^{13}C NMR Analysis of Electrostatic Interactions between NAD$^+$ and Active Site Residues of UDP-galactose 4-Epimerase: Implications for the Activation Induced by Uridine Nucleotides†

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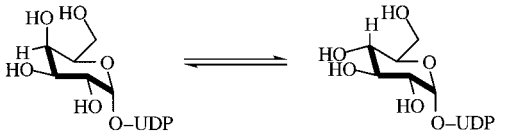
ABSTRACT: UDP-galactose 4-epimerase contains the coenzyme NAD$^+$ bound tightly at the active site. NAD$^+$ functions as the coenzyme for the interconversion of UDP-galactose and UDP-glucose by reversibly mediating their dehydrogenation to the common intermediate UDP-4-ketohexopyranoside. The epimerase structure and spectrophotometric data indicate that NAD$^+$ may engage in electrostatic interactions with amino acid side chains that may regulate the reactivity of NAD$^+$. In this work, we carried out NMR studies of [*nicotinamide*-4-^{13}C]NAD$^+$ bound to wild-type epimerase and epimerases mutated at amino acid residues in contact with NAD$^+$. The 4-^{13}C NMR chemical shifts revealed the following: The 4-^{13}C chemical shift in wild-type epimerase is 149.9 ppm; mutation of Ser 124 to Ala changes it slightly by 0.2 ppm to 150.1 ppm; mutation of Tyr 149 to Phe results in a downfield perturbation of 2.7 ppm to 152.6 ppm; and the simultaneous mutation of Ser 124 to Ala and Tyr 149 to Phe also causes a downfield perturbation of 2.8 ppm to 152.7 ppm. Mutation of Lys 153 to Met results in a ^{13}C chemical shift of 150.8 ppm, which is 0.9 ppm downfield from that of wild type and 1.8 ppm upfield from that of Y149F-epimerase. The ^{13}C chemical shifts of nicotinamide C4 of NAD$^+$ in these epimerases are correlated with their respective reactivities with $NaBH_3CN$. In addition, reactivity of NAD$^+$ in wild-type and S124A-epimerases displays pH dependence, with higher rates at lower pH where Tyr 149 in these two enzymes is protonated. The results support an electrostatic model in which repulsion between positively charged Lys 153 and N1 of the nicotinamide ring increases the reactivity of NAD$^+$, while the phenolate of Tyr 149 opposes the positive electrostatic field and attenuates the reactivity of NAD$^+$. Ser 124 has very little effect on the electron distribution within the nicotinamide ring or the reactivity of NAD$^+$. The effects of binding the substrate analogue P^1-uridyl-P^2-methyl diphosphate (Me-UDP) on the 4-^{13}C chemical shifts are opposite to those induced by the mutations. MeUDP perturbs the 4-^{13}C chemical shift 2.9 ppm downfield in the wild-type and S124A-epimerases but has little or no effect in the cases of Y149F- or K153M-epimerases. The results support the postulate that NAD$^+$ activation induced by uridine nucleotides is brought about by a conformational change of epimerase that repositions Tyr 149 at an increased distance from nicotinamide N1 of NAD$^+$ while maintaining the electrostatic repulsion between Lys 153 and nicotinamide N1 of NAD$^+$.

UDP-galactose 4-epimerase (epimerase) catalyzes the interconversion of UDP-Gal[1] and UDP-Glc according to eq 1 and is an essential enzyme in the metabolism of galactose (*1*). The enzyme from *Escherichia coli* is a homodimer in which each subunit contains tightly bound NAD$^+$,[1] which functions as a coenzyme (*2*, *3*). The reaction proceeds through hydride abstraction from C4 of the pyranosyl ring



(1)

by NAD$^+$, resulting in a UDP-4-ketoglucopyranoside intermediate and NADH, as illustrated in Scheme 1. Rotation of the ketopyranosyl intermediate about the P_β—O bond connecting UDP with the anomeric oxygen atom of the keto sugar allows the opposite face of the 4-keto group to

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[1] Abbreviations: NAD$^+$, nicotinamide adenine dinucleotide; NADH, reduced NAD$^+$; [4-^{13}C]NAD$^+$, [*nicotinamide*-4-^{13}C]NAD$^+$; UDP-Glc, uridine 5′-diphosphate glucose; UDP-Gal, uridine 5′-diphosphate galactose; UMP, uridine 5′-monophosphate; UDP, uridine 5′-diphosphate; Me-UDP, P^1-uridyl-P^2-methyl diphosphate; epimerase, UDP-galactose 4-epimerase; ANS, 8-anilino-1-naphthalenesulfonate; EDTA, ethylenediaminetetraacetate; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; NMR, nuclear magnetic resonance; ns, number of scans.

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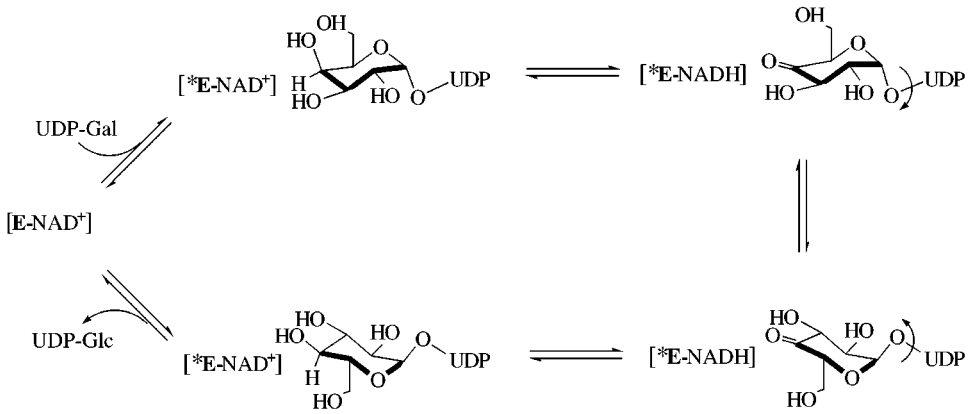
Scheme 1



approach NADH and accept the hydride, thus forming UDP-Glc and NAD^+ (*4*). NAD^+ and NADH remain bound to the enzyme throughout the catalytic cycle. Structural evidence supporting flexibility in the binding of glycosyl moieties has been presented (*5*). Extensive kinetic evidence for a uridine nucleotide-induced conformational change (**E** to **E*** in Scheme 1) has been presented, and this transition markedly increases the reactivity of NAD^+. Proton abstraction from the pyranosyl C4(OH) concomitant with hydride transfer is believed to be driven by Tyr 149 acting as the general base catalyst (*6*, *7*).

Crystal structures of epimerase show that two potentially charged amino acid side chains positioned near NAD^+ may be engaged in electrostatic interactions with the coenzyme (*2*, *8*, *9*). Lys 153 binds to the 2′- and 3′-OH groups of the nicotinamide ribosyl ring and is separated from nicotinamide N1 by 5.3 Å. Tyr 149 is positioned near both Lys 153 and nicotinamide N1 of NAD^+, and it exists as its phenolate ion in neutral solutions, as is known by its ionization with a pK_a value of 6.1 (*6*, *7*). The electrostatic interactions may play important roles in regulating epimerase activity. Electrostatic repulsion between the ϵ-aminium group of Lys 153 in UDP-galactose 4-epimerase and nicotinamide N1 of NAD^+ should polarize the π-electron system of NAD^+, increasing positive charge on C4 and enhancing its reactivity (*10*), whereas the phenolate of Tyr 149 should decrease the reactivity of NAD^+ by attenuating the positive electrostatic field between Lys 153 and nicotinamide N1 of NAD^+ (*6*).

Previous studies have established that the uridine nucleotide moiety of the substrate contributes most of the binding affinity with the enzyme (*11*, *12*) and that the binding of uridine nucleotides to the enzyme induces a conformational change in the protein that enhances the reactivity of NAD^+ as a hydride acceptor (*11*, *13*−*17*). UMP increases the reactivity of epimerase−NAD^+ toward $NaBH_3CN$ by 860-fold (*15*), and the complex of UMP−epimerase−NAD^+ is 130 times more reactive with $NaBH_3CN$ than free NAD^+ in solution (*16*). The enhanced reactivity of NAD^+, a result of the conformational change, may occur through altered interactions of Lys 153 and Tyr 149 with NAD^+ (*6*, *18*).

To probe whether the effects of neighboring residues on the reactivity of the coenzyme involve modulation of the electron distribution within the nicotinamide ring, NAD^+ enriched with ^{13}C at nicotinamide C4 has been synthesized and incorporated into the coenzyme site of wild-type and mutated versions of epimerase. The perturbations of electron distribution within the nicotinamide ring caused by mutations of surrounding amino acids were investigated by NMR spectroscopy and correlated with the reactivity displayed by NAD^+ toward $NaBH_3CN$. In an effort to elucidate the mechanism of epimerase activation upon uridine nucleotide binding, the effects of MeUDP binding on the chemical shifts of nicotinamide C4 of NAD^+ bound to these epimerase variants were studied.

EXPERIMENTAL PROCEDURES

Materials. NAD^+, UDP-Glc dehydrogenase, UDP-Gal, bicine, and UMP-morpholidate were purchased from Sigma. HEPES (enzyme grade), NaOH, and NaCl were purchased from Fisher Scientific. Ethyl formate-^{13}C was from Isotec Inc. Piperazine, methyl dichlorophosphate, and all other chemicals were purchased from Aldrich.

Enzyme Purification and Activity Assay. Site-specific mutations in UDP-galactose 4-epimerase were generated by cassette mutagenesis using the cloning vector pTZ19RB as previously described (*6*, *19*). The vector expressing S124A/Y149F-epimerase was generously donated by Dr. James Thoden. The mutated enzymes were expressed in *E. coli* BL21(DE3)pLysS cells, which lack the *gal* operon in their genome, and purified as described (*2*, *6*). The system allowed a high level of epimerase expression, so that more than 1 g of enzyme was produced by 50 g of pelleted cells. The purified homogeneous epimerase contained various amounts of inactive, abortive complexes consisting of epimerase−NADH−uridine nucleotide (*20*). The fractions from the last column were assessed for abortive complexes by spectroscopic and kinetic analysis (*3*). The enzyme fractions containing no or very little abortive complexes were used for NMR and kinetic studies. The concentration of UDP-galactose 4-epimerase was determined by dividing the absorbance at 280 nm by 1.81 mL mg^{-1} cm^{-1} (*3*). UDP-galactose 4-epimerase was assayed as described by Wilson and Hogness (*21*). UV/vis absorbance was measured with a Shimadzu UV-1601PC spectrophotometer.

Synthesis of MeUDP. MeUDP was synthesized from methyl dichlorophosphate and UMP-morpholidate and purified as previously described (*10*). The final product was characterized by HPLC, mass spectrometry, and ^1H and ^{13}C NMR. The concentration of the Me-UDP stock solution was determined spectrophotometrically by use of the extinction coefficient 10.0 mM^{-1} cm^{-1} at 262 nm at pH 7.0.

Synthesis of ^{13}C-Enriched NAD$^+$. [4-^{13}C]Nicotinamide with more than 99% isotopic enrichment was synthesized by following a previously described method (*22*). The labeled NAD$^+$ was produced in an exchange reaction between [4-^{13}C]nicotinamide and 3-thio-NAD$^+$ catalyzed by NAD$^+$ glycohydrolase (*23*). The product was further purified by chromatography as described (*10*). Typically, a batch of 250 mg of crude NAD$^+$ was applied to a DEAE-Sephadex A-25 column (2.5 × 30 cm) in the chloride form. The column was eluted with a linear salt gradient formed from 1 L of 10 mM LiCl and 1 L of 150 mM LiCl. NAD$^+$-containing fractions were pooled, evaporated, and precipitated with ice-cold ethanol. The precipitate was collected by vacuum filtration, washed with ice-cold ethanol until the filtrate was negative to chloride as determined by testing with 5% $AgNO_3$, and dried in vacuo.

Exchange of [4-^{13}C]NAD$^+$ into Epimerase. The [4-^{13}C]-NAD$^+$ was incorporated into epimerase following the method described previously (*20*). Briefly, the enzyme was partially denatured with 2.65 M urea in the presence of a 10-fold molar excess of the [4-^{13}C]NAD$^+$. After the exchange was allowed to proceed for 4 h at 25 °C, the enzyme sample was diluted 2-fold with 20 mM potassium phosphate buffer containing 1 mM EDTA at pH 7.0. This solution was then concentrated to the desired volume by use of an Amicon YM30 membrane and dialyzed at 4 °C against 100 mM phosphate buffer (pH 7.0) containing 1 mM EDTA for 15−24 h, during which the dialysis buffer was changed twice. The specific activity of the enzyme after exchange was the same as that before exchange. D_2O was added to 10% of the total volume prior to NMR analysis.

NMR Spectroscopy of [4-^{13}C]NAD$^+$ Bound to Epimerase. Proton-decoupled ^{13}C NMR spectra were obtained at 32 °C in 10 mm tubes on a Bruker DMX 500 MHz spectrometer. The spectra were collected at a spectral width of 20 kHz with a 90° pulse and a 1 s relaxation delay. Protein spectra were processed with 40 or 60 Hz Lorentzian line broadening. ^{13}C chemical shifts were reported as parts per million (ppm) downfield from 3-(trimethylsilyl)propanesulfonic acid sodium salt, although they were referenced to an external standard of 10% dioxane in water with 10% D_2O, which was assigned a shift of 62.99 ppm. The specific activity of the enzyme was monitored by epimerase assay with small aliquots of the enzyme removed from the NMR sample and found to remain constant throughout the NMR experiments.

Titration of K153M-Epimerase. Solutions of K153M-epimerase (2.4 mg/mL) were prepared in 0.050 M HEPES/piperazine buffer in the pH range of 5.5−10.0, and the ionic strength was adjusted with NaCl to 0.1. Absorbance of each solution at 320 and 345 nm was measured to monitor the charge-transfer interaction between Tyr 149 and nicotinamide N1 of NAD$^+$.

Reduction of NAD$^+$ in Epimerase by $NaBH_3CN$. All experiments were performed in triplicate with 0.02−0.05 mM epimerase and 1−15 mM $NaBH_3CN$ in 1.0 mL cuvettes. Initial rates of reduction were measured by following the increase of absorbance at 340 nm upon reduction of epimerase-bound NAD$^+$ to NADH by $NaBH_3CN$ by using $\epsilon_{340} = 6.22$ mM^{-1} cm^{-1} for NADH. Second-order rate constants (k) were calculated from the initial rates and the concentrations of epimerase and $NaBH_3CN$ used in the reaction.

Measurement of K_d for MeUDP. The dissociation constants of MeUDP for epimerase variants were determined by the fluorescence titration method of Wong and Frey (*24*). Briefly, ANS binding to the epimerase substrate site results in prominent fluorescence emission at 475 nm (excitation at 375 nm). MeUDP competes for the binding site, and the displacement of ANS by MeUDP leads to the quenching of fluorescence. The fluorescence measurements were recorded with increasing amounts of ANS (0−100 μM) added to a solution containing epimerase (0.3−0.4 mg/mL) and various concentrations of Me-UDP (0−50 μM). The assays were carried out in 0.10 M phosphate buffer (pH 7.0) at room temperature with a Perkin-Elmer MPF-3 fluorescence spectrophotometer. The K_d values were calculated by fitting these data to a competitive binding equation (*24*).

RESULTS

^{13}C NMR Spectroscopy of [4-^{13}C]NAD$^+$ in Epimerases. The proton-decoupled ^{13}C NMR spectra of the ^{13}C-enriched NAD$^+$ in epimerase incorporating mutations at Tyr 149, Ser 124, and Lys 153 are compared in Figure 1 with that of wild-type epimerase. The chemical shift in the wild type was determined to be 149.9 ppm, very close to the previously reported value of 149.8 ppm (*10*). The slight difference may be due to minor variations in experimental conditions. The value of 149.9 ppm was used in this paper for comparison with mutated epimerases because the values were determined in experiments conducted side by side. As shown in Figure 1, mutation of Ser 124 to Ala changed the chemical shift only slightly by 0.2 ppm to 150.1 ppm. Mutation of Tyr 149 to Phe resulted in a downfield perturbation of 2.7 ppm to 152.6 ppm. Double mutation of Ser 124 to Ala and Tyr 149 to Phe had an effect similar to that of the single mutation of Tyr 149 to Phe and caused a downfield perturbation of 2.8 ppm to 152.7 ppm. The mutation of Lys 153 to methionine resulted in a chemical shift of 150.8 ppm for nicotinamide C4 of NAD$^+$, which is 0.7−0.9 ppm downfield from those of the S124A-epimerase and wild-type enzymes but 1.8−1.9 ppm upfield from those of the Tyr 149 mutants. Therefore, mutations of Tyr 149, Ser 124, and Lys 153 had distinctly different effects on the ^{13}C chemical shift of nicotinamide C4 in NAD$^+$ bound at the active site.

Titration of K153M-Epimerase. Both the kinetic and thermodynamic values of pK_a for Tyr 149 in wild-type epimerase have been found to be 6.1 (*6*, *7*). At neutral or higher pHs, a charge-transfer band arises from the interaction between the phenolate of Tyr 149 and the nicotimamide ring of NAD$^+$; however, the band is quenched upon protonation of phenolate of Tyr 149 at lower pHs. In the present work, we undertook to determine the pK_a of Tyr 149 in K153M-epimerase by titrating the enzyme solutions while monitoring the absorbance between 320 and 340 nm. However, even with K153M-epimerase solutions at high concentration, no charge-transfer band was observed, suggesting that the phenolic −OH of Tyr 149 is protonated in this mutated enzyme. A residual absorbance at 345 nm, which is likely due to the trace amount of NADH or other contaminants in the protein preparation, remained constant in a wide pH range up to 9.5. Above this point, absorbance at 320 and 345 nm started to increase, indicating the appearance of a charge-transfer band as a result of the ionization of Tyr 149 (data not shown). Unfortunately, the instability of the protein above

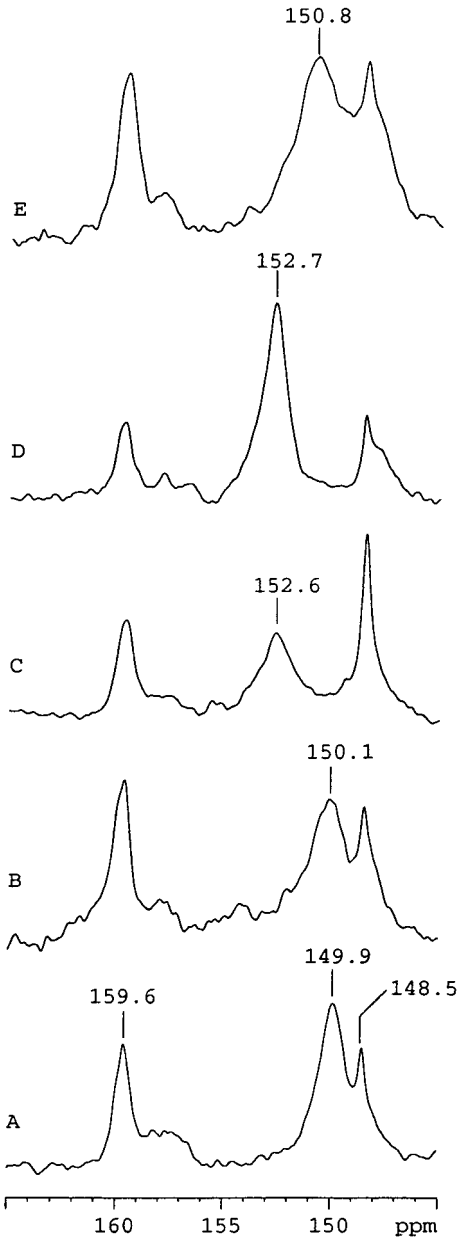


FIGURE 1: ^{13}C NMR signals of [4-^{13}C]NAD^+ bound to epimerases. Sample preparation and ^{13}C NMR analysis are described in Experimental Procedures. Spectra: A, wild type, ns = 12 000; B, S124A-epimerase, ns = 15 000; C, Y149F-epimerase, ns = 15 000; D, S124A/Y149F-epimerase, ns = 17 000; E, K153M-epimerase, ns = 15 000. Line broadening is 40 Hz for all spectra. The sharp peak at 148.5 ppm is due to free [4-^{13}C]NAD^+ and the peak at 159.6 ppm to protein backbone carbon atoms.

pH 10 prevented an accurate determination of pK_a for Tyr 149 in K153M-epimerase.

Reduction of NAD^+ in Epimerase Variants by $NaBH_3CN$. NAD^+ bound to the epimerase active site is subject to reduction by sodium cyanoborohydride, a reducing agent that, unlike a substrate, does not require general base catalysis in hydride transfer (*6*, *16*). Rates of reductive inactivation by sodium cyanoborohydride are expressed as second-order rate constants in Table 1. For the wild-type enzyme, the rate constant is 0.23 ± 0.06 M^{-1} min^{-1}. Mutation of Tyr 149 to Phe increases the reactivity of NAD^+ by 90-fold to 20.4 ± 1.9 M^{-1} min^{-1}, in agreement with earlier results (*6*). The effect of Ser 124 mutation on NAD^+ reactivity was much less pronounced, a modest increase of 2-fold to 0.45 ± 0.04 M^{-1} min^{-1}. The double mutation of Ser 124 to Ala and Tyr 149 to Phe had an effect analogous to that of the mutation of Tyr 149 alone and increased the reactivity by 45-fold to 10.2 ± 0.4 M^{-1} min^{-1}. The results show that the mutation of Tyr 149 to Phe markedly enhances the reactivity of the coenzyme NAD^+ toward reduction by $NaBH_3CN$; that is, NAD^+ becomes a better hydride acceptor. Apparently, Tyr 149 in the native enzyme attenuates the reactivity of NAD^+ toward $NaBH_3CN$. In addition, the mutation of Lys 153 to Met increased NAD^+ reactivity by 5.3-fold to 1.22 ± 0.42 M^{-1} min^{-1}.

Table 1: Correlation of ^{13}C Chemical Shifts of Nicotinamide C4 and Second-Order Reaction Rate Constants for Reduction of Epimerase−NAD^+ by $NaBH_3CN$ in Variant Epimerases

	nicotinamide C4		$NaBH_3CN$ reactivity of NAD^+	
	^{13}C chemical shift (ppm)	perturbation from wild type (ppm)	rate constant k (M^{-1} min^{-1})[a]	change from wild type (fold)
wild type	149.9		0.23 ± 0.06	
S124A	150.1	+0.2	0.45 ± 0.04	2.0
Y149F	152.6	+2.7	20.4 ± 1.9	90
S124A/Y149F	152.7	+2.8	10.2 ± 0.4	45
K153M	150.8	+0.9	1.22 ± 0.42[b]	5.3

[a] Rates were measured in 0.1 M phosphate buffer (pH 7.0) at 22 °C. [a] Value was calculated from that reported in ref *18*.

pH Dependence of the Reduction Rate of Epimerase−NAD^+ by $NaBH_3CN$. The cofactor NAD^+ in epimerase is subject to reduction by $NaBH_3CN$. We have determined the pH dependence of the reaction rate. Because the reduction of the free epimerase−NAD^+ complex is extremely sluggish, UMP, which activates the reduction of NAD^+ in epimerase, was included in each reaction at 5 mM, a saturating concentration. The plots of the logarithm of second-order rate constants versus pH are shown in Figure 2. NAD^+ in both wild-type and S124A-epimerase displayed pH dependence in the rates of reduction by $NaBH_3CN$. The rates dramatically decreased with increasing pH and were fitted to eq 2. In fitting the data, k values were the second-order

$$\log k = \log[c/(1 + K_a/[H^+])] \quad (2)$$

reaction rate constants, c was the pH-independent value arising from the fitting procedure, and K_a values were the fitted values of acid association constants. The pK_a values of 6.5 and 6.7, respectively, were obtained for wild-type and S124A-epimerase. The reduction of free NAD^+ by $NaBH_3CN$ did not display any pH dependence in this range.

K_d Values of MeUDP for Epimerase Variants. MeUDP is a substrate analogue and, like other uridine nucleotides, binds to the epimerase active site. Employing a fluorescence assay, we measured the dissociation constants for the binding of MeUDP at pH 7.0 by S124A-, Y149F-, and K153M-epimerases (*24*). The K_d values of MeUDP were found to be 29 ± 1 μM, 22 ± 2 μM, and 42 ± 1 μM, respectively. The K_d values indicate that MeUDP can effectively bind to all of these epimerase variants. These values are also close to that for the binding of MeUDP by the wild-type enzyme (30 μM; ref *20*), suggesting that the mutations did not significantly change the structure of the substrate binding

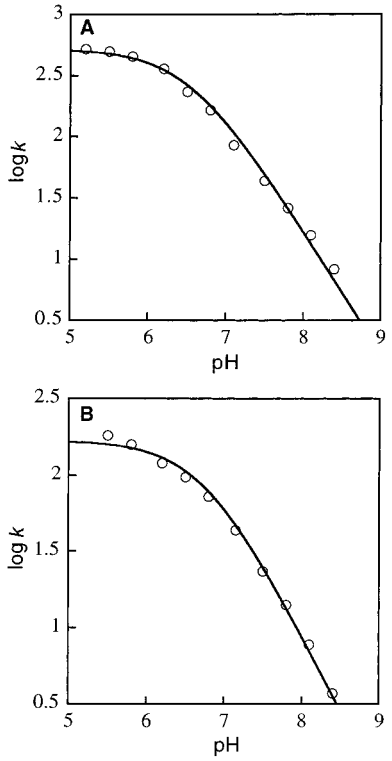


FIGURE 2: pH dependence of second-order rate constants for UMP-dependent reduction of epimerase−NAD^+ by $NaBH_3CN$. The reactions were buffered with 0.050 M HEPES/piperazine, and the ionic strength was adjusted with NaCl to 0.1. Panel A is a plot of log *k* vs pH for wild-type epimerase; panel B is for S124A-epimerase. Units for the second-order rate constants *k* are M^{-1} min^{-1}. The two sets of data are each fitted to eq 2 and give pK_a values of 6.5 and 6.7 for wild-type and S124A-epimerase, respectively.

site. On the basis of these results, MeUDP was used to study the effects of urdine nucleotide binding on the ^{13}C NMR spectra of epimerase-bound NAD^+.

Effect of MeUDP Binding on the Chemical Shift of Nicotinamide C4. In wild-type epimerase, binding of uridine nucleotides causes a downfield perturbation of the ^{13}C chemical shift of nicotinamide C4 (*10*). We have sought to study the effect of uridine nucleotide binding on the ^{13}C chemical shifts of [4-^{13}C]NAD^+ in mutated epimerases. As shown in Figure 3, a new signal for [4-^{13}C]NAD^+ in S124A-epimerase appeared downfield at 152.9 ppm when MeUDP was added, while the intensity of the original peak at 150.1 ppm was diminished. The new signal represents NAD^+ in epimerase species with an activated conformation induced by MeUDP binding. Increased MeUDP concentration further increased the size of the new signal at the expense of the one at 150.1 ppm. The behavior of the ^{13}C NMR signal toward MeUDP addition in S124A-epimerase is similar to that of the wild-type epimerase (*10*).

As shown in Figure 4, addition of MeUDP to 50% saturation did not cause significant perturbation on the ^{13}C NMR spectra of [4-^{13}C]NAD^+ bound to Y149F-epimerase. The ^{13}C signals observed in Figure 4B for this variant in the absence of MeUDP remained essentially at the same position after addition of MeUDP in Figure 4C. Further addition of MeUDP led to an increase in the free [4-^{13}C]NAD^+ and a decrease in the enzyme-bound cofactor. It seems that some

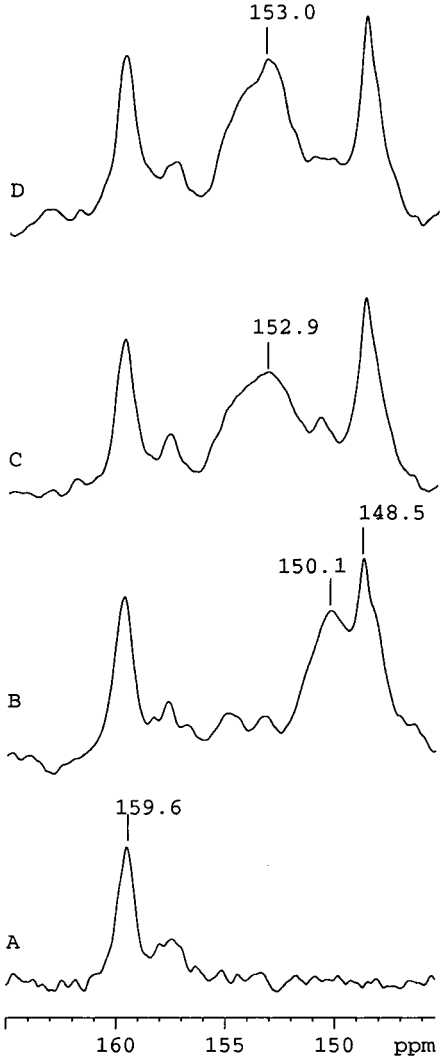


FIGURE 3: Effect of MeUDP binding on the ^{13}C NMR spectrum of [4-^{13}C]NAD^+ in S124A-epimerase. Spectra: A, enzyme with natural abundance NAD^+ as control; B, 1 mM enzyme dimers with [4-^{13}C]NAD^+; C, 1 mM MeUDP added to B (50% saturation); D, 3.5 mM MeUDP added to B (95% saturation). Line broadening is 40 Hz for spectrum A and 60 Hz for spectra B, C, and D; ns is 15 000 for all spectra.

of the cofactor at the enzyme active site is released into the solution.

The effect of MeUDP on the spectra of [4-^{13}C]NAD^+ in K153M-epimerase is analogous to that on Y149F-epimerase. As illustrated in Figure 5, the addition of MeUDP to K153M-epimerase did not cause a significant change in the chemical shift of the nicotinamide C4 signal. However, the prominence of the signal for free [4-^{13}C]NAD^+ was increased with a concomitant decrease in the signal of the enzyme-bound cofactor. Addition of MeUDP to 95% saturation further decreased the signal for enzyme-bound [4-^{13}C]NAD^+ and increased the signal for free [4-^{13}C]NAD^+. It seems that the mutation of Tyr 149 to Phe or Lys 153 to Met weakens the binding of NAD^+ and makes it susceptible to displacement or structural perturbation by MeUDP. Weakened binding of NAD^+ is a reasonable consequence of the change of Tyr 149 to Phe, or Lys 153 to Met, given the electrostatic interaction between the phenolate group of Tyr 149 and the positively charged nicotinamide ring and the hydrogen bonding between the ϵ-aminium ion of Lys 153 and the 2′-

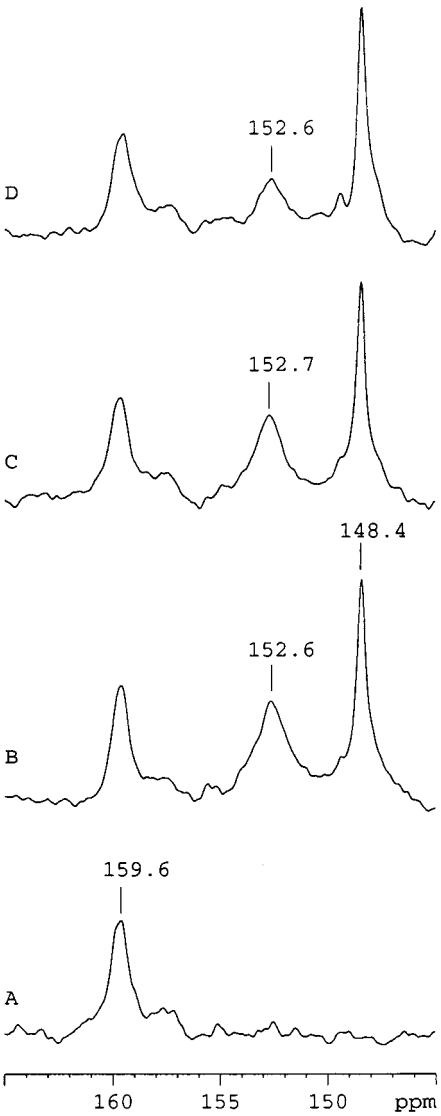


FIGURE 4: Effect of MeUDP binding on the ^{13}C NMR spectrum of [4-^{13}C]NAD^+ in Y149F-epimerase. Samples were prepared and spectra accumulated as described in Experimental Procedures. Spectra: A, enzyme with natural abundance NAD^+ as control; B, the same as Figure 1C, 1.5 mM enzyme dimers with [4-^{13}C]NAD^+; C, 1.5 mM MeUDP added to B (50% saturation); D, 2.5 mM MeUDP added to B (95% saturation). For all spectra, line broadening is 40 Hz; ns = 15 000.

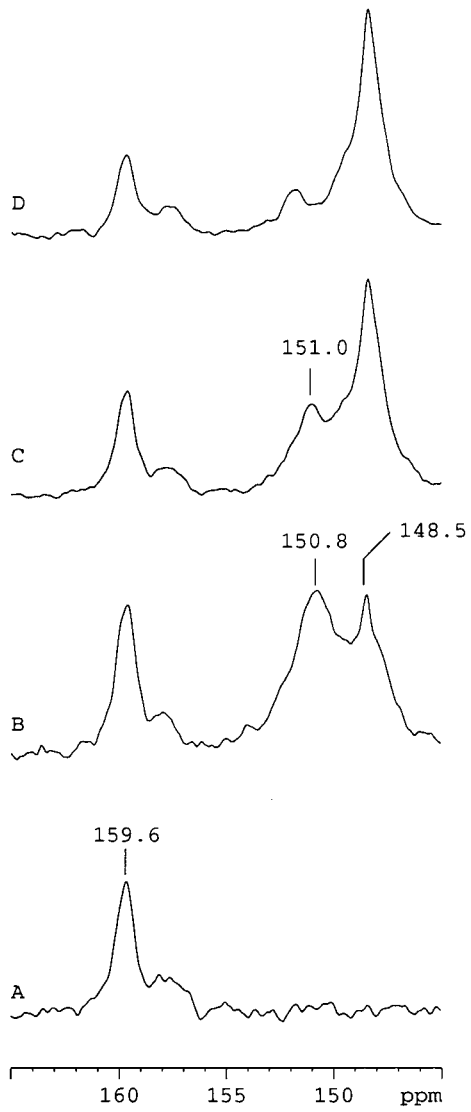


FIGURE 5: Effect of MeUDP binding on the ^{13}C NMR spectrum of [4-^{13}C]NAD^+ in K153M-epimerase. Samples were prepared and spectra accumulated as described in Experimental Procedures. Spectra: A, enzyme with natural abundance NAD^+ as control; B, the same as Figure 1E, 1.3 mM enzyme dimers with [4-^{13}C]NAD^+; C, 1.3 mM MeUDP added to B (50% saturation); D, 3.2 mM MeUDP added to B (95% sauration). For all spectra, line broadening is 40 Hz; ns = 15 000.

and 3′-hydroxyl groups of the nicotinamide ribosyl ring. However, MeUDP does not bring about a significant field shift in the signal for [4-^{13}C]NAD^+ bound to either Y149F- or K153M-epimerase.

DISCUSSION

The X-ray crystal structures of epimerase in complex with UDP or UDP-Glc reveal that the active site contains Lys 153, Tyr 149, and Ser 124 interacting directly with NAD^+ or/and the C4(OH) of the hexopyranose ring (*8*, *9*). The interactions of Lys 153, Tyr 149, and Ser 124 with NAD^+ and with one another are illustrated in Figure 6. The positively charged ϵ-aminium group of lysine and the phenolate oxygen of Tyr 149 are both placed near N1 of the nicotinamide ring of NADH (*8*, *9*). The proximity of Lys 153 and Tyr 149 to the positively charged nicotinamide N1 in epimerase suggests that they engage electrostatically. The following experimental observations support the significance of such interactions: First, the value of the pK_a for Tyr 149 of 6.1 (*6*) is 4 units lower than the corresponding value of 10.1 in aqueous solution. Much of the phenolate anion stabilization implied by the downward pK_a perturbation can be attributed to the positive electrostatic field created by the nearby positive charges on the nicotinamide ring and Lys 153. Second, a charge-transfer band arises from the interaction between the phenolate oxygen of Tyr 149 and the nicotinamide ring of NAD^+, and removal of phenolate by substitution of Phe in place of Tyr 149 in epimerase abolishes the band without inducing a significant change in the overall structure (*6*).

The electrostatic interactions of Lys 153 and Tyr 149 with NAD^+ have profound effects on the chemical reactivity of NAD^+. Lys 153 increases the reactivity of NAD^+ (*18*). It has been postulated that it does so by perturbing the electron distribution in the nicotinamide ring toward N1 and away

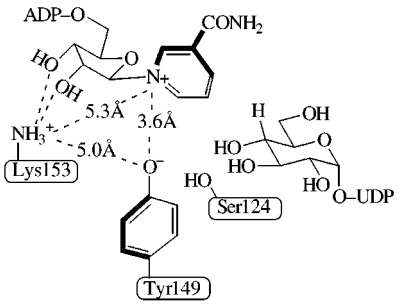


FIGURE 6: Interactions among Lys 153, Tyr 149, Ser 124, and NAD^+ at the active site of epimerase. The distances shown are taken from the structure of the abortive complex of epimerase−NADH−UDP-Glc (PDB file 1XEL).

from C4 (*10*). On the other hand, the phenolate of Tyr 149 attenuates the reactivity of NAD^+ (*6*), and it is hypothesized to do so by partially shielding nicotinamide N1 from the electrostatic field created by Lys 153. Because the ^{13}C chemical shift measures the electron density and the degree of shielding on the carbon atom, NMR spectroscopy is well suited to investigate the effects of the active site amino acids on the electron distribution within the nicotinamide ring. Nicotinamide C4 is the hydride acceptor in either the epimerase-catalyzed reaction or the nonspecific reduction by reducing agent such as $NaBH_3CN$. Therefore, [4-^{13}C]NAD^+ was synthesized and exchanged into the NAD^+ binding site and used to probe the electron density at nicotinamide C4.

If the π-electron density in the nicotinamide ring is modulated by the charged amino acids at the epimerase active site, mutations of these amino acids to those with uncharged side chains would abolish such interactions and change the ^{13}C chemical shift of nicotinamide C4 in predictable ways. The present experiments verify that the electrostatic interaction of the phenolate group of Tyr 149 with nicotinamide N1 increases the electron density at nicotinamide C4, and its removal decreases the electron density. Since Ser 124 has an uncharged side chain, the mutation of this residue should not significantly perturb the electron density or the chemical shift of nicotinamide C4, and this is also verified.

The mutation of the positively charged Lys 153 to Met is expected to decrease the repulsion between the two positive charges of the Lys 153 side chain and nicotinamide N1 and to increase the electron density on nicotinamide C4. In reality, the effects of this mutation are more complex. The aminium group of Lys 153 is 5.0 Å from the phenolic group in Tyr 149. Mutation of Lys 153 to Met should attenuate the positive electrostatic field surrounding the phenolic group in Tyr 149 and increase its pK_a. In this work, the pK_a was determined to be above 9.5, several pH units higher than that of Tyr 149 in the wild-type epimerase. As Tyr 149 in K153M-epimerase is protonated at neutral pH, the effects of Lys 153 to Met mutation are 2-fold. On one hand, it removes a positive charge of the Lys 153 side chain; on the other hand, it abolishes a negative charge of the phenolate of Tyr 149. At the active site of K153M-epimerase, the net change in the total electrostatic charges around NAD^+ is zero compared to that of wild-type epimerase. However, these two opposite effects could not simply cancel. Because Tyr 149 lies near both Lys 153 and NAD^+ and is closer to nicotinamide N1 (*8*, *9*), removal of the negative charge of Tyr 149 should have a greater impact on the electron density of the nicotinamide ring than removal of the positive charge of Lys 153. This is shown to be the case, as the chemical shift of NAD^+ in K153M-epimerase is perturbed downfield by 0.9 ppm from the wild-type epimerase. This means that the removal of the two opposite charges led to an overall decrease in electron density on nicotinamide C4. To gain direct insight into the effect of Lys 153 on the electron distribution on the NAD^+ nicotinamide ring, we can compare the NMR spectra of K153M- and Y149F-epimerase. In both enzymes, the negative charge of the phenolate of Tyr 149 is deleted, either through direct mutation of Tyr 149 or through the secondary effect of mutating Lys 153. With the phenyl or protonated phenolic group as the side chain of the amino acid at position 149, the difference at this position between Y149F- and K153M-epimerase is small except for an extra −OH in the latter enzyme. The two uncharged amino acids should not have significantly different effects on the electron distribution on the nicotinamide ring of NAD^+, and the differences between the spectra of [4-^{13}C]NAD^+ in the two enzymes can be primarily attributed to the presence or absence of Lys 153. In Y149F-epimerase where Lys 153 is present, the chemical shift of nicotinamide C4 is 1.6 ppm downfield from that in K153M-epimerase, where Lys 153 is changed to an uncharged amino acid. It is obvious from this analysis that the positive charge of the Lys 153 side chain decreases the electron density at nicotinamide C4.

The nicotinimide C4 accepts hydride in either epimerase action or reduction by $NaBH_3CN$. Because the electron density about it dictates the electrophilic reactivity of this atom, and the chemical shift measures the degree of shielding at this atom, the value of the chemical shift of C4 is correlated with the reactivity of NAD^+ (*10*). Summarized in Table 1 are the chemical shifts of nicotinamide C4 and the reactivities toward $NaBH_3CN$ of wild-type and mutated epimerases. Mutation of Ser 124 to Ala led to a relatively small change in reactivity of NAD^+, consistent with the very small perturbation of $\delta^{13}C$ of nicotinamide C4. Both single mutation of Tyr 149 to Phe and double mutation to S124A/Y149F-epimerase led to a large downfield perturbation of $\delta^{13}C$ of nicotinamide C4 and greatly activated NAD^+. The effects of Lys 153 to Met mutation were larger than Ser 124 to Ala mutation but smaller than the mutation of Tyr 149 to Met in terms of perturbation on $\delta^{13}C$ of nicotinamide C4 and increase in NAD^+ reactivity. Therefore, the $\delta^{13}C$ values of nicotinamide C4 in epimerase-bound NAD^+ are directly correlated with their reactivities toward $NaBH_3CN$. It is also worth noting the correlation between the reactivity of NAD^+ in an epimerase with the abortive complex (E−NADH−uridine nucleotide) content of the purified enzyme. We found that preparations of wild-type and S124A-epimerases contained similar small amounts of abortive complexes, whereas the purified Y149F- and S124A/Y149F-epimerases contained substantially more. Apparently, NAD^+ bound in Tyr 149 variants is more reactive and susceptible to adventitious reduction within bacterial cells than the wild-type or S124A-epimerases. It is evident that the mutation of Tyr 149 to Phe leads to deshielding, or decreased electron density at nicotinamide C4, rendering NAD^+ more reactive as a hydride acceptor.

In this work, the pH dependence of $NaBH_3CN$ reduction rates of NAD^+ in both the wild-type and S124A-epimerase was studied. Because of the proximity of the phenolic group of Tyr 149 to nicotinamide N1, its ionization state is expected

to affect the reactivity of NAD^+. As shown in this work, the reaction rates dramatically decreased when the pH was increased from the lower range where the phenolic −OH of Tyr 149 was protonated to higher values where it was deprotonated. A pK_a value of 6.7 was obtained for Tyr 149 in S124A-epimerase. In the case of the wild-type epimerase, a value of 6.5 was obtained in this work, whereas a pK_a value of 6.1 was previously reported for Tyr 149 in the free enzyme. In the present work, saturating UMP was included in each reaction to facilitate the measurement of the otherwise slow reduction rates. Therefore, the pK_a value of 6.5 refers to the complex of epimerase with UMP and not to free epimerase. In any case, the protonation of Tyr 149 greatly increases the reaction rates, similar to the effect of mutating Tyr 149 to Phe. In both cases, the negative charge of the phenolate of Tyr 149 in NAD^+ is removed. The results provide further evidence that a charge-transfer interaction exists between the phenolate of Tyr 149 and nicotinamide N1 and that this interaction modulates NAD^+ reactivity.

The electrostatic field between Lys 153 and nicotinamide N1 has been implicated in the mechanism of NAD^+ activation upon uridine nucleotide binding (*10*, *18*). Kinetic and spectroscopic evidence indicates that uridine nucleotide binding induces a conformational change in epimerase (*10*−*18*, *25*). As shown by the fact that UMP or UDP bleaches the charge-transfer band (*6*), the uridine nucleotide-induced conformational change may increase the distance between Tyr 149 and NAD^+ and thus reduce the interaction. Consistent with this explanation, uridine nucleotides induce downfield perturbations on the ^{13}C NMR signal for nicotinamide C4 of NAD^+ in the wild-type epimerase (*10*). It is likely that the increased distance between Tyr 149 and NAD^+, induced by binding a uridine nucleotide, decreases the charge-transfer interaction and consequently decreases the electron density at nicotinamide C4, making NAD^+ more reactive as a hydride acceptor.

In this work, we sought to test the above hypothesis. If altered interaction between the phenolate group of Tyr 149 and the positively charged nicotinamide N1 of NAD^+ is responsible for NAD^+ activation as a result of uridine nucleotide-induced conformational change, NAD^+ activation would not be observed when Tyr 149 is substituted with an uncharged amino acid. This prediction is corroborated by the present results.

The spectroscopic and kinetic results implicate Tyr 149 in two essential functions in the mechanism of action of UDP-galactose 4-epimerase. The earlier structural, mutagenic, and kinetic experiments implicated Tyr 149 in general acid−base catalysis of hydride transfer (*6*). The structure of the abortive complex of the epimerase with NADH and UDP-Glc implicates Tyr 149 in interactions between the hexopyranosyl group and NAD^+ (*8*). Mutation of Tyr 149 severely impairs both epimerase activity and the rate of the UMP-dependent reduction by glucose (*6*). On the other hand, the results of UV/vis, ^{13}C NMR, and kinetic experiments with $NaBH_3CN$ document its role in the uridine nucleotide-induced electrostatic control of the reactivity and reduction potential of NAD^+ (*6*, *10*, this work). The present spectroscopic and kinetic experiments directly implicate Tyr 149 in the substrate-induced epimerase activation. Therefore, Tyr 149 plays dual roles in the mechanism of epimerase action in the wild-type enzyme.

The structures of Y149F-, S124A-, and Y149F/S124A epimerases have been published (*6*, *19*, *26*) and found to be structurally similar to that of the wild-type epimerase, except for the absence of the oxy groups of Tyr 149 and Ser 124. The present results extend the evidence of electrostatic interactions among Lys 153, Tyr 149, and NAD^+ by documenting, through ^{13}C NMR analysis, the consequences of perturbations on the electron density and reactivity on nicotinamide C4 in NAD^+. The results in this paper directly support the presence of charged interactions of NAD^+ with Tyr 149 and Lys 153 and their absence with the neutral Ser 124. The results also verify that the mechanism of epimerase activation through a uridine nucleotide-induced conformational change involves an alteration in the relative positioning of Tyr 149 and the nicotinamide ring of NAD^+, while maintaining the electrostatic repulsion between Lys 153 and nicotinamide N1.

The structural details of the uridine nucleotide-induced repositioning of Tyr 149 and NAD^+ are not known. The NMR, spectrophotometric, and kinetic results suggest that the protein conformational change either increases the distance between Tyr 149 and the nicotinamide ring or alters the angle of their interaction. Any uridine nucleotide-induced repositioning of the Tyr 149 phenolate and nicotinamide rings would entail a motion of one or the other ring or of both. This could be a translational motion. Alternatively, a torsional motion of the nicotinamide ring about the *N*-ribosyl bond could be involved. Differences in the syn/anti orientation of the nicotinamide ring have been documented in structures of various epimerase complexes. Thus, the nicotinamide ring is syn with respect to the ribosyl oxygen in the epimerase−NAD^+−UDP and epimerase−NADH−UDP-Glc complexes, and it is anti in the epimerase−NADH−UDP and epimerase−NADH−UDP−phenol complexes (*8*, *9*, *27*). In the anti complexes, the carboxamide group of the nicotinamide ring is hydrogen bonded to Ser 124 and Tyr 149, and in the syn complexes, it is hydrogen bonded to Lys 84 and the β-phospho group of the ADP moiety of the nicotinamide coenzyme. Any rotational or translational reorientation of these rings could alter the reduction potential and kinetic reactivity of the nicotinamide ring.

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