UDP-Galactose 4-Epimerase: NAD^+ Content and a Charge-Transfer Band Associated with the Substrate-Induced Conformational Transition†

Yijeng Liu,‡,§ Janeen L. Vanhooke,‡ and Perry A. Frey*,‡,§

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

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ABSTRACT: UDP-galactose 4-epimerase from *Escherichia coli* contains tightly bound NAD^+, which participates in catalyzing the interconversion of UDP-galactose and UDP-glucose through its redox properties. The purified enzyme is a dimer of identical subunits that consists of a mixture of catalytically active subunits designated **E**·NAD^+ and inactive, abortive complexes designated **E**·NADH·uridine nucleotide, in which the uridine nucleotide may be UDP-glucose, UDP-galactose, or UDP [Vanhooke, J. L., & Frey, P. A. (1994) *J. Biol. Chem. 269,* 31496−31404]. The abortive complexes are transformed into active **E**·NAD^+ by denaturation of the purified enzyme at 4 °C in 6 M guanidine hydrochloride buffered at pH 7.0 in the presence of 0.126 mM NAD^+ for 3 h, followed by dilution of guanidine hydrochloride to 0.18 M and of NAD^+ to 0.076 mM for 2 h. The renatured enzyme is fully active and contains negligible amounts of NADH and uridine nucleotides. The extinction coefficent of the epimerase at 280 nm is 1.81 ± 0.15 mL mg^{-1} cm^{-1} (ϵ_{280} = 137 ± 11 mM^{-1} cm^{-1}), as determined by quantitative amino acid analysis and spectrophotometric measurements. This value allows the value of the extinction coefficient for the reduced enzyme (**E**·NADH)to be calculated as ϵ_{344} = 5.7 mM^{-1} cm^{-1}. On the basis of the new value of ϵ_{280}, analytical measurements of the NAD^+ content of epimerase show that there are two molecules of NAD^+ per dimer, which confirms conclusions from X-ray crystallography and revises the earlier bioanalytical determinations. The ultraviolet/visible absorption spectrum of **E**·NAD^+ from denaturation−renaturation experiments reveals the presence of a broad absorption band extending from 300 nm to beyond 360 nm that cannot be attributed to NADH and appears to be a charge-transfer band. This band is partially bleached by UMP and almost totally abolished by UDP, indicating that the interactions leading to the charge-transfer band are altered by the uridine nucleotide-induced conformational change in this enzyme. This conformational change is associated with control of the chemical reactivity of NAD^+ in the reaction mechanism.

UDP-galactose 4-epimerase (EC 5.1.3.2), hereafter referred to as epimerase, catalyzes the interconversion of UDP-galactose[1] and UDP-glucose, the third reaction in the Leloir pathway for the transformation of galactose into glucose-1-P. In the first two steps, galactose is phosphorylated to galactose-1-P by the action of galactokinase and ATP, and hexose-1-P uridylyltransferase catalyzes the reaction of UDP-glucose with galactose-1-P to produce UDP-galactose and glucose-1-P. Epimerase uses NAD^+ as the coenzyme for its catalytic action. As purified from *Escherichia coli*, epimerase is a dimer of identical subunits that contains tightly bound NAD^+, and original reports indicated the presence of one molecule of NAD^+ per enzyme dimer (Wilson & Hogness, 1964, 1969). However, the X-ray crystal structure shows that each subunit contains electron density corresponding to a molecule of a pyridine nucleotide in each subunit (Bauer et al., 1992).

The original analyses for NAD^+ in UDP-galactose 4-epimerase were complicated by three factors, one being that the purified enzyme contains NADH associated with abortive complexes (Vanhooke & Frey, 1994). In this paper we show that two other factors introduced complications into the original measurements of NAD^+. The value of the extinction coefficient for the enzyme at 280 nm led to incorrectly high values for the enzyme concentration in bioanalytical experiments. We now also find that the enzyme exhibits a broad chromophore extending from 300 to >360 nm. This appears to be a charge-transfer band, and it interferes with simple measurements of enzyme-bound NADH. Taking these factors into account, we now show that homogeneous, maximally active enzyme contains approximately two molecules of NAD^+ per dimer. The charge-transfer band is bleached by the presence of UDP or UMP, which shows that it is related to the uridine nucleotide-induced conformational change.

EXPERIMENTAL PROCEDURES

Materials. NAD^+, UDP-glucose dehydrogenase, UDP-galactose, NAD^+, and MOPS were purchased from Sigma.

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‡ Institute for Enzyme Research, The Graduate School.

§ Department of Biochemistry, College of Agricultural and Life Sciences.

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[1] Abbreviations: NAD^+, nicotinamide adenine dinucleotide; NADH, reduced nicotinamide adenine dinucleotide; UDP, uridine 5′-diphosphate; UMP, uridine 5′-phosphate; UDP-glucose, uridine 5′-diphosphate glucose; UDP-galactose, uridine 5′-diphosphate galactose; ATP, adenosine 5′-triphosphate; ADP-ribose, adenosine 5′-diphosphoribose; TCA, trichloroacetic acid; KP_i, potassium phosphate; MOPS, 3-(*N*-morpholino)propanesulfonic acid; EDTA, ethylenediaminetetraacetate; HPLC, high-performance liquid chromatography; PAGE, polyacrylamide gel electrophoresis.

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Prior to being used in renaturation experiments, NAD^+ was purified by chromatography through DEAE Sephadex A-25 to decrease the ADP-ribose content to <0.05%, as determined by HPLC. Guanidine hydrochloride and urea were purchased from GIBCO BRL. Tetramethylammonium hydrogensulfate and tetrabutylammonium hydroxide for ion-pairing HPLC analysis were purchased from Fluka. HPLC grade organic solvents were used without further purification. Standard amino acids were purchased from Sigma and PIERCE. [*adenine*-2,8-^{3}H]NAD^+ was purchased from New England Nuclear (DU PONT) and purified as follows. [*adenine*-2,8-^{3}H]NAD^+ was diluted with chromatographically purified NAD^+ (see below) and desalted by Bio-Gel P2 gel filtration chromatography. The concentration of the purified NAD^+ was determined spectrophotometrically at 260 nm by use of the extinction coefficient 18 mM^{-1} cm^{-1}. Phenylisothiocyanate was purchased from PIERCE. UDP and UMP were purchased from Sigma and purified by DEAE Sephadex A-25 chromatography.

Enzyme Purification and Assay. *E. coli* cells of strain BL21(DE3) transformed with pLysS and the overexpression vector pTZ18R containing the *E. coli* epimerase gene were grown in 2× YT medium and harvested after 8 h at 37 °C. Epimerase was purified as described by Bauer et al. (1991) with the exception that Q-Sepharose Fast Flow ion exchanger was substituted for DEAE-Sephadex in the final chromatography step, as described earlier (Vanhooke & Frey, 1994). The elution gradient used was from 20 to 300 mM KP_i at pH 8.5. Specific activities of fractions eluted from this column ranged from 117 to 345 units mg^{-1} after Q-Sepharose chromatography (Vanhooke & Frey, 1994). The homogeneity of the purified epimerase was estimated by gel electrophoresis to be more than 95% prior to denaturation and much more than 95% after renaturation.

Epimerase activity was assayed by the method of Wilson and Hogness (1964) in solutions containing 0.125 M potassium bicinate buffer at pH 8.5, 1.25 mM NAD^+, 0.05 mM UDP-galactose, and 0.04 unit of UDP-glucose dehydrogenase at 27 °C. Initial rates of NADH formation were measured at 340 nm. Epimerase concentrations were originally calculated by use of 1.05 mL mg^{-1} as the extinction coefficient at 280 nm. The extinction coefficient has been redetermined in the present work and was found to be 1.81 ± 0.15 mM^{-1} cm^{-1} (see below and Results). Specific activities in this paper have been calculated by use of this new value. The original activity unit was defined as the amount of enzyme required to produce 1 μmol of NADH per hour under the standard conditions. Given the change in extinction coefficient, we have taken the opportunity to redefine the activity unit as the international unit, the amount of enzyme required to produce 1 μmol of NADH per minute under the standard conditions. All specific activities in this paper have been calculated by use of the new extinction coefficient and activity unit. Multiplication of all previously reported specific activities by 0.0287 will correct them to the new extinction coefficient and activity unit.

Denaturation and Renaturation of Enzyme. The epimerase used in denaturation−renaturation experiments was purified as described (Vanhooke & Frey, 1994). Final column fractions were pooled to give a specific activity between 175 and 240 unit mg^{-1}. The denaturing buffer contained 50 mM MOPS (pH 7.0), 1 mM EDTA, 6 M guanidine hydrochloride, and 0.126 mM NAD^+. Epimerase was added to the denaturing solution to a final concentration of 0.58 mg mL^{-1} and set aside for 3 h at 4 °C. Renaturation was carried out by 33-fold dilution with 50 mM MOPS containing 0.076 mM purified NAD^+ at pH 7.0 and 4 °C. After 2 h, the solution was diluted 5-fold with deionized distilled water, and Q-Sepharose Fast Flow anion exchange resin (about 80 mL) was added to adsorb the enzyme. The resin was filtered through a fritted glass funnel, packed into a glass tube, and eluted with 0.3 M KP_i at pH 8.5. The eluted protein was diluted 10-fold with water and rechromatographed through a Q-Sepharose column as in the original purification. Renatured epimerase was concentrated by Amicon Centriprep or Centricon and filtered through either a Cameon A25 or a Gilson Z-spin filter.

UV and Fluorescence Spectra. UV/vis absorbance spectra were recorded in a Hewlett Packard 8452A diode array spectrophotometer. Fluorescence spectra were obtained in a Perkin Elmer MPF-3 fluorescence spectrophotometer.

Determination of Nucleotides Associated with Epimerase. For quantitative measurement of enzyme-bound uridine nucleotides, epimerase was denatured by addition of 20% TCA, and the precipitate was removed by centrifugation. TCA was extracted from the supernatant fluid with ether, and the aqueous layer was filtered through a Gilson Z-spin filter and lyophilized. The dried samples were dissolved in double-distilled water for HPLC analysis in a 3.9 mm × 15 cm stainless steel Novapak C18 reverse phase column eluted at a flow rate of 0.8 mL min^{-1}. The HPLC gradient was controlled by a Waters automated gradient controller, and all the nucleotides were detected spectrophotometrically at 260 nm. ADP-ribose, NAD^+, and NADH were separated by tetramethylammonium paired ion HPLC using a gradient formed from solvents A and B. Solvent A contained 5 mM tetramethylammonium hydrogensulfate and 10 mM KP_i at pH 5.8, and solvent B was a 1:1 mixture of methanol and water. Solvent B was increased by the gradient controller to 5% in 3 min with a convex gradient and to 20% in 5 min with a concave gradient. UDP was separated from other nucleotides by tetrabutylammonium paired ion chromatography. Solvent A was 2.5 mM tetrabutylammonium formate at pH 7.1 in methanol:H_2O (1:9), and solvent B was tetrabutylammonium formate at pH 7.1 in methanol:H_2O (6:4). The gradient started with 0% solvent B, reaching 60% in 8 min with a concave gradient and 70% in 19 min with linear gradient. UDP-galactose and UDP-glucose were identified by enzymatic assays using UDP-glucose dehydrogenase and UDP-galactose 4-epimerase in 0.125 M potassium bicinate at pH 8.5 and 27 °C. UDP was identified enzymatically using pyruvate kinase and L-lactate dehydrogenase.

Impact of Uridine Nucleotides on Absorbance Spectra of Epimerase: Studies of Difference Spectra. Difference spectra were acquired in a Hewlett Packard model 8452A diode array spectrophotometer as described below. Epimerase was placed in one chamber of a split cell and a uridine nucleotide solution in the other, both in the same buffer. Spectra were recorded before and after mixing the solutions in the two chambers. A difference spectrum was computed from the two spectra. Epimerase used for difference spectroscopy had been subjected to the denaturation and renaturation activation and exhibited a specific activity of at least 350 units mg^{-1}. The concentration of epimerase was

5.74 μM (subunits), that of UDP varied from 0.024 to 4.89 mM, and that of UMP varied from 0.32 to 12.8 mM.

Measurement of Extinction Coefficient of Epimerase. Samples of epimerase were purified with or without the denaturation−renaturation and exhibited specific activities of 260, 320, and 350 units mg^{-1}. The molar concentrations of epimerase samples used for the calculation of extinction coefficient at 280 nm were determined by quantitative amino acid analysis. The absorbance at 280 nm of an epimerase sample was measured, and 100 μL of the solution was transferred to a vacuum hydrolysis tube (Pierce) together with 100 μL of constant boiling HCl. The tube was sealed under vacuum (0.05 Torr) and placed at 110 °C for 25 h. The hydrolysis mixture was transferred to a microcentrifuge tube, rinsed several times with double-distilled water, and dried under vacuum. The amino acids were transformed into their phenylthiocarbamyl derivatives according to Heinrikson and Meredith (1984) and dissolved in 100 μL of 0.05 M ammonium acetate at pH 6.8. Standard amino acids were derivatized by the same procedure. The phenylthiocarbamyl derivatives of aspartate, glutamate, serine, glycine, proline, arginine, methionine, phenylalanine, and lysine were separated by HPLC over a Novapak C18 reverse phase column (Waters). Solvent A contained 0.05 M ammonium acetate at pH 6.8, and solvent B consisted of 44% acetonitrile, 10% methanol, and 46% H_2O containing 0.1M ammonium acetate at pH 6.8. The flow rate was 1 mL min^{-1}, and solvent B was increased in a linear gradient from 0% to 15% within 20 min, to 50% solvent B in 65 min, and to 100% in 75 min. In addition to running standard amino acids to calibrate the elution profile, two control experiments were performed to correct the profile for background, one by substituting 100 μL of phosphate buffer in place of the enzyme sample and another in which no sample was included in the analysis. The amino acid content of each sample was used to calculate its concentration based on the amino acid composition of epimerase (Lemaire & Müller-Hill, 1986; Bauer et al., 1992), and this was used with the absorbance at 280 nm to calculate the extinction coefficient.

NAD$^+$ Content of Epimerase. The ratio of coenzyme and enzyme was determined radiochemically by exchanging [*adenine*-2,8-^{3}H]NAD$^+$ into the epimerase. Exchange took place in solutions containing 2.9 mg of epimerase mL^{-1}, 20 mM KP_i at pH 7.0, 2.65 M urea, 0.63 mM [*adenine*-2,8-^{3}H]NAD$^+$ at 25 °C for 2−6 h. The solution was dialyzed against 20 mM KP_i at pH 7.0 overnight at 4 °C. Epimerase was then rechromatographed on a Q-Sepharose column with stepwise elution by 62 and 300 mM KP_i at pH 7.0. Epimerase fractions were pooled and partially desalted by repeated ultrafiltration and dilution, first with water and finally with 20 mM KP_i at pH 7.0. The [*adenine*-2,8-^{3}H]-NAD$^+$ associated with epimerase was measured by liquid scintillation (Packard Tri-Carb 4640).

RESULTS AND DISCUSSION

UDP-galactose 4-epimerase purified from *E. coli* exhibits variable activity. The highest specific activity reported to date is 400 units mg^{-1} for the most active chromatographic fractions (Wilson & Hogness, 1964). However, the enzyme purified from an operator constitutive strain typically exhibits a specific activity of 280−320 units mg^{-1} (Wong et al., 1978), and the specific activity of enzyme purified from bacteria carrying the epimerase gene on an overexpression vector is typically 115−230 units mg^{-1} (Vanhooke & Frey, 1994). Less than optimal activity is caused by the presence of abortive complexes that contain NADH and tightly bound UDP, UDP-glucose, or UDP-galactose (Vanhooke & Frey, 1994).

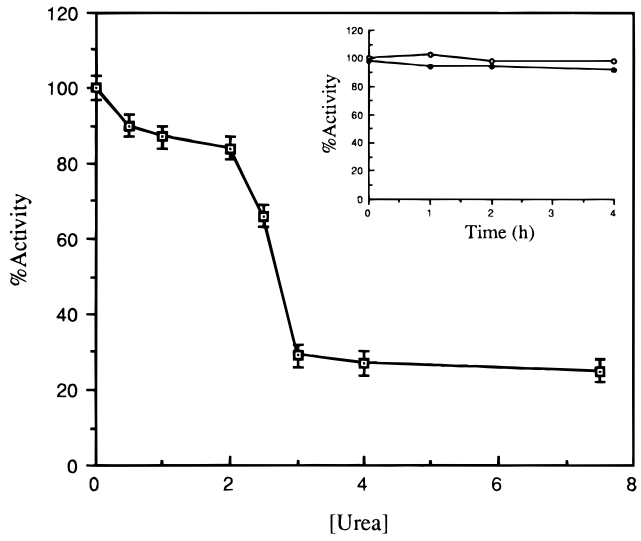

FIGURE 1: Effects of urea and NAD$^+$ on the activity of UDP-galactose 4-epimerase. Solutions of 1.00 mL volume containing 3.3 μM (0.26 mg, 200 units mg^{-1}) epimerase and 0−7.42 M urea in buffer (20 mM KP_i containing 1 mM tetrasodium EDTA at pH 7.0) were incubated at 25 °C for 1 h. A 20 μL aliquot of each solution was removed and diluted into 980 μL of the same buffer at 0 °C. Aliquots of the diluted samples were assayed immediately for epimerase activity. Inset: Two solutions containing 3.3 μM (0.26 mg, 168 units mg^{-1}) epimerase in the same buffer used above were supplemented with 113 μM NAD$^+$ and either 0 or 2.50 M urea and placed at 25 °C for 4 h. Aliquots of 20 μL were removed at 0, 1, 2, and 4 h, diluted into 980 μL of cold buffer, and immediately assayed for epimerase activity.

The essential coenzyme of epimerase is NAD$^+$, which is tightly bound to the purified enzyme and does not exchange spontaneously with free NAD$^+$. The epimerase was originally reported to contain 1 mole of NAD$^+$ per mol of dimeric enzyme (Wilson & Hogness, 1964), and this enzyme was for many years considered to represent an extreme case of negative cooperativity in binding NAD$^+$. Recently, the crystal structure of epimerase revealed the presence of two NAD$^+$ binding domains as well as electron density corresponding to a nicotinamide dinucleotide bound to each subunit of the dimer (Bauer et al., 1992). The finding of two nicotinamide coenzymes per dimer called into question the analysis of epimerase for NAD$^+$. The presence of abortive complexes in most preparations complicates the measurement of NAD$^+$ in epimerase; however, the original analytical data of Wilson and Hogness on the most highly purified fractions has repeatedly been reproduced in this laboratory.

Effect of Free NAD$^+$ on Denaturation of Epimerase. In order to develop conditions for replacing NADH in abortive complexes such as **E**·NADH·UDP-glucose with NAD$^+$ or isotopically labeled NAD$^+$, we evaluated the effect of urea on the activity of epimerase in the presence and absence of free NAD$^+$. The data in Figure 1 show that incubation of epimerase with urea for 1 h decreases its activity by about 35% at 2.5 M and by almost 80% at 3 M urea. The inset shows that free NAD$^+$ protects epimerase from the effects

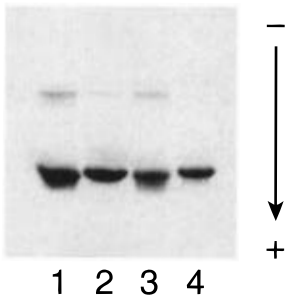

FIGURE 2: Effect of denaturation−renaturation in the presence of NAD^+ on the homogeneity of epimerase. Samples of epimerase before (lanes 1 and 3) and after (lanes 2 and 4) denaturation−renaturation were subjected to PAGE. Loading was 4.3 μg in lanes 1 and 2 and 2.2 μg in lanes 2 and 4. Note the sharpness of the major, epimerase band after denaturation−renaturation and the decrease in contaminating bands relative to the original sample. The corresponding diffuse band in the original sample represents homogeneous protein consisting of active epimerase (**E**·NAD^+) contaminated with inactive abortive complexes (**E**·NADH·uridine nucleotide).

of 2.5 M urea. The simplest rationale for protection by NAD^+ is that urea induces partial reversible denaturation of epimerase to one or more states designated $\mathbf{E}^\dagger$·NAD^+ that can release NAD^+ (eq 1a,b). Denaturation can become

$$\mathbf{E}\cdot NAD^+ \rightleftharpoons \mathbf{E}^\dagger\cdot NAD^+ \quad (1a)$$

$$\mathbf{E}^\dagger\cdot NAD^+ \rightleftharpoons \mathbf{E}^\dagger + NAD^+ \quad (1b)$$

irreversible when the enzyme is very dilute, presumably because of side reactions by $\mathbf{E}^\dagger$. The presence of free NAD^+ maintains NAD^+ in its binding site and prevents irreversible denaturation. The mechanism of eq 1a,b also accounts for the fact that low concentrations of urea facilitate the exchange of radiochemically labeled NAD^+ into epimerase, as is shown in a later section.

Activation of Abortive Complexes by Denaturation and Renaturation. Inasmuch as free NAD^+ protects epimerase from irreversible denaturation by urea, it may facilitate renaturation of the extensively unfolded enzyme. In fact, NAD^+ is known to facilitate the refolding of epimerase from yeast (Bhattacharyya, 1993). Partial denaturation in the presence of free NAD^+ offers the potential for activating preparations of epimerase that exhibit low activity owing to the presence of abortive complexes such as **E**·NADH·UDP-glucose. Activation would result from denaturant-induced release of UDP-glucose and NADH and replacement by NAD^+ followed by dilution of the denaturant and reconstitution of the native structure. We have found that denaturation of epimerase in 6 M guanidine hydrochloride containing 0.126 mM free NAD^+, followed by dilution with water to 0.18 M guanidine hydrochloride and 0.076 mM NAD^+ dramatically activates epimerase and removes nearly all of the NADH and uridine nucleotides. In six experiments, samples of epimerase displaying specific activities of 175 or 240 units mg^{-1} were activated to 368 ± 12 units mg^{-1} after rechromatography over Q-Sepharose. The method is described in the Experimental Procedures and allows activation of 30−100 mg of low-activity epimerase with a recovered yield of 50%−75%.

In addition to being highly active, the denatured−renatured epimerase is more homogeneous than the enzyme purified by the usual procedure, as shown by the native polyacrylamide gels in Figure 2. Note that faint auxiliary bands in the original sample are absent in the renatured sample, presumably because NAD^+ facilitates the renaturation of epimerase but not of impurities. The diffuse bands for purified epimerase in lanes 1 and 3 of Figure 3 are sharpened following denaturation−renaturation (lanes 2 and 4). This may be due to the presence of the abortive complexes (**E**·NADH·uridine nucleotide) in the purified enzyme, which are removed by denaturation−renaturation. The renatured enzyme is essentially free of NADH, as is shown by the UV and fluorescence spectra in Figure 3. Denaturation−renaturation also decreases the uridine nucleotide content of low-activity epimerase. In one experiment, the specific activity was increased from 239 to 350 units mg^{-1}, and the uridine nucleotide content was decreased from 0.35 per subunit to less than 0.11 per subunit after renaturation, as determined by enzymatic and HPLC analyses.

NAD^+ Content and Extinction Coefficient of UDP-Galactose 4-Epimerase. Conventional enzymatic assays for NAD^+ released from the most active fractions of epimerase by acidification indicate 1 NAD^+ per dimer of identical subunits (Wilson & Hogness, 1964). In all such experiments to date the epimerase concentration has been assayed either by measurement of absorbance at 280 nm, using an extinction coefficient of 1.05 mL mg^{-1} cm^{-1} (ϵ_{280} = 78.0 mM^{-1} cm^{-1} for dimeric epimerase) for the most active sample, or by

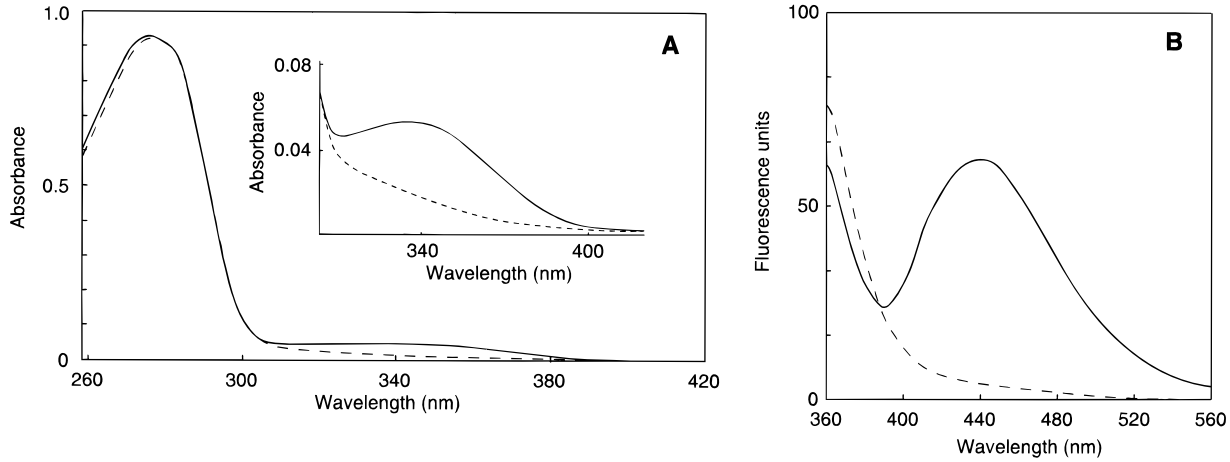

FIGURE 3: UV and fluorescence spectra of epimerase before and after denaturation−renaturation. A. Ultraviolet/visible absorption spectra of epimerase before (—) and after (− −) denaturation−renaturation. Inset is an expansion of the region from 300 to 400 nm. B. Fluorescence emission spectra of epimerase (0.45 mg/mL) before (—) and after (− −) denaturation−renaturation. Excitation wavelength is 280 nm.

measuring the increase in absorbance at 345 nm upon UMP-dependent reductive inactivation by glucose (Kalckar et al., 1970; Kang et al., 1975), assuming the value of 6.22 mM^{-1} cm^{-1} as ϵ_{340} for NADH. The two methods have given comparable results. In recent experiments the apparent difference extinction coefficient ($\Delta\epsilon_{344}$) in the reduction of **E**·NAD$^+$ to **E**·NADH was reported to be 3.3 mM^{-1} cm^{-1} (Vanhooke & Frey, 1994). Calculation of $\Delta\epsilon_{344}$ depended upon the use of 1.05 mL mg^{-1} cm^{-1} as the extinction coefficient at 280 nm. On the basis of the latter value for $\Delta\epsilon_{344}$, the two methods give very different enzyme concentrations.

Estimation of the ϵ_{280} nm for epimerase on the basis of its amino acid and NAD$^+$ content indicated that it should be substantially higher than 1.05 mL mg^{-1} cm^{-1}. Therefore, we measured the ϵ_{280} in six samples of epimerase by quantitative analysis for nine amino acids and measurement of absorbance at 280 nm. Three different preparations of enzyme displaying specific activities of 260, 320, and 350 units mg^{-1} gave results that were indistinguishable within experimental error. On the basis of these analyses, the extinction coefficient at 280 is 1.81 ± 0.15 mL mg^{-1} cm^{-1} (ϵ_{280} = 137 ± 11 mM^{-1} cm^{-1}). The new value has been used for all calculations of epimerase concentrations in this paper, and we now use it to revise the value of $\Delta\epsilon_{345}$, originally reported by Vanhooke and Frey (1994), from 3.3 to 5.7 mM^{-1} cm^{-1}.

In the measurement of NAD$^+$ content, epimerase was placed at 2.9 mg mL^{-1} in 2.65 M urea at pH 7.0 with 0.63 mM [*adenine*-2,8-^{3}H]NAD$^+$ at 25 °C for 2, 4, or 6 h. After dialysis and rechromatography, its molar content of [*adenine*-2,8-^{3}H]NAD$^+$ was determined by radiochemical analysis and measurement of absorbance at 280 nm. The data revealed the presence of 1.71 ± 0.18, 2.08 ± 0.23, and 1.79 ± 0.21 mol of NAD$^+$ per mole of dimeric epimerase in the three experiments. HPLC analysis for NAD$^+$ released from epimerase by TCA precipitation of the protein gave 1.96, 2.02, and 1.91 mol of NAD$^+$ per mole of enzyme in three determinations. Recalculation of the NAD$^+$ content reported by Wilson and Hogness (1964) using the new value for the extinction coefficient of the enzyme gave 1.8 NAD$^+$ per dimer of epimerase.

Bleaching of the Racker Band by Uridine Nucleotides. The UV spectrum of denatured−renatured epimerase in Figure 3 shows the presence of a shoulder extending from 300 nm to beyond 360 nm, which is most likely a charge-transfer band. Other NAD$^+$ dependent dehydrogenases display analogous spectral features, which have become known as "Racker bands" (Rizzo et al., 1987). Because they generally appear upon mixing a dehydrogenase with NAD$^+$, charge-transfer bands are usually ascribed to noncovalent interactions of the nicotinamide ring in NAD$^+$ with amino acid side chains. However, they could just as well arise from interactions of amino acid side chains that are induced by conformational changes coupled to the binding of NAD$^+$. The charge-transfer interactions of NAD$^+$-dependent dehydrogenases have not been structurally characterized.

The difference spectra in Figure 4 show that the intensity of the charge-transfer band associated with epimerase is decreased by the presence of UDP or UMP. Bleaching by UDP is greater than by UMP. Epimerase undergoes a uridine nucleotide-induced conformational change that leads to an increase in the reactivity of NAD$^+$ toward reducing agents.

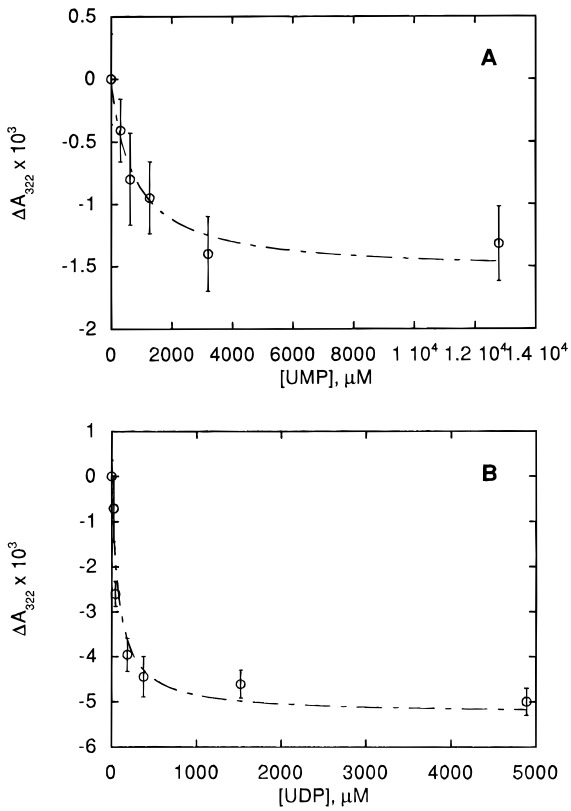

FIGURE 4: Bleaching of the charge-transfer band in epimerase by UMP or UDP. The absorbance of epimerase (5.74 μM subunits) at 322 nm was measured as a function of the concentration of UMP (A) or UDP (B) in 25 mM KP_i (pH 7.0) and ionic strength 0.2 adjusted by addition of KCl at 27 °C. The dashed curves are calculated values for ΔA_{322} based on the known values of K_d and the maximum ΔA_{322} observed at saturating UMP or UDP. The values of K_d were determined kinetically in UMP- or UDP-activated reductions of **E**·NAD$^+$ to **E**·NADH·uridine nucleotide by glucose to be 783 μM for UMP and 84 μM for UDP.

Evidence for the conformational transition includes the observation of UMP-dependent reductive inactivation (Kalckar et al., 1970; Kang et al., 1975), UMP-dependent perturbation of the ^{31}P NMR spectrum of **E**·NAD$^+$ (Konopka et al., 1989), UDP-dependent perturbations of the ^{13}C and ^{15}N NMR chemical shifts in the nicotinamide ring of **E**·NAD$^+$ (Burke & Frey, 1993), and a difference in secondary structure between **E**·NAD$^+$ and **E**·NADH·UMP as determined by circular dichroism (Wong et al., 1978). The bleaching of the charge-transfer band by UDP or UMP is an additional spectroscopic signal of the uridine nucleotide-induced conformational transition.

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