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Reactivity and Function of Sulfhydryl Groups in Horse Liver Alcohol Dehydrogenase*

Ting-Kai Li† and Bert L. Vallee

ABSTRACT: Iodoacetate inhibits horse liver alcohol dehydrogenase (LADH) by selectively carboxymethylating two of the twenty-four SH groups of the molecule. DPNH protects the enzyme both against the inhibition [T.-K. Li and B. L. Vallee, 1961, *Biochem. Biophys. Res. Commun. 12*, 44] and the alkylation of these two cysteine residues. DPNH and ethanol still interact with the inactive carboxymethylated enzyme as shown by spectral and rotatory dispersion titration and by zinc exchange; hence these SH groups are not indispensible for coenzyme and substrate binding. However, both the dissociation constant of the carboxymethylated LADH·DPNH complex and the rate of zinc exchange are increased. This suggests that in the three-dimensional array of the enzyme the cysteinyl residues are situated in close proximity to the zinc and coenzyme binding sites.

Iodine similarly inhibits the enzyme when, on the average, six SH groups per mole of protein are oxidized, but DPNH and isobutyramide only *partially* protect the enzyme against inhibition. Optical rotatory dispersion and ultracentrifugation indicate that iodination denatures one fraction of the enzyme molecules; thus iodine reacts both with catalytically essential residues and with others involved in maintaining structural stability of the enzyme.

We have reported briefly (Li and Vallee, 1963) that preferential carboxymethylation of two out of twenty-four thiol groups[1] in liver alcohol dehydrogenase (LADH)[2] results in complete loss of catalytic activity. DPN^+ and DPNH protect the enzyme against inactivation and chemical modification indicating that these groups are situated at the active centers. Peptides containing these active center residues have been isolated and their sequences characterized (Li and Vallee, 1964a; Harris, 1964).

The present report describes the effects of this modification upon the binding of coenzymes and substrates to liver alcohol dehydrogenase as well as the enzymatic and physicochemical consequences of oxidation of the SH groups with iodine. Like iodoacetate, iodine preferentially reacts with the active center SH groups but, in addition, it also induces a pronounced change in the structure of the protein. A preliminary communication containing some of the data has been published (Li and Vallee, 1963).

Materials and Methods

Crystalline alcohol dehydrogenase of horse liver was

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[1] The sulfhydryl content of different preparations of the enzyme has varied from 20 to 28 groups per mole of protein. The sulfhydryl titer of the enzyme employed in this study was 24 moles SH per mole of protein.

[2] Abbreviations: LADH, liver alcohol dehydrogenase; CM-LADH, carboxymethylated liver alcohol dehydrogenase; [(LADH)$^{65}Zn_2$], liver alcohol dehydrogenase with the two functional zinc atoms replaced by ^{65}Zn; DPN^+ and DPNH, oxidized and reduced forms of diphosphopyridine nucleotide, respectively.

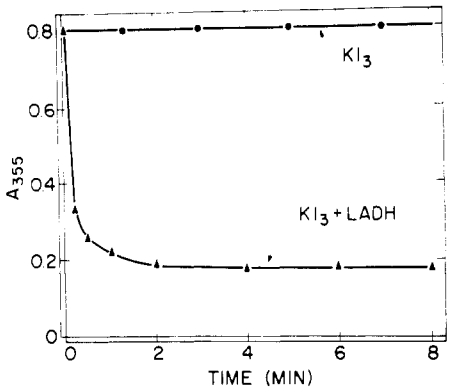

FIGURE 1: The reaction of iodine with horse liver alcohol dehydrogenase. Absorbance at 355 mμ is plotted against time. Enzyme (0.084 mg/ml) (▲—▲) was exposed to 6.2 meq/ml of KI_3 (●—●) in 0.2 M KI and 0.15 M phosphate, *p*H 6.5, 5°.

obtained from C.F. Boehringer und Soehne, Mannheim, W. Germany. Before use, the enzyme was dialyzed for 5 days against 0.1 M sodium phosphate buffer, *p*H 7.5, at 4°, to remove low molecular weight impurities which absorb radiation at 280 mμ. The concentration of protein was determined by measurement of the molar absorptivity at 280 mμ based upon an absorbance index of 0.455 mg^{-1} cm^2 (Bonnichsen, 1950) and according to the method of Lowry *et al.* (1951). Molar concentrations are based on a molecular weight of 83,300 (Ehrenberg and Dalziel, 1958). The enzyme was monodisperse upon centrifugation in 0.1 *M* phosphate buffer, *p*H 7.5, 20°. In 1.7×10^{-2} M pyrophosphate buffer, at *p*H 8.8, 20°, with 1.7×10^{-3} M DPN^+, 1.7×10^{-2} M ethanol, and 3.3 μg of enzyme per ml, the turnover number of enzyme preparations varied from 480 moles to 530 moles DPN^+ per minute per mole of protein.

Reduced diphosphopyridine nucleotide (β-DPNH, 98%) was obtained from the Sigma Chemical Co. Solutions of DPNH in metal-free distilled water at *p*H 6, 4°, were prepared daily from the desiccated stock powder in order to minimize the formation of inhibitor due to the decomposition of DPNH (Fawcett *et al.*, 1961). The concentration of DPNH was determined by measurement of its absorbance at 340 mμ (Kaplan, 1960). DPN^+ (Pabst Laboratories) was dissolved in 0.05 M phosphate buffer, *p*H 7.5. Acetaldehyde (Eastman Organic Chemicals) was distilled at 24° and diluted with metal-free water. Potassium iodide, iodoacetic acid, sodium dihydrogen phosphate, sodium pyrophosphate, isobutyramide, and ethanol were reagent grade chemicals and were used without further purification. Iodine was resublimed daily, dissolved in 0.2 M KI, and stored at 4° in the dark. The concentration of iodine in solution was measured from the absorbance of potassium triiodide at 355 mμ (Cunningham and Nuenke, 1959). [1-^{14}C]Iodoacetate (2 mc/mmole) was obtained from New England Nuclear Corp. and diluted with unlabeled iodoacetate before use. Dialyses were carried out in cleaned (Hughes and Klotz, 1956) cellulose casings (Visking Corp.). The purification of water and cleaning of glassware have been described (Vallee and Hoch, 1955).

Iodination was performed as described by Cunningham and Nuenke (1959). Solutions of KI_3 were added to the enzyme in 0.2 M KI and 0.15 M phosphate buffer at *p*H 6.5 and 5°. The reaction was followed by measurement of the decrease in absorbance at 355 mμ in a Beckman DU spectrophotometer at 5°. The modified enzyme was separated from the KI solution by passage over Sephadex G-25 (Pharmacia, Sweden) contained in a 30- × 1-cm column and equilibrated with 0.1 M phosphate buffer, since iodide ions interfered both with the *p*-mercuribenzoate titrations and with the interaction of LADH with DPNH (Li *et al.*, 1963).

Carboxymethylation with iodoacetate was carried out in 0.1 M phosphate buffer at *p*H 7.5 and 21°. The reaction was terminated by passage of the solution over Sephadex G-25.

The SH titer of the native and modified enzymes was measured with *p*-mercuribenzoate by spectrophotometric titration (Boyer, 1954), and by amperometric titration with silver (Benesch *et al.*, 1955). DPNH binding was measured spectrophotometrically (Theorell and Bonnichsen, 1951) and by rotatory dispersion titration (Li *et al.*, 1962). Substrate binding to the native and carboxymethylated enzymes was determined by their capacity to retard the exchange of $Zn^{2+} \rightleftharpoons {}^{65}Zn^{2+}$ in [(LADH)$^{65}Zn_2$] which was prepared by equilibrium dialysis (Druyan and Vallee, 1964).

Optical rotation was measured by means of a Model 200S-80Q photoelectric spectropolarimeter (O. C. Rudolph and Sons) using as light source a high-pressure mercury lamp (A-H6, General Electric Co.) as described by Li *et al.* (1962). Specific rotations calculated on the basis of enzyme concentrations are precise to ±1.0°. The dispersion constant, λ_c, was calculated as suggested by Yang and Doty (1957).

Sedimentation was performed in a Model E Spinco ultracentrifuge. Amino acid analysis after acid hydrolysis (5.7 N HCl at 110° for 24 hours) of the native and carboxymethylated enzymes was performed in a Model 120B Beckman amino acid analyzer according to the method of Spackman *et al.* (1958). $^{65}Zn^{2+}$ activity was measured by γ-ray counting in a well-type scintillation detector (Tracerlab) and ^{14}C activity by standard procedures of liquid scintillation counting. The catalytic activity of the enzyme for both the oxidation of ethanol and the reduction of acetaldehyde was measured spectrophotometrically (Li and Vallee, 1964b).

Results

Iodination. Iodine reacts instantaneously with liver alcohol dehydrogenase in 0.2 M KI and 0.15 M phosphate, *p*H 6.5, 5°. The reaction is virtually complete within 3 minutes as measured by the decrease in absorbance of KI_3 at 355 mμ. Thus, when 6.2×10^{-5}

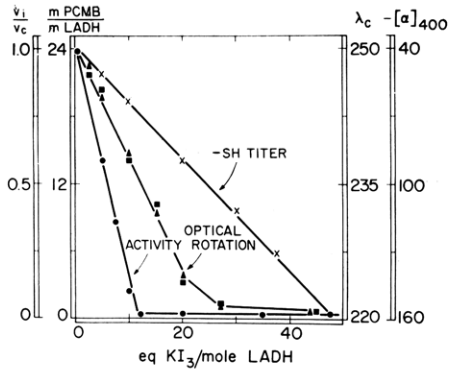

FIGURE 2: The enzymatic activity, SH titer, and optical rotatory dispersion of liver alcohol dehydrogenase as a function of iodine concentration. Fractional residual activity, V_i/V_c (●—●), number of free SH groups remaining per mole of protein, moles *p*-mercuribenzoate per mole of LADH (×—×), dispersion constant, λ_c (▲—▲), and specific levorotation at 400 mμ, $-[\alpha]_{400}$ (■—■) are plotted against equivalents of KI_3 added per mole of enzyme. V_c is the control enzymatic activity and V_i is the activity of the enzyme in the presence of iodine. Enzyme (2.5 mg/ml) was exposed to increasing concentrations of iodine in 0.2 M KI and 0.15 M phosphate, *p*H 6.5, 5°.

meq KI_3/ml are added to 1.0×10^{-6} M enzyme, 4.8×10^{-5} meq KI_3/ml are consumed (Figure 1). When less than 48 eq of KI_3 per mole of LADH are present, all of the iodine is decolorized within 3 minutes.

The rapid rate of the reaction indicates that iodine reacts with thiol groups of the protein (Cunningham and Nuenke, 1959). Direct titration of the modified protein with *p*-mercuribenzoate supports this conclusion. The remaining number of free thiol groups decreases as a direct function of the number of equivalents of iodine taken up. When 48 eq of KI_3 per mole of enzyme have been consumed, none of the twenty-four thiol groups of LADH reacts with *p*-mercuribenzoate (Figure 2). The stoichiometry of 2 eq of KI_3 per mole of SH is in accord with that observed previously for the oxidation of SH groups of other proteins with iodine (Cunningham and Nuenke, 1959,1960).

Iodine instantaneously and irreversibly inhibits the enzyme. Activity is inversely proportional to the concentration of iodine and is lost completely when 12 eq of KI_3 have reacted with each mole of protein (Figure 2). Thus, enzymatic activity is abolished completely when an average of only 6 out of the 24 free SH groups per mole of protein have been oxidized; thus, out of the total, iodine preferentially modifies a limited number of SH groups which may be involved in activity either specifically or nonspecifically.

To differentiate between these possibilities the effects of DPNH and the substrate homolog, isobutyramide, upon the iodination reaction were measured. In the absence of these agents, 10 eq of iodine per mole of enzyme reacts with 5 SH groups abolishing 90% of the

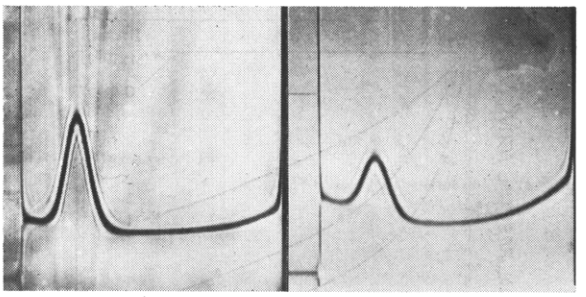

FIGURE 3: The sedimentation pattern of iodinated liver alcohol dehydrogenase after 33 minutes at 59,780 rpm in 0.1 M phosphate, *p*H 7.5, and 1% KCl, 20°. Protein concentration, 7.4 mg/ml. A: native enzyme. B: enzyme iodinated with 10 eq KI_3/mole protein, as under Methods, and passed over Sephadex G-25 equilibrated with 0.1 M phosphate, *p*H 7.5, 20°.

enzymatic activity. The addition to the reaction mixture of both DPNH and isobutyramide in excess of 2 moles per mole of enzyme does not significantly alter the number of SH groups modified but preserves between 45 and 55% of the original activity (Table I).

The effect of iodination upon the structural integrity of the enzyme was studied to distinguish active center thiol residues from others involved perhaps in stabilizing

TABLE I: Effect of DPNH and Isobutyramide upon Iodination of Liver Alcohol Dehydrogenase.[a]

DPNH (M × 10^6)	Isobutyramide (M × 10^6)	V_i/V_c	SH Groups Oxidized[b] (moles/mole protein)
0	0	0.09	5.0
15	15	0.45	
45	45	0.53	4.8
90	90	0.55	4.7
150	150	0.52	

[a] LADH, 7.5×10^{-6} M; KI_3, 7.5×10^{-5} M in 0.2 M KI and 0.15 M sodium phosphate, *p*H 6.5, 5°. [b] Measured by titration with *p*-mercuribenzoate of the free SH groups remaining in the modified enzyme.

structure. When LADH is exposed to successively increasing concentrations of iodine, the specific levorotation of the protein at 400 mμ increases from 40 to 160° while its dispersion constant, λ_c, decreases from 250 to 220 mμ (Figure 2), changes which are consistent with denaturation (Ulmer *et al.*, 1961; Li *et al.*, 1962). However, at a ratio of 10 eq of KI_3 per mole of LADH when enzymatic activity is already less than 10% of that originally present, the changes in optical rotatory

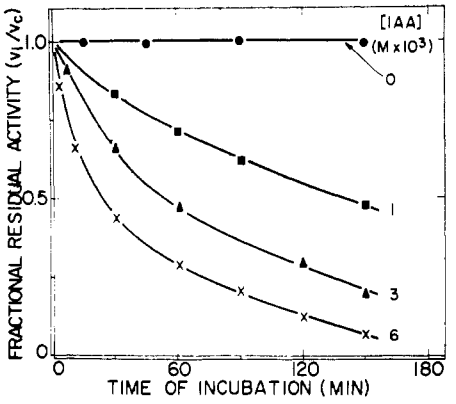

FIGURE 4: The activity of liver alcohol dehydrogenase as a function of iodoacetate concentration. Fractional residual activity, V_I/V_c, is plotted against time of incubation when 0.84 mg per ml of enzyme (●–●) is exposed to 1×10^{-3} M (■–■), 3×10^{-3} M (▲–▲), and 6×10^{-3} M (×–×) iodoacetate in 0.1 M phosphate, *p*H 7.5, 20°.

dispersion are only approximately 40% of those maximally possible on denaturation.

Ultracentrifugation of the enzyme modified with this amount of iodine reveals the presence of two distinct components. The first sediments rapidly even prior to the attainment of maximum rotor speed; the second appears as a slower symmetrical peak, with a sedimentation coefficient identical with that of the native protein. The area under this peak is approximately 58% of that of the unmodified native enzyme centrifuged under otherwise identical conditions (Figure 3). Thus, at a point where the enzyme is 90% inhibited, both optical rotatory dispersion and ultracentrifugation indicate that only about 40% of the enzyme molecules have undergone structural alterations.

Carboxymethylation. In contrast to the instantaneous reaction of iodine with LADH, iodoacetate at *p*H 7.5 and 21° inhibits both the oxidation of ethanol and reduction of acetaldehyde at a first-order rate which is governed by the concentration of the reagent employed. A 600-fold M excess of iodoacetate decreases the activity to 10% of the control in 150 minutes (Figure 4). In 180 minutes the enzyme is inhibited completely. During this period an average of approximately 2.5 SH groups are carboxymethylated, as measured by the moles of [1-^{14}C]iodoacetate per mole of protein incorporated and the amount of *S*-carboxymethylcysteine formed and by *p*-mercuribenzoate titration of the modified protein (Table II, column 1). No amino acid residues other than cysteine are modified. The optical rotatory dispersion and sedimentation coefficient of the inactive carboxymethylated protein are virtually identical with that of the native enzyme (Li and Vallee, 1963); thus, as gauged by these criteria, such carboxymethylation of this enzyme does not change its protein structurre

Iodoacetamide also inhibits LADH. However, moe. than a 1500-fold M excess of the reagent is required in order to attain complete inactivation within 3 hours of incubation.

TABLE II: Reaction of Iodoacetate with LADH and LADH·DPNH.[a]

	LADH	LADH·DPNH	Difference
Enzymatic activity (% of control)	0	95	95
[^{14}C]Carboxymethyl incorporated per mole of protein	3.0	0.6	2.4
SH groups per mole protein	21.6	23.7	2.1
S-Carboxymethylcysteines formed per mole protein	2.4	0.3	2.1

[a] LADH, 6×10^{-5} M; DPNH, 1.2×10^{-3} M; iodoacetate, 3.6×10^{-2} M in 0.1 M phosphate, *p*H 7.5, 21°.

Both DPN^+ and DPNH protect the enzyme against inactivation by iodoacetate (Li and Vallee, 1963), and in their presence only about 0.4 SH group are carboxymethylated (Table II, column 2). The differences between the values in columns 1 and 2 of Table II demonstrate that DPNH protects two SH groups which are essential for catalytic function.

The reactivity toward iodoacetate of the SH groups which DPNH does not protect was investigated further. When the LADH DPNH complex is exposed to iodoacetate for as much as 18 hours, at the maximum only about three additional SH groups react, but without affecting activity. However, when the enzyme is denatured with 0.01 M sodium dodecyl sulfate first and then exposed to iodoacetate, within a period of 2.5 hours twenty thiol groups per mole of protein react with iodoacetate and within 6 hours virtually all of the free SH groups are carboxymethylated (Table III).

To elucidate the mechanism of inhibition by iodoacetate, the interaction of coenzymes and substrates with the modified protein was studied. The LADH DPNH complex exhibits a characteristic negative Cotton effect centered at 327 mμ (Ulmer *et al.*, 1961). Addition of 6×10^{-5} M DPNH to 2×10^{-5} M CM-LADH results in a Cotton effect which is identical in sign, shape, and position with that of the complex of the native enzyme with DPNH (Figure 5). Thus DPNH still binds to the carboxymethylated, inactive enzyme although less firmly than to the native enzyme as indicated by the smaller amplitude and breadth of the Cotton effect.

Spectral titration of the enzymes with DPNH confirms this. On binding to the native enzyme, absorption of DPNH undergoes hypsochromic and hypochromic shifts; this circumstance has served to measure the dissociation constant of the LADH DPNH complex (Theorell and Bonnichsen, 1951). Similarly, formation

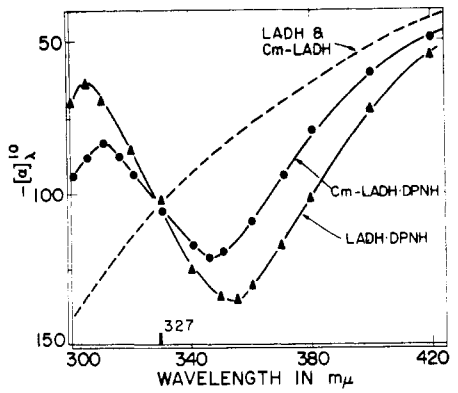

FIGURE 5: The effect of DPNH on the optical rotatory dispersion of native and carboxymethylated liver alcohol dehydrogenase. Specific levorotation, $-[\alpha]_\lambda^{10}$, is plotted against wavelength. The optical rotatory dispersions of 1.7 mg/ml of LADH and CM-LADH are superimposable (---). DPNH (6×10^{-5} M) was exposed to the identical concentrations of LADH (▲-▲) and of CM-LADH (●-●).

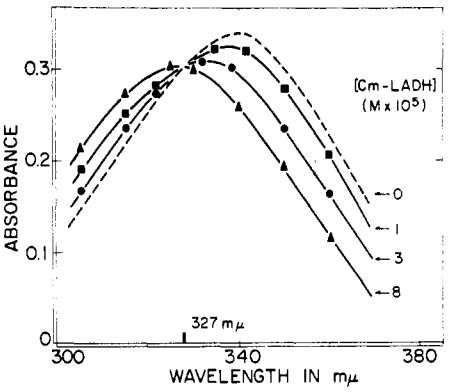

FIGURE 6: The effect of carboxymethylated liver alcohol dehydrogenase on the absorption spectrum of DPNH. Absorbance is plotted against wavelength; 5.5×10^{-5} M DPNH (----) was exposed to 0.84 mg/ml (■-■), 2.5 mg/ml (●-●), and 6.7 mg/ml (▲-▲) of CM-LADH in 0.1 M phosphate, pH 7.5, 20°.

TABLE III: Reaction of Iodoacetate with LADH·DPNH and Denatured LADH.[a]

	Time of Incubation (hours)	SH Groups Alkylated[b] (moles/mole protein)
LADH·DPNH	2.5	0.4
	6	1.8
	18	3.2
Denatured LADH[c]	2.5	20
	6	>22

[a] LADH, 6×10^{-5} M; DPNH, 1.2×10^{-3} M; iodoacetate, 3.6×10^{-2} M in 0.1 M phosphate, pH 7.5, 21°. [b] Measured by titration with *p*-mercuribenzoate of the free SH groups remaining in the modified enzyme. [c] LADH was dialyzed against 0.01 M sodium dodecyl sulfate in 0.1 M Tris, pH 8, 4°, for 24 hours, and then against 0.1 M phosphate, pH 7.5.

of the CM-LADH·DPNH complex shifts the absorption from 340 mμ, typical of free DPNH, to 325 mμ, characteristic of the complex (Figure 6). Two moles of DPNH binds to each mole of CM-LADH with a dissociation constant of 4×10^{-5} M.

Since the interaction of coenzymes and substrates (homologs) at the zinc atoms of LADH retards the rates of exchange of the protein-bound zinc atoms with ionic zinc (Druyan and Vallee, 1964), the capacity of the inactive CM-LADH to form such ternary complexes was studied by this means. [(Cm-LADH)$^{65}Zn_2$] was prepared by equilibrium dialysis against $^{65}Zn^{2+}$ (Druyan and Vallee, 1964), then carboxymethylated and dialyzed against an equimolar concentration of stable Zn^{2+}. Carboxymethylation accelerates the exchange of ^{65}Zn under all conditions studied; the half-life of exchange, $t_{1/2}$, for the carboxymethylated enzyme alone is 14 as opposed to 22 hours in the presence of 1.75×10^{-3} M DPNH. The presence of both DPNH and 0.1 M ethanol further prolongs exchange to 42 hours (Figure 7). These values may be compared with identical conditions for the native enzyme where $t_{1/2}$ was 20, 38, and 72 hours, respectively.

Discussion

Thiol groups have been shown to be essential constituents of the active centers of several dehydrogenases (Racker and Krimsky, 1955; Li and Vallee, 1963; DiSabato and Kaplan, 1963; Whitehead and Rabin, 1964). Their identity has been established through chemical modification with site-specific reagents and the subsequent isolation and characterization of the peptides containing the modified amino acid residues (Harris *et al.*, 1963; Li and Vallee, 1964a,b; Harris, 1964). The success of such procedures in general has depended importantly upon the selective interaction of site-specific reagents with functional side chains of a molecule, as contrasted with their failure to attack chemically identical but functionally inert groups. While the chemical basis of this selectivity is not understood, it is thought to reflect, among other factors, differences in the microscopic environment of the amino acid side chains.

The difference in the reactivity of the SH groups in horse liver alcohol dehydrogenase toward several SH reagents serves to illustrate what has become a common type of observation. Sulfhydryl groups have long been considered essential in the catalytic function of this enzyme (Theorell and Bonnichsen, 1951). However, past

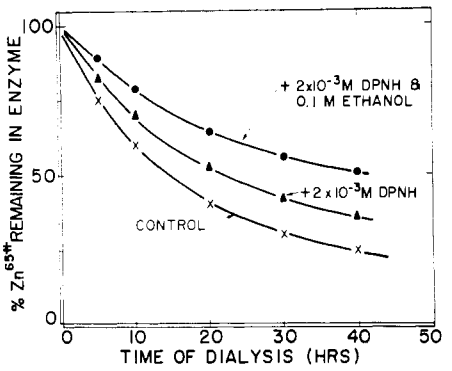

FIGURE 7: Effect of DPNH and ethanol on $^{65}Zn^{2+} \rightleftharpoons Zn^{2+}$ exchange in carboxymethylated liver alcohol dehydrogenase labeled with ^{65}Zn, [(Cm-LADH)$^{65}Zn_2$]. The per cent of ^{65}Zn remaining in the enzyme is plotted against time of dialysis. ^{65}Zn-labeled enzyme, prepared by equilibrium dialysis of the native enzyme against $^{65}Zn^{2+}$ (Druyan and Vallee, 1964), was carboxymethylated with a 600 M excess of iodoacetate for $2^3/_4$ hours as described under Methods. [(Cm-LADH)-$^{65}Zn_2$] (2.1 mg) in 1 ml was dialyzed against 50 ml of 0.1 M Tris-succinate buffer, *p*H 6.1, 4°, containing 4×10^{-5} M Zn^{2+} (X--X), Zn^{2+} plus 2×10^{-3} M DPNH (▲-▲), and Zn^{2+}, DPNH plus 0.1 M ethanol (●-●).

efforts to delineate the number which are directly involved in enzymatic activity have met with limited success. The SH groups in LADH, unlike those in lactic dehydrogenase (DiSabato and Kaplan, 1963) and glyceraldehyde 3-phosphate dehydrogenase (Velick, 1953), do not exhibit differential rates of reactivity toward *p*-mercuribenzoate and other similar reagents. These agents inhibit the enzyme only after the majority of the SH groups have reacted; substrates or coenzymes provide little if any protection against the inhibition (Witter, 1960; Li *et al.*, 1962; Yonetani and Theorell, 1962). Moreover, the ensuing structural changes raise the possibility that the nonspecific effects of denaturation account for the inhibition (Li *et al.*, 1962). In contrast, the present report demonstrates that iodine and, even more selectively, iodoacetate react preferentially with a limited number of functional SH groups of LADH. While the factors which render these groups more reactive toward iodine and iodoacetate than others are not clear at this juncture, iodoacetamide has been shown to be a competitive inhibitor of LADH (Woronick, 1961), a kinetic manifestation of the "affinity" of this agent for the active centers.

The oxidation of SH groups and substitution of the conjugated aromatic rings of tyrosine and histidine residues are generally thought to be the two major reactions of iodine with proteins. Limited concentration of the reagent, low temperature, and neutral *p*H virtually confine its reaction to the oxidation of SH groups (Cunningham and Nuenke, 1959). Under these conditions, 2 eq of iodine per mole of SH reacts instantaneously with LADH, suggesting the formation of sulfenyl iodide rather than of disulfide as the major pathway of the oxidation process (Cunningham and Nuenke, 1960). When six out of the twenty-four SH groups per mole of LADH have been oxidized inhibition is complete. Thus not more than six of these groups are essential to activity. But since the calculated number of SH groups modified per mole of protein represents a statistical average, correlation of this number with inhibition does not necessarily signify that each molecule of enzyme requires all six SH groups for catalytic function. In fact, the studies with iodoacetate show that catalytic function can be abolished solely by modification of two out of all the free SH groups present. It appeared therefore that the mechanism of inhibition with iodine might be complex, constituting the combined results both of site-specific modification of active center groups and of less specific effects on protein structure, analogous to the effects of *p*-mercuribenzoate on this enzyme (Li *et al.*, 1962).

To investigate these possibilities, the effect of iodine on the inhibition was measured in the presence of DPNH and isobutyramide which form a stable but catalytically inactive ternary complex with LADH (Winer and Theorell, 1960). Their interaction with the active center of the enzyme would be expected to protect its catalytically active groups against modification and hence prevent inhibition. Even though the same total number of SH groups are modified, a substantial fraction of enzymatic activity (50–60%) is preserved, indicating that catalytically essential groups have been partially protected against modification (Table I). Such a finding is not unexpected since the SH groups are present in large excess and iodine concentration is limiting during the reaction.

Optical rotatory dispersion and ultracentrifugation demonstrate that the activity changes on iodination are accompanied by changes in protein structure. The structural changes however are not sufficiently extensive to account for the degree of inhibition. Notably, when more than 90% of activity is lost, the change in optical rotatory dispersion amounts to only 40% of that maximally attained with iodination (Figure 3). Coincident with this, about 40% of the initial material is denatured at this juncture and ultracentrifugation demonstrates its aggregation. The remaining 60% is enzymatically active, apparently protected by DPNH and isobutyramide, and the sedimentation is identical with that of the native enzyme. The site-specific modification of active center SH groups apparently does not cause structural changes detectable by these methods, but the coenzyme and substrate homolog fails to protect those enzyme molecules which are denatured by iodination. Thus, interpretation of the functional significance of chemical modification depends importantly upon the separation of site-specific effects from those which have analogous functional consequences but are due to nonspecific changes in protein structure, as demonstrated here and in other instances (Elöidi, 1960; Li *et al.*, 1962; Bethune *et al.*, 1964).

Iodoacetate inhibits the enzyme at a first-order rate and as a direct function of the concentration of iodo-

acetate; it reacts more selectively with the catalytically functional SH groups of LADH than does iodine. When two to three SH groups per mole of protein have been alkylated, inactivation is complete and changes in protein structure cannot be measured (Li and Vallee, 1963). DPNH prevents both the inhibition and also the modification of two SH groups, indicating that one of these groups per active site is essential for catalytic function. Isotopic labeling and amino acid analysis confirm that carboxymethylcysteine is the only derivative formed, and that DPNH prevents the alkylation of the two cysteinyl residues. The active center cysteinyl peptides of this enzyme labeled with [^{14}C]iodoacetate have been isolated and characterized (Li and Vallee, 1964a; Harris, 1964).

DPNH does not protect SH groups of LADH which are unessential to catalysis. Hence, in the enzyme·DPNH complex, their reactivity with iodoacetate can be studied apart from enzyme activity. These groups react with iodoacetate at a considerably slower rate than those involved in the activity of the native enzyme. Once the enzyme is denatured, however, all of them react at the same rate (Table II). Due to the time dependence of the iodoacetate reaction, this difference in reactivity is demonstrated more readily with iodoacetate than with iodine and suggests that the catalytically nonessential SH groups are less accessible to the reagent, perhaps owing to intramolecular bonding in the native protein. Conversely, their modification with *p*- mercuribenzoate and iodine induces conformational changes suggesting that such bonding plays an important role in maintaining the structural stability of the protein.

It has been postulated that SH groups constitute one of the points of attachment of DPN(H) to LADH (Theorell and Bonnichsen, 1951). The efficacy of DPNH in blocking the reaction indicates that these particular SH groups which are reactive to iodoacetate indeed are either at, or closely adjacent to, the binding site. Direct measurement by spectral titration and rotatory dispersion titration shows that the inactive carboxymethylated enzyme still binds 2 moles of DPNH per mole of protein, albeit less firmly. This decrease in affinity does not account for the inhibition observed, since catalytic activity is not restored by increasing the concentration of the coenzyme in the reaction mixture. The spectral shifts and the sign, shape, and spectral location of the Cotton effects in the LADH·DPNH and the CM-LADH·DPNH complexes are identical. Since the Cotton effect reflects the steric orientation of the coenzyme in its interaction with the enzyme, carboxymethylation alters the geometry of the binding site minimally, if at all. The SH groups attacked by iodoacetate do not seem to be involved in coenzyme binding directly. They may be situated in close proximity to the binding site so that the affinity of the coenzyme for the enzyme is weakened perhaps through the introduction of the negatively charged carboxymethyl group.

Substrates or substrate homologs in conjunction with DPN^+ or DPNH retard the exchange of Zn^{2+} with [(LADH)$^{65}Zn_2$]. This phenomenon has been attributed to the formation of a ternary complex of enzyme·coenzyme·substrate (homolog) at or near the zinc site and constitutes a means to measure substrate binding (Druyan and Vallee, 1964). Carboxymethylation does not displace zinc from LADH. The rate of exchange of zinc, however, is accelerated, the $t_{1/2}$ of exchange for the carboxymethylated enzyme being 14 hours as compared to 20 hours for the native enzyme. Addition of ethanol and DPNH to the carboxymethylated enzyme increases the $t_{1/2}$ of exchange from 14 to 42 hours, an increase of 28 hours over that of the control and 20 hours over that of DPNH alone. This retardation of exchange by ethanol beyond that caused by DPNH alone shows that the CM-LADH·DPNH complex, though inactive, retains the capacity to interact with substrates. Therefore, the cysteinyl residues which are carboxymethylated do not appear to participate in the formation of a ternary complex with the substrate.

The catalytic mechanisms of enzymes have been viewed as consisting of two fundamental steps, i.e., the binding of coenzymes and substrates followed by catalysis. The present data indicate that the cysteinyl residues at the active centers are not indispensible to coenzyme and substrate binding. It is not possible to distinguish at this juncture whether they participate in catalysis directly or whether their modification interferes indirectly with the catalytic mechanism through steric factors not detectable by currently available physical methods. The concurrent acceleration of zinc exchange and weakening of DPNH binding on carboxymethylation suggests that all three loci must be in close proximity to one another in the three-dimensional array of the enzyme.

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