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Activity of Liver Alcohol Dehydrogenase with Various Substituents on the Amino Groups†

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ABSTRACT: Modification of amino groups at the active sites of horse liver alcohol dehydrogenase increased the activity of the enzyme up to tenfold, as assayed at pH 7 with high concentrations of substrates. Reductive alkylation and amidination by a variety of imido esters (new compounds are described) activated the most. Carbamylation changed the activity little, and succinylation inactivated. As the sizes of positively charged substituents on the amino groups were increased, the Michaelis and inhibition constants for all four substrates and the turnover numbers for the reactions catalyzed by the modified enzymes generally increased. Substituents that changed the net charge of the amino groups decreased the rates of binding of NAD^+ or NADH to modified enzymes; most substituents increased the rates of dissociation of the enzyme–coenzyme complexes. Modification of amino groups also differentially affected the binding of ethanol and acetaldehyde.

Most attempts to accelerate ethanol metabolism have given small or inconsistent effects (Lundquist, 1971). Since liver alcohol dehydrogenase catalyzes a rate-limiting step in the metabolism (Hawkins and Kalant, 1972), activation of the enzyme could accelerate ethanol metabolism.

Picolinimidylation of one ϵ-amino group at each active site of horse liver alcohol dehydrogenase increases the turnover number for the reaction of NAD^+ and ethanol about tenfold. The modified enzyme has an ordered bi–bi mechanism, in which the rate of dissociation of NADH (the rate-limiting step for the native enzyme) is so fast that transfer of hydrogen in the ternary complex becomes at least partially rate limiting (Plapp, 1970; Plapp *et al.*, 1973). The ordinary imido esters probably cannot be used to activate the enzyme *in vivo* because they nonspecifically modify most ϵ-amino groups of proteins (Hunter and Ludwig, 1972).

As part of a program to study the role of amino groups in enzymic activity and to design specific active-site directed activators of liver alcohol dehydrogenase, we have modified the amino groups with substituents of varied size, shape, and charge and studied the effects on the activity and kinetics of the enzyme.

Experimental Section

Chemistry. Many of the imido esters used in this study have been prepared previously (Hunter and Ludwig, 1962; Mc-

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Elvain and Nelson, 1942; Brooker and White, 1935; Schaefer and Peters, 1961). The preparation of new imido esters is described below. All products were dried over P_2O_5 and Na_2CO_3 *in vacuo*. Melting points were determined in open capillary tubes in an oil bath and are uncorrected. Elementary analyses were performed by Galbraith Laboratories, Knoxville, Tenn. Nuclear magnetic resonance (nmr) spectra were recorded on a Varian A-60 instrument, using tetramethylsilane as a reference. Infrared (ir) spectra were recorded on a Perkin-Elmer 21 instrument (KBr pellet) and ultraviolet (uv) spectra were recorded on a Cary 118C.

Methyl Octanimidate Hydrochloride. Octanonitrile (13.8 g, 0.11 mol) was mixed with absolute CH_3OH (3.5 g, 0.11 mol) and cooled to 0°. Dry HCl gas (7.6 g, 0.21 mol) was added, and the solution was stirred for 1 hr. Crystallization began after 12 hr at 0° and continued 3 more days. Absolute diethyl ether (150 ml) was added, and the white crystals were collected by filtration, washed with absolute ether, and dried to give 16 g (75%) of product: mp 100–102°; ir 1668 cm^{-1} (C=N); nmr (Me_2SO-d_6) δ 4.08 (s, OCH_3). For elementary analysis, the amidine was prepared. Absolute CH_3OH (200 ml) was saturated with NH_3 at 0°, and the octanimidate (9.7 g, 50 mmol) was added and allowed to react for 18 hr at 4°. A small amount of precipitate was removed by filtration, and the solvent was removed *in vacuo* at room temperature. Addition of 100 ml of ether resulted in formation of two phases. HCl (25 ml of 2 N) was added and the ether removed *in vacuo*. The crystalline residue was washed with diethyl ether and dried to give 6.1 g (55%) of product: mp 101–103°. *Anal.* Calcd for $C_8H_{19}N_2Cl$: C, 53.76; H, 10.72; N, 15.68; Cl, 19.84. Found: C, 53.66; H, 10.52; N, 15.59; Cl, 20.02.

Methyl 2-Naphthimidate Hydrochloride. 2-Naphthonitrile (7.7 g, 50 mmol) in 100 ml of CH_3OH and $NaOCH_3$ (0.27 g, 5 mmol) reacted for 25 hr at room temperature. Acetic acid (0.3 g, 5 mmol) was added and CH_3OH was removed at 30° *in vacuo*. The residue was triturated with diethyl ether, and undissolved sodium acetate was filtered off. HCl gas was introduced into the ethereal solution; the resulting suspension was stored over P_2O_5 and Na_2CO_3 overnight and the white crystals collected and dried to give 1.75 g (16%) of product: mp 193–194°; ir 1640 cm^{-1} (C=N); nmr (Me_2SO-d_6) δ 3.09 (s, OCH_3). *Anal.* Calcd for $C_{12}H_{12}ONCl$: C, 65.00; H, 5.46; N, 6.32; Cl, 16.02. Found: C, 65.13; H, 5.31; N, 6.26; Cl, 15.90.

Methyl Isonicotinimidate. 4-Cyanopyridine (26 g, 0.25 mol) in 225 ml of dry CH_3OH and $NaOCH_3$ (1.35 g, 25 mmol) reacted for 48 hr at room temperature. Acetic acid (1.45 ml, 25 mmol) was added, and CH_3OH was evaporated *in vacuo* at 30°. Solid sodium acetate was removed by filtration, and the filtrate was distilled *in vacuo*: bp 78–79° (0.45 mm). The yellowish liquid crystallized after 36 hr at −16° to give 17 g (50%) of methyl isonicotinimidate: mp 48–50°; ir 1653 cm^{-1} (C=NH); nmr ($CDCl_3$) δ 3.95 (s, OCH_3). For analysis the amidine was prepared: 1.36 g (10 mmol) of imidate was dissolved in 15 ml of CH_3OH, 535 mg (10 mmol) of NH_4Cl was added, and the suspension was stirred for 12 hr, after which the NH_4Cl had dissolved. CH_3OH was removed *in vacuo* (30°), and the residue was recrystallized several times from ethanol–ethyl acetate to give 1.2 g (76%) of isonicotinamidine hydrochloride: mp 102–104°. *Anal.* Calcd for $C_6H_8N_3Cl$: C, 45.70; H, 5.10; N, 26.66; Cl, 22.50. Found: C, 45.64; H, 5.29; N, 26.65; Cl, 22.43.

Methyl 1-Methylisonicotiniumimidate Iodide. Methyl isonicotinimidate (1.8 g, 13 mmol) was dissolved in 25 ml of CH_3NO_2. The solution was cooled to 0° and CH_3I (1.9 g, 13 mmol) in 25 ml of CH_3NO_2 was added slowly with stirring. After 5 days at 5° the solution was concentrated *in vacuo* at 30°, and a brown precipitate removed. Absolute diethyl ether (100 ml) was added, and after 12 hr at −20° yellow crystals formed, were collected, and dried to give 0.62 g (17%) of product: mp 161–163°; ir 1650 cm^{-1} (C=NH); nmr (Me_2SO-d_6) δ 3.96 (s, OCH_3), 4.53 (s, NCH_3); uv (H_2O) λ_{max} 264 nm. *Anal.* Calcd for $C_8H_{11}N_2OI$: C, 34.45; H, 3.98; N, 10.08; I, 45.50. Found: C, 34.47; H, 4.06; N, 9.95; I, 45.45.

Methyl 1-Ethylisonicotiniumimidate Iodide. Methyl isonicotinimidate was treated with CH_3CH_2I as above. Addition of absolute diethyl ether to the dark brown solution produced two phases. The lower phase crystallized on washing with more ether, and the yellow crystals were collected and dried to give 17 g (59%) of product: mp 144–145°; ir 1645 cm^{-1} (C=NH); nmr (Me_2SO-d_6) δ 3.92 (s, OCH_3), 4.80 (q, NCH_2), 1.60 (t, CH_2CH_3); uv (H_2O) shoulder at 264 nm. *Anal.* Calcd for $C_9H_{13}N_2OI$: C, 37.00; H, 4.48; N, 9.59; I, 43.46. Found: C, 37.09; H, 4.58; N, 9.57; I, 43.59.

Methyl 1-Methylnicotiniumimidate Iodide. Methyl nicotinimidate was prepared (Schaefer and Peters, 1961) and distilled *in vacuo* (49% yield): bp 67° (0.27 mm); ir 1655 cm^{-1} (C=NH); nmr (Me_2SO-d_6) δ 3.98 (s, OCH_3). Treatment with CH_3I as above gave, after 12 hr, long, yellow needles, which were recrystallized from cold CH_3OH (dissolved at room temperature, cooled to −20°) and dried to give a 35% yield of product: mp 157–158° dec; ir 1660 cm^{-1} (C=NH); nmr (Me_2SO-d_6) δ 3.92 (s, OCH_3), 4.52 (s, NCH_3); uv (H_2O) λ_{max} 267 nm, forming peaks at 262 and 334 nm in the presence of KCN. *Anal.* Calcd for $C_8H_{11}N_2OI$: C, 34.45; H, 3.98; N, 10.08; I, 45.40. Found: C, 34.63; H, 4.03; N, 10.07; I, 45.61.

Methyl 1-Ethylnicotiniumimidate Iodide. Methyl nicotinimidate was treated with CH_3CH_2I as above. The concentrated material was fractionated with diethyl ether to give an 18% yield of yellow crystals: mp 133–135° (dec above 137°); ir 1659 cm^{-1} (C=NH); nmr (Me_2SO-d_6) δ 3.91 (OCH_3), 4.83 (q, NCH_2), 1.61 (t, CH_2CH_3); uv (H_2O) λ_{max} 268 nm, forming peaks at 263 and 334 nm in the presence of KCN. *Anal.* Calcd for $C_9H_{13}N_2OI$: C, 37.00; H, 4.48; N, 9.59; I, 43.46. Found: C, 37.22; H, 4.36; N, 9.59; I, 43.51.

Enzymology. Crystalline horse liver alcohol dehydrogenase and chromatographically purified NAD^+ were purchased from Boehringer Mannheim. NADH was obtained from Sigma. The protein concentration was determined by amino acid analysis (Plapp, 1970). A Gilford 240 spectrophotometer was used for kinetic studies (Plapp *et al.*, 1973) as was a Cary 118C spectrophotometer, except that 0.02- and 0.05-*A* scales were used with native and some modified enzymes, which have small Michaelis constants for the coenzymes. Kinetic data were analyzed as described previously (Plapp, 1970) using the computer programs of Cleland (1963a, 1967).

Results

Since horse liver alcohol dehydrogenase has 30 lysine residues per subunit (Jörnvall, 1970), differential labeling was required if only the amino groups at the active sites were to be modified. The amino groups outside of the active sites were acetimidylated while the active sites were protected by the formation of the enzyme–NAD^+–pyrazole complex. Removal of the NAD^+ and pyrazole gave partially acetimidylated enzyme, which was used for all of the studies described here. Methyl picolinimidate reacts with one amino group per active site of the partially acetimidylated enzyme and activates it at about the same rate that the reagent activates native

enzyme (Plapp *et al.*, 1973). The partially acetimidylated enzyme is useful because we can determine and compare the activities of enzymes that are subsequently modified at the active site.

The partially acetimidylated enzyme was treated with a variety of reagents that react relatively specifically with amino groups under the conditions described below. The results are given in Table I. For most reactions, 10 μl of the reaction mixture was added directly to 0.99 ml of assay solution for determination of enzymic activity. The reagents changed the activity of the enzyme by a process dependent on time and reagent concentration. The assay for enzyme activity was not noticeably affected by the concentrations of reagents present. The activity (NAD^+ reduction) of modified alcohol dehydrogenases was measured at pH 9 with high concentrations of ethanol (Plapp, 1970), and under more physiological conditions, at pH 7 and 37° with 50 mM ethanol. The concentration of alcohol (50 mM) corresponds to about 250 mg/100 ml, which is sufficient to intoxicate 99% of all humans (Krantz and Carr, 1969). The native or partially acetimidylated enzymes were about 2.5 times as active in the pH 7 assay as in the pH 9 assay. The fold activation reported is the maximal activation observed. Under other conditions it is possible that more activation would be observed, but complete modification of some derivatives was indicated by the lack of further activation by methyl picolinimidate.

For modification by the imido esters, partially acetimidylated enzyme (about 1 mg/ml) in 0.5 M *N*-ethylmorpholine-HCl or triethanolamine-HCl buffer (pH 8.0) was treated with 0.1 M methyl imidates (except 0.01 M methyl 2-naphthimidate hydrochloride was used) at 25° for the times given in Table I. Higher (0.2 M) or lower (0.05 M) concentrations of the imido ester decreased or increased the time required for activation, but affected only slightly the fold activation. (The chloroacetimidylated enzyme was as stable in storage as the acetimidyl derivative, so there was no evidence for a cross-linking reaction as found with pancreatic ribonuclease (Olomucki and Diopoh, 1972).)

The alkylated derivatives were prepared by reduction with $NaBH_4$ of the Schiff base formed between the enzyme and the appropriate aldehyde or ketone at 0° (Means and Feeney, 1968) in 45 mM sodium pyrophosphate and 25 mM sodium phosphate buffer (pH 9.3) containing 0.19 mM EDTA and 5 mM NaCl. Before assays, the modified enzyme was freed of reagents by gel filtration. Native enzyme was activated 18-fold in the pH 9 assay and 4.8-fold in the assay of Dalziel (1957) by reductive methylation, which yields ϵ-dimethyllysine residues (Means and Feeney, 1968).

The carbamylated derivative was prepared with KNCO (Plapp, 1970). The ethyl- and phenylcarbamyl derivatives were prepared by the addition of 2 μl of the isocyanates diluted tenfold with dioxane to 0.4 ml of the enzyme in 0.5 M *N*-ethylmorpholine buffer (pH 8) at 25°. After 5 min of reaction, the isocyanate has decomposed (Brown and Wold, 1973) and the modified enzyme was assayed. Use of more or less isocyanate gave less activation or inactivation. (Incorporation of 1.1 equiv of fluorescein isothiocyanate per subunit of the native enzyme by the procedure of Weiner (1968) did not change the activity of enzyme in the pH 9 assay.)

Partially acetimidylated enzyme was acylated at 25° with maleic and succinic anhydrides (Butler and Hartley, 1972; Klapper and Klotz, 1972) by the addition of 5 mg of anhydride to about 1 mg of enzyme in 1 ml of 0.5 M triethanolamine-HCl buffer (pH 8). After 10 min, 5 mg of anhydride was added again. (Smaller amounts of anhydride (1 mg) inactivated in a stepwise manner, with the logarithm of the remaining activity being proportional to the amount added.) The pH was maintained between 7.5 and 8.0 by the addition of triethanolamine.

TABLE I: Activity of Modified Liver Alcohol Dehydrogenases.[a]

Substituent on Amino Groups[b]	Time of Reaction (hr)	Fold Activation[c]	
		pH 9	pH 7
Imidyl, $RC(NH_2^+)$			
CH_3	1	18	4.6
$ClCH_2$	1	29	5.6
CH_3CH_2	5	11	2.0
$CH_3(CH_2)_2$	2.4	9.3	1.9
$CH_3(CH_2)_6$	28	11	1.9
C_6H_5	24	28	5.0
2-Naphthyl	48	25	3.1
$(CH_3CH_2)_2NCH_2$	4	12	2.9
$(CH_3)_2NCH_2CH_2$	46	11	1.8
2-Pyridyl	4	45	5.7
3-Pyridyl	24	41	6.8
4-Pyridyl	6	49	3.5
3-(1-Methylpyridinium)	27	12	0.71
4-(1-Methylpyridinium)	24	12	0.65
3-(1-Ethylpyridinium)	48	12	0.71
4-(1-Ethylpyridinium)	26	11	N. D.
Alkyl, R			
$(CH_3)_2$	1	29	10
CH_3CH_2	1	17	7.0
$(CH_3)_2CH$	1	38	6.5
Carbamyl, RNHCO			
H	3.5	3.9	0.17
CH_3CH_2	0.1	3.7	0.8
C_6H_5	0.1	9.0	1.0
Acyl, R			
Succinyl	0.33	0.09	0.013
Maleyl	0.33	0.044	0.045

[a] Partially acetimidylated enzyme was modified with various reagents as described in the text so that the varied substituents should modify only the amino groups at the active sites. [b] R is varied as indicated. [c] Activity was determined in one assay mixture containing 85 mM $Na_4P_2O_7$, 6.5 mM semicarbazide-HCl, 18 mM glycine, 550 mM ethanol, and 1.75 mM NAD^+, at pH 9.0, and 25°, and in the other mixture containing 46 mM sodium phosphate, 10 mM semicarbazide, 10 mM NaCl, 50 mM ethanol, and 1.75 mM NAD^+, at pH 7.0 and 37°.

The steady-state kinetic parameters (Table II) for some of the modified enzymes (freed of excess reagents) were determined by product inhibition studies (Plapp, 1970). The patterns of inhibition for all modified enzymes were consistent with the ordered bi–bi mechanism (Cleland, 1963b; Plapp, 1970; Plapp *et al.*, 1973). The kinetic constants for the native enzyme in Table II agree with the constants found by Dalziel (1963b) at pH 8, except for K_a and K_{iq} which are 9 and 5 times larger in our studies. Using NAD^+ that was freshly purified by chromatography (Dalziel, 1963a) or leaving out the EDTA in the buffers did not change our results. The kinetic constants of partially acetimidylated enzyme were very similar to those for native enzyme (unpublished results of D. C. Sogin).

TABLE II: Kinetic Constants for Modified Alcohol Dehydrogenases.[a]

Constant[b]	Native	Dimethyl	Isopropyl	Acetimidyl	Benzimidyl	Picolinimidyl	Nicotinimidyl	1-Methylnicotiniumimidyl	Diethylaminoacetimidyl	Carbamyl	Acetimidyl[c]	Nicotinimidyl[c]
K_a, μM	26 ± 3	36 ± 2	26 ± 2	25 ± 5	420 ± 20	420 ± 20	360 ± 40	630 ± 80	1600 ± 120	200 ± 30	34 ± 3	500 ± 100
K_b, mM	0.35 ± 0.03	7.9 ± 0.5	28 ± 2	7.4 ± 0.6	12 ± 1	18 ± 1	18 ± 4	10 ± 2	19 ± 3	4.3 ± 0.4	8 ± 1	36 ± 8
K_p, mM	0.40 ± 0.05	5.8 ± 0.3	14 ± 1	0.78 ± 0.10	4.1 ± 0.2	5.7 ± 0.3	10 ± 1	6.5 ± 0.8	2.7 ± 0.3	1.4 ± 0.1	1.8 ± 0.2	1.4 ± 0.4
K_q, μM	5.8 ± 1.3	70 ± 8	170 ± 30	14 ± 3	140 ± 10	120 ± 10	340 ± 50	170 ± 20	230 ± 20	190 ± 40	11 ± 2	65 ± 10
K_{ia}, μM	27 ± 5	220 ± 20	210 ± 20	30 ± 5	750 ± 30	640 ± 20	1600 ± 200	900 ± 60	2800 ± 200	1500 ± 200	72 ± 9	640 ± 80
K_{ib}, mM	27 ± 3	20 ± 2	32 ± 8	130 ± 40	28 ± 2	26 ± 3	22 ± 5	*d*	39 ± 9	21 ± 5	210 ± 60	N. D.
K_{ip}, mM	0.52 ± 0.10	6 ± 4	*d*	*d*	12 ± 1	14 ± 2	18 ± 8	*d*	*d*	*d*	*d*	N. D.
K_{iq}, μM	3.3 ± 0.3	5.2 ± 0.2	8.2 ± 0.4	3.4 ± 0.6	38 ± 1	32 ± 1	35 ± 3	82 ± 9	140 ± 10	46 ± 6	3.0 ± 0.2	25 ± 3
K_bK_{ia}/K_a, mM	3.8 ± 0.6	25 ± 2	46 ± 5	11 ± 2	24 ± 2	31 ± 2	46 ± 7	33 ± 2	36 ± 6	4.8 ± 0.5	35 ± 4	14 ± 3
K_pK_{iq}/K_q, μM	14 ± 1	380 ± 30	1100 ± 60	84 ± 6	640 ± 50	830 ± 60	1300 ± 300	960 ± 100	540 ± 40	220 ± 10	66 ± 11	880 ± 170
V_1/E_t, sec^{-1}	3.4 ± 0.6	29 ± 1	26 ± 1	9.0 ± 0.7	29 ± 1	32 ± 1	38 ± 1	7.4 ± 0.2	22 ± 2	1.2 ± 0.2	6.8	5.7 ± 0.4
V_2/E_t, sec^{-1}	44 ± 4	790 ± 10	1100 ± 200	110 ± 10	530 ± 50	560 ± 10	800 ± 80	180 ± 2	150 ± 30	51 ± 8	240 ± 10	380 ± 30

[a] Except for native enzyme, all of the modified enzymes were partially acetimidylated on amino groups outside of the active sites and then modified at the active sites as indicated. [b] Values of K ± standard error, values of V/E ± range. Letters a, b, p, and q represent **NAD**$^+$, ethanol, acetaldehyde, and **NADH**, respectively. K is a Michaelis constant, K_i an inhibition constant; V_1/E_t is the turnover number for the reaction of **NAD**$^+$ and ethanol and V_2/E_t for **NADH** and acetaldehyde. [c] In 67 mM sodium phosphate buffer, pH 7.0 and 25°; other determinations in 33 mM sodium phosphate, pH 8.0 and 25°. Buffers contained 0.25 mM EDTA. [d] These values were not obtained because the product inhibition patterns appeared competitive. This probably indicates that the intercept inhibition constant (K_{ip}) is at least 50 times larger than the slope inhibition constant (K_pK_{iq}/K_q).

Discussion

Table I shows that alcohol dehydrogenase is activated when it is modified by a variety of reagents. Small aliphatic or aromatic substituents that retain the net positive charge of the amino group activate 17- to 49-fold at pH 9. Bulkier aliphatic or doubly charged aromatic substituents activate about 12-fold. Substituents that lack any charge activate 4- to 9-fold, and those that have a net negative charge inactivate. The relative fold activation observed at pH 9 is usually about 5 times the activation at pH 7, which can be explained as follows. In the assays at pH 9, native enzyme is inhibited about 55% by the high concentration of ethanol, whereas modified enzymes are much less inhibited. At pH 7 with 50 mM ethanol, native enzyme is only slightly inhibited by substrate (Dalziel and Dickinson, 1966). Furthermore, the maximum velocity for picolinimidylated enzyme depends upon an unprotonated group with a pK of 7.2 so this derivative should be 40% as active at pH 7 as at pH 9 (Plapp *et al.*, 1973). Native enzyme is 70% as active at pH 7.1 as at pH 9 (Dalziel, 1963b).

The kinetic constants for positively charged derivatives of the enzyme generally increase as the size of the substituent increases (Table II). Of particular interest, however, is that the Michaelis (K_a, K_q) and inhibition constants (K_{ia}, K_{iq}) for **NAD**$^+$ and **NADH** obtained with the acetimidyl derivative are similar to the values found with native enzyme. Apparently the active site is able to accommodate this small substituent on the amino group without markedly affecting coenzyme binding. On the other hand, even this substituent increases the Michaelis (K_b) and inhibition (K_{ib}) constants for ethanol. These results may indicate that the amino group of the native enzyme is closer to the binding site for ethanol than it is to the site for the nicotinamide ring of the coenzymes (Plapp, 1970). Alternatively, small changes in coenzyme binding could amplify changes in ethanol binding. The dimethyl and isopropyl derivatives also have small substituents, but these increase most of the kinetic constants. The small, neutral carbamyl substituent increases the kinetic constants for the coenzymes even more than the small, positively charged substituents do. Thus, the size and shape of the substituent on the amino group and its charge localization differentially affect the interaction of modified enzymes with substrates.

Since the homogeneity of the enzyme derivatives has not been determined, the effect of contaminating, unmodified or inactive enzyme forms on the magnitudes of the kinetic constants should be evaluated. The modified enzymes generally have larger kinetic constants than the partially acetimidylated enzyme. Therefore, a mixture of active enzyme forms should give nonlinear Lineweaver–Burk plots. Linear plots were observed, however, with the high concentrations of substrates and inhibitors used for the kinetic studies. Thus, the modified enzymes contributed the most to the activity observed, and the Michaelis and inhibition constants should closely approximate the true values. On the other hand, the turnover numbers could be less accurate because they should be proportional to the (undetermined) fraction of the enzyme that was modified. Experiments with incompletely picolinimidylated enzyme support these arguments. Recent work has shown that a unique lysine residue in partially acetimidylated alcohol dehydrogenase is amidinated or reductively alkylated (unpublished work of R. T. Dworschack and D. C. Sogin).

On the assumption that the mechanisms of the modified enzymes are ordered bi–bi, the kinetic data in Table II can be used to calculate rate constants for coenzyme binding and

dissociation constants for other substrates (Cleland, 1963b; Plapp, 1973). The rates of binding of NAD^+ and NADH to modified enzymes vary somewhat with the different derivatives, but decrease markedly if the substituent is uncharged (carbamyl) or at least partially doubly charged (methylnicotiniumimidyl, diethylaminoacetimidyl). The rates of dissociation of NAD^+ and NADH are affected less by the charge and more by the size or shape of the substituent. The acetimidyl group markedly increases the apparent dissociation constant calculated for ethanol. All substituents increase the apparent dissociation constants calculated for acetaldehyde. Knowledge of the three-dimensional structure of the enzyme (Brändén *et al.*, 1973) and its ternary complexes will facilitate further explanation of the data.

If modification of liver alcohol dehydrogenase *in vivo* were to be effective in accelerating ethanol metabolism, the modified enzyme must have suitable kinetic properties. The acetimidyl, dimethyl, and isopropyl derivatives seem to be appropriate because their Michaelis constants for NAD^+ are small, and their Michaelis constants for ethanol are suitable for highly intoxicating concentrations of ethanol. The picolinimidyl and other derivatives probably would not be suitable because their Michaelis constants for NAD^+ are so large that they could not compete for the free NAD^+ in the cell.

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