Inactivation of Horse Liver Alcohol Dehydrogenase by Modification of Cysteine Residue 174 with 3-Bromopropionic Acid†

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ABSTRACT: Horse liver alcohol dehydrogenase is inactivated with Michaelis kinetics at pH 7 and 25 °C by 3-bromopropionic acid. In the absence of NAD^+, the K_i is 2 mM, and the pseudo bimolecular rate constant (k_3/K_i) is 0.03 M^{-1} s^{-1}; in the presence of 1 mM NAD^+, K_i is 2.3 mM, and k_3/K_i is 0.006 M^{-1} s^{-1}. 3-Bromopropionic acid is a competitive inhibitor, K_i of 0.4 mM, against ethanol as a substrate. Inactivation was prevented in the ternary complexes with NAD^+·pyrazole and NADH·isobutyramide, was retarded by NAD^+, NADH, or bipyridine, and was almost unaffected by imidazole and AMP. Carboxyethylated enzyme did not detectably (as observed spectrophotometrically) bind bipyridine, NAD^+, or NADH. Enzyme was inactivated with radioactive 3-bromopropionic acid, aminoethylated, and digested with trypsin and chymotrypsin. Analysis of the labeled peptides showed that Cys-174 was predominantly modified. In the presence of 1 mM NAD^+, the reaction was much less specific. The interaction of the carboxyl group of 3-bromopropionic acid with the guanidino group of Arg-369 probably facilitates the selective reaction with Cys-174, which is ligated to the zinc at the active site. Carboxyethylation apparently inactivates by interfering with the proper binding of the pyrophosphate of the coenzyme to the enzyme.

Active-site-directed reagents have provided information about the structure and mechanism of liver alcohol dehydrogenase (Brändén et al., 1975). The sulfur of Cys-46 (a ligand to the active-site zinc) is selectively alkylated by α-halo acids (Harris, 1964; Li & Vallee, 1964; Jörnvall, 1970; Dahl & McKinley-McKee, 1977). Iodoacetate inactivates 53 times faster than does iodoacetamide (Dahl & McKinley-McKee, 1981), and it has been suggested that the ionic interaction of the carboxyl group with the guanidino group of Arg-47 facilitates the reaction (Zeppezauer et al., 1975). Inactivation by 3-bromopropionic acid is also facilitated and is assumed to be due to reaction of Cys-46 (Dahl & McKinley-McKee, 1981). The rate of inactivation by haloacetates or haloacetamides is reduced 100-fold by NAD^+ or NADH (Li & Vallee, 1963; Fries et al., 1975), whose pyrophosphate groups bind to Arg-47 and Arg-369 and can prevent access of the reagent to Cys-46 (Brändén et al., 1975; Eklund et al., 1981). In contrast, the rate of inactivation by 3-bromopropionic acid is reduced only to one-third by NAD^+ (Fries et al., 1975), which could indicate that Cys-46 is not being modified. Inactivation by long ω-bromoacetamido-substituted fatty acids is facilitated by NAD^+, and Met-306 in the substrate binding pocket could be alkylated (Chen & Plapp, 1978). Therefore, we have investigated the reaction of 3-bromopropionic acid in the presence and absence of NAD^+.

Experimental Procedures

Materials. Crystalline horse liver alcohol dehydrogenase, NAD^+, and NADH (grade I) were purchased from Boehringer Mannheim; 3-bromopropionic acid was obtained from Eastman; β-[1-^{14}C]alanine was from New England Nuclear. Other chemicals were of the highest grades available from commercial sources.

3-Bromo[1-^{14}C]propionic Acid. This was synthesized according to a slightly modified procedure reported for the synthesis of 2-bromo[4-^{13}C]succinic acid (Ott, 1981). To an ice-cold solution of β-[1-^{14}C]alanine (8.9 mg, 0.1 mmol, 50 μCi) and NaBr (44 mg, 0.43 mmol) in 93 μL of 2.1 M HBr was added well-powdered $NaNO_2$ (12.4 mg, 0.17 mmol) over a period of 2 h with occasional shaking. The reaction mixture was kept at 0 °C for another 20–30 min and treated with 19 μL of 40% H_2SO_4, stirred for 15 min, and extracted with five 1-mL portions of ether. The ether extract was dried over anhydrous $MgSO_4$ and the ether evaporated under reduced pressure. The residue was triturated with 1 mL of CCl_4 and the solvent again evaporated to yield 9.3 mg of white product (61% yield). A larger preparation, starting with 2 g of β-alanine, gave the product as colorless, shiny crystals, mp 61–62 °C (lit. mp 62.5 °C; Heilbron & Bunbury, 1959), undepressed by an authentic sample.

(Carboxyethyl)cysteine. Cysteine hydrochloride (18 mg, 0.01 mmol) and 3-bromopropionic acid (15 mg, 0.01 mmol) were dissolved in 1 mL of water, and the pH of the solution was adjusted to 8.0 with 1 M NaOH. The reaction mixture was kept 2 days at room temperature, and then 1 mL of 0.2 N sodium citrate buffer, pH 2.0, was added, and the suspended particles were removed by filtration. (Carboxyethyl)cysteine appeared on the amino acid analyzer as a shoulder in front of glutamic acid.

(Carboxyethyl)histidine Derivatives. 3-Bromopropionic acid (0.73 g, 4.8 mmol) was added to a solution (4.8 mmol) of histidine hydantoin (Stark, 1967) in 5 mL of water. The pH of the reaction mixture was adjusted to 8.0, and the reaction was allowed to proceed at 50 °C for 24 h. The products were hydrolyzed with 6 M HCl at 110 °C for 96 h and subjected to amino acid analysis. N^π- and N^τ-(carboxyethyl)-histidines appeared after phenylalanine whereas N^π,N^τ-(dicarboxyethyl)histidine eluted at the position of isoleucine on the amino acid analyzer, as presumed from the positions of the corresponding carboxymethyl derivatives (Crestfield et al., 1963).

Carboxyethylation. Crystalline horse liver alcohol dehydrogenase was freed from endogenous ethanol by filtration through a column of Sephadex G-50 equilibrated with 46 mM sodium phosphate buffer, pH 7.0, containing 0.25 mM ethylenediaminetetraacetic acid (EDTA). The enzyme (1–8 mg/mL) was carboxyethylated with sodium 3-bromo-

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propionate (1.6–20 mM) at 25 °C. Enzyme activity at 25 °C was measured by the addition of 10-μL samples of the reaction mixture to a 1-mL assay mixture (Plapp, 1970). Carboxyethylated enzyme was freed from excess reagent by gel filtration.

Binding Studies. NADH binding in the absence or presence of isobutyramide was determined by fluorescence measurements using a Hitachi MPF-2A spectrofluorometer with excitation at 330 nm and emission at 440 nm (Yonetani & Theorell, 1962). NADH binding in the presence of isobutyramide was also determined by spectrophotometric titration at 355 nm using the difference extinction coefficient between NADH and the enzyme·NADH·isobutyramide complex of 2500 M^{-1} cm^{-1} (Taniguchi et al., 1967). NAD^+ binding was determined by spectrophotometric titration at 300 nm in the presence of 10 mM pyrazole, where the difference extinction coefficient between the enzyme·NAD^+ and the enzyme·NAD^+·pyrazole complexes is 7200 M^{-1} cm^{-1} (Theorell & Yonetani, 1963). The modified enzyme was also titrated with 2,2′-bipyridine, which gives a difference extinction coefficient of 11 000 M^{-1} cm^{-1} at 308 nm (Sigman, 1967).

Dissociation constants and the number of coenzyme binding sites were obtained by analyzing the data with the methods of Scatchard or Stinson & Holbrook (1973) and by fitting to a straight line with the least-squares programs NONLIN (C. M. Metzler, The Upjohn Co., Kalamazoo, MI) or FLKI (L. Cocco, Department of Pharmacology, St. Jude Children's Research Hospital, Memphis, TN). K_d values and extinction coefficients for bipyridine binding were calculated by using the HYPER program, or if the double-reciprocal plot of the data was not linear, by the TWOONE program (Cleland, 1979).

Other Analytical Procedures. Normal (per subunit) concentrations of liver alcohol dehydrogenase were calculated by using an absorption coefficient of 18 200 N^{-1} cm^{-1} at 280 nm (Bonnichsen, 1950; Jörnvall, 1970). Total sulfhydryl content after inactivation was determined in 8 M urea by the method of Ellman (1959). Spectrophotometric analyses were carried out with a Cary 118C recording spectrophotometer at 25 °C. Peptides were hydrolyzed in glass-distilled 5.7 M HCl in vacuo at 110 °C for 24 h and analyzed on a Beckman Model 121 MB analyzer by using an extended time for the second buffer and the protein hydrolysate or physiological fluid systems. Liquid scintillation counting was performed in 5 or 10 mL of Bray's solution (Bray, 1960). A Beckman 890C sequencer was used for the Edman degradation. Polybrene (6 mg) was used to minimize loss of sample during solvent extractions (Klapper et al., 1978). A Sequemat P-6 autoconverter was used to convert the anilinothiazolinone derivatives to the phenylthiohydantoins, which were identified by chromatography on a Beckman Ultrasphere RP-18 column (4.6 × 250 mm, 5 μm) developed with a gradient of 20–70% methanol in 10 μM sodium acetate, pH 5.

Results and Discussion

Inactivation by 3-Bromopropionic Acid. 3-Bromopropionic acid slowly inactivates liver alcohol dehydrogenase (Figure 1). The pattern of protection by various ligands (at close to saturating concentrations) shows that the inactivation probably occurs by reaction at the active site but that it is qualitatively different than the reactions with iodoacetate. The enyzme is only partially protected against inactivation by NAD^+ and NADH but is completely protected by NAD^+ and pyrazole or NADH and isobutyramide. The partial protection by bipyridine suggests that the reaction is near the active-site zinc, which bipyridine chelates (Boiwe & Brändén, 1977). Imidazole binds to the zinc and stimulates the rate of inactivation

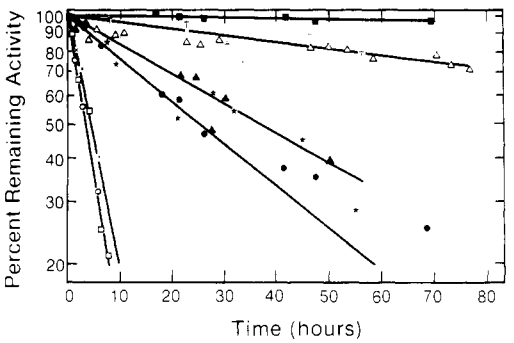

FIGURE 1: Effect of nucleotides and other ligands on the rate of inactivation of liver alcohol dehydrogenase by 3-bromopropionic acid. Enzyme (1 mg/mL, 25 μN) was incubated at 25 °C in 46 mM sodium phosphate buffer and 0.25 mM EDTA, pH 7.0, with 20 mM sodium 3-bromopropionate in the absence of any ligand (□) or in the presence of 1 mM pyrazole plus 1 mM NAD^+ (†), 10 mM isobutyramide plus 0.2 mM NADH (△), 3.2 mM bipyridine (▲), 0.2 mM NADH (*), 1 mM NAD^+ (●), 10 mM imidazole (×), or 1 mM AMP (○). The percent activity relative to the control (■) without reagent at zero time is presented. The lines represent first-order reactions.

Table I: Rates of Inactivation by 3-Bromopropionic Acid in the Presence of Coenzymes and Other Protecting Ligands[a]

additions	second-order rate constant ($\times 10^5$ M^{-1} s^{-1})
$BrCH_2CH_2COOH$	260 ± 20[b]
+1 mM AMP	270
+10 mM imidazole	230
+1 mM NAD^+	43 ± 3[b]
+0.2 mM NADH	27
+3.2 mM bipyridine	26
+1 mM NAD^+ + 1 mM pyrazole	4.8
+0.2 mM NADH + 10 mM isobutyramide	4.8
$BrCH_2CH_2CONH_2$	14
+1 mM pyrazole + 1 mM NAD^+	1.4
+0.20 mM NADH	≦3.1
+10 mM isobutyramide + 0.2 mM NADH	≦3.1
+1 mM NAD^+	≦3.1

[a] The enzyme was treated with 20 mM reagent in 46 mM sodium phosphate buffer, pH 7, containing 0.25 mM EDTA, at 25 °C. The apparent second-order rate constant for inactivation was calculated with the assumption that the reaction was first order in reagent. [b] The mean and standard deviation from four experiments.

by iodoacetic acid, which reacts with Cys-46, but does not stimulate the reaction of 3-bromopropionic acid (Dahl & McKinley-McKee, 1981). The lack of protection by AMP is in contrast to the results observed with iodoacetic acid (Evans & Rabin, 1968; Reynolds et al., 1970. The inactivation reaction requires the carboxyl group of the bromopropionic acid, as 3-bromopropionamide reacted about 20 times more slowly than 3-bromopropionic acid. The apparent second-order rate constants for inactivation are summarized in Table I.

In the absence of NAD^+, carboxyethylation could reduce activity to 2.4% in 25 h, or to 0.3% if the reaction was allowed to proceed for 72 h. In the presence of NAD^+, activity was reduced to about 20% after reaction for 65–70 h, but the kinetics were not first order as indicated by the fact that the apparent rate constant of inactivation decreased with time.

The effect of pH on inactivation by 20 mM bromopropionic acid was determined with various buffers over the pH range from 6 to 9.1 in the absence of NAD^+; the rate of inactivation was about half as fast at pH 9 as at pH 7, but there was no simple pH dependence.

As with other short- and long-chain fatty acids (Winer & Theorell, 1960), 3-bromopropionic acid is a competitive in-

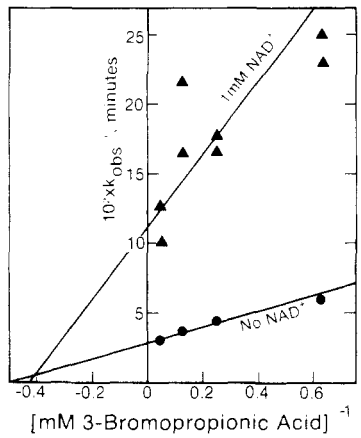

FIGURE 2: Dependence of the rate of inactivation of liver alcohol dehydrogenase on the concentration of 3-bromopropionic acid in the absence or presence of 1 mM NAD^+. The enzyme at a concentration of about 1 mg/mL was treated with 1.6, 4, 8, and 20 mM sodium 3-bromopropionate with (▲) and without (●) 1 mM NAD^+ in 46 mM sodium phosphate and 0.25 mM EDTA buffer, pH 7, at 25 °C. The apparent rate constants were calculated from plots such as those shown in Figure 1. The lines are computed fits (Cleland, 1979) to the equation $k_{obsd} = k_3[I]/(K_i + [I])$, which describes the saturation kinetics of the active-site-directed mechanism (Kitz & Wilson, 1962; Plapp, 1982):

$$E + I \overset{K_i}{\rightleftharpoons} E{\cdot\cdot}I \xrightarrow{k_3} E\text{-}X$$

hibitor of alcohol dehydrogenase activity when tested against varied concentrations of ethanol. At pH 7 and 25 °C, 3-bromopropionic acid gave a slope inhibition constant of 0.41 ± 0.04 mM, which is the dissociation constant for the ternary enzyme·NAD^+·acid complex.

Since 3-bromopropionic acid could inactivate the enzyme by an active-site-directed mechanism, we determined the rate of inactivation with varied concentrations of 3-bromopropionic acid in the presence or absence of 1 mM NAD^+. The reactions were assumed to fit pseudo-first-order kinetics, at least to 50% inactivation. The results in Figure 2 show that the inactivation proceeds as expected for an active-site-directed mechanism. In the absence of NAD^+, the value of K_i was 2.0 ± 0.5 mM, and the pseudobimolecular rate constant (k_3/K_i) was 0.029 M^{-1} s^{-1}. These values are about the same as the values determined previously in a different buffer (Dahl & McKinley-McKee, 1981). The bimolecular rate constant is about half as large as the constant for the reaction of 3-bromopropionate with the thiolate anion of cysteine, but 240 times larger than the constant for the reaction with the mercaptoethanol–zinc complex (Dahl & McKinley-McKee, 1981) and 46 times faster than the facilitated reaction of the halo acid with His-119 in ribonuclease A (Heinrikson et al., 1965). These comparisons suggest that inactivation of alcohol dehydrogenase by 3-bromopropionic acid is facilitated by the binding of reagent to the enzyme.

In the presence of NAD^+, the enzyme reacts with 3-bromopropionic acid with a K_i value of about 2.3 ± 1.1 mM and a k_3/K_i value of 0.0063 M^{-1} s^{-1}. NAD^+ may simply protect the enzyme against inactivation since the overall rate of inactivation is decreased to a fifth of its previous value. This could happen if only 80% of the active sites were occupied by NAD^+, which is possible under the conditions used, since the dissociation constant for NAD^+ is about 250 μM. On the other hand, in the presence of 3-bromopropionic acid, which binds to the enzyme·NAD^+ complex, the apparent dissociation constant for NAD^+ should be smaller, and we would expect that the fraction of active sites that were free would be much less than 20%. Further studies, with varied concentrations of NAD^+, would be required to determine if the enzyme·NAD complex can be inactivated by 3-bromopropionic acid, but the deviation from first-order kinetics during the long reaction times would compromise the conclusions. It was noted previously that the enzyme catalyzes the release of iodide from 3-iodopropionate (Biellmann et al., 1979).

Characterization of Carboxyethylated Enzymes. The residual activities of the carboxyethylated enzymes could be due to the modified enyzmes themselves or to the fraction of unmodified enzyme. To obtain information on this point, we characterized the enzyme carboxyethylated in the absence or presence of NAD^+ with residual activities of 6% or 21%, respectively. The Michaelis constants for ethanol were not significantly different with the modified enzymes as compared to the native enzyme, even though the turnover numbers were reduced in proportion to the extent of inactivation. The turnover numbers were the same with deuterated ethanol. The enzyme carboxyethylated in the presence of NAD^+ showed increasing activity when the pH was increased from 6 to 10 just as did the native enyzme. These results are in clear contrast to those found for carboxymethylated enyzme, where the enzyme with 2% residual activity had increased Michaelis constants for ethanol, an altered pH dependence, and an isotope effect with deuterated ethanol as substrate, due to rate-limiting hydride transfer (Reynolds & McKinley McKee, 1975).

We also determined if the active sites of the modified enzymes could bind coenzyme and various ligands. Carboxyethylated alcohol dehydrogenase, with residual activity of 5% or less, did not detectably bind 2,2′-bipyridine, NAD^+ in the presence of 10 mM pyrazole, or NADH in the presence of 0.1 M isobutyramide, as measured by changes in absorbance at 355 nm or by fluorescence of the NADH. The lack of binding by 2,2′-bipyridine suggests that the zinc in the active site has been affected by the modification, and the lack of detectable binding for the coenzymes suggests that the environment for the nicotinamide ring, which is near the catalytic zinc, has been greatly disturbed or that coenzyme does not bind.

In contrast, the enzyme carboxyethylated in the presence of NAD^+, which had about 20% residual activity, had some remaining binding activity. On the basis of the protein concentration, about half of the active sites could bind NAD^+ in the presence of pyrazole, but with a dissociation constant of 1 μM compared to a K_d of 0.1 μM for the native enzyme. Likewise, about half of the active sites could bind NADH in the presence of 0.1 M isobutyramide as measured by a spectrophotometric titration. About 20% of the sites of enzyme carboxyethylated in the presence of NAD^+ could bind NADH as determined by fluorescence enhancement, with a K_d that was about 0.12 μM, as compared to 0.02 μM for the native enzyme. About 80% of the sites could bind 2,2′-bipyridine, with a K_d of 11 mM, which is much larger than the 0.4 mM observed for the native enzyme, but the other 20% bound bipyridine with the normal K_d value. Although it is difficult to establish, it appears that enzyme carboxyethylated in the presence of NAD^+ is heterogeneous in the binding of various ligands. Some of the residual binding may be due to unmodified enzyme, but the fact that the K_d values for the binding are increased in general can also indicate that modified enyzme has residual binding activity.

Identification of Modified Residues. Amino acid analyses of carboxyethylated enzymes showed no significant differences in composition as compared to native enzyme except for possible decreases in cysteine and histidine content. However, (carboxyethyl)cysteine chromatographed under glutamic acid, and the presumed N^{τ}-(carboxyethyl)histidine derivative appeared as a separate peak after phenylalanine but near the

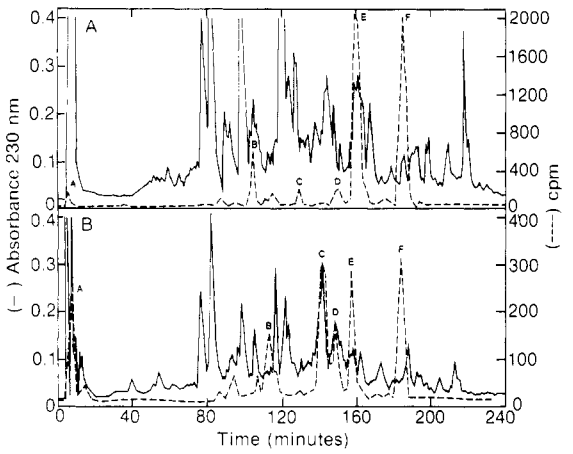

FIGURE 3: Preparation of labeled peptides from proteolytic digests of labeled alcohol dehydrogenases. The enzyme (40 mg) was inactivated with 3-bromo[1-^{14}C]propionic acid to 6% residual activity [in the absence of NAD^+ (A)] or to 18% [in the presence of NAD^+ (B)]. The dialyzed enzyme was aminoethylated (Cole, 1967) and digested with trypsin and chymotrypsin (each totaling 1% of the concentration of the protein, added at 0 and 4 h) for 8 h in 0.2 M *N*-ethylmorpholine acetate buffer, pH 8.1, at 37 °C. Digestion was stopped by addition of acetic acid, and the peptides were passed through a Sephadex G-50 column (1.6 × 93 cm) equilibrated with 0.1 M acetic acid, without obtaining separation of radioactive peptides. Fractions from the radioactive peak [90% of the total radioactivity in (A) and 100% in (B)] were pooled. Samples of 0.5 mL (about 10 mg of peptides) were injected onto a Beckman Ultrasphere RP-18 (4.6 × 250 mm, 5 μm) column and developed with a linear gradient from 0 to 50% acetonitrile in 0.1% (v/v) trifluoroacetic acid at room temperature and a flow rate of about 0.6 mL/min (Mahoney & Hermodson, 1980). The peptides were detected by the absorbance at 230 nm and by scintillation counting of 0.1 mL of the 1.4-mL fractions.

Table II: Distribution and Recovery of Labeled Peptides from Carboxyethylated Alcohol Dehydrogenases

	distribution (% of recovered) for peak (on RP-18 column)						recovered (% of applied)
	A	B	C	D	E	F	
Figure 3A	2.8	4.1	2.1	3.1	40	34	75
Figure 3B	11	10	17	8	12	13	88

change to the third buffer. In several samples of carboxyethylated enzyme, there appeared to be a peak at this position, but it could have been an artifact. When assayed for total sulfhydryl content (Ellman, 1959), carboxyethylated enzyme had 12.3 thiol groups per subunit, as compared to 13.2 determined for the native enzyme, which has 14 cysteine residues. Enzyme carboxyethylated in the presence of NAD^+ had 12.1 thiol groups per subunit. Accessible histidine residues were determined with diethyl pyrocarbonate, which modifies up to five of the seven histidine residues in the enzyme (Hennecke & Plapp, 1983); five could also be modified in carboxyethylated enzyme.

To establish which amino acid residues were modified, enzyme was inactivated with radioactively labeled 3-bromopropionic acid. In the absence of NAD^+, about 1.5 equiv of reagent was incorporated at 90% inactivation, and in the presence of NAD^+, about 1.0 equiv was incorporated at 80% inactivation. The labeled enzymes were digested with trypsin and chymotrypsin, and the peptides from this mixture were separated by chromatography on an octadecyl reverse-phase column as shown in Figure 3. Carboxyethylated enzyme (Figure 3A) had two major labeled peptides, E and F, whereas enzyme labeled in the presence of NAD^+ (Figure 3B) had several peptides in significant amounts.

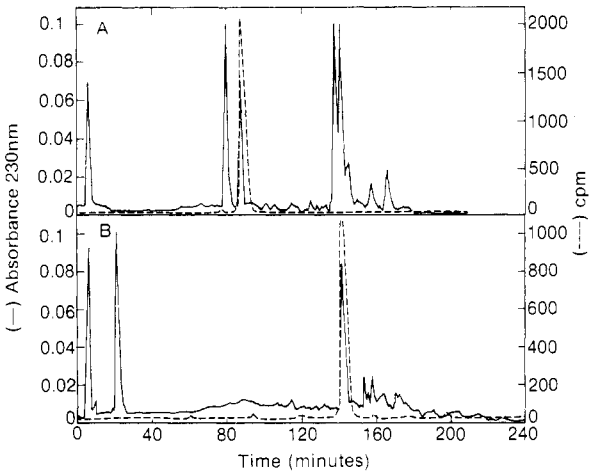

FIGURE 4: Rechromatography of the major peptides E (panel A) and F (panel B) from Figure 3A. A gradient from 0 to 50% (v/v) acetonitrile in 0.1 M sodium perchlorate containing 5 mM sodium phosphate buffer, pH 7, was used to develop the RP-18 column (Meek, 1980).

Table II summarizes the peptide purification. Most of the radioactivity applied to the column could be recovered. About 74% of the material recovered from purification of the peptides from carboxyethylated enzyme (Figure 3A) was distributed in peptides E and F. On the other hand, enzyme carboxyethylated in the presence of NAD^+ had a wide distribution of radioactive peptides. Only 25% of the label was in peptides E and F, and the number and amount of the other peptides indicate that the reaction was not specific.

The labeled materials isolated from the columns shown in Figure 3 were further purified by rechromatography under different conditions, as shown for two examples in Figure 4. In each case, the labeled material chromatographed with a single peak of material that absorbed at 230 nm. Since the columns shown in Figure 4 were developed with buffers containing sodium perchlorate, the peptides were desalted by chromatography again under the conditions shown in Figure 3.

Amino acid analyses showed that peptides E and F originated from the sequence 171–176 in alcohol dehydrogenase, Leu-Ile-Gly-Cys-Gly-Phe (Jörnvall, 1970). As was found previously for enzyme inactivated by diazonium-1*H*-tetrazole (Sogin & Plapp, 1976), this sequence yielded two peptides, apparently due to slow cleavage by chymotrypsin. Peptide F corresponds to residues 171–176 and peptide E to residues 172–176. The compositions clearly indicated that Cys-174 had been modified. This identification was confirmed by sequence analysis of peptide F from carboxyethylated enzyme. The amino acids expected at each cycle of the Edman degradation were found, and (carboxyethyl)cysteine was obtained in the fourth cycle, associated with more than two-thirds of the radioactivity extracted in the six cycles [the phenylthiohydantoin of (carboxyethyl)cysteine chromatographed near the position of the phenylthiohydantoin of methionine]. It appears that enzyme in the absence of NAD^+ is quite selectively carboxyethylated at Cys-174 and that inactivation is due to modification of this residue.

In the presence of NAD^+, carboxyethylation of Cys-174 is retarded, and modification of other sites occurs during the long reaction time. The compositions of peptides B, C, and D were unfortunately not identifiable with any known sequence in alcohol dehydrogenase. The only peptide obtained from enzyme carboxyethylated in the presence of NAD^+ in sufficient amounts for sequence analysis, peptide C, had a composition

consistent with the sequence 48–57, but it was refractory to Edman degradation. Although we were not able to determine what other sites were modified in this enzyme, it is clear that it would be difficult to ascribe inactivation to modification of any particular residues.

Basis for Selective Reaction and Inactivation. From inspection of the three-dimensional structure of native horse liver alcohol dehydrogenase (Eklund et al., 1976), it appears most reasonable to suggest that 3-bromopropionic acid binds to the free enzyme with its carboxyl group interacting with the guanidino group of Arg-369. Model-building experiments show that if 3-bromopropionic acid were bound to this arginine, the methylene would be in a suitable position to react with the sulfur of Cys-174. Nevertheless, Cys-46 is also nearby, and it is not obvious why 3-bromopropionic acid would not react with its sulfur. It has been suggested that iodoacetate interacts with Arg-47 while selectively alkylating Cys-46 (Zeppezauer et al., 1975), but our view of the structure suggests that iodoacetate could also bind to Arg-369 while reacting with Cys-46. It has also been suggested that the carboxyl group of (*S*)-2-chloro-3-(imidazol-5-yl)propionate binds to both Arg-47 and Arg-369 during its stereospecific reaction at Cys-46 (Dahl et al., 1983).

Cys-174 also reacts with diazonium-1*H*-tetrazole (Sogin & Plapp, 1976). Two alkylating coenzyme analogues, 3-[4-(bromoacetyl)pyridinio]propyldiphosphoadenosine and nicotinamide 4-methyl-5-(bromoacetyl)imidazole dinucleotide, react with Cys-174, but 4-[3-(bromoacetyl)pyridinio]butyldiphosphoadenosine reacts with Cys-46 (Woenckhaus et al., 1979). The homologous cysteine residues in yeast alcohol dehydrogenase (Cys-43 and Cys-153; Jörnvall et al., 1978) can also be modified selectively with different reagents. The coenzyme analogues all react with Cys-43, but iodoacetate, iodoacetamide, and butyl isocyanate react with either cysteine residue, the ratio of products depending upon pH (Belke et al., 1974). Styrene oxide reacts with both cysteine residues in the yeast enzyme (and probably in the liver enzyme), but the yeast enzyme is inactivated 10 times faster than is the liver enzyme (Klinman et al., 1977). The differing reactivities of these reagents suggest that the structures of the enzymes are different, but the selectivities of the reaction have not been explained in terms of these structures.

Arg-369 in the horse liver enzyme participates in the binding of coenzyme by interacting with the phosphate attached to the nicotinamide ribose (Eklund et al., 1981); thus, carboxyethylation of Cys-174 could inactivate the enzyme by blocking the binding of coenzyme. Furthermore, modification of Cys-174 should interfere with the binding of bipyridine to the zinc as does carboxymethylation of Cys-46 (Hennecke & Plapp, 1983). Crystallographic investigations would be required to determine how the carboxyethyl group affects ligand binding.

In the complexes of the enzyme with coenzyme, access of 3-bromopropionic acid to Cys-174 would be severely restricted. Some of the modification that occurs in the presence of NAD^+ is probably due to the reaction of free enzyme, which does not have bound NAD^+. However, it appears that inactivation in the presence of NAD^+ can also occur because of reaction at other sites. One such site could be His-51, which is on the surface of the enzyme and is thought to be involved in a proton relay system (Eklund et al., 1982). Modification of five histidine residues with diethyl pyrocarbonate leads to inactivation of the enzyme, probably due to modification of His-51 (Hennecke & Plapp, 1983). If His-51 were carboxyethylated, activity should be decreased, but the modified enzyme could still bind coenzymes, albeit with decreased affinities.

Acknowledgments

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Registry No. NAD, 53-84-9; alcohol dehydrogenase, 9031-72-5; Cys, 52-90-4; 3-bromopropionic acid, 590-92-1; 3-bromo[1-^{14}C]-propionic acid, 4615-72-9.

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Subunit Binding in the Pyruvate Dehydrogenase Complex from Bovine Kidney and Heart†

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ABSTRACT: Binding of pyruvate dehydrogenase (E_1) and dihydrolipoamide dehydrogenase (E_3) to the isolated dihydrolipoamide acetyltransferase (E_2) core of the pyruvate dehydrogenase complex from bovine heart and kidney was investigated with equilibrium, competitive binding, and kinetic methods. E_2, which consists of 60 subunits arranged with icosahedral 532 symmetry, apparently possesses six equivalent, noninteracting binding sites for E_3 dimers. It is proposed that each E_3 dimer extends across 2 of the 12 faces of the E_2 pentagonal dodecahedron. The equilibrium constant (K_d) for dissociation of E_3 from E_2 is about 3 nM, and the dissociation rate constant is about 0.057 min^{-1}. For E_1, K_d is about 13 nM, and the dissociation rate constant is about 0.043 min^{-1}. Extensive phosphorylation of E_1 (about three phosphoryl groups per E_1 tetramer) increases K_d to about 40 nM.

The mammalian pyruvate dehydrogenase complex is composed of multiple copies of three major components: pyruvate dehydrogenase (E_1),[1] dihydrolipoamide acetyltransferase (E_2), and dihydrolipoamide dehydrogenase (E_3). The E_2 component forms a structural core, composed of 60 subunits arranged with icosahedral 532 symmetry in a pentagonal dodecahedron like particle, to which E_1 and E_3 are bound by noncovalent bonds [for a review, see Reed & Pettit (1981)]. In solution, uncomplexed E_1 is a tetramer ($\alpha_2\beta_2$) and E_3 is a homodimer. The bovine kidney pyruvate dehydrogenase complex contains about 20 E_1 tetramers and about 6 E_3 dimers, whereas the heart complex contains about 30 E_1 tetramers and about 6 E_3 dimers. The kidney complex can bind about 10 additional E_1 tetramers, but neither complex can bind additional E_3 dimers (Barrera et al., 1972; L. Hamilton, P. Munk, and L. J. Reed, unpublished data). It is difficult to reconcile the presence of only six E_3 dimers (or 12 E_3 polypeptide chains) with the apparent icosahedral 532 symmetry of the E_2 core.

It is interesting to note in this connection that in the native pyruvate dehydrogenase complex from *Escherichia coli*, 24 E_1 chains and 12 E_3 chains are attached to the E_2 core, which is composed of 24 subunits arranged with 432 symmetry in a cubelike particle (Eley et al., 1972). E_3 is present in the complex as dimers (Reed et al., 1975; Coggings et al., 1976). One E_3 dimer is thought to be located on each of the six faces of the E_2 core (Oliver & Reed, 1982). Binding studies (Reed et al., 1975) indicated that the *E. coli* E_2 core can bind up to 24 E_1 dimers in the absence of E_3 and up to 24 E_3 dimers in the absence of E_1. When both E_1 and E_3 are present, the binding stoichiometry approaches 12 E_1 dimers and 6 E_3 dimers. This stoichiometry is the same as that of the native complex and produces maximum activity. Thus, although there appears to be a binding site (domain) on each of the 24 E_2 subunits for an E_1 dimer and a separate binding site on each E_2 subunit for an E_3 dimer, steric hindrance between the relatively bulky E_1 and E_3 dimers apparently prevents the E_2 core from binding 24 molecules of each ligand.

To gain further insight into the molecular basis of the unequal ratio of E_3 and E_2 subunits in the mammalian pyruvate dehydrogenase complex, we studied binding of E_3 and E_1

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[1] Abbreviations: E_1, pyruvate dehydrogenase; E_2, dihydrolipoamide acetyltransferase; E_3, dihydrolipoamide dehydrogenase; M_r, molecular weight; EDTA, ethylenediaminetetraacetate.

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