Sweeney, W. V., Bearden, A. J., and Rabinowitz, J. C. (1974), *Biochim. Biophys. Res. Commun. 59,* 188.
Tamiya, N., and Miller, S. L. (1963), *J. Biol. Chem. 238,* 2194.
Weaver, P., Tinker, K., and Valentine, R. C. (1965), *Biochem. Biophys. Res. Commun. 21,* 195.
Yagi, T. (1970), *J. Biochem. (Tokyo) 68,* 649.
Yphantis, D. A. (1964), *Biochemistry 3,* 297.

The Interaction of an Epoxide with Yeast Alcohol Dehydrogenase: Evidence for Binding and the Modification of Two Active Site Cysteines by Styrene Oxide†

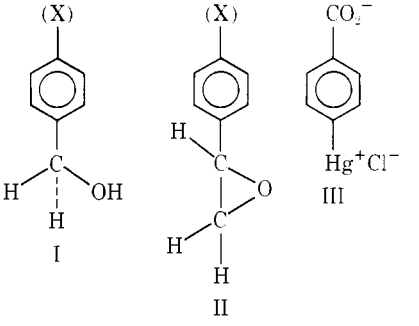
Judith P. Klinman

ABSTRACT: Yeast alcohol dehydrogenase is inactivated and alkylated by styrene oxide in a single exponential kinetic process. The concentration dependence of half-times for inactivation indicates the formation of an enzyme inhibitor complex, $K_I = 2.5 \times 10^{-2}$ *M* at pH 8.0. Reduced nicotinamide adenine dinucleotide (NADH), at a concentration of 3×10^{-4} *M* where $K_d \simeq 1 \times 10^{-5}$ *M*, has a small effect on kinetic parameters for inactivation. Although benzyl alcohol and acetamide-NADH increase the K_I for styrene oxide in a manner consistent with their dissociation constants, substrate also increases the rate of inactivation at high styrene oxide concentrations. The reciprocal of half-times for inactivation, extrapolated to infinite styrene oxide concentration, increases with pH between 7.6 and 9.0, p$K \simeq 8.5$. The stoichiometry of alkylation by [^{3}H]styrene oxide is 2.2 mol of reagent incorporated/mol of subunit, and is accompanied by the loss of 1.9 mol of sulfhydryl/mol of subunit; prior alkylation with iodoacetamide reduces the stoichiometry to 0.88:1, and increases the rate of labeling. Tryptic digests of enzyme modified with [^{14}C]iodoacetamide or [^{3}H]styrene oxide produce two major peptides which cochromatograph, indicating that styrene oxide and iodoacetamide modify the same cysteine residues. Previous investigators have reported that iodoacetate, iodoacetamide, and butyl isocyanate alkylate either of two reactive cysteines of yeast alcohol dehydrogenase; both cysteines cannot be modified simultaneously [Belke et al. (1974), *Biochemistry 13,* 3418]. The inactivation of enzyme by *p*-chloromercuribenzoate (PCMB) is reported here to be accompanied by the incorporation of 2.3 mol of PCMB/mol of enzyme subunits, in analogy with styrene oxide; the planarity of the alkylating agent appears to be an important factor in determining the stoichiometry of labeling.

The modification of enzymes by chemically reactive substrate analogs can provide insight into the nature of the active site side chains involved in catalysis. Inactivation of yeast alcohol dehydrogenase by iodoacetate and iodoacetamide (Whitehead and Rabin, 1964; Harris, 1964), and butyl isocyanate (Twu and Wold, 1973; Twu et al., 1973) has been studied by previous investigators in some detail. These three reagents are characterized by the following common properties: (i) the kinetics of the inactivation process do not indicate the reversible formation of enzyme–inhibitor complexes prior to inactivation; (ii) the coenzymes, NADH and NAD, protect against inactivation at concentrations consistent with their dissociation constants; (iii) inactivation is accompanied by the incorporation of 1 mol of reagent/mol of enzyme subunit; (iv) inactivation results in the alkylation of either of two reactive cysteines, depending on the reagent and the conditions of inactivation; and (v) both cysteines cannot be alkylated simultaneously, presumably due to overlap in sites.

In an effort to affinity label yeast alcohol dehydrogenase at the substrate binding site, and thereby obtain information concerning the side chain, pK = 8.25, which has been implicated in acid–base catalysis of the hydride transfer step (Klinman, 1975), a study of the inactivation of yeast alcohol dehydrogenase by styrene oxide, an analog of the aromatic substrate benzyl alcohol (Scheme I), was undertaken. Preliminary experiments indicated that the inactivation of yeast alcohol dehydrogenase by styrene oxide was characterized by saturation kinetics, suggesting the formation of a reversible enzyme–inhibitor complex, and that

Scheme I: Structural Similarity of Benzyl Alcohol (I), Styrene Oxide (II), and *p*-Chloromercuribenzoate (III).



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NADH at a concentration 30 times greater than its dissociation constant did not appreciably depress the rate of this inactivation.

In this paper the properties of the styrene oxide inactivation and alkylation of yeast alcohol dehydrogenase are described. Although the kinetic properties of styrene oxide inactivation are shown to be markedly different from those of iodoacetamide and butyl isocyanate, styrene oxide is reported here to alkylate the same cysteine residues. In contrast to iodoacetamide and butyl isocyanate, however, styrene oxide alkylates both reactive cysteines simultaneously. The data presented here are discussed in terms of a possible role for active center cysteines in the mechanism of yeast alcohol dehydrogenase.

Experimental Procedures

Materials

Yeast alcohol dehydrogenase was obtained from Boehringer as an ammonium sulfate suspension. Trypsin, treated with L-1-tosylamido-2-phenylethyl chloromethyl ketone, was from Worthington. Styrene oxide (Matheson Coleman and Bell) was vacuum distilled prior to its use (bp 73–75° (10 mm)). 1′,2′-Epoxy[1′-$^3H_1$]ethylbenzene was synthesized by New England Nuclear, specific activity 1.8×10^6 cpm/μmol. The radiochemical purity of this material was >99% initially, as ascertained by thin-layer chromatography. Over a period of 10 months, the radiochemical purity declined to 73% of the original. Iodoacetamide was purchased from Eastman. [1-^{14}C]Iodoacetamide, obtained from New England Nuclear, had a specific activity of 1.1×10^7 cpm/μmol. NADH and NAD^+ were purchased from Sigma and ethyl alcohol was from Pharmco. Benzyl alcohol (Fisher) was vacuum distilled (bp 91° (10 mm)). 5,5′-Dithiobis(2-nitrobenzoate) (Nbs_2)[1] was obtained from Sigma and *p*-chloromercuribenzoate (PCMB) from Mann Research Lab. Sephadex G-25 and SP-Sephadex-25 were obtained from Pharmacia.

Methods

Kinetic Studies of Inactivation. Due to the low solubility of styrene oxide in water, the maximum concentration of stock solutions was 20 m*M*. The concentration of stock solutions was determined by the addition of 0.1–0.5 ml of styrene oxide solution to an equal volume of 0.4 *M* sodium thiosulfate, to which is added a drop of phenolphthalein. The resultant production of base is titrated with a standardized solution of approximately 0.1 *N* acetic acid. In a typical inactivation experiment, 0.06 mg of yeast alcohol dehydrogenase was added to a 1-ml solution containing PP_i buffer (40 m*M* PP_i–140 m*M* glycine–5 m*M* KCl (pH 8.5), μ = 0.22) and 2–16 m*M* styrene oxide at 25°. At appropriate time intervals, 5 μl of the reaction mixture was assayed for loss of activity. Assay mixtures contained PP_i buffer, 9 m*M* NAD^+ and 0.8 *M* ethanol. The rate of appearance of NADH was followed at 340 nm on a Cary 118B spectrophotometer temperature thermostated at 25°. Half-times for inactivation, at a given concentration of styrene oxide, were obtained from semilog plots of enzyme activity vs. time interval of inactivation. The data were corrected for small losses in enzyme activity upon prolonged incubation in the absence of styrene oxide.

Preparation of Yeast Alcohol Dehydrogenase Labeled with Tritiated Styrene Oxide. In a typical experiment, 3 mg of YADH was added to a PP_i buffered solution containing 8–12 m*M* 1′,2′-epoxy[1′-$^3H_1$]ethylbenzene. At the appropriate times, 0.1–0.2 ml of this reaction mixture was withdrawn and either added directly to a G-25 medium Sephadex column, 1 × 25 mm, or diluted first with an equal volume of an 0.4 *M* sodium thiosulfate solution, to hydrolyze unreacted epoxide. In general, it was possible to obtain virtually complete separation of the protein peak from a subsequent peak of tritiated styrene oxide. A blank was run in the absence of protein to determine the percentage of counts eluting in the protein peak due to polymers of styrene oxide or other high molecular weight radioactive impurities; this correction amounted to no more than 10% of the total counts in the protein peak. Radioactivity was determined by counting in toluene-based scintillation fluid on a Packard TriCarb liquid scintillation spectrophotometer. Protein concentrations were determined either by the method of Lowry et al. (1951), or by absorbance at 280 nm, ϵ = 1.26 for an 0.1% solution (Hayes and Velick, 1954).

Preparation of Carboxamidomethylated Yeast Alcohol Dehydrogenase. In a typical preparation, 15 mg of enzyme, dissolved in 0.5 ml of PP_i buffer, was added to 0.5 ml of 3 m*M* iodoacetamide in PP_i buffer. The resulting solution was incubated at 25° for approximately 1 hr, or until the total enzyme activity decreased to <1% of the original activity. The derivatized protein was separated from unreacted iodoacetamide by Sephadex G-25 chromatography or by precipitation two times with 70% ammonium sulfate. Treatment of carboxamidomethylated enzyme with tritiated styrene oxide was carried out as described above for native protein.

Titration of Sulfhydryl Groups. The method of Vanaman and Stark (1970) was followed. Titration of cysteine with 5,5′-dithiobis(2-nitrobenzoate) indicated an extinction coefficient, ϵ_{441} 10.5 mM^{-1} cm^{-1}, for the 5-thio-2-nitrobenzoate anion under the conditions of experiments described here. For the experiment illustrated in Figure 3, yeast alcohol dehydrogenase, 1.4 mg/ml, was incubated with 11 m*M* styrene oxide in PP_i buffer at 25°. A control without styrene oxide was carried out in a parallel experiment. At the time points indicated, 0.2 ml of the reaction mixture or control was added to 0.7 ml of a solution containing 8 *M* guanidine hydrochloride, 0.2 *M* Tris hydrochloride, 0.02 *M* EDTA (pH 8.2); following the addition of 0.1 ml of a 10 m*M* Nbs_2 solution to this mixture, absorbance was read at 441 nm against a blank without enzyme.

Inactivation with p-Chloromercuribenzoate. For the experiment illustrated in Figure 5, 3 mg of yeast alcohol dehydrogenase in 1 ml of PP_i buffer was titrated with a 1.3 m*M* solution of *p*-chloromercuribenzoate. The enzyme solution underwent a 9% increase in volume in the course of this titration. The concentration of PCMB solutions was determined as described by Boyer (1954). Enzyme activity was assayed as described earlier in this section.

Tryptic Digestion of Modified Enzyme and Chromatography on SP-Sephadex. In parallel experiments 5 mg of enzyme modified either with ^{14}C-labeled iodoacetamide or tritiated styrene oxide was dissolved in 3 ml of a solution containing 6 *M* guanidine hydrochloride, 0.1 *M* P_i (pH 8.0), and 10 m*M* EDTA. A 10:1 molar excess of dithiothreitol was added, the pH adjusted to 9, and the solution bubbled with N_2. After a 3-hr incubation in the dark, the pH was

[1] Abbreviations used are: Nbs_2, 5,5′-dithiobis(2-nitrobenzoate); PCMB, *p*-chloromercuribenzoate; PP_i buffer, buffer containing 40 m*M* PP_i, 140 m*M* glycine, and 5 m*M* KCl (pH 8.5), μ = 0.22.

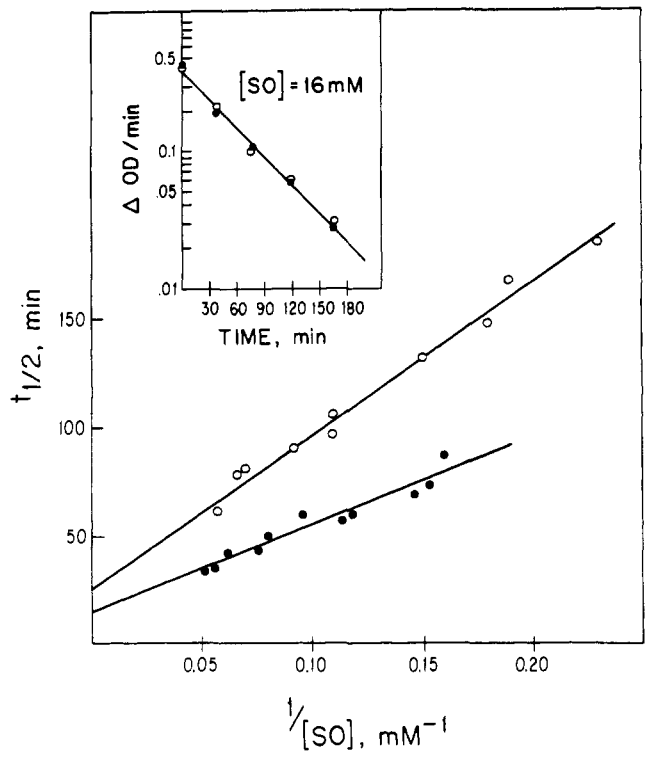


FIGURE 1: Inset: Semilog plot of the rate of inactivation of yeast alcohol dehydrogenase by 16 m*M* styrene oxide (SO). The inactivation was carried out at 25° in the presence of 80 m*M* KP_i (pH 8.0), μ = 0.22. Bottom curve: (●) A plot of the half-time of inactivation ($t_{1/2}$) of yeast alcohol dehydrogenase as a function of the reciprocal of the styrene oxide (SO) concentration at 25°, in the presence of 80 m*M* KP_i (pH 8.0), μ = 0.22. (○) The same experiment as described above with the exception that NADH was present at a concentration of 0.3 m*M*.

readjusted to 8, and a 20:1 molar excess of iodoacetate added. The pH was maintained at 8 for 5 min, followed by the addition of a 10:1 molar excess of 2-mercaptoethanol to iodoacetate. The reaction of 2-mercaptoethanol with iodoacetate was carried out at pH 7.0. Reduced, carboxymethylated samples were then dialyzed against distilled water, followed by 0.5% ammonium bicarbonate.

Trypsin, 0.2 ml of a 2-mg/ml solution, was added to each sample and incubated at 37° for 5 hr. Half of each sample was lyophilized and dissolved in 1.5 ml of 0.05 *M* pyridine acetate buffer (pH 2.5). Samples were mixed at this point and then added to a jacketed SP-Sephadex column, 0.9 × 22 cm, thermostated at 37°, which had been equilibrated with 0.05 *M* pyridine acetate buffer (pH 2.5). A four chamber pyridine acetate gradient was used for the elution: 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). A total volume of 720 ml was run through the column at a flow rate of 30 ml/hr. Fractions, 3 ml each, were monitored for carbon-14 and tritium using settings on a Packard TriCarb scintillation counter such that there was *no* overlap of tritium in the carbon-14 channel; carbon-14 overlapped in the tritium channel by a factor of 2.06. A total of 53,000 cpm of tritiated labeled peptides and 13,000 cpm ^{14}C-labeled peptides were added to the column; 68% of counts in tritium and 62% of counts in carbon-14 were recovered from the column.

Results

Kinetics of Inactivation of Yeast Alcohol Dehydrogenase by Styrene Oxide. The inactivation of yeast alcohol dehydrogenase by excess styrene oxide was observed to be a pseudo-first-order kinetic process up to 93% inactivation (Figure 1, inset); further incubation overnight led to a >98% loss of activity. The effect of varying concentrations of styrene oxide on the half-time of inactivation ($t_{1/2}$) was investigated. As illustrated in Figure 1, $t_{1/2}$ varies linearly with the reciprocal of the styrene oxide concentration; the upper curve in Figure 1 represents the inactivation by styrene oxide in the presence of 0.3 m*M* NADH. Table I summarizes slopes, intercepts, and inhibitor constants, K_I, for curves such as shown in Figure 1 at pH 7.6–9.0. Due to the limited solubility of styrene oxide in water, it was not possible to study the inactivation of enzyme above 20 m*M* styrene oxide. In order to increase the accuracy of intercept and K_I values a large number of data points were obtained for a single double reciprocal plot; correlation coefficients, *r*, for the best lines through these points are also indicated in Table I. The observation of saturation kinetics for the inactivation of yeast alcohol dehydrogenase by styrene oxide indicates that styrene oxide binds to enzyme. The value of K_I = 37 m*M* for styrene oxide at pH 8.5 is similar to the kinetically determined K_d = 27 m*M* for the binding of the substrate benzyl alcohol to free enzyme (Klinman, unpublished results). Although the presence of NADH at a concentration of 0.3 m*M*, $K_d = 1 \times 10^{-5}$ *M* (Dickinson, 1970), brings about a small increase in both slope and intercept values, Figure 1 and Table I, this high level of NADH does not appreciably protect enzyme against inactivation. The effect of an aromatic substrate, benzyl alcohol, on the inactivation parameters is shown in Table I; it can be seen that benzyl alcohol does not protect against inactivation in a strictly competitive manner since both the slope and intercept of double reciprocal plots are changed. From the increase in the apparent K_I, a dissociation constant of 56 m*M* is calculated for benzyl alcohol. In the enzyme-catalyzed reduction of acetaldehyde by NADH at pH 8.5, acetamide is characterized by an inhibition constant of 70 m*M* (J. P. Klinman, unpublished results). The presence of acetamide in the inactivation of enzyme-NADH by styrene oxide affects both the slope and intercept of double reciprocal plots (Table I). From the increase in the apparent K_I of styrene oxide a dissociation constant of 100 m*M* is calculated for the binding of acetamide to enzyme-NADH. It can be seen

Table I: Kinetic Constants for Inactivation of Yeast Alcohol Dehydrogenase.

pH[b]	NADH, 0.3 m*M*	Slope (*min*, m*M*)	Intercept (min)	K_I (m*M*)	n[a]	r[a]
7.6	+	692	33.5	21	9	0.84
8.0	–	393	15.6	25	12	0.96
	–, +$PhCH_2OH$, 45 m*M*	493	11.0	45	12	0.97
	+	695	25	28	12	0.99
	+, +acetamide, 100 m*M*	1030	18.5	56	12	0.95
8.5	–	407	11.1	37	20	0.97
	+	630	11.5	55	26	0.99
9.0	+	406	7.7	53	6	0.99

[a] The constant n refers to the number of data points per double reciprocal plot while r is the correlation coefficient for the best line drawn through these points. [b] The buffers used for these pH studies were: pH 7.6, 88 m*M* KP_i; pH 8.0, 80 m*M* KP_i; pH 8.5, 40 m*M* KPP_i–140 m*M* glycine–5 m*M* KCl; pH 9.0, 30 m*M* KPP_i–110 m*M* glycine; μ = 0.22.

Table II: Labeling of Carboxamidomethylated Yeast Alcohol Dehydrogenase with Tritiated Styrene Oxide, Specific Activity = 1.8×10^6 cpm/μmol.[a]

Time (hr)	Specific Activity of Protein	μmol of Styrene Oxide/μmol of Enzyme
0 63	6.4×10^6	3.5
1.60	6.3×10^6	3.5
3 17	6.4×10^6	3.5

[a] The enzyme was carboxamidomethylated and then incubated with styrene oxide as described in the Experimental Section. At each time point, the specific activity of labeled protein was determined as described after separation of approximately 2.6 nmol of protein from radioactive styrene oxide on Sephadex G-25 columns.

that while benzyl alcohol and acetamide protect yeast alcohol dehydrogenase against inactivation at low styrene oxide concentration, the effect of these reagents is to increase the rate of inactivation at high styrene oxide concentrations.

In Figure 2, the reciprocal of intercepts for inactivation in the presence of coenzyme are plotted as a function of pH. In this figure the solid line represents a theoretical titration curve, pK = 8.5. In a previous publication, the hydride transfer step in the yeast alcohol dehydrogenase reduction of aldehydes and oxidation of alcohols was demonstrated to depend on a single active site side chain, pK = 8.25 (Klinman, 1975). The data in Table I and Figure 2 indicate a pH dependence for the styrene oxide inactivation process which is similar to the pH dependence of the catalytic interconversion step.

Demonstration and Properties of Alkylation of Yeast Alcohol Dehydrogenase by Styrene Oxide. Treatment of yeast alcohol dehydrogenase with tritiated styrene oxide gives rise to radioactively labeled protein. The time course of the incorporation of counts into enzyme at pH 8.5 in the presence of 9 mM styrene oxide (specific activity = 1.8×10^6 cpm/μmol) is illustrated in Figure 3. The incorporation of counts increases to a final value of 4.1×10^4 cpm/2.6 nmol of enzyme, corresponding to the incorporation of 8.8 mol of reagent/mol of enzyme. Yeast alcohol dehydrogenase is a tetramer and has previously been demonstrated to incorporate 1 mol of iodoacetamide or butyl isocyanate per subunit. The data presented here indicate the incorporation of 2.2 mol of styrene oxide/subunit. Although these data indicate the alkylation of approximately two functional groups per subunit of enzyme, the kinetics of alkylation can be fit by a single rate constant which is obtained from the semilog plot illustrated in the inset in Figure 3, $t_{1/2}$ Alk = 43 min. The half-time for inactivation of enzyme at pH 8.5 in the presence of 9 mM styrene oxide is $t_{1/2}$ Inac = 53 min. A test for the loss of sulfhydryl groups concomitant with the introduction of styrene oxide into enzyme was carried out using 5,5′-dithiobis(2-nitrobenzoate). As shown in Figure 3, the time course for the loss of sulfhydryl groups parallels the incorporation of radioactive styrene oxide into enzyme. After 2 hr a total of 7.7 mol of sulfhydryl was lost per mol of enzyme, corresponding to a loss of 1.9 mol/subunit.

Wold and coworkers have reported the presence of two distinct reactive sulfhydryl groups in yeast alcohol dehydrogenase. Although either sulfhydryl group can be preferentially alkylated by iodoacetate, iodoacetamide, or butyl isocyanate, they report that enzyme does not become doubly labeled with these reagents (Twu et al., 1973; Belke et al., 1974). To obtain information regarding the relationship between the sulfhydryl group alkylated by iodoacetamide and the two sulfhydryls modified by styrene oxide, yeast alcohol dehydrogenase was carboxamidomethylated with iodoacetamide prior to incubation with tritiated styrene oxide. The results of such an experiment are summarized in Table II. It can be seen that pretreatment with iodoacetamide reduces the extent of incorporation and increases the rate of incorporation of radioactive styrene oxide into enzyme. The data indicate that carboxamidomethylated enzyme incorporates styrene oxide at a stoichiometry of 3.5 mol/mol of enzyme or 0.88 mol/mol of enzyme subunits.

In order to determine whether the same amino acid side chains are modified by iodoacetamide and styrene oxide, yeast alcohol dehydrogenase was inactivated with [^{14}C]iodoacetamide and [^{3}H]styrene oxide in parallel experiments. Labeled protein was subjected to trypsin digestion as described in the Experimental Section, mixed, and chromato-

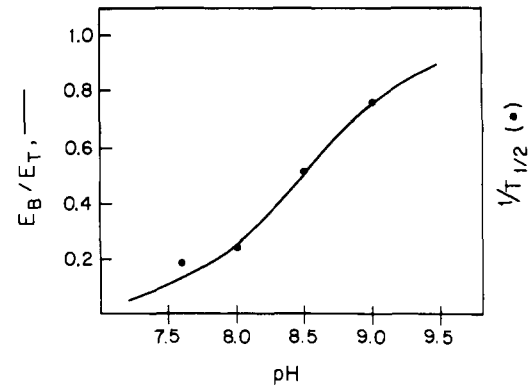


FIGURE 2: The pH dependence of the reciprocal of intercepts for enzyme inactivation in the presence of NADH ($1/T_{1/2}$) (Table I). Values for $1/T_{1/2}$ at pH 7.6, 8.1, and 8.5 are relative to the value of $1/T_{1/2}$ at pH 9.0. The solid line represents a theoretical titration curve, pK = 8.5, where E_B/E_T refers to the fraction of enzyme in the free base form.

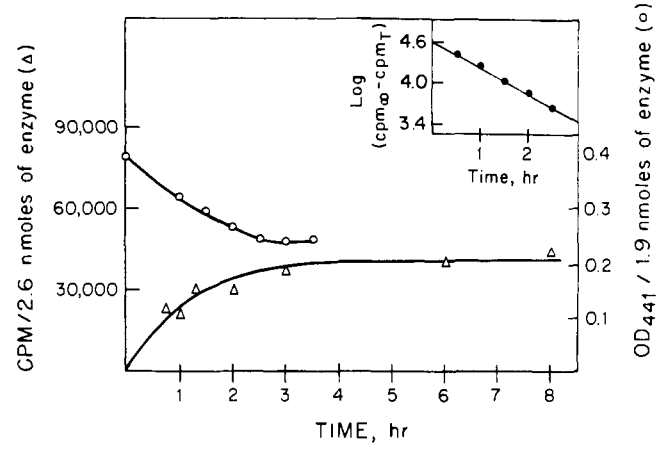


FIGURE 3: (Δ) Time course for the incorporation of tritiated styrene oxide (specific activity = 1.8×10^6) into yeast alcohol dehydrogenase at 25°, pH 8.5, μ = 0.22. Buffer contained 40 mM KPP_i, 140 mM glycine, and 5 mM KCl. (O) Time course for loss of free sulfhydryl groups upon reaction of yeast alcohol dehydrogenase with styrene oxide under the conditions described above. The concentration of sulfhydryl groups was determined with 5,5′-dithiobis(2-nitrobenzoate) where the extinction coefficient for the 5-thio-2-nitrobenzoate anion was determined to be ϵ_{441} 10.5 mM^{-1} cm^{-1}. For further details concerning the experiments illustrated in these curves, see the Experimental Section. The inset represents a semilog plot of the rate of incorporation of tritiated styrene oxide into enzyme.

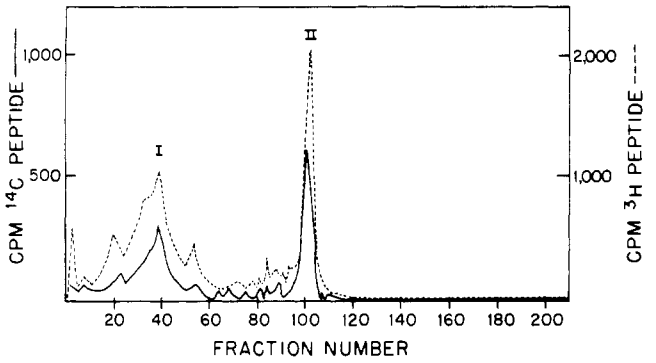


FIGURE 4: Elution pattern of tryptic peptides, derived from yeast alcohol dehydrogenase modified with ^{14}C-labeled iodoacetamide (—) or tritium-labeled styrene oxide (- - -) from an SP-Sephadex column. This experiment is described in detail in the Experimental Section.

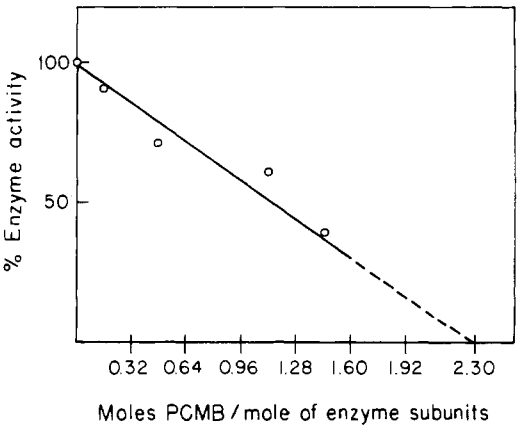


FIGURE 5: Titration of yeast alcohol with *p*-chloromercuribenzoate. Experimental details are contained in the Experimental Section.

graphed on an SP-Sephadex-25 column. In Figure 4 the elution pattern of radioactive peptides is illustrated. Inactivation with either iodoacetamide or styrene oxide gives rise to two major labeled peptides which cochromatograph. On the basis of the data in Figure 4, it is concluded that styrene oxide and iodoacetamide modify the same cysteine residues.

Treatment of Yeast Alcohol Dehydrogenase with p-Chloromercuribenzoate. The stoichiometry of labeling of yeast alcohol dehydrogenase by *p*-chloromercuribenzoate was determined. As illustrated in Figure 5, enzyme has incorporated 1.1 mol of PCMB/mol of enzyme subunits after a 50% loss of enzyme activity. Extrapolation to zero enzyme activity indicates the incorporation of 2.3 mol of PCMB/mol of enzyme subunits.

Discussion

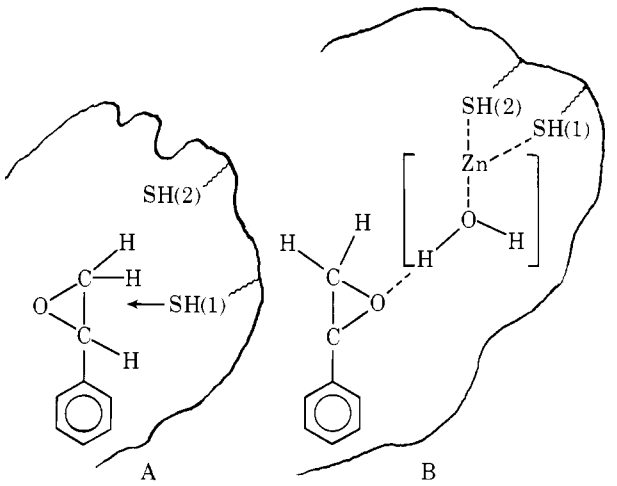
The data presented in this paper indicate that the styrene oxide inactivation of yeast alcohol dehydrogenase involves the alkylation of two cysteine residues/subunit of enzyme (Figure 3). The inactivation process has been shown to be a single exponential process up to 93% inactivation (Figure 1, inset). The $t_{1/2}$ = 53 min for enzyme inactivation in the presence of 9 m*M* styrene oxide (pH 8.5) is close to $t_{1/2}$ = 43 min for enzyme alkylation under the same conditions (Figure 3). The inactivation of yeast alcohol dehydrogenase by iodoacetamide, iodoacetate, and butyl isocyanate has been shown by previous investigators to involve the alkylation of either of two cysteines (Belke et al., 1974); the same cysteines appear to be modified by styrene oxide and iodoacetamide (Figure 4). If both cysteine residues were characterized by similar intrinsic reactivities toward styrene oxide and were alkylated independent of one another, one would expect to lose approximately 75% enzyme activity at 50% alkylation; in the event that the alkylation of both cysteine residues by styrene oxide were required for enzyme inactivation 50% alkylation would result in a 25% loss of activity. The observed close similarity of rate constants for inactivation and alkylation suggests that the modification of the first cysteine residue is accompanied by the rapid alkylation of a second residue. Cooperativity in the alkylation process is also suggested by the observation that pretreatment of yeast alcohol dehydrogenase with iodoacetamide activates enzyme toward alkylation with styrene oxide. At the earliest time point studied, 0.63 hr, the incorporation of tritiated styrene oxide into iodoacetamide inactivated enzyme was complete.

The inability to label both cysteines simultaneously with iodoacetamide suggests steric hindrance between cysteine residues in the case of aliphatic alkylating reagents. The close structural similarity of *p*-chloromercuribenzoate to styrene oxide (Scheme I) led to a determination of the stoichiometry of the PCMB reaction with yeast alcohol dehydrogenase. As indicated in Figure 5, at 100% loss of enzyme activity, approximately 2 mol of PCMB are incorporated per mol of subunit. It is of interest that Heitz and Anderson (1968) found the same stoichiometry of 2 per site in the titration of yeast alcohol dehydrogenase by fluorescein mercuric acetate. Although considerably larger than PCMB (Karush et al., 1964), fluorescein mercuric acetate is a planar molecule. Thus, the planarity of the alkylating reagent appears to be an important factor in determining the stoichiometry of labeling.

It is also reported in this paper that styrene oxide inactivation is characterized by saturation kinetics, indicating the formation of a reversible enzyme–inhibitor complex. At pH 8.0, the presence of NADH (0.3 m*M*, $K_d \simeq 1 \times 10^{-5}$ *M*) has little effect on the apparent K_I, while benzyl alcohol (45 m*M*, $K_d \simeq 27$ m*M*) and acetamide (100 m*M*, $K_d \simeq 70$ m*M*) increase K_I. Although the kinetic mechanism for the yeast alcohol dehydrogenase catalyzed interconversion of acetaldehyde and ethanol is preferred ordered with coenzyme binding first, the binding of aromatic substrates to free enzyme has been shown to be kinetically significant (Klinman, 1972; and unpublished results). Thus, the inactivation of enzyme by styrene oxide in the absence of coenzyme, together with the differential effects of NADH and benzyl alcohol and acetamide on the apparent K_I for styrene oxide, are consistent with a role for styrene oxide at the substrate site. It is necessary to explain, however, why benzyl alcohol and acetamide decrease the half-time for inactivation at infinite styrene oxide concentration (Table I).

As Baker (1967) has discussed, the observation of saturation kinetics in the inactivation of an enzyme by a substrate analog could be the result of either the formation of an enzyme–inhibitor complex which leads to inactivation, or the binding of inhibitor to enzyme where binding protects against a second-order inactivation of free enzyme by inhibitor in solution. It is possible to propose two mechanisms for the yeast alcohol dehydrogenase inactivation by styrene oxide which are consistent with the data presented in this paper. According to Scheme IIA, styrene oxide alkylates a cysteine residue, -SH(1), when bound to enzyme at or near the substrate site via a first-order kinetic process. Alternatively another cysteine residue, -SH(2), can be alkylated in a second-order process, where the alkylation of either

Scheme II: Alternative Modes for Styrene Oxide Binding[a]



[a] In A, binding leads to inactivation; in B, binding protects against inactivation.

-SH(1) or -SH(2) leads to inactivation and the rapid alkylation of the second cysteine residue. The observed rate constant for such a process would be described by

$$k_{obsd} = k_1/(1 + K_I/I) + k_2I \quad (1)$$

where k_1 is the rate constant for a first-order inactivation and k_2 is the rate constant for a second-order inactivation. Although eq 1 does not describe a straight line at all inhibitor concentrations, for the range of styrene oxide concentrations studied curvature would not be observed when $k_1 > k_2$. In the presence of benzyl alcohol or acetamide, the apparent K_I for styrene oxide would increase, decreasing the contribution of the k_1 term to k_{obsd} and the intercept of plots of half-times of inactivation vs. the reciprocal of styrene oxide concentrations.

An alternative mechanism is illustrated in Scheme IIB and involves the binding of styrene oxide to an active site residue where this binding protects both reactive cysteine residues from alkylation. Although the actual spatial relationship between the binding site for styrene oxide and cysteines cannot be determined from the data reported here, Eklund et al. (1974) have recently described the crystal structure of liver alcohol dehydrogenase at 2.4 Å; their results indicate two cysteines as ligands for an active site Zn atom, which is also liganded to water or hydroxide ion. A possible role for a $Zn-H_2O$ is indicated in brackets in Scheme IIB. The rate equation for a mechanism of inactivation involving protective binding by styrene oxide is given by

$$k_{obsd} = k_2K_I/(1 + K_I/I) \quad (2)$$

where k_2 is the rate constant for a second-order inactivation. According to eq 2, substrate binding will result in an increase in slope and a decrease in intercept of plots of half-times of inactivation vs. the reciprocal of styrene oxide concentrations under conditions where substrate gives partial protection against a second order inactivation process.[2]

[2] In the presence of substrate, under conditions where substrate gives complete protection against inactivation, the slope of plots of $t_{1/2}$ for inactivation vs. the reciprocal of styrene oxide concentration is $(1 + S/K_S)/k_2$; the intercept of such plots is unchanged and is equal to $1/k_2K_I$. If substrate does not protect at all against inactivation, the intercept of plots of $t_{1/2}$ vs. the reciprocal of styrene oxide concentration is $1/k_2K_I(1 + S/K_S)$; the slope is unchanged and is equal to $1/k_2$. Thus, under conditions of partial protection by substrate, both an increase in slope and a decrease in intercept are predicted to occur.

In attempting to distinguish between IIA and -B, it is of great value to compare the properties of styrene oxide and iodoacetamide inactivation. Although iodoacetamide and styrene oxide alkylate the same cysteine residues, iodoacetamide rapidly inactivates enzyme under conditions where there is no evidence for complex formation of iodoacetamide with enzyme prior to alkylation (Whitehead and Rabin, 1964). Furthermore, Whitehead and Rabin have demonstrated that the rate of inactivation of yeast alcohol dehydrogenase by iodoacetamide is independent of pH between pH 5 and 10 in contrast to the pH dependency of styrene oxide inactivation. In light of the kinetic differences between iodoacetamide and styrene oxide inactivation, it is suggested that styrene oxide binds to yeast alcohol dehydrogenase in a pH dependent process, $pK \simeq 8.5$, and that this binding protects the enzyme against a second-order, pH independent alkylation by styrene oxide. Reuben and Bruice (1974) have reported rate constants at 30° for the addition of mercaptides to benzene oxide, where $k_{RS^-} = 0.055$–$0.217\ M^{-1}\ sec^{-1}$. The rate constant for the attack of a cysteine side chain of yeast alcohol dehydrogenase on styrene oxide can be obtained from the data reported here. For Scheme IIA, the rate constant is obtained from the intercept of double reciprocal plots, Table I and eq 1, $k = 0.0010\ sec^{-1}$ at pH 8.5. For Scheme IIB the rate constant is obtained from the slope of double reciprocal plots, Table I and eq 2, $k = 0.042\ M^{-1}\ sec^{-1}$ at pH 8.5; the similarity of this rate constant to values reported by Reuben and Bruice also suggests that Scheme IIB is operative. Additional studies on the inactivation of alcohol dehydrogenases by epoxides are in progress; these studies should elucidate further the mode of interaction of styrene oxide with yeast alcohol dehydrogenase, and the possible role of an active site $Zn-H_2O$ vs. cysteine in the catalytic mechanism.

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References

Baker, B. R. (1967), Design of Active-Site Directed Irreversible Enzyme Inhibitors, New York, N.Y., Academic Press.

Belke, C. J., Chin, C. C. Q., and Wold, F. (1974), *Biochemistry 13,* 3418.

Boyer, P. D. (1954), *J. Am. Chem. Soc. 76,* 4331

Dickinson, F. M. (1970), *Biochem. J. 90,* 821.

Eklund, H., Nordstrom, B., Zeppezauer, E., Soderland, G., Ohlsson, I., Bouve, J., and Brändén, C. (1974), *FEBS Lett. 44,* 200.

Harris, I. (1964), *Nature (London) 203,* 30.

Hayes, J. E., and Velick, S. F. (1954), *J. Biol. Chem. 207,* 225.

Heitz, J. R., and Anderson, B. M. (1968), *Arch. Biochem. Biophys. 127,* 637.

Karush, F., Klinman, N. R., and Marks, R. (1964), *Anal. Biochem. 9,* 100.

Klinman, J. P. (1972), *J. Biol. Chem. 247,* 7977.

Klinman, J. P. (1975), *J. Biol. Chem. 250,* 2569.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall,

R. J. (1951), *J. Biol. Chem. 193,* 265.
Reuben, D. M. E., and Bruice, T. C. (1974), *J. Chem. Soc., Chem. Commun. 113.*
Twu, J., Chin, C. C. Q., and Wold, F. (1973), *Biochemistry 12,* 2865.
Twu, J., and Wold, F. (1973), *Biochemistry 12,* 381.
Vanaman, T. C., and Stark, G. R. (1970), *J. Biol. Chem. 245,* 3565.
Whitehead, E. P., and Rabin, B. R. (1964), *Biochem. J. 90,* 532.

The Two-Step Reversible Denaturation of Lactate Dehydrogenase at Low pH†

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ABSTRACT: Upon exposure to conditions of low pH, beef B_4 lactate dehydrogenase rapidly loses enzymatic activity, but this process can be completely reversed yielding 100% of the original activity if the enzyme is immediately returned to neutral conditions. As the time of exposure to low pH is increased, the fraction of activity recovered declines to a value of 50–60% and remains nearly constant over a period of many hours. Correlated with this behavior is a change in the kinetics of the recovery of activity. Recovery of activity has been shown to be a second-order process for enzyme exposed to low pH for brief periods of time (Anderson, S., and Weber, G. (1966), *Arch. Biochem. Biophys. 116,* 207). After several minutes at low pH recovery of activity is found to become first order and to occur at a considerably slower rate. Gel filtration chromatography at low pH separates the protein into two fractions. The lower molecular weight fraction is found to be primarily monomeric, as indicated by equilibrium ultracentrifugation, and is capable of recovering enzymatic activity. The higher molecular weight fraction is generated from the lower molecular weight fraction, and is incapable of recovering activity. These results are interpreted to indicate that the enzyme exists sequentially in three denatured forms at low pH, the first two capable of being restored to the native state, and the third irreversibly denatured.

Lactate dehydrogenase is a tetrameric protein of molecular weight equal to about 140,000 (Appella and Markert, 1961; Jaenicke and Knof, 1968; Adams et al., 1970). It appears to undergo reversible dissociation to subunits under a variety of conditions, as indicated by the technique of isozyme hybridization (Markert, 1963; see reviews by Jaenicke, 1970; Everse and Kaplan, 1973). In general, dissociation is accompanied by irreversible denaturation of the protein, behavior which has complicated the interpretation of direct physicochemical data on the dissociated state. A number of reports have been published indicating that fully reversible dissociation of lactate dehydrogenase may be induced by exposure of the enzyme to low pH (Anderson and Weber, 1966; Levitzki, 1972; Levitzki and Tenenbaum, 1975). Loss of catalytic activity at low pH may be completely reversed upon neutralization; and, if two isozymes are exposed together to such treatment, the formation of hybrid isozymes results. Recovery of activity was found to follow second-order kinetics in the case of beef A_4[1] and B_4 lactate dehydrogenase (Anderson and Weber, 1966; Levitzki and Tenenbaum 1975; S. Anderson, personal communication), further evidence that these isozymes become dissociated at low pH. For all isozymes examined, full recovery of the native condition may be obtained only if exposure of the enzyme to acidic conditions is of very short duration, exposure for longer than several minutes resulting in irreversible loss of activity. This suggests that the dissociated state is unstable and indicates that interpretation of physicochemical data must still be approached with caution.

Direct characterization of the enzyme at low pH has shown the molecular weight to be lower than that of the native tetramer (Deal et al., 1963; Jaenicke and Knof, 1968; Anderson and Weber, 1966; Millar et al., 1969). Millar and coworkers using enzyme prepared from beef heart presented evidence from equilibrium ultracentrifugation indicating the existence of a number of different molecular weight species at low pH in rapidly reversible equilibrium. The minimum molecular weight observed was approximately 18,000, about half the previously reported monomer molecular weight (Appella and Markert, 1961). The centrifugation data were found to be consistent with a scheme involving the reversible association of the 18,000 species to polymeric forms of a size as large as the native tetramer. In contrast to the full recovery of activity obtained with enzyme briefly exposed to low pH, enzyme used in this study was found to be incapable of recovering activity. This has suggested to us that two different denatured forms of the ezyme may have been under investigation in the different studies, a short-lived form capable of complete renaturation, and an irreversibly denatured form present after the

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[1] Abbreviations used are: LDH, lactate dehydrogenase; A_4 and B_4 LDH refer to the homopolymeric isozymes, composed of four A or four B subunits, and are equivalent to M_4 and H_4 LDH, respectively.