UDP-galactose 4-Epimerase. Phosphorus-31 Nuclear Magnetic Resonance Analysis of NAD^+ and NADH Bound at the Active Site†

John M. Konopka,‡ Christopher J. Halkides,‡ Janeen L. Vanhooke,‡ David G. Gorenstein,§ and Perry A. Frey*,‡

Institute for Enzyme Research, Graduate School, and Department of Biochemistry, College of Agriculture and Life Sciences, University of Wisconsin—Madison, Madison, Wisconsin 53705, and Department of Chemistry, Purdue University, West Lafayette, Indiana 47907

Received May 6, 1988; Revised Manuscript Received October 3, 1988

ABSTRACT: The phosphorus atoms of NAD^+ bound within the active site of UDP-galactose 4-epimerase from *Escherichia coli* exhibit two NMR signals, one at $\delta = -9.60 \pm 0.05$ ppm and one at $\delta = -12.15 \pm 0.01$ ppm (mean ± standard deviation of four experiments) relative to 85% H_3PO_4 as an external standard. Titration of epimerase·NAD^+ with UMP causes a UMP-dependent alteration in the chemical shifts of the resulting exchange-averaged spectra, which extrapolate to $\delta = -10.51$ ppm and $\delta = -11.06$ ppm, respectively, for the fully liganded enzyme, with an interconversion rate between epimerase·NAD^+ and epimerase·NAD^+·UMP of at least 490 s^{-1}. Conversely, the binding of 8-anilinonaphthalene-1-sulfonate, which is competitive with UMP, causes a significant sharpening of the epimerase·NAD^+ resonances but very little alteration in their chemical shifts, to $\delta = -9.38$ ppm and $\delta = -12.16$ ppm, respectively. UMP-dependent reductive inactivation by glucose results in the convergence of the two resonances into a single signal of $\delta = -10.57$ ppm, with an off-rate constant for UMP dissociation from the epimerase·NADH·UMP complex estimated at 8 s^{-1}. Reductive inactivation by borohydride under anaerobic conditions yields a single, broad resonance centered at about $\delta = -10.2$ ppm. The data are consistent with, and may reflect, the activation of NAD^+ via a protein conformational change, which is known from chemical studies to be driven by uridine nucleotide binding. Incubation of epimerase·NAD^+ with UMP in the absence of additional reducing agents causes a very slow reductive inactivation of the enzyme with an apparent pseudo-first-order rate constant of 0.013 ± 0.001 h^{-1}, which appears to be associated with liberation of inorganic phosphate from UMP.

UDP-galactose[1] 4-epimerase (EC 5.1.3.2) catalyzes the interconversion of UDP-galactose and UDP-glucose. The enzyme from *Escherichia coli* can be purified to homogeneity as a dimer of M_r 79 000 which consists of identical subunits and contains 1 mol of NAD^+/mol of dimers. NAD^+ is essentially irreversibly bound and immobilized within the active site but is released under denaturing conditions such as perchloric acid (Wilson & Hogness, 1964) or boiling water (Nelsestuen & Kirkwood, 1971) in either the oxidized (Wilson & Hogness, 1964) or reduced forms (Nelsestuen & Kirkwood, 1971). The enzyme catalyzes epimerization by reversible oxidation of either substrate, with hydride transfer from the glycosyl C-4 to NAD^+, yielding a UDP-4-ketopyranose intermediate (Nelsestuen & Kirkwood, 1971; Maitra & Ankel, 1971; Wee & Frey, 1973; Adair et al., 1973). Subsequent reduction by hydride transfer from NADH to either face of the 4-ketopyranose moiety completes the catalytic cycle (Kang et al., 1975). In the presence of UMP, the active site NAD^+ can be reduced by various sugars (Bertland et al., 1971; Kang et al., 1975) or by $NaBH_4$ or $NaBH_3CN$ (Davis et al., 1974), to produce an inactive enzyme. UMP-dependent reductive inactivation by sugars, first described by H. M. Kalckar and his co-workers (Bertland et al., 1966) is a well-known and extensively studied phenomenon [for a recent review see Frey (1987)]. In the case of these sugars, as well as in the case of $NaBH_3CN$, the presence of UMP is absolutely required, while in the case of $NaBH_4$ the rate of inactivation is greatly accelerated by UMP. It therefore appears that the binding of uridylyl moieties to the active site "activates" the NAD^+ toward reduction by these agents. This activation is probably caused by destabilization of the nicotinamide ring, possibly driven by a protein conformational change, which is known to occur on the basis of circular dichroism studies (Wang et al., 1978).

In this paper we present data consistent with the above model which have been obtained from ^{31}P NMR studies of the active site NAD^+ of UDP-galactose 4-epimerase from *E. coli* under resting conditions, under conditions of the binding of UMP and of 8-anilinonaphthalene-1-sulfonate (ANS), and under conditions of both UMP-dependent and UMP-inde-

† Supported by Grants GM 30480 (to P.A.F.) and GM 36281 (to D.G.G.) from the National Institute of General Medical Science, and by Predoctoral Fellowships RCD-86-51-883 and RCD-85-50-798 (to C.J.H.), Grant BBS8614177 (to D.G.G.), and a Minority Graduate Fellowship (to J.L.V.) from the National Science Foundation. Assistance was also provided by Purdue University Biochemical Magnetic Resonance Laboratory, which is supported by the National Institutes of Health (Grant PR01077 from the Biotechnology Resources Program of the Division of Research Resources). This study made use of the National Magnetic Resonance Facility at Madison which is supported in part by NIH Grant RR02301 from the Biomedical Research Technology Program, Division of Research Resources. Equipment in the facility was purchased with funds from the University of Wisconsin, the NSF Biological Instrumentation Program (Grant PCM-845048), NIH Biomedical Research Technology Program (Grant RR02301), NIH Shared Instrument Program (Grant RR02781), and the U.S. Department of Agriculture.

‡ University of Wisconsin—Madison.

§ Purdue University.

[1] Abbreviations: ANS, 8-anilinonaphthalene-1-sulfonate; FID, free induction decay; EDTA, ethylenediaminetetraacetate; EGTA, ethyleneglycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; NAD^+, nicotinamide adenine dinucleotide (oxidized); NADH, nicotinamide adenine dinucleotide (reduced); NMR, nuclear magnetic resonance; Tris, tris(hydroxymethyl)aminomethane; UDP, uridine diphosphate; UMP, uridine monophosphate.

0006-2960/89/0428-2645$01.50/0

pendent reductive inactivation. In the course of these investigations, we have also discovered evidence that UMP itself will reductively inactivate the enzyme very slowly in the absence of additional reducing agents.

Experimental Procedures

Materials. UDP-galactose 4-epimerase was purified by the procedure of Wilson and Hogness (1964) with two modifications. The hydroxylapatite column was equilibrated and eluted with 10 mM potassium phosphate, pH 7.1, rather than pH 6.5 as originally described, since the enzyme is retained by the column at the lower pH originally specified. The final enzyme was passed through a large column of Sephadex G-150 equilibrated with 50 mM Tris-HCl buffer at pH 8.5 to remove catalase, which copurifies with UDP-galactose 4-epimerase and is present when the *E. coli* cell growth approaches the stationary phase.

UMP (sodium salt) was obtained from Sigma. UMP utilized in kinetic studies of the reductive inactivation of epimerase by glucose was used without further purification. UMP utilized in all other experiments was further purified by ion-exchange chromatography using procedures previously described for nucleotides (Richard & Frey, 1982). The resulting mono(triethylammonium) salt, dissolved in water, was converted to the sodium salt by passage through a column of Sp-Sephadex C-25 (Na^+ form) and dried by rotary evaporation.

8-Anilinonaphthalene-1-sulfonate (ANS, Mg^{2+} salt), obtained from Eastman Kodak, was recrystallized six times from hot water following treatment with activated charcoal. It was converted to the sodium salt by passage through a column of Sp-Sephadex C-25 (Na^+ form) and dried by rotary evaporation.

ACRO LC3A (0.45-μm) disposable microfilters were purchased from Gelman. D_2O (100.0% D, low in paramagnetic impurities) was obtained from Aldrich. Potassium arsenate (monobasic) was a product of either Merck & Co. or Sigma. Potassium phosphate (dibasic) was from Mallinckrodt. Tetrasodium EDTA and EGTA (free acid) were obtained from Sigma, as were all enzymes, coenzymes, and substrates used for epimerase assays. Other materials were obtained commercially and used without further purification.

Assays. UDP-galactose 4-epimerase was assayed as described by Wilson and Hogness (1964), who define 1 unit of epimerase activity as the amount that will convert 1 μmol of UDP-galactose to UDP-glucose in 1 h at 27 °C, except that an assay temperature of 32 °C was utilized for these studies. Enzyme concentrations were determined by absorbance at 280 nm assuming an extinction coefficient of 1.05 $(mg/mL)^{-1}$ cm^{-1} (Wilson & Hogness, 1964). For enzyme samples containing UMP, concentrations of epimerase and UMP were determined from absorbance measurements at 262 and 280 nm and by solving simultaneous equations using appropriate extinction coefficients. For epimerase, ϵ_{262} was calculated from ϵ_{280} and the observed A_{262}/A_{280} prior to UMP addition. Extinction coefficients for UMP were determined to be 9.84 mM^{-1} cm^{-1} at 262 nm and 3.76 mM^{-1} cm^{-1} at 280 nm in 10 mM dipotassium phosphate. This procedure gave excellent agreement with values expected on the basis of dilution considerations. In samples containing both UMP and glucose, epimerase concentrations were determined by multiplying the concentration prior to glucose addition by the appropriate dilution factor. This was necessary due to the observation that reductive inactivation of epimerase by glucose in the presence of UMP resulted in significant hypochromicity in the ultraviolet absorbance, the precise extent of which is currently undetermined with respect to the extinction coefficients of UMP and epimerase at the wavelengths measured. Concentrations of ANS and epimerase in mixed samples were calculated from absorbance measurements at 262 and 280 nm and solution of simultaneous equations as above. Extinction coefficients at 19.3 mM^{-1} cm^{-1} at 262 nm and 13.7 mM^{-1} cm^{-1} at 280 nm were used for ANS; these were determined experimentally relative to absorbance at 350 nm, on the basis of ϵ_{350} = 6.3 mM^{-1} cm^{-1} (Kolb & Weber, 1975).

Specific activities of NMR samples were monitored by removing aliquots (generally 10 μL) directly from the NMR tube and diluting to 1 mL with 10 mM dipotassium phosphate and 0.1 mM EDTA, immediately freezing on powdered dry ice, and storing at −70 °C until thawing for assay at a later time. Unless stated otherwise, specific activities reported with error estimates are the mean ± standard deviation of three activity determinations.

Preparation of NMR Samples. UDP-galactose 4-epimerase was dialyzed extensively vs 100 mM potassium arsenate and 1 mM EGTA, pH 8.0 at 4 °C. This was followed by extensive dialysis vs 5 mM potassium arsenate and 0.1 mM EGTA, pH 8.0. Dialyzed enzyme was shell frozen in a pear-shaped flask on powdered dry ice and lyophilized and then redissolved to one-tenth the original volume with D_2O (a similar procedure was used for Tris-HCl-buffered samples). Following redissolution, samples were prepared for NMR experiments by force-filtering through an ACRO LC3A disposable microfilter (0.45 μm) directly into NMR tubes using disposable plastic syringes. Several milliliters of air was forced through the filter following the sample to ensure quantitative delivery. Samples were stored at 4 °C until needed for NMR, at which time they were warmed to room temperature prior to placement within the superconducting solenoid (same day). Redissolved, lyophilized residues which were not used for NMR experiments the same day were frozen in powdered dry ice and stored at −70 °C until needed.

Buffered 10 mM solutions of NAD^+ and NADH were prepared for NMR by lyophilizing 1 mM solutions in 5 mM potassium arsenate and 0.1 mM EDTA, pH 8.0, redissolving in one-tenth the original volume in D_2O, and then filtering into NMR tubes as above. A buffered solution of ANS (Na^+ salt) was prepared by rotary evaporation of the appropriate concentration in the same buffer and redissolved in one-tenth the original volume in D_2O. A buffered solution of glucose for addition to NMR tubes already containing epimerase was prepared by lyophilizing an appropriate concentration in the above buffer (substituting EGTA for EDTA) and redissolving in one-tenth the original volume. An unbuffered solution of 7.1 mM UMP for addition to NMR tubes was prepared by redissolving the repurified sodium salt in 30% D_2O with 0.08 mM EGTA, the pH being adjusted to 8.6 (pH meter reading) with NaOH. A 0.2 M solution of UMP for addition to NMR tubes was prepared by redissolution of a lyophilized, repurified sample in 100% D_2O with a final concentration of 0.8 mM EGTA adjusted to pD 8.7 with KOH.

Determination of Kinetic Constants for the UMP-Dependent Reductive Inactivation of UDP-galactose 4-Epimerase by Glucose under NMR Conditions. Assays were performed in 50 mM potassium arsenate and 1 mM EGTA, pH 8.4 at 32 °C, by using a Cary 118C recording spectrophotometer with temperature control in the sample compartment by liquid circulation from a Forma Scientific Model 2160 bath and circulator. A 0.2-mL aliquot of epimerase (8 mg/mL) in the above buffer was diluted to 0.8 mL with the same buffer in a 1-mL quartz cuvette and the absorbance at 280 nm measured

vs a buffer blank. One-tenth milliliter of buffered UMP of varying concentration was added and the sample allowed to equilibrate to the chamber temperature for 10 min. reactions were started by addition of 0.1 mL of a 32 °C solution of glucose in the assay buffer. Final concentrations were 1.6 mg/mL epimerase, 0.05–4 mM UMP, and either 50 or 200 mM glucose. The initial rate of increase in A_{345} (λ_{max} of epimerase-bound NADH; Wilson & Hogness, 1964) was recorded and normalized for the precise concentration of epimerase. Double-reciprocal plots of initial velocity vs UMP concentration at changing glucose, and vs glucose at changing UMP, were analyzed by linear regression. Values of the true dissociation constants for UMP and glucose, their K_m's at a saturating concentration of the other substrate, and the V_{max} at saturating concentrations of both were determined by analysis of slope and intercept replots vs reciprocal substrate concentration by using the equations for rapid equilibrium random bireactant systems described by Segel (1975).

Reductive Inactivation of UDP-galactose 4-Epimerase by UMP. A sample of 8.9 mg/mL epimerase in 5 mM dipotassium phosphate was lyophilized and redissolved in D_2O to give 69 mg/mL epimerase in 39 mM dipotassium phosphate, and 50 μL was placed in each of three 0.5 × 5 cm Pyrex test tubes. At zero time, 10 μL of a 6.2 mM solution of purified UMP in D_2O was added to one tube, 10 μL of a similar solution of commercial UMP was added to a second tube, and 10 μL of D_2O was added to the third (control). The concentrations in the complete reaction mixture were 58 mg/mL epimerase (0.73 mM based on a molecular weight of 79 000; Wilson & Hogness, 1964, 1968; Imae et al., 1964), 32 mM dipotassium phosphate, and 1 mM UMP. The samples were mixed, and 10 μL was removed from each and diluted to 1 mL with ice-cold 10 mM dipotassium phosphate. The tubes were then sealed with a rubber septum and placed on a heating block at 32 °C for 24 h (heating was employed to mimic the conditions of epimerase samples during NMR data acquisition, which was performed at this temperature in order to enhance signal-to-noise ratios). Additional 10-μL aliquots were removed and diluted at appropriate times. Diluted aliquots were assayed immediately to determine enzyme-specific activities and absorbances at 345 nm, which were converted to extinction coefficients on the basis of a path length of 1 cm and the millimolar concentrations of epimerase. The mean and standard deviation of the specific activities of all three zero-time aliquots plus the original concentrated enzyme sample was defined as the zero-time control of 8720 ± 350 (4%) units/mg. At subsequent time points, the specific activities of aliquots removed from the tubes containing UMP were divided by the specific activities of the aliquots removed from the control tube at the same time point in order to normalize the data for non-UMP-specific inactivation (the control activity had decreased 14 ± 6% by the end of the 24-h experiment). The zero-time standard deviation was applied to all such ratios in data presentation as an error estimate. A similar procedure was followed for the extinction coefficient data, which are, however, presented as the difference from control values, rather than as ratios.

200-MHz ^{31}P NMR. The proton-decoupled ^{31}P NMR spectra of UDP-galactose 4-epimerase samples, relative to an external standard of 85% H_3PO_4, were obtained on a Bruker AM-500 spectrometer operating at 202.5 MHz at 4 and 32 °C. For 5-mm tubes, a 30° pulse of 4 μs, a block size of 8192 bytes, spectral width of 6024 Hz (resulting acquisition time = 0.68 s; digital resolution = 1.5 Hz or 0.0073 ppm), and a relaxation delay of 0.190 s were used. For 10-mm tubes, a 30° pulse of 8 μs was employed. Six to forty-five thousand transients were accumulated, depending on the experiment. In experiments in which FID's were acquired before and after the addition of ligand solutions whose volumes significantly diluted the epimerase, the number of transients after ligand addition was increased relative to the number acquired prior to addition. In order to maintain a theoretically constant signal-to-noise ratio, it was assumed that signal to noise decreases linearly with concentration and increases as the square root of the number of transients accumulated. The resulting FID's were zero-filled with an additional 8192 bytes of computer memory and exponentially multiplied prior to Fourier transformation using a line-broadening factor of 2, 15, or 20 Hz. The spectra of NAD^+, NADH, and UMP were obtained similarly, except that line-broadening factors of 0–2 Hz were used and fewer transients were accumulated.

Line widths at half-height for UMP were obtained by using a Lorentzian line fitting program on spectra that had been exponentially multiplied by use of several different line-broadening factors and extrapolating to zero line broadening.

All chemical shifts are reported by using the convention that shifts downfield from the H_3PO_4 standard are given positive values. Unless otherwise specified, chemical shifts for broad resonances are quoted from the tallest point of the signal.

Effect of Temperature on the ^{31}P NMR Spectrum of UDP-galactose 4-Epimerase. The ^{31}P NMR spectrum of a 1.9-mL sample of 57 mg/mL epimerase in 50 mM potassium arsenate and 1 mM EGTA, pD 8.5, was obtained in a 10-mm tube at 32 °C as described earlier, collecting 6101 transients (total acquisition time ~ 1.5 h). The probe temperature was then reduced to 4 °C and the sample allowed to equilibrate to this temperature for 30 min. Another FID was then acquired, accumulating 45 237 transients (total acquisition time ~ 11 h). Aliquots of 10 μL were removed, diluted, and stored for later assay prior to FID accumulation at 32 °C and following FID acquisition at 4 °C.

Effect of UMP Binding on the ^{31}P NMR Spectrum of UDP-galactose 4-Epimerase. Following the acquisition of the ^{31}P NMR spectrum of epimerase at 4 °C (described above), the probe temperature was again raised to 32 °C and the sample allowed to equilibrate to this temperature in the probe for 1 h. A 5-μL aliquot of unbuffered 200 mM UMP and 0.8 mM EGTA, pD 8.7 was added (resulting concentrations: 58 mg/mL epimerase, 0.6 mM UMP) and an FID obtained. This was followed by addition of 20 μL of UMP (58 mg/mL epimerase, 2.9 mM UMP) and accumulation of a second FID. A third FID was acquired following addition of a further 40 μL of UMP (56 mg/mL epimerase, 7.6 mM UMP, 48 mM potassium arsenate) and a fourth and final FID obtained following addition of 140 μL of UMP (final concentrations: 50 mg/mL epimerase, 22.5 mM UMP, 45 mM potassium arsenate, pD 8.4). FID's in each case were composed of 3000 transients with total acquisition times of 43 min. The total time of exposure to UMP was about 7 h, including time between FID acquisitions for sample preparation and magnet shimming. Following accumulation of each FID, 10-μL aliquots were removed for later assay. For comparison, 10 μL of the same UMP solution was added to 3 mL of 50 mM potassium arsenate and 1 mM EGTA, pD 8.5, in D_2O in a 10-mm tube (final concentration 0.7 mM UMP), and an FID of 1000 transients was obtained.

Effect of the Binding of 8-Anilinonaphthalene-1-sulfonate on the ^{31}P NMR Spectrum of UDP-galactose 4-Epimerase. The ^{31}P NMR spectrum of a 0.5-mL sample of 87 mg/mL epimerase in 50 mM potassium arsenate and 1 mM EGTA,

pD 8.4, in a 5-mm tube was acquired by collecting 10 100 transients. Aliquots (10 μL) were removed both before and after acquisition, diluted, and stored for later assay. A 0.1-mL volume of buffered 7.3 mM ANS in D_2O was added with mixing (final concentrations: 72 mg/mL epimerase, 1.2 mM ANS) and a second FID, consisting of 14 747 transients, accumulated. Following acquisition, a final 10-μL aliquot was removed, diluted, frozen, and stored for later assay.

Effect of UMP-Dependent Reductive Inactivation by Glucose on the ^{31}P NMR Spectrum of UDP-galactose 4-Epimerase. The ^{31}P NMR spectrum of a 0.5-mL sample of 98 mg/mL epimerase in 50 mM potassium arsenate and 1 mM EGTA, pD 8.4, was obtained as described (10 500 transients accumulated). Aliquots (10 μL) were removed from the NMR tube both before and after acquisition for later assay. A 0.1-mL aliquot of the 7.1 mM unbuffered solution of UMP was removed from the NMR tube in which its spectrum had recently been obtained and added with mixing (resulting epimerase concentration = 83 mg/mL), and a second FID was immediately acquired (15 120 transients, total time of exposure to UMP about 4 h). Following removal of an additional 10-μL aliquot, 0.1 mL of the buffered 0.8 M glucose solution was added, mixed (resulting epimerase concentration = 69 mg/mL), and allowed to incubate at room temperature for 30 min. A third FID consisting of 20 580 transients was acquired. A final 10-μL aliquot was obtained following signal acquisition.

Effect of Borohydride Reduction on the ^{31}P NMR Spectrum of UDP-galactose 4-Epimerase. A sample of 50 mg of epimerase in 4 mL of 75 mM Tris-HCl and 0.8 mM EGTA, pH 8.2, was shell-frozen on powdered dry ice in a pear-shaped flask and lyophilized to dryness. The flask was sealed with a rubber septum and the atmosphere within exchanged for argon gas. The lyophilized residue was then redissolved in 3 mL of 100% D_2O (resulting concentrations: 14 mg/mL epimerase, 100 mM Tris-HCl, 1 mM EGTA) which had been sequentially degassed under high vacuum and sparged with nitrogen several times. The redissolved epimerase sample was drawn from its flask with a nitrogen-filled syringe, a Gelman LC3A microfilter was fitted between the syringe body and needle, and the sample was injected into a 10-mm NMR tube that had been sealed with a Parafilm-covered rubber septum and filled with argon. A 50-μL aliquot, which had been left in the lyophilization flask, was diluted to 1 mL with 10 mM dipotassium phosphate and 0.1 mM EDTA, frozen on powdered dry ice, and stored at −70 °C for later assay.

The absorbance at 345 nm of the epimerase sample in the anaerobic NMR tube was then measured on a Gilford spectrophotometer by placing the tube into a tightly fitting 3-mL plastic cuvette within a sample holder in the light path and covering with a black cloth. The ^{31}P NMR spectrum of the sample was then obtained at 32 °C by using 12 000 transients, following which the absorbance at 345 nm was again measured. A 20-μL aliquot of 3 M potassium borohydride in 0.1 M KOH was then added by syringe and the change in A_{345} monitored for 10 min, by which time the increase had leveled off. A second FID of 12 000 transients was acquired, following which A_{345} was rechecked.

Results

Effect of Temperature on the ^{31}P NMR Spectrum of UDP-galactose 4-Epimerase. The 200-MHz ^{31}P NMR spectrum of the active-site-bound NAD^+ of 57 mg/mL epimerase from *E. coli* in 50 mM potassium arsenate, 1 mM EGTA, and 100% D_2O, pD 8.5 at 32 °C, is presented in Figure 1A, which exhibits two distinct resonances of approximately equal intensity and area. Chemical shifts are $\delta_1 = -9.59$ ppm for the downfield peak and $\delta_2 = -12.14$ ppm for the upfield peak. The ^{31}P NMR spectrum of native epimerase is also shown for other samples in Figures 6A, 7A, and 9A, the chemical shift values for which are quoted in the appropriate places in the text. A mean ± standard deviation for the shifts of these four spectra gives $\delta_1 = -9.60 \pm 0.05$ ppm for the downfield and $\delta_2 = -12.15 \pm 0.01$ ppm for the upfield resonance. These standard deviations give some indication of the inherent precision of chemical shift measurements in these experiments, given the line widths, noise levels, and line-broadening factors involved. In comparison, the ^{31}P NMR spectrum of free NAD^+ under similar conditions appeared as a very sharp AB quartet, with chemical shifts of $\delta_1 = -10.53$ ppm and $\delta_2 = -10.85$ ppm and a phosphorus–phosphorus coupling constant of $J_{P-P} = 20.7$ Hz.

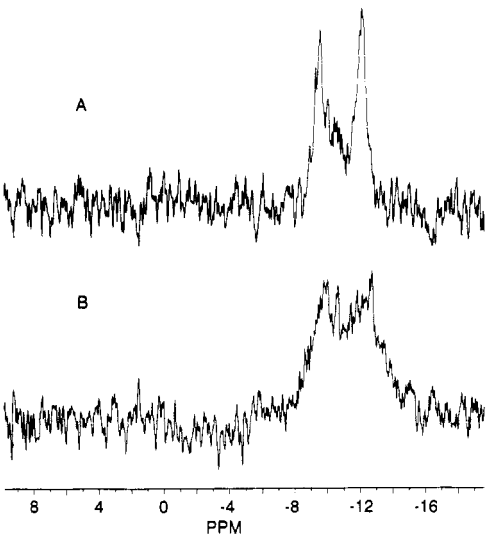

FIGURE 1: Effect of temperature on the ^{31}P NMR spectrum of UDP-galactose 4-epimerase. The ^{31}P NMR spectrum of phosphorus atoms of the active site NAD^+ of UDP-galactose 4-epimerase was determined at 32 °C and at 4 °C at 200 MHz in 50 mM potassium arsenate and 1 mM EGTA, pD 8.5. The sample contained 57 mg/mL epimerase in 1.9 mL in a 10-mm tube. FID's were composed of the indicated number of transients and exponentially multiplied by using a line-broadening factor of 20 Hz. (A) 0.73 mM epimerase at 32 °C, 6101 transients accumulated over 1.5 h, specific activity prior to signal acquisition = 7700 ± 600 units/mg (six determinations). (B) The same sample at 4 °C, 45 237 transients accumulated over 11 h, specific activity = 9400 ± 300 units/mg following signal acquisition.

The ^{31}P NMR spectrum of the same epimerase sample at 4 °C is shown in Figure 1B, which apparently still exhibits two resonances of roughly equal intensity at $\delta_1 = -9.81$ ppm and $\delta_2 = -12.74$ ppm (tallest point in each case), although the signals are substantially broader than at the higher temperature.

Due to the substantial sharpening of the epimerase·NAD^+ signals at the higher temperature, all subsequent spectra were accumulated at 32 °C. In light of the above results and other preliminary data at 22 °C not presented here,[2] it was judged that this gave a roughly optimal combination of minimum temperature and signal-to-noise ratio.

Reductive Inactivation of UDP-galactose 4-Epimerase by UMP. Results of time-course studies investigating the reductive inactivation of epimerase by 1 mM UMP in the absence of any other reducing agents are presented in Figure 2, which demonstrates a UMP-dependent decrease in specific activity over time with a concomitant increase in ϵ_{345} due to

[2] J. M. Konopka, unpublished data.

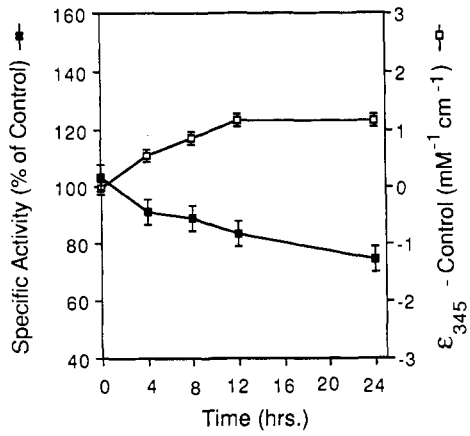

FIGURE 2: Activity time course of reductive inactivation of UDP-galactose 4-epimerase by UMP. D_2O solutions of 0.73 mM epimerase in 32 mM dipotassium phosphate with and without (control) 1 mM UMP were incubated at 32 °C in sealed tubes. The percent of control specific activity (■) and change in ϵ_{345} (□) were determined as described under Experimental Procedures. One hundred percent of control specific activity represents 8720 ± 350 units/mg. Specific activity of the control sample decreased to 7460 ± 120 units/mg at 24 h.

reduction of epimerase-bound NAD^+, the two processes being correlative within the indicated experimental error. The pseudo-first-order rate constant for the loss of activity was found to be 0.013 ± 0.001 h^{-1}. Data obtained in a similar experiment utilizing 1.9 mM UMP in 55 mM potassium arsenate buffer and 1.1 mM EDTA, pD 8.4, were within experimental error of those presented here.

In light of these results, conditions in experiments employing UMP were chosen with the goal of minimizing the exposure time of epimerase to UMP sufficiently so that experimental data and the conclusions drawn from them were not significantly affected by this phenomenon.

Kinetic Parameters of UMP-Dependent Reductive Inactivation of UDP-galactose 4-Epimerase by Glucose under NMR Conditions. The kinetics of the epimerase inactivation were studied by initial velocity assays as described under Experimental Procedures in 50 mM potassium arsenate and 1 mM EGTA, pH 8.4 at 32 °C. Kinetic parameters for this reaction were determined to be as follows: for UMP, K_{dis} = 0.4 mM, K_m = 0.08 mM; for glucose, K_{dis} = 1.6 M, K_m = 0.3 M; k_{cat} = 0.05 s^{-1}. Comparison with values obtained previously in 0.1 M Tris-HCl, pH 8.5 at 27 °C (Kang et al., 1975), indicates that, whereas the kinetic constants for glucose have not changed within experimental error, the parameters for UMP have decreased, as has the value of k_{cat}. However, assuming a 2-fold increase with a 10 °C rise in temperature, the value of k_{cat}/K_m(UMP) has not changed within the experimental uncertainties, suggesting that the buffer change results in substantially tighter binding of UMP to the enzyme without a significant alteration in the chemical mechanism.

Effect of UMP Binding on the ^{31}P NMR Spectrum of UDP-galactose 4-Epimerase. The change in the ^{31}P spectrum of epimerase-bound NAD^+ with increasing concentrations of UMP in the absence of reducing agents is shown in Figure 3. The two signals for epimerase·NAD^+ approach each other as the UMP concentration is increased; chemical shift values obtained are given in the legend to Figure 4. The pD of the final sample was 8.4, down from an original value of 8.5 in the sample prior to addition of UMP (Figure 1). The change in chemical shift for the two epimerase resonances with the increase in the fraction of epimerase·NAD·UMP complex formed relative to total epimerase, based on the concentrations

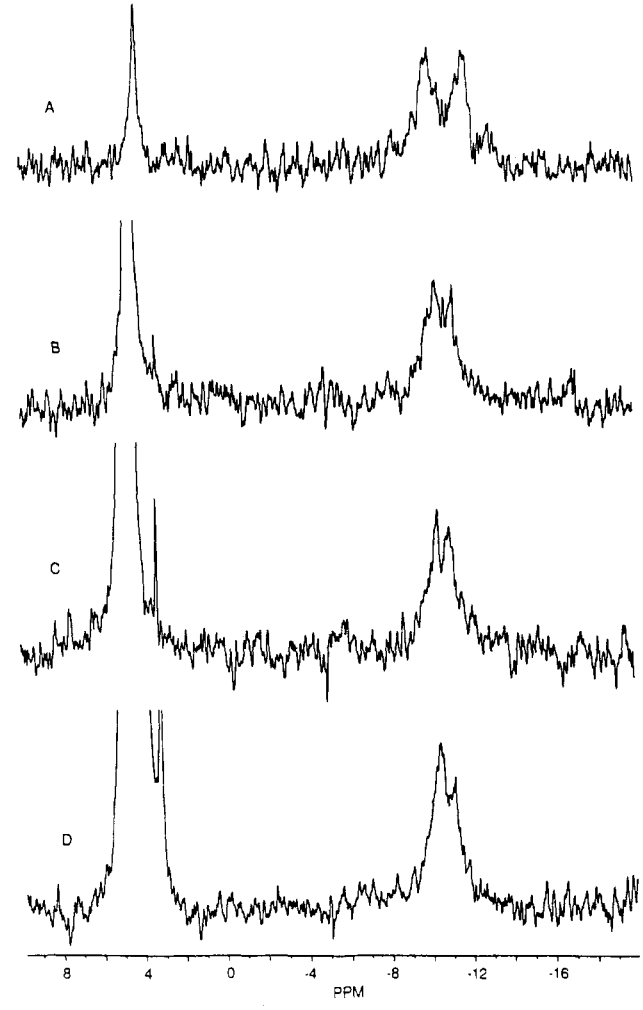

FIGURE 3: Effect of UMP binding on the ^{31}P NMR spectrum of UDP-galactose 4-epimerase. Increasing amounts of UMP were added to the epimerase sample used in Figure 1 in the same 10-mm tube and 200-MHz spectra acquired at 32 °C, accumulating 3000 transient FID's, which were exponentially multiplied prior to Fourier transformation using a line-broadening factor of 15 Hz. (A) 0.73 mM epimerase + 0.6 mM UMP (specific activity = 9700 ± 500 units/mg, 8 determinations); (B) 0.73 mM epimerase + 2.9 mM UMP (specific activity = 10000 ± 600 units/mg, 8 determinations); (C) 0.71 mM epimerase + 7.6 mM UMP (specific activity = 9600 ± 700 units/mg, 12 determinations); (D) 0.64 mM epimerase + 22.5 mM UMP, 45 mM potassium arsenate, 0.9 mM EGTA, pD 8.4 (specific activity = 9900 ± 500 units/mg, 8 determinations).

of epimerase and UMP present (listed in Figure 3) and a dissociation constant of 0.4 mM, is illustrated in Figure 4 (the chemical shift values for [E·NAD^+·UMP]/[total epimerase] = 0 were taken from Figure 1A). The computer-generated least-squares lines extrapolate to δ_1 = −10.51 ppm and δ_2 = −11.06 ppm when epimerase is fully liganded with UMP ([E·NAD^+·UMP]/[total epimerase] = 1).

The signals for UMP are partially off scale in parts B–D of Figure 3 owing to its high concentrations in these experiments. The values obtained for δ and line width are given in the legend to Figure 5; in contrast, 0.7 mM UMP in 50 mM potassium arsenate, 1 mM EGTA, and 100% D_2O, pD = 8.5, gave a chemical shift of δ = 4.48 ppm with a line width at half-height of 2.9 Hz. Figure 5 illustrates the change in the chemical shift of UMP relative to the fraction of the total present bound to epimerase (the chemical shift value for [E·NAD^+·UMP]/[total UMP] = 0 was taken from the value obtained for 0.7 mM UMP in the absence of epimerase, quoted above). The computer-fitted least-squares fit to the data extrapolates to δ = 3.88 ppm for the value of epimerase-bound

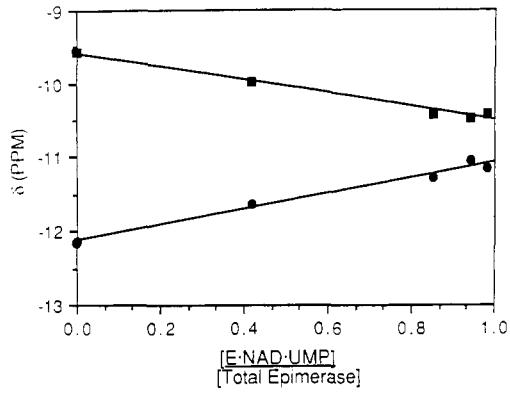

FIGURE 4: Dependence of the ^{31}P NMR chemical shifts of active site NAD^+ in UDP-galactose 4-epimerase on the degree of liganding by UMP. Chemical shift values were taken from Figure 3, except for that corresponding to [E·NAD^+·UMP]/[total epimerase] = 0, which was taken from Figure 1A. Chemical shift values are as follows: from Figure 1A, $\delta_1 = -9.59$ ppm and $\delta_2 = -12.14$ ppm; from Figure 3A, $\delta_1 = -9.99$ ppm and $\delta_2 = -11.64$ ppm; from Figure 3B, $\delta_1 = -10.43$ ppm and $\delta_2 = -11.26$ ppm; from Figure 3C, $\delta_1 = -10.48$ ppm and $\delta_2 = -11.04$ ppm; and from Figure 3D, $\delta_1 = -10.41$ ppm and $\delta_2 = -11.13$ ppm. Values for [E·NAD^+·UMP]/[total epimerase] were calculated on the basis of measured concentrations of UMP and epimerase and a dissociation constant of 0.4 mM. Lines are computer-generated linear least-squares fits of the data, which have the following solutions: for δ_1 (■), $y = -9.595 - 0.914x$, with a squared correlation coefficient (R^2) = 0.982; for δ_2 (●), $y = -12.123 + 1.067x$, with $R^2 = 0.985$.

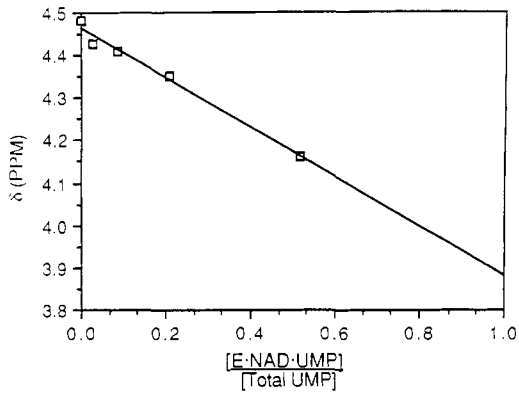

FIGURE 5: Dependence of the ^{31}P NMR chemical shift of UMP on the degree of binding to UDP-galactose 4-epimerase. Chemical shift values and line widths at half-height ($\Delta\nu_{1/2}$) are taken from Figure 3, except for the value corresponding to [E·NAD^+·UMP]/[total UMP] = 0, which was obtained as described in the text. The value from Figure 3A is $\delta = 4.16$ ppm (UMP, $\Delta\nu_{1/2} = 46$ Hz), those from Figure 3B are $\delta = 4.35$ ppm (UMP, $\Delta\nu_{1/2} = 13.9$ Hz) and $\delta = 3.17$ ppm (inorganic phosphate), those from Figure 3C are $\delta = 4.41$ ppm (UMP, $\Delta\nu_{1/2} = 7.0$ Hz) and $\delta = 3.17$ ppm (inorganic phosphate), and those from Figure 3D are $\delta = 4.43$ ppm (UMP, $\Delta\nu_{1/2} = 3.16$ Hz) and $\delta = 3.16$ ppm (inorganic phosphate). Values for [E·NAD^+·UMP]/[total UMP] were calculated as in Figure 4. The computer-generated least-squares line has the equation $y = 4.464 - 0.583x$, $R^2 = 0.990$.

UMP ([E·NAD^+·UMP]/[total UMP] = 1); however, this is a long extrapolation.

Effect of 8-Anilinonaphthalene-1-sulfonate on the ^{31}P NMR Spectrum of UDP-galactose 4-Epimerase. Wong and Frey (1978) demonstrated that ANS binds to epimerase·NAD^+ in competition with UMP and much more tightly, with a dissociation constant of about 27 μM. The effect of such binding on the ^{31}P NMR spectrum of epimerase is presented in Figure 6. Resonances for 86 mg/mL epimerase·NAD^+ in the absence of ANS appear at $\delta_1 = -9.61$ ppm and $\delta_2 = -12.16$ ppm in Figure 6A. Following ANS addition to a level that is expected to give 93% of the total epimerase present as the epimerase·NAD^+·ANS complex (72 mg/mL epimerase, 1.23

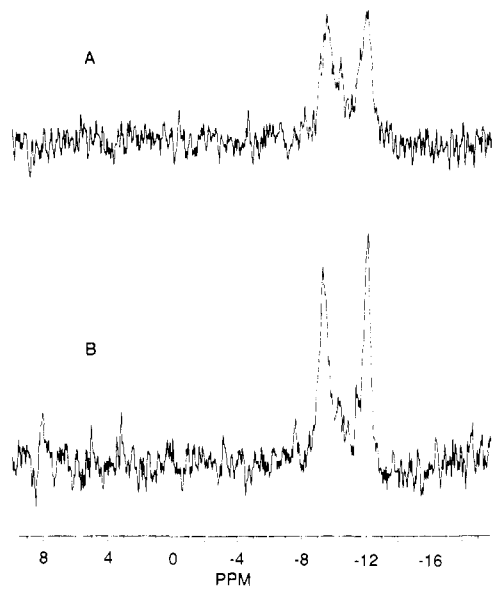

FIGURE 6: Effect of ANS binding on the ^{31}P NMR spectrum of UDP-galactose 4-epimerase. A 0.1-mL aliquot of 7.3 mM ANS in 50 mM potassium arsenate and 1 mM EGTA, pD 8.4 in D_2O, was added to 0.5 mL of epimerase in the same buffer in a 5-mm tube. FID's were accumulated at 32 °C and exponentially multiplied prior to Fourier transformation using a line-broadening factor of 15 Hz. (A) 1.1 mM epimerase (specific activity prior to signal acquisition = 8200 ± 500 units/mg, six determinations; specific activity following acquisition of 10100 transients = 8600 ± 1100 units/mg, three determinations); (B) 0.91 mM epimerase + 1.23 mM ANS (specific activity following acquisition of 14747 = 9100 ± 200 units/mg, three determinations).

mM ANS, assuming a dissociation constant of 27 μM), δ_1 is seen to shift slightly downfield to $\delta_1 = -9.38$ ppm, while δ_2 remains constant at $\delta_2 = -12.16$ ppm (Figure 6B). It is also apparent that both resonances sharpen upon binding ANS. The areas under the two signals are essentially equivalent in Figure 6A (±5%) and do not change significantly relative to each other in Figure 6B. A similar experiment, performed earlier at 80 MHz on a Nicolet NTC-200 spectrometer gave similar results, except that the chemical shift of δ_2 shifted upfield by 0.1 ppm, while δ_1 remained unchanged.[2] These small divergences of the chemical shifts of the two resonances may or may not be significant given the roughly ±0.05 ppm inherent precision of the experiments. However, their relative constancy is in striking contrast to their substantial convergene upon binding UMP (Figure 3) in light of the fact that kinetic and fluorescence-monitored binding experiments indicate that ANS and UMP are directly competitive in all respects, leading to the conclusion that they bind to the same site on epimerase·NAD^+ (Wong & Frey, 1978).

Effect of UMP-Dependent Reduction by Glucose on the ^{31}P NMR Spectrum of UDP-galactose 4-Epimerase. Figure 7 shows the alteration of the ^{31}P NMR spectrum of UDP-galactose 4-epimerase upon reductive inactivation by glucose in the presence of UMP. Resonances for epimerase·NAD^+ appear in Figure 7A at chemical shifts $\delta_1 = -9.53$ ppm and $\delta_2 = -12.15$ ppm. Upon addition of UMP in part B the spectrum changes to $\delta_1 = -10.38$ ppm and $\delta_2 = -11.34$ ppm. Both signals are consistent with the graph in Figure 4. The signal for UMP appears at $\delta = 4.16$ ppm. A small amount of inorganic phosphate also appears, at $\delta = 3.10$ ppm. Upon reductive inactivation by added glucose, shown in Figure 7C, the epimerase signals apparently merge into a single resonance at $\delta = -10.57$ ppm (tallest point; the resonance is roughly centered at $\delta = -10.5$ ppm). By comparison, free NADH in the same buffer system exhibits a single, sharp resonance at

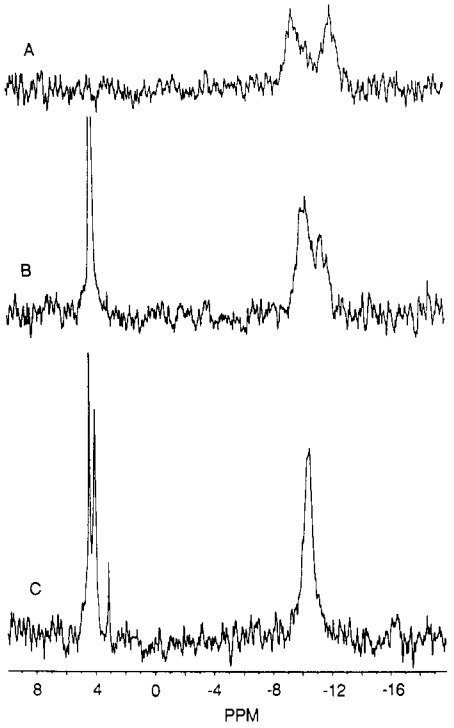

FIGURE 7: ^{31}P NMR spectra of the UMP-dependent reductive inactivation of UDP-galactose 4-epimerase by glucose. Following acquisition of the spectrum of a 0.5-mL solution of epimerase in 50 mM potassium arsenate and 1 mM EGTA, pD 8.4, in a 5-mm tube, 0.1 mL of 7.1 mM UMP and 0.08 mM EGTA in 30% D_2O, pH 8.6, and 0.8 M glucose in the NMR buffer were added sequentially to the same tube and FID's accumulated following addition of each. Accumulated FID's were exponentially multiplied by using a line-broadening factor of 15 Hz. (A) 1.2 mM epimerase (7100 ± 700 units/mg, 10 502 transients); (B) 1.0 mM epimerase (8000 ● 300 units/mg) + 1.3 mM UMP (15 120 transients); (C) 0.9 mM epimerase (450 ± 60 units/mg) + 1.1 mM UMP + 0.12 M glucose (20 580 transients).

δ = −10.49 ppm. The UMP signals appears as two distinct resonances at δ = 4.37 ppm and δ = 4.00 ppm. A slightly increased level of inorganic phosphate is also evident at δ = 3.06 ppm.

Spectra A and B of Figure 8 show the expanded UMP regions of spectra B and C, respectively, of Figure 7, using a line-broadening factor of 2 Hz for exponential multiplication of the FID's rather than 15 Hz as used in Figure 7. Figure 8A is the UMP region for epimerase·NAD^+ and UMP and shows a single broad resonance for UMP at δ = 4.17 ppm, with inorganic phosphate at δ = 3.09 ppm. The line width at half-height of the UMP resonance was determined to be 19 Hz. By way of comparison, the sample of UMP from which an aliquot had been taken and added to this sample showed a single resonance at δ = 4.37 ppm with a line width at half-height of 0.5 Hz. Figure 8B shows the spectral region for UMP of a solution of epimerase·NADH containing both UMP and glucose. There are two distinct and nearly resolved populations of UMP, with chemical shifts at δ = 4.37 ppm and δ = 3.98 ppm. The line width at half-height was found to be 8.5 Hz for the downfield resonance and 27 Hz for the upfield resonance.

In a similar experiment, addition of further glucose resulted in no significant alteration in the signal intensities or line shapes of the two UMP signals in Figure 8B. Addition of more UMP increased the intensity of the downfield peak, while the integral of the upfield peak retained a 1:1 stoichiometry with enzyme on a molar basis. In light of this evidence, we conclude that the downfield and upfield resonances represent populations

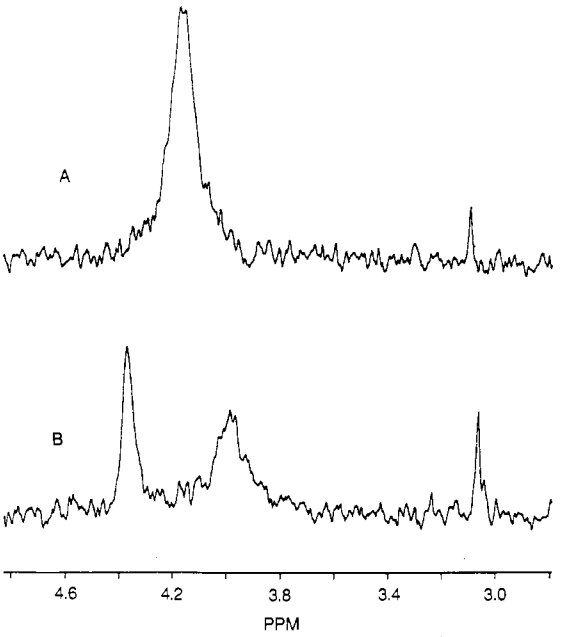

FIGURE 8: High-resolution ^{31}P NMR spectra of UMP in the presence of UDP-galactose 4-epimerase ± glucose. The same FID's used to generate spectra B and C of Figure 7 were exponentially multiplied with a line-broadening factor of 2 Hz and the downfield regions expanded. (A) 1.3 mM UMP + 1.0 mM epimerase; (B) 1.1 mM UMP + 0.9 mM epimerase + 0.12 M glucose.

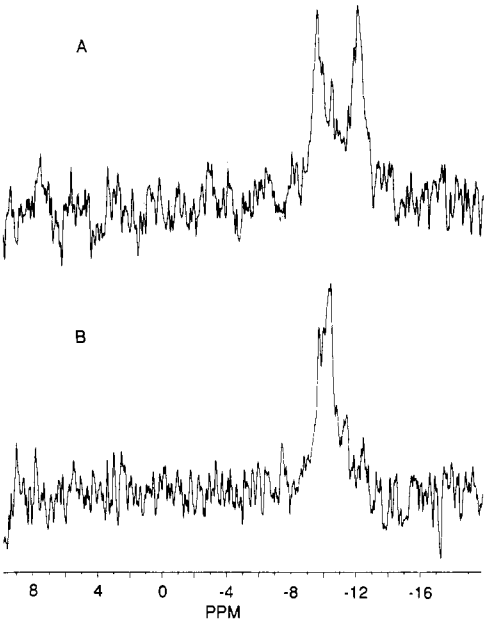

FIGURE 9: Effect of reduction by borohydride on the ^{31}P NMR spectrum of UDP-galactose 4-epimerase. A 20-μL aliquot of 3 M potassium borohydride in 0.1 M KOH was added to a 3-mL anaerobic solution of 0.18 mM epimerase in 100 mM Tris-HCl and 1 mM EGTA in a 10-mm tube. FID's composed of 12 000 transients were accumulated at 32 °C and exponentially multiplied prior to Fourier transformation using a line-broadening factor of 20 Hz. (A) Epimerase in anaerobic solution (specific activity prior to signal acquisition = 8300 ● 500 units/mg, six determinations); (B) epimerase + 20 mM borohydride, total time following borohydride addition = 4 h.

of free UMP and UMP bound to reduced enzyme in an epimerase·NADH·UMP complex, respectively.

Effect of Borohydride Reduction on the ^{31}P NMR Spectrum of UDP-galactose 4-Epimerase. Davis et al. (1974) reported that epimerase·NAD^+ can be reduced to epimerase·NADH in the absence of UMP by borohydride if an excess of borohydride is used in an anaerobic medium. Furthermore,

the enzyme is reoxidized to epimerase·NAD^+ by reintroduction of air. The effect of borohydride reduction on the ^{31}P NMR spectrum of epimerase is shown in Figure 9. The resonances for epimerase·NAD^+ (14 mg/mL in 100 mM Tris-HCl, 1 mM EGTA, and 100% D_2O, pH 8.2, prior to lyophilization and redissolution in D_2O) in an anaerobic environment appear in Figure 9A at $\delta_1 = -9.65$ ppm and $\delta_2 = -12.13$ ppm. The absorbance of the sample at 345 nm was measured directly in the NMR tube as described under Experimental Procedures and was found to be 0.7 prior to FID acquisition and 1.0 following acquisition. (The method of measuring A_{345} used here was necessarily crude in order to obtain independent confirmation of epimerase reduction without jeopardizing the anaerobic integrity of the sample. The uncertainty in A_{345} is estimated to be ±0.1.) Upon addition of 20 mM potassium borohydride to the sample (while maintaining anaerobic conditions), A_{345} increased to 1.9 within 10 min. This increase of 0.9–1.2 absorbance units was consistent with an expected increase of about 1.0 for complete reduction of 0.18 mM epimerase·NAD^+, assuming an extinction coefficient of 6.2 mM^{-1} cm^{-1} (Wilson & Hogness, 1964) and a path length of 0.9 cm (interior diameter of the 10-mm NMR tube). The ^{31}P NMR spectrum of the resulting epimerase·NADH is shown in Figure 9B which can be interpreted as a single broad resonance centered at about $\delta = -10.2$ ppm; the apparent fine structure near the top of the signal is probably noise. The excess borohydride, being unstable at the pD employed, degenerated during the course of signal acquisition, liberating hydrogen gas. However, since the bubbles did not adhere to the walls of the spinning NMR tube, this did not cause any problems of field inhomogeneity. The A_{345} was determined to be 1.8 following FID acquisition, indicating that the sample had not undergone significant reoxidation during the 4 h following borohydride addition.

The signals for epimerase·NAD^+ are upfield and downfield of that for epimerase·NADH and appear to be absent in Figure 9B. The signal for epimerase·NADH is downfield 0.4 ppm from that for both NADH in the epimerase·NADH·UMP complex and free NADH. We cannot explain the greater width of the signal for NADH in epimerase·NADH in Figure 9B, compared with those for NAD^+ in epimerase·NAD^+ and NADH in the epimerase·NADH·UMP complex.

Discussion

During the course of preliminary experiments for this study, it was discovered that UDP-galactose 4-epimerase was reductively inactivated when subjected to an 8-h FID acquisition at 32 °C in the presence of 2 mM UMP in 50 mM Tris-HCl and 1 mM EDTA, pD 8.4, in spite of there being no known reducing agents present. Subsequent investigation revealed that most of this inactivation could be eliminated upon changing to phosphate buffer,[2] indicating that the Tris buffer itself was the apparent source of the reducing equivalents for this inactivation process. This discovery demonstrates the degree to which the active site NAD^+ of epimerase is activated toward reduction by the binding of uridine nucleotides (Bertland et al., 1971; Davis et al., 1974; Kang et al., 1975). A residual, low rate of reductive inactivation was found to be due to the presence of UMP (Figure 2). The mechanism of this process is unknown at this time. The source of the reducing equivalents would presumably be the ribose ring of UMP. Oxidation of the ribose ring could conceivably occur at the 3′-position, yielding the keto form, for example, which would liberate the 5′-phosphate by β-elimination. This might explain the appearance of inorganic phosphate with time in Figures 3 and 7. UDP, in contrast, was found to cause no significant inactivation relative to controls over a 24-h period. However, the use of UDP rather than UMP for the study of alterations in the ^{31}P NMR spectrum of epimerase·NAD^+ on binding uridine nucleotides presented problems owing to the overlap of the α-phosphorus signal of UDP with that of epimerase·NAD^+.[2] We therefore elected to pursue these studies utilizing UMP, but to employ conditions that would minimize this reductive inactivation. To this end, enzyme concentrations, sample size, and NMR parameters were chosen to maximize signal-to-noise ratios while minimizing the exposure time of epimerase to UMP. A temperature of 32 °C was retained due to the substantial enhancement of epimerase signal intensity that this provided (Figure 1). An experimental temperature of 22 °C was likewise found inadequate for our purposes.[2] The state of the enzyme in these experiments was monitored by the determination of specific activity. As will be seen from inspection of these data, the extent of reductive inactivation was minimal in all cases, including the experiment illustrated in Figure 3, in which the UMP concentration was the highest and the total exposure time the longest. We therefoe conclude that our precautions in this respect have been adequate in the experiments presented here and that the conclusions drawn from our data have not been materially affected by this phenomenon.

There are three reasonable interpretations for the existence of two broad, widely separated ^{31}P resonances in the spectrum of epimerase·NAD^+. First, they may represent the individual phosphorus atoms of the NAD^+ pyrophosphate backbone, whose chemical shifts differ from their values in free solution due to their unique magnetic environments within the active site. Second, each resonance, which is broad enough to encompass an entire free NAD^+ AB quartet, may represent a different conformational state of the enzyme, reflected in differential magnetic environments for the pyrophosphate. Third, the two signals may represent different aggregation states of the enzyme.

Of these possibilities, we consider the first to be the most likely for the following reasons. If the two resonances represent two different conformational states of the enzyme that interconvert slowly on the NMR time scale, then the ratio of their areas represent the equilibrium constant for the interconversion. Determination of the integrals of the downfield vs upfield resonances in each of the spectra of native epimerase·NAD^+ (Figures 1A, 6A, 7A, and 9A) shows them to be essentially equivalent in all cases (±5%). This equivalence is also observed in all other spectra of native epimerase obtained so far, in spite of differences in solvent composition (20%[3] vs 100% D_2O), pH (pH 7.0[3] vs pD 8.4 and 8.8), buffer composition and ionic strength (50[3]–100 mM Tris-HCl vs 50 mM potassium arsenate), and temperature (4, 22,[2] and 32 °C). It is unlikely that the two conformations would remain thermodynamically equivalent ($K_{eq} = 1$) regardless of the conditions employed. A constant integration ratio is also observed upon the binding of 8-anilino-1-naphthalenesulfonate (Figure 6), as well as, for the most part, upon binding UMP (Figure 3). The apparent nonequivalence of the integrals and peak intensities in Figure 7B is somewhat anomalous. All other spectra obtained so far under similar conditions exhibited equivalent signals.[2] Finally, the integration ratio is independent of the enzyme concentration (14 mg/mL in Figure 9A to 98 mg/mL in Figure 7A, and up to 150 mg/mL[2]). This excludes the possibility that the two signals could arise from aggregation effects.

[3] D. Gorenstein, unpublished data.

On the other hand, equivalent peak areas under all conditions are expected if the two resonances represent the individual phosphorus atoms of the NAD^+ pyrophosphate linkage. The divergence of the ^{31}P NMR chemical shifts of nucleotides upon binding to enzymes has precedent (Cohn & Rao, 1979; Hyde et al., 1980). In fact, the values obtained here for the chemical shifts of the phosphorus atoms of the NAD^+ pyrophosphate bound to epimerase are remarkably similar to the values found for $NADP^+$ upon binding to NADPH–cytochrome P-450 reductase (Otvos et al., 1986). We, therefore, tentatively conclude that the two ^{31}P NMR resonances observed with UDP-galactose 4-epimerase represent the individual phosphorus atoms of the pyrophosphate linkage of the tightly bound active site NAD^+, although we cannot assign the signals to specific phosphorus centers in NAD^+.

The fact that the chemical shifts are so different from those for NAD^+ in free solution indicates that they reside in a very different environment or are differently conformed when bound within the epimerase active site. By contrast, it appears that the magnetic environments of the phosphorus atoms of epimerase-bound NADH are equivalent (or at least nearly so in light of the line widths involved, Figures 7C and 9B), just as they are in free solution. This finding was unexpected, since the bound NAD^+ exhibits a spectrum quite different from that seen in free solution. It is even more surprising, given that magnetic inequivalence of the two phosphorus nuclei of bound NADH is often observed (Mas & Colman, 1984; Feeney et al., 1975). The fact that there are substantial relative differences between the epimerase-bound vs unbound spectra for NAD^+, but substantial similarities between the spectra for epimerase-bound vs unbound NADH, is an indication of alterations in the microenvironment of epimerase-bound NAD^+ upon reduction. The similarities in the spectra of the epimerase·NADH·UMP complex and free NADH suggest that the active site environment of bound NADH is more akin to that found in free solution than is the environment of bound NAD^+. However, fluorescence studies have shown that the reduced coenzyme is essentially immobilized in the epimerase active site, apparently residing within a substantially hydrophobic region, quite possibly consisting of aromatic protein side chains (Wong & Frey, 1977).

Figure 3 demonstrates that the binding of UMP to epimerase induces a substantial alteration in the ^{31}P NMR spectrum of the active site NAD^+. The degree of alteration in the chemical shifts of the spectra of the resulting mixed populations of epimerase·NAD^+ and epimerase·NAD^+·UMP follows a linear relationship relative to the fraction of epimerase·NAD^+·UMP formed (Figure 4). This fact indicates that the rate of interconversion of the two forms is sufficiently fast relative to the differences in the resonance frequencies of their respective ^{31}P NMR signals to create exchange-averaged spectra. Using the average value for $\delta_2 = -12.15$ ppm obtained for native epimerase·NAD^+ in these experiments and the extrapolated value $\delta_2 = -11.06$ ppm for epimerase·NAD^+·UMP (Figure 4 and relevant accompanying text), one calculates a minimum rate of exchange necessary for convergence of about 490 s^{-1} (Campbell et al., 1979). The actual rate could in fact be significantly faster than this, given the relative constancy of signal intensities.

Additional evidence for the conclusion that a fast exchange rate is operative is obtained from a consideration of the UMP signals in Figure 3, which exhibit single signals for UMP throughout the titration experiment and whose chemical shifts also show a linear relationship relative to the fraction of UMP bound to epimerase (Figure 5). It is well-known that the observed chemical shift of the nucleus of a ligand undergoing a binding process is $\nu = \nu_f P_f + \nu_b P_b$ in the fast-exchange limit, where $P_b = [EL]/[L]_t$ and $P_f = [L]_f/[L]_t = 1 - P_b$ (Campbell et al., 1979). Feeney, Roberts, and co-workers have pointed out that this equation does not apply in the intermediate exchange region (Feeney et al., 1979; Hyde et al., 1980). They conclude that values of K_{dis} and $\nu_f - \nu_b$ can be greatly in error if they are obtained from a graph of $\nu - \nu_f$ vs $[L]_t$ and if the assumption of fast exchange is made. We determined K_{dis} independently, however, and this value allows us to calculate P_f and P_b. Moreover, these same authors state that, in the fast-exchange limit, a plot of the line width of a ligand vs P_b should be linear with an intercept equal to the free ligand's line width. If the line width is principally controlled by exchange (intermediate exchange), a graph of line width vs $P_b(P_f)^2$ should be linear. We have plotted such graphs and analyzed them by linear regression, and although the resulting intercepts are slightly negative in both cases, indicating that the data at hand are insufficiently precise for a rigorous treatment, the squared linear correlation coefficient for line width vs P_b was 0.976, while that for line width vs $P_b(P_f)^2$ was 0.412. This indicates that the fast-exchange assumption is more consistent with the data than that of intermediate exchange.

In contrast to the rapid exchange of UMP with epimerase·NAD^+, the rate of exchange between free and bound UMP is dramatically reduced upon reduction of the enzyme with glucose. This is clearly illustrated in Figure 8, which shows the separation of the UMP resonance into distinct signals for the free and epimerase-bound populations. Assuming a dissociation constant of 12 μM (Wong & Frey, 1977), and comparing the line width of free UMP in the presence and absence of reductively inactivated epimerase, an off-rate constant for UMP dissociation from epimerase·NADH·UMP is calculated at 8 s^{-1} (Campbell et al., 1979).

Previous studies indicated that the binding of ANS to epimerase·NAD^+ was directly competitive with UMP, leading to the conclusion that the two substances bound to the same site on the enzyme. However, whereas the affinity of epimerase for UMP increased upon reduction, it was concluded that ANS had no detectable affinity for reduced epimerase, since it could not displace UMP from epimerase·NADH·UMP. These data were interpreted to mean that, although UMP and ANS bind to the same site, UMP binding drives an enzyme conformational change, in effect binding to an altered conformation consistent with that of reduced epimerase (E*·NADH), while the affinity of ANS is solely for that of the native conformation of epimerase·NAD^+ (Wong & Frey, 1978). The data presented here are fully consistent with these conclusions. Figure 6 demonstrates that the chief effect of ANS binding is to sharpen the native epimerase·NAD^+ signals, which might be expected of the binding of a ligand which merely stabilized the resting conformation. On the other hand, UMP binding causes substantial changes in the chemical shift values, whereby δ_2 (shifting 1.08 ppm) closely approaches, and δ_1 (shifting –0.92 ppm) actually achieves, the chemical shift value for epimerase·NADH·UMP of $\delta \approx -10.5$ ppm.

A comparison of the spectra of epimerase·NADH (Figure 9B) with that of epimerase·NADH·UMP (Figure 7C) indicates that the change in the environment of the backbone phosphates of the active site coenzyme is much smaller ($\sim$–0.3 ppm resonance center to resonance center) on UMP binding to epimerase·NADH than on its binding to epimerase·NAD^+. These data are consistent with a model in which at least a large part of the conformational change has been achieved upon

reduction, converting the enzyme from E·NAD^+ to E*·NADH, with UMP binding at this point mostly contributing to the stabilization of the E* conformation.

The conclusion that the rate of interconversion of the epimerase·NAD^+ and epimerase·NAD^+·UMP forms occurs at a rate of at least 490 s^{-1} is significant in light of the determination that the k_{cat} for the epimerization reaction is 500 s^{-1} (Wilson & Hogness, 1964), indicating that, if a nucleotide-driven conformational change is involved, it is probably occurring fast enough to support maximal activity. This result also suggests that, in the case of UMP-dependent reductive inactivation by glucose, which proceeds under NMR conditions with $k_{cat} = 0.05$ s^{-1}, the mechanism follows a rapid equilibrium random pathway as previously determined by kinetic studies (Kang et al., 1975).

In conclusion, the data presented here demonstrate that the ^{31}P NMR signals of epimerase·NAD^+ are sensitive to ligand binding and the oxidation state of the nicotinamide ring. The observed changes are consistent with the hypothesis that the binding of uridine nucleotides activates the active site NAD^+ toward reduction via an enzyme conformational change. The UMP- and reduction-dependent changes in the ^{31}P NMR signals for epimerase·NAD^+ suggest that the signals for bound NAD^+ sense the protein conformational changes, although other interpretations are not necessarily excluded.

ACKNOWLEDGMENTS

We express our gratitude to Ed Mooberry and W. Milo Westler of the NMR Facility at Madison for their technical and advisory assistance, to Angela Smallwood and Alan Bauer for skillfully purifying the epimerase enzyme, and to George Flentke for generously providing recrystallized ANS and for his contributions in pertinent discussions.

Registry No. UMP, 58-97-9; NAD, 53-84-9; 8-anilinonaphthalene-1-sulfonate, 20096-52-0; glucose, 50-99-7; borohydride, 16971-29-2; UDP-galactose 4-epimerase, 9032-89-7.

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