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Substituent Effects in Alkylated Liver Alcohol Dehydrogenases

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Alcohol dehydrogenase from horse liver was reductively alkylated with aldehydes having varied alkyl substituents. Kinetic studies of alkylated liver alcohol dehydrogenases which were modified in the absence and in the presence of NADH indicate that the alkylation of the specific lysine residues generally activates the enzyme by increasing Michaelis and inhibition constants for substrates and maximum velocities for the reactions. These kinetic parameters were analyzed in terms of electronic, steric, and hydrophobic effects of alkyl substituents. The hydrophilic character of the lysine residues is the most important factor which affects all kinetic parameters, particularly K_{ia} and V_2. In addition, the nucleophilic character of the lysine residues enhances the enzyme activity by increasing the maximum velocity of ethanol oxidation and the affinity of alcohol dehydrogenase for NADH and acetaldehyde. The steric interaction at the lysine residues favors the affinity of the enzyme for NADH and ethanol.

INTRODUCTION

Lysine residues have been implicated in the catalytic activity of alcohol dehydrogenase (EC 1.1.1.1) from horse liver (LADH).[1] Thus, picolimidination (*1*) and reductive methylation (*2*) enhance the catalytic activity, whereas pyridoxal phosphate modification (*3*) inactivates LADH. Zoltobrocki *et al.* (*4*) demonstrated that this differential effect of lysine modification was mainly due to the alternation in the net charge of Lys-228, which was involved in hydrogen bonding with Asp-223 at the coenzyme binding site (*5*).

Factors other than the net charge effect are known to affect enzymic reactions. Reactivities of substrates or inhibitors of enzymes have been successfully analyzed by substituent effects in a number of systems (*6–11*). Although a similar approach has not been applied to the correlation of enzyme structures and functions, alkylated LADH is particularly suitable for such a study for the following reasons: (a) Alkylated LADH can be easily prepared, and various substituents can be readily introduced to the specific lysine residue. (b) Alkylation results in an increase in activity which can be conveniently monitored and analyzed. Alcohol dehydrogenase from horse liver was reductively alkylated, and kinetic parameters of alkylated LADH were analyzed for electronic, steric, and hydrophobic effects of substituents introduced at the lysine residue, which interacts with NADH. The results are discussed in terms of the factors which are responsible for the activation in alkylated enzymes and the role of the lysine residue in LADH.

[1] Abbreviations used: LADH, liver alcohol dehydrogenase(s); NAD, nicotinamide adenosine dinucleotide; NADH, reduced nicotinamide adenosine dinucleotide.

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MATERIALS AND METHODS

Alcohol dehydrogenase from horse liver (once crystallized, 1–2 units/mg), NAD and NADH, were obtained from Sigma Chemical Corp. Aldehydes were purchased from Aldrich Chemical Co., and liquid aldehydes were purified by distillation.

Alkylation of LADH was carried out by a slight modification of the procedure previously described (2). One-half milliliter of freshly prepared $NaBH_4$ solution (5.0 mg/ml) was mixed with 4.5 ml of solution containing 0.30 μmol of the dehydrogenase in 0.10 *M* sodium pyrophosphate buffer (pH 9.0) in ice. Two-tenths milliliter (6.0 μmol) of aldehyde solution (methanal, ethanal, propanal, and 3,3,3-trichloroethanal in water, butanal, and 2-butenal in 50% aqueous dioxane, and 3-methylbutanal, pentanal, 3,3-dimethylpropanal, phenylmethanal, and 3-phenylpropenal in dioxane) was added in six portions over a 60-min period. The reaction mixture, after being kept in ice for an additional 15 min, was exhaustively dialyzed against three changes of distilled water and lyophilized. NADH protected samples were prepared in an identical manner in the presence of 7.5 μmol of NADH. Free amino groups were determined by the 2,4,6-trinitrobenzene sulfonic acid procedure (*12*) at pH 9.5. Isoelectric points of alkylated enzymes were determined by starch gel electrophoresis according to the method of Fine and Costello (*13*). Mobilities from the origin were plotted against pH. The pH value at which the enzyme showed zero mobility was taken as its isoelectric point.

Kinetic studies were carried out by monitoring changes in optical densities at 340 nm in a Perkin–Elmer spectrophotometer (Coleman Model 124) equipped with a variable output recorder (Coleman Model 165) and a thermostat circulator maintained at 25 ± 0.5°C. In the forward reaction (ethanol oxidation), ethanol concentrations varied from 0.50 to 10 m*M* and NAD from 0.10 to 1.0 m*M* in 0.10 *M* sodium pyrophosphate buffer at pH 9.0. In the reverse reaction (acetaldehyde reduction), acetaldehyde concentrations varied from 0.50 to 5.0 m*M* and NADH from 0.010 to 0.10 m*M* in 0.10 *M* sodium pyrophosphate buffer at pH 9.0. Reactions were initiated by the addition of 0.125 n*M* enzyme solution, and initial velocities were analyzed graphically (*14*) according to the homeomorphic equations, Eqs. (1) and (2), for bisubstrate enzymic reactions such that

$$V = \frac{V_1 AB}{K_{ia} K_b + K_b A + K_a B + AB} \tag{1}$$

for ethanol oxidation, and

$$V = \frac{V_2 PQ}{K_{iq} K_p + K_q P + K_p Q + PQ} \tag{2}$$

for acetaldehyde reduction, where A, B, P, and Q are NAD, ethanol, acetaldehyde, and NADH, respectively. V_1, V_2, K_a, K_b, K_p, K_q, K_{ia}, and K_{iq} are maximum velocities, Michaelis constants, and inhibition constants, respectively (*15*).

Kinetic parameters were analyzed for substituent effects according to

$$\log V_i^r \text{ or } K_i^r = d + \rho^* \sigma^* + S E_s + P\pi \tag{3}$$

using a computer program (Carleton University Science Program Library, "REGRESS") for multiple linear regression by ordinary least-squares and stepwise

forward regression analyses (*16*). V_i^r or K_i^r are ratios of corresponding kinetic parameters of alkylated LADH to NADH protected alkylated enzymes. ρ^*, S, and P represent the contribution of electronic, steric, and hydrophobic variables to a given kinetic parameter resulting from the alkylation of LADH. The linear intercept is d. σ^*, E_s, and π (Table 1) are corresponding electronic constants (*17*), steric constants (*18*) and hydrophobic constants (*19*) for given substituents of alkylated LADH, respectively. The hydrophobic constants (π) were taken as defined and as discussed by Leo and coworkers (*20*) using logarithms of partition coefficients of alcohols as models.

TABLE 1

SUBSTITUENT CONSTANTS USED IN REGRESSION ANALYSES OF ALKYLATED LADH

Substituents[a]	σ^*	E_s	π
H	0.490	1.24	–0.66
CH_3	0.000	0.00	–0.16
CH_3CH_2	–0.100	–0.07	0.34
$CH_3(CH_2)_2$	–0.115	–0.36	0.84
$(CH_3)_2CHCH_2$	–0.125	–0.93	1.14
$CH_3(CH_2)_3$	–0.130	–0.39	1.34
$(CH_3)_3C$	–0.300	1.54	1.36
$CH_3CH{=}CH$	0.360	–0.36[b]	0.54[c]
$C_6H_5CH{=}CH$	0.410	–0.38[b]	1.80[c]
C_6H_5	0.600	–2.58[b]	1.10
Cl_3C	2.65	–2.06	2.00[d]

[a] Substituents (R) in RCH_2.
[b] Assumed identical to those of corresponding saturated compounds.
[c] Calculated according to Ref. (*20*).
[d] Taken from Ref. (*21*).

RESULTS

Alcohol dehydrogenase from horse liver was reductively alkylated in the presence and the absence of NADH in order to investigate factors which are responsible for the enhanced activity of the alkylated LADH and the role of the lysine residue. Table 2 shows that ethylation of lysine residues activates LADH 3- to 4-fold, whereas carboxymethylation decreases the enzyme activity by 50%. This is in agreement with the observation (*4*) that the introduction of negative charges to lysine residues results in the inactivation of LADH. As expected, ethylation does not change the mobility of LADH in electrical field, whereas carboxymethylation lowers the isoelectric point of the enzyme.

Kinetic parameters for ethanol oxidation and acetaldehyde reduction catalyzed by alkylated LADH were determined (Tables 3 and 4). In general, alkylation increases maximum velocities for the reactions as well as Michaelis and inhibition constants for substrates. However, the kinetic parameters listed in Tables 3 and 4 do not show a definite trend of changes with respect to substituents. This may be due to the opposing

TABLE 2

CHARGE EFFECT ON ALKYLATED ALCOHOL DEHYDROGENASES[a]

Alkylated enzymes	Initial velocity (μM ml^{-1} min^{-1})	Isoelectric point	Lysine remaining (%)
Control	8.9	6.9	100
Ethylated LADH	26.1	6.8	62
Carboxymethylated LADH	5.4	6.2	68

[a] Liver alcohol dehydrogenase was reductively ethylated and carboxymethylated with acetaldehyde and glyoxylic acid, respectively. The control was prepared by replacing aldehyde with water. Initial velocities of ethanol oxidation were measured by following the change in optical densities at 340 nm of reaction mixtures containing 0.50 mM NAD, 5.0 mM ethanol, and 0.125 nM alkylated LADH in 0.10 M sodium pyrophosphate buffer, pH 9.0.

TABLE 3

KINETIC PARAMETERS FOR ETHANOL OXIDATION CATALYZED BY ALKYLATED LIVER ALCOHOL DEHYDROGENASE

Sample	Alkylated–LADH RCH_2^-	V_1 (μM min^{-1})	K_a (μM)	K_b (mM)	K_{ia} (μM)
	NADH protected[a]	15.4 ± 0.20	45.1 ± 1.6	0.793 ± 0.06	50.1 ± 2.4
	NADH protected[b]	10.4 ± 0.10	47.0 ± 1.0	0.715 ± 0.060	46.1 ± 4.8
	NADH protected[c]	12.0 ± 0.20	48.8 ± 1.2	0.768 ± 0.039	69.0 ± 6.7
1	H	129 ± 8.6	353 ± 24	3.97 ± 0.22	446 ± 21
2	CH_3	107 ± 2.0	177 ± 3.0	2.09 ± 0.12	293 ± 12
3	CH_3CH_2	80.1 ± 3.2	268 ± 10	3.78 ± 0.25	313 ± 28
4	$CH_3(CH_2)_2$	34.0 ± 1.0	110 ± 6.0	1.26 ± 0.070	212 ± 18
5	$(CH_3)_2CHCH_2$	58.8 ± 1.0	232 ± 8.0	1.50 ± 0.080	353 ± 24
6	$CH_3(CH_2)_3$	34.5 ± 1.2	120 ± 7.0	1.34 ± 0.16	280 ± 27
7	$(CH_3)_3C$	18.3 ± 0.60	75.8 ± 4.0	0.977 ± 0.056	94.2 ± 18
8	$CH_3CH{=}CH$	47.0 ± 1.5	165 ± 8.0	2.27 ± 0.15	189 ± 16
9	$C_6H_5CH{=}CH$	12.7 ± 0.10	39.4 ± 2.6	0.562 ± 0.040	72.4 ± 8.0
10	C_6H_5	36.7 ± 1.3	186 ± 11	2.05 ± 0.19	175 ± 17
11	Cl_3C	10.3 ± 0.10	48.3 ± 2.0	0.647 ± 0.010	118 ± 11

[a] The control for aldehydes (samples 1, 2, 3, and 11) used in water.
[b] The control for aldehydes (samples 4 and 8) used in 50% aqueous dioxane.
[c] The control for aldehydes (samples 5, 6, 7, 9, and 10) used in dioxane.

effects of alkyl groups introduced to various lysine residues of LADH. To correct the number of modified lysines which may affect the enzyme activity differently from one alkylated LADH to another, the ratio of kinetic parameters for the alkylated LADH

TABLE 4

KINETIC PARAMETERS FOR ACETALDEHYDE REDUCTION CATALYZED BY ALKYLATED LIVER ALCOHOL DEHYDROGENASE

LADH sample	Alkylated LADH RCH_2^-	V_2 (μM min^{-1})	K_p (mM)	K_q (μM)	K_{iq} (μM)
	NADH protected[a]	16.6 ± 0.30	0.407 ± 0.014	3.99 ± 0.10	11.9 ± 0.80
	NADH protected[b]	13.1 ± 0.70	0.309 ± 0.010	2.80 ± 0.10	12.7 ± 0.90
	NADH protected[c]	12.8 ± 0.30	0.389 ± 0.013	2.98 ± 0.08	10.9 ± 0.90
1	H	304 ± 20	1.92 ± 0.17	37.2 ± 2.1	45.3 ± 3.8
2	CH_3	236 ± 14	1.28 ± 0.10	40.2 ± 2.8	19.1 ± 2.4
3	CH_3CH_2	82.0 ± 5.0	1.05 ± 0.11	15.3 ± 1.0	13.7 ± 1.6
4	$CH_3(CH_2)_2$	42.9 ± 1.0	0.655 ± 0.013	10.1 ± 0.50	11.4 ± 1.1
5	$(CH_3)_2CHCH_2$	61.6 ± 1.0	0.564 ± 0.049	14.5 ± 0.55	19.7 ± 2.7
6	$CH_3(CH_2)_3$	37.8 ± 0.80	0.538 ± 0.018	6.05 ± 0.40	9.17 ± 0.84
7	$(CH_3)_3C$	21.3 ± 0.80	0.453 ± 0.043	3.69 ± 0.45	12.6 ± 1.4
8	$CH_3CH{=}CH$	127 ± 9.0	1.54 ± 0.40	21.9 ± 1.1	14.8 ± 1.3
9	$C_6H_5CH{=}CH$	25.6 ± 0.80	0.455 ± 0.013	5.06 ± 0.11	12.5 ± 1.5
10	C_6H_5	67.1 ± 1.3	0.770 ± 0.034	13.9 ± 1.4	7.54 ± 0.87
11	Cl_3C	14.7 ± 0.40	0.369 ± 0.026	9.78 ± 0.92	15.5 ± 1.8

[a] The control for aldehydes (samples 1, 2, 3, and 11) used in water.
[b] The control for aldehydes (samples 4 and 8) used in 50% aqueous dioxane.
[c] The control for aldehydes (samples 5, 6, 7, 9, and 10) used in dioxane.

and its corresponding NADH protected control was taken. Therefore, only effects of the two alkylated lysine residues which interacted with NADH were compared. These ratios were then analyzed for electronic (σ^*), steric (E_s), and hydrophobic (π) effects of alkyl substituents.

TABLE 5

STEPWISE REGRESSION ANALYSIS OF SUBSTITUENT EFFECTS ON KINETIC PARAMETERS

	Regression coefficient of			Correlation coefficient,	Standard error of estimate,
	σ^*	E_s	π	r	s
log V_2^r			−0.456	0.922	0.164
	−0.0348		−0.443	0.924	0.171
		−0.0279	−0.482	0.923	0.172
	−0.0423	−0.0371	−0.475	0.926	0.180
log V_1^r			−0.385	0.900	0.159
	−0.115		−0.342	0.936	0.136
	−0.123	−0.0432	−0.380	0.940	0.141
log K_{iq}^r		0.117		0.627	0.163
	0.0920	0.146		0.719	0.154
	0.0800		−0.175	0.668	0.165
		0.0781	−0.0717	0.660	0.167
	0.100	0.100	−0.0885	0.761	0.154

Stepwise forward regression analyses of kinetic parameters according to Eq. (3) suggest that the hydrophobic effect of substituents is the most important factor affecting V_1, V_2, K_a, K_b, K_p, K_q, and K_{ia}. The σ^* and E_s effects were added, in turn, to improve both correlation coefficients (r) and standard errors of estimates (s) as exemplified in Table 5. Although the inclusion of σ^* and E_s slightly improves the correlation coefficient for V_2, the standard error of the estimates increases unfavorably. Therefore, π is taken as the factor which affects V_2 in the correlation analysis. The same argument, however, favors the correlations of V_1 and K_{iq} with two and three parameters, respectively (Table 5). The results of these three examples are illustrated in Figs. 1, 2, and 3.

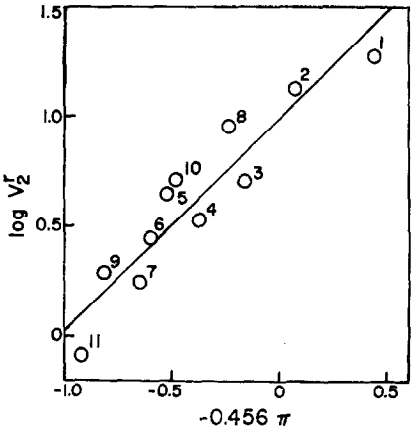

FIG. 1. Substituent effect on maximum velocities of acetaldehyde reduction catalyzed by alkylated liver alcohol dehydrogenases. The numbers pertain to alkyl groups introduced at the lysine residues listed in Table 4.

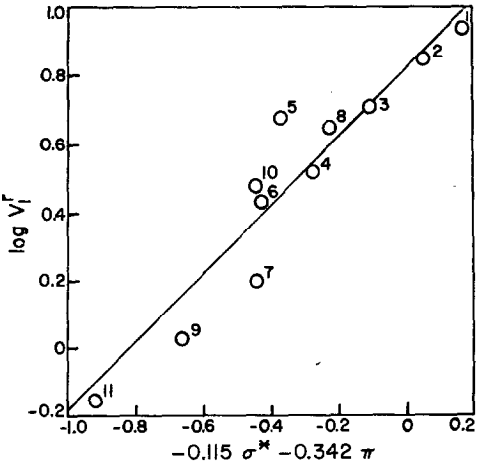

FIG. 2. Substituent effects on maximum velocities of ethanol oxidation catalyzed by alkylated liver alcohol dehydrogenases. The numbers pertain to alkyl groups introduced at the lysine residues listed in Table 3.

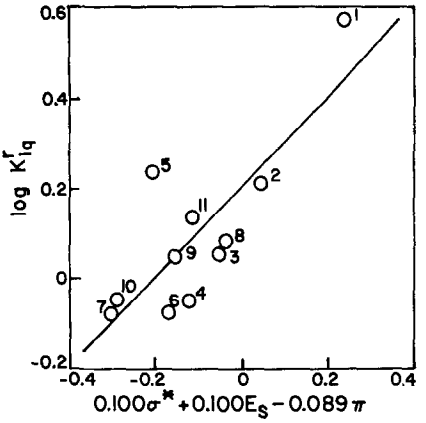

FIG. 3. Substituent effects on inhibition constants of NADH for alkylated liver alcohol dehydrogenases. The numbers pertain to alkyl groups introduced at the lysine residues listed in Table 4.

The above approach was used to correlate all kinetic parameters of alkylated LADH with substituent effects. Results are summarized in the following expressions:

$$\log V_i^r = 0.823 - 0.115\,\sigma^* - 0.342\,\pi$$

n	r	s	
11	0.936	0.136	(4)

$$\log K_a^r = 0.742 - 0.089\,E_s - 0.402\,\pi$$

n	r	s	
11	0.859	0.176	(5)

$$\log K_b^r = 0.580 - 0.0716\,E_s - 0.365\,\pi$$

n	r	s	
11	0.895	0.137	(6)

$$\log K_{iq}^r = 0.798 - 0.284\,\pi$$

n	r	s	
11	0.801	0.181	(7)

$$\log V_2^r = 0.922 - 0.456\,\pi$$

n	r	s	
11	0.922	0.164	(8)

$$\log K_p^r = 0.541 + 0.0149\,\sigma^* - 0.281\,\pi$$

n	r	s	
11	0.895	0.125	(9)

$$\log K_q^r = 0.847 + 0.106\,\sigma^* - 0.346\,\pi$$

n	r	s	
11	0.858	0.174	(10)

$$\log K_{iq}^r = 0.214 + 0.100\,\sigma^* + 0.100\,E_s - 0.0885\,\pi$$

n	r	s	
11	0.761	0.154	(11)

where n, r, and s are the number of alkylated LADH analyzed, the correlation coefficient, and the standard error of the estimates, respectively. In general, good correlations are obtained except K_{ia} and K_{iq} which, incidentally, are subject to a greater experimental uncertainty in the kinetic determination.

DISCUSSION

The modification of lysine residues which interact with nicotinamide coenzyme either activates or inactivates LADH. The introduction of a negative charge to the lysine residues decreases LADH activity, whereas the treatment without changing their net charge generally increases the enzyme activity (Tables 2–4). To elucidate the role of the lysine residues and the mechanism of activation upon modification, LADH was alkylated reductively by introducing various alkyl groups which did not alter the net charge but differed in electronic affinity, size, and hydrophobicity.

Kinetic studies of alkylated LADH indicate that the enhanced LADH activity is associated with an increase in values of kinetic parameters. The increased rate of dissociation of the enzyme–coenzyme complex was considered to be the factor for the enhanced activity of acetimidated LADH (*4*). However, K_{ia} and K_{iq} of trichloroethylated LADH are greater than those of the NADH-protected control in spite of a lower overall activity for the trichloroethylated enzyme. Presumably, the enhanced activity of alkylated LADH is due to the interplay of kinetic parameters which are subject to substituent effects of alkyl groups introduced at the lysine residues.

Stepwise forward regression analyses of substituent effects for kinetic parameters at a 95% F level of acceptance indicate that the hydrophobicity of substituents is the most important variable affecting all kinetic parameters except K_{iq}. The inclusion of the electronic and/or steric effects in the regression analyses normally improve the correlation coefficient but do not always minimize the standard error of estimates. Therefore, the regression analyses were optimized for the correlation coefficients and standard errors of estimates according to Eq. (3).

The negative coefficient for π indicates that all kinetic parameters increase with the hydrophilicity of the lysine residues which interact with NADH. Although π is the only predominant variable affecting K_{ia} and V_2 (Eqs. (7) and (8)), good two-variable correlations were observed for most of kinetic parameters (Eqs. (4), (5), (6), (9), and (10)). The ρ^* values of those kinetic parameters which are affected by σ^* suggest that the nucleophilicity of the lysine residues increases V_1 but decreases K_p and K_q. Michaelis constants, K_a and K_b, are subject to steric effects. The negative coefficient for E_s implies that K_a and K_b increase with an increased steric hindrance at the lysine residues. Although σ^*, E_s, and π show an equivalent influence on K_{iq}, other unidentified factors may be involved because of the poor correlation coefficient.

Various factors determine the activity of an enzyme. The present study illustrates an approach by which the structure–activity relationship of an enzyme with respect to the amino acid residue can be obtained via chemical modification. For example, the contribution to the LADH activity of the lysine residues which interact with NADH can be rationalized in terms of the charge, electronic, steric, and hydrophobic effects at the sites. The introduction of negative charges to the lysine residues is detrimental to LADH

activity, whereas the modifications without changing the charge character of the lysine residues generally increase the enzyme activity. The hydrophilic character of the lysine residue affects the catalytic activity of LADH by favorably increasing V_1 and V_2 while unfavorably decreasing the affinity of the enzyme for its substrates. The nucleophilic character of the lysine residues enhances LADH activity by increasing V_1 as well as affinities of the enzyme for NADH and acetaldehyde. It is interesting to note that K_p and K_q are affected by an electronic effect whereas K_a and K_b are affected by a steric effect. Presumably, the affinity of LADH for NAD and ethanol is favored by the steric interaction at the lysine residues via spacialization.

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