Activation of Liver Alcohol Dehydrogenases by Imidoesters Generated in Solution†

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ABSTRACT: Various ω-halogenated carboxy acids and amides were evaluated as potential active-site-directed reagents for alcohol dehydrogenase. 2-Bromoacetamide and bromoacetic and 3-bromopropionic acids inactivated the enzyme; AMP, NAD^+, and NADH markedly decreased the rate of inactivation. Some ω-halogenated carboxyamides, $X(CH_2)_nCONH_2$, increased the activity of the enzyme with the rate and extent of activation depending on the number of methylene units (n) in the order $3 > 4 > 2$ and on X in the order Br > Cl. 4-Chlorobutyramide (0.1 M) activated the horse liver enzyme 20-fold in 24 hr at pH 8.0 and 25°. The activation was not prevented by AMP or 2,2-bipyridine, but was by NADH. The kinetic constants and turnover numbers for human and horse liver alcohol dehydrogenases treated with chlorobutyramide were increased markedly compared to those for native enzymes. Alcohol dehydrogenase treated with chlorobutyramide was not further activated by methyl picolinimidate, an imidoester which activates native enzyme by modifying amino groups in the active sites. Chlorobutyramide does not appear to react directly with the enzyme but cyclizes in the reaction medium to form an intermediate imidoester, 2-iminotetrahydrofuran, which reacts with most of the amino groups of the enzyme.

Liver alcohol dehydrogenase (EC 1.1.1.1) catalyzes a rate-limiting step in ethanol metabolism (Hawkins and Kalant, 1972; Plapp, 1975). Therefore, reagents that specifically increase or decrease the activity of this enzyme in vivo might be used to change the rate of ethanol metabolism. When ε-amino groups of lysine residues at the active sites of horse liver alcohol dehydrogenase are alkylated or amidinated with substituents that retain the positive charge, the maximum velocities of the enzymatic reactions are increased by up to tenfold (Plapp, 1970; Plapp et al., 1973; Zoltobrocki et al., 1974). The modification of lysine residue 228 is responsible for the activation (Sogin and Plapp, 1975a; Dworschack et al., 1975). Carboxymethylation of cysteine residue 46 almost completely inactivates the enzyme (Harris, 1964; Li and Vallee, 1964; Reynolds and McKinley-McKee, 1970) as does reaction of cysteine-174 with diazonium-1H-tetrazole (Sogin and Plapp, 1975b) or nicotinamide 5-bromoacetyl-4-methylimidazole dinucleotide (Jörnvall et al., 1975). Since the reagents used for the modifications described above can react readily with most proteins, none of them could be used to modify alcohol dehydrogenase specifically in vivo.

Fatty acids and amides form stable ternary complexes with alcohol dehydrogenase and NAD^+ or NADH (Winer and Theorell, 1960; Woronick, 1963). Therefore, some ω-halogenated acids and amides were tested as active-site-directed reagents, in which the acid or amide group might bind to the zinc at the active site while the alkyl halide reacted with nearby amino acid residues, activating or inactivating the enzyme.

Experimental Procedure

Materials. The halogenated amides were prepared by the method used by Hamilton and Simpson (1929) for the synthesis of 3-bromopropionamide. For example, 4-chlorobutyramide was made by adding 13 g (90 mmol) of 4-chlorobutyryl chloride, dropwise and with vigorous stirring, to 20 ml of concentrated NH_4OH and 10 ml of H_2O cooled in an ice-salt bath. White crystals formed slowly. These were collected, dried in air, and dissolved in dry acetone. The insoluble ammonium chloride was removed by filtration, and the acetone was evaporated at reduced pressure to give 7.2 g (65%) of the amide, which was washed with hexane and dried in vacuo: mp 96–98°; ir 1630 cm^{-1} (NH_2CO–); NMR (Me_2SO-d_6) δ 3.7 (t, $ClCH_2$), 2.2 (t, CH_2CO), 1.9 (quintet, $CH_2CH_2CH_2$). Anal. Calcd for C_4H_8ONCl: C, 39.51; H, 6.63; N, 11.52; Cl, 29.16. Found: C, 39.62; H, 6.56; N, 11.47; Cl, 29.15. 4-Bromobutyramide was prepared similarly: mp 72–74° immediately after preparation. The compound was hygroscopic and unstable at room temperature. It was stored at −20°; nevertheless, it decomposed slowly. Anal. Calcd for C_4H_8ONBr: C, 28.93; H, 4.86; N, 8.44. Found: C, 28.08; H, 4.63; N, 8.21. 5-Bromovaleramide was prepared in 58% yield, mp 84–85°. Anal. Calcd for $C_5H_{10}ONBr$: C, 33.29; H, 5.41; N, 7.83. Found: C, 33.35; H, 5.60; N, 7.78. 6-Bromocaproamide was prepared in 94% yield, mp 105–107°. Anal. Calcd for $C_6H_{12}ONBr$: C, 37.13; H, 6.23; N, 7.22. Found: C, 37.40; H, 6.46; N, 7.31. 5-Chlorovaleramide and 6-chlorocaproamide were prepared similarly; the properties of the ω-chloroamides have been described by Nohira et al. (1965). 2-Bromoacetamide was prepared previously (Plapp et al., 1968). The acid chlorides were commercially available (Aldrich or ICN-K&K) or were prepared from the acids with PCl_3.

Bromoacetic and bromovaleric acids were purchased from Aldrich and recrystallized from diethyl ether–hexane and hexane, respectively. Bromocaproic and bromopropionic acids were purchased from Eastman. Bromobutyric acid was prepared by hydrolysis of bromobutyryl chloride with 48% hydrobromic acid and recrystallized from hexane. Melting points of recrystallized acids agreed with literature values.

Butyrolactimidate hydrochloride (2-iminotetrahydrofur-

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Table I: Activation of Liver Alcohol Dehydrogenase by Halo Amides.[a]

$X(CH_2)_nCONH_2$		$10^3 k_{obsd}$	Fold
X	n	(M^{-1} min^{-1})	Activation
Cl	3	80	20
	4	20	7
Br	2	4	3
	3	1000	20
	4	40	10

[a] The horse enzyme at a concentration of about 0.2 mg/ml in 0.5 M triethanolamine hydrochloride buffer (pH 8.0) was treated with the reagent at a concentration of 0.1 M at 25°. Samples of 10 μl were taken for assay of enzymatic activity at various times. The fold activation was calculated from the maximum activity observed relative to a control that did not contain the reagent. The observed, pseudo-first-order rate constants were estimated graphically by use of the expression $\ln (A_{max} - A_t) - \ln (A_{max} - A_0) = -kt$, where A_{max}, A_t, and A_0 are the activities at maximum activation, time t, and time zero, respectively. The second-order rate constants were calculated on the assumption that the reaction was first order in reagent.

Table II: Inactivation of Liver Alcohol Dehydrogenase by Bromoacetamide and Bromo Acids.[a]

	k_{obsd} (M^{-1} min^{-1})			
Reagent	No Addition	+AMP	+NAD^+	+NADH
$BrCH_2CONH_2$	1.6	0.11	0.010	0.0059
$BrCH_2COOH$	8.2	0.14	0.092	0.082
$Br(CH_2)_2COOH$	0.038	0.036	0.013	0.0096

[a] The horse enzyme, at a concentration of about 0.25 mg/ml in 0.1 M $Na_4P_2O_7$ adjusted to pH 8.0 with H_3PO_4, was treated with the reagent at a concentration of 0.1 M at 25°. The bromo acids were neutralized before addition to the enzyme. The final concentrations of nucleotides were 2 mM AMP, 1 mM NAD^+, and 0.2 mM NADH. Samples of 10 μl were taken for assay at various times. The loss of activity was pseudo first order in enzyme concentration and the reaction was assumed to be first order in reagent concentration.

an hydrochloride) was prepared by the method of Nohira et al. (1965). γ-Butyrolactone was obtained from Aldrich; γ-butyrolactam from ICN-K&K. 4-Hydroxybutyramide was prepared by ammonolysis of butyrolactone and its structure was confirmed by elementary analysis and NMR. AMP, NAD^+, NADH, and ε-aminocaproic acid were obtained from Sigma.

The horse liver enzyme was purified by a modification of the method of Theorell et al. (1966). The human liver enzyme was partially purified (27-fold) by fractionation with ammonium sulfate and chromatography on DEAE-cellulose and CM-cellulose by procedures adapted from von Wartburg et al. (1964) and Blair and Vallee (1966). Enzymatic activity was determined in 1-ml assays containing 0.55 M ethanol and 1.75 mM NAD^+ at pH 9 and 25° (Plapp, 1970).

Modification of Alcohol Dehydrogenase with 4-Chlorobutyramide for Kinetics and Structural Studies. The enzyme was freed of ethanol by filtration through Sephadex G-50 equilibrated with 33 mM sodium phosphate buffer (pH 8.0). The solution of enzyme was made 0.5 M in triethanolamine hydrochloride buffer (pH 8.0) by the addition of 1/3 volume of the 2.0 M buffer, and 12 mg of solid chlorobutyramide was added to each milliliter of solution. When maximal activation was obtained, the reaction mixture was again filtered through Sephadex G-50 equilibrated with 33 mM sodium phosphate buffer (pH 8.0) and 0.25 mM EDTA. The modified enzyme was quite stable when stored at 5° and could be used for kinetics studies for several days. For subsequent modification with methyl picolinimidate, the solution was again made 0.5 M in triethanolamine buffer, and treated as previously described (Plapp, 1970). The incorporation of picolinimidyl groups was determined spectrophotometrically (Plapp et al., 1973). The protein was hydrolyzed in 6 M HCl in sealed, evacuated tubes at 110° for 22 and 46 hr (Moore and Stein, 1963), and analyzed for amino acids on a Beckman 120C analyzer.

Results

Reaction of Alcohol Dehydrogenases with Halo Amides and Halo Acids. Treatment of horse liver alcohol dehydrogenase with some haloamides significantly increased the enzymatic activity (Table I). In general, the extent of activation was in the order butyryl > valeryl > propionyl. The bromo amides reacted faster than the chloro amides. The human liver enzyme was activated 14-fold by chlorobutyramide as assayed at pH 9 in 0.55 M ethanol (Plapp, 1970). Except for some reversible inhibition, the chloro- and bromocaproamides did not change the activity of the enzyme over a 20-hr treatment; however, the solubility of these compounds was less than 0.1 M.

As expected from previous work (Plapp et al., 1968), bromoacetamide inactivated the enzyme (Table II), but the rate of inactivation in the 0.5 M triethanolamine hydrochloride buffer (0.2 M^{-1} min^{-1}) was much slower than in 0.1 M sodium pyrophosphate adjusted to pH 8 with H_3PO_4 (1.6 M^{-1} min^{-1}). Perhaps the effect is due to the chloride ions, which markedly retard carboxymethylation (Reynolds and McKinley-McKee, 1969). Bromoacetic acid (2 mM) also rapidly inactivated the enzyme; again, the rate in triethanolamine hydrochloride buffer (1 M^{-1} min^{-1}) was slower than in the pyrophosphate–phosphate buffer (8 M^{-1} min^{-1}). In the pyrophosphate–phosphate buffer, 3-bromopropionic acid inactivated slowly, and the 4, 5, and 6-carbon bromo acids inactivated very slowly (<0.004 M^{-1} min^{-1}). None of the acids activated the enzyme in either buffer.

The specificity of the reaction of the chloro and bromo amides and the bromo acids with the active site of the enzyme was tested by adding AMP, NAD^+, and NADH at concentrations (2, 1, and 0.2 mM, respectively) that would almost saturate the binding sites. In general, AMP only protected slightly against activation by the halo amides listed in Table I. In contrast, NADH almost completely prevented the activation by these amides. The results for 4-chlorobutyramide are shown in Figure 1. In the presence of NADH, the amides reversibly inhibited the enzyme, indicating that enzyme–NADH–amide complexes formed, but apparently covalent reaction of the amide was not facilitated in the complexes. For comparison, the activation of the enzyme by the imidoesters, ethyl acetimidate and methyl picolinimidate (Plapp, 1970), was studied under the conditions of Figure 1; AMP slightly retarded the activation, but NADH essentially prevented the activation. The bromo acids and bromoacetamide generally inactivated the enzyme much more slowly in the presence of AMP, NAD^+, and NADH than in their absence (Table II), as might be predicted from studies with iodoacetic acid (Li and Vallee, 1963; Reynolds and McKinley-McKee, 1969) or bromoace-

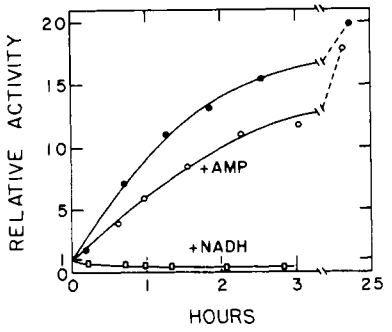

FIGURE 1: Activation of liver alcohol dehydrogenase by 4-chlorobutyramide in the presence of various ligands. The horse enzyme at a concentration of about 0.11 mg/ml in 0.5 *M* triethanolamine hydrochloride buffer (pH 8.0) was treated at 25° with 0.1 *M* 4-chlorobutyramide alone (●) or in the presence 2 m*M* AMP (○) or 0.2 m*M* NADH (□). The activity relative to a control without reagents is presented.

tamide (Plapp et al., 1968). Since bromoacetamide binds reversibly to the enzyme-NADH complex with a dissociation constant of about 4 m*M* (Woronick, 1963), the active sites were saturated under the conditions of our experiments. Apparently no nucleophilic amino acid residues at the active site are close enough to react with the bound bromoacetamide. The dissociation constant for bromoacetate from the enzyme-NAD^+-bromoacetate complex is probably about 4 m*M* also (Winer and Theorell, 1960), but again the bound bromoacetate did not appear to react. The lack of reaction was not due to hydrolysis of the bound bromoacetate since the addition of excess cysteine after 94 hr of reaction still gave 80% of the expected yield of carboxymethylcysteine.

The possibility that the halo amides bind to the zinc ion in the active site during the activation was tested by protecting the enzymes with compounds that bind to the zinc. But concentrations of 2,2′-bipyridine, imidazole, and *n*-butyramide (3.3, 4, and 500 m*M*, respectively) much higher than the dissociation constants (Sigman, 1967) did not change the rate or extent of activation by 4-chlorobutyramide.

Characterization of Enzyme Modified with Chlorobutyramide. Associated with the activation of the enzyme by chlorobutyramide are substantial increases in the kinetic constants for the enzyme-catalyzed reactions (Table III). The increased Michaelis and inhibition constants require that high substrate concentrations be used in assays if the increased activity is to be observed. A measure of the amount of activation that is independent of substrate concentrations is given by the turnover numbers (V/E_t), which for the modified horse enzyme are roughly 10 times the values published for the native enzyme (Zoltobrocki et al., 1974). The human enzyme is affected similarly. As discussed previously (Plapp, 1970), such increases in activity are probably due to increased rates of dissociation of the enzyme-coenzyme complexes, the rate-limiting steps for the native enzyme.

The Michaelis constants for NAD^+ and ethanol with the human enzyme are increased so much by the modification that it is doubtful that chlorobutyramide could be used to accelerate ethanol metabolism in vivo; the concentration of NAD^+ and ethanol would be too low relative to the constants. Furthermore, as assayed at pH 7 and 37° with 50 m*M* ethanol and 1.75 m*M* NAD^+ (Zoltobrocki et al., 1974) human enzyme treated with chlorobutyramide was inactivated by 66%; similarly, the horse enzyme was inactivated by 90%.

Table III: Kinetic Constants for Alcohol Dehydrogenases Activated with Chlorobutyramide.[a]

Constant[b]	Horse Modified	Human Modified	Human Native
K_a (μM)	89 ± 7	600 ± 40	17 ± 3
K_b (mM)	48 ± 3	90 ± 10	1.8 ± 0.3
K_p (mM)	17 ± 2	16 ± 2	1.5 ± 0.2
K_q (μM)	180 ± 10	190 ± 20	10 ± 1
K_{ia} (μM)	460 ± 20	1100 ± 100	49 ± 5
K_{ib} (mM)	310 ± 30	*c*	*c*
K_{ip} (mM)	*c*	*c*	*c*
K_{iq} (μM)	19 ± 1	34 ± 2	2.1 ± 0.3
K_bK_{ia}/K_a (mM)	70 ± 20	70 ± 10	9 ± 2
K_pK_{iq}/K_q (mM)	3.3 ± 0.4	3.5 ± 0.4	0.23 ± 0.06
V_1/E_t (sec^{-1})	24 ± 3	22 ± 2[d]	1.4 ± 0.1[d]
V_2/E_t (sec^{-1})	820 ± 60	350 ± 20	34 ± 2

[a] The kinetic constants were determined from product inhibition studies as described previously (Plapp, 1970), except that a Cary 118C spectrophotometer with 0.02–0.10 *A* scales was used. The buffer used was 33 m*M* sodium phosphate (pH 8.0), containing 0.5 m*M* EDTA, at 25°. [b] The values and their standard errors were computed with Cleland's programs (1967) and corrected for the concentration of the nonvaried substrate on the assumption of an Ordered Bi Bi mechanism. *K* is a Michaelis constant, K_i an inhibition constant; a, b, p, and q represent NAD^+, ethanol, acetaldehyde, and NADH, respectively. V_1/E_t is the turnover number for the reaction of NAD^+ and ethanol, and V_2/E_t for NADH and acetaldehyde. [c] These values were not obtained because the product inhibition patterns appeared to be competitive, probably because the intercept inhibition constants are much larger than slope inhibition constants. [d] The turnover numbers were calculated on the assumption that the pure human enzyme had a specific activity of 2.5 μmol per mg per min (André Dubied, personal communication) in the assay described by Lutstorf et al. (1970) and an equivalent weight of 40000. In the assay used by Plapp (1970), the specific activity would then be 3.0 μmol per mg per min, and in an assay under conditions used by Dalziel (1957) but using initial velocities, 3.8 μmol per mg per min. The latter value is close to the value of 3.5 found by Mourad and Woronick (1967) for crystalline enzyme.

The product of the reaction of chlorobutyramide and alcohol dehydrogenase was also examined chemically. The amino acid compositions of the modified and native enzymes hydrolyzed in 6 *M* HCl at 110° and 22 hr were the same. All of the lysine was recovered as free lysine, and no ε-carboxypropylated lysine was observed. But of course, one modified lysine out of the 30 lysines per subunit (Jörnvall, 1970) might not have been detected directly or by difference. Thus, it might appear that few of the lysines were modified; however, most of the lysines were modified as shown by the following experiments. The modified protein had only about two amino groups free to react with the imidoester, methyl picolinimidate, as shown by the increased absorbance at 262 nm after modification. With the native enzyme, 25 of the 30 lysines can react with methyl picolinimidate as shown by spectral changes or by amino acid analysis (Plapp, 1970). The utility and sensitivity of this method for determining the number of free amino groups have been documented previously for partially acetimidoylated alcohol dehydrogenase and bovine pancreatic deoxyribonuclease (Plapp et al., 1971, 1973). Furthermore, methyl picolinimidate did not increase the activity of the enzyme treated with chlorobutyramide, and chlorobutyramide did not increase the activity of enzyme previously activated eightfold by treatment with ethyl acetimidate. These results probably indicate that chlorobutyramide activates the enzyme by

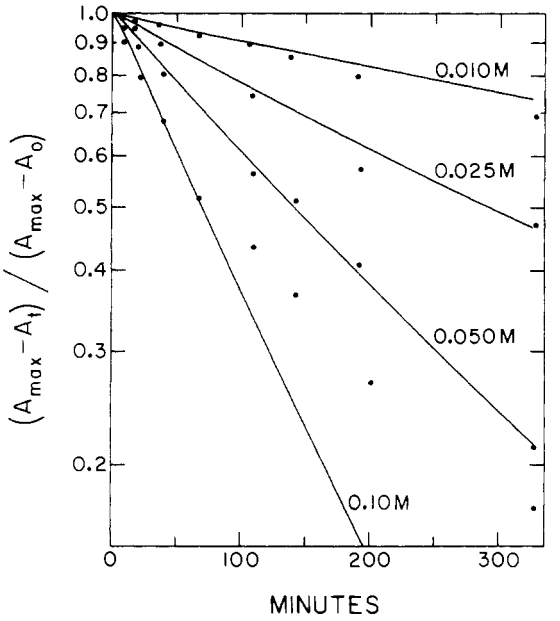

FIGURE 2: Kinetics of activation of liver alcohol dehydrogenase by 4-chlorobutyramide. The experiments were carried out as described in Table I except that the concentration of chlorobutyramide was varied as indicated on the figure. The points are experimental and the lines were generated from eq 1 using values of $k_1 = 6.6 \times 10^{-4}$ min^{-1}, $k_2 = 0.45$ min^{-1}, and $k_3 = 72$ M^{-1} min^{-1} as discussed in relation to Scheme I.

modifying lysine residue 228. Apparently most of the amino groups were blocked by substituents that were completely removed during acid hydrolysis in 6 *M* HCl at 110° for 22 hr. Since amidines, such as acetamidine and picolinamidine, are labile to acid hydrolysis (Plapp, 1970; Plapp and Kim, 1974), it seemed possible that the chlorobutyramide had in some way amidinated the enzyme to form a derivative that was rapidly hydrolyzed in 6 *M* HCl at 110°.

Generation of Imidoesters in Solution. The cyclization of N-substituted chloro and bromo amides to form imidoesters may be promoted by pyrolysis, silver tetrafluoroborate, triethylenediamine, or methoxide (Stirling, 1960; Schmir and Cunningham, 1965; Zaugg et al., 1966; Heine, 1956). By analogy, the halobutyramides could form imidoesters that would hydrolyze or amidinate alcohol dehydrogenase according to Scheme I. Support for such a scheme comes from a variety of experiments. When chlorobutyramide was dissolved in unbuffered D_2O, it decomposed with a rate constant of 9×10^{-4} min^{-1} and simultaneously formed butyrolactone. (The disappearance of the NMR signal at δ 4.4 ($ClCH_2$) and the appearance of the signal at δ 5.4 were followed in a Varian A-60 spectrometer. No intermediate imidoester was detected.) The rate of decomposition of the bromo and chlorobutyramides in 0.5 *M* triethanolamine-HNO_3 buffer was also determined by titration of halide ion

Scheme I

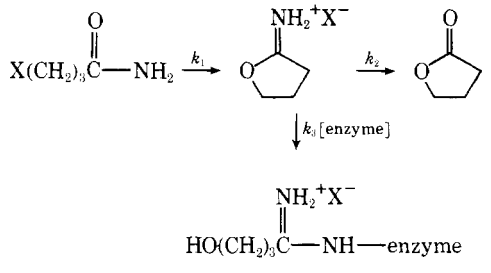

Table IV: Summary of Kinetic Constants for Reactions of Halobutyramides.[a]

Reagent	$10^3 k_1$ (min^{-1})[b]	k_2 (min^{-1})[c]	k_3 (M^{-1} min^{-1})[c]
Chloro	0.66 ± 0.01	0.45 ± 0.05	72 ± 4
Bromo	14 ± 1	0.62 ± 0.11	68 ± 4

[a] See Scheme I for identification of rate constants. Reactions were carried out in 0.5 *M* triethanolamine hydrochloride (or HNO_3) buffer at pH 8.0, 25°. [b] A 0.04 or 0.05 *M* solution of the haloamide was allowed to decompose. Samples of 1 ml were taken at various times and titrated with standardized 10 m*M* $AgNO_3$ with 0.5 ml of 0.05 *M* K_2CrO_4 as an indicator (Waser, 1964). Silver ions did not catalyze decomposition significantly, for bromopropionamide did not give a precipitate even after 10-min exposure to $AgNO_3$. The logarithm of the maximal titrant consumed minus the titrant consumed at any time were fitted to the equation for a first-order reaction by nonlinear least-squares program with calculation of the standard errors (NONLIN, C. M. Metzler, The Upjohn Company). [c] Data (such as in Figure 1) for each concentration of reagent were fitted with NONLIN with the value of k_1 fixed. The mean values and their standard deviations are presented.

with silver nitrate. The first-order rate constants (k_1) are given in Table IV.

For the evaluation of k_2 and k_3, the kinetics of activation of alcohol dehydrogenase were studied. The results in Figure 2 show that the rate of activation approximates pseudo first order at each concentration of chlorobutyramide. Using the values of k_1 as constants, the data for each concentration were fitted by an iterative, nonlinear least-squares curve-fitting procedure to the equation derived by Dunn et al. (1974) describing the overall kinetics:

$$\frac{A_{max} - A_t}{A_{max} - A_0} = e^{-R_0}\left[\left(\frac{-k_3}{k_1 - k_2}\right)(1 - e^{-k_1 t}) + \left(\frac{k_1 k_3}{k_2(k_1 - k_2)}\right)(1 - e^{-k_2 t})\right] \quad (1)$$

where A refers to enzyme activity and R_0 to the initial concentration of reagent. The values in Table IV were obtained over a tenfold range of R_0 and for two different reagents. The good agreement of the values for k_2 and k_3 between reagents is consistent with the mechanism proposed in Scheme I.[1] (Note also that the calculated lines show a lag in reaction rate at very short reaction times and that the rate slows down at longer reaction times.)

Most importantly, the cyclic imidoester, butyrolactimidate, was prepared by another method and was found to activate the enzyme 20-fold. At pH 8.0 and 25° with 0.1 *M* imidoester, the apparent second-order rate constant was 4 M^{-1} min^{-1}, which is faster than the rates for the halobutyramides in Table I. (Varying the concentration of imidoester showed that the reaction was not strictly first order in reagent, but the reaction was too fast with higher concentrations of reagent to obtain reliable rate constants and did not give 20-fold activation at lower concentrations of reagent. Therefore we were unable to compare the rate constant obtained to that predicted from k_2 and k_3 in Table IV.) In

[1] At 0.1 *M* reagent, the differences between the experimental points and the calculated line suggest that the dependence of the rate on the concentration of reagent is not strictly first order, but instead shows a saturation effect. This effect cannot be explained in terms of Scheme I, but it may be related to the mechanism of the reaction of the cyclic imidoester with the enzyme.

contrast, butyrolactone, butyrolactam, and 4-hydroxybutyramide did not activate the enzyme. Enzyme that had been fully activated by treatment with butyrolactimidate also was subjected to hydrolysis in 6 *M* HCl at 110° and analyzed for its amino acid composition. After hydrolysis times as short as 12 or 18 hr, all 30 (±3) or the lysine residues per subunit were recovered as free lysine. As with enzyme activated with chlorobutyramide, no unidentified peaks were observed that could represent modified lysine residues. It appears that the hydroxybutyramidine is quite unstable to acid hydrolysis.

Further confirmation of this conclusion was obtained from studies with a model system. ϵ-Aminocaproic acid was treated in water at room temperature with a fivefold molar excess of 4-chlorobutyramide added in portions over 5 days while the pH was maintained at 10 with triethylamine. The reaction mixture was evaporated to dryness and washed with acetone to remove by-products. The oily product was further purified by dissolving it in anhydrous ethanol, filtering off insoluble impurities, and removing the ethanol completely by evaporation under vacuum. The residual oil slowly solidified. (Attempts to crystallize the compound from boiling 1-propanol–ethyl acetate failed because the product was converted to compounds that were insoluble in 1-propanol.) The product was not pure for it contained some ϵ-aminocaproic acid, but after hydrolysis in 6 *M* HCl at 110°, almost the theoretical amount of amino acid was found upon analysis on the short column of the amino acid analyzer. The kinetics of the appearance of ϵ-aminocaproic acid during the acid hydrolysis were first order and the half-time was 3 hr. This is considerably faster than the half-time of 57 hr for ϵ-acetimidyllysine (Plapp and Kim, 1974) or of 17 hr for ϵ-picolinimidyllysine (Plapp, 1970) and may reflect intramolecular catalysis of hydrolysis by the hydroxybutyryl group.

Discussion

Although carboxylic acids and amides bind to the alcohol dehydrogenase–coenzyme complexes, chemical reaction of the corresponding ω-chloro and bromo compounds does not appear to be facilitated within the ternary complexes. In fact, the coenzymes protect against the inactivation produced with bromoacetamide and bromoacetic acid and the activation obtained with the chloro- and bromobutyramides. AMP protects against inactivation by bromoacetic acid, perhaps because the phosphate group interacts with the guanidinium group of Arg-47, whose positive charge may facilitate carboxymethylation by attracting the carboxylate group of the reagent while the sulfur of Cys-46 displaces the halide (Eklund et al., 1974). The protection by AMP or ADP (Plapp et al., 1968) against bromoacetamide is not so easily explained. Likewise, the lack of protection by AMP against the activation by imidoesters, including those generated from chloro- or bromobutyramide, is puzzling because the adenosyl group is bound very close to the amino group of Lys-228, which has been identified as the site modified during the activation by amidination or alkylation.

The activation of the alcohol dehydrogenases by the haloamides probably results from the amidination of Lys-228 by a cyclic imidoester generated in solution as shown in Scheme I. Cyclization of the haloamides is reasonable since cyclic imidoesters have been proposed as intermediates in the selective cleavage of peptide chains in aqueous solutions (Witkop, 1961). Furthermore, certain haloamides are in equilibrium with the imidoesters in polar solvents (Zaugg et al., 1966), and butyrolactimidate produces chlorobutyramide upon heating and butyrolactone upon hydrolysis (Nohira et al. 1965). The order of reactivity in Table I is consistent with the expected rate of formation of cyclic structures: 5 > 6 > 4 membered rings, and Br > Cl leaving groups.

Although the reagents tested in this work are not specifically directed toward the active site of liver alcohol dehydrogenase, the discovery that imidoesters generated in solution can be used to modify proteins leads us to suggest that similar reagents could be useful biochemical and pharmacological agents. For instance, such imidoesters may be useful antisickling agents (Lubin et al., 1975). Although a simple imidoester, ethyl acetimidate, can penetrate the human erythrocyte membrane, more polar reagents cannot (Whiteley and Berg, 1974). If specific imidoesters were generated inside the cell, permeability problems might be solved. Furthermore, the production of reactive reagent over a period of time could increase the specificity and yield of the desired reaction.

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