BIOORGANIC CHEMISTRY 9, 176–186 (1980)

Studies on Methylated Yeast Alcohol Dehydrogenase

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Received June 12, 1978

The incubation of yeast alcohol dehydrogenase with formaldehyde in the presence of $NaBH_4$ methylates lysine residues to form $\epsilon N,\epsilon N$-dimethyl lysine with a concurrent decrease in enzymic activity which is not alleviated by the presence of coenzymes. The modification causes structural change(s) in yeast alcohol dehydrogenase as evidenced by a hyperchromic shift in the uv spectrum, the sensitivity to heat inactivation, the reactivity to sulfhydryl reagents, and a change in Stokes' radius. Kinetic studies indicate that the reduced activity of the methylated enzyme to oxidize alcohols is associated with decreased maximum velocities by retarding the interconversion of the ternary complexes. The catalytic efficiency of the control enzyme to oxidize primary alcohols is affected by the steric interaction which is absent in the methylated enzyme.

INTRODUCTION

It has been observed for horse liver alcohol dehydrogenase (LADH,[1] alcohol:NAD^+ oxidoreductase, EC 1.1.1.1) that reductive methylation of lysine residues results in conformational changes with a concurrent increase in its activity to oxidize ethanol (*1*). Detailed kinetic studies of methylated LADH indicate that the methylation facilitates the enzyme ability to oxidize primary alcohols by enhancing the dissociation of the enzyme–NADH–alcohol complex but, at the same time, reduces its ability to oxidize secondary alcohols by retarding the interconversion between the ternary complexes (*2*). Since yeast alcohol dehydrogenase (YADH) catalyzes an identical reaction without forming the enzyme–NADH–alcohol complex (*3*), it offers a desirable source of study for obtaining further information concerning the methylation effect observed in LADH.

Although both LADH and YADH catalyze the same reaction and possess presumably similar binding and catalytic domains (*4*), the two enzymes differ in many aspects. The dehydrogenase from yeast is tetrameric whereas that from liver is dimeric. The two enzymes are homologous in less than 40% of the sequence (*5*). A broad substrate specificity characterizes both enzymes (*6*), however YADH is active toward primary alcohols and those secondary alcohols to which both alkyl groups attached are not larger than methyl (*7*). Furthermore the activity of primary alcohols decreases with an elongation in the carbon chain.

[1] Abbreviations used: ADPR, adenosine diphosphate ribose; LADH, horse liver alcohol dehydrogenase; NMN, nicotinamide mononucleotide; TNBS, 2,4,6-trinitrobenzene sulfonic acid; YADH, yeast alcohol dehydrogenase.

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This is attributed to a smaller catalytic site pocket for the yeast enzyme (*4*). Alcohol dehydrogenase from horse liver is, structurally and kinetically, one of the best-characterized enzymes (*4*). In addition, considerable knowledge concerning the methylation or related modifications of LADH has been accumulated recently (*2, 8–10*). Therefore, studies on methylated YADH may provide additional comparative characteristics for the two enzymes.

MATERIALS AND METHODS

Alcohol dehydrogenases from horse liver ($1.7–3.4 \times 10^{-8}$ kat/mg) and yeast (3.4×10^{-6} kat/mg), bovine serum albumin, α-chymotrypsin, cytochrome *c*, lysozyme, NAD^+, NADH, nicotinamide mononucleotide (NMN), adenosine diphosphate ribose (ADPR), and phenyl methane sulfonyl fluoride were obtained from Sigma Chemical Corp. ϵN-Monomethyl-L-lysine and $\epsilon N,\epsilon N$-dimethyl-L-lysine were purchased from Cyclo Chemical Corp. [^{14}C]Formaldehyde (10 mCi/mol in 1.0% aqueous solution) was supplied by New England Nuclear. Primary alcohols were products of Fisher's Chem. Comp. and purified by distillation prior to the use.

Methylated YADH was prepared by a slight modification of the procedure previously described for LADH (*9*). Freshly prepared $NaBH_4$ solution (0.5 ml of 10 mg/ml) was mixed with 4.2 ml of solution containing 0.30 μmol of yeast alcohol dehydrogenase (based on a molecular weight of 1.50×10^5) in 0.10 *M* sodium pyrophosphate buffer, pH 9.0 on ice. Formaldehyde solution (12 μmol in total volume of 0.3 ml) was added in six portions over a 2-hr period. The reaction mixture was dialyzed overnight and subject to gel filtration in a column (1.5×30 cm) packed with Bio Gel P200 employing water as an elution solvent. The fractions corresponding to tetrameric methylated YADH were pooled and lyophilized. The control was prepared by replacing formaldehyde with water. ^{14}C-labeled methylated YADH was prepared in an identical manner using 0.01 μCi/μmol [^{14}C]formaldehyde.

Free amino groups were determined by the 2,4,6-trinitrobenzene sulfonic acid (TNBS) procedure (*11*). The reaction with 5,5′-dithiobis(2-nitrobenzoate) (DTNB) was performed as described (*12*). The heat inactivation was carried out by incubating the enzyme solution (1.0 mg/2.0 ml in 0.10 *M* sodium phosphate buffer, pH 8.0) in a water bath (Neslab, model TEV 40) equipped with a temperature programmer (Neslab, model TP2). Samples were withdrawn for assaying the enzymic activity. The methylated YADH was subjected to sucrose gradient centrifugation by layering 50 μl of the enzyme solution (5.0 mg/ml) on the top of a tube containing sucrose gradient prepared from an equal volume (2.2 ml) of 5 and 20% (w/v) sucrose solutions. References were prepared in an identical manner. Tubes of the sample and references were centrifuged at 45,000 rpm for 12 hr. At the end of centrifugation, 10-drop fractions were collected for protein determination at 280 nm and enzymic assays.

The enzymic activity was assayed by following absorbancy changes at 340 nm of a reaction mixture (1.0 ml) containing an appropriate amount of YADH

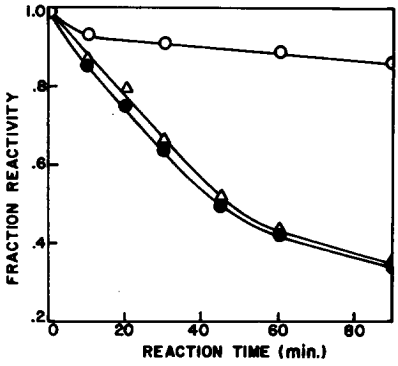


FIG. 1. Time-dependent inactivation of YADH by reductive methylation. Yeast alcohol dehydrogenase (0.30 μmol) was incubated with 6.0 μmol of formaldehyde in the presence of 2.0 mg $NaBH_4$ (methylation, ●) and 11.4 μmol of NAD^+ (NAD^+ protection △). The control (○) was prepared by replacing formaldehyde with water. At time intervals, 0.5-ml aliquots were withdrawn and filter through a column (1.0 × 12.5 cm) of BioGel P6. Filtrates were assayed for protein and YADH activity.

(A_{280} = 0.001 for the control and 0.05 for the methylated enzyme), 0.50 μmol NAD^+ and 1.0 μmol ethanol in 0.10 *M* sodium pyrophosphate buffer (pH 9.0) using a Beckman DB spectrophotometer at 30 ± 0.5°C. Kinetic studies were carried out in a Perkin-Elmer spectrophotometer (Coleman, model 124) equipped with a variable output recorder (Coleman, model 165) and a temperature circulator maintained at 30 ± 0.2°C. The reaction was initiated by the addition of YADH (5.0×10^{-3} A_{280}-units corresponding to 33.8 n*M*) or methylated YADH (5.0×10^{-2} A_{280}-units corresponding to 275 n*M*) to a reaction mixture containing 0.050 to 2.0 m*M* NAD^+, 1.0 to 200 m*M* primary alcohols in 0.10 *M* sodium phosphate buffer, pH 8.0. The rate of NADH formation was followed at 340 nm. Initial velocities were analyzed graphically according to:

$$v = \frac{VAB}{AB + K_a B + K_b A + K_{ia} K_b} \qquad [1]$$

where V, K_a, K_b, and K_{ia} are maximum velocity, Michaelis constant for NAD^+ (A), Michaelis constant for alcohol (B), and inhibition constant for NAD^+, respectively (*13*).

RESULTS

Figure 1 shows that treatment of YADH with formaldehyde in the presence of $NaBH_4$ caused a gradual loss in catalytic activity. Neither NADH nor NAD^+ affords protection to the yeast enzyme from inactivation under the experiment conditions, while the coenzymes protect LADH from activation (Table 1). Furthermore studies with coenzyme constituents do not appear to implicate any part of the coenzyme molecule which is structurally essential for the protection. These results suggest that the modified lysine residues are unlikely to be situated at the catalytic or NAD^+ binding site. The inactivation of YADH follows first

TABLE 1

EFFECT OF COENZYMES ON REDUCTIVE METHYLATION OF LIVER AND YEAST ALCOHOL DEHYDROGENASES[a]

Protecting agents	Ethanol oxidation by methylated enzymes v(μmol min^{-1})	
	LADH (1.0 mg)	YADH (0.10 mg)
Control	9.0	17.0
NAD^+	10.5	2.1
NADH	10.1	2.5
NMN	14.8	1.8
ADPR	12.5	2.0
None	40.8	1.6

[a] Alcohol dehydrogenase from horse liver and yeast was methylated (see text) for 1.0 hr in the presence of 38-fold excess of protecting agents. Initial velocities for ethanol oxidation were measured as described in the text.

order kinetics (k = 0.787 min^{-1}) for approximately 1 hr to reach a residual activity of 20–30% in 1.5 hr. Within a 1 hr-period, 10–20% of ε-amino groups corresponding to 2–4 of 24 lysine residues per subunit were modified as assayed by TNBS. Lysine is the only amino acid residue methylated (*1*). Figure 2 shows that more than 95% of the radioactivity from ^{14}C-labeled methylated YADH is associated with ε*N*,ε*N*-dimethyl lysine.

To examine the effect of methylation on the subunit structure of YADH, the

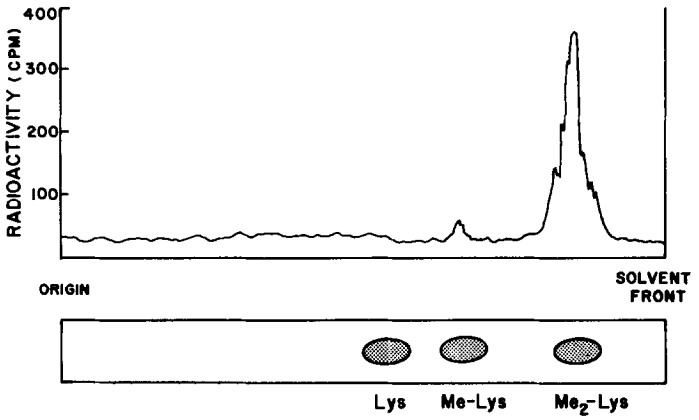


FIG. 2. Identification of ε*N*,ε*N*-dimethyl lysine. ^{14}C-labeled methylated YADH was prepared as described in the text and hydrolyzed with 6.0 *N* HCl for 18 hr in a sealed tube. The hydrolysate, after being freed of HCl by repeated evaporations, was spotted on Whatman No. 1 filter paper which was developed with a solvent system, water-conc. NH_4OH-propan-1-ol (1 : 3 : 6 by volume) in a descending manner. Reference compounds, lysine (Lys), ε*N*-monomethyl lysine (MeLys), and ε*N*,ε*N*-dimethyl lysine (Me_2Lys) were run in parallel. Amino acids were visualized by ninhydrin spray and radioactivity was detected by means of Packard Radiochromatogram Scanner.

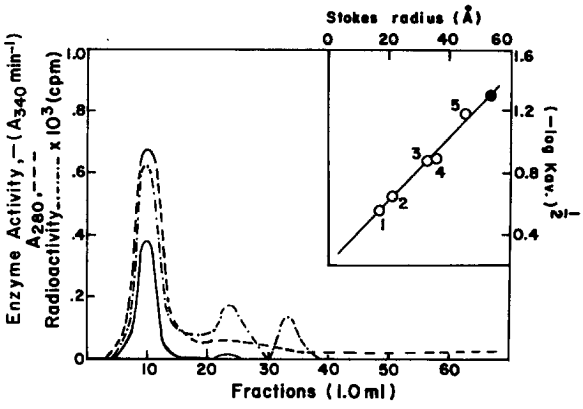


FIG. 3. BioGel P200 filtration chromatography of methylated YADH. Yeast alcohol dehydrogenase was methylated for 1.5 hr. The methylated sample was analyzed on BioGel P200 (1.5 × 30 cm). One-milliliter fractions were collected. Inset shows a plot for the estimation of Stokes' radius of the methylated YADH (●). The column was calibrated with reference proteins, i.e., 1, cytochrome *c*; 2, α-chymotrypsin; 3, LADH; 4, bovine serum albumin; and 5, YADH.

methylated sample was subjected to filtration chromatography on Bio Gel P200 (Fig. 3). The major component of the methylated YADH was eluted ahead of the native YADH. This is due presumably to change in the tetrameric molecule rather than aggregation since the same sample, when subjected to a sucrose gradient centrifugation, sedimented at an identical rate as the native enzyme (Fig. 4). Furthermore, the rechromatography of the tetrameric methylated YADH gave a single peak and the enzymic activity was linearly related to enzyme concentration. In a number of experiments, protein species with smaller molecular size corresponding to dimeric and monomeric forms were detected in the methylated sample (Figs. 3 and 4). The presence of these dissociated products was particularly noticeable in the ^{14}C-labeled methylated sample. A similar elution profile was obtained in the presence of 1.0 m*M* phenyl methane sulfonyl fluoride suggesting

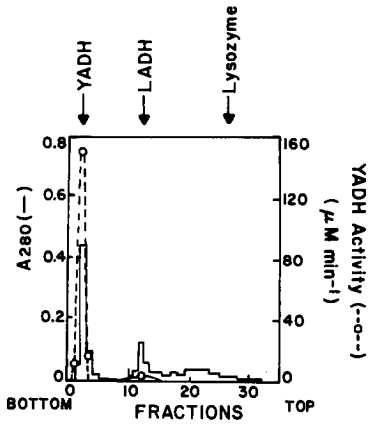


FIG. 4. Sucrose gradient centrifugation of methylated YADH. Bands corresponding to reference proteins are indicated by arrows.

that the dissociated species did not arise from nonspecific proteolysis. Frequently a very low enzymic activity (less than 1/500 of the control) was found to associate with the dimeric molecule. However, we were neither able to reproduce an identical result (the active dimer) in every modification nor able to isolate the enzymatically active dimer for characterization. Nevertheless the tetrameric methylated protein (to be referred as the methylated YADH) can be isolated for further studies, though a loss in activity occurred after lyophilization and storage.

The methylated YADH exhibits a slight uv hyperchromicity ($\epsilon_{280} = 1.82 \times 10^5$ cm M^{-1}) and an increased Stokes' radius (54.5 Å) based on the relationship between Stokes' radii and distribution coefficients, K_{av} (*14*) of reference proteins as shown in the inset of Fig. 3. The methylated enzyme which was completely inactivated at 67.5°C is less stable to heat denaturation than the control YADH which was denatured completely at 72.5°C. However the modified YADH remained soluble during heat inactivation, while the native enzyme became turbid at 57.5 ± 1.5°C. 5,5′-Dithiobis(2-nitrobenzoate) instantaneously reacted with 2 sulfhydryl groups per mole of the methylated YADH ($k = 0.997$ min^{-1}), an identical reaction took approximately 1 hr for the control enzyme ($k = 0.0322$ min^{-1}).

Initial rate studies of the methylated and control YADH reveal interesting differences though both enzymes follow the same mechanism (see discussion). At high concentrations, NAD^+ inhibits the methylated YADH but not the control (Fig. 5). High ethanol concentrations activate the control but inhibit the methylated enzyme whereas high concentrations of allyl alcohol inhibit the control but activate the modified YADH. In the asymptotic region of NAD^+ concentrations ($NAD^+ \leq 1.0$ mM) plots of v^{-1} versus $[\text{ethanol}]^{-1}$ or $[\text{allyl alcohol}]^{-1}$ give a linear asymptotic region ([ethanol] $\leq$ 50 mM; [allyl alcohol] $\leq$ 5.0 mM) and a concave region ([ethanol] $\geq$ 50 mM; [allyl alcohol] $\geq$ 5.0 mM). Table 2 lists kinetic parameters for the respective regions (*2*). The substrate activation is associated with K'_b whereas the substrate inhibition, with K'_{-b}(*2*). The methylation affects YADH by lowering V and V' as well as altering $K'_b(-b)$.

To assess factors affecting V of YADH on methylation, kinetic studies were

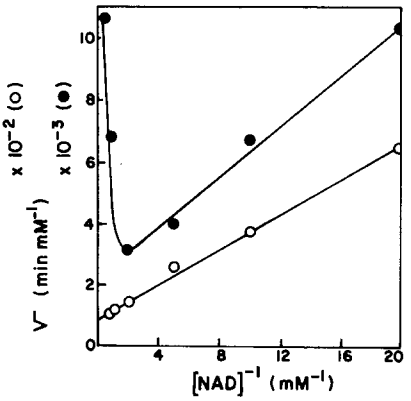


FIG. 5. Initial velocities for oxidation of pentan-1-ol (5.0 mM) with varied NAD^+ concentrations. -○-○-, Control YADH; -●-●-, methylated YADH.

TABLE 2

YEAST ALCOHOL DEHYDROGENASES (CONTROL AND METHYLATED) CATALYZED OXIDATION OF ETHANOL AND ALLYL ALCOHOL[a]

Kinetic parameters	Control YADH		Methylated YADH	
	CH_3CH_2OH	$CH_2{=}CHCH_2OH$	CH_3CH_2OH	$CH_2{=}CHCH_2OH$
Asymptotic				
V(mM min^{-1})	0.254	0.212	2.77×10^{-3}	1.33×10^{-3}
K_a(mM)	0.377	0.496	0.271	0.0867
K_b(mM)	3.54	1.24	5.58	3.65
K_{ia}(mM)	0.540	0.711	0.258	0.210
Concave				
V'(mM min^{-1})	0.263	0.0347	182×10^{-3}	9.09×10^{-3}
K'_a(mM)	0.132	0.833	0.364	0.0455
K'_b(mM)	37.9			69.6
K'_b(mM)		0.583	11.5	
K'_{ab}(mM)	0.353	0.655	0.329	0.0869

[a] E_t = 33.8 nM for the control and 275 nM for the methylated YADH. Kinetic parameters in the concave region were evaluated as described (*2*). The concave upward plot (substrate inhibition) is associated with lower V' and K'_b.

carried out in the asymptotic region using primary alcohols (RCH_2OH) with various substituents (R). The kinetic parameters are summarized in Tables 3 and 4. Methylation greatly lowers V without significantly affecting K_a, K_b, and K_{ia}. Therefore V/E_t values were analyzed for the substituent effects according to

$$\log V/E_t = \rho^*\sigma^* + SE_s + p\pi + d \qquad [2]$$

as described previously (*9*). σ^*, E_s, and π are corresponding electronic constants, steric constants, and hydrophobic constants, respectively, for given substituents of primary alcohols (*9*). ρ^*, S, and P are fitted contributions to electronic, steric, and hydrophobic effects. Stepwise regression analysis indicates that the steric effect is the most important factor affecting V/E_t of the control (Fig. 6) though the inclusion of the electronic effect ($\rho^* = 0.689$) slightly improves the correlation coefficient ($r = 0.866$). The positive coefficient of E_s ($S = 2.05$) suggests an unfavorable steric contribution.

Three substituent variables were required to give an acceptable but poor correlation ($r = 0.756$) for the methylated YADH (Fig. 7). The negative coefficient for E_s is probably fortuitous. Nevertheless it is important to note the absence of the deleterious steric hindrance.

DISCUSSION

Formaldehyde in the presence of $NaBH_4$ which specifically methylates lysine residues activates LADH to oxidize ethanol (*1*). The coenzymes protect LADH from modification. An identical treatment inactivates YADH. Neither NAD^+ nor

TABLE 3

KINETIC PARAMETERS FOR YADH (CONTROL) CATALYZED OXIDATION OF ALCOHOLS

R in RCH_2OH	V (mM min^{-1})	K_a (mM)	K_b (mM)	K_{ia} (mM)	V/E_t (min^{-1})
CH_3	0.320	0.550	3.07	0.590	9.47
CH_3CH_2	0.215	0.572	7.66	0.547	6.36
$CH_2{=}CH$	0.314	0.496	2.03	0.711	9.29
$CH_3(CH_2)_2$	0.133	0.596	9.68	0.610	3.94
$(CH_3)_2CH$	0.930×10^{-3}	0.204	5.02	0.525	0.0275
$CH_3(CH_2)_3$	0.0328	0.399	8.74	0.323	0.970
$(CH_3)_2CHCH_2$	0.747×10^{-3}	0.290	3.65	0.571	0.0221
$(CH_3)_3C$	0.511×10^{-3}	0.168	2.18	0.800	0.0151

NADH provide protection. Not only the responses of the two enzymes to reductive methylation differ, the two modified enzymes behave differently in several aspects. While methylated LADH exhibits an increased stability to heat and sulfhydryl reagent inactivation, methylated YADH shows an increased sensitivity toward heat and sulfhydryl reagent inactivation implicating subunit changes or an exposure of the catalytic site pocket of YADH upon methylation. While the methylated LADH remains dimeric, the reductive methylation causes partial dissociation of YADH in agreement with the dissociability of the tetrameric yeast enzyme (*15–17*).

Both alcohol dehydrogenases from liver and yeast catalyze the reversible oxidation of alcohols presumably *via* a partial random-partial ordered mechanism (*3, 18*). However, the kinetic mechanism of LADH allows the formation of the nonproductive enzyme–NADH–alcohol (EBQ) complex at high substrate concentrations. This nonproductive complex is absent from the YADH catalysis. The proposed kinetic mechanism for YADH catalysis is presented in Scheme 1 where the catalytic interaction of ternary complexes, EAB and EPQ is rate-limiting (*19*).

The steady-state rate equation for Scheme 1 can be obtained from that of

TABLE 4

KINETIC PARAMETERS FOR METHYLATED YADH CATALYZED OXIDATION OF ALCOHOLS

R in RCH_2OH	V (mM min^{-1})($\times 10^{-3}$)	K_a (mM)	K_b (mM)	K_{ia} (mM)	V/E_t (min^{-1})($\times 10^{-3}$)
CH_3	1.84	0.320	2.73	0.338	6.69
CH_3CH_2	1.34	0.196	5.43	0.236	4.87
$CH_2{=}CH$	1.70	0.0867	4.44	0.233	6.18
$CH_3(CH_2)_2$	0.878	0.230	7.26	0.338	3.19
$(CH_3)_2CH$	0.455	0.135	2.07	0.202	1.65
$CH_3(CH_2)_3$	0.494	0.0835	3.42	0.506	1.80
$(CH_3)_2CHCH_2$	0.602	0.0940	4.27	0.411	2.19
$(CH_3)_3C$	1.35	0.133	4.80	0.310	4.91

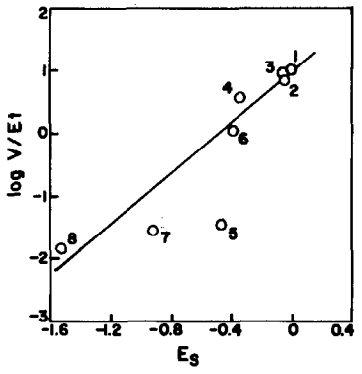


FIG. 6. Substituent effect on turnover number of YADH (control) catalyzed oxidation of primary alcohols. Substituent groups, R of primary alcohols RCH_2OH are: 1, CH_3; 2, CH_3CH_2; 3, $CH_2{=}CH$; 4, $CH_3(CH_2)_2$; 5, $(CH_3)_2CH$; 6, $CH_3(CH_2)_3$; 7, $(CH_3)_2CHCH_2$; and 8, $(CH_3)_3C$. The slope of the regression, $S = 2.05 \pm 0.508$ and the correlation coefficient, $r = 0.855$.

LADH by deleting steps leading to and from EBQ (2). In the absence of products (P and Q), it becomes

$$v = \frac{(n_{ab}AB + n_{a_2b}A^2B + n_{ab_2}AB^2)E_t}{d_0 + d_aA + d_bB + d_{ab}AB + d_{a_2}A^2 + d_{b_2}B^2 + d_{a_2b}A^2B + d_{ab_2}AB^2} \quad [3]$$

where E_t, A, and B are enzyme concentration, concentrations of NAD^+, and alcohol, respectively. The kinetic coefficients n_{x_i} and d_{x_i} are numerator and denominator terms, respectively, associated with substrate x of ith degree (*2*). Equation [3] describes v^{-1} versus A^{-1} plot to be linear (control YADH) or concave upward (methylated YADH) depending on the relationship,

$$(n_{ab}B + n_{ab_2}B_2)/(d_0 + d_bB + d_{b_2}B^2) > n_{a_2b}B/(d_a + d_{ab}B + d_{ab_2}B^2)$$

or

$$(n_{ab}B + n_{ab_2}B^2)/(d_a + d_{ab}B + d_{ab}B^2) > n_{a_2b}B/(d_{a_2} + d_{a_2b}B).$$

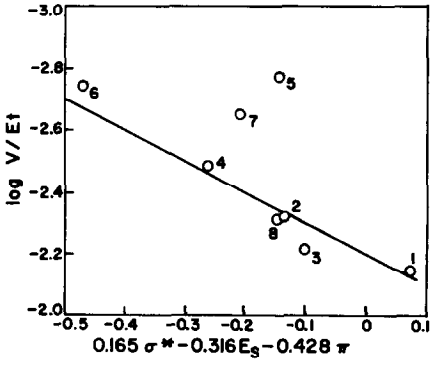
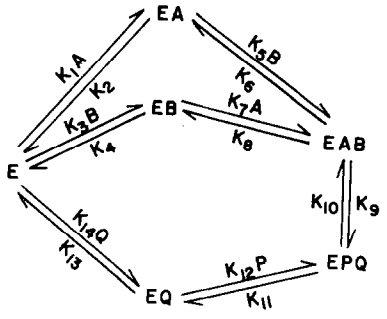


FIG. 7. Substituent effects on turnover number of methylated YADH-catalyzed oxidation of primary alcohols. The numbers denote substituent groups (R) of primary alcohols (RCH_2OH) listed in Fig. 6. The correlation coefficient $r = 0.756$.



SCHEME 1

Likewise, plots of v^{-1} versus B^{-1} may be concave upward (methylated YADH with ethanol or control YADH with allyl alcohol) or concave downward (control YADH with ethanol or methylated YADH with allyl alcohol) depending on whether $(n_{ab}A + n_{a_2b}A^2)/(d_b + d_{ab}A + d_{a_2b}A^2)$ is larger or smaller than $n_{ab_2}A/(d_{b_2} + d_{ab_2}A)$, respectively. The concave upward plots may also result from the dead-end inhibition at high substrate concentrations. However, this is not the case when the diagnostic test (plots of vB^{λ} versus B where λ = integer) of Bardsley and Child (*20*) was applied. It seems, therefore, that Scheme 1 adequately represents the kinetic mechanism of the methylated YADH despite different responses for the control and methylated enzymes to high substrate concentrations due to the difference in the magnitude of kinetic coefficients.

In the asymptotic region, both the control and methylated YADH prefer the ordered mechanism. A comparison of kinetic parameters between the control and methylated enzymes in the asymptotic region indicates that V is the only parameter which is significantly affected by the methylation. Since the interconversion between EAB and EPQ is rate-limiting, the methylation of lysine residues presumably deactivates YADH to oxidize alcohols by decreasing V via retardation of the catalytic interconversion between the ternary complexes. Stepwise regression analysis of the contributions of electronic, steric, and hydrophobic effects to V/E_t indicates that the steric interaction of alcohols with the control YADH is the most important factor. The insensitivity of the control YADH to the electronic variable may arise from the opposing effects on the rate constants which constitute V/E_t $k_9k_{11}k_{13}/k_9k_{11} + k_9k_{13} + k_{11}k_{13} + k_{10}k_{13})$. The positive coefficient of E_s indicates a deleterious effect of bulky groups of the substrates. YADH possesses a smaller catalytic pocket (*4*) which may account for the steric susceptibility and a narrower substrate specificity than LADH. Although the correlation is poor, the regression analysis of the substituent effect of alcohols on V/E_t of the methylated YADH is presented to demonstrate the absence of a deleterious steric effect. Thus methylation abolishes the steric susceptibility of YADH presumably due to changes in the catalytic site pocket as a result of added methyl groups on lysine residues.

ACKNOWLEDGMENT

This work was supported by a grant from Carleton University.

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