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Kinetics of Native and Activated Isozymes of Horse Liver Alcohol Dehydrogenase†

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ABSTRACT: The major isozymes of horse liver alcohol dehydrogenase (EC 1.1.1.1), EE, ES, and SS, have been separated by chromatography on phosphocellulose. Product inhibition studies showed that the kinetic behavior of EE and SS isozymes was consistent with the ordered BiBi mechanism. The different primary structures of the E and S subunits were expressed with higher Michaelis constants for ethanol and acetaldehyde and lower activity for the SS isozyme when compared with the EE isozyme. The differences for SS isozyme are reflections of slower rates of association and dissociation of coenzymes and slower rates of hydrogen transfer, not of affinities for the substrates. The contributions of each subunit in ES isozyme to the kinetic constants were not additive, indicating that the subunits may not act independently. Activation of the isozymes by amidination and alkylation suggested that lysine residues were present at the active sites of both E and S subunits. Kinetic studies indicated that isonicotinimidylation increased enzyme activity of the three isozymes by increasing the rates of dissociation of the enzyme–coenzyme complexes.

Horse liver alcohol dehydrogenase (EC 1.1.1.1) is heterogeneous; 12 isozymic forms have been reported [(Lutstorf et al., 1970; Pietruszko and Theorell, 1969; Pietruszko and Ryzewski, 1976); reviews: Pietruszko, 1975; Brändén et al., 1975]. Three of the major isozymes are dimers of two distinct subunits (Pietruszko et al., 1969; Pietruszko and Theorell, 1969). Jörnvall (1970) showed that the E subunit (active toward ethanol) and the S subunit (ethanol and steroid substrates) differed by at least six amino acid substitutions. The structures of the remaining isozymes have not been determined.

Kinetic studies have shown that the major isozymes (EE, ES, and SS) differ in Michaelis constants and specificities for various coenzymes, alcohols, and aldehydes and in pH dependencies (Lutstorf et al., 1970; Gurr et al., 1972; Theorell et al., 1970; Pietruszko, 1973), but the kinetic mechanisms of the ES and SS isozymes have not been determined. The E and S subunits act independently of each other in the binding of NADH[1] (Theorell et al., 1970), but they may interact with one another in catalyzing the oxidation of ethanol and cyclohexanol (von Wartburg et al., 1974).

The predominant EE isozyme is generally purified by modifications of the methods of Dalziel (1958, 1960), Theorell et al. (1966), and Taniguchi et al. (1967). Seven isozymes have been separated by chromatography on CM-cellulose and DEAE-cellulose (Lutstorf et al., 1970). Affinity chromatography on N^6-(6-aminohexyl)-AMP-Sepharose can be used to separate purified EE and SS isozymes (Andersson et al., 1974) and to purify SS isozymes (Andersson et al., 1975).

In order to study the ES and SS isozymes further, we used a simple method to isolate the major isozymes. The kinetics

† From the Department of Biochemistry, The University of Iowa, Iowa City, Iowa 52242. *Received March 19, 1976.* This work was supported by United States Public Health Service Grant AA00279 from the National Institute on Alcohol Abuse and Alcoholism.
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§ Supported by Research Scientist Development Award (K02AA00010) from the National Institute on Alcohol Abuse and Alcoholism.

[1] Abbreviations used: NAD, nicotinamide adenine dinucleotide; NADH, reduced NAD; EDTA, ethylenediaminetetraacetic acid; CM, carboxymethyl; DEAE, diethylaminoethyl; AMP, adenosine monophosphate; Tris, tris(hydroxymethyl)aminomethane; NADD, 4-deuterio-NADH.

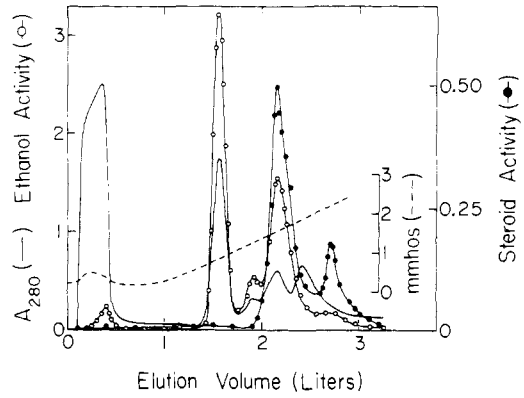


FIGURE 1: Chromatography of the isozymes of alcohol dehydrogenase on phosphocellulose. The sample was applied to a 2 × 50 cm column of Bio-Rad Cellex-P (0.89 mequiv/g) equilibrated with 2 mM sodium phosphate, pH 7.0, at 4 °C. The column was developed at 65 ml/h with a gradient formed with 1 l. each of sodium phosphate buffers, pH 7.0 (2, 10, and 50 mM) contained in three identical cylinders connected in series. Activity toward steroid was determined with 100 μM 3-keto-5β-cholanoic acid (Steraloids, Inc.) and 200 μM NADH in 46 mM sodium phosphate buffer, pH 7.0, at 25 °C.

TABLE I: Purification of Liver Alcohol Dehydrogenase.[a]

Step		Total Enzyme Units	Spec. Act. (units/mg)
Extract supernate		2800	0.04
50–80% $(NH_4)_2SO_4$ precipitate		2300	0.08
Heated supernate		2100	0.12
DEAE-cellulose		2100	0.47
Phosphocellulose:	EE	880 (730)	1.2 (1.8)
	ES	480 (310)	1.0 (1.3)
	SS	80 (50)	0.35 (0.50)
Crystallization:	EE	644	2.4

[a] These results are based on the purification of enzyme from 1 kg of frozen horse liver. Activity toward ethanol is the difference between the activities in the standard assay (Plapp, 1970) in the presence and absence of 10 mM pyrazole. Protein was determined (Lowry et al., 1951) with crystalline serum albumin as standard and from the extinction of a 1 mg/ml alcohol dehydrogenase solution (A_{280} = 0.455; Bonnichsen, 1950). The values in parentheses represent the particular values for the fractions after rechromatography on phosphocellulose.

of the isozymes were studied to determine the effects of the amino acid substitutions on the mechanism, kinetic parameters, and the independence of the subunits during catalysis. The amino groups of the SS isozyme were amidinated and alkylated to determine whether enzyme activity can be increased, as found previously for modification of the amino groups of Lys-228 of EE isozyme (Dworschack et al., 1975; Sogin and Plapp, 1975).

Experimental Procedure

Purification of the Major Isozyme Forms. The purification is based on an unpublished procedure obtained from Å. Åkeson, S. Taniguchi, and J. D. Shore. Our contribution to the procedure is to substitute gradient elution on phosphocellulose for stepwise elution from CM-cellulose.

Frozen horse liver was partially thawed, coarsely ground in a meat grinder, and suspended in twice its weight of distilled water. The slurry was adjusted to pH 7.0 with 4 N NH_4OH, allowed to stand at 4 °C for 2 h, and centrifuged for 1 h at g_{max} = 20 000. Alcohol dehydrogenase was precipitated with solid ammonium sulfate (enzyme grade, Schwarz/Mann) at 4 °C and pH 7.0. The material precipitating between 50 and 80% saturation was resuspended in a minimal volume of 46 mM sodium phosphate buffer, pH 7.0, and dialyzed at 4 °C against 2 mM sodium phosphate buffer, pH 7.8, for 24 h. The dialysate was heated in 200-ml batches to 52 °C with constant stirring in an 85 to 90 °C water bath, held for 15 min in a 52 °C water bath, and rapidly cooled. The suspension was centrifuged and the supernate was applied to a column of Whatman DE-52 cellulose (300 ml of packed cellulose per kg of original liver, 0.7 mequiv/g) equilibrated with 2 mM sodium phosphate buffer, pH 7.8, at 4 °C. The alcohol dehydrogenase that did not absorb to the column was adjusted to pH 7.0 with H_3PO_4 and chromatographed on phosphocellulose (Figure 1). The first large activity peak containing the major EE isozymes was followed by ES and SS isozymes. These isozymes were readily distinguished by using the ratio of their activities toward ethanol and the steroid: EE, >100; ES, 4–5, SS, 1.2. Protein loading of 25–45 mg of protein/ml of resin (12–21 enzyme units/ml of resin) appeared to be optimal for successful chromatography. Much lower loads resulted in poor yields. Active material was rechromatographed on smaller columns (0.9 to 2.0 cm in diameter) with correspondingly smaller gradients when the purest isozymes were desired. Protein loading was 5–15 mg of protein (6–26 enzyme units) per ml of resin. After rechromatography, each of the major isozymes was shown to be free from contamination by the other isozymes by electrophoresis on cellulose acetate. (Titan III plates, 2.5 × 7.5 cm, from Helena Laboratories were used, and electrophoresis was carried out in 25 mM Tris-HCl buffer, pH 8.5, at 200 V for 30 min at room temperature.) Enzyme was located with an activity stain (Pietruszko and Theorell, 1969).) The rechromatographed fractions were suitable for the kinetic studies described later, but the ES and SS isozyme preparations still contain inactive, extraneous protein if it is assumed that the extinction coefficients of the E and S subunits are the same.

The EE and ES isozymes were concentrated by crystallization in 35% ethanol at −20 °C. EE isozyme was crystallized in 46 mM sodium phosphate buffer, pH 7.0, and ES isozyme was crystallized in 6.6 mM buffer, pH 8.0. The EE isozyme was recrystallized as described by Dalziel (1958) except that 10% ethanol in 46 mM buffer, pH 7.0, was used. ES isozyme was recrystallized in 25% ethanol in 6.6 mM buffer, pH 8.0. The EE isozyme was purified 60-fold with a 23% yield (Table I).

Kinetic Studies. A Cary 118C spectrophotometer or a Hitachi MPF-2A spectrofluorimeter with excitation wavelength at 340 nm and emission wavelength at 460 nm was used. The fluorimeter was modified with a zero suppression circuit so that it was possible to measure small changes in NADH concentration in the presence of up to 2.5 μM NADH. At a sensitivity setting of 3, 1 μM NADH gave 12 in. of recorder deflection when the excitation slit was 7 mm and the emission slit was 40 mm. NAD^+ used for the fluorimetric studies was purified at 5 °C by chromatography on a column of Bio-Rad Cellex-D (2 × 50 cm) with a 500-ml linear gradient from 5 to 200 mM sodium phosphate buffers, pH 6.0. The data were analyzed as described previously (Plapp, 1970) using the computer programs of Cleland (1967).

The specific activities of EE, ES, and SS isozymes were calculated from activities on ethanol (Plapp, 1970) and the concentration of active sites was determined by fluorimetric titrations with NADH in the presence of 100 mM isobutyramide (Theorell and McKinley-McKee, 1961) and by spec-

TABLE II: Relative Activity of Isozymes with Modified Amino Groups.[a]

Modification	EE	ES		SS	
	Ethanol	Ethanol	Steroid	Ethanol	Steroid
Acetimidylation	7.7	6.8	5.9	8.7	3.8
Methylation	16	16	1.4	6.5	1.6
4-Hydroxybutyrimidylation	20	17	5.5	16	3.8
Picolinimidylation	20	17	3.5	30	3.4
Isonicotinimidylation	22	26		19	3.7

[a] The enzymes were modified as described by Zoltobrocki et al. (1974)—except that native, not partially acetimidylated, enzyme was used—and Fries et al. (1975) and assayed on ethanol (Plapp, 1970) or steroid (legend to Figure 1).

trophotometric titrations with NAD^+ in the presence of pyrazole (Theorell and Yonetani, 1963). The specific activities for the isonicotinimidylated isozymes could not be easily determined by the titration methods; consequently, specific activities were based on the value for native enzyme and the increase in activity upon modification.

Results and Discussion

Activation of Isozymes and Implication of Amino Groups in Activity. Various modifications of the amino groups of the isozymes increased the enzyme activity toward both ethanol and steroid substrates (Table II). Any one amidination increased the activity of all isozymes toward ethanol to about the same extent. Steroid activities were also increased comparably upon modification of the ES and SS isozymes. Methylation differentially activated; activity toward steroid was only slightly increased.

The lower activation observed with steroid as substrate was not due to a shift to a larger K_m with the modified enzyme. The K_m values of native and isonicotinimidylated SS isozyme toward 3-keto-5β-cholanoic acid were estimated to be 5.1 ± 0.8 and 7.2 ± 0.9 μM, respectively, at 200 μM NADH in 46 mM sodium phosphate buffer, pH 7.0 at 25 °C. (Waller et al. (1965) also obtained a K_m of 5 μM under similar conditions.) Therefore, velocities at the steroid concentrations used in the assays were near maximum for both native and modified SS isozymes. Assays performed in the same buffer with 200 μM NADH and 200 μM NADD (Rafter and Colowick, 1957) indicated that there was no isotope effect for this steroid substrate (4 to 20 μM 3-keto-5β-cholanoic acid) with native and modified SS isozymes. Thus the lower activation suggests that dissociation of the steroid alcohol product may be at least partially rate limiting for the overall reaction (cf. Waller et al., 1965).

The observation that modification of the SS isozyme increases its activity suggests that the S subunit has at least one lysine residue involved in the enzyme mechanism, as demonstrated previously for the EE isozyme. Presumably, the lysine residues in the E and S subunits are in homologous locations in the active sites. The following kinetic studies indicate that the lysine residues probably have the same roles in the isozymes and that modification affects the same steps in the mechanisms.

Product Inhibition Studies and Enzymatic Mechanism. The kinetics of the isozymes and their isonicotinimidylated derivatives were compared by means of product inhibition studies. Figures 2 and 3 present the inhibition patterns for the native ES and SS isozymes. Product and dead-end inhibition results for EE isozyme were reported previously (Zoltobrocki et al., 1974); in those studies the substrate concentrations were

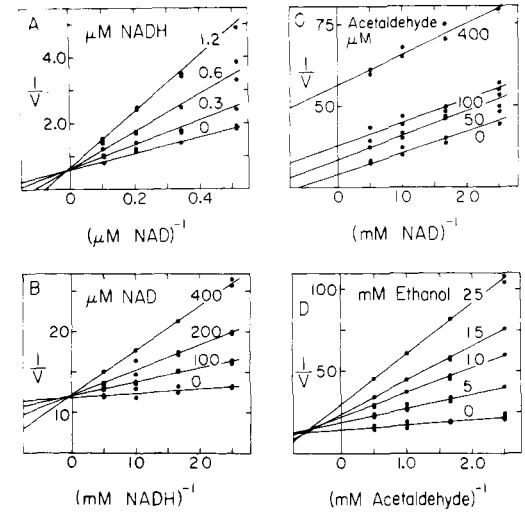


FIGURE 2: Product inhibition studies of ES isozyme. The buffer was 33 mM sodium phosphate, pH 8.0, containing 0.25 mM EDTA. Each figure is a Lineweaver-Burk plot of the primary data; V has units of ΔF per min for (A) and ΔA_{340} per min for B-D. The lines are calculated from least-square fits of the data to a hyperbola. Note that the values of the ordinate at the intersection with the abscissa may not be zero. (A) Inhibition by NADH against varied concentrations of NAD^+ at 5 mM ethanol; enzyme, 78 nN. (B) Inhibition by NAD^+ against varied concentrations of NADH at 4 μM acetaldehyde; enzyme, 7.5 nN. (C) Inhibition by acetaldehyde against varied concentrations of NAD^+ at 8 mM ethanol; enzyme, 80 nN. (D) Inhibition by ethanol against varied concentrations of acetaldehyde at 200 μM NADH; enzyme, 8.0 nN.

similar to those used in Figures 2 and 3. Product inhibition of isonicotinimidylated enzymes was studied using concentrations of substrates up to 20 times higher. All inhibition patterns for the native and isonicotinimidylated isozymes are summarized in Table III, where it may be seen that the results are consistent with the ordered BiBi mechanism. The uncompetitive patterns obtained for ethanol and acetaldehyde inhibition against variable concentrations of the coenzymes eliminate rapid equilibrium random and Theorell-Chance mechanisms (Cleland, 1963). However, the product inhibition results do not eliminate other random mechanisms, which are present to some extent, as evidenced by initial velocity (Dalziel and Dickinson, 1966; Hanes et al., 1972) and isotope exchange studies (Silverstein and Boyer, 1964; Ainslie and Cleland, 1972). It appears that the coenzyme binding sites are not drastically altered by the substitution of amino acid residues or the modification of the lysine residues so that the compulsory binding order (i.e., coenzyme followed by substrate) is essentially maintained. It is interesting that the substitution of Glu-336 in the E subunit for a lysine residue in the S subunit

TABLE III: Product Inhibition Patterns for Native and Isonicotinimidylated Isozymes.[a]

Isozyme	Q vs. A	P vs. B	P vs. A	B vs. P	B vs. Q	A vs. Q
Native EE[b]	C	NC		NC		C
ES	C	C	UC	NC		C
SS	C	NC		NC	UC	C
Isonicotinimidylated EE	C	C	NC	NC		C
ES	C	NC		NC		C
SS	C	C	NC	C	UC	C
Ordered Bi Bi mechanism[c]	C	NC	NC or UC	NC	NC or UC	C

[a] Each series is stated as inhibitor against variable concentrations of substrate. The letters, A, B, P and Q, represent NAD^+, ethanol, acetaldehyde, and NADH, respectively. The abbreviations used are as follows: C, competitive; NC, noncompetitive; UC, uncompetitive. [b] Data from Zoltobrocki et al. (1974). [c] Cleland (1963). It may be noted that uncompetitive inhibition patterns are obtained for P against A and B against Q only if the concentrations of the nonvaried substrate (i.e., B and P, respectively) are saturating.

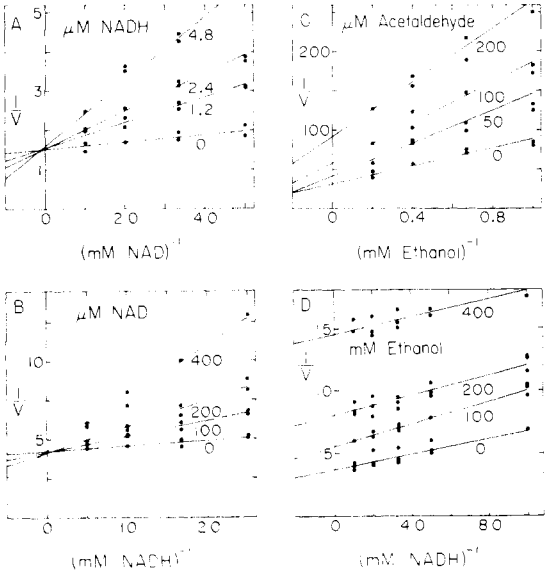


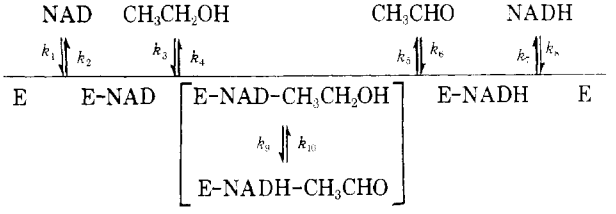
FIGURE 3: Product inhibition studies of SS isozyme. The buffer was 33 μM sodium phosphate, pH 8.0, containing 0.25 mM EDTA. V has units of ΔF per min for (A) and ΔA_{340} per min for B–D. (A) Inhibition by NADH against varied concentrations of NAD^+ at 25 mM ethanol; enzyme 500 μN. (B) Inhibition by NAD^+ against varied concentrations of NADH at 5 mM acetaldehyde; enzyme, 200 nN. (C) Inhibition by acetaldehyde against varied concentrations of ethanol at 2 mM NAD^+; enzyme, 400 nN. (D) Inhibition by ethanol against varied concentrations of NADH at 5 mM acetaldehyde; enzyme, 200 nN.

(the only known lysine substitution) has no effect on the kinetics of the SS isozyme with modified amino groups.

Comparison of Kinetic Characteristics. The data from the product inhibition studies were used to calculate the kinetic constants for an ordered BiBi mechanism (Table IV). The native isozymes have comparable Michaelis and dissociation constants for the coenzymes, but the SS isozyme has increased values of K_b and K_p. (The similarity of K_b and K_p values for EE and ES isozymes is probably due simply to the fact the E subunit contributes most of the activity of the ES isozyme and therefore dominates the kinetics.) The isonicotinimidylated isozymes generally have much larger constants than those for the native isozymes, but the Michaelis constant for ethanol has increased so much for the isonicotinimidylated SS isozyme that some apparent constants could not be properly corrected for the effect of the subsaturating concentration of nonvaried substrate.

The contributions of the E and S subunits to the turnover number for the reduction of acetaldehyde appear to be additive for both native and isonicotinimidylated isozymes, whereas the turnover numbers for the oxidation of ethanol are not additive (i.e., the values of V_1/E_t for native and modified ES isozyme are not the arithmetic means of those for EE and SS isozymes). The nonadditivity is also reflected in the specific activities, which were based on assays at pH 9 with 550 mM ethanol (Plapp, 1970) and active site titrations: EE, 2.4; ES, 2.3; and SS, 1.3 enzyme units per mg. These results may indicate that the subunits of the enzyme do not act independently, in agreement with previous conclusions (von Wartburg et al., 1974). However, it is clear that further work is required to establish and characterize the postulated interaction of E and S subunits and to clarify their possible relationship to "half-of-the-sites" reactivity observed in EE isozyme (Bernhard et al., 1970).

In order to interpret the kinetic constants for the various enzymes studied, the data from Table IV were used to calculate dissociation constants for ethanol and acetaldehyde and rate constants for coenzyme binding (Table V). (The Haldanes for native and modified isozymes are comparable to the equilibrium constant at 25 °C, 9.7×10^{-12} M (Bäcklin, 1958), which indicates that the kinetic constants are reasonably self-consistent and suitable for further analysis.) Comparing native SS with EE isozymes, it may be noted that the dissociation constants for ethanol, $K_{EA,B}$, are essentially the same. As discussed previously (Plapp, 1973), this constant is a good approximation of the ratio k_4/k_3.



Thus it appears that amino acid substitutions in SS isozyme do *not* alter the affinity for ethanol. However, the magnitudes of k_3 and k_4 could be increased or decreased proportionately. (Unfortunately, k_3 and k_4 cannot be calculated from the available data.) Such proportional changes can be demonstrated in the rate constants for coenzyme binding to EE and SS isozymes; the dissociation constants, K_{ia} and K_{iq}, are similar, but the rate constants for the SS isozyme have all decreased by 4- to 10-fold ($K_{ia} = k_2/k_1$, $K_{iq} = k_7/k_8$). The lower values of k_2 and k_7 are reflected in the smaller turnover numbers of SS isozyme. Dissociation of coenzymes from the E·NAD or E·NADH complexes is rate limiting for EE isozyme

TABLE IV: Kinetic Constants for Native and Isonicotinimidylated Isozymes.[a]

	EE	ES	SS
	Native		
K_a (μM)	3.9 ± 0.3[b]	4.2 ± 0.5	2.9 ± 0.7
K_b (mM)	0.35 ± 0.03	0.38 ± 0.02	1.9 ± 0.43
K_p (mM)	0.40 ± 0.05	0.22 ± 0.04	1.6 ± 0.39
K_q (μM)	5.8 ± 1.3	4.0 ± 0.9	9.8 ± 1.7
K_{ia} (μM)	27 ± 5	35 ± 8	48 ± [illegible]
K_{ib} (mM)	27 ± 3	20 ± 4	22 ± 11
K_{ip} (mM)	0.52 ± 0.10	0.45 ± 0.02	0.13 ± 0.05
K_{iq} (μM)	0.50 ± 0.03[b]	0.42 ± 0.05	0.52 ± 0.08
$K_{ia}K_b/K_a$ (mM)	3.8 ± 0.6	2.6 ± 0.5	19 ± 7.8
$K_{iq}K_p/K_q$ (μM)	14 ± 1	16 ± 1	98 ± 44
V_1/E_t (s^{-1})	3.5 ± 0.2	2.4 ± 0.6	0.38 ± 0.04
V_2/E_t (s^{-1})	47 ± 7	29 ± 4	7.9 ± 0.4
	Isonicotinimidylated		
K_a (μM)	474 ± 28	620 ± 40	450 ± 45[c]
K_b (mM)	42 ± 5	70 ± 8	4600 ± 2600[c]
K_p (mM)	19 ± 2	11 ± 1	5.0 ± 0.62
K_q (μM)	268 ± 36	180 ± 20	110 ± 15
K_{ia} (μM)	977 ± 81	450 ± 40	800 ± 70
K_{ib} (mM)	180 ± 54	33 ± 6	430 ± 55[c]
K_{ip} (mM)	65 ± 25	20 ± 3	76 ± 7.7[c]
K_{iq} (μM)	55 ± 3	32 ± 1	340 ± 100
$K_{ia}K_b/K_a$ (mM)	61 ± 8	49 ± 7	66 ± 7.8
$K_{iq}K_p/K_q$ (μM)	2900 ± 370	1700 ± 190	43 000 ± 4900
V_1/E_t (s^{-1})	29 ± 2	31 ± 4	9.9 ± 0.5
V_2/E_t (s^{-1})	920 ± 30	500 ± 30	67 ± 7

[a] The standard errors of the constants, K, from the computer fits and the standard deviations of the constants, V/E_t, from two or more determinations are reported. The subscripts a, b, p, and q for the Michaelis constants (K) and the inhibition constants (K_i) represent NAD^+, ethanol, acetaldehyde, and NADH, respectively. V_1/E_t represents the turnover number for the oxidation of ethanol, whereas V_2/E_t is that for the reduction of acetaldehyde. E_t is the normality of enzyme. [b] K_a and K_{iq} values for the native isozymes were obtained from kinetic studies performed with the fluorimeter: these values for EE isozyme agree with those obtained previously by Dalziel (1963) and should be substituted for those reported earlier (Zoltobrocki et al., 1974). The K_a and K_{iq} values for native enzyme determined spectrophotometrically (even with a 0.02A scale on a Cary 118C) are about five times too high, probably because accurate initial velocities are not obtained. K_a and K_{iq} values for the modified isozymes, obtained from studies performed with the spectrophotometer, were the same as those obtained with the fluorimeter. [c] These values could not be corrected for the subsaturating concentrations of ethanol due to the large difference between the concentration of ethanol used in the study and the K_b.

TABLE V: Haldane Relationships, Dissociation Constants, and Rate Constants for Ethanol and Acetaldehyde for Native and Isonicotinimidylated Isozymes.[a]

	EE	ES	SS
	Native		
Haldane (M)	16×10^{-12}	6.0×10^{-12}	4.4×10^{-12}
	1.6×10^{-12}	1.6×10^{-12}	0.5×10^{-12}
$K_{EA,B}$ (mM)	52	28	28
$K_{EQ,P}$ (mM)	0.45	0.37	0.12
k_1 ($\mu M^{-1}\ s^{-1}$)	0.90	0.60	0.13
k_2 (s^{-1})	24	21	6.3
k_7 (s^{-1})	4.0	3.0	0.42
k_8 ($\mu M^{-1}\ s^{-1}$)	8.1	7.3	0.81
	Isonicotinimidylated		
Haldane (M)	8.0×10^{-12}	6.9×10^{-12}	(0.7×10^{-12})
	2.0×10^{-12}	6.0×10^{-12}	(9.4×10^{-12})
$K_{EA,B}$ (mM)	2800	733	1600
$K_{EQ,P}$ (mM)	9.9	7.1	39
k_1 ($\mu M^{-1}\ s^{-1}$)	0.06	0.05	0.03
k_2 (s^{-1})	60	22.5	13
k_7 (s^{-1})	90	87	210
k_8 ($\mu M^{-1}\ s^{-1}$)	3.4	2.7	0.61

[a] Haldanes and rate constants were calculated according to Cleland (1963) and dissociation constants according to Ainslie and Cleland (1972).

(Dalziel, 1963) and for SS isozyme as indicated by the near equalities of V_1/E_t to k_7 and V_2/E_t to k_2.

Why the Michaelis constant for ethanol is higher with SS isozyme than with EE isozyme remains to be explained. Since Michaelis constants are complex functions of several rate constants, it is clearer to consider first the expression

$$V_1/K_bE_t = k_3k_5k_9/(k_4k_5 + k_4k_{10} + k_5k_9)$$

(Plapp, 1973). If we assume that k_4 and k_5 are the predominant terms in the denominator (since both are perhaps rapid dissociation reactions, whereas k_9 and k_{10} are limited by slower chemical reactions), the expression simplifies to k_3k_9/k_4. Since k_3/k_4 is similar for EE and SS isozymes (see above), it is reasonable to ascribe the 50-fold decrease in V_1/K_bE_t for SS isozyme to a decrease in k_9. Brooks et al. (1972) have determined a value of 150 s^{-1} for k_9 with EE isozyme and, therefore, even a value of 3 s^{-1} for SS isozyme would not make k_9 rate limiting for V_1/E_t. Then it is interesting that the fivefold increase in K_b, where

$$K_b = \frac{k_7(k_4k_5 + k_4k_{10} + k_5k_9)}{k_3(k_5k_7 + k_5k_9 + k_7k_9 + k_7k_{10})} \approx \frac{k_4k_7}{k_3k_9}$$

can be explained by disproportionate changes in k_7 and k_9, rate constants that do not reflect the affinity of the alcohol for the enzyme. By similar reasoning, changes in kinetic constants involving acetaldehyde can be explained by a 10- to 20-fold decrease in k_{10}, coupled with the changes already calculated. It is not obvious how the differences in rate constants can be explained in terms of the known differences in structures between EE and SS isozymes, but the substitution of a leucine residue for Phe-110 in the site proposed for substrate binding may be involved (Eklund et al., 1976).

Isonicotinimidylation of the isozymes increases the maximum velocities because k_2 and k_7 (rate limiting for native isozymes) are increased. The dissociation constants for coenzymes are increased for the same reason. As shown previously, by both steady state and transient kinetics, modification of amino groups of EE isozyme decreases k_1 but does not change k_8 significantly. The same result is observed with SS isozyme. From these kinetic results, it is not possible to determine whether modification affects direct interactions between amino groups and the coenzymes or the rate of a conformational alteration. It may be noted that the calculated rates of dissociation of NAD^+ (k_2) are substantially slower than the overall turnover number for the modified, but not the native, isozymes. This inconsistency (turnover cannot be faster than the slowest step) has been interpreted previously as evidence for an isomerization of the enzyme·NAD^+ complex prior to coenzyme dissociation (Cleland, 1963; Wratten and Cleland, 1963; Plapp, 1970).

Acknowledgment

The technical assistance of David P. Bohlken was greatly appreciated.

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