibit generally similar properties, except that the binding of DPN^+ by mammalian enzymes is reversible, though still very tight, and DPN^+ in the yeast enzyme can exchange with DPN^+ under conditions of low ionic strength (Gabriel et al., 1975; Langer and Glaser, 1974).

There remains the possibility that our depolarization of fluorescence experiments might have been insensitive to a small degree of dihydropyridine mobility. To be missed, such a motion would have to be associated with a relaxation time substantially longer than 200 ns. It is possible that this could be the case if such a motion corresponded to the rate-limiting catalytic process for this enzyme; the turnover number is reported to be 500 s^{-1} (Wilson and Hogness, 1964) corresponding to a relaxation time of 2×10^3 ns.

The data on uridine nucleotide binding contrast sharply with those on DPN^+ binding. Tables II and III clearly show that the major binding interactions between epimerase·DPN^+ and UDP-pyranoses and between epimerase·DPNH and UDP-4-ketopyranoses involve the nucleotide components. Those between the pyranosyl rings and the respective enzyme species are relatively weak. This binding model is further strengthened by the fact that UDP-hexoses activate reduction of epimerase DPN^+ by glucose, showing that epimerase·DPN^+·UDP-hexose·glucose complexes are possible. These findings are also in accord with the lack of sugar specificity exhibited by this enzyme in catalyzing the epimerizations of UDP-xylose (Ankel and Maitra, 1968), UDP-fucose (Spencer et al., 1973), and UDP-2-deoxyglucose (Druzhinina et al., 1975), which shows that there can be no essential binding interactions involving the C-6 hydroxymethyl group or the C-2 hydroxyl group.

Our findings support the mechanistic model for nonstereospecific action set forth in a recent paper from this laboratory. According to this proposition, DPN^+ and DPNH are rigidly bound within a highly specific site, while substrates are bound at a single adjacent site. The basis for nonstereospecificity is rotational mobility of the keto sugar in UDP-4-keto sugar intermediates. The major binding interactions involve the uridylpyrophosphoryl groups, which serve as binding anchors, while the 4-ketopyranosyl groups bind much less strongly and can undergo rotation about the bond connecting the glycosyl oxygen atom and the β-phosphorus atom of the pyrophosphoryl linkage, resulting in the projection of opposite faces of the 4-ketopyranosyl ring toward the dihydropyridine ring of DPNH. Epimeric substrates are productively bound with opposite faces of their pyranosyl rings projecting toward DPN^+.

More complex models invoking second binding sites for both the nicotinamide ring of DPN^+ and the pyranosyl rings of substrates, together with bond rotational mobility for both DPN^+ and substrates, cannot be definitely excluded. However, the lack of any experimental basis for mobility on the part of DPN^+ and the stereochemical feasibility of binding two substrate conformations at one site (Kang et al., 1975) lead us to favor the simpler model.

Our epimerization model, and the data supporting it, imply that this enzyme acts as an efficient catalyst for glycosyl interconversion without the involvement of highly specific, tight binding, interactions between the active site and the glycosyl components of either substrates or intermediates. Several theories of enzyme action postulate that such binding interactions between active sites and reaction centers of substrates and intermediates, or of transition states, constitute an important part of the mechanisms of rate acceleration by enzymes (Koshland, 1958; Storm and Koshland, 1970; Wolfenden, 1972; Lienhard, 1973). It is possible to impose interpretations upon our results which would accommodate our binding data with some of these theories. This would require that we postulate the expenditure of glycosyl-binding energy to drive

conformational distortions of the active site or of the glycosyl moieties of substrates and intermediates, or of both. We are reluctant to make such interpretations at this time, particularly in view of the fact that large rate accelerations by enzymes can, in favorable cases, be explained by losses of overall translational and rotational entropy resulting from the binding process itself, without reference to the particular loci of strong binding interactions (Page and Jencks, 1971; Jencks, 1975). However, our models for binding and epimerization are not intended to imply that there are not intimate and strong binding interactions between the active site and glycosyl moieties in the transition states for hydrogen transfer. In the transition states, the glycosyl components and the tightly bound pyridine nucleotide are bridged by partial covalent bonding to the H atom being transferred. This is likely to constitute tight binding of the transition states of the glycosyl moieties.

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