The Importance of Binding Energy in Catalysis of Hydride Transfer by UDP-Galactose 4-Epimerase: A ^{13}C and ^{15}N NMR and Kinetic Study†

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ABSTRACT: UDP-galactose 4-epimerase contains NAD^+ irreversibly but noncovalently bound to the active site. Uridine nucleotides bind to the substrate site and induce a protein conformational change that increases the chemical reactivity of NAD^+ at the coenzyme site. Activation of NAD^+ by uridine nucleotides perturbs the ^{15}N and ^{13}C NMR chemical shifts of selectively enriched NAD^+ bound to the coenzyme site. The proton-decoupled ^{15}N NMR signal for enzyme-bound [*carboxamide*-^{15}N]NAD^+ does not change upon addition of UDP, indicating that activation is not brought about by a change in the binding of the carboxamide group. The ^{15}N NMR signal of enzyme-bound [*nicotinamide*-1-^{15}N]NAD^+ is shifted upfield 3.0 ppm and the ^{13}C NMR signal for [*nicotinamide*-4-^{13}C]NAD^+ is shifted downfield 3.4 ppm downfield by the binding of UDP at the substrate site. These changes are consistent with the induction of a distortion into the nicotinamide ring, in which positive charge is transferred from N-1 to C-4. The kinetic and thermodynamic effects of these perturbations are significant, as indicated by the nonenzymatic chemical reactivities of a series of *N*-alkyl nicotinamides differing in the inductive electron withdrawing effects of the alkyl substituents. A downfield change of 3.4 ppm in the 4-^{13}C chemical shifts brought about by electron withdrawal in the model compounds corresponds to a 3200-fold increase in the rate of reduction by $NaBH_3CN$ in water, a 15 000-fold increase in 86% ethanol, and a 152 mV more positive reduction potential in this series. The distortion of NAD^+ by the binding of UDP is a long-range effect that is transmitted from the substrate binding site to the coenzyme through the protein conformational change. This apparently distorts the π-electron distribution in the nicotinamide ring and reduces the activation energy for its reduction. Activation of enzyme-bound NAD^+ toward reduction apparently arises from a destabilization in the nicotinamide ring structure rather than from a stabilization of the transition state through attractive interactions between the nicotinamide ring and the enzyme.

UDP-galactose 4-epimerase (EC 5.1.3.2) catalyzes the interconversion of UDP-galactose and UDP-glucose in microorganisms, plants, and animals. This is an integral step in the metabolism of galactose into glucose 6-phosphate for use as an energy source in glycolysis, and it is the essential step in the production of galactosyl units from glucosyl units in all organisms. The enzyme from *Escherichia coli* is a dimer of identical subunits with an overall molecular weight of 79 000, and it contains NAD^+ irreversibly but noncovalently bound to the coenzyme site (Wilson & Hogness, 1964, 1969). The chemical mechanism proceeds through the reduction of the coenzyme to NADH and the concomitant oxidation of the substrate into enzyme-bound UDP-4-ketoglucopyranose (Nelsestuen & Kirkwood, 1971; Maitra & Ankel, 1971; Wee & Frey 1973; Adair *et al.*, 1973). The ketone intermediate then undergoes nonstereospecific reduction by NADH to yield either UDP-glucose or UDP-galactose. This process is presumably facilitated through the interconversion of 4-ketopyranosyl rotamers of UDP-4-ketoglucopyranose, so that hydride transfer from NADH takes place to either face of the ketone (Kang *et al.*, 1975; Frey, 1987). In constrast, hydride transfer from C-4 of UDP-glucose or UDP-galactose to NAD^+ proceeds specifically to the *si*-face (B-face) of nicotinamide-C-4.

It has been shown that the uridine nucleotide portion of the substrate contributes the greater amount to the binding interactions with the enzyme, while the sugar moiety contributes very little (Kang *et al.*, 1975; Wong and Frey, 1977). Moreover, the NAD^+ in the active site can be reduced by various sugars or sodium cyanoborohydride at greatly increased rates when uridine nucleotides are present (Bhaduri *et al.*, 1965; Bertland *et al.*, 1971; Davis *et al.*, 1974; Kang *et al.*, 1975). These facts, together with circular dichroism studies (Wong *et al.*, 1978), indicate that binding of the uridine nucleotide moiety of substrates induces an enzyme conformational change that increases the reactivity of NAD^+. The energy required for the conformational change is derived

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[1] Abbreviations: NAD^+, nicotinamide adenine dinucleotide; NADH, reduced NAD^+; UDP-glucose, uridine-5′-diphosphate glucose; UDP-galactose, uridine-5′-diphosphate galactose; UMP, uridine-5′-monophosphate; UDP, uridine-5′-diphosphate; MeUDP, P^1-uridyl-P^2-methyl diphosphate; EDTA, ethylenediaminetetraacetate; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-(2-hydroxypropanesulfonic acid); NMR, nuclear magnetic resonance.

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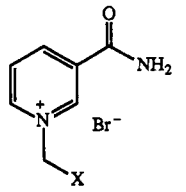
from the binding energy made available through interactions between the enzyme and the uridine diphosphoryl moiety of substrates. The conformational change alters the interactions between the enzyme and NAD^+ at the coenzyme site and causes NAD^+ to become more reactive or "activated" toward reduction. The rate increase resulting from this activation is on the order of 10 000-fold in the case of the reduction with glucose (Frey, 1987).

Spectroscopic evidence supporting activation of the coenzyme through a conformational change in the enzyme is provided by ^{31}P NMR studies of the pyrophosphate group of NAD^+ in UDP-galactose 4-epimerase, E·NAD^+, which indicate that the environment about the phosphoryl groups of NAD^+ changes when UMP binds to the substrate site (Konopka *et al.*, 1989). In this paper, we provide a more detailed spectroscopic investigation of the nature of the uridine nucleotide-induced activation of E·NAD^+. Various species of NAD^+ enriched with ^{15}N or ^{13}C in the nicotinamide moiety have been synthesized and incorporated into the coenzyme site of the enzyme. These samples of the enzyme–coenzyme complex have been used in ^{15}N and ^{13}C NMR experiments to observe changes in the signals upon binding UDP or MeUDP to the substrate binding site.

In order to evaluate the significance of chemical shift perturbations in E·NAD^+



1a X = CH_3 **1d** X = CO_2CH_3
1b X = C_6H_5 **1e** X = CN
1c X = *p*-C_6H_4CN

caused by the binding of uridine nucleotides, we have correlated the nonenzymatic reactivities for a series of N-substituted nicotinamides **1a–e** with the ^{13}C chemical shifts ($\delta^{13}C$) for C-4 in the nicotinamide ring. According to this correlation, the perturbations induced $\delta_{^{13}C}$ for E·[4-^{13}C]NAD^+ indicate that the binding of uridine nucleotides increases the intrinsic reduction reactivity of NAD^+ in E·NAD^+ by a large factor.

EXPERIMENTAL PROCEDURES

Materials. UDP-galactose 4-epimerase from *E. coli* was purified to homogeneity by use of the procedure of Bauer *et al.* (1991). UDP (sodium salt) was obtained from Sigma and further purified by ion-exchange chromatography according to the general method of Richard & Frey (1982) for nucleotides to yield the triethylammonium salt which was then converted to the sodium salt by passage through a column of SP-Sephadex C-25-120 followed by lyophilization. UMP-morpholidate (4-morpholine-*N*,*N*′-dicyclohexylcarboxamide salt) was obtained from Sigma. [^{15}N]NH_4OH (6 M, 99.9% ^{15}N) was purchased from Isotec.

Assays. Unless otherwise noted, UDP-galactose 4-epimerase was assayed and the enzyme concentrations determined as described by Wilson and Hogness (1964). For those enzyme samples containing UDP or MeUDP, the concentrations of uridine nucleotide and enzyme were determined by measuring the absorbance at 262 and 280 nm and then solving simultaneous equations using the appropriate extinction coefficients for epimerase (Konopka *et al.*, 1989) and either UDP (ϵ_{262} = 9.24 mM^{-1} cm^{-1}, ϵ_{280} = 3.84 mM^{-1} cm^{-1}) or MeUDP (ϵ_{262} = 9.11 mM^{-1} cm^{-1}, ϵ_{280} = 3.65 mM^{-1} cm^{-1}) in 100 mM potassium phosphate containing 1 mM EDTA at pH 7.0.

The enzyme was assayed at the time each experiment described in this paper was carried out, and the activity ranged between 8000 and 6000 units per mg of protein. The specific activity of the purified enzyme was about 8000 units per mg of protein. This was somewhat lower than the highest specific activity that has been reported (Wilson and Hogness, 1964), which is 12 000 units per mg. Although the enzyme was homogeneous, the lower activity was due to the fact that UDP-galactose 4-epimerase obtained from overexpression systems contains a larger fraction of its pyridine nucleotide in the form of NADH than the most active epimerase. NADH bound to the epimerase does not exchange with NAD^+ under the conditions in which enzyme-bound NAD^+ exchanges with isotopically enriched samples of NAD^+ such as [^{14}C]NAD^+ or [4-^{13}C]NAD^+. In the NMR experiments described in this paper, the presence of NADH at some of the coenzyme sites of UDP-galactose 4-epimerase does not interfere with the observations of NMR signals for [^{15}N]NAD^+ or [^{13}C]NAD^+ at the other coenzyme sites. Specific enzymatic activities of NMR samples were monitored by removing 10-μL aliquots from the NMR samples and diluting to 1 mL with buffer (100 mM potassium phosphate containing 1 mM EDTA at pH 7.0) and freezing until it could be assayed at a later time. The enzymatic activities decreased slightly in the course of the NMR experiments, generally by not more than 20%.

Synthesis of ^{13}C- and ^{15}N-Enriched NAD^+. The synthetic methods previously reported for [1-^{15}N]nicotinamide (Oppenheimer *et al.*, 1978), [2-^{13}C]nicotinamide (Bryson *et al.*, 1974b), [4-^{13}C]nicotinamide (Oberfrank *et al.*, 1984), and [6-^{13}C]nicotinamide (Bryson *et al.*, 1974a) were applied to the syntheses of these compounds. [*carboxamide*-^{15}N]Nicotinamide was synthesized from ethyl nicotinate and 6 M [^{15}N]NH_4OH (LaForge, 1928). All labeled nicotinamides were over 99% isotopically enriched at the positions indicated. Each labeled nicotinamide was exchanged into NAD^+ by use of NAD^+ glycohydrolase and thio-NAD^+ according to the procedure of Williams *et al.* (1976) and further purified by chromatography, typically by applying 250 mg 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. Fractions were collected and analyzed by measurements of A_{260}. NAD^+-containing fractions were pooled, evaporated to a syrup, and precipitated with ice-cold ethanol. The resulting lithium salt of NAD^+ was isolated by vacuum filtration and washed with ice-cold ethanol until the filtrate was negative to chloride as determined by testing with 5% $AgNO_3$. It was dried *in vacuo* to remove any remaining ethanol.

Synthesis of N-Substituted Nicotinamides. 1-Ethylnicotinamide bromide (**1a**), 1-benzylnicotinamide bromide (**1b**), 1-(4-cyanobenzyl)nicotinamide bromide (**1c**), 1-(carbomethoxymethyl)nicotinamide bromide (**1d**), and 1-(cyanomethyl)nicotinamide bromide (**1e**) were synthesized as previously described (Norris & Steward, 1977; Bunting & Sindhuatmadja, 1981; Hirayama *et al.*, 1985).

Synthesis of P^1-Uridyl-P^2-methyl Diphosphate (MeUDP). Methyl dichlorophosphate (0.8 mL, 8 mmol) was added to a solution of 9 mL of water and 1 mL of pyridine. After stirring at room temperature for 30 min, the solution was washed with ether (2 × 25 mL), and the water was removed by rotary evaporation. The residue was dried by repeated (3 times) addition of 10 mL of anhydrous pyridine and rotary evapo-

ration. To the resulting pyridinium salt of methyl phosphate was added a solution of UMP-morpholidate (1.1 g, 1.6 mmol) in 10 mL of anhydrous pyridine. The solution was again evaporated to dryness, and the residue was dissolved in 15 mL of anhydrous pyridine and stirred at room temperature under nitrogen for 20 h. Pyridine was removed by rotary evaporation, and the residue was dissolved in 30 mL of 0.6 M lithium acetate and washed with chloroform (2 × 30 mL). The aqueous layer was diluted to 500 mL and applied to a Bio-Rad AG1-X4 anion exchange column (2.5 × 42 cm, 100–200 mesh, chloride form). The column was eluted by use of a linear gradient formed from 1.8 L of 50 mM LiCl and 1.8 L of 500 mM LiCl. MeUDP emerged between 1.8 and 2.2 L of the effluent and was detected by measuring the absorbance at 260 nm. The product-containing fractions were pooled and evaporated to dryness. The residue was suspended in 300 mL of cold ethanol, filtered, and washed with cold ethanol until the filtrate was free of chloride as tested with 5% $AgNO_3$. The isolated solid was dried *in vacuo* to yield the desired product as the lithium salt (250 mg, 36%): ^{1}H NMR (500 MHz, D_2O) δ 3.59 ppm (d, J_{P-H} = 11 Hz, 3 H), δ 4.13–4.37 ppm (m, 5 H), δ 5.90 ppm (d, J = 8 Hz, 1 H), δ 5.94 ppm (d, J = 4 Hz, 1 H), δ 7.90 ppm (d, J = 8 Hz, 1 H); ^{13}C NMR (101 MHz, D_2O) δ (ppm) 56, 67, 72, 77, 86, 91, 105, 144, 154, 169; ^{31}P NMR (162 MHz, D_2O) δ −11.40 ppm (d, J_{P-P} = 21 Hz), −9.62 ppm (dq, J_{P-H} = 11 Hz, J_{P-P} = 21 Hz).

Exchange of ^{13}C- and ^{15}N-Enriched NAD$^+$ into UDP-Galactose 4-Epimerase. ^{13}C- and ^{15}N-enriched NAD$^+$ were incorporated into the enzyme by use of the procedure of Vanhooke and Frey,[2] in which the enzyme is partially denatured with 2.65 M urea in the presence of 10-fold molar excess of the ^{13}C- or ^{15}N-enriched NAD$^+$. After allowing the exchange 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 ultrafiltration unit equipped with a YM30 membrane, and dialyzed against 110 mM KP_i buffer containing 1 mM EDTA at pH 7.0 (pH adjusted at room temperature) for 15 h (changing dialysate twice) at 4 °C. The specific activity of each sample of dialyzed enzyme obtained following the exchange of labeled NAD$^+$ was at least as high as that of the enzyme prior to undergoing the exchange process. Therefore, the effects of 2.65 M urea did not cause any loss of enzymatic activity. Prior to analysis by NMR, D_2O was added to a concentration of 10%.

NMR Spectroscopy of ^{13}C- and ^{15}N-Enriched NAD$^+$. Proton-decoupled ^{13}C NMR spectra were obtained at 32 °C in 10-mm tubes at either 100.6 MHz on a Bruker AM-400 spectrometer, 125.7 MHz on a Bruker AM-500 spectrometer, or 150.9 MHz on a Bruker AM-600 spectrometer using 10-mm broad-band probes. Data were collected at spectral widths of 20, 25, and 30 kHz at 100.6, 125.7, and 150.9 MHz, respectively, with 50–60° pulse angles and a 2-s relaxation delay. Protein spectra were processed with 10- to 75-Hz Lorentzian line broadening (LB). ^{13}C chemical shifts are reported as ppm downfield from 3-(trimethylsilyl)propanesulfonic acid, sodium salt (DSS), although they were referenced to external 10% dioxane in water (10% D_2O), which was assigned a shift of 69.22 ppm. The chemical shift of the reference was determined both before and after the acquisition of each spectrum.

^{15}N NMR spectra were obtained at 32 °C in either 10- or 20-mm tubes at 40.5 MHz on a Bruker AM-400 spectrometer using either a 10- or 20-mm broad-band probe. Using the 10-mm probe, data were collected at a spectral width of 8.5 kHz with a 30° pulse angle and a 2-s relaxation delay. Using the 20-mm probe, data were collected at a spectral width of 9 kHz with a 50° pulse angle and a 3-s relaxation delay. Proton decoupling was not employed in experiments using [1-^{15}N]NAD$^+$ in order to avoid signal loss due to a negative nuclear Overhauser effect. Protein spectra were processed with 4–30-Hz Lorentzian line broadening. ^{15}N chemical shifts are reported as ppm downfield from NH_3, although they were referenced to external $^{15}NH_4Cl$ (23 mM) in 1 M HCl (10% D_2O), which was assigned a shift of 24.93 ppm (Levy & Lichter, 1979). The chemical shift of the reference was determined both before and after the acquisition of each spectrum. Line-shape analyses were performed by use of a Gaussian resolution enhancement (Bruker software) to obtain line widths at half-height, $\Delta\nu_{1/2}$.

^{13}C NMR Spectroscopy of N-Substituted Nicotinamides. Proton-decoupled ^{13}C NMR spectra of 35 mM aqueous (50% D_2O) and 5 mM ethanolic (86% ethanol, 7% D_2O) solutions of each model nicotinamide were obtained at 100.6 MHz on a Bruker AM-400 spectrometer with a 10-mm broad-band probe at 25 °C. Chemical shifts for ^{13}C were determined from the natural abundance of the isotope and referenced as described for ^{13}C-enriched NAD$^+$. In order to assign the ^{13}C NMR resonances accurately, two-dimensional ^{13}C–^{1}H correlation spectra of some of the aqueous model nicotinamide solutions were collected on a Bruker AM-500 (125.7 MHz) spectrometer with a 5-mm inverse broad-band probe by use of the pulse sequence of Zuiderweg (1990).

Determination of Inhibition Constants for UDP and MeUDP. Solutions of UDP-galactose 4-epimerase (0.26 nM) containing 1 mM EDTA, 1.25 mM NAD$^+$, and UDP-glucose dehydrogenase (0.04 units/mL) in either 100 mM KP_i buffer at pH 7.0 or 50 mM sodium pyrophosphate buffer at pH 9.8 were incubated at 32 °C with varying concentrations of UDP-galactose (five concentrations ranging from 43 to 324 mM) at different fixed concentrations of either UDP (0–110 mM) or MeUDP (0–100 mM). The change in absorbance at 340 nm, corresponding to the formation of NADH from NAD$^+$ is proportional to twice the epimerase activity (Wilson & Hogness, 1964). Kinetic cnstants were obtained from nonlinear regression analyses of the data (Cleland, 1979).

Reduction of N-Substituted Nicotinamide Salts by Sodium Cyanoborohydride. The rates of reduction of the model nicotinamides in aqueous solution were measured by adding 100 μL of 500 mM sodium cyanoborohydride in 100 mM HEPES buffer at pH 7.0 to 900 μL of a 2.22 mM solution of each nicotinamide. The analogous rates of reduction of the model nicotinamides in 86% ethanol were also measured by adding 100 μL of 500 mM sodium cyanoborohydride in 100 mM HEPES buffer at pH 7.0 to 900 μL of a 2.22 mM solution of each nicotinamide in 95% ethanol except for **1e** (X = CN), in which a 50 mM solution of sodium cyanoborohydride was used instead, owing to the rapid rate of reduction of this compound. Initial rate measurements (less than 5% of complete reaction) at 25 °C were made by monitoring the change in absorbance at 362 nm as a function of time by use of a Hewlett-Packard 8452A diode array spectrophotometer. The rates were calculated by use of the appropriate extinction coefficients at 362 nm, which were determined for each reduced model nicotinamide by reducing 0.16 mM aqueous and ethanolic (86%) solutions with sodium borohydride and measuring the ΔA_{362}.

[2] J. L. Vanhooke & P. A. Frey, personal communication.

Table I: ^{15}N and ^{13}C NMR Chemical Shifts for Complexes of [^{15}N]NAD^+ and [^{13}C]NAD^+ with UDP-Galactose 4-Epimerase[a]

enriched NAD^+	δ (ppm)			
	free	E·NAD^+	E·NAD^+·UDP	E·NAD^+·MeUDP
[1-^{15}N]NAD^+	221.66	225.93	222.94	223.28
[$CONH_2$-^{15}N]NAD^+	111.35	99.97	99.95	
[4-^{13}C]NAD^+	148.56	149.82	153.2	
		149.58		152.30
		149.73 (pH 9.8)		152.15 (pH 9.8)
[6-^{13}C]NAD^+	145.12	151.38	150.07	
[2-^{13}C]NAD^+	142.55	139.29	140.65	

[a] Phosphate buffer at pH 7 or pyrophosphate buffer at pH 9.8.

Determination of Reduction Potentials of N-Substituted Nicotinamide Salts. The general method of Wallenfels and Diekmann (1959) was followed, in which solutions of the model nicotinamide (0.067 mM) in 50 mM Tris buffer at pH 8.0 was combined with KCN at various concentrations (0–2 M) while keeping the ionic strength at 2 M with KCl. After allowing equilibration at 20 °C for 15 min, the equilibrium constant was calculated from the absorbance at ~340 nm which corresponds to the formation of the 4-cyano-1,4-dihydropyridine adduct. By comparing the equilibrium constant of a model nicotinamide to that for NAD^+, which has a reduction potential of −348 mV at pH 8.0, versus the normal hydrogen electrode at 20 °C (Rodkey, 1955), and assuming that this exchange is identical to that for direct hydride exchange, a value for the reduction potential for the model compound can be calculated.

RESULTS

Inhibition of UDP-Galactose 4-Epimerase by UDP and MeUDP. The inhibition constants for the inhibition of UDP-galactose 4-epimerase by UDP and MeUDP in 100 mM potassium phosphate containing 1 mM EDTA at pH 7.0 and 32 °C were required to interpret the NMR data. The following inhibition constants were obtained: for UDP, K_i = 34 μM and $K_{mUDPGal}$ = 6 μM; for MeUDP, K_i = 8 μM and $K_{mUDPGal}$ = 8 μM. At pH 9.8 in 50 mM sodium pyrophosphate the following results were obtained for MeUDP; K_i = 110 μM and $K_{mUDPGal}$ = 216 μM.

^{15}N NMR Spectra of [1-^{15}N]NAD^+ *and* E·[1-^{15}N]NAD^+. The changes in the ^{15}N NMR spectrum of the coenzyme enriched with ^{15}N at N-1 ([1-^{15}N]NAD^+) upon binding to the enzyme are shown in Figure 1 together with the effects on the spectrum of increasing the concentration of UDP. The signal for the enzyme-bound coenzyme appears downfield by 4.27 ppm relative to its position in solution. The chemical shift values obtained are given in Table I, and the observed line widths ($\Delta\nu_{1/2}$) are in the legend to Figure 1. The ^{15}N-signal is shifted upfield as the UDP concentration is increased. The ^{15}N chemical shifts for mixtures of UDP and E·[1-^{15}N]-NAD^+ are linearly correlated with the percent of epimerase complexed with UDP at the substrate site, as calculated using the inhibition constant of 34 μM for the dissociation constant at pH 7.0. A short extrapolation to saturation indicates a maximum upfield perturbation of 3.0 ppm on the ^{15}N chemical shift upon saturation with UDP. Subsequent reduction of this enzyme sample by addition of glucose resulted in a shift of the nitrogen signal to 112.5 ppm, a value that is very similar to that for N-1 in free NADH.

An upfield perturbation is also brought about by MeUDP as the activating uridine nucleotide. An analysis similar to that in Figure 1 showed that MeUDP brings about an upfield perturbation of 2.65 ppm upon binding to E·[1-^{15}N]NAD^+. The values of the ^{15}N chemical shifts are given in Table I.

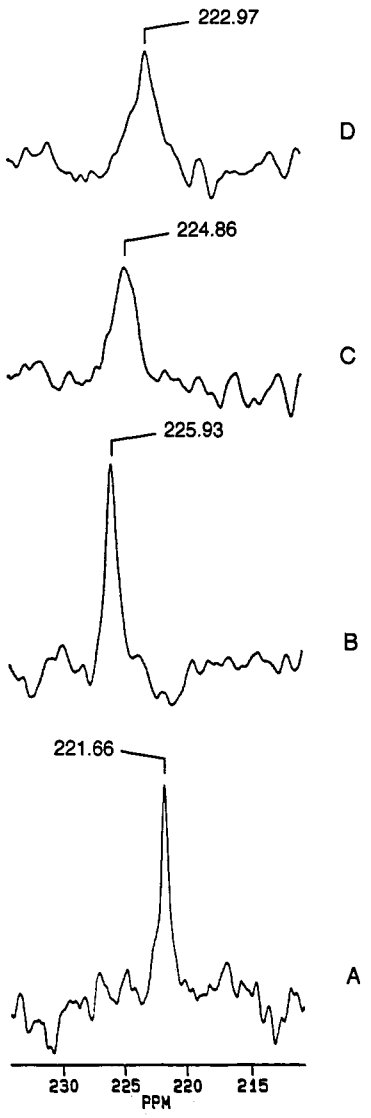


FIGURE 1: ^{15}N NMR spectra of [1-^{15}N]NAD^+, its complex with UDP-galactose 4-epimerase (E·[1-^{15}N]NAD^+), and the effect of UDP. The following samples were prepared and their spectra obtained in 20-mm tubes as described in the Experimental Procedures: (A) 4 mM [1-^{15}N]NAD^+ in 100 mM potassium phosphate containing 1 mM EDTA at pH 7.0, NS = 500, LB = 20 Hz, $\delta^{15}N$ = 221.66 ppm, $\Delta\nu_{1/2}$ = 43 Hz; (B) E·[1-^{15}N]NAD^+ (1.08 mM) in the same buffer, NS = 10 000, LB = 30 Hz, $\delta^{15}N$ = 225.93 ppm, $\Delta\nu_{1/2}$ = 51 Hz; (C) E·[1-^{15}N]NAD^+ (1.08 mM) plus 0.44 mM UDP, NS = 10 070, LB = 30 Hz, $\delta^{15}N$ = 224.86 ppm, $\Delta\nu_{1/2}$ = 84 Hz; (D) [E·[1-^{15}N]NAD^+ (1.06 mM) plus 3.87 mM UDP, NS = 10 470, LB = 30 Hz, $\delta^{15}N$ = 222.97 ppm, $\Delta\nu_{1/2}$ = 92 Hz.

^{13}C NMR Spectra of [4-^{13}C]NAD^+ *and* E·[4-^{13}C]NAD^+. The proton-decoupled ^{13}C NMR spectrum (100.6 MHz) of the coenzyme enriched in ^{13}C at nicotinamide C-4 ([4-^{13}C]-NAD^+) bound to the enzyme are shown in Figure 2, together with the effect of increasing concentrations of UDP on $\delta^{13}C$

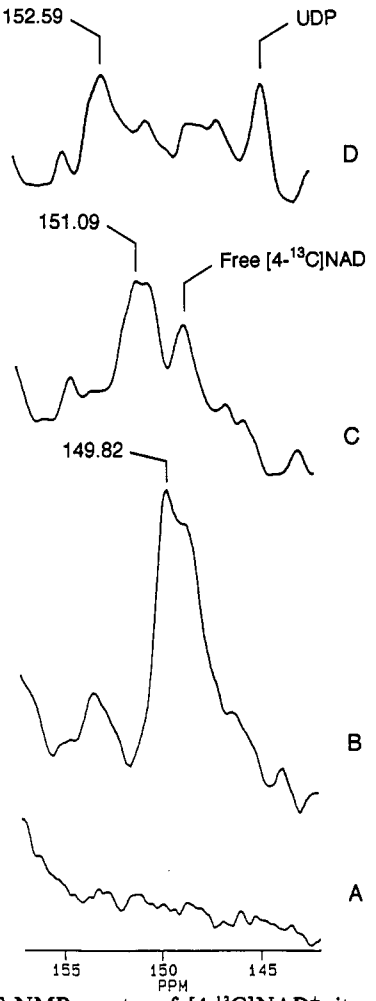


FIGURE 2: ^{13}C NMR spectra of [4-^{13}C]NAD^+, its complex with UDP-galactose 4-epimerase (E·[4-^{13}C]NAD^+), and the effect of UDP. The following samples were prepared and spectra obtained at 100.6 MHz as described in the Experimental Procedures: (A) epimerase (0.79 mM) in 100 mM K-phosphate containing 1 mM EDTA at pH 7.0, NS = 19 100, LB = 50 Hz; (B) E·4-^{13}C]NAD^+ (0.75 mM) in the same buffer, NS = 13 100, LB = 70 Hz, $\delta^{13}C$ = 149.82 ppm, $\Delta\nu_{1/2}$ = 146 Hz; (C) E·[4-^{13}C]NAD^+ (0.75 mM) plus 0.40 mM UDP (49% saturation), NS = 14 000, LB = 70 Hz, $\delta^{13}C$ = 151.09 ppm, $\Delta\nu_{1/2}$ = 185 Hz; (D) [E·[4-^{13}C]NAD^+ (0.66 mM) plus 3.87 mM UDP (99% saturation), NS = 15 700, LB = 70 Hz, δ^{13} = 152.59 ppm, $\Delta\nu_{1/2}$ = 195 Hz.

for E·[4-^{13}C]NAD^+. The signal for the enzyme-bound coenzyme was shifted downfield by 1.26 ppm from its position in solution. The chemical shift values obtained are given in Table I and the observed line widths, $\Delta\nu_{1/2}$ in the legend to Figure 2. The signal for E·[4-^{13}C]NAD^+ was then shifted further downfield as the UDP concentration increased. A plot of $\delta^{13}C$ versus the percent of epimerase bound to UDP (not shown) was linear and showed a maximum downfield perturbation of 2.8 ppm upon saturation with UDP.

As shown in Figure 2, the ^{13}C signal from E·[4-^{13}C]NAD^+ becomes increasingly broadened as it is shifted downfield by increasing concentrations of UDP. If a simple binding system such as that described by eq 1 were in effect, and the exchange

$$E{\cdot}NAD^+ + UDP \rightleftharpoons E'{\cdot}NAD^{+}{*}{\cdot}UDP \quad (1)$$

between the two forms of the enzyme were in the intermediate to fast exchange region as compared with the difference in their chemical shifts, we would expect a single exchange-averaged signal. In such a system, upon going from zero UDP to 49% saturating levels of UDP the line width should increase due to an exchange-broadened contribution (Drago, 1977). However, one would expect that increasing the UDP concentration to 99% saturation should cause the signal to be sharpened because of the fact that the exchange-broadening contribution due to the free enzyme would become very small.

The fact that the UDP-saturated signal remains broadened can be rationalized by an intermediate exchange rate between two putative distorted conformers that constitute the UDP-saturated enzyme. This is described by the model of eq 2, in which UDP binds

$$E{\cdot}NAD^+ + UDP \rightleftharpoons E{\cdot}NAD^{+}{\cdot}UDP \rightleftharpoons E'{\cdot}NAD^{+}{*}{\cdot}UDP \quad (2)$$

to the enzyme to form a complex that is in equilibrium with another species having the same chemical composition but a different conformation. In the first complex the coenzyme is in a resting state, and in the second complex an enzyme conformational change destabilizes the coenzyme into an activated state symbolized in eq 2 by $NAD^{+}{*}$. In this model, the observed $\delta^{13}C$ is an exchange-averaged signal for the two complexes, even at saturating levels of UDP, and is broadened due to an intermediate-to-fast-exchange process. If this model is correct, the ^{13}C chemical shift for the hyperreactive coenzyme in E'·$NAD^{+}{*}$·UDP is shifted farther downfield from the *observed* exchange-averaged UDP-saturated signal.

Increasing the field strength to 125.7 MHz (Figure 3B) resulted in a further broadening of the ^{13}C NMR signal for the UDP-saturated epimerase. Further increasing the field strength to 150.9 MHz (Figure 3C) appeared to give two distinct signals in place of an exchange-averaged one, presumably owing to the fact that the rate of the interconversion between the two states is now slower than the NMR time scale at the field strength of 150.9 MHz. The two signals consist of a larger one at 153.2 ppm and a smaller one at 151.06 ppm. The signal at 153.2 ppm is perturbed upfield by 3.4 ppm relative to that for E·[4-^{13}C]NAD^+ in the absence of UDP. From the difference in the chemical shifts and the fact that a single exchange-averaged signal is seen at 100.6 MHz, the rate of exchange between the two forms can be calculated as described by Drago (1977) to be between 475 and 720 s^{-1}; these values are in the range of the value of k_{cat} for the enzymatic reaction. The signals at 144.4 and 154.4 ppm are due to ^{13}C at natural abundance in free UDP.

A similar perturbation of the ^{13}C NMR chemical shift for E·[4-^{13}C]NAD^+ is brought about by MeUDP as the activating uridine nucleotide, as shown in Figure 4. At 100.6 MHz, an analysis similar to that in Figure 2 showed that the binding of MeUDP perturbs the chemical shift upfield. The maximum upfield perturbations by MeUDP at pH 7.0 and 9.8 are given in Table I and in the same direction as those observed for the binding of UDP. The perturbed signal induced by MeUDP is considerably less broadened at 100.6 MHz than those induced by UDP, indicating that the exchange rates corresponding to eq 2 are much faster in the case of MeUDP than when UDP is the activating nucleotide. Inasmuch as this is probably an exchange-averaged signal, the observed perturbation is a minimum value that corresponds to a composite for two species in equilibrium.

^{13}C NMR Spectra of [6-^{13}C]NAD^+ and E·[6-^{13}C]NAD^+. The changes in the principal proton-decoupled ^{13}C NMR chemical shift of [6-^{13}C]NAD^+ obtained at 100.6 MHz upon binding to the enzyme at pH 7.0, together with the effect of increasing concentrations of UDP, are given in Table I. The binding of [6-^{13}C]NAD^+ to the enzyme perturbs the chemical shift downfield by 6.3 ppm compared with its value in solution.

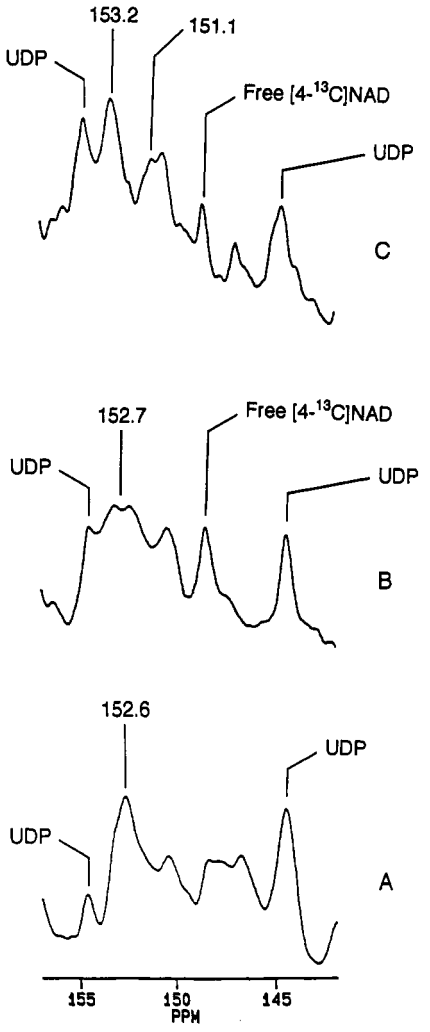


FIGURE 3: Effect of field strength on the proton-decoupled ^{13}C NMR spectrum of the complex E·[4-^{13}C]NAD$^+$·UDP. Enzyme samples were prepared and spectra obtained as described in the Experimental Procedures: (A) sample identical to Figure 2D, spectra obtained at 100.6 MHz, NS = 15 700, LB = 70 Hz, $\Delta\nu_{1/2}$ = 195 Hz; (B) identical sample, spectra obtained at 125.7 MHz, NS = 16 700, LB = 60 Hz, $\Delta\nu_{1/2}$ = ~380 MHz; (C) identical sample, spectra obtained at 150.9 MHz, NS = 7000, LB = 75 Hz.

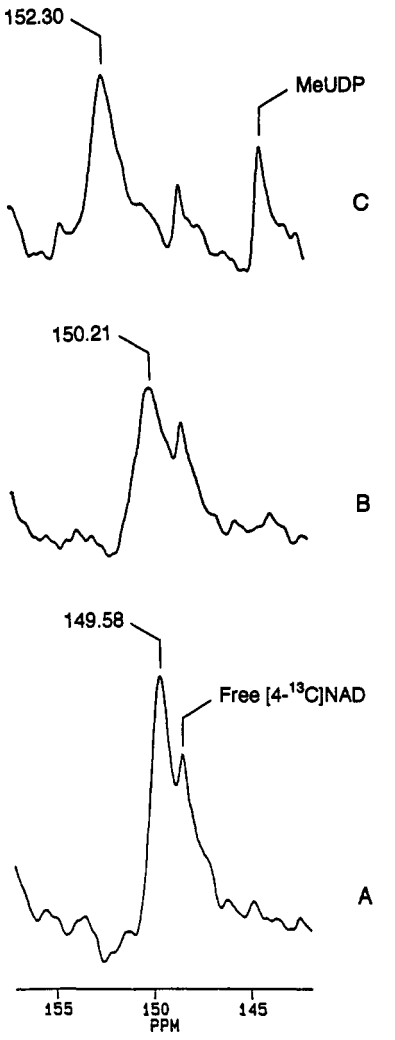


FIGURE 4: Effect of MeUDP on the proton-decoupled ^{13}C NMR spectrum of E·[4-^{13}C]NAD$^+$. Enzyme samples were prepared and spectra obtained at 100.6 MHz as described in the Experimental Procedures: (A) [4-^{13}C]NAD$^+$ bound to epimerase (0.72 mM) in 100 mM potassium phosphate, 1 mM EDTA, pH 7.0; NS = 29 900, LB = 50 Hz, $\delta^{13}C$ = 149.58 ppm, $\Delta\nu_{1/2}$ = 76 Hz; (B) [4-^{13}C]NAD$^+$ bound to epimerase (0.71 mM) with 0.20 mM MeUDP (28% saturation), NS = 30 200, LB = 50 Hz, $\delta^{13}C$ = 150.21 ppm, $\Delta\nu_{1/2}$ = 142 Hz. (C) [4-^{13}C]NAD$^+$ bound to epimerase (0.63 mM) with 3.59 mM MeUDP (100% saturation), NS = 34 415, LB = 50 Hz, $\delta^{13}C$ = 152.30 ppm, $\Delta\nu_{1/2}$ = 106 Hz.

The signal for E·[6-^{13}C]NAD$^+$ is perturbed slightly upfield (1.3 ppm) by saturation with UDP.

^{13}C NMR Spectra of [2-^{13}C]NAD$^+$ and E·[2-^{13}C]NAD$^+$. The change in the principal ^{13}C-chemical shift of [2-^{13}C]-NAD$^+$ obtained at 100.6 MHz, upon binding to the enzyme at pH 7.0 is given in Table I, together with the effect of saturating UDP on the principal $\delta^{13}C$ exhibited by E·[2-^{13}C]-NAD$^+$. The signal for the enzyme-bound coenzyme was shifted upfield by 3.3 ppm from its position in solution, and that for the enzyme-bound coenzyme was perturbed downfield 1.4 ppm by saturating UDP. The reduced complex E·[2-^{13}C]NADH·UDP, obtained by reducing the enzyme sample with 0.2 M glucose, gave a signal at 134.4 ppm. The signal for nicotinamide-C-2 of NADH in solution appears at 141.0 ppm (spectrum not shown).

^{15}N NMR Chemical Shift of [carboxamide-^{15}N]NAD$^+$. The difference in the proton-decoupled ^{15}N NMR spectrum of [*carboxamide*-^{15}N]NAD$^+$ in solution compared with that in the coenzyme binding site of UDP-galactose 4-epimerase at pH 7.0 is given in Table I. The signal for the enzyme-bound coenzyme is upfield 11.6 ppm relative to its position in solution. The signal for the enzyme-bound coenzyme did not change when UDP was added to 79% saturation and then to saturating levels of UDP. This signal did not change when the coenzyme was subsequently reduced with 0.2 M glucose in the presence of 3.9 mM UDP.

Correlation of the Reduction Rates of N-Substituted Nicotinamides with ^{13}C Chemical Shifts of Carbon-4. The second-order rate constants governing the reduction of N-substituted nicotinamides **1a–e** by sodium cyanoborohydride in aqueous solutions and in 86% ethanol are given in Table II, together with the values of $\delta^{13}C$ assigned to nicotinamide-C-4 for each compound in water and 86% ethanol$_{(aq)}$. Table II shows that the rates of reduction are enhanced by increasing the electron-withdrawing effect of the substituent X. This indicates, as expected, a significant increase in the electron density in the nicotinamide ring upon proceeding to the transition state from the reactant nicotinamide cation. Changes in the value of $\delta^{13}C$ at C-4 should represent changes in the electron density at this center. The relationship between the second-order rate constants for reduction and the values of $\delta^{13}C$ is *logarithmic*. Semilogarithmic plots of the rate constants for reduction against $\delta^{13}C$

Table II: Chemical Shifts for C-4, Second-Order Rate Constants, and Reduction Potentials for Reduction of *N*-Alkylnicotinamides by $NaBH_3CN$[a]

compound	X–	$k \times 10^3$ [b] (M^{-1} s^{-1})	$k \times 10^3$ [c] (M^{-1} s^{-1})	$\delta^{13}C$[b] (ppm)	$\delta^{13}C$[c] (ppm)	$E°$ [e] (mV)
1a	CH_3–	0.14 ± 0.03	1.18 ± 0.06	146.40	145.98	–424 ± 8
1b	Ph–	0.44 ± 0.13	5.84 ± 0.56	146.93	146.40	–394 ± 6
1c	*p*-NCPh–	1.57 ± 0.28	22.7 ± 1.0	147.35	146.82	–376 ± 11
1d	CH_3O_2C–	3.53 ± 0.25	69.2 ± 3.6	147.93	147.56	–359 ± 6
1e	NC–	49.2 ± 2.2	1600 ± 160[d]	148.83	148.41	–319 ± 7

[a] Rates of reduction of 2 mM solutions of *N*-alkylnicotinamide compounds **1a–e** by 50 mM $NaBH_3CN$ were measured in triplicate and second-order rate constants calculated. Second-order kinetics was verified for compound **1b** (X = Ph). [b] Measured in water. [c] Measured in 86% ethanol. [d] Rate measured using 5 mM $NaBH_3CN$. [e] The $E°$ values were measured at pH 8.0 and 20 °C. They were calculated from cyanide equilibrium data and based on a value of –348 mV for NAD^+ at pH 8.0. Each value is the mean of three determinations and includes the 90% confidence limits. See the Experimental Procedures for details.

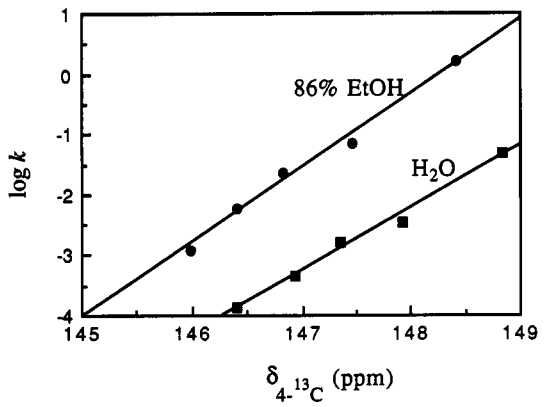


FIGURE 5: Correlation of 4-^{13}C chemical shifts for *N*-alkylnicotinamides with reactivity toward sodium cyanoborohydride. The logarithms of the second-order rate constants for the reduction of compounds **1a–e** by sodium cyanoborohydride are plotted versus the values of the ^{13}C chemical shifts for C-4 in the same molecules. The filled square symbols refer to the reaction in water (slope = 1.03) and the filled circles to the reaction in 86% ethanol$_{(aq)}$ (slope = 1.23). The rate measurements are described in the Experimental Section.

for these model compounds are straight lines, as shown in Figure 5, for the reaction both in aqueous solutions and in 86% ethanol$_{(aq)}$ (squared linear correlation coefficients of 0.99). The plot of log k versus $\delta^{13}C$ of data obtained in aqueous solution gives a slope of 1.03, and that of data obtained in 86% ethanol$_{(aq)}$ gives a slope of 1.23.

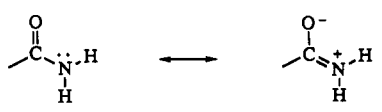
Correlation of C-4 Chemical Shifts with Reduction Potentials for N-Substituted Nicotinamides. Two-electron reduction potentials for the N-substituted nicotinamides were calculated using an indirect method, in which aqueous solutions of the nicotinamide salts were allowed to equilibrate with cyanide ion at 20 °C (Wallenfels & Diekmann, 1959). It is known that the addition of cyanide is reversible, that it takes place at the 4-position, and that free energy changes associated with cyanide addition correspond well with the free energy changes of direct hydride transfer (Wallenfels, 1959). The equilibrium constant was determined by studying the equilibrium at different cyanide concentrations. Therefore, by comparing the equilibrium constant for reaction of cyanide with a model nicotinamide to that for NAD^+, which has a reduction potential of –348 mV versus the normal hydrogen electrode at 20 °C and pH 8 (Rodkey, 1955), the reduction potential for the model compound can be approximated. Two-electron reduction potentials calculated using this cyanide affinity method have been shown to agree very well with those measured both enzymatically and potentiometrically (Wallenfels, 1959; Blankenhorn, 1976).

The reduction potentials ($E°$) for the series of model N-substituted nicotinamides (**1a–e**) are included in Table II. As expected, an increase in the electron-withdrawing capacity of the substituent makes the reduction potential more positive. The relationship between reduction potential and $\delta^{13}C$ of C-4 is *linear* for compounds **1a–e**, and the slope of the plot is 44.7 mV ppm^{-1} (squared linear correlation coefficient of 0.99).

DISCUSSION

Microenvironmental Effects of the NAD^+ Binding Site on ^{15}N and ^{13}C NMR Signals for Nicotinamide-Labeled NAD^+. The principal objective of the present research was to probe the interactions between the uridine nucleotide binding site and the NAD^+ binding site of UDP-galactose 4-epimerase. The experiments provided auxiliary information about the difference between the microenvironment of NAD^+ in its binding site and the aqueous environment of free NAD^+. The data in Table I show significant differences between the chemical shifts for ^{15}N and ^{13}C in the nicotinamide moiety of NAD^+ in the enzymatic site compared with their values in solution.

Experiments with [1-^{15}N]NAD^+ showed that the signal for the enzyme-bound coenzyme at pH 7.0 is very narrow, presumably owing to the fact that N-1 is not directly bonded to a hydrogen atom and, therefore, the spin–spin relaxation time is long (Oppenheimer & Davidson, 1980). The signal was found to lie 4.3 ppm downfield from its position in solution. Nitrogen-1 is quaternary and positively charged in NAD^+ and is presumably less solvated in the active site; it is known that desolvation of positively charged ^{15}N atoms moves their NMR signals downfield (Duthaler & Roberts, 1979; Schuster et al., 1979; Oppenheimer & Davidson, 1980). Reduction of the complex E·[1-^{15}N]NAD^+·UDP by glucose shifted the signal upfield to 112.5 ppm, which is similar to the value for [1-^{15}N]NADH in aqueous solution (Oppenheimer & Davidson, 1980; as calculated by subtracting the difference between the reported $\delta^{15}N$ of [1-^{15}N]NAD^+ and [1-^{15}N]NADH from the value of $\delta^{15}N$ for NAD^+ in Figure 1). The ^{15}N NMR signal for enzyme-bound [*carboxamide*-^{15}N]NAD^+ appeared 11.6 ppm upfield from its position in solution. This difference is most probably due to hydrogen bond donation from the amide protons to amino acid residues in the active site, which would make the charge-separated resonance form shown below less important in the carboxamide structure (Gattegno *et al.*, 1976). The crystal structure of the enzyme with P^1-uridyl-



P^2-phenyl diphosphate bound at the substrate site shows the amide protons within hydrogen bonding distances of the main chain peptide carbonyl groups of phenylalanine 178 and tyrosine 177 (Frey *et al.*, 1992).

The ^{13}C chemical shift for [4-^{13}C]NAD^+ bound to the coenzyme site was found to be very similar to that in solution, whereas those for [2-^{13}C]NAD^+ and [6-^{13}C]NAD^+ differed significantly from the solution values. Inasmuch as NAD^+ bound to the coenzyme site is reduced exclusively at C-4 by sodium [^3H]borohydride to [4-β-^3H]NAD^+ (Nelsestuen & Kirkwood, 1971; Wee & Frey, 1973), nicotinamide C-4 may be partially exposed to the solution, so that the ^{13}C chemical shifts for [4-^{13}C]NAD^+ in solution and in the coenzyme site are similar. In contrast, the signal for [6-^{13}C]NAD^+ at the coenzyme site was found to be downfield by 6.3 ppm and that for [2-^{13}C]NAD^+ upfield by 3.3 ppm from their positions in solution. The microenvironments of C-6 and C-2 in the coenzyme site clearly differ from each other and from the solution environment, indicating that the nicotinamide ring is partially buried. This is proven to be the case by the crystal structure, which shows it to be buried with no fixed water molecules in close proximity to the nicotinamide ring (Bauer *et al.*, 1992). Carbon-6 is deshielded by its microenvironment in the coenzyme site to an extent that is comparable to the deshielding of N-1, presumably through desolvation. Carbon-2 is more shielded at the coenzyme site than in solution; this may be caused by a ring-current effect from Tyr 177, which is near C-2 and hydrogen bonded to the carboxamide group.

Effects of Uridine Nucleotides on Signals for [^{15}N]NAD^+ and [^{13}C]NAD^+ in the Coenzyme Site. The mechanism by which uridine nucleotides increase the reactivity of NAD^+ bound to the coenzyme site of UDP-galactose 4-epimerase is of interest in the context of theories of the mechanism of enzymatic catalysis. Activation of **E**·NAD^+ toward reducing agents is very likely to be associated with a fundamental aspect of the mechanism by which this enzyme catalyzes hydride transfer, a process that is poorly understood for reactions of pyridine nucleotides. *A priori*, the most probable means by which this increase in reactivity might be brought about could be expected to be one or both of the following: (a) The binding of uridine nucleotides induces an enzyme conformational change that aligns catalytic groups engaged in binding the reducing agent and that promote its reaction with NAD^+. (b) The conformational change induced by the binding of uridine nucleotides increases the intrinsic reactivity of NAD^+ toward reducing agents.

The NAD^+ site does not overlap the uridine diphosphoryl binding locus of the substrate binding site (Bauer *et al.*, 1992), so that any effect of UDP on the reactivity of the nicotinamide ring cannot be attributed to direct contacts between UDP and NAD^+. Both effects (a) and (b) above require the uridine nucleotide to induce a conformational change in the protein, but they differ in the consequences of this conformational change. Effect (a) is conventionally expected to be important in mechanistic enzymology and corresponds to the stabilization of the transition state for hydride transfer through interactions with the reducing substrate, that is, to binding the transition state. In the case of uridine nucleotide dependent reduction of **E**·NAD^+, the process should be more complex because of the fact that the activation mechanism increases the reactivity of **E**·NAD^+ toward *nonspecific* reducing agents such as sodium cyanoborohydride (Davis *et al.*, 1974). The alignment of catalytic groups that could catalyze hydride transfer from an alcoholic group such as galactosyl-C-4 of UDP-galactose cannot be expected to promote the reaction of sodium cyanoborohydride. Effect (b), the chemical activation of NAD^+, can be expected to be important for promoting the reaction with reducing agents in general, including nonspecific agents.

The present results show that the binding of uridine nucleotides by UDP-galactose 4-epimerase perturbs the structure of the nicotinamide ring of NAD^+ bound to the coenzyme site. The perturbation is such that it polarizes the electron distribution in the nicotinamide ring toward N-1 and away from C-4, and this should increase the intrinsic chemical reactivity of NAD^+ toward reducing agents. Mechanism (a) would not entail perturbations in the electronic structure of NAD^+; therefore, we conclude that the observations in this paper support mechanism (b) and do not provide information about mechanism (a). These conclusions follow from the effects of the binding of uridine nucleotides on the chemical shifts of [1-^{15}N]NAD^+ and [4-^{13}C]NAD^+ bound in the coenzyme site of the enzyme. The 3 ppm upfield perturbations of $\delta^{15}N$ in Table I indicate an increase in the nuclear shielding of N-1, and the 2.8–3.4 ppm downfield perturbations of $\delta^{13}C$ for C-4 indicate a decrease in the nuclear shielding brought about by binding UDP or MeUDP in the substrate site. The simplest means by which these changes could be brought about are through a perturbation in the electron density surrounding these nuclei. A polarization of the π-electrons toward N-1 and away from C-4 would lead to the observed changes in chemical shifts.

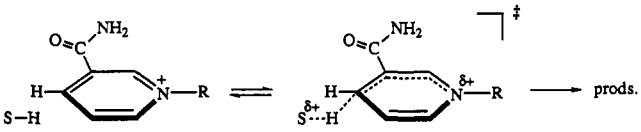
The broad signal for ^{13}C within **E**·[4-^{13}C]NAD^+ at 100.6 MHz in Figure 2 indicated that two species might be present, and this signal was resolved at 150.9 MHz (Figure 3) into two signals, one at 153.2 ppm and another at 151.1 ppm. The composite broad signal at 152.6 ppm in Figure 2D corresponds to these two and may represent slightly different conformational states in equilibrium. If the maximum perturbed value of $\delta^{13}C$ is 152.6 ppm as in Figure 2D, the UDP-induced perturbation is 2.8 ppm downfield; and if it is 153.2 ppm as in Figure 3B, the maximum UDP-induced perturbation is 3.4 ppm downfield. These are significant, easily measured perturbations. The maximum value for the perturbation by UDP is 3.4 ppm, and, because the interactions of UDP cannot be expected to mimic those of a true substrate perfectly, this should be regarded as the minimum value that can be expected for a substrate. The fact that the substrate analogs UDP and MeUDP elicit the perturbations enables us to observe them in isolation from the chemical changes that would take place in the presence of a substrate.

How much of an increase in chemical reactivity can these chemical shift perturbations represent? The answer to this question is the subject of the next section, which shows that the downfield displacement of $\delta^{13}C$ for C-4 of NAD^+ corresponds to a substantial increase in the intrinsic chemical reactivity toward reduction of the nicotinamide ring.

Relationship between Chemical Reactivity and $\delta^{13}C$ for C-4 of N-Substituted Nicotinamides. The data in Table II show that, as expected, the ^{13}C-chemical shifts for C-4 of N-substituted nicotinamides are sensitive to the electronic properties of the substituents X– in the compounds **1a–e**. Moreover, also as expected, the rates at which these compounds are reduced by sodium cyanoborohydride increase with increasing electron withdrawal by the substituent X–. The plots of log k versus $\delta^{13}C$ in Figure 5 give slopes of 1.03 for reduction in water and 1.23 for reduction in 86% ethanol$_{(aq)}$. These slopes allow us to estimate an effect on kinetic reactivity that can be expected from a structural change that induces a 2.8- or 3.4-ppm downfield perturbation in $\delta^{13}C$ for **E**·[4-^{13}C]NAD^+. The slope of 1.03 for reduction in water means that a 2.8 ppm downfield perturbation will correspond to an

increase of 770-fold in kinetic reactivity toward sodium cyanoborohydride, and a 3.4 ppm perturbation corresponds to a 3200-fold increase in reactivity.

The coenzyme binding site of UDP-galactose 4-epimerase is not aqueous; indeed, there is no water in contact with the nicotinamide ring of the enzyme-bound coenzyme. Therefore, if the reduction of N-substituted nicotinamides is sensitive to solvents, rate correlations in water should not be valid for reactions taking place at the active site. The data for 86% ethanol$_{(aq)}$ in Table II may be more representative of an apolar environment such as that in the active site. The rates in 86% ethanol$_{(aq)}$ are substantially faster than those in water; moreover, the slope of the plot of log k versus δ^{13}C in Figure 5 is also larger, 1.23, meaning that the rate in ethanol is more sensitive to downfield changes in chemical shift than it is in water. Enhanced reactivity in ethanol can be attributed to the fact that charge is dispersed in the transition state for hydride transfer, as illustrated below, so that the reaction is more favored in less polar media than in water. The slope of

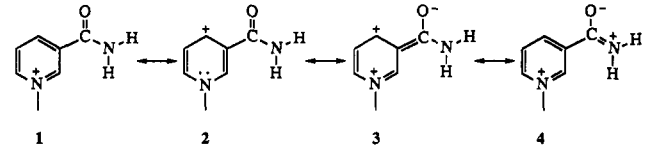


1.23 means that a downfield perturbation of 2.8 ppm in δ^{13}C for C-4 of N-substituted nicotinamides corresponds to a 2800-fold increase in the rate of reduction by sodium cyanoborohydride, and a downfield perturbation of 3.4 ppm corresponds to a 15 000-fold increase in rate.

Because of the fact that we do not know the polarity of the coenzyme site in UDP-galactose 4-epimerase precisely, the data in Table II and the rate correlations in the preceding paragraphs do not lead us to a definitive assignment of the rate enhancement that can be correlated with the downfield perturbation of δ^{13}C for E·[4-^{13}C]NAD$^+$ brought about by UDP. However, the polarity of the site should be more similar to that of 86% ethanol$_{(aq)}$ than to water, and it may be even less polar than 86% ethanol$_{(aq)}$. Therefore, we believe that the rate enhancement should be on the order of 3000–15 000, depending on whether we take the UDP-induced perturbation on δ^{13}C for E·[4-^{13}C]NAD$^+$ to be 2.8 (at 100.6 MHz) or 3.4 (at 150 MHz). The spectra in Figure 3 indicate a perturbation of 3.4 ppm for a major fraction of the enzyme, and this would correspond to a 15 000-fold rate enhancement in 86% ethanol$_{(aq)}$. An enhancement of 3000–15 000 in the intrinsic reactivity of E-NAD$^+$ brought about through binding interactions between the substrate site and the uridine pyrophosphoryl moiety of substrates is a crucial aspect of catalysis by this enzyme; in its absence a cell would require a correspondingly larger amount of enzyme to accomplish the essential functions of UDP-galactose 4-epimerase.

The data in Table II show that the reduction potentials of N-substituted nicotinamides are, as expected, sensitive to the electronic properties of the substituents X– in compounds **1a**–**e**. The slope of a plot of $E°$ versus δ^{13}C for these compounds is 44.7 mV ppm^{-1}. This slope corresponds to a 152-mV positive change in the reduction potential that can be expected from a downfield perturbation of 3.4 ppm in the 4-^{13}C chemical shift of an N-substituted nicotinamide. This change in the reduction potential should be expressed as a decrease of 7.0 kcal mol^{-1} in the activation energy for reduction. We conclude from the data in Table II and Figure 5 that the ^{15}N and ^{13}C chemical shift changes induced by uridine nucleotides in Figures 1–4 and Table I are clearly significant and serve as indicators of important aspects of the mechanism of enzymatic hydride transfer.

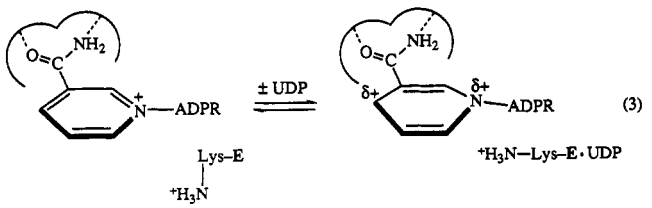
Activation of NAD$^+$ through Enzymatic Interactions. The present data indicate that the binding of UDP and methyl-UDP to UDP-galactose 4-epimerase·NAD$^+$ increases the shielding of nicotinamide-N-1 and decreases the shielding of nicotinamide-C-4. This would be brought about by changes in the enzyme–NAD$^+$ interactions that polarize the π-electron structure of the nicotinamide ring in such a way as to decrease the positive charge on N-1 and increase the charge on C-4. One way of representing such changes is to consider the nicotinamide structure in terms of resonance forms. Many resonance forms can be written, but only a small number of them contain positive charges on N-1 and C-4. They are the most relevant forms for describing a decrease in charge on N-1 and an increase on C-4 and are shown below. Form **1** is generally regarded as the most important contributor to the structure of the nicotinamide ring. The ^{15}N and ^{13}C NMR



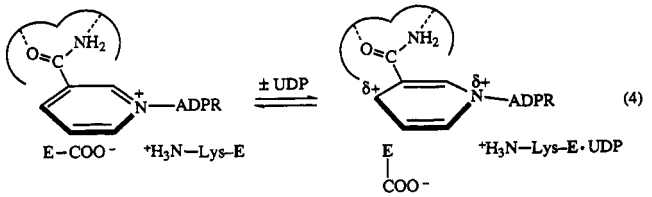
data in this paper indicate that the binding of UDP or MeUDP to UDP-galactose 4-epimerase induces a conformational change in the enzyme that alters the electronic structure of the nicotinamide ring to one in which the importance of resonance form **2** is increased. A structure in which the importance of resonance form **1** is decreased and that of form **2** is increased could be brought about by the positioning of a positively charged amino acid side chain near nicotinamide-N-1.

The ^{13}C NMR data alone would also be consistent with an increase in the importance of resonance form **3** for NAD$^+$, but this would not account for the ^{15}N NMR data. Moreover, form **3** would be favored by strong hydrogen bond donation from one or two positively charged amino acid residues, or from one or two peptide amide groups, to the carbonyl oxygen of the carboxamide group. However, the carbonyl oxygen is hydrogen bonded to the neutral hydroxyl groups of serine 124 and tyrosine 149 (Frey *et al.*, 1992). These interactions should be less effective in favoring resonance form **3** than interactions with positively charged or amide hydrogen bond donors. The data on [*carboxamide*-^{15}N]NAD$^+$ indicate that the importance of form **4** in the structure of NAD$^+$ is decreased upon binding to the coenzyme site, but the binding of UDP at the substrate site has no further effect. Thus, we have no evidence for a uridine nucleotide-induced change in hydrogen bonding to the carboxamide group.

A protein conformational change driven by uridine nucleotide binding might place the positive charge of a basic amino acid residue in proximity to the positive center at N-1 of the nicotinamide ring. This might be the ϵ-ammonium group of lysine 153, which is hydrogen bonded to both the 2′-OH and 3′-OH groups of the nicotinamide riboside of moiety of NAD$^+$ when the enzyme is in its activated conformation (Frey *et al.*, 1992). Because the coenzyme is highly immobilized within its site, as indicated by circular dichroism and fluorescence polarization studies and the three-dimensional structure of the enzyme (Wong and Frey, 1977; Wong *et al.*, 1978; Bauer *et al.*, 1992), electrostatic repulsion between the two positive centers would be relieved through a transfer of electron density from C-4 to N-1. This is illustrated in a simplified and exaggerated way by the structures in eq 3. Another mechanism



by which an electrostatic destabilization by lysine 153 could be brought to bear as a result of the binding of a uridine nucleotide is illustrated in eq 4. In this process, the positive



electrostatic field created by the proximity of lysine 153 and the nicotinamide ring of NAD^+ is partially discharged in the resting state by the carboxylate group of an acidic amino acid. The conformational change induced by the binding of a uridine nucleotide relocates the carboxylate group to another site and allows electrostatic repulsion between the ϵ-ammonium group of lysine 153 and nicotinamide-N-1 to polarize the π-electrons of the ring.

The ϵ-ammonium group of lysine 153 in the active conformation of the enzyme is hydrogen bonded to the 2′-OH and 3′-OH groups of the nicotinamide riboside moiety of NAD^+ and to two fixed water molecules as well as to another fixed species either water or chloride ion. This ammonium group is only 5.3 Å from N-1 but more than 7 Å from C-4. It could be exerting a significant electrostatic effect on the structure of the nicotinamide ring. A positive charge proximal to N-1 in the active site, which fluorescence data shows to be highly apolar relative to an aqueous environment (Wong and Frey, 1977), should perturb the positively charged ring in such a way as to decrease the positive charge on N-1 and increase the positive charge on C-4. This lysine residue is conserved in all the known epimerase sequences (Poolman *et al.*, 1990; Bauer, 1991).

Further support for the participitation of a protonated lysine residue in uridine nucleotide-dependent reduction of E·NAD^+ is provided by the pH dependence for the reduction of enzyme-bound coenzyme by P^1-5′-uridine-P^2-glucose-6-yl pyrophosphate, a substrate analog, which shows that two essential groups with pK_a values between 9.2 and 9.3 must be protonated for optimal coenzyme reduction (Arabshahi *et al.*, 1988). One of these groups could be the ϵ-ammonium group of a lysine residue.

In order to examine further whether a protonated lysine residue may participate in the structural distortion of the coenzyme, we performed the ^{13}C NMR experiments with E·[4-^{13}C]NAD^+ at pH 9.8. At this pH, a smaller fraction of the lysine residues should be protonated than at pH 7.0, and an unprotonated lysine residue in the active site would be less able to exert a strong electrostatic repulsion. This experiment was conducted using MeUDP instead of UDP in order to maintain the charge of −2 in the pyrophosphate moiety at pH 9.8. The downfield shift upon going from free enzyme to MeUDP-saturated enzyme at pH 9.8 was, indeed, 0.30 ppm less than at pH 7.0 (2.42 vs 2.72 ppm shift). Model studies of 1-substituted nicotinamide bromide salts (*vide infra*) indicated that a decrease of 0.3 ppm in the perturbation of $\delta^{13}C$ for C-4 would correspond, approximately, to a 2.3-fold decrease in reactivity. This correlates reasonably to the decrease of 2.2-fold in k_{cat} determined for the epimerase-catalyzed reaction of UDP-galactose upon going from pH 6.9 to 9.7.

Given the location of the ϵ-ammonium group of Lys 153, the foregoing electrostatic scenario is the simplest interpretation of the present results. However, the possibility must be considered that changes in chemical shifts for N-1 and C-4 might be caused by alterations in the magnetic environment surrounding the coenzyme rather than by a perturbation of the electronic structure of the nicotinamide ring. This appears to be unlikely because the effect of a ring current, which is the same for ^{13}C and ^{15}N as for ^1H, is normally not much greater than ~1 ppm (Stothers, 1972; DuVernet & Boekelheide, 1974). Larger ring current shifts such as those observed here would require one of two types of conformational changes. One type would lead to the imposition of complementary and large additive ring current effects from a system consisting of more than one interacting aromatic ring. The structure of the enzyme does not reveal the presence of multiple, closely interacting aromatic amino acids near the coenzyme. The other type would be a structural change that moves N-1 from a deshielding lateral interaction with the edge of an aromatic ring to a shielding interaction with its face (Levin & Roberts, 1973; Perkins & Dwek, 1980). The same structural change would have to have the opposite effect on C–4, that is, a change from interaction with the face of an aromatic ring to interaction with its edge. Such a change is conceivable geometrically through an interaction of the nicotinamide ring with a single aromatic ring that becomes reoriented by the conformational change. However, this relationale cannot be reconciled with the fact that the value of $\delta^{13}C$ for E·[4-^{13}C]NAD^+ (149.8 ppm) is slightly downfield from that for free [4-^{13}C]NAD^+ (148.6 ppm). The nicotinamide-C-4 of enzyme-bound NAD^+ is solvent exposed and accessible to $NaBH_4$. Thus ^{13}C-4 in E·[4-^{13}C]NAD^+ does not exhibit an upfield ring current effect. Upon binding UDP, $\delta^{13}C$ is shifted *further downfield*. This is difficult to reconcile with a scenario in which the complementary chemical shift perturbations of N-1 and C-4 are attributed to differential ring current effects. Moreover, no such aromatic amino acid residue appears in the structure of the enzyme in its active conformation. However, we cannot be confident of the structural basis for the chemical shift changes until the structure of the resting enzyme (E·NAD^+) is available.

The present results show that the conformational change induced in UDP-galactose 4-epimerase by the binding of UDP perturbs the structure of the nicotinamide ring in NAD^+ and increases its reactivity toward reduction by a large factor. We propose that this is the mechanism by which binding energy to the nonreacting portions of substrates is made available for reducing the activation energy of hydride transfer from glycosyl-C-4 of substrates to NAD^+. We postulate that the chemical reactivity of NAD^+ is increased in the activated conformation of the enzyme by electrostatic repulsion between nicotinamide-N-1 and the ϵ-ammonium group of lysine 153. Thus, binding between lysine 153 and nonreacting portions of the nicotinamide riboside, the 2′- and 3′-hydroxyl groups, potentiate the increase in chemical reactivity of the reacting portion, the nicotinamide ring, by holding the ϵ-ammonium ion of lysine 153 near N-1 of the nicotinamide ring. That lysine 153 plays a critical role in catalysis and UMP-dependent reductive inactivation of this enzyme is proven by the experiments in the following paper (Swanson & Frey, 1993).

Calculations published recently by Almarsson *et al.* (1992) indicate that a structural perturbation corresponding to a 15° out of plane bending of C-4 toward a boat conformation can contribute 20 kcal mol^{-1} toward decreasing the activation energy for reducing NAD^+ to NADH. These researchers carried out molecular dynamics calculations for reactions of lactate dehydrogenase and concluded that the driving force for a deformation of the nicotinamide ring in that case could be provided by interactions between the nicotinamide ring and hydrophobic amino acid side chains. In the case of UDP-galactose 4-epimerase, it may be that the driving force is an electrostatic interaction between the nicotinamide ring of NAD^+ and the ϵ-ammonium group of lysine 153.

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