Epoxide Inhibition of Alcohol Dehydrogenases. Identification of Modified Cysteines in Yeast Alcohol Dehydrogenase and Demonstration of Reversible and Irreversible Inhibition of Liver Alcohol Dehydrogenase by Styrene Oxide†

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ABSTRACT: The inactivation of yeast alcohol dehydrogenase by styrene oxide leads to the simultaneous alkylation of two cysteines per subunit (Klinman, J. P. (1975), *Biochemistry 14*, 2568). The amino acid composition of one of two major radioactive peptides, obtained by tryptic digestion of enzyme alkylated with tritiated styrene oxide (StyO), indicates that cysteine-43 has been modified. Subsequent digestion of the second labeled tryptic peptide with pepsin leads to an octapeptide (StyO-Cys-Ala-Gly-Ile-Thr-Val-Tyr-Lys) which corresponds to positions 152–160 in the protein sequence. These results establish that styrene oxide alkylates cysteine-43 and -152, two of the three residues implicated as ligands for an active-site zinc. Horse liver alcohol dehydrogenase is found to be slowly inactivated by styrene oxide, $t_{1/2}$ = 7.8 h at pH 8.0, 25 °C, 10 mM styrene oxide. In analogy with the yeast enzyme, inactivation is accompanied by the incorporation of 2 mol of styrene oxide per subunit, and prior alkylation with iodoacetate reduces this stoichiometry to 1 mol per subunit. The observation of saturation kinetics for inactivation in the presence of 1 mM NADH (K_i = 9.6 mM) is attributed to "nonproductive" binding by styrene oxide, since second-order rate constants for inactivation are essentially the same both in the presence and absence of NADH, $k = 2.0\text{–}2.4 \times 10^{-3}$ M^{-1} s^{-1}. The horse liver alcohol dehydrogenase catalyzed reduction of acetaldehyde by NADH is reversibly inhibited by styrene oxide in a noncompetitive manner. The binding of styrene oxide to enzyme–NADH (obtained from slope replots) is observed to be biphasic, indicating inhibitor constants of K_i(slope) = 1.2 mM and K_i'(slope) = 4.6 mM at pH 8.6; the similarity between K_i'(slope) and the K_i derived from enzyme inactivation suggests that the lower affinity epoxide binding site competitive with acetaldehyde is responsible for the observation of saturation kinetics in enzyme inactivation. K_i(slope) is essentially unchanged at 1.0–1.1 mM in the pH range 6.0–9.1, whereas the binding of styrene oxide to enzyme–NAD^+ (obtained from intercept replots) increases from 1.2 mM at pH 6.0 to 4.8 mM at pH 9.1. These differences in the magnitude and pH dependence of K_i(intercept) vs. K_i(slope) are attributed to a stabilization of bound epoxide by hydrogen bonding to the conjugate acid of an active-site functional group of $pK \simeq 7.8$ in the enzyme–NAD^+ complex vs. $pK > 9$ in the enzyme–NADH complex.

Inactivation of yeast alcohol dehydrogenase by styrene oxide is characterized by saturation kinetics, an increase in rate with increasing pH, and the simultaneous alkylation of two cysteine residues per mole of active site (Klinman, 1975). These properties of styrene oxide inactivation contrast with the irreversible inhibition of enzyme by iodoacetamide and butyl isocyanate, reagents which alkylate either cysteine-43 or -152 in second-order, pH-independent kinetic processes (Harris, 1964; Twu et al., 1973). Cysteines-43 and -152 of the yeast enzyme are contained in peptides homologous to those containing the active-site cysteines of horse liver alcohol dehydrogenase (Eklund et al., 1976), and have been concluded, by analogy, to function as ligands for an active-site zinc (Jörnvall, 1977a). Previously, tryptic peptides of yeast alcohol dehydrogenase labeled with [^{14}C]iodoacetamide or tritiated styrene oxide were shown to cochromatograph, suggesting that styrene oxide and iodoacetamide alkylate the same cysteine residues at different stoichiometries (Klinman, 1975). A subsequent determination of the amino acid compositions of these peptides indicated that one of the two labeled peptides contained five cysteines. Further characterization of styrene oxide containing peptides was carried out to distinguish the simultaneous alkylation of cysteines-43 and -152 from the alkylation of one active-site cysteine together with a second cysteine distant from the active site.

Evolutionary differences between yeast and horse liver alcohol dehydrogenases include significant changes in primary sequence, subunit molecular weight, quaternary structure, and affinity for zinc (Brändén et al., 1975; Jörnvall, 1977b; Veillon and Sytkowski, 1975; Klinman and Welsh, 1976). A large number of aliphatic and aromatic electrophilic reagents have been demonstrated to modify either of two active-site cysteines (-46 or -174) of horse liver alcohol dehydrogenase (Harris, 1964; Li and Vallee, 1964; Reynolds and McKinley-McKee, 1969; Jörnvall et al., 1975; Sogin and Plapp, 1976). In this paper we report the stoichiometry and kinetic properties of liver alcohol dehydrogenase inactivation by styrene oxide for comparison with the yeast enzyme. Although the rate of liver alcohol dehydrogenase alkylation by styrene oxide is found to occur at only 10% of the rate for yeast alcohol dehydrogenase inactivation, the stoichiometry of alkylation (2 mol of styrene oxide per mol of active site) is the same for both enzymes. In addition to enzyme inactivation, the reversible inhibition of liver alcohol dehydrogenase activity by styrene oxide has been characterized.

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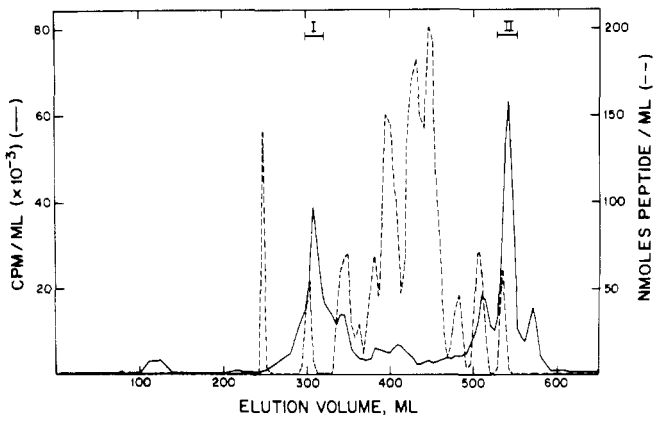


FIGURE 1: Elution profile of a tryptic digest of yeast alcohol dehydrogenase labeled with tritiated styrene oxide from Sp-Sephadex. Derivatized yeast alcohol dehydrogenase was obtained by reacting 236 mg of enzyme (6.3 μmol of enzyme subunits) in 40 mM KPP_i–150 mM glycine–5 mM HCl, pH 8.5, with 190 μmol of styrene oxide, sp act. = 1.3×10^6 cpm/μmol, in a total volume of 19 mL. The reaction was run to 80% inactivation and protein precipitated by the addition of ammonium sulfate (final concentration 70%); this suspension was centrifuged, and the precipitate was redissolved in pH 8.5 buffer. Protein was further separated from unreacted epoxide by gel filtration on a Sephadex G-25 column (2.5 × 30 cm) eluted with water; a total of 10.2×10^6 cpm eluted, corresponding to a 76% yield of protein (estimated on the basis of two reactive side chains per subunit and 80% inactivation). Labeled protein was reduced and carboxymethylated as described previously (Klinman, 1975). Following dialysis against ammonium bicarbonate (0.5%), 6 mg of trypsin was added, and the reaction was incubated for 6 h at 37 °C. Of the total radioactivity recovered, 78% was solubilized. A sample containing 4.91×10^6 cpm (~2 μmol of protein) was added to an Sp-Sephadex column, 1.8 × 50 cm, thermostated at 37 °C. Elution was carried out with 800 mL of a four-chamber pyridine-acetate buffer: chamber 1, 0.05 M pyridine-acetate, pH 2.5; chamber 2, 0.2 M, pH 3.1; chamber 3, 0.5 M, pH 3.75; and chamber 4, 2.0 M, pH 5.0. The flow rate was 23 mL/h, and 2.3-mL fractions were collected. The two major radioactive peptides, TpI and TpII, were pooled individually as indicated by bars.

Experimental Procedures

Materials. Yeast alcohol dehydrogenase was obtained from Boehringer as an ammonium sulfate suspension. Crystalline liver alcohol dehydrogenase was purchased from Worthington. Trypsin (treated with L-1-tosylamido-2-phenylethyl chloromethyl ketone), pepsin, and carboxypeptidases A and B were from Worthington. Styrene oxide (Matheson Coleman and Bell) was vacuum distilled prior to use [bp 73–75 °C (10 mm)]. Tritiated styrene oxide (1′,2′-[1′-^{3}H]epoxyethylbenzene) was synthesized by New England Nuclear (sp act. = 5.0×10^6 cpm/μmol). The radiochemical purity of this material was >99%, as ascertained by thin-layer chromatography. Iodoacetic acid (Sigma) was recrystallized from cold petroleum ether (bp 30–60 °C) before use. NADH[1] and NAD^+ were purchased from Sigma and ethyl alcohol was from Pharmco. Acetaldehyde (Matheson Coleman and Bell) was distilled immediately preceding kinetic studies. L-Cysteine ethyl ester was from Sigma. SP-Sephadex and gel filtration media were obtained from Pharmacia, and polyamide sheets were from Gallard-Schlessinger. Pyridine (Baker) was distilled after addition of solid ninhydrin (1 g/L). Fluram reagent was purchased from Pierce Chemicals. The following buffers were used in a study of the effect of pH on liver alcohol dehydrogenase inhibition by styrene oxide: 200 mM K P_i, pH 6.0; 160 mM KP_i, pH 6.6; 80 mM KP_i, pH 7.6; 31 mM KPP_i–109 mM glycine–5 mM HCl, pH 8.6 (PP_i buffer); and 24 mM KPP_i–41 mM glycine, pH 9.1 ($\mu = 0.20 \pm 0.03$).

Methods. Kinetic studies of enzyme inactivation were carried out as described previously (Klinman, 1975). The concentration of alcohol dehydrogenase was determined by absorbance at 280 nm, $A = 0.45$ for a 0.1% solution of the liver enzyme (Taniguchi et al., 1967) and $A = 1.26$ for a 0.1% solution of the yeast enzyme (Hayes and Velick, 1954). The methodology used for the preparation of radioactively labeled protein and peptides is described in the appropriate figure legend or table of this paper. Radioactivity was determined in toluene–ethanol cocktails on an Intertechnique scintillation spectrometer. Peptides were detected by the fluorescent method of Nakai et al. (1974). Amino acid analyses were performed on a Durrum D-500 amino acid analyzer. Samples were prepared for analysis by hydrolyzing in constant boiling HCl under reduced pressure at 110 °C for 24 h or the times stated in the text. An adduct of the ethyl ester of L-cysteine and styrene oxide was prepared by reacting 10 mM L-cysteine ethyl ester and 10 mM styrene oxide at pH 8.5, 25 °C, for 6–7 h; at this time approximately 89% of the cysteine initially present had reacted. Varying concentrations of this adduct were hydrolyzed in constant boiling HCl for 1–24 h and analyzed on the amino acid analyzer. The N-terminal amino acid sequence of the peptide TpI–PpII was carried out according to Edman (1956). Newly exposed amino terminal residues were dansylated according to the techniques of Hartley (1970) and Gray and Smith (1970). Dansylated amino acid derivatives were identified by chromatography on polyamide sheets by a modification of a procedure described by Woods and Wang (1967) and Angeletti et al. (1973); in addition, aliquots of the Pth amino acids released were counted on a liquid scintillation counter. C-Terminal amino acids were determined in the amino acid analyzer after incubation for 5 min with 0.5 μg of CPB, and 20 and 40 min in the presence of 0.5 μg of CPB plus 1 μg of CPA; controls were run to determine the amino acids released by CPB and CPA in the absence of peptide.

Results

Isolation and Purification of Labeled Tryptic Peptides from Yeast Alcohol Dehydrogenase. Yeast alcohol dehydrogenase was inactivated with tritiated styrene oxide, separated from unreacted styrene oxide by ammonium sulfate precipitation and Sephadex G-25 chromatography, and treated with trypsin to provide the elution pattern of labeled peptides illustrated in Figure 1. Two major radioactive peaks were obtained: TpI in 18% yield and TpII in 21% yield, indicating a 39% recovery of radioactive material from the SP-Sephadex column in these two peaks. The remaining counts (58%) were eluted from the column in multiple, minor peaks, which we attribute to incomplete proteolytic digestion, a small degree of nonspecificity in side-chain modification, and instability of the styrene oxide modified residues. TpI and TpII were pooled individually as indicated by the bars in Figure 1, and were further purified by gel filtration. Their elution profiles are shown in Figures 2A and 2B, respectively. Although the peptide and radioactivity peaks coincide in Figure 2B, TpI is only a shoulder in the peptide profile, Figure 2A. However, the amino acid composition of TpI was found to be distinct from that of fraction 53 and constant in fractions 35, 40, and 45, indicating little or no contamination by the major peptide peak. The amino acid analyses of pooled fractions from these gel filtration columns are summarized in Table I.

Due to the absence of a ninhydrin-positive StyO–cysteine

[1] Abbreviations used are: Tp, tryptic peptide; Pp, peptic peptide; Pth, phenylthiohydantoin; StyO, styrene oxide; CPA, carboxypeptidase A; CPB, carboxypeptidase B; CM, carboxymethyl; EBH, the conjugate acid of an enzyme-bound functional group; EB, the conjugate base of an enzyme-bound functional group; NAD^+ and NADH, oxidized and reduced nicotinamide adenine dinucleotide.

TABLE I: Amino Acid Composition of TpI, TpII, and TpI–PpII.

Amino acid	Residues/mol		
	TpI	TpII	TpI–PpII
StyO-cysteine[c]	(1)	(1)	(1)
CM-Cysteine	3.9		
Aspartic acid	7.0	3.2	0.20
Threonine[a]	3.9	1.8	1.1
Serine[a]	3.8	0.86	0.26
Glutamic acid	8.4	2.1	0.22
Proline	2.6	3.2	
Glycine	6.0	7.3	1.2
Alanine	9.9	2.1	1.2
Valine[b]	4.1	5.2	1.0
Methionine	1.0	0.78	
Isoleucine[b]	2.0	0.14	1.0
Leucine[b]	4.9	4.0	
Tyrosine	5.3	0.96	0.8
Phenylalanine	1.0	0.01	
Histidine	2.7	3.8	
Lysine	1.0	2.0	1.1
Arginine			
Total	69	38	8

[a] Values were extrapolated to zero time of hydrolysis. [b] Values at 72 h of hydrolysis were used. [c] Calculated from the specific activity of hydrolyzed peptide.

TABLE II: Amino Acid Sequence of TpI-PpII.[a]

Sequence:	*StyO-Cys-Ala-Gly-Ile*-Thr-*Val-Tyr-Lys*
Carboxypeptidase digestion:	CPB, 5′: Tyr, 0.81; Lys, 1.0
	CPA, 20′: Val, 0.06; Tyr, 0.82; Lys, 1.0
	CPA, 40′: Val, 0.74; Tyr, 0.83; Lys, 1.0

[a] A total of 20 nmol of TpI–PpII was used for sequence analysis, carried out as described in detail in the Methods section.

derivative from the amino acid compositions in Table I, a model StyO-cysteine ethyl ester derivative was prepared as described in the Methods section. This derivative eluted immediately preceding tyrosine in the amino acid analyzer. After 1 h of hydrolysis at 110 °C in constant boiling 6 N HCl, only cysteine and cystine remained; more extensive hydrolysis reduced the yields of these amino acids. These results indicate the instability of a StyO-cysteine derivative to the hydrolysis conditions, consistent with the absence of a modified amino acid in peptide hydrolysates.

Peptic Digest of TpI. The presence of four carboxymethylated cysteines, in addition to a styrene oxide modified residue in TpI, necessitated further digestion to determine the precise location of the modified residue. The elution profile of a peptic digest of TpI from Sp-Sephadex (Figure 3) revealed two major radioactive peptides. The first eluted at the same position as TpI from the original SP-Sephadex column of the tryptic digest (Figure 1), and is concluded to correspond to undigested peptide. The second peak, TpI–PpII, was further purified by gel filtration (Figure 3, inset); the coincidence of the peptide and radioactivity peaks indicates that it is a pure peptide.

Characterization of TpI–PpII. The amino acid composition of the purified TpI–PpII peptide is shown in Table I. The value for StyO-cysteine was estimated from the specific activity of the hydrolyzed peptide. The amino acid sequence of TpI–PpII was determined by a combination of Edman degradation and carboxypeptidase digestion (Table II). Ninety-five percent of the radioactivity in the peptide was released in the first Pth-derivative extraction, and StyO-cysteine can be assigned to

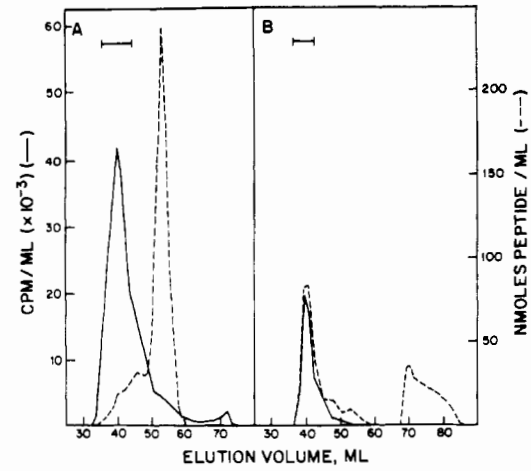


FIGURE 2: Gel filtration of TpI and TpII. The two major radioactive peptides from Figure 1, TpI and TpII, were further purified on Sephadex G-25 superfine columns (0.9 × 142 cm) equilibrated and eluted with 0.05 M pyridine–acetate (pH 2.5). The flow rate was 5 mL/h and 1.2-mL fractions were collected. The elution profile for TpI is shown in A and TpII is shown in B.

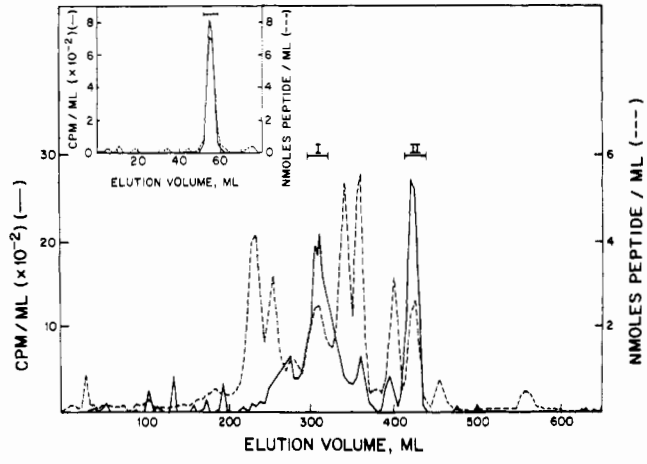


FIGURE 3: Elution profile of a peptic digest of TpI from Sp-Sephadex. A sample containing 0.2 × 10^6 cpm (~0.190 μmol) of TpI was incubated with 20 μg of pepsin at ambient temperature for 2 h in 5% formic acid. The sample was dried down, dissolved in 0.05 N pyridine–acetate (pH 2.5), and added to an SP-Sephadex column, 1.7 × 49 cm; elution was carried out with the four-chamber gradient described for Figure 1. (Inset) The second of the two major peptides from Figure 3, TpI–PpII, was rechromatographed on Sephadex G-25 superfine (0.9 × 142). Elution was carried out with 0.05 M pyridine–acetate (pH 2.5). The flow rate was 5 mL/h and 1.2-mL fractions were collected.

the amino terminus of the peptide. The internal threonine was not directly confirmed.

Inactivation of Horse Liver Alcohol Dehydrogenase by Styrene Oxide. Horse liver alcohol dehydrogenase is slowly inactivated by styrene oxide. Semilog plots of percent enzyme activity remaining vs. time permitted the determination of half-times for inactivation as a function of styrene oxide concentration, ±NADH. Due to the slow rates, inactivation was normally only followed up to the first half-time. As illustrated in Figure 4, the inactivation is a second-order process in the absence of coenzyme, $k = 2.4 \times 10^{-3}$ M^{-1} s^{-1}. In the presence of 1 mM NADH the inactivation shows saturation kinetics and an apparent $K_i = 9.6$ mM for styrene oxide. The stoichiometry of enzyme alkylation by tritiated styrene oxide was compared to enzyme inactivation. As summarized in Table III, the loss of enzyme activity and incorporation of tritiated epoxide in the absence of NADH proceeded as parallel processes, with a

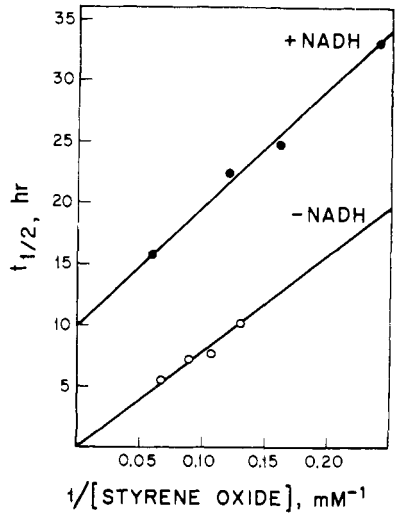


FIGURE 4: The relationship between the half-time for horse liver alcohol dehydrogenase inactivation by styrene oxide and the reciprocal of the styrene oxide concentration. The data were obtained under conditions of 25 °C, 80 mM P_i, pH 8.0, and 2–16 mM styrene oxide either in the absence or presence of 1 mM NADH. Incubations contained 0.6 mg/mL of enzyme. At appropriate time intervals 10 μL of the reaction mixture was removed and assayed for loss of enzyme activity. Values of $t_{1/2}$ were obtained from semilog plots of enzyme activity remaining vs. time.

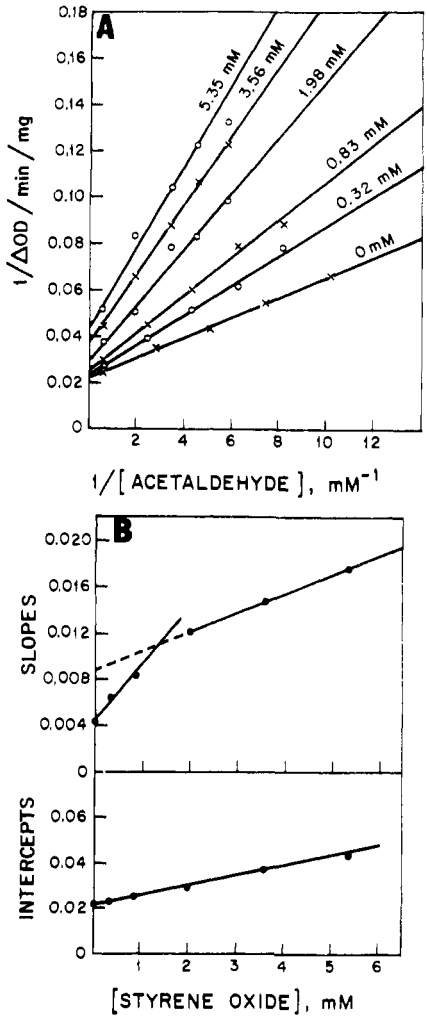


FIGURE 5: Styrene oxide inhibition of the liver alcohol dehydrogenase catalyzed reduction of acetaldehyde by NADH. (A) Primary data were obtained at 25 °C, PP_i buffer, pH 8.6, in the presence of 0.3 mM NADH with acetaldehyde varied between 0.10 and 2.0 mM. (B) Replots of the slopes and intercepts as a function of styrene oxide concentration.

TABLE III: Stoichiometry of Labeling of Liver Alcohol Dehydrogenase by Tritiated Styrene Oxide as a Function of Percent Inactivation.[a]

Time (h)	Fraction of enzyme act. lost	Sp act. of labeled enzyme subunits (cpm/μmol)	μmol of epoxide incorp./μmol of enzyme subunits
1.6	0.27	2.12×10^5	0.46
4.5	0.41	4.07×10^5	0.86
6.8	0.48	4.47×10^5	0.96
23.5	0.82	8.99×10^5	1.9

[a] 2–3 mg/mL (0.05–0.075 μmol of enzyme subunits) of liver alcohol dehydrogenase was incubated with 10 mM styrene oxide (sp act. = $4–5 \times 10^5$ cpm/μmol) in 80 mM P_i, pH 8.0. Aliquots were withdrawn at the appropriate times and added to G-25 columns (1 × 25 cm). Labeled protein was eluted with water and characterized with regard to its specific activity. It was possible to obtain virtually complete separation of the protein peak from a subsequent peak of tritiated epoxide. Blanks, run in the absence of protein to determine the percentage of counts eluting in the protein peak, due to polymers to styrene oxide or other high molecular weight radioactive impurities, amounted to no more than 10% of the total counts in the protein peak.

TABLE IV: Stoichiometry of Labeling of Carboxymethylated Liver Alcohol Dehydrogenase by Tritiated Styrene Oxide.[a]

Time (h)	Sp act. of labeled enzyme subunits (cpm/μmol)	Fraction of enzyme subunits labeled
1.0[b]	0.13×10^5	0.031
1.5	0.39	0.092
3.25	0.60	0.141
6.0	2.99	0.70
3.25[c]	1.91	0.37
6.0	2.14	0.41
9.0	2.35	0.45
22.5	4.67	0.90

[a] Carboxymethylated liver alcohol dehydrogenase was prepared by incubation of enzyme, 10 mg/mL (0.25 μmol of enzyme subunits), with 5 mM iodoacetate in 80 mM P_i, pH 8.0, for 19 h. At this point greater than 90% of enzyme activity was lost. Unreacted iodoacetate was removed from protein by gel filtration on Sephadex G-25. Modified enzyme, 3 mg/mL, was then incubated with 10 mM tritiated styrene oxide for 1–23 h, rechromatographed on Sephadex G-25, and characterized with regard to its specific activity. [b] Sp act. of styrene oxide = 4.26×10^5 cmp/μmol. [c] Sp act. of styrene oxide = 5.21×10^5 cpm/μmol.

constant ratio of 2 mol of styrene oxide incorporated per mol of enzyme activity lost. The time course for incorporation of tritiated epoxide indicates a $t_{1/2}$ = 6.8 h at 10 mM styrene oxide for comparison with the $t_{1/2}$ = 7.8 h determined from Figure 5 (−NADH).

To determine whether prior carboxymethylation prevents labeling by styrene oxide, liver alcohol dehydrogenase was inactivated with iodoacetate, followed by incubation with 10 mM styrene oxide. The relationship between time of incubation and fraction of protein labeled is given in Table IV. The data indicate the incorporation of 0.9 mol of styrene oxide into carboxymethylated enzyme after 22.5 h of incubation. On the assumption of 1 mol of styrene oxide incorporated into carboxymethylated enzyme at infinite time, a semilog plot of the fraction of liver alcohol dehydrogenase remaining unlabeled vs. time is linear and indicates a $t_{1/2}$ = 6.8 h for the incorporation of tritiated styrene oxide into carboxymethylated liver alcohol dehydrogenase.

Inhibition of Horse Liver Alcohol Dehydrogenase by Styrene Oxide. The slow rate of irreversible inactivation of enzyme

by styrene oxide facilitated inhibition studies. The effect of increasing styrene oxide concentrations on the rate of the enzyme-catalyzed reduction of acetaldehyde under conditions of pH 8.6 and high NADH (0.3 mM where K_d = 0.5 μM) is illustrated in Figure 5A. Replots of slopes and intercepts, Figure 5B, indicate a single K_i(intercept) = 4.5 mM; in contrast, the slope replot undergoes a break above 1 mM styrene oxide, K_i(slope) = 1.2 mM and K_i'(slope) = 4.6 mM. In an effort to elucidate the mode of epoxide binding, K_i was determined in the pH range 6.0–9.1. These results indicate that K_i(slope) is essentially independent of pH: K_i = 1.0, 1.4, 0.73, and 1.1 mM at pH values of 6.0, 6.6, 7.6, and 9.1, respectively. The magnitude of K_i'(slope) at pH 9.1 was 6.1 mM; the lower concentrations of styrene oxide employed below pH 8.6 ($\leq$3 mM) did not permit an accurate estimate of this constant across the pH range. K_i(intercept) was found to increase fourfold between pH 6.0 and 9.1: K_i = 1.2, 1.6, 2.2, and 4.8 mM at pH values 6.0, 6.6, 7.6, and 9.1, respectively.

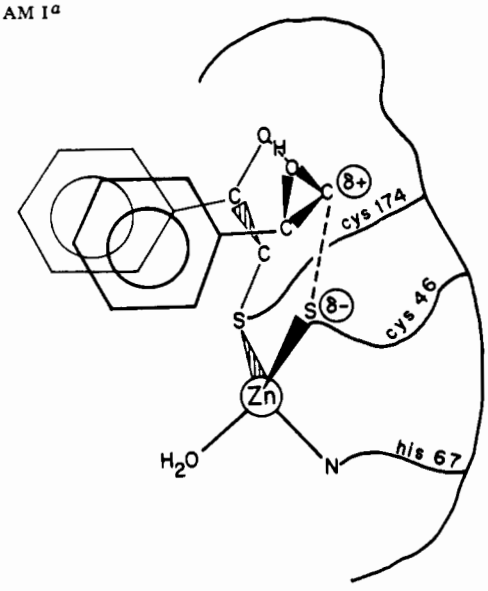
Discussion

Identity of Modified Residues. In a previous study, styrene oxide was demonstrated to alkylate two cysteine residues per subunit of yeast alcohol dehydrogenase (Klinman, 1975). In this paper, the two major labeled peptides obtained from tryptic digestion of inactivated enzyme have been purified and shown to have the amino acid compositions in Table I. The composition of TpII is the same as a peptide obtained from iodoacetamide inactivation of yeast alcohol dehydrogenase (Harris, 1964), indicating that one of the two cysteines alkylated by styrene oxide is cysteine-43. Since TpI contains four cysteines in addition to the styrene oxide modified residue, it was subjected to limited proteolysis by pepsin. Treatment of TpI in this fashion results in an octapeptide whose sequence (Table II) places it in positions 152–160 in the alcohol dehydrogenase sequence determined by Jörnvall (1977a). These results establish that styrene oxide alkylates cysteines-43 and -152, two of the three residues implicated as ligands for an active-site zinc (Jörnvall, 1977a).

In analogy with yeast alcohol dehydrogenase, horse liver alcohol dehydrogenase can be inactivated by a variety of reagents, e.g., iodoacetate and iodoacetamide, which modify cysteine-46 (Harris, 1964), and nicotinamide 5-bromoacetyl-4-methylimidazole dinucleotide and diazonium-1*H*-tetrazole, which modify cysteine-174 (Jörnvall et al., 1975; Sogin and Plapp, 1976). We find that inactivation by styrene oxide is accompanied by the incorporation of 2 mol of epoxide per subunit (Table III). Prior alkylation of cysteine-46 reduces the stoichiometry of labeling to 1 mol of epoxide per subunit (Table IV). These results suggest that the unique ability of styrene oxide to alkylate both active-site cysteines of yeast alcohol dehydrogenase pertains to the horse liver enzyme as well. Several properties of styrene oxide could facilitate the rapid incorporation of a second mole of reagent, subsequent to the initial inactivation event. These include a stacking of planar, aromatic rings, and electrophilic catalysis via hydrogen bonding between a newly formed hydroxyl group of a covalently bound styrene oxide and the epoxide oxygen of a second, attacking molecule of styrene oxide (Diagram I). The mechanism in Diagram I is based on the reported active-site configuration of the horse liver enzyme (Eklund et al., 1976). The assumption has been made that both epoxide molecules undergo nucleophilic attack by sulfur at the less sterically hindered epoxide carbon.

Kinetic Properties of Horse Liver Alcohol Dehydrogenase Inactivation. A number of important properties distinguish the inactivation of horse liver alcohol dehydrogenase from the yeast enzyme: (1) a considerably slower rate of inactivation ($t_{1/2}$ = 7.8 h at pH 8.0, 10 mM styrene oxide vs. $t_{1/2}$ = 1 h for the yeast enzyme under the same conditions); (2) a second-order inactivation process in the absence of NADH, pH 8.0 (vs. saturation kinetics for the yeast enzyme, K_i = 25 mM); and (3) an essentially unaltered rate of incorporation of styrene oxide into enzyme carboxymethylated at cysteine-46 (the incorporation of styrene oxide into carboxamidomethylated yeast alcohol dehydrogenase was complete on a time scale in which native enzyme is only 50% inactivated). This last property of liver alcohol dehydrogenase may indicate that styrene oxide inactivation involves a rate-determining attack on cysteine-174 followed by rapid alkylation of cysteine-46.

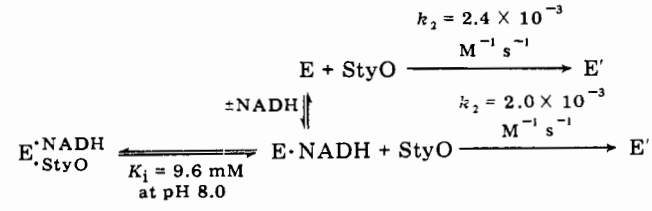
DIAGRAM I[a]



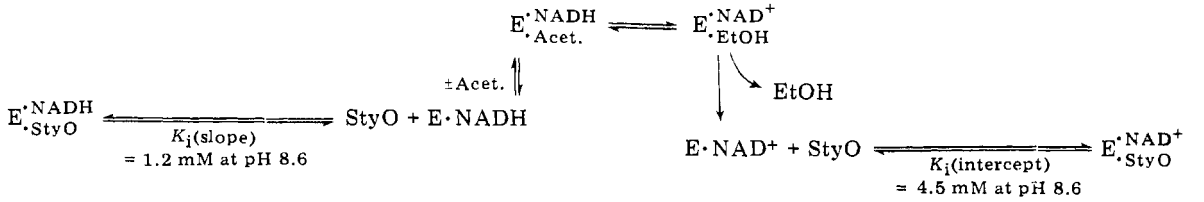
[a] Alkylation of *both* active-site cysteines could be due to a stacking of aromatic rings and electrophilic catalysis via hydrogen bonding between a newly formed hydroxyl group of a covalently bound styrene oxide and the epoxide oxygen of a second, attacking molecule.

The failure to observe saturation kinetics for horse liver alcohol dehydrogenase inactivation in the absence of NADH (Figure 4) is consistent with a highly preferred order of binding in which coenzyme adds before substrate in the enzyme-catalyzed reduction of acetaldehyde (Theorell and Chance, 1951). It can be seen from Figure 5 that the addition of 1 mM NADH results in a nonzero value for $t_{1/2}$ at infinite styrene oxide concentration (K_i = 9.6 mM). As discussed previously, the observation of saturation kinetics in the inactivation of an enzyme by a substrate analogue can result from the formation of an enzyme–inhibitor complex which either leads to inactivation or protects against inactivation (Baker, 1967; Klinman, 1975). If the interaction of styrene oxide with the liver alcohol dehydrogenase–coenzyme complex is "nonproductive", i.e., binding protects against inactivation, the rate constant for inactivation is obtained from the slope rather than intercept of plots of $t_{1/2}$ vs. [styrene oxide]$^{-1}$. Although the observation that NADH leads to *essentially no change in slope* [k_2 = 2.4 $\times$ 10^{-3} M^{-1} s^{-1} from Figure 5 (−NADH) vs. k_2 = 2.0 $\times$ 10^{-3}

SCHEME I: Proposed Kinetic Mechanism for Horse Liver Alcohol Dehydrogenase Inactivation by Styrene Oxide.



SCHEME II: Proposed Kinetic Mechanism for Horse Liver Alcohol Dehydrogenase Inhibition by Styrene Oxide.



M^{-1} s^{-1} from Figure 5 (+NADH)] could be fortuitous, it is most likely indicative of a second-order inactivation process which can be prevented by styrene oxide but is largely insensitive to the presence of NADH (Scheme I).

The apparent ability of iodoacetamide and styrene oxide to alkylate the same cysteines of yeast alcohol dehydrogenase (at different stoichiometries), together with the observation that iodoacetamide inactivation is a second-order, pH-independent process whereas styrene oxide indicates saturation kinetics and a pH dependence ($pK \simeq 8.5$), previously led to the proposal that styrene oxide binds "nonproductively" to this enzyme (Klinman, 1975).

Kinetic Properties of Horse Liver Alcohol Dehydrogenase Reversible Inhibition. In contrast to the requirement for high concentrations of styrene oxide and long incubation times to observe irreversible inhibition of horse liver alcohol dehydrogenase, enzyme activity is sensitive to relatively low epoxide concentrations. The data in Figure 5 indicate noncompetitive inhibition by styrene oxide. It is well established that the steady-state rate of the horse liver alcohol dehydrogenase catalyzed reduction of acetaldehyde is limited by NAD^+ release (Theorell and Chance, 1951). The observed pattern for styrene oxide inhibition (Figure 5) suggests (1) a competition between styrene oxide and acetaldehyde for E·NADH, K_i (slope) = 1.2 mM at pH 8.6, and (2) a binding of styrene oxide to E·NAD^+ subsequent to ethanol departure from the ternary complex, K_i(intercept) = 4.5 mM at pH 8.6 (Scheme II).

The above scheme is an oversimplification, since it does not take into account the biphasic nature of the slope replots. Figure 5B indicates two classes of binding sites for the interaction of styrene oxide with E·NADH. The observation of a twofold increase in K/V upon extrapolation to zero styrene oxide from high vs. low inhibitor concentration may indicate a differential affinity of styrene oxide for each of the two subunits of liver alcohol dehydrogenase. Previous investigators have proposed nonequivalent subunits for this enzyme, as an explanation for their failure to observe a burst of bound coenzyme production stoichiometric in enzyme subunits under conditions of aromatic aldehyde reduction (Dunn and Bernhard, 1971; McFarland and Bernhard, 1972) and alcohol oxidation (Luisi and Bignetti, 1974). More recently, Hadorn et al. (1975) and Weidig et al. (1977) have been able to obtain amplitudes equal to 90–100% of the enzyme subunit concentration by trapping the enzyme-bound NADH formed in the direction of benzyl alcohol oxidation. Weidig et al. argue that less than stoichiometric amplitudes in the liver alcohol dehydrogenase transient burst kinetics can be rationalized from the interrelationship of rate constants for the chemical interconversion and product release steps. Although it does not appear necessary to propose a mechanism in which product dissociation from one subunit is necessary for the other subunit to react, these authors point out that they are not able to exclude a biphasic oxidation of bound NADH.

It is of interest that we observe K_i'(slope) = 4.6 mM to be within a factor of two of the inhibitor constant derived from enzyme inactivation, K_i = 9.6 mM. Thus, the lower affinity epoxide site competitive with acetaldehyde may be the binding site leading to protection against irreversible inactivation, Scheme I. The eightfold discrepancy between K_i(slope) = 1.2 mM and the inhibitor constant obtained from enzyme inactivation indicates that the higher affinity epoxide site competitive with acetaldehyde is ineffective in either leading to or preventing enzyme inactivation. These results suggest that self-protection against irreversible inactivation may occur at epoxide concentrations sufficiently high to saturate the second of two enzyme subunits, characterized by an unequal affinity for styrene oxide.

Shore et al. (1974) have demonstrated the perturbation of an enzyme functional group from a pK_a greater than 9 to 7.6, upon addition of NAD^+; in the case of NADH binding, the pK_a appears to be unperturbed. It has been suggested that this functional group is the proton-donor-acceptor in the enzyme-catalyzed interconversion of NADH–aldehyde to NAD^+–alcohol. The approximately fourfold tighter binding of styrene oxide to enzyme–NADH than enzyme–NAD^+ at pH 8.6, together with a fourfold increase in K_i(intercept) from pH 6.0 to pH 9.1, may reflect different prototropic forms for enzyme–NADH (EBH) vs. enzyme–NAD^+ (a mixture of EBH and EB which depends on pH), and suggests a stabilization of bound epoxide by hydrogen bonding to EBH. Using limiting values for K_i(intercept) of 1.2 mM at pH 6.0 and 4.8 mM at pH 9.1, a plot of K_i(intercept) vs. pH indicates a $pK \simeq 7.8$ for the binding of epoxide to the enzyme–NAD^+ complex.

Bloxam et al. (1975) reported an increased binding of 2,3-epoxy butyrate and 2,3-epoxy propionate to the binary complex of lactate dehydrogenase and NADH with decreasing pH (pK = 6.8), interpreting their results in terms of hydrogen binding between the active-site histidine of lactate dehydrogenase and the epoxide oxygen.[2] In the case of liver alcohol dehydrogenase inhibition by styrene oxide, we find K_i(slope) to be essentially unchanged in the pH range 6 to 9. This insensitivity is analogous to the reported pH independence of the pre-steady-state rate constant and substrate K_m for β-naphthaldehyde reduction (McFarland and Chu, 1975), and the steady-state rate constant for benzaldehyde reduction, catalyzed by hydroxybutyrimidylated liver alcohol dehydrogenase (Dworschack and Plapp, 1977). Rather than ruling out a role for EBH in epoxide binding, these observations most likely reflect the high pK_a of a critical functional group in the enzyme–NADH complex.

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[2] These authors failed to observe any inactivation of lactate dehydrogenase by the competitive inhibitor 2,3-epoxy butyrate in the presence or absence of NADH after a period of 48 h.

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Effect of Coenzymes and Temperature on the Process of in Vitro Refolding and Reassociation of Lactic Dehydrogenase Isoenzymes†

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ABSTRACT: Dissociation and deactivation of the H_4 and M_4 isoenzymes of lactic dehydrogenase in strong denaturants may be reversed with a yield of reactivation up to 100%. The products of reconstitution are indistinguishable from the native enzymes as far as the Michaelis constants and the dissociation constants for substrate and coenzyme as well as spectral and hydrodynamic properties are concerned. The presence of NAD^+ and NADH does not affect either the conformational state of the product of reconstitution, or the kinetics of reactivation, using the pure apoenzymes as a reference. At 20 °C the kinetics of reactivation for LDH-M_4 in the presence and absence of coenzyme may be quantitatively described by a second-order rate equation ($k_2 = 23.4 \pm 2.6\ mM^{-1}\ s^{-1}$) while LDH-$H_4$ is characterized by a uni-bimolecular reaction sequence ($k_1 = 1.45 \pm 0.45 \times 10^{-3}\ s^{-1}$, $k_2 = 5 \pm 1\ mM^{-1}\ s^{-1}$), in agreement with earlier observations (Rudolph, R., et al. (1977), *Biochemistry 16,* 3384–3390). Regarding the influence of temperature on the rate of reactivation no significant anomalies are detectable within the range of 0–25 °C. The (apparent) activation energies, taken from the linear Arrhenius plots, are 58 kcal/mol for the association reaction of LDH-M_4, and 41 kcal/mol for the transconformation reaction of LDH-H_4.

The role of the amino acid sequence in the attainment of the three-dimensional structure of proteins is an established fact (Sela et al., 1957; Anfinsen, 1973). On the other hand, solvent parameters, like temperature and salts, or specific ligands (e.g., substrates or coenzymes) were shown to play an important role in the acquisition of the native structure of enzymes (Wetlaufer and Ristow, 1973). A number of mechanisms were proposed to define possible kinetic constraints in the thermodynamically determined process of reconstitution (divergency of stable structures, nucleation, ligand binding to unfolded polypeptide chains, stabilization of the native state, etc.).

In the case of NAD-dependent dehydrogenases, reactivation

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