Identification of Lysine 153 as a Functionally Important Residue in UDP-Galactose 4-Epimerase from *Escherichia coli*†

Barbara A. Swanson and Perry A. Frey*

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

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ABSTRACT: The role of lysine 153 in the action of UDP-galactose 4-epimerase from *Escherichia coli* has been investigated by site specific mutagenesis and kinetic and spectrophotometric analysis of the mutant enzymes. The crystal structure of UDP-galactose 4-epimerase shows that the binding of NAD^+ to the coenzyme site includes the hydrogen bonded interaction of the ϵ-ammonium group of lysine 153 with the 2′- and 3′-hydroxyl groups of the nicotinamide riboside. Mutation of this residue to methionine or alanine decreases the catalytic activity of the enzyme by a factor of more than 10^3. The NAD^+ associated with the wild type enzyme is subject to UMP-dependent reduction by sugars such as glucose and arabinose, but the mutant proteins K153M and K153A are not reduced by sugars in the presence or absence of UMP. NAD^+ associated with the wild type enzyme is also subject to UMP-dependent reduction by sodium cyanoborohydride. However, although the mutant proteins bind UMP very well, the rate at which NAD^+ associated with them is reduced by sodium cyanoborohydride is almost insensitive to the presence of UMP. The purified wild type enzyme contains significant amounts of NADH bound to the coenzyme site; however, the purified mutants K153M and K153A contain very little NADH. We conclude that lysine 153 plays an important role in increasing the chemical reactivity of enzyme-bound NAD^+ in the uridine nucleotide-dependent conformational change associated with reductive inactivation and the catalytic activity of UDP-galactose 4-epimerase.

UDP-galactose 4-epimerase (EC 5.1.3.2), hereafter referred to as epimerase, catalyzes the interconversion of UDP-galactose and UDP-glucose.[1] It is found in most organisms, including bacteria, plants, and animals, and is an enzyme in the Leloir pathway for the metabolism of galactose. The epimerase isolated from *E. coli* is a dimer of identical subunits with a molecular weight of 79 000 (Wilson & Hogness, 1964, 1969). Until recently, it was believed that the epimerase contained one NAD^+ per dimer (Wilson & Hogness, 1964). However, the crystal structure of the enzyme shows that there is one NAD^+ or related nicotinamide dinucleotide tightly bound per monomer (Bauer *et al.*, 1992).

In the mechanism by which this epimerase interconverts UDP-galactose and UDP-glucose, the substrate is bound at the active site and oxidized by NAD^+ to the intermediate UDP-4-ketoglucose. This is accompanied by the transfer of the pyranosyl 4-hydride to the *si*-face (B-face) of NAD^+ to form NADH (Maitra & Ankel, 1971; Nelsestuen & Kirkwood, 1971; Wee & Frey, 1973; Adair *et al.*, 1973; Ketley & Schellenberg, 1973). It has been proposed that the 4-keto-pyranosyl moiety of the intermediate rotates around the bond linking the glycosyl-O-1 and the β-phosphorus atom of the nucleotide, allowing the hydride to be returned to the opposite face of the sugar to form the product and NAD^+ (Kang *et al.*, 1975). The product is released in the final step.

The binding of substrate or uridine nucleotide to the active site of the epimerase induces a protein conformational change that causes the reactivity of the NAD^+ in the coenzyme binding site to increase toward reducing agents (Bertland & Kalckar, 1968; Kang *et al.*, 1975). In the preceding article it was proposed that this activation results from the destabilization of the nicotinamide ring and that destabilization is brought about by electrostatic repulsion between the positively charged nicotinamide ring and a positively charged amino acid residue (Burke and Frey, 1993). The only available crystal structure of the epimerase is that of the activated conformation, in which the competitive inhibitor P^1-uridyl-P^2-phenyl diphosphate is bound in the active site (Bauer *et al.*, 1992). In this structure, there are two positively charged residues in the vicinity of the nicotinamide ring, lysine 84 and lysine 153 (Bauer *et al.*, 1992). The ϵ-ammonium group of lysine 153 is 5.3 Å from the nicotinamide-N-1 and is hydrogen bonded to the nicotinamide ribosyl 2′- and 3′-hydroxyl groups. This lysine residue is conserved in all of the known epimerase sequences, ranging from bacteria and yeast (Poolman *et al.*, 1990) to rat (Zeschnigk *et al.*, 1990). The ϵ-ammonium group of lysine 84 is hydrogen bonded to one of the phosphoryl groups of NAD^+ and is 7 Å from nicotinamide-N-1. Inasmuch as several epimerase sequences have a serine at this position, this residue is not conserved.

It is proposed in the preceding paper in this issue (Burke & Frey, 1993) that the positively charged ϵ-ammonium group of lysine 153 may perturb the electron distribution in the nicotinamide ring of NAD^+ through charge repulsion upon substrate binding. The pH rate profile for reductive inactivation of the epimerase·NAD^+ by P^1-5′-uridine-P^2-glucose-6-yl pyrophosphate, which irreversibly reduces the NAD^+ to NADH (Arabshahi *et al.*, 1988), is consistent with a lysine-ϵ-ammonium group playing a role in the reaction. To test whether lysine 153 participates in the UMP-dependent

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[1] Abbreviations: UDP-glucose, uridine-5′-diphosphate glucose; UDP-galactose, uridine-5′-diphosphate galactose; UMP, uridine-5′-phosphate; NAD^+, nicotinamide adenine dinucleotide; NADH, reduced NAD^+; PAGE, polyacrylamide gel electrophoresis; ANS, 8-anilinonaphthalene-1-sulfonate; EDTA, ethylenediaminetetraacetic acid.

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reduction of NAD^+, this lysine has been mutated using site-specific mutagenesis to two uncharged residues, alanine and methionine. The methionine residue is nearly isosteric with lysine, and neither residue should disrupt the α-helix in which residue 153 is found. The enzymatic activities of the mutants K153A and K153M are severely reduced compared to that of the wild type enzyme. The rates of reduction of NAD^+ associated with the mutant and wild type enzymes by sodium cyanoborohydride in the presence and absence of UMP are also compared.

EXPERIMENTAL PROCEDURES

Materials. *E. coli* strain DH5α was obtained from Gibco BRL Life Technologies (Gaithersburg, MD), and BL21-(DE3)pLysS was obtained from Novagen, Inc. (Madison, WI). Plasmid pI24 was a generous gift from Dr. Teresa Field, and pTZ18R was purchased from U.S. Biochemicals. Restriction enzymes were purchased from New England Biolabs and used according to the manufacturers specifications. Bacto tryptone and bacto yeast extract were obtained from Difco Labs (Detroit, MI). Ampicillin, UDP-glucose dehydrogenase, UMP, UDP-galactose, UDP-glucose, and Q-Sepharose were purchased from Sigma (St. Louis, MO). Chloramphenicol was obtained from Boehringer-Mannheim (Indianapolis, IN). A kit for site-directed mutagenesis including *E. coli* CJ236 dut$^-$ ung$^-$, helper phage M13K07, and necessary enzymes was purchased from Bio-Rad (Hercules, CA) and applied according to the specifications to the preparation of specific epimerase mutants. Plasmid DNA was purified by the use of tip-20 columns from Qiagen (Chatsworth, CA) and analyzed on a Fisher agarose gel electrophoresis box. DNA oligomer primers were either synthesized on a Biosearch 8600 DNA synthesizer or in the DNA synthesis facility of the Department of Biochemistry, University of Wisconsin—Madison. Protein gels were run on a Pharmacia PhastSystem (Piscataway, NJ). UV–vis spectra were obtained on either a Hewlett-Packard 8452A diode-array spectrophotometer or a Cary 118 spectrophotometer. Fluorescence spectra were obtained by use of a Perkin-Elmer MPF-3 spectrofluorimeter. Streptomycin sulfate, bovine serum albumin, mono and dibasic potassium phosphate, and perchloric acid (65%) were from Mallinckrodt; sodium cyanoborohydride (95%) was obtained from Aldrich and used without further purification.

Liquid 2xYT medium (Sambrook *et al.*, 1989) containing 100 mg/mL ampicillin and 100 mg/mL chloramphenicol and LB plates containing 100 mg/mL ampicillin were used unless otherwise specified. All enzymatic reactions were carried out in 100 mM potassium phosphate, 1 mM EDTA, pH 7.0 at 27 °C except for activity assays, which were performed in the standard assay buffer consisting of 125 mM potassium bicinate, pH 8.5.

Construction of the Epimerase Expression Vector pT7E2. An expression vector containing the gene for UDP-galactose 4-epimerase was constructed in a manner similar to the construction of the pT7E epimerase expression vector (Bauer *et al.*, 1991). The 2100 bp region containing the gal promoter and the epimerase gene was excised from pI24 (Majumdar & Adhya, 1987) by the actions of EcoRI and PvuII and purified on a low melting agarose gel. The expression vector pTZ18R containing the T7 promoter was digested with EcoRI and HincII and the linearized plasmid purified on low melting agarose. The plasmid and gene were ligated in a 1:2 ratio and transformed into DH5α cells. Plasmids containing the desired insert were identified by their mobility through an agarose gel and by restriction analysis. The pT7E2 plasmid was ultimately transformed into BL21(DE3)pLysS cells, which do not contain the galactose operon in their genome.

Mutagenesis of the Epimerase Gene. The desired epimerase mutants were produced by use of the Kunkel method of mutagenesis (Kunkel, 1985). First, single stranded plasmid was prepared by transforming the pT7E2 plasmid into CJ236 dut$^-$ ung$^-$ competent cells and infecting with helper phage M13K07 following the protocol from Bio-Rad. The single stranded plasmid was purified from the supernatant by the procedure from Qiagen. The purity and concentration of purified plasmid was determined by agarose gel electrophoresis.

The mutagenic second strand was synthesized using complementary phosphorylated 24-mer primers containing the codon for the desired mutation. The alanine codon GCG was employed because of its high frequency of appearance in the epimerase gene; ATG was employed for methionine. Single stranded pT7E2 (0.04 or 0.14 pmol) and a 100-fold excess of phosphorylated primer were annealed and then incubated with T7 polymerase, T4 DNA ligase, and Bio-Rad synthesis buffer. Competent DH5α cells were transformed with aliquots of the reaction mixture and plasmids from 6–8 successfully transformed colonies purified by use of Qiagen tip-20 columns. The K153A mutants were identified by sequencing the region of mutation using the dideoxy chain termination method of Sanger (Sanger *et al.*, 1977). The K153M colonies were screened for mutation by restriction digestion with SphI, which cut in the introduced methionine codon. Plasmids containing the desired alanine or methionine mutation were then transformed into BL21(DE3)pLysS cells, colony-purified on LB plates, and the plasmids sequenced in the region of the mutation.

Expression of UDP-Galactose 4-Epimerase. Wild type and mutant epimerases were monitored for expression by inoculating flasks containing 100 or 200 mL of 2xYT medium and 100 mg/mL ampicillin with 1 mL of overnight stock grown from single colonies. Typically four separate cultures were grown for each mutant. After growing 8–9 h at 37 °C with ampicillin (100 mg/mL) added every 3 h, the cells were harvested and frozen in liquid nitrogen. Crude epimerase was prepared by following the procedure of Bauer *et al.* (1991) through the dialysis step following ammonium sulfate precipitation. The level of epimerase expression was determined by measuring the total amount of protein present, the total epimerase activity by enzymatic assays, and by native PAGE (8–25%). For each mutant and wild type epimerase, the colony stock which produced the greatest amount of epimerase was selected to initiate large scale growth.

Purification of Wild Type and Mutant Epimerases. The wild type, K153A, and K153M epimerases were all purified from 6 L growths according to the procedure of Bauer *et al.* (1991), except that a 4.5 × 30 cm Q-Sepharose column was substituted for the DEAE column previously described. The Q-Sepharose column was operated at a flow rate of 3 mL/min and eluted by use of a linear gradient of potassium phosphate at pH 8.5 (3 L) increasing in concentration from 20 to 300 mM. All three enzymes exhibited similar chromatographic properties and appeared to be about 95% homogeneous as estimated by native PAGE.

Epimerase Activity Assay. Epimerase activity was determined by use of the coupled assay system of Wilson and Hogness (1964). The assay reactions consisted of 125 mM potassium bicinate buffer at pH 8.5, UDP-galactose (250 μM), UDP-glucose dehydrogenase (0.04 unit/mL), NAD^+ (1.25 mM), and epimerase. Initial rates were determined by monitoring the increase in absorbance at 340 nm for the first

few minutes of the reaction. The concentration of UDP-galactose 4-epimerase was determined by measuring the absorbance at 280 nm and dividing by 1.05 mL/mg (Wilson & Hogness, 1964).

Rate Measurements. The steady-state kinetic parameters for wild type, K153A, and K153M epimerases with UDP-galactose as the substrate were evaluated from duplicate rates obtained by use of at least six substrate concentrations that bracketed the value of K_m. The concentration ranges for UDP-galactose were as follows: wild type, 2.5–35 μM; K153A, 25–195 μM; K153M, 20–165 μM. The kinetic parameters were evaluated by use of a nonlinear regression analysis (Cleland, 1979).

Measurements of K_d for UMP. The binding of UMP to wild type and mutant epimerases was observed according to the method of Wong and Frey (1978). The concentration of epimerase was about 0.15 mg/mL in all binding experiments. Briefly, the K_d for ANS binding to the wild type and mutant epimerases was evaluated by measuring the increase in fluorescence emission at 475 nm (excitation at 375 nm) upon addition of aliquots of ANS (5–80 μM). Knowing the K_d for ANS, the enhancement of fluorescence for ANS upon binding to the active site, and that UMP displaces ANS from the epimerase, the K_d for UMP could be determined by observing the quenching of ANS-fluorescence upon addition of UMP to a mixture of epimerase and ANS. Data required for an evaluation of K_d for UMP was obtained by adding aliquots of UMP (0.1–1 mM) to a solution containing epimerase and ANS (20 or 30 μM) and measuring the decrease in fluorescence at 475 nm. The K_d value for UMP was graphically evaluated from these data as described (Wong & Frey, 1978).

Assay of NAD^+ Bound to Epimerase. The NAD^+ content of wild type and mutant epimerases was measured according to the procedure of Wilson and Hogness (1964).

Fluorescence Spectroscopy of Epimerases. The fluorescence spectra presented in this paper are uncorrected. The concentration of wild type, K153A, and K153M epimerase samples (2.5 mL) were adjusted to 0.9 mg/mL, as calculated from their A_{280} and the extinction coefficient ϵ_{280} of 1.05 (Wilson & Hogness, 1964). Emission spectra were obtained by exciting the samples at 345 nm (4-nm slit width) and monitoring emissions from 380 to 630 nm (8-nm slit width); scan speed, 30 nm/min. The height at maximal emission was measured for each sample.

Excitation spectra were obtained by exciting the samples from 240–450 nm (4-nm slit width) and monitoring fluorescence at 460 nm (8-nm slit width); scan speed 30 nm/min.

The mutant epimerases K153A and K153M (3.5 mg) in 1 mL of buffer were each reduced by addition of 2 mM UMP and 25 mM sodium cyanoborohydride. Reduction was monitored at 344 nm until completion (additional small aliquots of sodium cyanoborohydride were added). Wild type epimerase was reduced under similar conditions by use of 10 mM sodium cyanoborohydride. The samples were passed through columns of Sephadex G-25 (fine, 1.5 × 25 cm) equilibrated with a buffer consisting of 100 mM potassium phosphate at pH 7.0 and 50 μM UMP, and fractions were collected. The fluorescence samples (2.5 mL) were made to 0.56 mg/mL and their excitation spectra determined.

Rates of Reduction of Epimerases by Sodium Cyanoborohydride. All experiments were performed at enzyme concentrations of 1 or 2 mg/mL in 0.7 or 1.0 mL reaction mixtures. Reductions of K153A and K153M were carried out by use of 15 mM sodium cyanoborohydride in the presence and absence of 2 mM UMP. Initial rates of reduction were measured by monitoring the change of absorbance at 344 nm, assuming $\epsilon_{344} = 3.7\ mM^{-1}\ cm^{-1}$.[2] Four or five sets of reduction experiments (in the presence and absence of UMP) were carried out for each mutant. The background rate before addition of sodium cyanoborohydride was subtracted from each rate. The initial rate of reduction of wild type epimerase was too fast to be measured under these conditions. Instead, sodium cyanoborohydride at a series of concentrations ranging from 0.25–5 mM was employed to measure initial rates, the reactions being initiated by the addition of UMP. The rate of reduction at 15 mM sodium cyanoborohydride in the presence of 2 mM UMP was determined by extrapolation from the slower rates measured at lower concentrations of the reductant. Two separate series of three reduction experiments were done. The rate of reduction in the absence of UMP was measured in quadruplicate in the presence of 15 mM sodium cyanoborohydride.

Table I: Specific Activities, Kinetic Parameters, and UMP-Binding Constants for Wild Type and Mutant Epimerases

epimerase	activity[a] (U/mg)	K_m UDPgal[b] (μM)	k_{cat}[b] (s^{-1})	k_{cat}/K_m[b]	K_d UMP[b] (mM)
wild type	8000	18 ± 0.6	24	1.3	1.0
K153A	12	72 ± 8	0.012	1.7×10^{-4}	0.7
K153M	7	83 ± 9	0.022	2.7×10^{-4}	0.8

[a] Assayed under standard conditions at pH 8.5. [b] Measured in 100 mM potassium phosphate containing 1 mM EDTA at pH 7.0. The kinetic and UMP-binding assays were performed as described in the Experimental Procedures.

RESULTS

Expression and Characterization of Epimerase. A high level of epimerase expression is obtained from the pT7E2 system. More than 2 g of the pure wild type enzyme and the mutant K153M can be obtained from 50 g of wet cells; the expression of K153A was not as high, but over 300 mg purified protein was obtained from 50 g of cells. All three epimerases exhibited similar behavior on native PAGE. The maximum specific activity of the wild type epimerase (11 000 U/mg protein) was similar to that previously reported (Wilson & Hogness, 1964); the epimerase employed in the present experiments exhibited specific activities of 8000–9000 U/mg protein.

The specific activities and kinetic parameters for the epimerase and the mutants K153M and K153A are listed in Table I. The mutant K153A is 1/700th as active as the wild type enzyme, while K153M is 1/1200th as active in the standard assay. The value of k_{cat}/K_m for K153A is 1/7800th and that for K153M is 1/4900th of the value for the wild type enzyme. The value of K_m for UDP-galactose is four to five times larger for the mutant proteins than for the wild type epimerase, whereas the values of K_d for UMP are slightly smaller in the case of the mutants compared with the wild type enzyme.

The NAD^+-contents of the wild type and mutant epimerases were found to be approximately one NAD^+ per dimer, when determined according to the method of Wilson and Hogness (1964). In this procedure, perchloric acid is used to precipitate the protein and release the noncovalently bound NAD^+. The released NAD^+ is assayed with UDP-glucose dehydrogenase. This method detects a 95% recovery of free NAD^+ when a known amount is added to a bovine serum albumin solution (20 mg/mL) and assayed. Under conditions of 1.9 mg of

[2] J. Vanhooke, A. J. Bauer, and P. A. Frey, unpublished results.

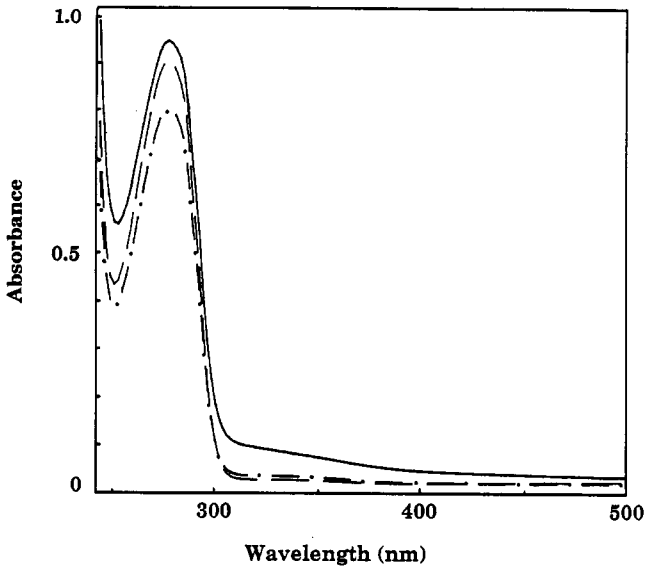

FIGURE 1: Ultraviolet and visible absorption spectra of wild type and mutant epimerases. The spectra of UDP-galactose 4-epimerase and the mutants K153A and K153M were recorded in 100 mM potassium phosphate containing 1 mM EDTA at pH 7.0. Symbols: (—), wild type; (– · –), K153A; (– –) K153M.

epimerase in 180 μL of buffer, 1.0 NAD^+ per dimer was recovered from wild type epimerase, 1.1 NAD^+ per dimer from K153A, and 0.7 per dimer from K153M.

The UV–visible spectra for wild type and mutant epimerases are shown in Figure 1. All exhibit protein absorbances at 276 nm and a small NADH absorbance around 344 nm. The ratios A_{276}/A_{344} nm are 17 for wild type, 43 for K153A, and 220 for K153M. Inasmuch as the NAD^+ contents of the mutant epimerases are similar to that of the wild type enzyme, the larger ratios A_{276}/A_{344} in the cases of the mutants show that they contain much less NADH than the wild type enzyme. This is important in the present context, because it supports other results showing that the mutant epimerases are less susceptible to uridine nucleotide-dependent reduction. This property presumably makes the mutant epimerases less susceptible to adventitious reduction within the bacterial cell than the wild type enzyme.

Fluorescence of Wild Type and Mutant Epimerases. The fluorescence properties of wild type and mutant epimerases were studied to compare relative amounts of NADH present in the enzymes and to detect any significant structural perturbations around the active site. Spectra were obtained after adjusting the concentration of the samples to 0.90 mg/mL. The emission spectra shown in Figure 2A were all obtained by exciting the samples at 345 nm and monitoring emission from 380 to 630 nm. All epimerase samples exhibit an emission maximum near 440 nm, 443 nm in the case of the wild type enzyme, and 435 nm in the cases of the mutants K153A and K153M. The fluorescence emission of K153A is attenuated 3.1-fold and that of K153M 5.2-fold relative to the wild type enzyme. The excitation spectra of the three epimerases, monitored at 460 nm, are shown in Figure 2B. All exhibit maxima at 295 nm and at a longer wavelength corresponding to the absorption of NADH, 357 nm in the case of the wild type enzyme, and 350 nm in the cases of K153A and K153M. Again, the mutants show reduced fluorescence; comparing the fluorescence intensities at 460 nm upon excitation at 345 nm, the wild type enzyme exhibits 3.7-fold more fluorescence than K153A and 6.3-fold more than K153M. The relative fluorescence among the three epimerases represent the composite effects of the relative

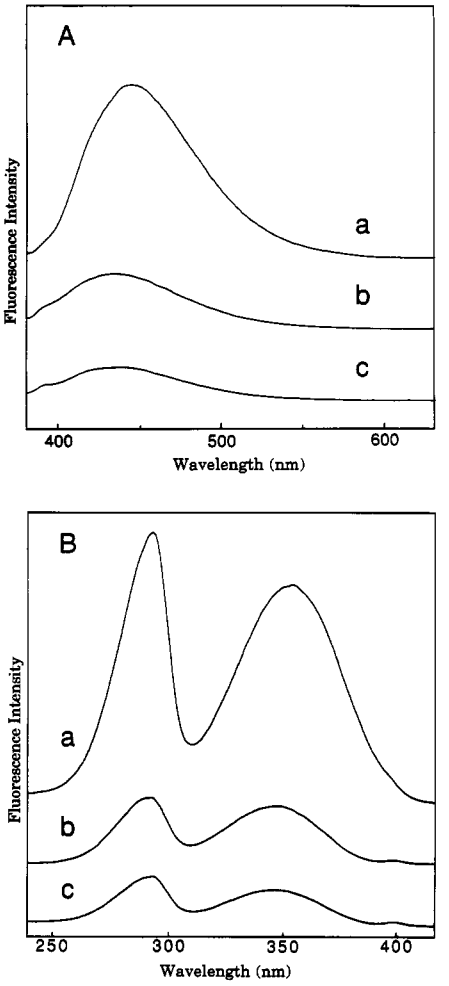

FIGURE 2: Fluorescence spectra of UDP-galactose 4-epimerase and lysine 153-mutants. A. Fluorescence emission spectra of (a) wild type, (b) K153A, and (c) K153M epimerases obtained by exciting the samples at 345 nm. The protein concentrations were 0.9 mg mL^{-1}. Spectra (a) and (b) are offset based on common fluorescence emission intensities at 630 nm. B. Fluorescence excitation spectra of (a) wild type, (b) K153A, and (c) K153M epimerases monitored for emission at 460 nm. The epimerase concentrations were 0.9 mg mL^{-1}. Spectra (a) and (b) are offset based on their common fluorescence emission at 430 nm.

amounts of NADH present in each enzyme and the environment around the dihydronicotinamide ring of NADH. Because the mutants contain less NADH than the wild type enzyme, as shown in Figure 1, part of the fluorescence differences must be attributed to the presence of less NADH in the mutants.

The wild type epimerase and the mutants K153A and K153M were fully reduced following the addition of UMP and sodium cyanoborohydride. Passage through a Sephadex G-25 column removed the excess UMP and reductant. A sample of each epimerase was then prepared at 0.56 mg/mL, and the fluorescence excitation spectrum was obtained from 240 to 450 nm by monitoring emission at 460 nm (not shown). The fluorescence intensities are lower for the mutant epimerases than for the wild type enzyme, 2.7-fold lower in the case of K153A and 3.5-fold lower in the case of K153M. The fluorescence differences between the mutants and wild type enzymes are much larger than the differences in NADH content. Based on the NADH contents estimated from the absorbances at 344 nm, the reduced mutants K153A and K153M contain about 90% and 80%, respectively, of the NADH in the reduced wild type enzyme. Therefore, the 2.7-

Table II: Reduction of Wild Type and Mutant Epimerases with Sodium Cyanoborohydride in the Presence and Absence of UMP

	initial rate of epimerase reduction (μmol NADH/min/mg epimerase)		
	wild type	K153A	K153M
complete[a]	$(4.4 \pm 1.4) \times 10^{-2}$ [b]	$(5.5 \pm 1.6) \times 10^{-4}$	$(2.2 \pm 0.8) \times 10^{-4}$
-UMP	$(0.87 \pm 0.2) \times 10^{-4}$	$(1.4 \pm 1.1) \times 10^{-4}$	$(2.3 \pm 1.6) \times 10^{-4}$
activation by UMP	500	4	1

[a] The complete reaction mixtures consisted of 1–2 mg mL^{-1} of wild type or mutant epimerases, 2 mM UMP, and 15 mM sodium cyanoborohydride in 0.1 M potassium phosphate buffer containing 1 mM EDTA at pH 7.0 and 27 °C. [b] The initial rate for the wild type enzyme is the value obtained by extrapolation of initial rates obtained between 0.2 and 5 mM sodium cyanoborohydride.

and 3.5-fold greater fluorescence of the reduced wild type enzyme compared with the mutants K153A and K153M, respectively, must be attributed to differences in the microenvironments of the dihydronicotinamide ring in the wild type and mutant enzymes.

The fluorescence differences between the *reduced* wild type and mutant epimerases are significantly smaller than those between the *unreduced* wild type and mutant epimerases. This confirms the earlier conclusion from the UV–visible spectra in Figure 1 that the unreduced mutant epimerases contain less NADH than the unreduced wild type enzyme.

Reduction of NAD⁺ Bound to Wild Type and Mutant Epimerases. The UMP-dependent reduction of wild type E·NAD⁺ to E·NADH by sugars could not be observed in the cases of the mutants K153M and K153A in the presence of 200 mM glucose and 3 mM UMP. This observation implicates lysine 153 in UMP-dependent reductive inactivation by sugars.

Wild type epimerase-NAD⁺ is also subject to UMP-dependent reductive inactivation to epimerase·NADH by sodium cyanoborohydride, and NAD⁺ bound to the mutant epimerases could be reduced slowly by sodium cyanoborohydride. Because of the time required for the complete reduction of the mutant epimerases by sodium cyanoborohydride—the half times were several hours—we measured the initial rates of reduction. The two mutants were reduced by 15 mM sodium cyanoborohydride both in the presence of 2 mM UMP and in the absence of UMP. As shown in Table II, there is very little difference in the rate at which sodium cyanoborohydride reduces K153M in the presence or absence of UMP. The mutant K153A shows about a 4-fold increase in reduction by the presence of UMP. The relatively large errors, especially for the reductions carried out in the absence of UMP, are at least partially attributable to the difficulty in measuring these slow rates.

In contrast to the mutant epimerases, the UMP-dependent reduction of wild type E·NAD⁺ under the conditions of Table II is too fast to measure. The rate enhancement induced by UMP under these conditions was, therefore, estimated by reducing the wild type epimerase at seven lower concentrations of sodium cyanoborohydride between 0.2 and 5 mM and linearly extrapolating the data to 15 mM. Under the conditions of Table II, the presence of UMP brings about a 500-fold increase in the rate at which sodium cyanoborohydride reduces the wild type epimerase·NAD⁺. A more direct comparison of the rate enhancement was obtained at 5 mM sodium cyanoborohydride. At this concentration, the rate of reduction in the absence of UMP was 3.5×10^{-5} μmol min^{-1} mg^{-1}; in the presence of 2 mM UMP it was 1.1×10^{-2} μmol min^{-1} mg^{-1}. Under these conditions the rate enhancement induced by UMP is 320-fold.

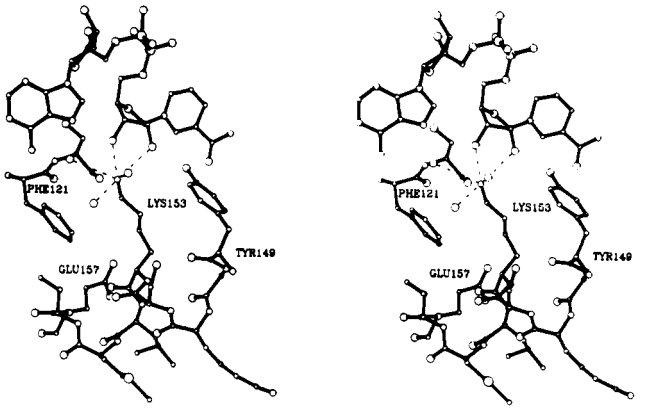

FIGURE 3: Environment of the ε-ammonium group of lysine 153 in UDP-galactose 4-epimerase. The environment of lysine 153 in the crystal structure of UDP-galactose 4-epimerase cocrystallized with P^1-uridyl-P^2-phenyl diphosphate is shown in this stereographic drawing. The ε-ammonium group of lysine 153 is shown connected by dashed lines to the species with which it is coordinated. These include the 2′- and 3′-hydroxyl groups of the nicotinamide riboside moiety of NAD⁺ and three other molecules that are fixed within 3.5 Å of this group. These fixed molecules are modeled in the structure as water molecules. The drawing was prepared through the courtesy of Dr. Hazel M. Holden and Dr. James B. Thoden.

DISCUSSION

The interactions of the ε-ammonium group of lysine 153 in the structure of UDP-galactose 4-epimerase from *E. coli* are shown in Figure 3. That the ε-ammonium group is protonated in the crystalline state at pH 6 is verified by the presence of three fixed molecules within 3.5 Å of this group in addition to the strong hydrogen bonded coordination of this group with the 2′- and 3′-hydroxyl groups of the nicotinamide ribosyl moiety of NAD⁺. The two closest fixed molecules are close enough to form strong hydrogen bonds and are likely to be water molecules; the third is about 3.5 Å from the ε-ammonium ion and could be water or a chloride ion. It is difficult to imagine how a neutral amino group could be hydrogen bonded to three fixed molecules in addition to the 2′- and 3′-hydroxyl groups. An ammonium ion can donate three hydrogen bonds, including the bifurated hydrogen bond to the 2′- and 3′-hydroxyl groups, and it can be associated by ion pairing with a counterion such as a chloride ion from the buffer. The distance between nicotinamide-N-1 and the ε-ammonium ion of lysine 153 is 5.3 Å when P^1-uridyl-P^2-phenyl diphosphate is bound at the active site.

To determine whether lysine 153 plays an important role in UMP-dependent reduction of epimerase-NAD⁺, we prepared the mutants K153A and K153M of epimerase, purified them, and compared them kinetically and spectroscopically with the wild type enzyme. The mutants were approximately 1/1000th as active as the wild type enzyme under the standard assay conditions at pH 8.5, and the values of k_{cat}/K_m at pH 7.0 were approximately 1/5000th (K153M) or 1/8000th (K153A) of the value for the wild type enzyme. Inasmuch as the K_m values of the mutants for UDP-galactose are several-fold higher than for the wild type enzyme, substrate binding has not been greatly perturbed; however, the differences are significant and rule out the possibility that the observed activity might be due to contamination by the wild type enzyme.

Because K153A and K153M contain 1.1 and 0.7 equiv, respectively, of NAD⁺, the large decrease in activity of the mutants is not due to loss of the coenzyme. However, there appears to be a partial loss of NAD⁺ during purification of K153M. The loss of the two hydrogen bonds from lysine 153 to the ribose-2′- and 3′-hydroxyl groups may decrease the

overall NAD^+ binding enough to allow a gradual loss of the cofactor. In the wild type enzyme, NAD^+ is essentially irreversibly bound (Wilson & Hogness, 1964).

All samples of epimerase purified from *E. coli* contain NADH in addition to NAD^+ (Wong *et al.*, 1978). It has often been observed in this laboratory that the wild type enzyme produced from an overexpression vector in *E. coli* contains an increased portion of NADH and is less active than that originally described by Wilson and Hogness (1964); that is, the specific activity is decreased and the NADH content increased relative to the epimerase purified from a regulatory mutant expressing the chromosomal epimerase (unpublished results). The NADH content can be estimated spectrophotometrically from the absorbance at 344 nm. As shown in Figure 1, both K153A and K153M contain much less NADH than the wild type enzyme (3- and 10-fold less, respectively). This lower NADH content is confirmed by the fluorescence spectra of wild type and mutants (Figure 2), suggesting that the mutants are less susceptible to reduction *in vivo* than the wild type enzyme. However, although the wild type and mutant enzymes contained comparable amounts of NAD^+, the fluorescence of the fully reduced mutants is approximately one-third of that of the wild type epimerase. This apparent difference in fluorescence efficiency indicates a difference in environment or mobility for the dihydronicotinamide ring in the mutant enzymes compared with the native enzyme.

The reducibility of the mutant and wild type epimerases was explored using sodium cyanoborohydride as the reductant. As shown in Table II, the NAD^+ associated with the wild type epimerase is reduced about 100–200 times faster than that bound to the mutant epimerases in the presence of UMP. The addition of 2 mM UMP to K153M does not detectably accelerate the reduction of the enzyme-bound NAD^+, while K153A exhibits a very modest rate enhancement of about 4-fold. In contrast, the reduction of wild type epimerase under the same conditions with UMP present is too fast to measure, so reductions at 0.25–5 mM sodium cyanoborohydride were carried out. Extrapolation of the rates to 15 mM sodium cyanoborohydride and comparison to the rates in the absence of UMP showed that the wild type epimerase is activated approximately 500-fold by UMP binding. This rate enhancement is an approximate factor that pertains to the conditions of Table II and should not be regarded as a precise estimate of the maximum effect that can be attained. The reduction in the absence of UMP is too slow to measure very accurately, and a small variability in the measured rate will significantly affect the apparent rate enhancement. Moreover, the UMP concentration in Table II is only about twice the value of K_d for the wild type epimerase, so that a 33% larger effect of UMP can be expected at saturation.

UMP enhances the rate at which sugars reduce the wild type E·NAD^+ to a greater degree than its effect on reduction by sodium cyanoborohydride. The enhancement factor for UMP-dependent reduction by glucose has been estimated to be on the order of 10 000-fold (Frey, 1987). Uridine nucleotides other than UMP, such as UDP and UDP-hexoses, also activate the reduction of E·NAD^+ to E·NADH, albeit to a lesser extent (Wee & Frey, 1973; Blackburn & Ferdinand, 1976; Wong & Frey, 1977). In contrast, we have not observed the reduction of either mutant K153A or K153M by sugars.

In the preceding paper in this issue by Burke and Frey (1993), the effect of UDP on the NMR properties of ^{15}N and ^{13}C in the nicotinamide ring of epimerase·NAD^+ was shown to rationalize the kinetic and thermodynamic enhancement of the reduction of NAD^+ to NADH at the active site. The spectroscopic perturbation of nicotinamide-4-^{13}C in epimerase·NAD^+ by UDP was correlated with an enhancement of 3 000–15 000-fold in the reactivity of *N*-alkylnicotinamides in solution. Because of its proximity to nicotinamide-N-1, charge repulsion between the ϵ-ammonium group of lysine 153 and the nicotinamide ring of NAD^+ was proposed to be responsible for most or all of this rate enhancement. Indeed, as shown in this paper, wild type epimerase·NAD^+ is reduced 2–3 orders of magnitude faster than K153A or K153M in the presence of UMP and sodium cyanoborohydride. This enhancement factor is somewhat smaller than predicted by the NMR studies of Burke and Frey (1993), probably owing to other factors which also affect the rates of reduction or inaccuracies in measuring the rates of reduction. The present results prove that lysine 153 plays a critical role in the catalytic activity of UDP-galactose 4-epimerase and in its uridine nucleotide-dependent reductive inactivation. Whether the promotion of charge repulsion between the ϵ-ammonium group of lysine 153 and the nicotinamide ring of NAD^+ is the function of lysine 153 in uridine nucleotide-dependent reduction remains to be proven.

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