Reaction of Uridine Diphosphate Galactose 4-Epimerase with a Suicide Inactivator†

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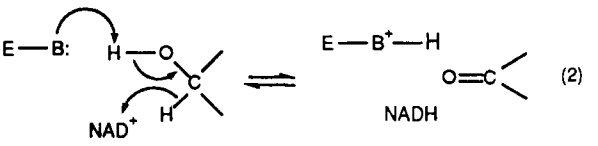
ABSTRACT: UDPgalactose 4-epimerase from *Escherichia coli* is rapidly inactivated by the compounds uridine 5′-diphosphate chloroacetol (UDC) and uridine 5′-diphosphate bromoacetol (UDB). Both UDC and UDB inactivate the enzyme in neutral solution concomitant with the appearance of chromophores absorbing maximally at 325 and 328 nm, respectively. The reaction of UDC with the enzyme follows saturation kinetics characterized by a K_D of 0.110 mM and k_{inact} of 0.84 min^{-1} at pH 8.5 and ionic strength 0.2 M. The inactivation by UDC is competitively inhibited by competitive inhibitors of UDPgalactose 4-epimerase, and it is accompanied by the tight but noncovalent binding of UDC to the enzyme in a stoichiometry of 1 mol of UDC/mol of enzyme dimer, corresponding to 1 mol of UDC/mol of enzyme-bound NAD^+. The inactivation of epimerase by uridine 5′-diphosphate [$^2H_2$]chloroacetol proceeds with a primary kinetic isotope effect (k_H/k_D) of 1.4. The inactivation mechanism is proposed to involve a minimum of three steps: (a) reversible binding of UDC to the active site of UDPgalactose 4-epimerase; (b) enolization of the chloroacetol moiety of enzyme-bound UDC, catalyzed by an enzymic general base at the active site; (c) alkylation of the nicotinamide ring of NAD^+ at the active site by the chloroacetol enolate. The resulting adduct between UDC and NAD^+ is proposed to be the chromophore with λ_{max} at 325 nm. The enzymic general base required to facilitate proton transfer in redox catalysis by this enzyme may be the general base that facilitates enolization of the chloroacetol moiety of UDC in the inactivation reaction.

UDPgalactose 4-epimerase catalyzes the interconversion of UDP-galactose[1] and UDP-glucose. The enzymes from *Escherichia coli* and yeast contain tightly but noncovalently bound NAD^+ at the active sites, and the NAD^+ undergoes transient reduction to NADH in an essential step of catalysis, concomitant with transient oxidation of the substrate to enzyme-bound UDP-4-ketoglucopyranose. In a subsequent step, the ketonic intermediate undergoes nonstereospecific reduction by NADH to either UDP-glucose or UDP-galactose, which are then released from the enzyme according to eq 1 (Frey, 1987; Nelsestuen & Kirkwood, 1971; Maitra & Ankel, 1971; Wee & Frey, 1973; Adair et al., 1973).

(1)

Transfers of hydride from alcohols to NAD^+ at the active sites of enzymes are thought to be facilitated by the actions of enzymic general bases, which abstract the alcoholic protons in concert with hydride transfers according to eq 2 (Holbrook et al., 1975). Kinetic studies of the epimerase from *E. coli*



(2)

show that the reaction rate is controlled by an enzymic ionization with a pK_a between 6.1 and 6.2 (Arabshahi et al., 1988). This may be the pK_a value for a general base in the active site acting in its unprotonated form as E–B: in eq 2. However, a pK_a value derived from a pH–rate profile for an enzymatic reaction may or may not reflect the action of a general base, since such an ionization may also control the enzyme activity through conformational effects; that is, the enzyme may be active in a single conformation that may change to an inactive conformation as a particular group undergoes ionization.

To learn more about the possible presence of a general base at the active site of UDPgalactose 4-epimerase, we have investigated the interaction of the enzyme from *E. coli* with the substrate analogues uridine 5′-diphosphate chloroacetol (UDC) and uridine 5′-diphosphate bromoacetol (UDB). These compounds have been found to be remarkably reactive inactivators of this enzyme, acting by an inactivation mechanism that

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[1] Abbreviations: NAD^+, nicotinamide adenine dinucleotide; NADH, reduced nicotinamide adenine dinucleotide; UDP-glucose, uridine 5′-diphosphate glucose; UDP-galactose, uridine 5′-diphosphate galactose; HEPES, *N*-(2-hydroxyethyl)piperazineethanesulfonic acid; MES, 2-(*N*-morpholino)ethanesulfonic acid; Bicine, *N,N*-bis(2-hydroxyethyl)glycine; UDC, uridine 5′-diphosphate chloroacetol; UDB, uridine 5′-diphosphate bromoacetol; UMP-morpholidate, uridine 5′-(phosphomorpholidate); ANS, 8-anilino-1-naphthalenesulfonic acid; DMF, dimethylformamide; NMR, nuclear magnetic resonance; UV, ultraviolet; TLC, thin-layer chromatography.

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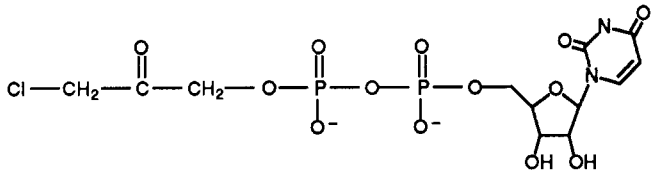
appears to require the function of an enzymic base.



UDC

Experimental Procedures

Materials. UDPgalactose 4-epimerase was purified from an operator constitutive strain of *E. coli* (ATCC 27797) as described by Wilson and Hogness (1964), except that phenylmethanesulfonyl fluoride was added to each buffer at 1 mM each day and the hydroxylapatite column was operated at pH 7.0. UDPglucose dehydrogenase (type III) was purchased from the Sigma Chemical Co. Biochemicals, specialized chemicals, and chromatographic media were purchased from commercial suppliers as follows: NAD^+, UDP-galactose, UMP-morpholidate, HEPES, MES, Bicine, and DEAE-Sephadex A-25 and A-50 from Sigma; acetonitrile, tri-*n*-octylamine, DMF, calcium hydride, [$^2H_2$]oxalic acid, D_2O, sodium 3-(trimethylsilyl)propionate, 4-(dimethylamino)-pyridine, and lithium from Aldrich; Dowex 50-X4, Dowex 1-X4, Bio-Gel P-2 and P-6, and Chelex 100 from Bio-Rad. Other chemicals were obtained from commercial sources in the highest available purity.

Pyridine and acetonitrile were dried overnight over CaH_2 and redistilled. Pyridine was stored over either KOH pellets or 4-Å molecular sieves. Acetonitrile was stored over 4-Å molecular sieves. Tri-*n*-octylamine was dried overnight over CaH_2, distilled under reduced pressure, and stored in a desiccator over $CaSO_4$. Methanol was made anhydrous by initially adding 100 mL of acetone-free methanol to 0.5 g of I_2 and 5 g of Mg turnings and allowing it to react for 3–5 h. Additional acetone-free methanol (900 mL) was added and the mixture refluxed overnight. Anhydrous methanol was then distilled and stored over 4-Å molecular sieves. DMF was dried over 4-Å molecular sieves, redistilled, and stored over molecular sieves. The Mg salt of 8-anilino-1-naphthalenesulfonic acid (ANS) was recrystallized six times from hot water with charcoal treatment.

Spectroscopy. Ultraviolet spectra were recorded on a Cary Model 118C spectrophotometer. Rates were measured spectrophotometrically with a Hitachi Model 100-81A kinetics system. Circular dichroic spectra were recorded on a Jasco J-41C spectrometer. ^{31}P NMR spectra were obtained with a Nicolet NT-200 WB spectrometer, and ^1H-NMR spectra were obtained with a Bruker WH-270 or a Bruker AM-400 spectrometer. All the NMR spectrometers were field-frequency locked onto deuterium in 80–99.8% D_2O.

Rate Measurements. The inactivation of UDPgalactose 4-epimerase by UDC was spectrophotometrically monitored by the appearance of the chromophore absorbing maximally at 325 nm. The pseudo-first-order rate constants were measured at various concentrations of UDC at pH 8.5 in 50 μM Na-Bicinate, 50 μM Na-HEPES, and 50 μM MES (μ = 0.1 adjusted with KCl) by plotting $A-A_\infty$ (325 nm) on semilog graph paper and determining the slopes by a linear unweighted regression procedure. The rate constants k_{obs} were calculated from the slopes and fitted to the equation $k_{obs} = k_{inact}[UDC]/(K_I + [UDC])$ to obtain values for k_{inact} and K_I. The data were computer fitted with the program of Cleland (1979).

Assays. UDPgalactose 4-epimerase was assayed as described by Wilson and Hogness (1964). The concentration of UDPgalactose 4-epimerase was routinely measured spectrophotometrically by its A_{280}, which is 1.05 for a solution 1.0 mg/mL in enzyme at pH 8.5 (Wilson & Hogness, 1964). UDPgalactose 4-epimerase concentrations in solutions containing UV-absorbing compounds were measured by the Lowry and Bradford methods, using standard solutions of UDPgalactose 4-epimerase as the colorimetric standards. NAD^+ released from UDPgalactose 4-epimerase was assayed enzymatically with alcohol dehydrogenase by the procedure of Klingenberg (1985). Inorganic phosphate in ashed samples was assayed by the method of Tashima and Yoshimura (1975). Samples were ashed by a modification of Ames' method (Ames, 1966). Ashed samples were hydrolyzed by addition of 0.5 mL of 1 M HCl and heating at 100 °C for 15 min. The samples were neutralized prior to assay for inorganic phosphate. Analyses of nucleotides by TLC were carried out on plates of Whatman PE SIL G/UV with 1-propanol–concentrated NH_3–H_2O (6:3:1) as the ascending phase and UV detection.

Synthesis of Haloacetol Dimethyl Ketals. Chloroacetol dimethyl ketal and bromoacetol dimethyl ketal were synthesized as described by Hartman (1970), with the following modifications: In the oxidation step twice the quoted amount of oxalic acid was used to ensure complete decomposition of unreacted dicyclohexylcarbodiimide. In the phosphorylation step, the pH of the quenched reaction mixture was adjusted to 8.5 by addition of cyclohexylamine before concentration by flash rotary evaporation, in order to avoid the formation of small but detectable amounts of halo ketones.

Synthesis of Uridine 5'-Diphosphate Chloroacetol (UDC). All evaporations were by rotary flash evaporation in vacuo. Chloroacetol phosphate dimethyl ketal was coupled to UMP-morpholidate by the general procedure of Khorana and Moffatt as described by Moffatt (1966). The dicyclohexylguanidinium salt of UMP-morpholidate (750 μmol) was dissolved in 5 mL of anhydrous pyridine and dried by evaporation from this solvent; this was repeated two additional times. The bis (cyclohexylammonium) salt of chloroacetol phosphate dimethyl ketal (3 mmol) was dissolved with heating in 30 mL of anhydrous pyridine, and 6 mmol of tri-*n*-octylamine was added with swirling until the solution became clear. (Alternatively, the cyclohexylammonium salt was converted to the pyridinium salt by passage through a column of SP-Sephadex C-25 in the pyridinium form; the pyridinium salt was evaporated to dryness, dissolved in methanol and combined with tri-*n*-octylamine, evaporated to dryness, and redissolved in anhydrous pyridine.) The cooled solution was evaporated to an oil and twice redissolved in 10 mL of anhydrous pyridine and evaporated. The UMP-morpholidate was dissolved in its flask with 5 mL of anhydrous pyridine and transferred into the flask containing the chloroacetol phosphate dimethyl ketal. The morpholidate flask was twice rinsed with 5 mL of pyridine, both rinses being combined with the chloroacetol phosphate dimethyl ketal. The pyridine was removed by evaporation, and the oil was twice more dissolved in pyridine and dried by evaporation. The dried oil was finally dissolved in 7.5 mL of anhydrous pyridine and heated to 80 °C. The reaction flask was sealed and placed at 70 °C for 3 days. After cooling to room temperature, the pyridine was removed by evaporation, and 10 mL of 0.6 M lithium acetate at pH 3.5 was added to the residue. The mixture was extracted with diethyl ether to remove tri-*n*-octylamine, and the aqueous layer was diluted to 500 mL and its pH adjusted to 3.5. This solution was loaded onto a 2.5 × 20 cm column of Dowex 1-X4 (100–200 mesh) anion exchange resin in the chloride form. The column was

eluted with a linear gradient of LiCl formed from 1 L of 0.01 M LiCl at pH 3.5 and 1 L of 0.5 M LiCl at pH 3.5 at a flow rate of 2 mL/min. Fractions were collected and analyzed by measurements of A_{260}. Analysis of peak fractions by TLC showed that the first two peaks were UMP and UMP-morpholidate and the third was the coupling product uridine 5′-diphosphate chloroacetol dimethyl ketal. Product-containing fractions were pooled, adjusted to pH 4.5, evaporated to a syrup, and applied to a 2.5 × 110 cm column of Bio-Gel P-2 (200–400 mesh), which was equilibrated and eluted with distilled, deionized water. All fractions exhibiting significant A_{260} that contained no chloride, as determined by testing with 5% $AgNO_3$, were pooled, concentrated, lyophilized, and stored as a powder at −20 °C. The yield was 189 mg (46%). Anal. Calcd for $C_{14}H_{21}N_2O_{14}P_2Li_2$: C, 30.42; H, 3.83; N, 5.06; Cl, 6.42. Found: C, 30.41; H, 4.11; N, 4.63; Cl, 6.67. The ^{31}P NMR spectrum consisted of two doublets at −11.3 ppm (P^1) and −10.8 ppm (P^2), relative to 85% H_3PO_4, with $J_{1,2}$ of 20.5 Hz. The ^1H NMR spectrum consisted of the following signals downfield from sodium 3-(trimethylsilyl)propionate (δ, multiplicity, J, integration, assignment): 7.99 ppm, d, 8.1 Hz, 1 H, uracil 5H; 6.01 ppm, d, 4.2 Hz, 1 H, ribose 1′H; 6.00 ppm, d, 8.0 Hz, 1 H, uracil 6 H; 4.2–4.5 ppm, m (unresolved coupling), 5 H, ribose 2′–5′; 4.06 ppm, d, 4.6 Hz, 2 H, methylene; 3.75 ppm, s (no coupling), 2 H, chloromethylene; 3.35 ppm, s (no coupling), 6 H, methyl.

Controlled acid hydrolysis of uridine 5′-diphosphate chloroactol dimethyl ketal afforded UDC in a yield of 40–50%. The stock precursor was hydrolyzed in small amounts to generate UDC in the quantities needed for the enzymic experiments. In a typical hydrolysis 10 mg of the precursor was dissolved in 5 mL of H_2O, the pH was adjusted to 1.6 by addition of 20 μL of 6 M HCl, and the solution was heated at 100 °C for 20 min. The hydrolysate was then immediately diluted with 25 mL of chilled H_2O and the pH adjusted to 3.5 by addition of 1 M LiOH. This solution was applied to a 1 cm × 8 cm column of Dowex 1-X4 resin (100–200 mesh) equilibrated with 0.2 M LiCl at pH 3.5. The product was eluted with 30 mL of 0.5 M LiCl at pH 3.5. UDC appeared in the second peak (A_{260}) to emerge, with the first peak containing UMP. The UDC-containing fractions were pooled, concentrated to 1 mL, and desalted by passage through a 1 cm × 100 cm column of Bio-Gel P-2 (200–400 mesh) equilibrated and eluted with water adjusted to pH 3.5 with HCl. The major A_{260} peak was salt-free UDC at a concentration of 4 mM and in a yield of 44%. The product, generated from 10–30 mg of the precursor, was obtained in solid form by lyophilization of pooled fractions from the Bio-Gel column. UDC was characterized by analysis of the uracil to phosphate ratio (1.00:2.04), UV absorption (λ_{max} 260 nm, ϵ_{260} 8900 $M^{-1}\cdot cm^{-1}$), and NMR analysis. The broad-band proton spin-decoupled ^{31}P NMR spectrum of UDC consisted of a single resonance at −10.36 ppm relative to 85% H_3PO_4. The ^1H NMR spectrum consisted of the following signals downfield from sodium 3-(trimethylsilyl)propionate (δ, multiplicity, J, integration, assignment): 7.98 ppm, d, 8.09 Hz, 1 H, uracil 5H; 6.04 ppm, d, 8.20 Hz, 1 H, uracil 6H; 6.03 ppm, d, 4.25 Hz, 1 H, ribose 1′H; 4.2–4.5 ppm, m (unresolved coupling), 5 H, ribose 2″–5′; 4.06 ppm, s (no coupling), 2 H, methylene; 3.75 ppm, s (no coupling), 2H, chloromethylene.

Synthesis of Uridine 5′-Diphosphate [$^2H_2$]Chloroacetol. Dideuterio-UDC was synthesized by a modification of the deblocking procedure used for the conversion of uridine 5′-diphosphate chloroacetyl dimethyl ketal to UDC, in which oxalic monoanion catalyzed the hydrolysis in D_2O. Oxalic acid monoanion catalyzed the exchange of the choromethylene protons with D_2O, and to a lesser extent the methylene protons of the chloroacetol moiety in UDC, as well as thehydrolysis of the precursor ketal group. The hydrolysis was carried out as described above for the preparation of UDC, except that the hydrolysis medium was 0.5 M oxalic acid in D_2O (99.8% ^2H) at pD 1.2. The ^1H NMR spectrum was the same as that of UDC, except that the integration of the chloromethylene group gave 0.3 H and that of the methylene group 1.5 H.

Synthesis of Uridine 5′-Diphosphate Bromoacetol (UDB). UDB was synthesized by the same sequence of reactions described above for UDC. However, the bromo substituent was too reactive to allow the coupling to be conducted in pyridine at high temperature over the lengthy time required for coupling; therefore, different conditions were used. And UDB was itself too reactive to be produced in pure form by acid hydrolysis of the ketal precursor, so it was obtained in impure form and in low yield. The bis(tri-*n*-octylammonium) salt of bromoacetol phosphate dimethyl ketal was prepared from 3.2 mmol of the bis(cyclohexylammonium) salt by passage of an aqueous solution through a 2.5 cm × 24 cm column of SP-Sephadex C-25 in the pyridinium form. The effluent was concentrated to a syrup, dissolved in methanol, combined with 6.4 mmol of tri-*n*-octylamine, evaporated to dryness, and dried overnight in vacuo over P_2O_5. The dicyclohexylguanidinium salt of UMP-morpholidate (0.8 mmol) and 4 g of 4-(dimethylamino)pyridine were dissolved together in 15 mL of anhydrous DMF and evaporated to dryness. The mixture was twice more dissolved in anhydrous DMF and evaporated to dryness to remove any traces of water. The bis(tri-*n*-octylammonium) salt of bromoacetol phosphate dimethyl ketal was similarly dried by dissolution in anhydrous DMF and evaporation. The UMP-morpholidate and bromoacetal phosphate dimethyl ketal were then dissolved together in 25 mL of DMF, evaporated to dryness, and twice more dissolved in 15 mL of DMF and evaporated. The mixture was finally dissolved in 16 mL of acetonitrile and stirred at 90 °C for 17 h. Analysis by TLC revealed the appearance of a product (R_f 0.37) and a trace of UMP (R_f 0.13). The solvent was removed by evaporation, and the residue was dissolved in 25 mL of water containing 9.4 mmol of lithium acetate. This solution was extracted three times with 25-mL aliquots of chloroform, diluted to 700 mL, adjusted to pH 3.5 by addition of 1 M HBr, and purified by anion exchange chromatography as described above for the chloroacetol compound, except that HBr was used in place of HCl to adjust the pH of the elution gradient to 3.5 and elution was with a LiBr gradient. Element analysis indicated that the product was impure and had suffered loss of Br. Hydrolysis of the ketal group at pH 1.6 in HBr as described above for UDC, followed by ion exchange chromatography, resulted in the formation of four major products, one of which contained the bromoketone group and gave, after desalting, an analysis for the phosphate to uridine ratio of 1.99:1.00. This was UDB, which was not further characterized owing to its instability and the fact that it was used solely to verify its similarity to UDC as an inactivator of UDPgalactose 4-epimerase.

Correlation of Residual Enzymatic Activity with Bound UDC. The reaction mixtures contained epimerase at 2 mg/mL and 25 or 100 μM UDC in 5 mM Na-HEPES buffer at pH 7.0. Reactions were initiated by addition of UDC. At various times 1-mL aliquots of the reaction mixtures were withdrawn and divided into halves, and each half was gel filtered by the centrifugation technique of Penefsky (1979), with Bio-Gel P-6 as the gel exclusion medium. The eluates were analyzed for

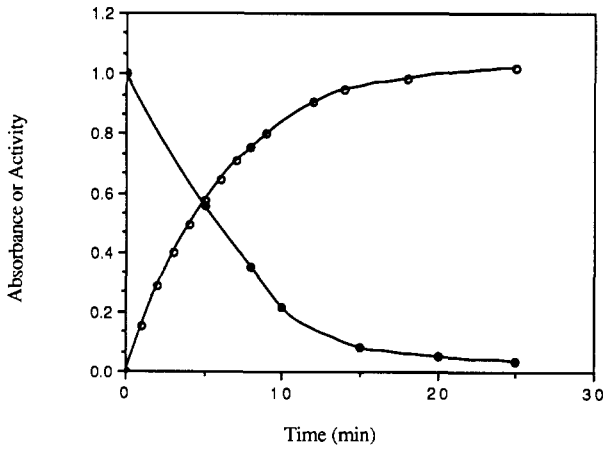


FIGURE 1: Inactivation of UDPgalactose 4-epimerase by UDC. Correlation of inactivation with the appearance of an enzymic chromophore absorbing at 325 nm. The reaction mixture contained 19 μM UDPgalactose 4-epimerase, 76 mM UDC, and 0.1 M Na-Bicinate buffer at pH 8.5, 27 °C. At the indicated times the A_{325} was measured and an aliquot diluted and assayed for enzymatic activity. Symbols: open circles, $A_{325} \times 10$; filled circles, relative activity.

residual enzymatic activity, protein content, and protein-bound total phosphate. The bound UDC was calculated by subtracting the phosphate attributable to bound NAD^+ from the total phosphate and dividing by 2 phosphates/UDC.

Results

Inactivation of UDPgalactose 4-Epimerase by UDC. UDPgalactose 4-epimerase is rapidly inactivated in the presence of UDC in a reaction that is accompanied by the appearance of a new enzymic chromophore with a λ_{max} of 325 nm with an extinction coefficient of 5200 $M^{-1} \cdot cm^{-1}$. As shown by the data in Figure 1, enzyme inactivation and the appearance of this chromophore are closely correlated in time. The enzyme is similarly inactivated by UDB with results similar to those in Figure 1, except that λ_{max} for the chromophore is 328 nm. UDPgalactose 4-epimerase is not inactivated by the synthetic precursor to UDC nor by chloroacetol phosphate.

Kinetics of Inactivation. The inactivation is a pseudo-first-order reaction when UDC is used in excess of enzyme. The observed first-order rate constants increase to a constant value as [UDC] is increased, consistent with saturation kinetics. When the rate constants are fitted to eq 3, the values

$$k_{obs} = \frac{k_{inact}[UDC]}{K_I + [UDC]} \quad (3)$$

of k_{inact} and K_I are 0.84 ± 0.04 min^{-1} and 0.110 ± 0.008 mM, respectively, at pH 8.5 and 27 °C. The fit is excellent and suggests that the kinetic mechanism is analogous to the Michaelis–Menten mechanism for enzyme catalysis, shown as eq 4 for the kinetic mechanism by which UDC inactivates

$$E + UDC \rightleftharpoons^{K_I} E \cdot UDC \xrightarrow{k_{inact}} E^* \quad (4)$$

UDPgalactose 4-epimerase, where E is active epimerase and E* is inactive epimerase. UDC presumably binds reversibly to the enzyme active site, with a dissociation constant K_I of 0.11 mM, and then undergoes an irreversible reaction that inactivates the enzyme and forms the 325-nm chromophore.

If UDC binds reversibly at the active site, competitive reversible inhibitors of enzyme activity should also inhibit inactivation by UDC. ANS is a competitive inhibitor of UDPgalactose 4-epimerase (Wong & Frey, 1978) with an inhibition constant of 25 μM (Wong & Frey, 1978). ANS also protects the enzyme against inactivation by UDC. In an inactivation reaction in which 3.8 μM epimerase in 0.1 M Na-Bicinate at pH 8.5 was subjected to inactivation by 0.1 mM UDC, the inactivation rate constant (k_{obs}) was 0.159 min^{-1} at 20 °C. In an otherwise identical reaction mixture, which also contained 25 mM ANS, the inactivation rate constant was 0.006 min^{-1}. Protection by the competitive inhibitor ANS to the expected degree indicates that UDC binds to the active site of the epimerase.

Characterization of the Inactivated Enzyme. Inactivation of the epimerase by UDC is accompanied by the incorporation of one diphosphate moiety of the inactivator into the complex of enzyme and pyridine nucleotide. This is shown by the fact that the increase in phosphate content of the enzyme is well correlated with the extent of inactivation by UDC, with the phosphate content of the inactivated enzyme increased by a factor of 2 relative to that of the native enzyme.

To generate UDC binding data for correlation with residual enzymatic activity, samples of the enzyme were incubated with UDC for various lengths of time, and the protein was then separated from unreacted UDC by gel filtration. The gel-filtered protein was assayed for residual enzymatic activity and also for total phosphate. The NAD^+ associated with the enzyme, 1 mol/mol of dimeric enzyme (Wilson & Hogness, 1964), served as an internal phosphate standard, and it gave assay values that confirmed the presence of one NAD^+ per enzyme dimer when compared with external phosphate standards. During the course of inactivation by UDC the phosphate content of the gel-filtered enzyme increased to a value twice that of the original epimerase–NAD^+ complex. After correction for the phosphate content of NAD^+ in the enzyme, the increased phosphate enabled us to calculate the molar increase in bound UDC. These calculations gave the following data [% residual activity, bound UDC (mol/mol NAD^+)]: 100, 0.00; 0.90, 0.18; 0.78, 0.23; 0.59, 0.42; 0.50, 0.54; 0.39, 0.63; 0.32, 0.67; 0.25, 0.81. When plotted against each other as residual activity versus bound UDC, the data were analyzed by standard nonweighted linear regression and gave a straight line (R value = 0.99) with a slope of –0.99 and an abscissa intercept corresponding to 1.0 UDC bound per molecule of NAD^+ in the enzyme complex.

Inactivation of the epimerase by UDC is essentially irreversible, since the enzymatic activity cannot be restored or increased by prolonged dialysis, nor does the A_{325} decrease during dialysis. Nevertheless, UDC in the inactive complex is not covalently bonded to the protein, since denaturation of the inactive complex releases all of the phosphate from the protein. The chromophore centered at 325 nm also is not stable to denaturation, as shown by the experiment in Figure 2, in which the absorption of the chromophore gradually decreases upon addition of urea at a denaturing concentration at pH 10.1. Under the conditions of Figure 2 NAD^+ is released from the enzyme. The chromophore is also completely abolished by heating the inactive complex at 100 °C for 2 min.

The nature of the 325-nm chromophore suggests that it involves NAD^+. The cyanide adduct of NAD^+, for example, absorbs maximally at 325 nm (Colowick et al., 1951). The chromophore is not fluorescent, since excitation at 325 nm does not elicit the emission of light. While the cyanide adduct of NAD^+ is fluorescent, the initial pyruvate adduct of NAD^+ bound to lactate dehydrogenase is not fluorescent. Moreover, the pyruvate adduct also absorbs light maximally at 322 nm (Griffin & Criddle, 1970). In two experiments, heat denaturation of the inactive complex released the theoretical amount of NAD^+ from the enzyme, 47 and 50 nmol, in two

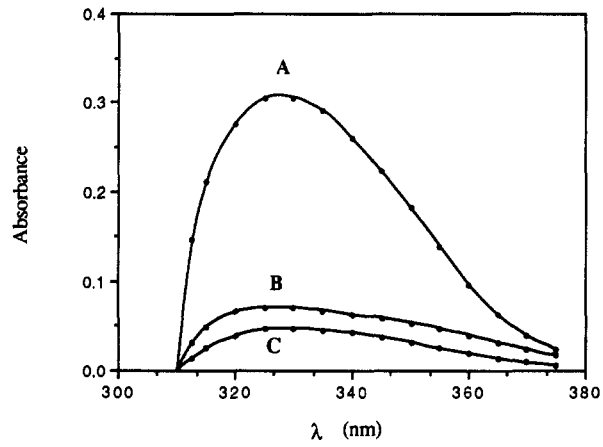


FIGURE 2: Decomposition of the 325-nm chromophore upon denaturation of the inactivated enzyme. Curve A is the difference spectrum of the UDC-inactivated enzyme prepared by reaction of 57 nmol of UDPgalactose 4-epimerase with 0.2 μmol of UDC in 0.80 mL of 4 mM Na-Bicinate buffer at pH 8.5, recorded against an otherwise identical solution from which UDC was excluded in the reference cuvette. To denature the protein, 0.1 mL of 1.0 M Na_2CO_3 and 480 mg of urea were added to each solution, and the difference spectrum was rerecorded. Curves B and C are the difference spectra recorded 10 and 30 min, respectively, after the addition of urea.

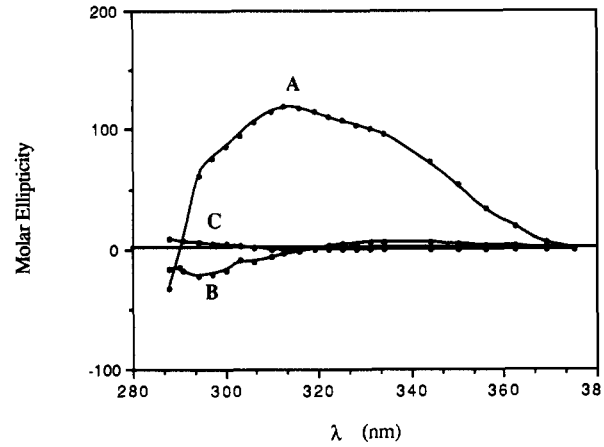


FIGURE 3: Circular dichroic spectrum for UDC-inactivated UDPgalactose 4-epimerase. The solutions used for circular dichroic analyses were the following: (A) UDC-inactivated enzyme prepared by incubating 133 nmol of epimerase with 3.5 μmol of UDC in 3.5 mL of 10 mM KP_i buffer at pH 8.5; (B) identical with (A) except that UDC was excluded; (C) identical with (A) except that enzyme was excluded. Molar ellipticities were calculated as deg·cm^2·dmol^{-1}, assuming a molecular weight of 79000 for UDPgalactose 4-epimerase (Wilson & Hogness, 1964) and 1:1 stoichiometry between UDC and the enzyme (Figure 2).

experiments, respectively, from 51 nmol of epimerase, as determined by enzymatic assays of free NAD^+. Denaturation of the inactivated complex also releases the UDC from the enzyme, since analysis of the denatured protein for phosphate showed that all phosphate is removed by denaturation of the UDC-inactivated complex. Therefore, if the inactivation product is an adduct between UDC and NAD^+, it must be stabilized at the active site of the enzyme and unstable to dissociation when released into solution.

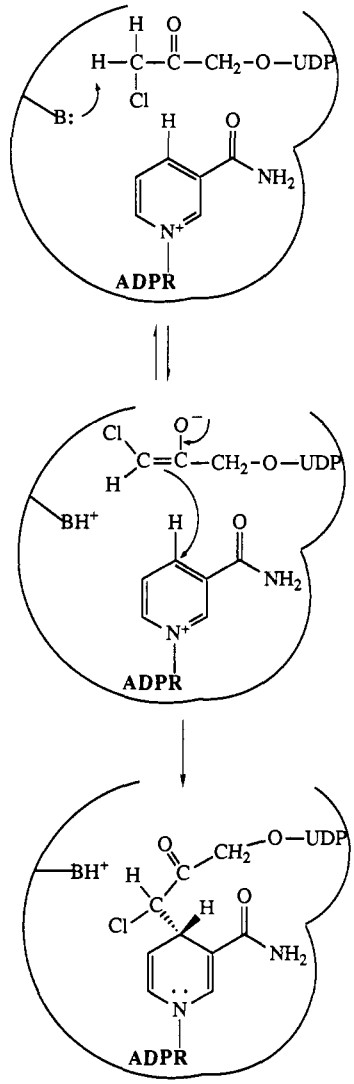
NADH in the epimerase–NADH–UMP complex exhibits a strong positive Cotton effect in the circular dichroic spectrum, in contrast to free NADH, which shows a weak negative Cotton effect (Wong et al., 1978). The strength of this Cotton effect is thought to reflect the interactions of the dihydronicotinamide ring with the highly asymmetric active site. An adduct between NAD^+ and UDC at the active site that is stabilized by binding interactions at this same site is also likely to exhibit a large Cotton effect. As shown in Figure 3, the inactivated complex exhibits large positive molar ellipticity between 290 and 370 nm, with a maximum at 315 nm and shoulders at 295 and 325 nm. The shoulder at 325 nm is probably associated with the 325-nm chromophore in the inactivated complex. The other maxima may reflect electronic transitions of protein chromophores such as tryptophan. However, since the circular dichroic spectrum of the free chromophore could not be obtained, owing to its instability, the origin of these shorter wavelength effects cannot be assigned. The maximum molar ellipticity is about 3 times that of NADH in the epimerase–NADH–UMP complex. The circular dichroic spectrum supports the contention that the chromophore in the inactivated complex is an adduct between NAD^+ and either UDC or some enzymic group.

Kinetic Isotope Effect in the Reaction of [$^2H_2$]UDC with UDPgalactose 4-Epimerase. If the chromophore generated by the reaction of UDC with this enzyme is an adduct between UDC and NAD^+, a bond in UDC must be broken in the reaction, presumably a C–H bond. The weakest C–H bonds in UDC are those to the chloromethylene protons, which are the most acidic protons.

To determine whether the inactivation reaction involves cleavage of one of these bonds, the rate of the inactivation by deuterio-UDC was compared with that by UDC itself. Deuterio-UDC containing 1.7 deuterons in the chloromethylene group and 0.3 deuteron in the methylene group of the chloroacetol moiety was prepared and purified by the same chromatographic procedure used for UDC itself. The rate constants (k_{obs}) for inactivation by deuterio-UDC were measured at inactivator concentrations from 0.04 to 0.4 mM and compared with data obtained with UDC over the same concentration range. Under the conditions of these rate measurements, the kinetic isotope effect $k_H/k_{D(app)}$ was 1.4, whereas $K_{I(H)}/K_{I(D)}$ was 1.0.

The isotope effect of 1.4 on k_{inact} is too large to be a secondary effect in this case and so must be a primary effect. Therefore, carbon–hydrogen bond cleavage is involved in the reaction and is at least partially rate limiting. Inasmuch as the effect is small and both the chloromethylene and the methylene groups of the chloroacetol moiety were partially deuterated, it is not obvious which carbon–hydrogen bond is broken. The chloromethylene group is the more acidic and, therefore, probably the reactive carbon. That this is in fact the case can be concluded from a further analysis of the measured isotope effect and the pattern of deuterium labeling in the molecule. The chloromethylene group in [$^2H_2$]UDC was 85% enriched with deuterium at each hydrogen position, and the methylene group was 15% enriched at each position. The inactivation rate constant for UDC (k_H) under the conditions of the experiment was 0.45 ± 0.03 min^{-1}, and the rate constant $k_{D(app)}$ for [$^2H_2$]UDC under the same conditions was 0.32 ± 0.02 min^{-1}. The inactivation rate constant for [$^2H_2$]UDC can be corrected to full deuterium enrichment at the reacting carbon as follows. The measured rate constant can be expressed as the sum $k_{D(app)} = \alpha k_H + \beta k_D$, where α and β are the fractions of H and ^2H, respectively, bonded to the reacting carbon. Inasmuch as values for $k_{D(app)}$ (0.32 min^{-1}), k_H (0.45 min^{-1}), α, and β are available, the corrected rate constant for fully deuterated inactivator can be calculated. If the chloromethylene group reacts, the values for α and β are 0.15 and 0.85, respectively, and the calculated value of k_D is 0.29 min^{-1}. This value for k_D gives a corrected isotope effect (k_H/k_D) of 1.6. A similar calculation for the methylene as the reactive group, in which α and β are 0.85 and 0.15,

Scheme I



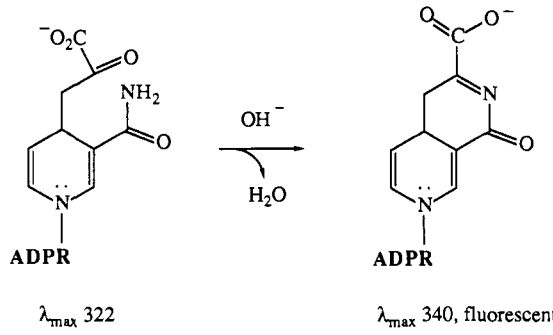
respectively, leads to a negative calculated value for k_D (−0.4 min^{-1}). Therefore, the methylene carbon is ruled out as the reactive group, since a negative rate constant is not possible.

Discussion

The experiments reported in this paper establish the following facts regarding the interaction of UDC and UDPgalactose 4-epimerase: (a) UDC efficiently inactivates the enzyme in a reaction that exhibits saturation kinetics via an E·UDC complex according to eq 3; (b) inactivation is prevented by ANS, a competitive reversible inhibitor; (c) inactivation is concomitant with the formation of a 325-nm chromophore; (d) the chromophore is not fluorescent but exhibits a large positive Cotton effect in the circular dichroic spectrum; (e) the phosphate content of the inactivated enzyme reflects the incorporation of 1 mol of UDC/mol of enzyme; (f) neither UDC nor NAD^+ is covalently bonded to the enzyme in the inactive complex; (g) the 325-nm chromophore is stabilized by binding interactions with the active site and decomposes when released from this site; (h) inactivation by [^{2}H]UDC proceeds with a primary kinetic isotope effect.

A chemical mechanism that accounts for enzyme inactivation and for the properties of the inactive complex is presented in Scheme I. In this mechanism UDC bound at the active site of UDPgalactose 4-epimerase is enolized by the action of a general base at the active site. The enolate is nucleophilic and adds to the nicotinamide ring of NAD^+, which is nearby in the active site, to form an adduct that blocks the site and is not released under nondenaturing conditions. This adduct is the 325-nm chromophore, which is stabilized by binding interactions between the enzyme and NAD^+ and between the enzyme and the UDP pyrophosphoryl moiety of UDC. The present results do not bear on the question of whether carbon-4 of the nicotinamide ring is the recipient carbon in the enolate addition. However, this carbon is known to be by far the most reactive toward $NaBH_4$, and reductions by this reagent and all other reducing agents proceed stereospecifically on the B-side (*si* face) of NAD^+ (Nelsestuen & Kirkwood, 1971; Wee & Frey, 1973). It is reasonable to suppose that enolate addition will follow a similar regiospecific and stereospecific course; therefore, the enolate is shown in Scheme I adding to carbon-4 from the *si* face.

Scheme II



The structure shown in Scheme I for the chromophore generated in the reaction of UDC with epimerase–NAD^+ is consistent with the properties of adducts of this type. The absorption maximum at 325 nm is similar to that of the cyanide adduct of NAD^+, as well as the initial adduct formed between pyruvate and NAD^+ at the active site of lactate dehydrogenase (Colowick et al., 1951; Griffin & Criddle, 1970). The addition of enolpyruvate to NAD^+ at the active site of lactate dehydrogenase is also formulated to proceed by addition of the enol carbon to carbon-4 of the nicotinamide ring in enzyme-bound NAD^+. This adduct also dissociates to pyruvate and NAD^+ when it is released from the enzyme in neutral solution (DiSabato, 1968). The pyruvate–NAD^+ adduct, which is nonfluorescent and has a λ_{max} at 322 nm, undergoes a secondary reaction when it is released from lactate dehydrogenase under alkaline conditions to form a fluorescent compound with a λ_{max} at 340 nm (Everse et al., 1971; Arnold & Kaplan, 1974). The new compound is formed by loss of water between the carbonyl group of the pyruvoyl moiety and the carboxamido nitrogen of the dihydronicotinamide ring in the initial adduct to generate a new fused ring, the structure of which is shown in Scheme II. No such secondary reaction occurs when the UDC–NAD^+ adduct is released from the active site of UDPgalactose 4-epimerase under the denaturing and alkaline conditions of Figure 2. Instead, the UDC–NAD^+ adduct dissociates as in neutral solution. The reason for the difference between the UDC and pyruvoyl adducts is not known, but it may be related to the fact that the carboxylate group of the secondary product derived from the pyruvoyl adduct, shown in Scheme II, can stabilize the fused ring by π-bond overlap with the double bond in the new ring. No such stabilization is possible in the case of the UDC–NAD^+ adduct.

According to Scheme I the enzyme catalyzes the enolization of UDC by providing a general base at the active site that abstracts a proton from the chloromethylene group. This step of the inactivation is supported by the kinetic isotope effect for the reaction of [$^2H_2$]UDC, which shows that cleavage of the carbon–hydrogen bond is involved in the rate-limiting step

of the reaction. This must be occurring at the active site, since UDC itself does not spontaneously undergo enolization in neutral aqueous solutions on the time scale of its reaction with UDPgalactose 4-epimerase. UDC does not, for example, undergo exchange of the chloromethylene protons with deuterons of D_2O in samples prepared for NMR analysis. Therefore, the putative enolization preceding adduct formation must occur at the enzymic active site. It is reasonable to propose that enolization is catalyzed by a general base at the active site. Such a base should catalyze proton transfer in the redox steps of the substrate epimerization mechanism, and it may be the same base that catalyzes the enolization of UDC.

UDC is a chloro ketone and as such has the chemical capacity to alkylate nucleophilic groups such as general bases in the active sites of enzymes. Examples of such alkylations by chloro ketones are the alkylations of histidine in the active site of α-chymotrypsin by phenylalanine chloromethyl ketone (Shaw, 1970) and of glutamate in the active site of triosephosphate isomerase by chloroacetol phosphate (Hartman, 1971). UDC does not alkylate a general base in the active site of UDPgalactose 4-epimerase. Alkylation of this enzyme is evidently prevented by the enolization of the chloro ketone and very rapid addition of the enolate to NAD^+ in the active site. The adduct shown in Scheme I is a chloro ketone and could, in principle, also alkylate a nucleophile if such a group is sterically accessible. No such alkylation occurs, either because no such group is sufficiently near the electrophilic carbon or because a nearby group is not nucleophilic. We note, in this connection, that the general base in Scheme I is in its protonated form subsequent to enolization and adduct formation. It is possible that this group remains protonated and unreactive when the bound pyridine nucleotide is in a dihydro form.

Numerous experiments designed to induce the chloro ketone moiety in UDC to alkylate an enzymic nucleophile in the active site failed to lead to covalent bonding of the inactivator to the enzyme. These experiments included the addition of UDC to solutions of the epimease–NADH complex, which could not undergo adduct formation, followed by analysis for phosphate covalently bound to the protein. No evidence for covalent bonding to UDC to the protein was obtained. Either an enzymic general base in the active site remained protonated when the bound pyridine nucleotide was in its dihydro form, or the chloro ketone group of UDC simply had no access to a nucleophilic group in the active site.

References

Adair, W. L., Jr., Gabriel, O., Ullrey, D., & Kalkcar, H. M. (1973) *J. Biol. Chem. 248*, 4635–4639.

Ames, B. M. (1966) *Methods Enzymol. 8*, 115–118.

Arabshahi, A., Flentke, G. R., & Frey, P. A. (1988) *J. Biol. Chem. 263*, 2638–2643.

Arnold, L. J., & Kaplan, N. O. (1974) *J. Biol. Chem. 249*, 652–655.

Cleland, W. W. (1979) *Methods Enzymol. 63*, 103–138.

Colowick, S. P., Kaplan, N. O., & Ciotti, M. M. (1951) *J. Biol. Chem. 191*, 447–459.

Di Sabato, G. (1968) *Biochem. Biophys. Res. Commun. 33*, 688–695.

Everse, J., Barnett, R. E., Thorne, C. J. R., & Kaplan, N. O. (1971) *Arch. Biochem. Biophys. 143*, 444–460.

Frey, P. A. (1987) in *Pyridine Nucleotide Coenzymes* (Dolphin, D., Poulson, R., & Avramovic, O., Eds.) Vol. 2B, pp 462–477, Wiley, New York.

Hartman, F. C. (1970) *Biochemistry 9*, 1776–1782.

Hartman, F. C. (1971) *Biochemistry 10*, 146–154.

Holbrook, J. J., Liljas, A., Steindel, S. J., & Rossmann, M. G. (1975) *Enzymes (3rd Ed.) 11*, 191–292.

Klingenberg, M. (1985) *Methods in Enzymatic Analysis*, 3rd ed. (Bergmeyer, H. U., Ed.) Vol. 2, pp 251–257, VCH, Berlin.

Maitra, U. S., & Ankel, H. (1971) *Proc. Natl. Acad. Sci. U.S.A. 68*, 1678–1683.

Moffatt, J. G. (1966) *Methods Enzymol. 8*, 136–142.

Nelsestuen, G. L., & Kirkwood, S. (1971) *J. Biol. Chem. 246*, 7533–7543.

Penefsky, H. S. (1979) *Methods Enzymol. 51*, 527–530.

Shaw, E. (1970) *Enzymes (3rd Ed.) 1*, 91–146.

Tashima, Y., & Yoshimura, N. (1975) *J. Biochem. (Tokyo) 78*, 1161–1169.

Wilson, D. B., & Hogness, D. S. (1964) *J. Biol. Chem. 239*, 2469–2481.

Wong, S. S., & Frey, P. A. (1978) *Biochemistry 17*, 3551–3556.

Wong, S. S., Cassim, J. Y., & Frey, P. A. (1978) *Biochemistry 17*, 516–520.