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Functional and Structural Responses of Liver Alcohol Dehydrogenase to Lysine Modifications

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Acetylation, glycosylation, and methylation, which modify lysine residues of horse liver alcohol dehydrogenase, have been investigated. Acetylation reacted with approximately two-third of the total lysines to induce the greatest structural changes of the enzyme. Glycosylation modified only one lysine residue selectively with indiscernible structural changes. The glycosylation effect was very specific with respect to diastereoisomers for aldopentoses, aldohexoses, and ketohexoses. Methylation produced the largest enhancement in the oxidative activity, which is related to the stability of the modified enzyme to prolonged modification and thermal denaturation. Kinetic studies revealed that a change in the maximal velocity was primarily responsible for the observed activity differences in the modifications.

INTRODUCTION

Chemical modifications have been extensively employed to elucidate the chemical basis of enzyme catalyses. Current methodologies have been devoted primarily to modifications of the active sites (*1, 2*) which lead to an inactivation of enzymes. The modifications of amino acid residues also take place *in vivo* subsequent to the translational process in protein synthesis (*3*). The post-translational modifications are integral steps in the processing of secretary proteins (*4, 5*), protein hormones (*6*), and viral proteins (*7*). They are important cellular regulatory mechanisms for a large number of enzymes (*8*) as well as for responding to certain physiological stresses (*9, 10*). These *in vivo* modifications, in contrast to the active site-directed modifications, normally cause desirable changes in the properties and/or activities of proteins without deleterious structural effects. Therefore, chemical modifications which mimic the post-translational modifications would be ideal approaches to manipulate enzymic activities and/or properties.

Earlier studies of alcohol dehydrogenase from horse liver revealed that both glycosylation (*11*) and methylation (*12*), which modified lysine residues, enhanced its oxidative activity. The present study was carried out to assess the potentiality of chemical modifications to impart enzymes with enhanced activities and favorable properties for biotechnological applications.

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MATERIALS AND METHODS

Alcohol dehydrogenase from horse liver (LADH),[1] NAD^+, D-arabinose, L-arabinose, 2-deoxy-D-glucose, D-fructose, D-glucose, L-glucose, maltotriose, D-tagatose, D-xylose, L-xylose, 2,4,6-trinitrobenzenesulfonic acid (TNBS) and glucose assay kit were obtained from Sigma Chemical Corporation. D-Ribose was a product of ICN Nutritional Biochemicals. D-Glucoheptose was provided by United States Biochemical Corporation. D-Lyxose was supplied by P-L Biochemicals Inc. D-Galactose, maltose, formaldehyde solution, acetic anhydride, and alkanols were supplied by Fischer Scientific Company. Absolute ethanol came from Consolidated Alcohols Ltd. [1-^{14}C]Acetic anhydride (10 mC_i/mmol), [^{14}C]formaldehyde (10 mC_i/mmol), and D-[U-^{14}C]glucose (2.2 mC_i/mmol) were purchased from New England Nuclear. Bio-gel P6 and Bio-gel P100 were acquired from Bio-Rad Laboratories.

Acetylation was performed by means of a pH stat assembly consisting of a Radiometer PHM62, a TTT60, and a ABU11. Acetic anhydride (1.5 μl) was delivered with constant stirring to a solution of LADH (0.25 μmol) in 5.0 ml 0.050 M sodium acetate adjusted to pH 8.0 on ice. Samples (100 μl) were withdrawn at time intervals and filtered through a column (1.0 × 30 cm) of Bio-Gel P6 for enzymatic assays. Glycosylations were carried out by mixing equal volumes of 250 nmol/ml LADH with 77.5 ± 2.5 μmol/ml glycose solutions in 0.20 M phosphate buffer, pH 8.0, at 25.0 ± 0.5°C. Samples (3.0 μl) were withdrawn at time intervals and assayed for dehydrogenase activity. Methylation was carried out as described previously (*12*). The pseudo-first-order rate constants for activation/inactivation during the initial phase of modifications were evaluated using a program written for the TI 59 calculator (*13*). The incorporations of acetyl, glucosyl, and methyl groups from [1-^{14}C]acetic anhydride (0.25 μC_i/ml), D-[U-^{14}C]glucose (0.20 μC_i/ml), and [^{14}C]formaldehyde (0.80 μC_i/ml), respectively, were followed. Aliquots of 10–20 μl were withdrawn at time intervals onto Whatman 3MM filter paper discs (2.3 cm diameter), which were successively washed with 10% cold trichloroacetic acid solution twice, and ethanol, ethanol–ether (1 : 1, v/v), and ether once each. The radioactivity of the air-dried discs was measured with a Beckman (LS150) scintillation counter.

The dehydrogenase activity was assayed spectrophotometrically (Beckman spectrophotometer Model 25) by following the rate of change in absorbance at 340 nm of the reaction mixture containing 1.0 mM NAD^+, 10 mM ethanol, and 20 ± 5.0 nM LADH in 0.10 M Tris buffer, pH 9.0, at 25°C. Kinetic studies were carried out in a similar manner (*14*) with varied concentrations of NAD^+ (0.10–1.0 mM) and ethanol (1.0–50 mM).

Free glucose was monitored by the colorimetric procedure of coupling glucose oxidase and peroxidase with *o*-dianisidine (Sigma glucose kit). Concentrations of the modified enzymes were measured with a microbiuret reagent (*15*) using LADH as the standard. Gel (Bio-Gel P100)-filtration chromatography was per-

[1] Abbreviations used: LADH, horse liver alcohol dehydrogenase; NAD^+, oxidized form of nicotinamide adenine dinucleotide; TNBS, 2,4,6-trinitrobenzenesulfonic acid, Ac, acetylated; Glu, glucosylated; Me, methylated.

formed to estimate Stokes' radius (*16*). Ultraviolet spectra were recorded with a Cary 14 spectrophotometer.

RESULTS

Chemical acetylation, glucosylation, and methylation, which modified lysine residues of LADH, were carried out. Figure 1 shows that an initial activation was followed by a rapid inactivation for the acetylation and a slight reduction in the enhanced activity of the glucosylated LADH, while the enhanced LADH activity remained relatively constant with the prolonged methylation.

The acetylation was pH sensitive. Only a narrow pH range (pH 8.0–10.0) offered the transient activation. A treatment of proteins with acetic anhydride in high concentrations of sodium acetate preferentially acetylates amino groups (*17*). This was shown by TNBS titration of a change in the free amino groups during acetylation in corroboration with an incorporation of 41 ± 9 [^{14}C]acetyl groups from [1-^{14}C]acetic anhydride.

In the presence of either D- or L-glucose, LADH underwent glucosylation with an accompanied enhancement in its oxidative activity. However, its diastereoisomers such as D-galactose and D-mannose were ineffective, though D[U-^{14}C]galactose was incorporated in the process. The diastereoisomeric effects of D-aldopentoses on the LADH activity are depicted in Fig. 2. Among the four D-aldopentoses, only D-arabinose exerted the enhancement effect. D-Xylose was ineffective while D-lyxose and D-ribose suppressed the oxidative activity of LADH. Table 1 summarizes pseudo-first-order rate constants for glycosylations which activate or inactivate LADH, respectively. The diastereoisomeric effect was also observed for D-ketohexoses. Only D-fructose exhibited a stimulatory effect. Of the three phosphate esters which are metabolic intermediates of D-

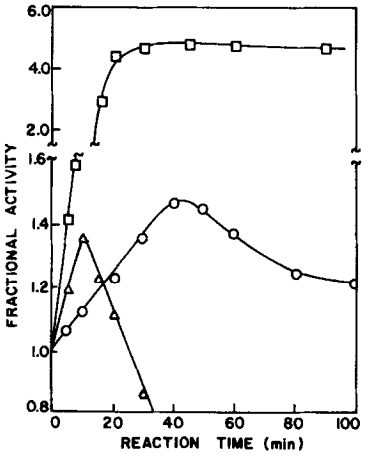


FIG. 1. Changes in LADH activity during acetylation (△), glucosylation (○), and methylation (□). The incorporation of [1-^{14}C]acetic anhydride, D-[U-^{14}C]glucose, and [^{14}C]formaldehyde was followed with the respective controls in parallel experiments (not shown).

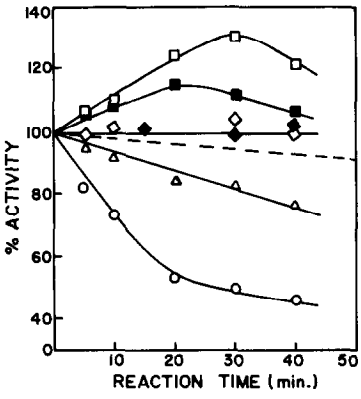


FIG. 2. Pentosylation of LADH. Changes in the oxidative activity were followed during the incubation of LADH with D-arabinose (□), L-arabinose (■), D-lyxose (△), D-ribose (○), D-xylose (◇), and L-xylose (◆).

fructose, only D-fructose 6-phosphate activated LADH. Within a narrow range of variation in chain length among D-arabinose, D-glucose, and D-glucoheptose, an optimal activation occurred with the hexose. Free glucose was not released from oligosaccharides of D-glucose during the activation. Double glycosylation experiments show that the oxidative activity of the glucosylated LADH was not affected by the subsequent pentosylation (Fig. 3A). In the reverse sequence, the glucosyla-

TABLE 1

RATE CONSTANTS FOR GLYCOSYLATION OF LIVER ALCOHOL DEHYDROGENASE

Glycose	k_{obs} (min^{-1} × 10^3)	
	Activation	Inactivation
Control		0.203 ± 0.012
D-Mannose		0.866
D-Galactose		1.12 ± 0.28
D-Glucose	1.85 ± 0.20	
L-Glucose	1.41 ± 0.15	
D-Glucoheptose	1.48	
2-Deoxy-D-glucose	1.10 ± 0.16	
D-Tagatose		0.277
D-Cellobiose	2.76	
D-Maltose	1.40	
Fructose 1-phosphate		0.195
Fructose 6-phosphate	1.28	
Fructose 1, 6-bisphosphate		0.145

Note. The reproducibility is given for the rate constants, which are the average values of more than three separate determinations. The control, in the absence of glycoses, was run in parallel experiments.

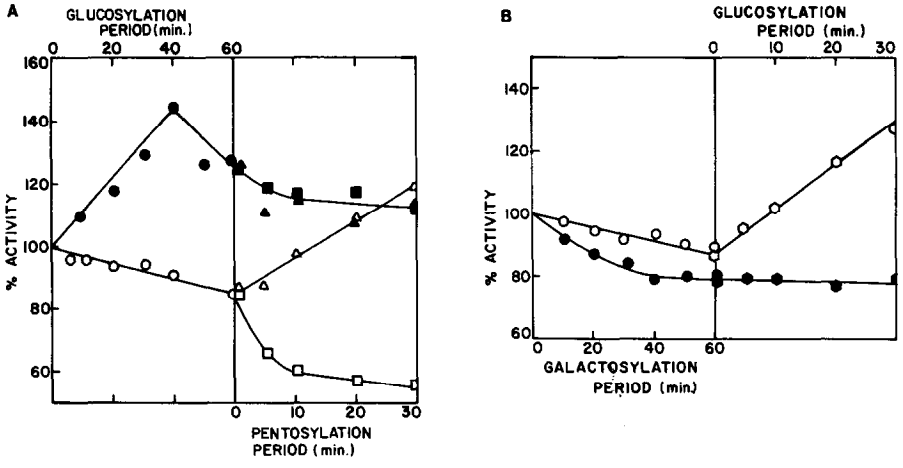


FIG. 3. Double glycosylation experiments. (A) The control (○) and glucosylated (●) LADH were prepared initially. Both the control (○) and glucosylated (●) enzymes were subsequently arabinosylated (△,▲) or ribosylated (□,■). The decreased activity of the glucosylated enzyme (▲,■) was at the control level. (B) The control (○) and galactosylated (●) LADH were prepared and subsequently glucosylated (○, from the control; and ●, from the galactosylated enzyme). The oxidative activity was monitored during the double glycosylation.

tion did not enhance the oxidative activity of the galactosylated LADH (Fig. 3B). These observations suggest that the common glycosylation site has been blocked by the initial glycosylation; therefore, the subsequent glycosylations fail to express their effects.

The maximally activated and modified enzymes were isolated for kinetic and structural studies. Table 2 lists kinetic parameters for ethanol oxidation catalyzed by the modified enzymes. A study on the substrate specificity of the glycosylated enzyme showed that the fructosylated LADH exhibited the enhanced oxidative activity (Table 3) toward both primary and secondary alcohols. Table 4 gives some structural characteristics of the modified enzymes. Sensitivities of the modified enzymes to thermal denaturation and solvent inactivation were compared in Figs.

TABLE 2

KINETIC PARAMETERS FOR ETHANOL OXIDATION CATALYZED BY MODIFIED ENZYMES AT pH 9.0

LADH (mod. time)	V (μM min^{-1})	K_a (μM)	K_b (μM)	K_{ia} (μM)	V/E_t (min^{-1})
Control	5.75	45.3	669	85.4	478
Ac (10 min)	6.04	61.9	354	237	504
Glu (40 min)	7.41	63.8	805	90.6	618
Me (30 min)	33.6	238	2580	441	2.80×10^3

Note. [LADH] (E_t) = 12.0 nM per active site based on subunit weight of 40,000. V, K_a, K_b, and K_{ia} are maximum velocity, Michaelis constant for NAD$^+$, Michaelis constant for ethanol, and inhibition constant for NAD$^+$, respectively.

TABLE 3

KINETIC PARAMETERS FOR ALCOHOL OXIDATIONS CATALYZED BY FRUCTOSYLATED HLADH AT pH 9.0

	V (μM min^{-1})	K_a (μM)	K_b (μM)	K_{ia} (μM)	V/E_t (min^{-1})
Control LADH					
Ethanol	11.2	39.2	616	70.6	467
Propan-1-ol	11.7	26.2	211	51.1	488
Propan-2-ol	1.46	54.3	1.44×10^3	270	60.8
Butan-1-ol	14.0	35.7	135	59.4	583
Butan-2-ol	4.87	36.2	1.29×10^3	262	203
Fructosylated LADH					
Ethanol	13.6	50.7	525	84.0	567
Propan-1-ol	12.8	49.6	319	104	533
Propan-2-ol	1.62	27.2	328	487	67.5
Butan-1-ol	14.2	29.2	169	258	592
Butan-2-ol	5.29	23.1	247	263	220

Note. [LADH] $(E_t)_a$ = 24.0 nM per active site. V, K_a, K_b, and K_{ia} are maximum velocity, Michaelis constant for NAD$^+$, Michaelis constant for alcohol, and inhibition constant for NAD$^+$, respectively.

4 and 5, respectively. The acetylated LADH underwent major structural changes, and was most sensitive to both thermal and solvent treatments. The structural responses of the glucosylated enzymes remained parallel to those of the control.

DISCUSSION

Lysine residues are implicated in various structural (*18, 19*) and catalytic (*20, 22*) functions of numerous enzymes. The ε-amino group of lysine residues, due to its chemical reactivity, is particularly suitable to *in vitro* chemical modifications (*17, 23–25*). It is also amenable to *in vivo* post-translational modifications such as

TABLE 4

CHARACTERIZATION OF MODIFIED LIVER ALCOHOL DEHYDROGENASES

LADH	Modif. time (min)	No. of lysine residues modified	λ_{max} (nm)	Stokes' radius (nm)
Control			278	334
Ac	10	41 ± 9	268	394
Glu	40	1	278	335
Me	30	26 ± 4	277	360

Note. Alcohol dehydrogenase from horse liver contains 60 lysine residues. The radioactive ^{14}C incorporation from [1-^{14}C]acetic anhydride, D-[U^{14}C]glucose and [^{14}C]formaldehyde, respectively, was followed to evaluate the number of lysine residues modified. Stokes' radii were estimated by means of gel-filtration chromatography.

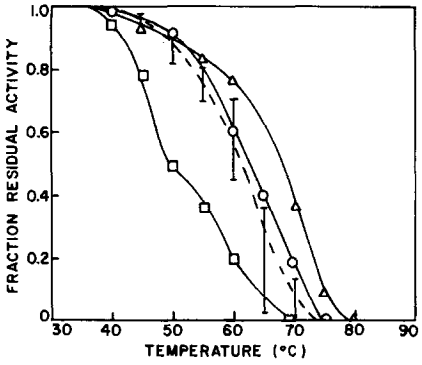


FIG. 4. Thermal denaturation of acetylated (□), glucosylated (○), and methylated (△) LADH at pH 7.0. The modified LADH (Table 4) were subjected to heat treatment in 5°C increments for 5-min periods prior to activity assays. The control (---) was run in parallel experiments with vertical bars indicating deviation.

acetylation (*26*), glycosylation (*27*), and methylation (*28*). All three chemical modifications, which modify lysines, enhance the LADH activity, though the acetylation provides only a transient activation. The maximum stimulation occurs with the methylation. The stimulatory effect of glycosylation on LADH is remarkably specific with respect to diastereoisomers for aldopentoses and aldohexoses, as well as ketohexoses. The common site is glycosylated presumably by different sugars with varied effects.

Scheme 1 depicts a plausible mechanism, analogous to chemical systems (*29*), for the glucosylation of LADH. This mechanism is consistent with the following observations (*11*): (a) identification of Lys-315 as the glucosylation site, (b) detection of both the *N*-glucosyl structure (**III**) and the Amadori product (**IV**) by ^{13}C NMR of the [^{13}C]glucosylated LADH, (c) abolition of glycosidase activity of

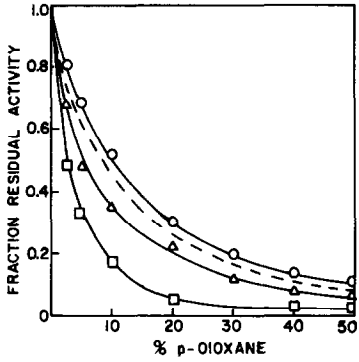
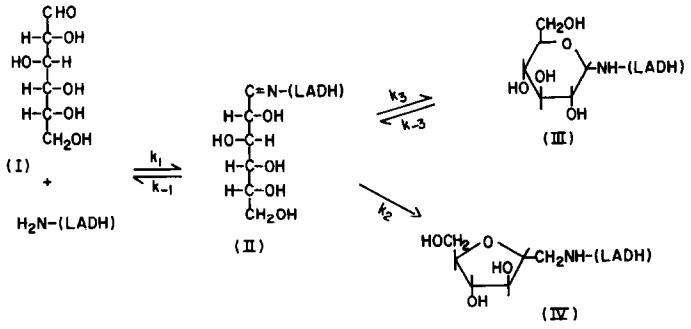


FIG. 5. Solvent inactivation of modified LADH. The acetylated (□), glucosylated (○), and methylated (△) LADH (Table 4) were mixed with different concentrations of *p*-dioxane in 0.10 M phosphate buffer, pH 7.0, at 25°C for 5 min, after which the activity was assayed. The control (---) was run in parallel experiments.



SCHEME 1. A plausible mechanism for glucosylation of LADH.

LADH by the glycosylation, and (d) activation of LADH by 2-deoxy-D-glucose, which does not undergo Amadori rearrangement. Although the open form of glycoses is implicated in the initial step of the modification, the observed glycosylation effects on LADH correlate with neither the percentage carbonyl group of various glycoses in solution (*30*) nor rates (k_1) of aldimine formation (*31*). This is not surprising in view of the recent kinetic result that k_1 [Glucose] > k_2 in the glucosylation of hemoglobin (*32*). Furthermore, the *N*-glycosyl structure (**III**) is responsible for the enhanced oxidative activity of the glycosylated LADH (*11*). Thus, k_3 is associated with the activation whereas k_2 with the inactivation. We can only speculate that the glycoses with $k_3 > k_2$ presumably enhance the oxidative activity of LADH while glycoses with $k_3 < k_2$ are likely suppressive. It is noted, however, that D-glycoses which enhance the oxidative activity of LADH possess the common triol chirality (i.e., *S,R,R*) for the nonreducing half of the molecules.

Steady-state kinetic studies indicate that the observed stimulatory effect of the lysine modifications is associated with the increased catalytic efficiency (V/E_t) of the modified LADH. Although the modifications also result in a general increase in Michaelis–Menten constants, no correlational trend is apparent. The fructosylated LADH exhibited an enhanced activity to oxidize both primary and secondary alcohols. This differs from the methylated LADH which displays an enhanced activity toward primary alcohols but a suppressed ability to oxidize secondary alcohols (*14*). The possibility of manipulating the substrate specificity of enzymes via chemical modifications would be particularly attractive to chemical applications of the modified enzymes in stereospecific organic syntheses (*33, 34*).

Among three chemical lysine modifications, the acetylation, which modifies 2/3 of the total lysine residues, is the least selective and the most drastic reaction. The treatment of LADH with formaldehyde and $NaBH_4$ methylates approximately half of the total lysine residues (*12*). D-Glycoses which specifically glycosylate Lys-315 located at the cleft of the subunit interaction region (*11*) are the least reactive but the most specific of the three modifications. It is interesting to note that the chemical reactivity–selectivity relationship (*35*) holds for the chemical modifications of LADH.

A further correlation exists between the selectivity of modifying reagents and structural effects which are manifested by a shift in uv absorption maximum,

Stokes' radius, and sensitivity to solvent denaturation of the modified enzymes. Extensive conformational changes of the acetylated LADH is evidenced by a large blue shift in the uv-absorption maximum, a large increase in Stokes' radius, and increased susceptibilities to solvent and thermal denaturations. The glucosylated LADH exhibits minimal structural changes. The experimental parameters aimed at detecting conformational changes of the glucosylated enzyme do not deviate from those of the control significantly. Changes in the structural parameters of the methylated LADH are intermediate between the glucosylated and acetylated enzymes except the improved thermal stability of the methylated enzyme.

Comparative studies of the acetylation, glycosylation, and methylation provide useful information concerning the functional and structural responses of LADH to chemical modifications which differ in reactivity and selectivity toward lysine residues. The present study demonstrates the potentiality of chemical modifications which can be advantageously employed to enhance activities and/or some desirable properties of enzymes for biotechnological applications.

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