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Equilibrium of 5,6-Hydration of NADH and Mechanism of ATP-Dependent Dehydration†

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ABSTRACT: At equilibrium, water addition to the 5,6 double bond of NADH was observed to favor the hydrate by a factor of ~100. Hydration generates two epimers of NADHX (β-6-hydroxy-1,4,5,6-tetrahydronicotinamide adenine dinucleotide). Only the 6*S* epimer of the hydrate was found to serve as a true substrate for an ATP-dependent dehydratase from yeast that regenerates NADH. Yet enzymatic conversion of both epimers of the hydrate to NADH was found to proceed essentially to completion in the presence of ATP and dehydratase. This is explained by the observed ability of the epimers to undergo rapid spontaneous equilibration, so that it is unnecessary to postulate a lack of stereospecificity in the dehydratase.

Glyceraldehyde 3-phosphate dehydrogenase (GPDH)[1] catalyzes the conversion of NADH to a hydrated compound that is inactive in the reactions normally catalyzed by this dehydrogenase (Rafter et al., 1954; Chaykin et al., 1956). Hydration of NADH also proceeds spontaneously at a slower rate under neutral or mildly acidic conditions, and the product is β-6-hydroxy-1,4,5,6-tetrahydronicotinamide adenine dinucleotide (NADHX) (Scheme I). Oppenheimer and Kaplan (1974b) established by ^{1}H NMR that the product consists of a mixture of C-6 epimers: about 60% *S* and 40% *R*.[2] These epimers can be separated by HPLC (Miksic & Brown, 1978; Margolis et al., 1978).

Hydration of NADH would presumably tend to result in depletion of this coenzyme, and the 5,6-hydrate of NADPH has been found to act as a strong inhibitor of glucose 6-

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[1] Abbreviations: NADHX, β-6-hydroxy-1,4,5,6-tetrahydronicotinamide adenine dinucleotide; c(THN)AD, cyclotetrahydronicotinamide adenine dinucleotide; PMSF, phenylmethanesulfonyl fluoride; GPDH, glyceraldehyde 3-phosphate dehydrogenase.

[2] The *S* epimer corresponds to hydroxylation on the "A" face of NADH, and the *R* epimer corresponds to hydroxylation on the "B" face.

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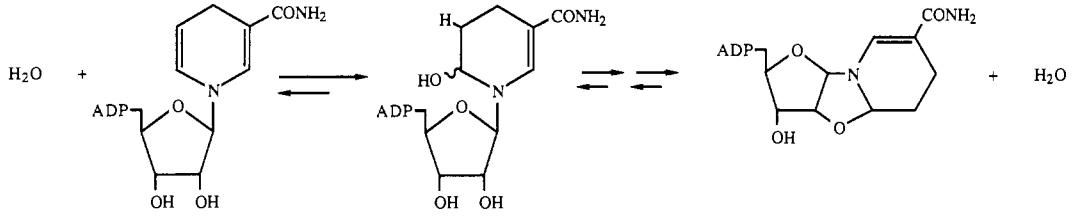
Scheme I



phosphate and lactate dehydrogenase (Yoshida & Dave, 1975). Physiological problems might therefore be expected to ensue from hydration of NADH. However, a possible solution was suggested by the discovery of an unusual enzyme that catalyzes the ATP-dependent dehydration of NADHX to NADH (Meinhart et al., 1956). This enzyme, which also catalyzes ATP-dependent conversion of NADHXP to NADPH (Requeiro et al., 1970), would presumably be useful in salvaging the hydrates.

The fact that hydrates arise spontaneously from NADH as a mixture of epimers raises questions about the chemical properties of any enzyme that might reverse this process. In the dehydration reaction, a likely function of ATP would be to phosphorylate the 6-hydroxyl substituent of NADHX, rendering it a better leaving group. An active site would need be unusually adroit in order to catalyze such a phosphorylation–elimination process for *both epimers* of NADHX (Scheme I). Does enzymatic dehydration of an epimeric mixture of hydrates proceed to completion, or is only one epimer dehydrated? If both epimers are dehydrated, does a single enzyme catalyze dehydration of both, or is a second activity present? If only one dehydratase is present, is it able to catalyze dehydration of both epimers? If it is active on only one epimer, what mechanism exists for removal of the second epimer? This paper addresses these questions and the position of equilibrium of the reaction that leads to covalent hydration of NADH.

Experimental Procedures

Materials. Nucleotides, Sephadex G-25, phenylmethanesulfonyl fluoride (PMSF), sodium lactate, sodium pyruvate, α-ketoglutarate, lactate dehydrogenase, glutamate dehydrogenase, and dried bakers' yeast were obtained from Sigma Chemical Co. Yeast glyceraldehyde 3-phosphate dehydrogenase was obtained from Boehringer Mannheim Biochemicals. 2-Mercaptoethanol was obtained from Fluka Chemical Corp. HPLC-grade methanol was obtained from Fisher Scientific. Ultrafiltration cones (type CF-25) were obtained from Amicon Corp. Agarose–hexane–adenosine 2′,5′-diphosphate type 2 affinity resin was obtained from Pharmacia Inc.

Methods of Analysis. Preparative HPLC separations were carried out on a C-18 (Whatman Partisil ODS-2) column (500 × 9.4 mm) at a flow rate of 4.0 mL/min, using a linear gradient from 0 to 20% methanol over 40 min. The aqueous phase contained sodium phosphate buffer (10 mM, pH 7.0). Analytical separations were carried out on an analytical column (250 × 4.1 mm) of the same material, at a flow rate of 1.0 mL/min, with the same gradient of methanol. The aqueous phase contained sodium phosphate buffer (40 mM, pH 7.0).

NADHX was prepared by incubating NADH (10 mg) in sodium phosphate buffer (0.5 mL, 0.5 M, pH 6.0) for 30 min at 35 °C. NADHX constituted ~35% of the resulting mixture and was isolated from this mixture by preparative HPLC in greater than 90% purity, as indicated by analysis by analytical HPLC. Both epimers of NADHX were eluted earlier than NADH and c(THN)AD (Figure 1), as demonstrated earlier by Miksic and Brown (1978). Solutions of NADHX, adjusted to pH 8–9 with 1.0 N sodium hydroxide and stored at 4 °C, were stable for several days. The concentration of NADHX was determined by using ϵ_{290} = 13 500 M^{-1} cm^{-1} (Chaykin et al., 1956). NADHX was also prepared by GPDH-catalyzed hydration of NADH (Chaykin et al., 1956; Oppenheimer & Kaplan, 1974b). This material was identical with the nonenzymatically generated compound in its chromatographic behavior and UV spectrum. The reason for this similarity became evident later, when the speed of interconversion of the epimers was appreciated (see below).

Preparation of Yeast Dehydratase. NADHX dehydratase was prepared from yeast by the following modification of the second method described by Meinhart et al. (1956). Dried bakers' yeast (20 g) was incubated at 38 °C for 4 h in 60 mL of 0.2 M ammonium phosphate buffer, pH 9.0, that contained 0.1% (v/v) 2-mercaptoethanol. Procedures following autolysis were carried out at 4 °C. The mixture was centrifuged for 30 min at 10000*g*, and the precipitate was discarded. To the supernatant (40 mL) was added PMSF (0.2 mL, 0.2 M in acetone) slowly with stirring, followed by ammonium sulfate (11.6 g). After centrifugation for 30 min at 10000*g*, the precipitate was discarded and ammonium sulfate (8 g) was added to the supernatant fluid. The precipitate, collected by centrifugation for 30 min at 10000*g*, was taken up in Tris-HCl buffer (15 mL, 0.02 M, pH 8.0). This solution was dialyzed against the same buffer (3 × 1 L) overnight. After dialysis, the solution (25 mL) was loaded onto a column (5-mL bed volume) of AG-2′,5′-ADP affinity resin that had been equilibrated with Tris-HCl buffer (0.02 M, pH 8.0). The column was washed with the same buffer (50 mL) until the A_{280} of the column effluent was less than 1% of the A_{280} of the protein solution loaded onto the column. Active enzyme was then eluted with Tris-HCl buffer (0.02 M, pH 8.0) that contained ATP (5.0 mM) and $MgSO_4$ (5.0 mM), in a total volume of 5–6 mL. This method yielded dehydratase free from contaminating NADH oxidase activity (Meinhart et al., 1956). The resulting enzyme preparation was stable for several weeks when frozen.

Enzyme Assays. The activity of affinity-purified dehydratase was assayed by monitoring the conversion of NADHX to NADH at 290 nm ($\Delta\epsilon_{290}$ = −11 400 M^{-1} cm^{-1}) or 340 nm ($\Delta\epsilon_{340}$ = 6200 M^{-1} cm^{-1}). In crude preparations containing large amounts of NADH oxidase activity, an approximate assay was used, based on conversion of NADHX to NAD^+ via NADH ($\Delta\epsilon_{290}$ = −12 700 M^{-1} cm^{-1}). Protein content was determined by A_{280}/A_{260} ratios (Layne, 1957). Initial velocities were determined at 25 ± 1 °C, during the first 10–15% of reaction when the rate of product formation was almost constant.

Determination of Rate of Epimerization. NADHX was prepared by incubation of NADH in sodium phosphate buffer

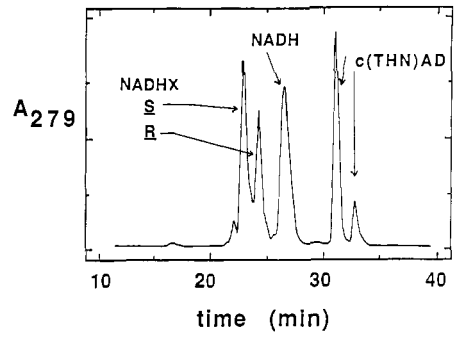


FIGURE 1: HPLC analysis of NADHX, NADH, and c(THN)AD. NADH was incubated in sodium pyrophosphate buffer (0.1 M, pH 6.0) at 25 °C for 6 h and analyzed by analytical HPLC as described under Experimental Procedures.

as described above. The trailing edge of the peak, corresponding to the *R* epimer of NADHX (Figure 1), was isolated by HPLC. This solution (0.1 mM in NADHX) was incubated at 25 °C, aliquots of 50 μL were removed at intervals and analyzed by HPLC on an analytical C-18 column (Whatman Partisil ODS-2), and the concentration of C-6 epimers was monitored from the integrated intensities of their absorbances at 279 nm.[3]

Determination of Equilibrium Constant for Hydration. Yeast glyceraldehyde 3-phosphate dehydrogenase (0.30 mL, 10 mg/mL) was desalted by ultrafiltration on an Amicon CF-25 ultrafiltration cone, which was centrifuged for 20 min at 750*g*, at 4 °C. Three successive additions of 5 mL of freshly prepared cysteine (30 mM, adjusted to pH 8.0 with 1 N NaOH) were followed by centrifugation. During the final wash, the enzyme was concentrated to its original volume. Hydration reactions were initiated by addition of 0.15 mL of this preparation to NADH (100 μM in 0.1 M sodium acetate, pH 5.5, containing 10 mM sodium pyrophosphate, 2.85 mL). At intervals beginning immediately after addition of the enzyme, aliquots (0.3 mL) were cooled with ice, adjusted to pH 8.0 with NaOH (10 μL, 1.0 N), subjected to ultrafiltration using an Amicon CF-25 cone to remove enzyme, and analyzed by analytical HPLC. The amounts of NADH, NADHX, and c(THN)AD present were determined from peak areas by using $\epsilon_{254}(\text{NADH})/\epsilon_{254}(\text{NADHX}) = 1.3$ and $\epsilon_{254}(\text{NADHX})/\epsilon_{254}[\text{c(THN)AD}] = 1.0$.

Under these conditions, the half-time for hydration was ~5 min. When the reaction was allowed to proceed for more than 50 min, the concentration of NADH approached a constant low level. The concentration of NADH present at 5, 10, 15, and 20 half-times was determined both by HPLC analysis and by a sensitive cycling assay using lactate and glutamate dehydrogenases (Lowry & Passonneau, 1972). The identity of the small "NADH" peak at long reaction times in HPLC elution profiles was confirmed by analysis of a 15-fold concentrate of a 3.00-mL hydration reaction solution. The hydration was allowed to proceed for ~15 half-times. The solution was adjusted to pH 8.0 with NaOH (1.0 N), subjected to ultrafiltration, and concentrated to dryness under vacuum at 25 °C. The residue was taken up in 0.20 mL of water and analyzed by HPLC. The HPLC eluant corresponding to the small NADH peak exhibited the characteristic wavelength maxima of 262 and 340 nm and when treated with sodium pyruvate and lactate dehydrogenase showed the absorption changes expected for oxidation of NADH to NAD^+.

[3] ^1H NMR analysis of mixtures of epimers and UV analysis of mixtures of epimers separated by HPLC indicate that the *S* and *R* epimers of NADHX have virtually identical molar extinction coefficients at 279 nm.

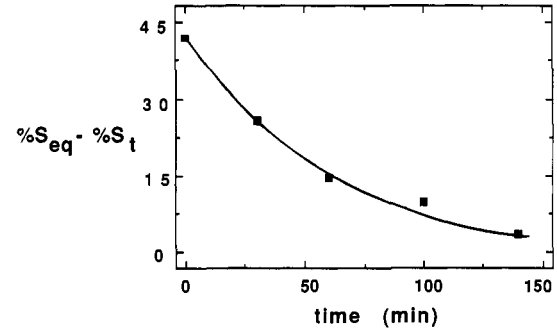


FIGURE 2: Approach to equilibrium for the epimerization of C-6 hydroxyl epimers of NADHX. The percentage of *S* epimer at the indicated times (% S_t) was determined by HPLC analysis as described under Experimental Procedures. The percentage of *S* epimer at equilibrium (% S_{eq}) was 60. The solid line is an exponential least-squares fit to the data.

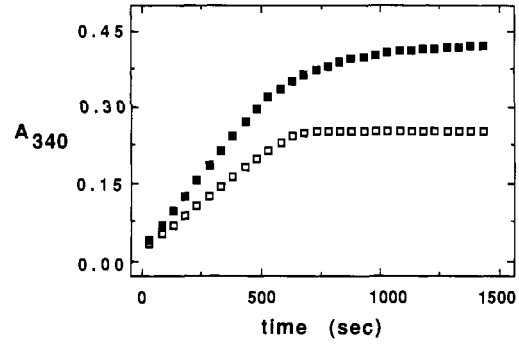


FIGURE 3: Enzymatic conversion of NADHX to NADH at 25 °C in 10 mM sodium phosphate buffers, pH 6.0 (■) and 8.0 (□), that contained 67 μM NADHX, 0.67 mM ATP, 0.67 mM $MgSO_4$, and 66 μg/mL protein.

RESULTS

Acid-Catalyzed Hydration of NADH. Under neutral or mildly acidic conditions, NADH was first converted to NADHX, then to c(THN)AD, and finally to a "secondary acid modification" product, in accord with earlier observations (Miksic & Brown, 1978; Margolis et al., 1978) and with the scheme proposed by Oppenheimer (1974a). At pH 7.0, formation of c(THN)AD from NADHX occurred ~50% more rapidly than formation of NADHX from NADH. Figure 1 shows a representative HPLC elution profile of a mixture of NADH, NADHX, and c(THN)AD. UV absorption spectra of NADHX and c(THN)AD were comparable with those reported earlier (Chaykin et al., 1956; Oppenheimer & Kaplan, 1974a,b).

Rate of Epimerization of NADHX. We examined the interconversion of the *R* and *S* epimers of NADHX by collecting the trailing edge of the peak corresponding to the *R* epimer (Figure 1), in buffer at pH 7.0, adjusting this to pH 6.0 with 1 N HCl or to pH 8.0 with 1 N NaOH, and analyzing these solutions by analytical reverse-phase HPLC. At pH 6, epimerization appeared to go to completion within a few minutes, whereas at pH 8, epimerization was incomplete after many hours. Figure 2 shows a plot of the reaction at pH 7.0 and 25 °C, which approached equilibrium with a half-time of 40 min.

Epimeric Specificity of the Yeast Dehydratase. If the yeast enzyme were specific for one of the two epimers of NADHX, then dehydration of an equilibrium mixture of epimers could not be expected to proceed to completion without spontaneous isomerization of the inactive to the active epimer. At pH 6.0, where epimerization had been found to occur rapidly compared

Table I: Equilibrium Constant for Hydration of NADH

	% composition						
	predicted[a]			observed[b]			
$t_{1/2}$	NADH	NADHX	NADHX/NADH	NADH	NADHX	c(THN)AD	NADHX/NADH
0.1	93.4	6.6	0.07	90	10	0.0	0.11
1.0	50.5	49.5	0.98	43	57	0.0	1.3
2.5	18.6	81.4	4.4	13	86	1.0	6.6
5.0	4.2	95.8	23	3.5	95	1.5	27
10.0	1.2	98.9	82	1.7	94	4.3	55
15.0	1.0	99.0	99	1.0	93	6.0	93
20.0	1.0	99.0	99	1.1	90	8.9	82

[a] Calculated on the basis of an approach to equilibrium according to K_{eq} = [NADHX]/[NADH][H_2O] = 99 with the usual convention of the water concentration taken as 1.0 M. [b] GPDH-catalyzed hydration of NADH was carried out as described under Experimental Procedures.

with enzyme-catalyzed dehydration (see above), one would then expect to observe complete conversion of both epimers to NADH. At pH 8.0, where epimerization proceeds at a very slow rate, the extent of enzyme turnover would be expected to reflect only the amount of active substrate that had been present at the outset.

Figure 3 shows the results of experiments with an equilibrium mixture of freshly isolated NADHX, incubated with dehydratase at 25 °C in 0.01 M sodium phosphate buffers at pH 6.0 and pH 8.0. At pH 6.0, the change in absorption observed at 340 nm was 94% of the change expected for complete consumption of both epimers of NADHX. Meinhart et al. (1956) observed a change in absorption at 340 nm corresponding to ~87% of the value expected for complete conversion. These differences from 100% conversion probably result from contamination of the NADHX with c(THN)AD. At pH 8.0, on the other hand, the change in absorption was 60% of the change observed at pH 6.0, corresponding to the approximate abundance of the *S* epimer in the equilibrium mixture. Thus, the enzyme appears to act preferentially on the *S* epimer.

Several additional observations confirmed this tentative assignment of stereospecificity. First, HPLC analysis of unreacted starting material and product gave results consistent with complete conversion of NADHX to NADH at pH 6.0 but consumption of only the *S* epimer of NADHX at pH 8.0. Second, initial rate measurements with enriched solutions of the epimers gave higher velocities for solutions enriched in the *S* epimer than for solutions enriched in the *R* epimer. These results do not rule out the possibility that the *R* epimer serves as a slow substrate for the dehydratase, but they can be explained on the simpler assumption that only the *S* epimer serves as a substrate.

Equilibrium Constant for Hydration of NADH. In principle, the free energy of hydration of NADH could be obtained directly, by determining the position of equilibrium of the hydration reaction, or indirectly, by determining the position of equilibrium of the ATP-dependent dehydration of NADHX. Our efforts to use the second of these methods were unsuccessful, since even with the highest possible concentrations of ADP (10^{-2} M) and P_i (0.75 M), dehydration proceeded to greater than 90% completion.

The equilibrium of hydration of NADH could not be determined directly in the absence of catalysts because of the instability of the hydrate, but GPDH-catalyzed formation of the hydrate was found to occur rapidly enough to allow the position of equilibrium to be established. Table I shows the composition of a mixture of NADH and NADHX equilibrating in the presence of GPDH at pH 5.5 as a function of time. Table I also shows the ratios predicted for a first-order approach to equilibrium with an equilibrium constant of 99. The amount of c(THN)AD arising from the hydrate remained small under these conditions, so that it did not significantly affect determination of the equilibrium constant. The equilibrium ratio of hydrate to NADH, obtained after 15 half-times from this and three similar experiments, was 96 ± 9.

Table II: Equilibria of C=C Hydration Reactions

A → H	(H)/(A)	(H)/(A)(H_2O)
phosphoenolpyruvate → 2-phosphoglycerate	0.16[a]	2.9×10^{-3}
crotonyl-CoA → 2-hydroxybutyryl-CoA	3.4[c]	6.2×10^{-2}
fumarate → l-malate	4.4[d]	8.0×10^{-2}
stearate → 10-hydroxystearate	30[e]	0.54
cis-aconitate → citrate	31[b]	0.56
NADH → NADHX	92[f]	1.66

[a] Wold and Ballou (1957). [b] Krebs (1953). [c] Stern and del Campillo (1956). [d] Bock and Alberty (1953). [e] Niehaus and Shroepfer (1965). [f] This study.

In view of experimental errors associated with estimating the small amounts of NADH present in these equilibrium mixtures by chromatographic analysis, NADH was also analyzed by the more sensitive and specific cycling assay of Lowry and Passoneau (1972), using glutamate and lactate dehydrogenases. By this method, NADH was found to approach a limiting value of 1.1% of the total nucleotide concentration present in the reaction mixture. From these results, the equilibrium ratio of hydrate to NADH was estimated as 90 ± 6.

Discussion

Taking water activity as unity, our best estimate of the equilibrium constant for NADH hydration is 92. Expressed alternatively, in terms of the actual molarity of water, the equilibrium constant for hydration is 1.66 M^{-1}. Table II compares these with values that have been reported for other C=C hydration reactions of biological importance. Of these reactions, only the hydration of stearic acid involves an isolated double bond, and if that reaction is taken as a benchmark, then the hydration products of NADH appear to be unusually favored relative to the unhydrated species.

In each of these reactions, C—H, C—C, and C—O bonds (with mean bond energies of 97.5, 84, and 88 kcal/mol, respectively) (March, 1985) can be considered to take the place of O—H and C=C bonds (with mean bond energies of 110.5 and 148.5 kcal/mol, respectively). The corresponding enthalpy of C=C hydration would be −10.5 kcal/mol, considerably more favorable than the value of −2.7 kcal/mol that would correspond to our observed free energy of hydration of NADH. Thus, the entropy of hydration is probably unfavorable, as expected from the loss of translational freedom that accompanies the combination of substrates to form a single hydrated product. The equilibrium constant for hydration of stearic acid, the only substrate with an unconjugated double bond, falls toward the high end of the values listed in Table II. This

might be attributed to the loss of resonance stabilization when conjugated substrates are hydrated, except for the fact that the equilibrium constant for hydration of NADH is even higher than that for stearic acid. These differences have more complex origins, possibly including variations in the amount of entropy that is lost when hydration occurs.

We are unaware of experimental evidence that might provide a direct answer to the interesting question of how the cell disposes of the cyclic derivative c(THN)AD when it is formed. However, in a manuscript in preparation, we report that (a) the first-order rate constant for disappearance of NADH (formation of NADHX) is (2.3–3.5) $\times 10^{-3}$ h^{-1} at 37 °C in each of several buffers of pH 7.4 and ionic strength 0.15, (b) the V_{max} (per milliliter of cells) for dehydratase in the human red cell is ~60 nmol h^{-1}, and (c) the K_M value of NADHX for the human red cell dehydratase is 6×10^{-6} M. Using these values, we calculate that, at steady state, the dehydratase should be able to return over 99.9% of the NADHX to NADH and that the concentration of NADHX would be $^1/_{3000}$th that of the NADH. The activity of the dehydratase is even higher in rat liver and in yeast.

Epimerization of NADHX appears to depend on hydrogen ion concentration, with a half-time in the neighborhood of 40 min at pH 7 and 4 min at pH 6. Its observed rapidity accounts for the ability of NADHX dehydratase to convert both epimers to NADH, making it unnecessary to posulate the existence of a second dehydratase of opposing stereospecificity. Epimerization may involve reversible loss of OH^- to form a quaternized intermediate or ring opening to form an aldehydic intermediate; these possibilities could be distinguished, at least in principle, by using ^{18}O-enriched water. It seems clear that epimerization does *not* involve dehydration followed by rehydration, because the intermediate would be NADH itself, a reasonably stable species. The detailed mechanism of action of the dehydratase is also unknown, but it seems probable that ATP is used to phosphorylate the OH group of NADHX, facilitating its departure. Whether this occurs in a stepwise or concerted manner remains to be determined.

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