Kinetics of Native and Modified Liver Alcohol Dehydrogenase with Coenzyme Analogues: Isomerization of Enzyme–Nicotinamide Adenine Dinucleotide Complex†

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ABSTRACT: Coenzyme analogues with the adenosine ribose replaced with *n*-propyl, *n*-butyl, and *n*-pentyl groups; coenzyme analogues with the adenosine replaced with 3-(4-acetylanilino)propyl and 6-(4-acetylanilino)hexyl moieties; and nicotinamide mononucleotide, nicotinamide hypoxanthine dinucleotide, and 3-acetylpyridine adenine dinucleotide were used in steady-state kinetic studies with native and activated, amidinated enzymes. The Michaelis and inhibition constants increased up to 100-fold upon modification of coenzyme or enzyme. Turnover numbers with NAD^+ and ethanol increased in some cases up to 10-fold due to increased rates of dissociation of enzyme-reduced coenzyme complexes. Rates of dissociation of oxidized coenzyme appeared to be mostly unaffected, but the values calculated (10–60 s^{-1}) were significantly less than the turnover numbers with acetaldehyde and reduced coenzyme (20–900 s^{-1}, at pH 8, 25 °C). Rates of association of coenzyme analogues also decreased up to 100-fold. When Lys-228 in the adenosine binding site was picolinimidylated, turnover numbers increased about 10-fold with NAD(H). Furthermore, the pH dependencies for association and dissociation of NAD^+ and turnover number with NAD^+ and ethanol showed the fastest rates above a p*K* value of 8.0. Turnover with NADH and acetaldehyde was fastest below a p*K* value of 8.1. These results can be explained by a mechanism in which isomerization of the enzyme–NAD^+ complex (110 s^{-1}) is partially rate limiting in turnover with NAD^+ and ethanol (60 s^{-1}) and is controlled by ionization of the hydrogen-bonded system that includes the water ligated to the catalytic zinc and the imidazole group of His-51.

Although the structure and mechanism of horse liver alcohol dehydrogenase have been extensively investigated (Brändén et al., 1975; Klinman, 1981), the kinetics of coenzyme binding have not been explained fully. It has been suggested that the rate of binding of coenzyme is controlled by the AMP moiety, whereas the rate of dissociation is controlled by the nicotinamide mononucleotide (NMN) moiety (Shore, 1969; Shore & Gilleland, 1970). These conclusions, however, have not been extended to coenzyme analogues with greatly varied adenosine substituents. Furthermore, there is evidence that the enzyme changes conformation upon binding coenzyme. Isomerization of enzyme–coenzyme complexes has been postulated to explain steady-state kinetic data that give a rate constant for the dissociation of the enzyme–nicotinamide adenine dinucleotide (NAD^+) complex that is less than the turnover number for the reaction of NADH and acetaldehyde (Wratten & Cleland, 1963), turnover with the 3-acetylpyridine analogue of NAD (Shore & Brooks, 1971; Kamlay & Shore, 1983), NMR data for dissociation of NADH (Czeisler & Hollis, 1973), fluorescence quenching of enzyme upon NAD^+ binding (Wolfe et al., 1977; Parker et al., 1978), and pressure relaxation studies on the enzyme–NAD^+ complex (Coates et al., 1977). The different structures of the apoenzyme and ternary complexes have even been determined by X-ray crystallography at high resolution (Eklund et al., 1981, 1984).

Coenzyme binding is also affected by chemical modifications. Amidination of the ϵ-amino group of Lys-228, which binds the 3′-hydroxyl group of the adenosine ribose, increases the turnover numbers about 10-fold by increasing the rates of dissociation of enzyme-coenzyme complexes. However, turnover with NADH and acetaldehyde is 10 times faster than the rate calculated for dissociation of the enzyme–NAD^+ complex (Plapp, 1970; Plapp et al., 1973). Using coenzyme analogues and modified enzyme, we have obtained evidence that a pH-dependent isomerization of the enzyme–NAD^+ complex can control the rate of NAD^+ binding and catalytic turnover.

Experimental Procedures

Materials. NAD^+, chromatographic grade I, was obtained from Boehringer; NADH, grade III, from Sigma; AcPAD[1] and NID, from P-L Biochemicals. The barium salts of Ac-

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[1] Abbreviations: AcPAD, 3-acetylpyridine adenine dinucleotide; NID, nicotinamide riboside inosine diphosphate; NAPrD, nicotinamide riboside 3-(adenin-9-yl)-*n*-propyl diphosphate; NABuD, nicotinamide riboside 4-(adenin-9-yl)-*n*-butyl diphosphate; NAPeD, nicotinamide riboside 5-(adenin-9-yl)-*n*-pentyl diphosphate; NAcAnPrD, nicotinamide riboside 3-(4-acetylanilino)-*n*-propyl diphosphate; NAcAnHeD, nicotinamide riboside 6-(4-acetylanilino)-*n*-hexyl diphosphate; AcPADH, NIDH, etc., reduced forms of the above; EDTA, ethylenediaminetetraacetic acid.

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PADH and NIDH were prepared (Rafter & Colowick, 1957) and further purified as the sodium salts (Dalziel, 1962). Activity with AcPAD was recorded at 363 nm (a_M = 9100 M^{-1} cm^{-1}) and activity for NID at 338 nm (a_M = 6200 M^{-1} cm^{-1}).

The syntheses of the nicotinamide riboside ω-(adenin-9-yl)-*n*-alkyl diphosphate analogues were described previously (Jeck & Wilhelm, 1973), as were the syntheses of the nicotinamide riboside ω-(4-acetylanilino)-*n*-alkyl diphosphate analogues (Vutz et al., 1980). NMN was produced by hydrolyzing NAD^+ according to Jeck et al. (1974). These analogues and NMN were reduced to the dihydro forms by treatment with sodium dithionite at pH 8 (Jeck & Wilhelm, 1973) and purified by chromatography on a Sephadex G-10 column (2 × 200 cm) developed with sodium glycine buffer, pH 9.3, at 10 mL/h. The reduced coenzyme was completely separated from byproducts and, in the case of the adenine alkyl analogues, was identified by an absorption spectrum with maxima at 260 and 340 nm (a_M = 14000 and 6200 M^{-1} cm^{-1}, respectively). The reduced analogues were used within 24 h, before the A_{260}/A_{340} ratio exceeded 2.9 (80% purity), due to decomposition of the dihydronicotinamide ring. For the (4-acetylanilino)alkyl analogues of NADH, a single maximum at 336 nm (a_M = 23 500 M^{-1} cm^{-1}) was observed, and the absorption at 265 nm was at a minimum (a_M = 1300 M^{-1} cm^{-1}). The solutions used for assays had an A_{336}/A_{265} of more than 15.8 (more than 90% purity). The oxidized forms had maxima at 265 and 336 nm (a_M = 5100 and 17 500 M^{-1} cm^{-1}, respectively). The coenzymatic activity for these analogues was measured at 265 nm, where the difference extinction coefficient is 3800 M^{-1} cm^{-1} (Vutz et al., 1980).

Methyl isonicotinimidate and methyl picolinimidate were prepared previously (Zoltobrocki et al., 1974). Crystalline horse liver alcohol dehydrogenase was obtained from Boehringer or purified (Dworschack & Plapp, 1977a). Partially acetimidylated enzyme was activated by modification of Lys-228 with the imidates (Zoltobrocki et al., 1974).

Steady-State Kinetic Studies. Activities were measured with a Cary 118C or a Gilford 240 spectrophotometer by using the 0.1*A* scale or less and 1- or 0.2-mL assay volumes. Redistilled 95% ethanol and freshly distilled acetaldehyde were used. The concentration of native enzyme was determined by using a specific activity of 2.4 units/mg in a standard assay (Plapp, 1970). The number of binding sites for NAD^+ was confirmed by titration in the presence of pyrazole (Theorell & Yonetani, 1963). Most of the kinetic studies were performed at 25 °C with 33 mM sodium phosphate buffer, pH 8.0, containing 0.25 mM EDTA, which stabilizes the enzyme. Activated, imidylated enzyme was diluted into 1 mg/mL bovine serum albumin and kept on ice during the few hours required for kinetics experiments. Initial velocities were determined graphically and fitted to the appropriate equations with estimation of standard errors (Cleland, 1979). The types of inhibition by products (competitive, noncompetitive, uncompetitive) were distinguished by using *t* tests that would indicate significant differences in slopes and intercepts of double-reciprocal plots (Johansen & Lumry, 1961).

For the pH dependency studies, 0.1 ionic strength sodium phosphate buffers with 0.25 mM EDTA were used, with 3.6 mM glycine added at pH 9 and 2.1 mM glycine at pH 9.9 for pH control (Dalziel, 1963). The p*K* values describing the kinetic data were fitted to

$$k_{obsd} = k_{max}/(1 + [H^+]/K_1 + K_2/[H^+])$$

by using a nonlinear least-squares program (C. M. Metzler, The Upjohn Co., Kalamazoo, MI). For some of the data, either K_1 or K_2 was not included in the final fit.

Results and Discussion

Kinetic Mechanism. The steady-state kinetic results for the modified coenzymes and enzymes are listed in Table I. Complete product inhibition or initial velocity studies of the reaction in both directions, with ethanol and acetaldehyde as substrates, were used to establish the kinetic mechanism and to allow calculations of rate constants. Since quantities of some of the analogues were limiting and the large Michaelis and inhibition constants required large amounts of material, many of the values were determined only once. The results were consistent with the sequential Bi mechanism in most cases and the ordered Bi-Bi mechanism when tested with product inhibition studies. Nevertheless, the data for NMN(H) fitted best to the rapid equilibrium ordered mechanism, with coenzyme binding first (Segel, 1975). In this case, the K_m for coenzyme approaches zero. The pentyl adenine analogue also appeared to fit the equilibrium ordered mechanism in the reaction of oxidized coenzyme and ethanol. These results suggest that coenzyme must bind first for structural reasons. The mechanism of isonicotinimidylated enzyme with the butyl adenine analogue appeared to be Theorell–Chance, which is a special case of the ordered Bi-Bi mechanism (Plowman, 1972). The values for the K_{eq} calculated from the Haldane relationships generally agreed with the values determined directly [10 pM for NAD or 300 pM for AcPAD (Sund & Theorell, 1963)], suggesting that the kinetic constants are approximately correct.

In general, the magnitudes of the Michaelis and inhibition constants for all substrates increased with the coenzyme analogues or with modified, activated enzyme. More significantly, in most cases the inhibition constants for the oxidized and reduced coenzymes, K_{ia} and K_{iq}, which represent the dissociation costants, increased by 1 order of magnitude. Furthermore, these constants are affected additively when both the coenzyme and the enzyme are modified. Alteration of either the coenzyme or enzyme disrupts some interactions in the complexes.

The turnover numbers also increased by up to 10-fold, due to increased rates of dissociation of product coenzyme (Plapp, 1970). The activating effects on turnover are not additive; for instance, the acetylpyridine analogue has the same V_1/E_t with all three enzymes, as does the butyl adenine analogue with native and isonicotinimidylated enzymes. The turnover numbers are also reduced with some of the analogues, i.e., the alkyl adenine derivatives and NMN, presumably because the enzyme cannot bind the coenzyme properly for hydrogen transfer. In general, modification of the enzyme increases the value of *R*, which indicates that the fraction of enzyme in central complexes has increased (Janson & Cleland, 1974). Others have also studied the kinetics with modified enzyme or coenzyme analogues, but the different conditions preclude ready comparison (Shore & Theorell, 1967; Baici et al., 1975; Tsai, 1978).

Rates of Association and Dissociation of Coenzymes. Rate constants for coenzyme binding can be calculated for the ordered Bi-Bi mechanism and used to explain the kinetic behavior. (See Table I for equations.) The calculated rate

$$E \underset{k_2}{\overset{k_1}{\rightleftharpoons}} E\cdot NAD \underset{k_4}{\overset{k_3}{\rightleftharpoons}}$$

$$(E\cdot NAD\cdot CH_3CH_2OH = E\cdot NADH\cdot CH_3CHO) \underset{k_6}{\overset{k_5}{\rightleftharpoons}}$$

$$E\cdot NADH \underset{k_8}{\overset{k_7}{\rightleftharpoons}} E$$

Table I: Steady-State Kinetic Constants for Native and Activated Liver Alcohol Dehydrogenases with Coenzyme Analogues[a]

	Native Enzyme									Isonicotinimidylated				Picolinimidylated	
Constant[b]	NAD	AcPAD	NID	NABuD	NAPeD	NAPrD	NAcAnHeD	NAcAnPrD	NMN	NAD	AcPAD	NID	NABuD	NAD	AcPAD
K_a, μM	3.9±0.3	38±3	4±4	270±70	*c*	160±60	150±20	1900±110	*c*	470±30	1200±60	2300±300	1000±200	420±20	410±30
K_b, mM	0.35±0.03	4.5±0.9	0.9±0.1	1.3±0.3	19±2	5±1	4.2±1.8	12±16	12±6	42±5	130±10	76±8	15±3	18±1	70±10
K_p, mM	0.40±0.05	9.1±0.3	0.51±0.06	0.34±0.09	1.3±0.4	0.6±0.1	3.2±1.0	1.9±0.3	750±200	19±2	13±1	5.7±2.2	4.1±0.9	5.7±0.3	5.2±0.6
K_q, μM	5.8±1.3	9.6±3.9	15±2	150±10	290±40	150±30	124±30	110±10	*c*	270±40	390±80	1300±300	220±30	120±6	100±20
K_{ia}, μM	27±5	67±7	150±20	740±50	420±40	4400±1000	640±320	4200±4400	370±200[d]	980±80	1800±100	8200±600	4500±1000	640±16	1000±100
K_{ib}, mM	27±3	31±1	5.9±1.5	670±170	*e*	*e*	*e*	*e*	*e*	180±50	31±8	4±2	67±10[f]	26±3	130±30
K_{ip}, mM	0.52±0.10	5±1	0.4±0.1	0.72±0.13	*e*	*e*	*e*	*e*	*e*	65±25	70±10	0.42±0.09	0.24±0.03[f]	14±2	----
K_{iq}, μM	0.50±0.03	5.1±1.6	7.7±0.8	43±11	660±300	2000±6000	17±11	310±60	13±1	55±3	300±20	420±30	700±230	32±1	120±12
V_1/E_t, s^{-1}	3.5±0.2	22±8	3.5±1.6	2.8±0.2	1.9±0.1	0.62±0.02	10±1	16±3	0.015±0.002	29±2	19±1	17±1	2.0±0.8	32±1	14±1
V_2/E_t, s^{-1}	47±7	71±9	48±11	27±6	90±5	18±1	160±30	220±9	0.24±0.06	920±30	23±2	250±10	60±1	560±13	13±1
k_1, $\mu M^{-1}s^{-1}$	0.87	0.65	0.09	0.01		0.004	0.071	0.015		0.06	0.016	0.0074	0.002	0.076	0.03
k_2, s^{-1}	24	44	14	7.5		17	45	62		60	30	60	9	48	30
k_7, s^{-1}	3.8	38	26	8	206	250	21	63		90	17	60	190	140	17
k_8, $\mu M^{-1}s^{-1}$	7.6	7.4	3.3	0.18	0.31	0.12	1.3	0.20		3.4	0.058	0.15	0.27	4.4	0.13
K_{eq} I, pM	16	480	20	15	23	19	14	89	1.4	8	140	2.6	13	14	100
K_{eq} II, pM	1.7	250	1.4	0.068						2	500	2.8	26[f]	5.3	----
K_{eq}, pM	8	300	4.4	8	8	8	8	8	8	8	300	4.4	8	8	300
$K_{EA,B}$, mM	50	39	20	2300						2800	24	16	----	300	57
$K_{EQ,P}$, mM	0.47	3.5	0.06	0.25						9.9	24	0.09		3.2	----
R[g]	0.034	0.17	0.84	0.34	---	0.96	0.35	0.60	---	0.38	0.047	0.54	0.77	0.18	0.38

[a] For abbreviations, see footnote 1. [b] Subscripts a, b, p, and q represent constants for NAD^+, ethanol, acetaldehyde, and NADH, respectively; $K_{eq}(I) = V_1K_pK_{iq}[H^+]/V_2K_bK_{ia}$, $K_{eq}(II) = (V_1/V_2)^2K_{ip}K_q[H^+]/K_{ib}K_a$, $K_{EA,B} = K_{ib}K_aV_2/K_{ia}V_1$, $K_{EQ,P} = K_{ip}K_qV_1/K_{iq}V_2$, $k_1 = V_1/E_tK_a$, $k_2 = k_1K_{ia}$, $k_7 = k_8K_{iq}$, and $k_8 = V_2/E_tK_q$. [c] Equilibrium ordered mechanism, $K_m \rightarrow 0$. [d] mM. [e] Not determined, as only sequential initial velocity studies were done. [f] Product inhibition patterns of ethanol against acetaldehyde, and vice versa, were competitive as for Theorell–Chance mechanism, so slope inhibition constants were reported, and $K_{eq}(II) = V_1K_{ip}K_q[H^+]/V_2K_aK_{ib}$. [g] R, fraction of enzyme in central complexes, is 0 for Theorell–Chance and 1 for rapid equilibrium.

Table II: pH Dependencies of Kinetic Constants for Picolinimidylated Alcohol Dehydrogenase[a]

constant	pH 5.8	6.07	6.4	6.7	7.0	7.4	8.0	9.0	9.9
K_a (μM)	660 ± 160	400 ± 140		490 ± 120	590 ± 160	890 ± 260	370 ± 90	530 ± 50	850 ± 150
K_b (mM)	60 ± 15	60 ± 17		64 ± 13	74 ± 18	82 ± 24	26 ± 7	9.8 ± 2	4 ± 4
K_p (mM)	3.3 ± 1.9	7.0 ± 0.8	3.0 ± 1.1		11 ± 6	7.7 ± 2.4	5.4 ± 2.1	1.8 ± 0.8	1.6 ± 24
K_q (μM)	99 ± 50	280 ± 140	110 ± 34		280 ± 150	190 ± 60	210 ± 70	28 ± 15	110 ± 91
K_{ia} (μM)	290 ± 110	630 ± 190		820 ± 160	660 ± 150	670 ± 170	880 ± 280	1000 ± 300	2400 ± 2800
K_{iq} (μM)	110 ± 50	70 ± 20	91 ± 30	32 ± 10	37 ± 12	47 ± 10	98 ± 25	170 ± 70	520 ± 610
V_1/E_t (s^{-1})	0.85 ± 0.11	1.4 ± 0.1		6.2 ± 0.6	9.1 ± 1.2	32 ± 5	26 ± 5	53 ± 2	44 ± 4
V_2/E_t (s^{-1})	530 ± 160	1000 ± 400	540 ± 100		1200 ± 500	900 ± 210	760 ± 190	110 ± 20	49 ± 23
k_1 (μM^{-1} s^{-1})	0.0013	0.0035		0.013	0.015	0.036	0.070	0.10	0.052
k_2 (s^{-1})	0.37	2.2		10	10	24	62	100	124
k_7 (s^{-1})	590	250	450		160	230	350	670	230
k_8 (μM^{-1} s^{-1})	5.4	3.6	4.9		4.3	4.8	3.6	3.9	0.44

[a] The kinetic constants for each pH were obtained from least-squares fits to the equation for a sequential Bi-Bi mechanism: $v = V_1AB/(K_{ia}K_b + K_aB + K_bA + AB)$. See Table I for definitions and calculations.

constants have about the same magnitudes as those determined by stopped-flow methods (Shore, 1969; Shore & Gilleland, 1970; Plapp et al., 1973; Dworschack, 1976). The results show that increases in V_1/E_t are accompanied by increases in k_7, as must be the case. On the other hand, k_7 can be increased without a corresponding increase in V_1/E_t, when coenzyme dissociation is no longer rate-limiting as for several coenzyme analogues with modified adenosine moieties. Turnover in the reverse reaction and values for k_2 will be discussed later.

The rate constants for association, k_1 and k_8 (calculated from V_1/K_a or V_2/K_q), decreased 10-fold or more by modification of the adenosine moiety or of the amino group of the enzyme. This result supports the previous interpretation (Shore, 1969; Shore & Gilleland, 1970). Furthermore, the effect of both modifications is generally additive.

Structural Interpretations. X-ray crystallography of enzyme–coenzyme complexes shows that the adenine ring fits in a hydrophobic pocket that can accommodate a variety of molecules (Eklund et al., 1981, 1984). Nevertheless, substitution of the adenine ring with hypoxanthine or the 6-(4-acetylanilino)hexyl moiety decreased affinity (K_{ia} and K_{iq}) about 1 order of magnitude. The adenosine ribose forms hydrogen bonds with the carboxylate of Asp-223, a residue that is conserved in NAD-dependent dehydrogenases, and the ϵ-amino group of Lys-228. Nevertheless, the 2′-deoxy and 3′-deoxy derivatives of NAD^+ have only 2-fold decreases in dissociation constants (Suhadolnik et al., 1977). Replacement of the ribose with an alkyl moiety has more drastic effects, and dissociation constants increase 10–1000-fold. However, the aliphatic chain connection allows considerable freedom of motion for hydrophobic interactions, and the butyl adenine and 6-(4-acetylanilino)hexyl derivatives have optimal lengths. The imidyl substituent on the amino group of Lys-228 sterically interferes with binding of the adenosine ribose and fits into the cleft between the two domains that move closer during the conformational change that occurs when enzyme forms a ternary complex (Plapp et al., 1983).

In the three-dimensional structure, the carboxamido group on the nicotinamide ring forms hydrogen bonds to the peptide backbone units: NH of the Phe-319 and CO groups of Val-292 and Gly-317 (Eklund et al., 1984). The acetyl group of the acetylpyridine analogue would have only one of these hydrogen bonds and should dissociate more rapidly. Enzyme modified on Lys-228 binds this coenzyme even less tightly, but turnover numbers do not increase, as steps other than coenzyme dissociation become rate limiting.

pH Dependencies of Kinetics with Picolinimidylated Enzyme. Many of the kinetic constants, summarized in Table II, show little systematic variation over the range from pH 5.8 to pH 9.9. Nevertheless, the turnover number in the forward direction, V_1/E_t, is maximal above a pK value of 8.0, as are the calculated rates of association (k_1) and dissociation (k_2) for NAD^+ (Figure 1). The shapes of the pH dependency curves are just the opposite of those found with native enzyme, for which binding of NAD^+, NADH, and adenosine diphosphoribose is fastest below a pK value of 9.6, and dissociation of enzyme–NAD^+ is most rapid below a pK value of 7.6 (Dalziel, 1963; Shore et al., 1977; DeTraglia et al., 1977; Kvassman & Pettersson, 1979, 1980). For modified enzyme, V_2/E_t is maximal at low pH, below a pK value of 8.1, as is K_b. The pK values and pH-independent rate constants are summarized in Table III.

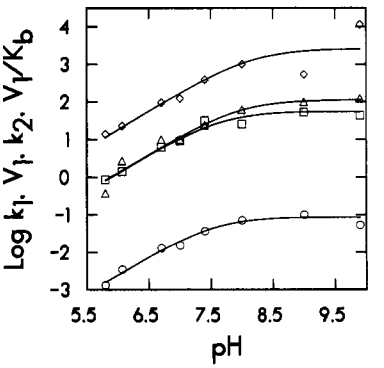


FIGURE 1: Dependence on pH of kinetic parameters for picolinimidylated liver alcohol dehydrogenase. The data from Table II were used to calculate the constants, using the logarithmic form of the pH dependency equation. The lines represent the fitted curves for $k_1 = k_{on}(NAD^+)$, calculated from V_1/E_tK_a (○); V_1/E_t (□); $k_2 = k_{off}(NAD^+)$, calculated from V_1K_{ia}/E_tK_a (△); and V_1/E_tK_b (◇).

Table III: pK Values for Picolinimidylated Alcohol Dehydrogenase[a]

constant	pK_1	pK_2	k_{max}
k_1 (μM^{-1} s^{-1})	7.9 ± 0.1	9.7 ± 0.1[b]	0.130 ± 0.005
k_2 (s^{-1})	7.9 ± 0.1		112 ± 5
V_1/E_t (s^{-1})	8.0 ± 0.2	10.4 ± 0.3[b]	60 ± 4
V_1/E_tK_b (μM^{-1} s^{-1})	8.1 ± 0.2		2600 ± 700
K_b (mM)		8.1 ± 0.3	69 ± 7
V_2/E_t (s^{-1})		8.1 ± 0.2	1200 ± 100

[a] Data from Table II were fitted to the pH dependency equation. [b] The pK value is only defined by a single point and therefore not adequately determined.

These data show that a group (or system) in picolinimidylated enzyme with a pK value of about 8 is critical for determining the rates of NAD^+ binding and release and ethanol oxidation. Hydroxybutyrimidylated enzyme was also found to have a group with a pK value of 8.4 that must be unprotonated for maximal rates of hydrogen transfer from

benzyl alcohol to NAD^+ (Dworschack & Plapp, 1977b), and native enzyme shows pK values ranging from 6.4 with ethanol and benzyl alcohol to 5.4 with 2-chloroethanol (Brooks et al., 1972; Kvassman et al., 1981). The kinetic and structural bases for the pH dependenceis are discussed below.

Rate-Limiting Isomerization of Enzyme–NAD^+ Complex. The values of k_2 calculated from the steady-state kinetic data (Tables I and II) are significantly lower than the observed values of V_2/E_t. This is inconsistent since the overall rate cannot be faster than the slowest step. Furthermore, the kinetic data in Table I show that the value of k_2 never exceeds 60 s^{-1}, despite large variations in coenzyme and enzyme structure and in V_2/E_t. An explanation for these results is that the enzyme–NAD^+ complex isomerizes, inserting an additional step in the mechanism:

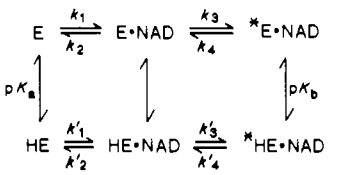
$$E \underset{k_2}{\overset{k_1}{\rightleftharpoons}} E{\cdot}NAD \underset{k_4}{\overset{k_3}{\rightleftharpoons}} {}^*E{\cdot}NAD \underset{k_6}{\overset{k_5}{\rightleftharpoons}} E{\cdot}NAD{\cdot}CH_3CH_2OH \underset{k_8}{\overset{k_7}{\rightleftharpoons}} E{\cdot}NADH{\cdot}CH_3CHO \underset{k_{10}}{\overset{k_9}{\rightleftharpoons}} E{\cdot}NADH \underset{k_{12}}{\overset{k_{11}}{\rightleftharpoons}} E$$

In this case, the value calculated for "k_2" from V_1K_{ia}/E_tK_a [the rate of dissociation of enzyme–NAD^+ complex, "k_{off}(NAD)"] is $k_2k_3k_4/(k_2 + k_3)(k_3 + k_4)$, which is less than the actual value for k_2 (Wratten & Cleland, 1963). For native enzyme, careful kinetic studies show that the discrepancy is a factor of 2 over the pH range 6–10, and it was concluded that the mechanism was not the simple ordered one (Dalziel, 1963; Wratten & Cleland, 1963). For the modified enzyme, the discrepancy can be as large as 10-fold at pH 8 or 1000-fold at low pH (e.g., 6), and the isomerization step must be considered as a rate-limiting factor in the mechanism. A similar case is that of *Bacillus subtilis* L-alanine dehydrogenase (Grimshaw & Cleland, 1981). Our results do not provide evidence for isomerization of the enzyme–NADH complex, but kinetic isotope and NMR experiments do (Cook & Cleland, 1981; Czeisler & Hollis, 1973).

If the isomerization of $E{\cdot}NAD^+$ is a slow step in the mechanism of picolinimidylated enzyme, the kinetic equations will simplify and the results can be explained. When k_2 and k_4 are relatively large compared to k_3 (and they must exceed 10^3 s^{-1}, the value of V_2/E_t at low pH), the value calculated for k_{off}(NAD) reduces to k_3. Likewise, "k_1" = k_{on}(NAD) = $V_1/E_tK_a = k_1k_3/(k_2 + k_3)$, which will simplify to k_1k_3/k_2, and $V_1/E_t = k_3k_7k_9k_{11}/(k_7k_9k_{11} + k_3k_7k_{11} + k_3k_7k_9 + k_3k_8k_{11} + k_3k_9k_{11})$ will reduce to $k_3k_7/(k_3 + k_7)$ if k_9 and k_{11} are much larger than the other rate constants. The rate of dissociation of the picolinimidylated enzyme–NADH complex, k_{11}, is faster than 200 s^{-1} (Plapp et al., 1973). The rate of release of acetaldehyde, k_9, is also very fast (Kamlay & Shore, 1983). The picolinimidylated enzyme exhibits significant primary kinetic isotope effects on turnover with NAD^+ and ethanol, indicating that k_7 is relatively slower than k_9 or k_{11} (Plapp, 1973; Dworschack & Plapp, 1977b).

With these simplifications, $k_{off}(NAD^+)$ calculated from V_1K_{ia}/E_tK_a is approximately k_3 and has a pH-independent value of 110 s^{-1} (Table III). Then, a value of 130 s^{-1} for k_7 can be calculated from V_1/E_t, and this value agrees well with the number determined by transient kinetic studies (Brooks et al., 1972). That the kinetic isotope effect (k_H/k_D) on V_1/E_t is about 3 with these modified enzymes, when an intrinsic isotope effect of about 6 is expected (Cook & Cleland, 1981), is consistent with the conclusion that V_1/E_t is controlled in part by a step not involving hydrogen transfer. Thus, all three kinetic parameters derived from V_1/E_t depend on k_3 and should show the same pH dependencies. On the other hand, the dissociation constant for NAD (K_{ia}, with a value of 860 μM) is pH-independent, since it is defined by $k_2k_4/k_1(k_3 + k_4)$ and reduces to k_2/k_1 if k_4 is larger than k_3.

Scheme I



The expression for V_2/E_t for this mechanism reduces to $k_4k_8/(k_4 + k_8)$ if k_2 and k_6 are large relative to k_4 and k_8. Thus, the pH dependency for V_2/E_t depends on k_4, rather than on k_3. The Michaelis constant for ethanol, K_b, also has k_4 in the numerator. Table III shows maximum values for V_2/E_t and K_b below a pK of about 8.1, the opposite of the pH dependences of the other kinetic values.

Pressure relaxation studies of native enzyme complexed with NAD^+ gave values of 300 s^{-1} for k_3, 60 s^{-1} for k_4, and 680 μM for k_2/k_1, at pH 7.7 (Coates et al., 1977). Furthermore, it has been suggested that the isomerization step, k_4, controls dissociation of the enzyme–NAD^+ complex and has the maximal rate below a pK value of 7.6 (Hardman, 1981). Since k_4 must be at least 1000 s^{-1}, chemically activated enzyme is clearly different than native enzyme. Thus, the modification has increased k_4 and decreased k_3, resulting in an altered isomerization equilibrium.

pH Dependence of Isomerization. The pH dependencies of kinetic constants for native and modified enzyme show that a group (or system) with a pK of about 8 controls the isomerization of the enzyme–NAD^+ complex. The results can be explained on the basis of Scheme I. We assume that pK_b is about 8. The isomerization rate constant, k_3, is largest when the enzyme is not protonated, whereas k_4 is largest when the enzyme is protonated. Because of the thermodynamics of this coupled system, the pK for HE·NAD must be larger than pK_b and could be the same as the pK of 9.6 determined for free enzyme. It would be desirable to detect the conformational change and determine these isomerization rate constants directly, as other mechanisms could explain the results.

The ionizing group(s) controlling the isomerization remains (remain) to be identified (Cleland, 1977). Perhaps the imidazole of His-51 must be unprotonated so that it can form a good hydrogen bond with the 2′-hydroxyl group of the nicotinamide ribose and allow the "open" form of the apoenzyme to "close up" to produce the conformation of the ternary complex. These structures have been described crystallographically (Eklund et al., 1976, 1981, 1982). On the other hand, ionization of the zinc–water to form the negatively charged zinc–hydroxide might facilitate proper binding of the positively charged nicotinamide ring of NAD^+. Since the pH dependence for inactivation by ethoxyformylation of apoenzyme shows a pK value of 9.6, the reactivity of His-51 may be controlled by the zinc–water ionization (Hennecke & Plapp, 1983), as these groups are coupled by hydrogen bonds. Thus, protonation of His-51 could disrupt the system and allow insertion of the 2′-hydroxyl group from the coenzyme into the system. Protonation of His-51 in the enzyme–coenzyme complex might destabilize the complex and allow faster rates of dissociation.

An alternative explanation is that ionization of the zinc–water causes a local conformational change, bringing the guanidinium group of Arg-47 into interaction with the zinc–hydroxide and preventing binding of coenzyme (Andersson et al., 1980). Arg-47 interacts with the AMP phosphate in the complex (Eklund et al., 1981). On the other hand, the zinc-

free apoenzyme and native enzymes show similar pH dependencies, and roles for amino acid residues must be considered (Dietrich et al., 1983).

The rates of association of NADH and adenosine diphosphoribose with native enzyme show the same pH dependency as does NAD^+ and can be explained by the same structural features. However, the conformation does not change when adenosine diphosphoribose binds (Nordström & Brändén, 1975). With picolinimidylated enzyme, the binding of NADH was pH-independent from pH 6 to pH 9, so the conformational change may not be observable due to faster isomerization or to the lack of charge on the nicotinamide ring. Indeed, with native enzyme, the isomerization of the $E{\cdot}NAD^+$ complex is relatively fast, whereas with modified enzyme or coenzyme, isomerization may have become a rate-limiting step. A structural explanation is that disruption of a few favorable interactions between enzyme and coenzyme hinders the coupling of binding with the conformational change (Plapp et al., 1983). When the adenosine moiety or Lys-228 is modified, the enzyme can still be a good catalyst, but when the AMP moiety is removed, as with NMN, the enzyme may not readily assume a conformation that binds the nicotinamide ring properly for catalysis. It remains to be determined if the enzyme must change conformation in order to catalyze dehydrogenation.

Registry No. NAD, 53-84-9; AcPAD, 86-08-8; NID, 1851-07-6; NABuD, 42188-24-9; NAPeD, 42188-25-0; NAPrD, 42188-23-8; NAcAnHeD, 75501-83-6; NAcAnPrD, 75501-82-5; NMN, 1094-61-7; NADH, 58-68-4; EtOH, 64-17-5; AcH, 75-07-0; alcohol dehydrogenase, 9031-72-5.

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Chick Neural Retina *N*-Acetylgalactosaminyltransferase/Acceptor Complex: Catalysis Involves Transfer of *N*-Acetylgalactosamine Phosphate to Endogenous Acceptors†

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ABSTRACT: Homogenates of embryonic chick neural retina prepared in 1% Triton X-100 have the ability to transfer *N*-acetyl[^{32}P]galactosamine ([^{32}P]GalNAc) from β-^{32}P-labeled uridine diphosphate *N*-acetylgalactosamine ([β-^{32}P]UDP-GalNAc) to endogenous macromolecular acceptors. The phosphotransferase activity sediments as three distinct peaks upon centrifugation on sucrose gradients. These peaks are coincident with the transferase/acceptor complexes previously described [Balsamo, J., & Lilien, J. (1982) *J. Biol. Chem. 257*, 345–354]. The parameters of the ^{32}P transfer reaction closely parallel those observed with UDP-[^{3}H]GalNAc as substrate when the densest particles, H, are used as a source of transferase/acceptors. Treatment of ^{3}H- and ^{32}P-labeled products with α-*N*-acetylgalactosaminidase removes [^{3}H]GalNAc residues and exposes ^{32}P-labeled groups. These data suggest that the sugar–phosphate is transferred intact, resulting in a terminal phosphodiester linkage. The resistance of the macromolecular products to digestion by endoglycosidase F and its sensitivity to hydrolysis under mild alkaline conditions suggest that the α-linked sugar is transferred to an oligosaccharide chain attached to the protein core via an *O*-serine or threonine residue. Characterization of the ^{32}P- and ^{3}H-labeled H particle products by sodium dodecyl sulfate–polyacrylamide gel electrophoresis reveals a series of coincident high molecular weight polypeptides.

Intact embryonic chick neural retina cells catalyze the incorporation of *N*-acetyl[^{3}H]galactosamine ([^{3}H]GalNAc)[1] from UDP-[^{3}H]GalNAc into endogenous macromolecular acceptors (Balsamo & Lilien, 1980). Both biochemical (Balsamo & Lilien, 1982) and immunological (Balsamo et al., 1986) approaches have verified the presence of enzyme and its acceptor at the cell surface. The enzyme and its endogenous acceptors can be obtained as three distinct complexes, stable in 1% Triton, each of which migrates to a defined density upon equilibrium centrifugation (Balsamo & Lilien, 1982). The properties of these complexes, in particular the densest, are similar to the properties of the enzyme as it exists at the cell surface. Catalysis of the transferase reaction among intact cells results in release of the glycosylated products into the reaction medium (Balsamo & Lilien, 1980), leaving the enzyme still associated with the cell surface and able to glycosylate exogenously added acceptors (Balsamo & Lilien, 1982). In the particulate complexes, the reaction catalyzed by the GalNAc-transferase results in the release of both the enzyme and the glycosylated reaction products into the reaction medium. Like the cell-surface enzyme, the released soluble GalNAc-transferase is now able to glycosylate exogenously added acceptors (Balsamo et al., 1986). The isolated complex thus provides an opportunity to study the properties of the transferase and its acceptor under more defined conditions.

Attempts to identify the GalNAc-transferase glycosylated products have suggested to us that some of the transferred GalNAc residues are very labile, possibly attached to the acceptor molecule via a phosphodiester bond. To investigate this possibility, we have synthesized [β-^{32}P]UDP-GalNAc and used it as a substrate for the particulate form of the enzyme. In this report, we demonstrate that catalysis of the cell-surface GalNAc-transferase/acceptor complex involves the transfer of GalNAc phosphate to endogenous glycoprotein acceptors.

Materials and Methods

Materials. UDP-[^{3}H]GalNAc (10.7 Ci/mmol; 0.0058 mg/mL), [^{3}H]acetic anhydride (50 mCi/mmol), [^{32}P]orthophosphate (25 mCi/250 μL), and Enlightening were purchased from New England Nuclear.

Antipain, leupeptin, chymostatin and PMSF (phenylmethanesulfonyl fluoride) were obtained from Sigma. Antipain and leupeptin were kept at −20 °C at 5 mg/mL in H_2O.

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[1] Abbreviations: GalNAc, *N*-acetylgalactosamine; GalNAc-transferase, *N*-acetylgalactosaminyltransferase; UDP-GalNAc, uridine diphosphate *N*-acetylgalactosamine; GalNAc-1-P, *N*-acetylgalactosamine 1-phosphate; UDP-Glc, uridine diphosphate glucose; Glc-1-P, glucose 1-phosphate; UDP-GlcNAc, uridine diphosphate *N*-acetylglucosamine; ATP, adenosine 5′-triphosphate; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; PMSF, phenylmethanesulfonyl fluoride; Me_2SO, dimethyl sulfoxide; DTT, DL-dithiothreitol; TCA, trichloroacetic acid; EDTA, ethylenediaminetetraacetic acid; PTA, phosphotungstic acid.

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