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Ambivalent Active-Site-Directed Inactivators of Liver Alcohol Dehydrogenase†

Wen-Sherng Chen‡ and Bryce V. Plapp*,§

ABSTRACT: A series of ω-(bromoacetamido) fatty acids [$BrCH_2CONH(CH_2)_nCOOH$] with n from 6 to 11, 4-(*p*-bromoacetamidophenyl)butyric acid and its amide, 4-(*p*-bromoacetamidophenyl)benzoic acid, and 4-(*p*-bromoacetamidophenoxy)benzoic acid were synthesized and evaluated as active-site-directed inactivators of horse liver alcohol dehydrogenase. These reagents were designed from a knowledge of the structure of the apoenzyme determined by X-ray crystallography so that their carboxylate or carboxamide groups could bind to the catalytic zinc ion in the enzyme–coenzyme complex while their bromoacetamido groups alkylated Met-306 14 Å away in the substrate-binding pocket, inactivating the enzyme by anchoring an inhibitor in the active site. In the absence of coenzyme, the carboxylate reagents could bind to the anion-binding site formed by Arg-47 and Lys-228 and alkylate Cys-46 or -174, ligands to the zinc. In the absence of nucleotides, all of the reagents inactivated the dehydrogenase as fast or faster than bromoacetic acid did (8.2 M^{-1} min^{-1} at pH 8, 25 °C). AMP, NAD^+, and NADH generally protected against inactivation, but 8-(bromoacetamido)octanoic acid and 4-(*p*-bromoacetamidophenyl)butyric acid inactivated as fast or faster in the presence of 1 mM NAD^+ as in the absence of nucleotide. In the presence of 0.2 mM NADH, 4-(*p*-bromoacetamidophenyl)butyramide inactivated with a pseudo bimolecular rate constant (400 M^{-1} min^{-1}) that was eight times larger than the rate constant in the absence of nucleotides and 67 000 times larger than the bimolecular rate constant for inactivation by bromoacetamide in the presence of NADH (0.006 M^{-1} min^{-1}). All of the reagents exhibited saturation kinetics, and the presence of coenzyme increased the binding of reagent by up to twofold. Amino acid analyses showed that enzyme inactivated by 4-(*p*-bromoacetamidophenyl)butyric acid or its amide in the absence of coenzyme contained one or more modified cysteine residues per subunit, whereas inactivation in the presence of NAD^+ or NADH modified about one methionine but no cysteine. These reagents react more specifically than bromoacetic acid (in the presence of NAD^+) or bromoacetamide (in the presence of NADH), which modify several cysteines and methionines during inactivation. It appears that 4-(*p*-bromoacetamidophenyl)butyric acid and its amide are ambivalent active-site-directed inactivators whose reaction specificity is determined by the coenzyme, as predicted above. Furthermore, it appears that the distance between the catalytic zinc and the sulfur of Met-306 is about 14 Å in both the apoenzyme and the enzyme–coenzyme complexes.

A variety of active-site-directed reagents have been used to investigate the structures and mechanisms of enzymes (Shaw, 1970; Jakoby & Wilchek, 1977). Baker (1967) has proposed that such reagents can be designed for chemotherapeutic purposes. Specific inactivators of liver alcohol dehydrogenase might be used to inhibit alcohol metabolism, for instance in treatment of methanol intoxication (Plapp, 1975). This enzyme can be inactivated by a variety of chemical reagents (Brändén et al., 1975). For instance, the sulfur atom of Cys-46 (a ligand to the zinc in the active site) reacts with iodoacetate (Harris, 1964; Li & Vallee, 1964; Jörnvall, 1970), which apparently is attracted to the active site by interaction with the guanidinium group of Arg-47 (Zeppezauer et al., 1975; Brändén et al., 1975). The ionic interaction facilitates the rate of reaction about tenfold, as evidenced by the slower reaction after arginine residues are modified (Lange et al., 1975) or when bromoacetamide is substituted for bromoacetate (Fries et al., 1975). It should also be noted that the rate of inactivation by haloacetates or haloacetamides is decreased about 100-fold by NAD^+ [1] or NADH (Fries et al., 1975; Li & Vallee, 1963; Reynolds et al., 1970), whose pyrophosphate groups apparently bind to Arg-47 and block access of the reagent to Cys-46 (Zeppezauer et al., 1975; Lange et al., 1975; Brändén et al., 1975). The anionic reagent diazonium-1*H*-

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[1] Abbreviations used: NAD^+, nicotinamide adenine dinucleotide; NADH, reduced NAD; EDTA, (ethylenedinitrilo)tetraacetic acid.

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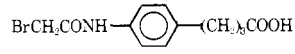
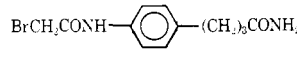
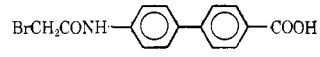
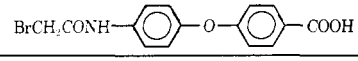
TABLE I: Synthesis and Characterization of Bromoacetamido Reagents.[a]

compd	structure	method	yield (%)	mp (°C)	R_f	recrystn solvent
1	$BrCH_2CONH(CH_2)_6COOH$	A	11	88-90	0.52	$CHCl_3$-petr ether
2	$BrCH_2CONH(CH_2)_7COOH$	B	4	64-66	0.61	$CHCl_3$-petr ether
3	$BrCH_2CONH(CH_2)_8COOH$	B	2	72-74	0.65	$CHCl_3$-petr ether
4	$BrCH_2CONH(CH_2)_{10}COOH$	C	10	83-85	0.69	$CHCl_3$-petr ether
5	$BrCH_2CONH(CH_2)_{11}COOH$	C	7	86-87	0.71	$CHCl_3$-petr ether
6[b]		C	60	133-135	0.51	THF-petr ether
7		D	31	150-152	0.48	CH_3OH-$CHCl_3$-petr ether
8[c]		C	45	278 (dec)	0.45	THF-petr ether
9[d]		C	75	225-227	0.47	THF-petr ether

[a] Melting points were determined on a Fisher-Johns apparatus and are not corrected. Thin-layer chromatography (TLC) was performed on Eastman silica gel chromatogram sheets in $CHCl_3$-CH_3OH-CH_3COOH (540:60:1) in which the R_f value of $BrCH_2COOH$ was 0.1. To detect the alkylating activity of the bromoacetamido function, a spray procedure was adapted from Epstein et al. (1955) and consisted of successive sprayings with (a) 0.05 M potassium biphthalate, (b) 2% 4-(*p*-nitrobenzyl)pyridine in acetone, and (c) 0.5 M NaOH, with drying by hot air for several minutes between each step. Alkylators showed up as purple to blue spots. Amino acids could be detected by a ninhydrin spray. Elemental analyses for C, H, and N were performed by Galbraith Laboratories, Knoxville, Tenn., and the Microanalytical Service, Department of Chemistry, University of Iowa, and were within ±0.4% of the theoretical values. [b] UV (H_2O) λ_{max} 252 nm (ϵ 9800; ϵ_{260} 9000; ϵ_{280} 4400). [c] UV (H_2O) λ_{max} 288 nm (ϵ 28 000; ϵ_{260} 12 800; ϵ_{280} 26 000). [d] UV (H_2O) λ_{max} 256 nm (ϵ 22 300; ϵ_{260} 21 800; ϵ_{280} = 11 300).

tetrazole inactivates the enzyme by modifying the sulfur of Cys-174 (Sogin & Plapp, 1976), which is also ligated to the catalytic zinc ion (Eklund et al., 1976).

Based on a knowledge of the three-dimensional structure of the coenzyme-free apoenzyme determined to a resolution of 2.4 Å (Brändén et al., 1975; Eklund et al., 1976), we have designed, prepared, and evaluated some reagents that should bind to and react with the enzyme-coenzyme complexes. The reagents contained a bromoacetamido function on the ω end of a fatty acid (or fatty acid amide) moiety, which could bind to the enzyme-NAD^+ (or enzyme-NADH) complex. The affinity moieties were chosen because fatty acids bind tightly to the enzyme-NAD^+ complex and are competitive inhibitors against ethanol, and fatty acid amides bind to the enzyme-NADH complex and are competitive against acetaldehyde (Winer & Theorell, 1960). It has been assumed for some years that the zinc ion in the active site binds the functional groups of these inhibitors and substrates (Theorell & McKinley-McKee, 1961). Direct evidence for the binding of the oxygen of a substrate has been recently obtained (Plapp et al., 1978). The reagents we made were about 14 Å long so that the molecule could bridge from the zinc and alkylate the sulfur of Met-306, which is furnished by the adjoining subunit of the dimeric enzyme and which forms part of the substrate-binding pocket (Brändén et al., 1975). Thus, in the presence of coenzyme we expected the reagents to react with Met-306 by exoalkylation (Baker, 1967), but in the absence of coenzyme we expected endoalkylation of Cys-46 or -174, since the reagents could bind in alternative ways, for instance with a carboxylate interacting with the anion-binding site furnished by the positively charged groups of Arg-47 or Lys-228, as in the inactivation by iodoacetate. The "ambivalence" of these reagents should be resolved, therefore, by the directing effect of the coenzyme. The purpose of this investigation was to test these predictions about the structure and reactivity of alcohol dehydrogenase as a step toward developing a better understanding of how enzymes interact with small molecules in the presence and absence of coenzymes.

Experimental Procedure

Chemistry. Most ω-amino fatty acids and other reagents were commercially available. 7-Aminoheptanoic acid and 9-aminononanoic acid were prepared by hydrolysis of 2-azacyclooctanone and 9-aminononanenitrile, respectively, in 6 M HCl at 110 °C for 1 day and recrystallized as the zwitterions from CH_3OH.

4-(*p*-Nitrophenoxy)benzoic acid was prepared by oxidizing 4-(*p*-nitrophenoxy)benzaldehyde (18.5 mmol) with Ag_2O (18.5 mmol) in 50 mL of H_2O containing 37 mmol of NaOH for 2 h at 4 °C with stirring. The black solid was removed by filtration and washed with several portions of H_2O. The combined filtrates and washings were acidified with concentrated HCl to pH 3.0, precipitating 2.4 g of 4-(*p*-nitrophenoxy)benzoic acid. Concentration of the mother liquors gave another 1.2 g of acid, for a total yield of 75%. The product was recrystallized from boiling absolute ethanol, mp 241-243 °C.

4-(*p*-Aminophenyl)butyramide was prepared by a series of reactions from 4-(*p*-nitrophenyl)butyric acid (46.8 mmol), which was heated under reflux with 7 mL (an excess) of $SOCl_2$ and 5 drops of PCl_3 in 150 mL of $CHCl_3$ for 22 h and concentrated to yield 10.2 g (96%) of crude liquid product. NH_3 was bubbled into a solution of 4-(*p*-nitrophenyl)butyryl chloride (40 mmol) in 150 mL of $CHCl_3$ at −10 °C with vigorous stirring for 1.5 h or until the product ceased to precipitate. The precipitate was filtered, washed with H_2O, and recrystallized from acetone-petroleum ether (1:3) in 94% yield, mp 136-138 °C. The solution of 4-(*p*-nitrophenyl)butyramide (34.5 mmol) in 140 mL of CH_3OH was hydrogenated at 45 psi of H_2 and 25 °C over 5% palladium on carbon (1 g) for 30 min. The filtrate was evaporated to dryness, and the residue was recrystallized from CH_3OH-Et_2O in 85% yield, mp 121-123 °C.

4-(*p*-Aminophenyl)butyric acid (recrystallized from boiling H_2O, mp 127-128 °C), 4-(*p*-aminophenyl)benzoic acid (recrystallized from DMF-H_2O, mp 245-247 °C), and 4-(*p*-aminophenoxy)benzoic acid (recrystallized from DMF-H_2O, mp 194-196 °C) were obtained by hydrogenation (H_2, Pd/C) of the corresponding *p*-nitro compounds, adding triethylamine if necessary to obtain salts soluble in CH_3OH.

The bromoacetamido compounds were synthesized by the following methods and are characterized in Table I.

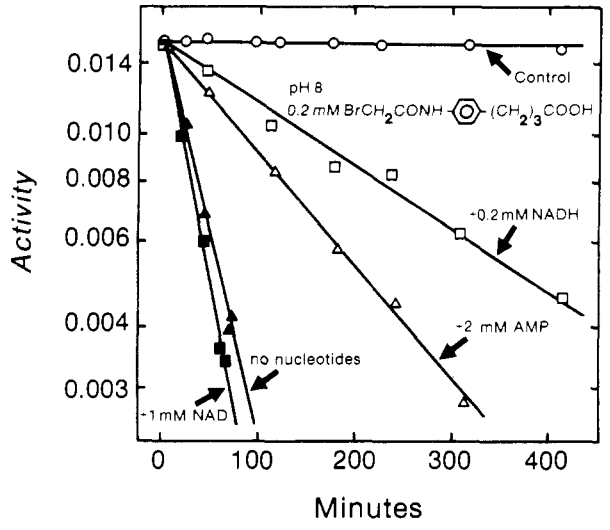


FIGURE 1: Effect of nucleotides on the inactivation of liver alcohol dehydrogenase by 4-(*p*-bromoacetamidophenyl)butyric acid.

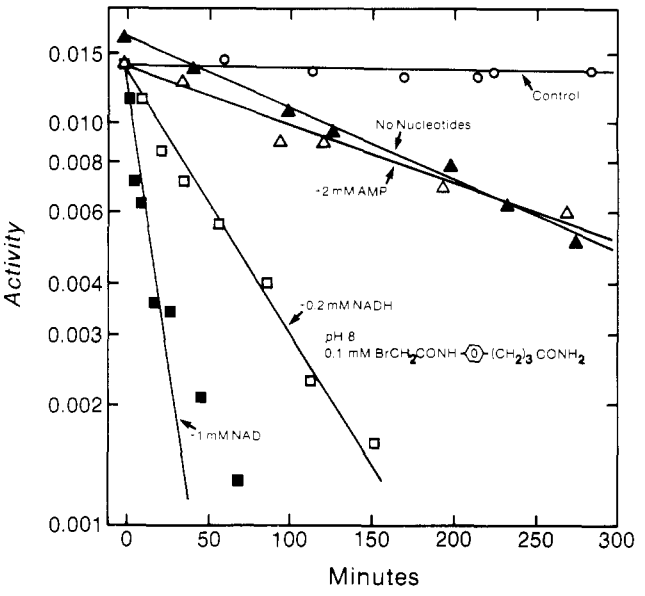


FIGURE 2: Inactivation of liver alcohol dehydrogenase by 4-(*p*-bromoacetamidophenyl)butyramide.

Method A. 7-(Bromoacetamido)heptanoic Acid (**1**). To a suspension of 7-aminoheptanoic acid (3.5 mmol) in 10 mL of dry tetrahydrofuran (THF) was added $BrCH_2COBr$ (3.5 mmol) with stirring at 25 °C. The suspension was stirred for 24 h and the remaining solid was removed by filtration. THF in the filtrate was evaporated, and the residue was dissolved in 1 mL of H_2O to convert any acyl halide into carboxylic acid and dehydrated by the addition of 19 mL of absolute ethanol and azeotropic distillation. The residue was purified on successive silica gel columns developed with $CHCl_3$–CH_3OH–CH_3COOH (450:50:1) and Et_2O–petroleum ether–CH_3COOH (450:50:1). The reactive product was recrystallized from $CHCl_3$–petroleum ether (1:2).

Method B. 8-(Bromoacetamido)octanoic Acid (**2**). To a solution of 8-aminocaprylic acid (10 mmol) in 20 mL of H_2O and $NaHCO_3$ (20 mmol) at 1–3 °C was added dropwise, with stirring, over a 20-min period $BrCH_2COBr$ (10 mmol). The reaction mixture was stirred for another 30 min, adjusted to pH 2.7 with HBr, and extracted with three 20-mL portions of Et_2O. The Et_2O was evaporated, and the residue was recrystallized from $CHCl_3$–petroleum ether (1:2).

Method C. 4-(p-Bromoacetamidophenyl)butyric Acid (**6**). A solution of 4-(*p*-aminophenyl)butyric acid (5.6 mmol) in 2 mL of $BrCH_2COBr$ was stirred at 25 °C for 24 h and poured onto 10 g of ice. The white precipitate was collected by filtration, washed with several 10-mL portions of H_2O, dried, and recrystallized from THF–petroleum ether (1:1).

Method D. 4-(p-Bromoacetamidophenyl)butyramide (**7**). A suspension of 5.6 mmol of 4-(*p*-aminophenyl)butyramide and 5.6 mmol of *N*-(bromoacetoxy)succinimide (Wilchek & Givol, 1977) in 20 mL of THF was stirred at 25 °C for 20 h. The filtrate from the suspension was evaporated to dryness, and the residue was washed with H_2O to remove *N*-hydroxysuccinimide. The product was applied to a column (0.9 × 45 cm) of silica gel (60–200 mesh), which was developed with $CHCl_3$–CH_3OH–CH_3COOH (450:50:1). The desired product (elution volume 86–98 mL, reactive with nitrobenzylpyridine spray) was collected, evaporated to dryness, and recrystallized from CH_3OH–$CHCl_3$–petroleum ether (1:3:5).

Enzymology. Horse liver alcohol dehydrogenase (EE or ES isozymes) was prepared by the method of Dworschack & Plapp (1977). Before each experiment the crystalline enzyme was dissolved in 33 mM sodium phosphate buffer (pH 8.2) containing 0.5 mM EDTA, and dialyzed in washed dialysis tubing against 33 mM sodium phosphate buffer (pH 8.0) containing 0.5 mM EDTA, for 1 day at 5 °C. This procedure removed most of the ethanol used to crystallize the enzyme. The remaining NAD^+ reductant was estimated by diluting the enzyme to 1 mg/mL in the pH 8 buffer and making the solution 1 mM in NAD^+. From the absorbance at 328 nm, produced by incubation at 25 °C for about 3 h, and the molar absorbance for enzyme-bound NADH (5.7 mM^{-1} cm^{-1}), it was estimated that various preparations of the enzyme could form about 0.5 equiv of NADH per subunit of enzyme. For evaluation of various inactivators, enzyme was diluted to 0.2 mg/mL with 0.1 M $Na_4P_2O_7$ buffer adjusted to pH 8.0 with H_3PO_4. If inactivation of enzyme in the presence of saturating concentrations of nucleotides (2 mM AMP, 1 mM NAD^+, 0.2 mM NADH) was studied, the nucleotides were added 1 min before the inactivator; under these conditions, NAD^+ was apparently not reduced to NADH, since the inactivators inhibited the NAD^+ reduction. Rates of inactivation of enzyme at 25 °C were measured by the addition of 10-μL aliquots of the reaction mixture to a 1-mL assay mixture (Plapp, 1970). The presence of low concentrations of inactivators in the assay produced no detectable inhibition.

Results

Identification of Specific Active-Site-Directed Inactivators. Each of the reagents was tested in a standard procedure in order to determine which ones fit the criteria expected for specific inactivation. Figure 1 shows that the pseudo-first-order rate of inactivation by **6** was slightly faster in the presence of NAD^+ and fourfold or more slower in the presence of AMP or NADH than in the absence of nucleotides. This is the pattern predicted for carboxylic acids, which should bind to the enzyme–NAD^+ complex but not bind to complexes containing AMP or NADH.

A correlative experiment with **7**, the amide of **6**, shows (Figure 2) that the rate of inactivation is about four times faster in the presence than in the absence of NADH. AMP did not protect against inactivation, presumably because **7** does not bind to the anion-binding site before reacting. Unexpectedly, the initial rate of inactivation was four to five times faster in the presence of NAD^+ than in the presence of NADH. After about 20 min, the rates of inactivation in the presence of

TABLE II: Inactivation of Liver Alcohol Dehydrogenase by Bromoacetamido Compounds.[a]

compd	app 2nd order rate constant (M^{-1} min^{-1})				
	no addition	2 mM AMP	0.2 mM NADH	1 mM NAD^+	NBP[b]
1	5.9	slow[c]	slow[c]	0.46	50
2	6.3	1.6	slow[c]	7.2	43
3	10	2.0	1.4	4.9	46
4	43	4.2	2.7	3.0	50
5	120	12	4.2	3.2	48
6	100	27	15	120	60
7	40	35	160	700[d]	57
8	140	31	13	24	63
9	320	31	12	17	58
$BrCH_2COOH$[e]	8.2	0.14	0.082	0.092	32
$BrCH_2CONH_2$[e]	1.6	0.11	0.0059	0.01	32

[a] The enzyme was treated with 0.2 mM reagent at pH 8 and 25 °C, and the apparent second-order rate constant for inactivation was calculated on the assumption that the reaction was first order in reagent. Nucleotides were added in the stated concentrations. [b] The relative chemical reactivity of the alkylating group of the reagent with NBP [4-(*p*-nitrobenzyl)pyridine] was determined by the method of Baker & Jordaan (1965). The reactivities are expressed as the rate of change of absorbance at 570 nm (1 cm cell) divided by the final concentration of the alkylator in the reaction mixture (at 37 °C). [c] Inactivation was so slow that the enzyme activity was the same as the control after 1 day of reaction. [d] The rate represents the initial, rapid phase of the inactivation. [e] Fries et al. (1975). Iodoacetate or iodoacetamide forms weak or undetectible reversible complexes with the enzyme (Evans & Rabin, 1968; Reynolds & McKinley-McKee, 1969), and it can be assumed that the apparent second-order rate constants for bromoacetate and bromoacetamide approximate the true bimolecular rate constants.

NAD^+ or NADH were about the same. Amides do bind moderately tightly to the enzyme-NAD^+ complex, and it is interesting that the complex has an absorption maximum at 315 nm (Sigman & Winer, 1970). Moreover, 4-(*p*-nitrophenyl)butyramide also rapidly, but partially, appeared to inactivate the enzyme in the presence of NAD^+. Thus, the initial, fast phase of "inactivation" may be due to the formation of a complex that dissociates slowly, as does the enzyme-NAD^+-pyrazole complex (Theorell et al., 1969). The slower phase of inactivation could be due to alkylation by the bromoacetamido group.

The possibility that curvature in inactivation kinetics might occur with an enzyme with structurally different active sites was tested by treating the heterodimeric ES isozyme with reagents **6** and **7**. The E subunit is active with ethanol but not steroid substrates, whereas the S subunit is active on both (Pietruszko, 1975; Brändén et al., 1975). For this experiment, the enzyme assay of Dalziel (1957) with 8 mM ethanol was used in order to differentiate activities of the subunits, which have different K_m values (Dworschack & Plapp, 1977). The patterns and rates of inactivations were essentially the same as those shown in Figures 1 and 2. It appears, therefore, that the active sites of the E and S subunits are similarly reactive.

The other reagents were tested by the standard procedure, and the apparent bimolecular rate constants are presented in Table II. Inactivation by the homologous series of reagents **1–5** in the absence of nucleotides increases as the length of the alkyl chain increases, as expected for the hydrophobic active site (Brändén et al., 1975; Hansch et al., 1972). The aryl or aralkyl reagents **6–9** inactivate even faster. AMP, NADH, and NAD^+ generally protect against inactivation, but reagents **2**, **6**, and **7** inactivate faster in the presence than in the absence

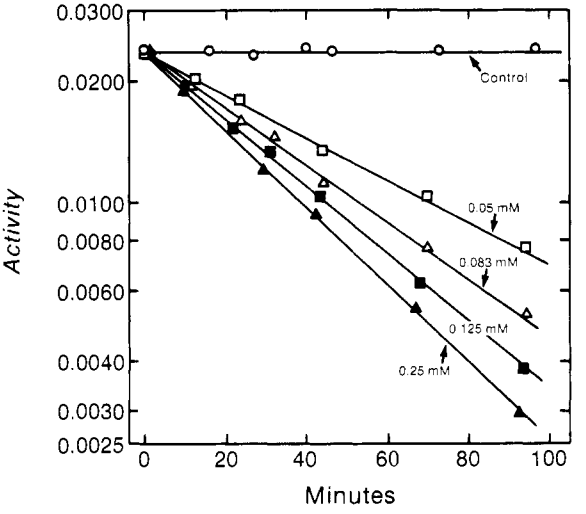


FIGURE 3: Kinetics of inactivation of alcohol dehydrogenase with different concentrations of 4-(*p*-bromoacetamidophenyl)butyric acid in the presence of 1 mM NAD^+.

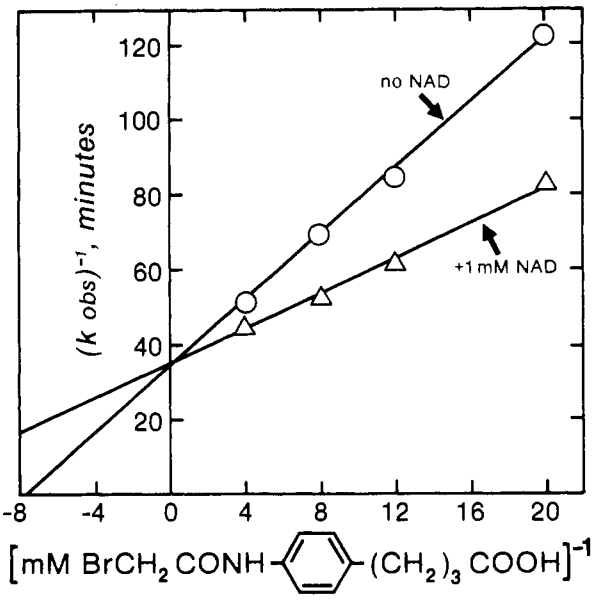


FIGURE 4: Dependence of the rate of inactivation of alcohol dehydrogenase in the absence or presence of 1 mM NAD^+ on the concentration of 4-(*p*-bromoacetamidophenyl)butyric acid. The lines are computed fits to the equation for a hyperbola (Cleland, 1967).

of NAD^+. It should be noted that the relative reactivities of the various alkylators with a model nucleophilic compound [4-(*p*-nitrobenzyl)pyridine] were the same within a factor of 2, which shows that the greatly enhanced reactivities of the new reagents, especially **2**, **6**, and **7** in the presence of coenzyme, are due to their specificities of interaction with the enzyme.

Kinetic Mechanism of Inactivation by Bromoacetamido Compounds. In order to determine if the reagents inactivate by a mechanism in which they first bind to the enzyme and then react chemically (Kitz & Wilson, 1962) and to determine the basis of the facilitation of inactivation by coenzymes, we studied the rates of inactivation by different concentrations of reagent in the absence and presence of coenzymes. An example, in Figure 3, shows that inactivation followed pseudo-first-order kinetics at each concentration. As shown in Figure 4, the reciprocal of the rate of inactivation was a linear function of the reciprocal of the concentration of reagent. Thus, the kinetics fit the expected active-site-directed mechanism. The

TABLE III: Kinetic Constants for Inactivation by Bromoacetamido Compounds in the Absence (and Presence) of Coenzyme.[a]

compd	$10^4 k_3$ (min^{-1})	K_I (μM)	k_3/K_I (M^{-1} min^{-1})
1	170 ± 40	2450 ± 830	7.1 ± 0.6
	(28 ± 1)	(2040 ± 140)	(1.40 ± 0.03)
2	67 ± 5	270 ± 50	25 ± 3
	(38 ± 1)	(180 ± 20)	(21 ± 1)
3	140 ± 2	800 ± 20	17.5 ± 0.2
	(24 ± 0.3)	(650 ± 10)	(3.80 ± 0.04)
4	310 ± 20	360 ± 50	87 ± 7
	(14 ± 3)	(180 ± 10)	(7.6 ± 0.4)
5	430 ± 30	160 ± 30	270 ± 40
	(16 ± 1)	(120 ± 5)	(13.6 ± 0.1)
6	290 ± 10	130 ± 10	230 ± 10
	(280 ± 9)	(64 ± 6)	(440 ± 25)
7	47 ± 3	97 ± 11	50 ± 3
	(390 ±10)[b]	(97 ± 6)[b]	(400 ± 10)[b]
8	400 ± 10	200 ± 10	200 ± 5
	(100 ± 4)	(190 ± 10)	(53 ± 2)
9	830 ± 70	220 ± 20	380 ± 14
	(28 ± 9)	(180 ± 11)	(16 ± 4)

[a] The constants were determined at pH 8 and 25 °C using varied concentrations of reagent in the absence or, for the constants in parentheses, in the presence of 1 mM NAD^+ as shown in Figures 3 and 4. The kinetics fit a Michaelis mechanism

$$E + I \overset{K_I}{\rightleftharpoons} E \cdots I \overset{k_3}{\rightarrow} E\text{-}I$$

where K_I corresponds to the dissociation constant, k_3 corresponds to the maximum rate of inactivation, and k_3/K_I corresponds to the pseudobimolecular rate constant. The constants were computed from fits of the pseudo-first-order rate constants for inactivation as a function of the concentrations of reagent to the equation for a hyperbola (Cleland, 1967). [b] Kinetic constants determined in the presence of 0.2 mM NADH. Since the kinetics were not pseudo first order in the presence of NAD^+, no valid constants could be determined.

results also show that one effect of NAD^+ on the inactivation by **6** is to tighten binding of the reagent. Similar experiments were performed with each of the other reagents, and the results are presented in Table III.

For most of the compounds, NAD^+ decreases the maximum first-order rate constants. But for compounds **6** and **7**, the k_3 values were the same or higher in the presence of NAD^+ or NADH. The presence of NAD^+ slightly increased, in general, the affinity ($1/K_I$) of the carboxylate reagents for the enzyme. The most significant effects are those on k_3/K_I, the pseudobimolecular rate constant of reaction, which gives the best measure of the comparative specificities of the reagents. For most of the carboxylic acids, k_3/K_I is much lower in the presence of NAD^+, but for **2** and **6**, k_3/K_I is the same or even larger in the presence of NAD^+. It appears that the enzyme-NAD^+ complex binds these reagents in a manner that facilitates reaction in the active site. The binding of the amide **7** is not enhanced by the presence of NADH, but, since k_3/K_I is eight times larger, it is clear that NADH facilitates reaction.

Amino Acid Analysis of Inactivated Enzyme. If reagents **6** and **7** inactivate by modifying Met-306 in the presence of coenzyme and Cys-46 in the absence of coenzyme, the modified enzyme should contain the corresponding *S*-carboxymethyl amino acid derivatives. (The amide linkage in the reagent would also be cleaved by acid.) Enzyme was inactivated by the various reagents to the extent of about 90%, hydrolyzed, and analyzed (Table IV). Enzyme inactivated by **6** and **7** in the presence of coenzyme lost one methionine per subunit (but such difference analyses are not very accurate) and gained about 0.6 equivalent of *S*-carboxymethylhomocysteine, a product obtained in 60% yield by hydrolysis of *S*-carboxymethylmethionine (Gundlach et al., 1959). In contrast, inactivation by **6** and **7** in the absence of coenzyme gave *S*-carboxymethylcysteine but no *S*-carboxymethylhomocysteine. That these reagents are specific is indicated by comparisons to enzyme inactivated by bromoacetate or bromoacetamide in the presence of coenzyme. Cysteine was extensively modified and some methionine derivatives were produced; these results do not allow us to identify which amino acid is essential for activity. Whether it is cysteine or methionine, however, the data show that **6** and **7** are much more specific (with or without coenzyme) and that the relative rates of inactivation (in Table III) can be used to estimate the rate-enhancement specificity. Although the amino acid analyses are consistent with the proposed modes of inactivation, analysis of labeled peptides or other evidence is required to support any conclusions about specificity of reagents.

Discussion

Structure and Reactivity of Liver Alcohol Dehydrogenase. The results presented here reveal new aspects about the interaction of alcohol dehydrogenase with analogues of fatty acids and fatty acid amides and demonstrate how NAD^+ and NADH affect these interactions. In the absence of coenzyme, the carboxylic acid **6** inactivates with a concomitant modification of one cysteine residue, which is probably either Cys-46 or Cys-174 on the basis of the following evidence.[2] AMP and NADH protect against inactivation and their phosphate groups are known to bind to the guanidinium group of Arg-47, which forms an anion binding site that is about 8 Å from the sulfur of Cys-46 (Brändén et al., 1975; Zeppezauer et al., 1975; Plapp et al., 1978). Reagent **6** reacts by a mechanism involving an intermediate reversible complex (expected for active-site-directed reagents), and the carboxylate probably binds to the anion-binding site before covalent reaction, as X-ray crystallographic results indicate for the reaction of iodoacetate with Cys-46 (Brändén et al., 1975). Moreover, previous work has shown that inactivation by iodoacetate is competitively prevented by decanoate, 4-biphenylcarboxylate, AMP, ADP, and adenosine 5′-diphosphoribose, which is consistent with a common site of binding for these reagents (Evans & Rabin, 1968; Reynolds & McKinley-McKee, 1969; Reynolds et al., 1970). The fact that **7**, the amide of **6**, inactivates about four times more slowly and reacts less specifically (six cysteine residues are modified in the inactivated enzyme) than **6** indicates that the carboxyl group of **6** is important in the reaction mechanism. However, the ionic interactions of the carboxyl group and the hydrophobic interactions of the phenylbutyryl group of **6** with the apoenzyme must not be very specific, since **6** inactivates only 28 times faster than does bromoacetic acid. Probably, **6** binds, in the absence of NAD^+, in various nonproductive modes in addition to at least one mode that results in the modification of a cysteine residue.

In contrast to the reactions in the absence of coenzyme, **6** and **7** bind, in the presence of NAD^+ or NADH, to another site on the enzyme, resulting in inactivation concomitant with the modification of one methionine residue per subunit. No other carboxymethylated derivatives were observed by amino acid analyses, which could resolve at least carboxymethylated derivatives of cysteine and histidine. Preliminary results with ^{14}C-labeled **6** show that only one molecule of reagent is in-

[2] Low resolution X-ray crystallographic studies show that **6** reacts at a site near Cys-46 with the enzyme crystallized in the absence of coenzyme, but in the presence of NAD^+ **6** reacts at a site near Met-306 in the substrate-binding pocket (B. V. Plapp, E. Zeppezauer, H. Eklund, & C.-I. Brändén, unpublished work).

TABLE IV: Amino Acid Composition of Inactivated Enzyme.[a]

reagent	concn	nucleotide	reaction time, (h)	amino acid res/subunit		
				S-CM-Cys	*S*-CM-Hcy[b]	Met
6	0.2 mM	1 mM NAD^+	3.3	0.1	0.73	7.9
6	0.2 mM	none	3.5	1.2	0.1	8.6
7	0.15 mM	1 mM NAD^+	1.5	0	0.58	7.9
7	0.15 mM	0.2 mM NADH	3.0	0	0.64	8.3
7	0.15 mM	none	26	5.8	0	8.6
bromoacetate	0.2 M	1 mM NAD^+	4.6	6.3	0.78	8.1
bromoacetamide[c]	0.1 M	0.2 mM NADH	118	7.5	3.7	3.9

[a] Enzyme was inactivated by 90 ± 2% under the conditions stated in the table. Samples were dialyzed against H_2O and hydrolyzed in 6 M HCl in sealed, evacuated tubes at 110 °C for 22 h (Moore & Stein, 1963) and analyzed for amino acids with a narrow-bore, single-column analyzer designed by Liao et al. (1973), using Durrum DC-4A resin and Fempto buffers and *o*-phthalaldehyde for fluorescent detection (Roth & Hampai, 1973; Hare, 1977). The fluorescent calibration constants for carboxymethylated amino acids were determined with samples that had been standardized on a Beckman 120C amino acid analyzer using ninhydrin for detection. Ninhydrin color values were taken from published values (Neumann et al., 1962). The fluorescent constant for *S*-carboxymethylcysteine was 1.1 times the constant for aspartic acid or 0.69 times the average of the constants for serine, glycine, alanine, methionine, isoleucine, leucine, phenylalanine, histidine, lysine, and arginine. The constants for the other derivatives were as follows: homoserine, 0.8× serine or 0.76× average; *S*-carboxymethylhomocysteine, 0.99× glutamic acid or 0.78× average. Homoserine lactone did not appear to react in the fluorescent system. [b] *S*-Carboxymethylhomocysteine is obtained in 59% yield upon acid hydrolysis of *S*-carboxymethylmethionine. Homoserine was obtained in 8% yield and methionine was regenerated in 13% yield. The enzyme contains nine methionines per subunit. [c] The exhaustive reaction also modified five of the seven histidine residues.

corporated during inactivation of each subunit of enzyme. X-ray crystallography has shown that the methionine closest to the catalytic zinc ion is Met-306, which is in the substrate binding pocket and which is not likely to be involved in catalysis (Brändén et al., 1975; Plapp et al., 1978). Therefore, it appears that reagents **6** and **7** inactivate by alkylating Met-306 and anchoring a good inhibitor into the active site rather than by modifying a residue absolutely required for catalytic reactions.[2] This means of inactivation is so effective that treatment of the enzyme with 0.2 mM **2** for 24 h inactivates by at least 96%. Similarly, enzyme with CM-Cys-46 has 2% residual activity, apparently due to the modified enzyme itself (Reynolds & McKinley-McKee, 1975).

The principal conclusion from these results is that these new reagents, **6** and **7**, can bind and react in two different ways with the enzyme. The ambivalence is resolved by coenzyme, which directs and facilitates the reaction by promoting binding of reagent in a favorably oriented mode.

Another conclusion is that the acid **6** (in the presence of NAD^+) and the corresponding amide **7** (in the presence of NADH) bind and react by a common mode, since both modify one methionine residue and inactivate the enzyme with about the same pseudobimolecular rate constant (420 M^{-1} min^{-1}). This could occur, for instance, if the terminal carbonyl group were ligated to the zinc while the bromoacetamido function was juxtaposed to the sulfur of Met-306.

Analysis of the rates of inactivation by the various reagents also permits some conclusions about the topography of the active site and how much this is altered by the formation of the enzyme–coenzyme complex. The distance between the catalytic zinc ion of one subunit and the sulfur of Met-306 from the other subunit, both of which are part of one active site, is 14 ± 1 Å in the apoenzyme structure (calculated from coordinates deposited in the Protein Data Bank, Brookhaven National Laboratory, Upton, N.Y. 11973). Reagents **6** and **7** have a maximum extensible length of about 13 Å from the terminal carbonyl group to the methylene carbon in the bromoacetamido moiety and could fit nicely between the zinc and the sulfur. Furthermore, **2** has about the same maximum length as **6** and **7**, and **2** is the reagent in the series **1** to **5** that exhibits maximal facilitation of inactivation by NAD^+. Reagent **2** also is anomalously more reactive (k_3/K_I) and more tightly bound ($1/K_I$) than reagents **1** or **3** in the presence of NAD^+. Using this series of reagents as chemical rulers, we would conclude that **1** is too short and **3**–**5** are too long to fit precisely and react in the active site. Bromoacetic acid and bromoacetamide appear to be too short for a facilitated reaction in the presence of coenzyme, since they react considerably more slowly than any of the new reagents. On the basis of these results, we conclude that the sulfur of Met-306 is about 14 Å away from the catalytic zinc ion in the enzyme complexed with coenzyme and reagent. It appears, therefore, that the conformational change that occurs when such a complex forms (Brändén et al., 1975) does not substantially alter the topographic relationship between Met-306 and the zinc ion.

Nevertheless, it is possible that the conformational change alters the active site without changing the distance between the zinc and Met-306. In that case the coenzyme might facilitate inactivation by **6** and **7** by causing exposure of a partially buried methionine residue rather than by promoting specific binding into the active site. The fact that bromoacetic acid and bromoacetamide react much more slowly than **6** and **7** with enzyme in the presence of coenzyme is evidence against the exposure mechanism. However, we cannot eliminate the possibility that coenzyme and **6** or **7** act synergistically to cause a conformational change that facilitates reaction. Inactivation of yeast alcohol dehydrogenase by N^1-(2-bromoacetamidoethyl)nicotinamide is thought to be facilitated by a conformational change caused by binding of AMP (Plapp et al., 1968).

Design and Reactivity of Active-Site-Directed Reagents. Based on a knowledge of the three-dimensional structure of alcohol dehydrogenase, we designed a series of reagents and predicted how truly active-site-directed reagents should react with the enzyme. As discussed above, some of the reagents did fit the required criteria: (a) they had affinity for the active site as evidenced by saturation kinetics of reaction, (b) they reacted at the active site and inactivated the enzyme, (c) they reacted more rapidly than a simple reagent that had the same chemical reactivity with a model compound, and (d) the specificity of their facilitated reactions depended upon the coenzyme that was present.

Our results indicate that specific reagents must be designed with a size and shape that is reasonably complementary to the active site and that empirical studies are required to obtain reagents that react with optimal facilitation. Our strategy was

to determine first the appropriate length of reagent by evaluating the series **1** to **5.** As discussed above, **2** appeared to fit optimally into the active site. As expected for reagents that should bind into the hydrophobic active site (Brändén et al., 1975; Hansch et al., 1972), the affinities ($1/K_I$) and reactivities (k_3/K_I) of the homologous reagents **1–5** generally increased as the length of the alkyl chain increased. The data for **1** and **3–5** fit a linear free-energy relationship with a value of $\Delta G°$ of 300 cal/mol per methylene unit bound in the presence or absence of NAD^+. Binding of fatty acids to the E·NAD^+ complex gave a value of 480 cal/mol (Winer & Theorell, 1960). **2** deviated from the relationship established by the other compounds, apparently because **2** is just the right size to fit into the active site.

The next step in our strategy was to increase the hydrophobicity of the reagent and to investigate the role of the size and shape of the reagent by evaluating the aralkyl and aryl reagents **6, 8,** and **9.** These reagents have about the same overall length, and **8** and **9** resemble known inhibitors of the enzyme, 4-biphenylcarboxylate and triiodothyroacetic acid (reviewed by Brändén et al., 1975). From an examination of models of the reagents and the enzyme, we expected that the rigid, linear biphenyl structure of **8** would not fit complementarily into the substrate binding pocket of the active site, the phenoxyphenyl group of **9** could interact more strongly, and the flexible phenylbutyryl group of **6** could fit well into the pocket between the zinc ion and Met-306. That inactivation (k_3/K_I) in the presence of NAD^+ by **8** or **9** is much slower than by **6** bears out our expectations about **8** (but not **9**) and emphasizes the requirement for good complementarity or at least some flexibility in specific reagents. Improvements in the size and shape of the reagents might give further enhancement of reaction rates.

The magnitude of the rate-enhancement specificity obtained with the affinity-labeling reagents can be estimated from our results. In the presence of NAD^+, **2** inactivates 230 times faster, and **6** 4800 times faster, than does bromoacetate. In the presence of NADH, **7** inactivates 67 000 times faster than does bromoacetamide! However, these values are only estimates, since rates of inactivation were measured and not the actual rates of modification of a specific amino acid residue, such as Met-306. It is not practicable at this time to determine the relative rates of modification, since bromoacetate or bromoacetamide modify several amino acid residues. Whatever the true values are, it is apparent that the new reagents are exceptionally potent inactivators, which are certainly better than nonspecific alkylators of methionine. We are therefore encouraged that the rational design of specific, active-site-directed agents is feasible.

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