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Rate Enhancements Brought About by Uridine Nucleotides in the Reduction of NAD^+ at the Active Site of UDP-Galactose 4-Epimerase

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UDP-galactose 4-epimerase catalyzes the interconversion of UDP-galactose and UDP glucose. In the course of the reaction, the galacto- and glucopyranosyl rings undergo reversible oxidation to the 4-keto-glucopyranosyl ring by reaction with the enzyme-bound NAD^+. The UDP-moiety of a substrate participates in catalysis by inducing a conformational change in the enzyme that enhances the chemical reactivity of NAD^+ toward reducing agents. This is modeled by UMP-dependent reductive inactivation of the epimerase-NAD^+ complex by various sugars as well as by borohydrides. The present work shows that UDP also activates the reduction of epimerase-bound NAD^+. Furthermore, the reduction of epimerase-NAD^+ by glucose at a very slow rate can be observed under anaerobic conditions in the absence of a uridine nucleotide. Comparisons of the second order rate constants for reduction of epimerase-NAD^+ by glucose in the presence and absence of uridine nucleotides have allowed the magnitude of the rate enhancements brought about by UMP and UDP to be estimated. The rate enhancements by UMP and UDP correspond to decreases of 5.7 and 4.1 kcal mol^{-1}, respectively, in the activation energy. A decrease of 4.0 kcal mol^{-1} in the activation energy for reduction by $NaBH_3CN$ was brought about by UMP-binding. The maximum increases in the reduction potential of epimerase-NAD^+ induced by UMP- and UDP-binding are estimated to be 120 and 90 mV, respectively. The results are well correlated with the perturbations of the nicotinamide-^{13}C NMR chemical shifts brought about by uridine nucleotides (Burke, J. R., and Frey, P. A. (1993) *Biochemistry* **32,** 13220–12230).

UDP-galactose 4-epimerase[1] from *Escherichia coli* is an NAD^+-containing enzyme that catalyzes the interconversion of UDP-glucose and UDP-galactose by a dehydrogenation mechanism, in which enzyme-bound NAD^+ is reversibly reduced by either UDP-galactose or UDP-glucose, with the intermediate production of a UDP-4-ketohexopyranose (*1*). The uridine nucleotide moiety of substrates is postulated to activate the enzyme by increasing the reactivity of the epimerase-bound NAD^+ toward reduction (*1*,*2*). The occurrence of this activation is supported by several lines of evi-

[1] Abbreviations used: UMP, uridine 5′-phosphate; UDP, uridine-5′-diphosphate; NAD^+, nicotinamide adenine dinucleotide; NADH, reduced nicotinamide adenine dinucleotide; Glc, glucose; P^2-methyl-UDP, P^1-5′-uridine-P^2-methyl diphosphate; Mops, 3-[*N*-morpholino]propanesulfonic acid; Tris, tris[hydroxymethyl] aminomethane; DEAE, diethylaminoethyl; NMR, nuclear magnetic resonance.

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dence: (1) The NAD^+ in epimerase-NAD^+ is rapidly reduced by D-glucose, D-fucose, L-arabinose, D-xylose, D-galactose, or $NaBH_3CN$ in the presence but not in the absence of UMP (*3–5*). (2) Epimerase-NAD^+ can also be reduced by $NaBH_4$ in the absence of UMP; however, the resulting epimerase-NADH undergoes autoxidation in the air (*6*). (3) UMP both stabilizes epimerase-NADH against oxidation and stimulates the reduction of epimerase-NAD^+ by $NaBH_3CN$ (*6*). (4) Electronic absorption and ^{31}P NMR spectroscopic results suggest that the conformation of epimerase is altered upon uridine nucleotide binding (*7,8*). (5) The uridine pyrophosphoryl moiety of UDP and P^2-methyl-UDP perturbs the ^{13}C- and ^{15}N-NMR chemical shifts of C4 and N1 in the nicotinamide ring of epimerase-NAD^+. These perturbations correspond to a positive change in the reduction potential and increased kinetic reactivity toward reducing agents, as shown by studies of model compounds (*2*).

The objective of the present research was to quantify the kinetic activation of NAD^+ brought about by uridine nucleotides binding to the active site of epimerase-NAD^+ and to compare the observed effects with those predicted by the correlation of ^{13}C NMR perturbations with chemical reactivity in model, nonenzymatic reactions (*2,9*).

EXPERIMENTAL PROCEDURE

Glucose, UMP, UDP, and $NaBH_3CN$ were purchased from Sigma. Glucose was used as supplied, and UMP and UDP were purified by chromatography over DEAE-Sephadex. Sodium cyanoborohydride was recrystallized, and the concentrations of stock solutions were determined by titration with iodine (*10*). UDP-galactose 4-epimerase for kinetic measurements was subjected to the denaturation-renaturation procedure to remove abortive complexes, as described elsewhere (*7*).

Rates of UMP- or UDP-dependent reduction of epimerase-NAD^+ by glucose were measured under aerobic conditions. Pseudo-first-order rate constants were determined by fitting the time course of increasing A_{344} to the first-order rate equation. Rates were measured in 25 mM K-phosphate buffer at pH 7.0 and 27°C at an ionic strength of 0.2 adjusted with KCl. In UMP-dependent reduction by glucose, the concentration ranges were 0.1 to 1 M for glucose and 0.108 to 3.57 mM for UMP. In UDP-dependent reduction by glucose, the concentration ranges were 0.13 to 0.5 M for glucose and 0.03 to 0.13 mM for UMP. The pseudo-first-order rate constants for reduction in the presence of UMP and UDP were fitted to Eq. [1].

$$k_{obs} = \frac{k[A][B]}{[A][B] + K_A[B] + K_B[A]} \qquad [1]$$

Uridine nucleotide-independent reduction of epimerase-NAD^+ by glucose was carried out anaerobically. Reaction solutions minus epimerase were made anaerobic by purging with argon for 1 h, and the enzyme solutions were stirred under argon for 1.5 h. Reduction reactions were carried out in anaerobic cuvettes, in which the oxygen-free solutions were mixed. The initial rates were measured spectrophotometrically at 344 nm at 27°C, either at pH 7.0 in 50 mM Mops buffer or at pH 8.5 in 0.1 M Tris buffer, with the ionic strength set at 0.2 by addition of KCl. After sufficient time to determine the initial rate of A_{344}-increase, UMP was added to the solutions to promote

the complete reduction to epimerase-NADH-UMP. The total change in A_{344} after addition of UMP was used to calculate the initial concentration of epimerase-NAD+, using the published extinction coefficient of 6.22 mM^{-1} cm^{-1} (*7*). The initial rates were calculated from the concentrations of enzyme and the initial ΔA_{344} at glucose concentration from 0.3 to 2.5 M. The data were plotted as initial rate divided by the concentration of epimerase-NAD+ against the concentration of glucose (Fig. 1).

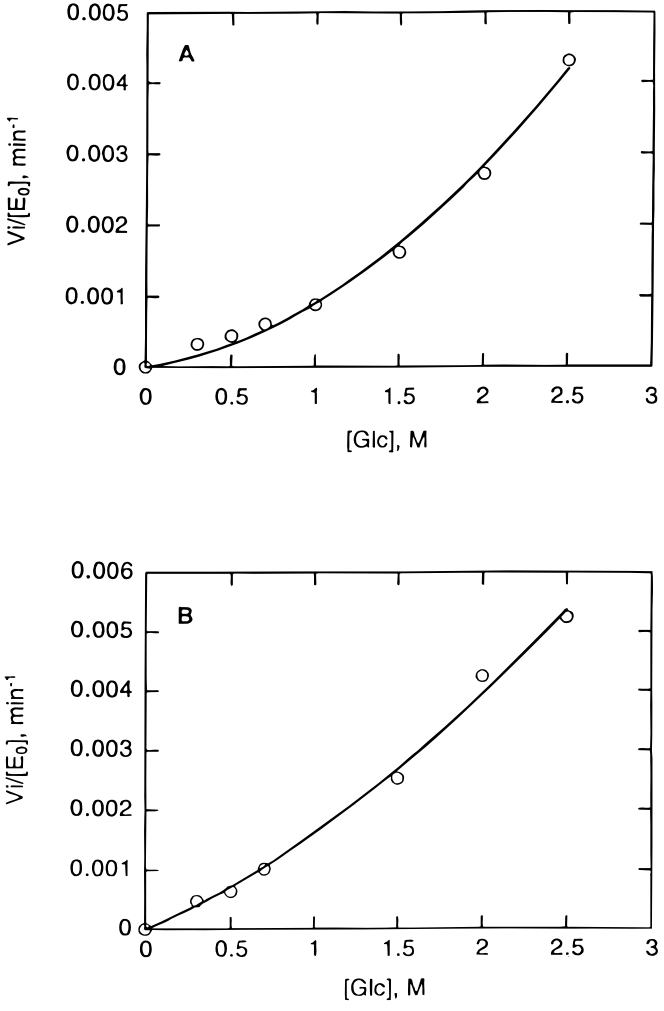

FIG. 1. Uridine nucleotide-independent reduction of epimerase-NAD+ by glucose. Initial rates acquired under anaerobic conditions at various glucose concentrations are plotted against glucose concentrations. Rates were measured spectrophotometrically at 344 nm. After measurement of the initial rate of A_{344}-increase, UMP was added to stimulate the complete reduction of epimerase-NAD+, and the total increase in A344 was used to calculate the total concentration of enzyme in that experiment. The results were then plotted as $v_i/[E_o]$ against [glucose]. The curves were obtained by fitting the data to Eq. [5]. (A) Reduction carried out in 50 mM Mops, ionic strength 0.2, pH 7.0, at 27°C. (B) Reduction carried out in 0.1 M Tris, ionic strength 0.2, pH 8.5, at 27°C.

Reduction of epimerase-NAD^+ by $NaBH_3CN$ was carried out in a buffer consisting of Mes, Mops, and *N*-methylpiperidine at 25, 25, and 50 mM, respectively, at pH 7.0 and 27°C. The ionic strength was adjusted to 0.2 by addition of KCl. UMP-independent reduction was carried out in an anaerobic cuvette, with solutions deoxygenated as described above. All kinetic data were acquired by use of a Hewlett-Packard Model 8452 spectrophotometer.

RESULTS

Uridine nucleotide-dependent reduction of epimerase-NAD^+ by glucose. Pseudo-first-order rate constants for UMP- or UDP-dependent reduction of epimerase-NAD^+ by glucose at pH 7.0 were first measured at glucose concentrations in the range of 0.1 to 1.5 M. It had been shown that at higher glucose concentrations the kinetics became complex at pH 8.5. We confirmed the earlier reported rates at pH 8.5 (*11*), in which downward curvature in plots of k_{obs}^{-1} against $[Glc]^{-1}$ was observed whenever glucose concentrations exceeded 1.6 M. The earlier studies had been, therefore, confined to less than 1.6 M glucose, where the double reciprocal plots were essentially straight lines (*11*). Therefore, glucose concentrations from 0.1 to 1.0 M were chosen for uridine nucleotide-dependent reductions at pH 7.0.

The dependence of the observed first-order rate constants on concentrations of glucose and UMP or UDP was consistent with ordered binding of the uridine nucleotide followed by glucose and then reduction of NAD^+ by glucose to form the epimerase-NADH-nucleotide complex according to Eqs. [2–4].

$$\mathbf{E}\text{-NAD}^+ + \text{nucleotide} \overset{K_{nuc}}{\rightleftharpoons} \mathbf{E}\text{-NAD}^+\text{-nucleotide} \quad [2]$$

$$\mathbf{E}\text{-NAD}^+\text{-nucleotide} + \text{Glc} \overset{K_{Glc}}{\rightleftharpoons} \mathbf{E}\text{-NAD}^+\text{-nucleotide-Glc} \quad [3]$$

$$\mathbf{E}\text{-NAD}^+\text{-nucleotide-Glc} \xrightarrow{k} \mathbf{E}\text{-NADH-nucleotide} + \text{gluconolactone} \quad [4]$$

Based on earlier reports, the uridine nucleotide remains bound to the reduced enzyme under the conditions of our experiments (*12,13*), and the glucose is oxidized at the reducing carbon to form gluconolactone (*11*). UMP has been extensively studied as the activator for reduction of epimerase-NAD^+ by sugars; however, we have found that UDP also potentiates the reaction, albeit at a slower rate. The kinetic parameters in Table 1 were obtained for the UMP- and UDP-dependent reductive inactivation of

TABLE 1

Kinetic Parameters for Uridine Nucleotide-Dependent Reduction of Epimerase-NAD^+ by Glucose

Parameter	UMP, pH 7.0	UDP, pH 7.0
k (min^{-1})	15.6 ± 1.2	1.24 ± 0.11
$K_{nucleotide}$ (mM)	0.88 ± 0.03	0.084 ± 0.001
K_{Glc} (M)	2.9 ± 0.3	2.9 ± 0.3