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Structure and Activity of Methylated Horse Liver Alcohol Dehydrogenase†

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ABSTRACT: Horse liver alcohol dehydrogenase is modified by formaldehyde in the presence of sodium borohydride. This reagent methylates 26 of its 60 lysine residues. Methylation of the enzyme causes increased activity as well as conformational changes. The modified liver alcohol dehydrogenase has a larger Stokes radius and exhibits a slight blue shift with an increased absorbance in the ultraviolet spectrum, an increased trough at 233 nm in the optical rotatory dispersion spectrum, and an increased stability toward heat and mercurial inactivations. Methylation partially desensitizes liver alcohol dehydrogenase to substrate inhibition by ethanol. NADH protects 4 of the 26 lysine residues from modification. Possible functions of lysine residues are discussed.

Kosower (1962) postulated that the positively charged ϵ-amino group of lysine found in dehydrogenases was involved in the binding of coenzyme. Chemical modifications of horse liver alcohol dehydrogenase (EC 1.1.1.1) with methyl picolinimidate (Plapp, 1970) and pyridoxal phosphate (McKinley-McKee and Morris, 1972) indicate that one or more lysine residues are at or near the site where the coenzyme is bound. Methyl picolinimidate modifies 50 of the 60 lysine residues causing an overall increase in enzyme activity. Pyridoxal phosphate, in the presence of sodium borohydride, modifies 11 residues causing an overall decrease in activity. The lack of the selectivity of these modifications and the difference in their effect on liver alcohol dehydrogenase raise the question concerning the specific function of the lysine residue in the dehydrogenase.

Formaldehyde, in the presence of sodium borohydride, was used as a specific reagent for amino groups (Means and Feeney, 1968; Rice and Means, 1971). In modifying liver alcohol dehydrogenase, this reagent may minimize structural perturbation due to the electronic neutrality and relatively small bulk of the methyl group introduced. Methylation of the dehydrogenase increases the activity[1] with concurrent conformational changes. Chemical and physical studies of the methylated dehydrogenase were carried out to assess the function of lysine residues in the dehydrogenase and the factors which are responsible for the enhancement of enzyme activity.

Materials and Methods

Chemicals. Horse liver alcohol dehydrogenase, NAD^+, NADH, and *p*-ClHgBzO were obtained from Sigma Chemical Co. Marker proteins were purchased from Worthington Biochemical Corp. Other chemicals were products of Fisher Chemical Co. Absolute ethanol and formaldehyde were redistilled prior to use.

Methylation of Liver Alcohol Dehydrogenase. Methylation of the dehydrogenase with formaldehyde in the presence of sodium borohydride was carried out as described by Means and Feeney (1968). One milliliter of freshly prepared sodium borohydride solution, 2.5 mg/ml, was mixed with a solution containing 25 mg of the dehydrogenase in 0.1 M sodium pyrophosphate buffer (pH 9.0). Portions (10 μl) of 9.25% formaldehyde solution were added every 10 min for 60 min. After the final addition of formaldehyde, the reaction mixture was kept in ice for an additional 30 min and then dialyzed for 18 hr against three changes of distilled water and lyophilized. The NADH protected sample was prepared by methylating liver alcohol dehydrogenase in the presence of 1.0 mM NADH. The control was prepared in an identical manner by replacing formaldehyde solution with water. The treatment of the dehydrogenase with formaldehyde alone by an identical procedure did not affect the enzyme activity.

Assay of Liver Alcohol Dehydrogenase. The assay mixture contained 1.0 mmol of sodium pyrophosphate buffer (pH 9.0), 1.0 μmol (low concentration assay) or 1.0 mmol (high concentration assay) of ethanol, 1.0 μmol of NAD^+, and 2.4 nmol of dehydrogenase or methylated dehydrogenase in a total volume of 3.0 ml. The rate of NADH formation was followed at 340 nm using a Beckman DB spectrophotometer

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[1] When this manuscript was in preparation, a communication reporting methylation of horse liver alcohol dehydrogenase appeared (Jörnvall, 1973).

[2] Abbreviations used are: NAD^+, nicotinamide adenosine dinucleotide; NADH, reduced nicotinamide adenosine dinucleotide; *p*-ClHgBzO, *p*-chloromercuribenzoate.

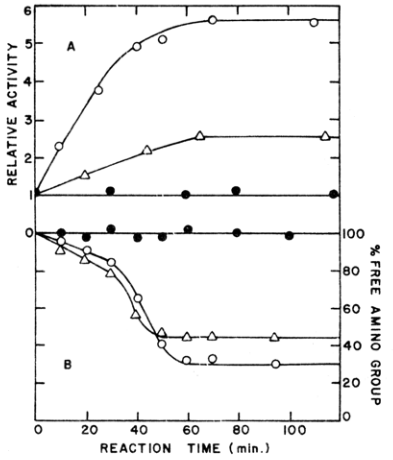

FIGURE 1: Methylation of horse liver alcohol dehydrogenase. Horse liver alcohol dehydrogenase was methylated by formaldehyde in the presence of sodium borohydride. Enzyme activities (A) were assayed at an ethanol concentration of 0.1 M and free amino groups (B) were determined by the 2,4,6-trinitrobenzenesulfonic acid procedure (Habeeb, 1966) at time intervals for dehydrogenase (●), the methylated dehydrogenase (○), and NADH-protected methylated dehydrogenase (Δ).

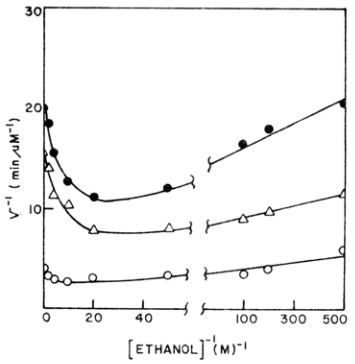

FIGURE 2: Double reciprocal plots for dehydrogenase (●), methylated dehydrogenase (○) and NADH-protected methylated dehydrogenase (Δ).

equipped with a thermostat-circulator set at 30 ± 0.5°. Initial velocities were expressed in micromoles per minute of NADH formed.

Analytical Methods. A Technican Model TM-120 amino acid analyzer was used for amino acid analyses. Samples were prepared according to the procedure of Moore and Stein (1963). Sodium dodecyl sulfate–polyacrylamide gel electrophoresis was carried out by the method of Weber and Osborn (1969). Gel chromatography was carried out in a column (25 mm × 365 mm) packed with Sephadex G-100 employing 0.1 M sodium pyrophosphate (pH 8.0) as an elution buffer. Marker proteins in eluents were measured at 280 nm except catalase which was measured at 405 nm. Both liver alcohol dehydrogenase and the methylated dehydrogenase (5 mg/ml in 0.1 M pyrophosphate buffer (pH 8.0)) were subjected to ultracentrifugation analysis at 60,000 rpm using a Spinco Model E analytical centrifuge.

Spectroscopic Analyses. Ultraviolet spectra were taken with a Cary 14 spectrophotometer. Optical rotatory dispersion spectra were obtained with a Cary 60 spectropolarimeter and mean residue rotations, $[m]_\lambda'$, were calculated from the observed rotations using 115 as the mean residue weight (Fasman, 1963). Protein solutions were clarified by centrifugation at 2700*g* for 15 min prior to analyses.

Results

The treatment of liver alcohol dehydrogenase with formaldehyde and sodium borohydride, in pyrophosphate buffer at pH 9.0, enhanced the enzyme activity (Figure 1A). NADH partially protected the dehydrogenase from the modification. The enhancement in enzyme activity during the modification paralleled a decrease in the number of free amino groups (Figure 1B). Amino acid analyses of the native and modified dehydrogenases indicated that 26 of the 60 lysine residues were methylated, under the experimental conditions. In the presence of NADH, 22 lysine residues were methylated.

The methylated dehydrogenase was five–six times as active as the native enzyme when assayed at high ethanol concentrations over the range of ethanol concentrations used. No such discriminatory effect was observed for the NADH-protected methylated dehydrogenase when assayed at different ethanol concentrations. Figure 2 shows that the greater activation of the methylated dehydrogenase, assayed at high ethanol concentrations, is due to partial desensitization of the dehydrogenase to substrate inhibition by ethanol.

Both the methylated dehydrogenase and liver alcohol dehydrogenase exhibit an identical mobility in sodium dodecyl sulfate gel electrophoresis. Based on the linear correlation between mobilities and the subunit weights of marker proteins (Weber and Osborn, 1969), dehydrogenase and the methylated dehydrogenase give a subunit weight of 3.81×10^4. This agrees with the reported mol wt of 84,000 for two subunits (Ehrenberg and Dalziel, 1958). When subjected to ultracentrifugation, both modified and unmodified enzymes gave identically sedimenting peaks (Figure 3).

Unexpectedly, the methylated dehydrogenase was eluted from Sephadex G-100 faster than the dehydrogenase. Since the modified and unmodified enzymes are identical in size, the difference in elution volumes probably arises from the difference in protein shape. Elution constants of macromolecules from Sephadex gel have been correlated with their Stokes radii (Acker, 1964; Siegel and Monty, 1966). A plot of $K_d^{1/3}$ *vs.* Stokes radii for the marker proteins gave a straight line from which Stokes radii for the dehydrogenase and the methylated dehydrogenase were estimated to be 33.4 and 36.0 Å, respectively.

Figure 4 shows that the absorption maximum of the ultraviolet spectrum of dehydrogenase shifts from 278 to 277 nm with an increased absorbance upon methylation. Both de-

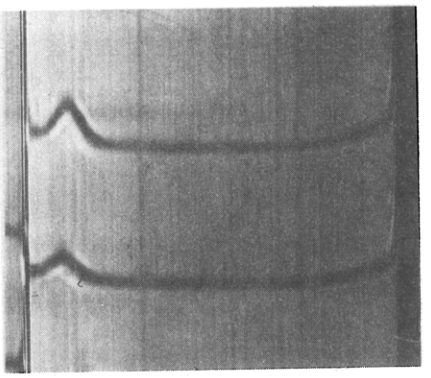

FIGURE 3: Ultracentrifugation of horse liver alcohol dehydrogenases. The ultracentrifugation of dehydrogenase (upper) and methylated dehydrogenase (lower) was carried out in a Spinco Model E ultracentrifuge at 20°. Photographs were taken 8 min after reaching the speed of 60,000 rpm.

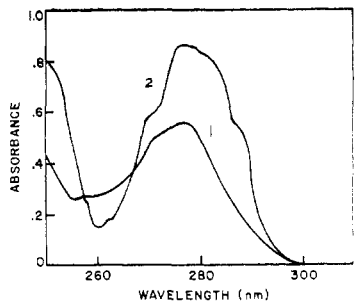

FIGURE 4: Ultraviolet spectra of horse liver alcohol dehydrogenases (12 mM) in 0.1 M acetate buffer (pH 4.0): (1) dehydrogenase; (2) methylated dehydrogenase.

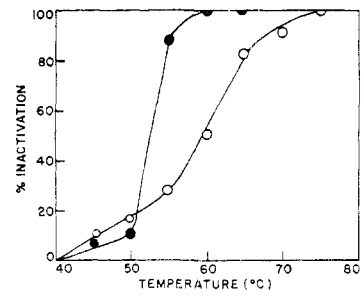

FIGURE 6: Heat inactivation of dehydrogenase (●) and methylated dehydrogenase (○). Enzyme solutions (0.8 mg/ml) in a 0.1 M pyrophosphate buffer (pH 9.0) were heated at a specified temperature for 5 min and enzyme activities were assayed.

hydrogenase and the methylated dehydrogenase exhibit a trough at 233 nm in optical rotatory dispersion spectra. The reduced mean residue rotation at 233 nm, $[m]_{233}'$, decreases from -2830 deg cm^2/dmol for the unmodified enzyme to -3310 deg cm^2/dmol for the modified enzyme (Figure 5).

The relative stabilities of dehydrogenase and the methylated dehydrogenase to inactivation by heat and sulfhydryl reagent were compared. Dehydrogenase and the methylated dehydrogenase were assayed for enzyme activities after being heated for 5 min. Figure 6 shows that methylation increases the heat stability of the enzyme. Both dehydrogenase and the methylated dehydrogenase were inactivated by *p*-ClHgBzO to a residual activity of 20% of the control in 30 min. However, they differ in the rate of inactivation. The pseudo-first-order rate constant of inactivation for dehydrogenase is 0.108 min^{-1}, whereas that for the methylated dehydrogenase is 0.058 min^{-1} until 70% of the activity was inactivated (Figure 7).

Discussion

It has been postulated that at least one lysine residue is situated at or near the binding site of the coenzyme in liver alcohol dehydrogenase (Kosower, 1962; Plapp, 1970). Picolinimidation activates the dehydrogenase (Plapp, 1970) whereas the treatment with pyrodoxal phosphate and sodium borohydride inactivates the enzyme (McKinley-McKee and Morris, 1972) despite the fact that both treatments modify lysine residues and NADH protects dehydrogenase from modifications. The modification of dehydrogenase with formaldehyde and sodium borohydride at 0° for 1 hr methylates 26 of the 60 lysine residues. The prolonged methylation does not affect the enzyme activity as well as free amino groups. This results in the enhancement of the enzyme activity and partial desensitization of the enzyme to substrate inhibition by ethanol. NADH protects 4 of the 26 lysine residues from methylation with a diminished enhancement in enzyme activities.

It appears that there are three classes of lysine residues in dehydrogenase. Class 1 lysines are those which are protected by NADH from modifications. There are two such lysines per subunit for methylation (present work), three per subunit for picolinimidation (Plapp, 1970) and one per subunit for pyridoxal phosphate modification (McKinley-McKee and Morris, 1972). It is likely that these lysines are situated at or near the binding site of NADH. Modifications of these residues produce pronounced effects on the enzyme activity. Class 2 lysines are those which are exposed and are subject to modifications. The methylation of these residues induces conformational changes in dehydrogenase structure. The remaining lysines which are not modified by the prolonged methylation belong to class 3. These are well masked and do not undergo modifications. Although NADH protects class 1 lysines from modifications, the treatment of dehydrogenase with lysine-specific reagents generally leads to nonselective modifications of lysines in classes 1 and 2. It should be pointed out that the division of amino acid residues based on their chemical reactivities is somewhat arbitrary. For example, the lysine residues of dehydrogenase behave differently to methyl-

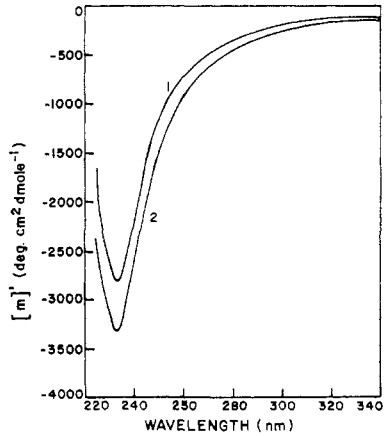

FIGURE 5: Optical rotatory dispersion spectra of horse liver alcohol dehydrogenases in 0.05 M phosphate buffer (pH 7.5): (1) dehydrogenase; (2) methylated dehydrogenase.

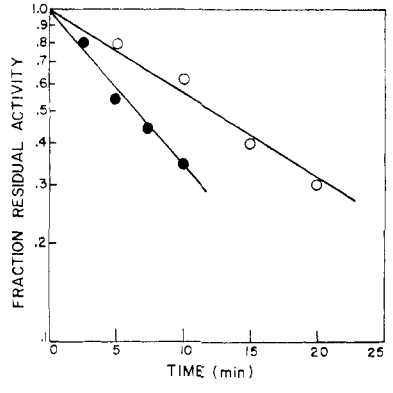

FIGURE 7: Mercurial inactivation of dehydrogenase (●) and methylated dehydrogenase (○). Enzyme solutions (0.8 mg/ml) in 0.1 M pyrophosphate buffer (pH 9.0) were incubated with 10^{-4} M *p*-ClHgBzO at 30°. At time intervals, an aliquot of 0.2 ml was withdrawn for the measurement of enzyme activites.

ation, picolinimidation, and pyridoxal phosphate reduction under various experimental conditions.[3]

The methylation does not seem to alter the subunit structure of dehydrogenase as indicated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and ultracentrifugation. However, dehydrogenase and the methylated dehydrogenase differ in Stokes radii as measured by Sephadex G-100 chromatography. A similar observation was made for the succinylation of bovine serum albumin and was attributed to conformational changes which affected the overall shape of the protein (Habeeb, 1967). Spectroscopic studies of the unmodified and modified enzymes provide evidence for the conformational changes of dehydrogenase. Methylated dehydrogenase exhibits a blue shift with an increased absorbance in the ultraviolet spectrum presumably due to the conformational changes in the protein structure which exposes the previously inaccessible chromophore to solvent (Yanari and Bovey, 1960). Methylated dehydrogenase, furthermore, displays an enhanced trough at 233 nm in the optical rotatory dispersion spectrum. Based on the empirical formula of Chen *et al.* (1972), the change in $[m]'_{233}$ represents an increase in the helical content of 3.7% when dehydrogenase is methylated. The methylation of dehydrogenase, moreover, increases the stability of the enzyme toward heat and mercurial inactivation suggesting that there is a difference in structures between dehydrogenase and methylated dehydrogenase.

Rosenberg *et al.* (1965) observed an increase in the helical content of the protein molecule when dehydrogenase formed the ternary complex with NADH and isobutyramide. The ternary complex is more resistant to the mercurial modification, and inactivation by acid, alkali, and heat treatments (Yonetani and Theorell, 1962). Although it does not imply directly that the methylated dehydrogenase and the ternary complex are conformationally related, an interesting inference can be made that the methylated dehydrogenase and the ternary complex undergo similar trends in structural changes.

The enhancement of the enzyme activity of the methylated dehydrogenase is more pronounced when assayed at a high ethanol concentration, suggesting that the release of the substrate inhibition is, in part, responsible for the observed enhancement. Although the methylation of the dehydrogenase partially desensitizes the enzyme to the substrate inhibition, the methylation fails to alleviate the NADH-protected methylated dehydrogenase to such inhibition. This implies that the lysine residues which interact with NADH may be situated near the abortive site of the dehydrogenase. Since the efficiency of substrate inhibition by alkanol decreases with an increasing bulk of substrates (Tsai, 1968), it seems that methylation of the dehydrogenase not only increases the steric hindrance for the interaction of NADH but also changes the conformation of the enzyme which may affect the formation of the abortive complex. However, further work is needed to resolve whether conformationl changes in the dehydrogenase structure are responsible for the enhancement in enzyme activity on methylation.

[3] Jörnvall (1973) methylated horse liver alcohol dehydrogenase at pH 8.5 by adding sodium borohydrate to the mixture of dehydrogenase and formaldehyde.

Acknowledgment

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