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Bromoacetophenone as an Affinity Reagent for Human Liver Aldehyde Dehydrogenase†

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ABSTRACT: Human liver aldehyde dehydrogenase isozymes E1 and E2 (EC 1.2.1.3) are both completely and irreversibly inactivated by bromoacetophenone (2-bromo-1-phenylethanone). Steady-state kinetics with both acetophenone and chloroacetophenone indicated interaction with the same enzyme form as the aldehyde substrate. Saturation kinetics with chloroacetophenone and bromoacetophenone indicated interaction at a specific site on the enzyme surface and gave a dissociation constant similar to that from steady-state kinetics, suggesting that the same processes were being observed by both methods and that the active site may be involved. Protection against inactivation was afforded by chloral and NAD together. Stoichiometry of inactivation showed the first 2 equiv per tetramer to abolish the majority of catalytic activity; 4 equiv inactivated both isozymes with complete loss of esterase, NAD-stimulated esterase, and dehydrogenase activities. Peptide mapping of enzyme modified with [*carbonyl*-^{14}C]bromoacetophenone of CNBr digests (E1) and tryptic digests (E1 and E2) showed one peptide to be preferentially labeled. The above results together with the similarity of bromoacetopenone to the substrate benzaldehyde suggest bromoacetophenone may react with a residue in the active site of aldehyde dehydrogenase. Amino acid analysis of the labeled E1 tryptic fragment indicated reaction with a different peptide from that with which iodoacetamide reacts.

The catalytic mechanism of aldehyde dehydrogenase is thought to involve an enzyme nucleophile that forms a covalent intermediate with the aldehyde substrate (Dunn & Buckley, 1985). Currently, it is suspected that the enzyme nucleophile is a cysteine residue. Evidence for this residue being a cysteine, however, is most circumstantial, arising from comparison with glyceraldehyde-3-phosphate dehydrogenase, sensitivity of the enzyme to sulfhydryl reagents, and the identification of a hyperreactive cysteine residue (Hempel & Pietruszko, 1981).

Chemical modification offers a strong tool for identification of catalytically important residues. Three approaches may be used in the chemical modification of protein. One involves the use of a reagent specific for a certain type of amino acid residue to selectively modify a hyperactive residue, which is often found in the active site (Baker, 1967). The second approach involves the design of a reagent that is structurally similar to a substrate or transition-state intermediate (Pauling, 1948) and contains a reactive functional group that is positioned on the reagent enabling formation of a covalent bond with residues positioned in the active site. This approach may or may not label the catalytic residue; however, it will always label residues within the active site (Baker, 1967; Singer, 1967; Vallee & Riordan, 1969; Shaw, 1970). Third is more specialized approaches including suicide inactivators (Abeles & Maycock, 1976) and photoaffinity reagents (Chowdry & Westheimer, 1979).

Previous work with human liver aldehyde dehydrogenase used the general reagent approach with the sulfhydryl reagent iodoacetamide (Hempel & Pietruszko, 1981). The work led

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to the identification of a hyperreactive residue; cysteine 302 of the E1 isozyme (Hempel et al., 1982b, 1984). However, the lack of total inactivation precluded assignment of this cysteine to the active site. In this investigation, work toward identification of residues located at the active site was performed by attempting to develop an active site directed reagent for aldehyde dehydrogenase. This work started with the previously established fact that acetophenone is a substrate-competitive inhibitor of aldehyde dehydrogenase (Deitrich & Hellerman, 1963). Along with acetophenone, experiments in this investigation used the reagents chloroacetophenone (2-chloro-1-phenylethanone) and bromoacetophenone (2-bromo-1-phenylethanone), both the E1 (cytoplasmic) and the E2 (mitochondrial) isozymes of aldehyde dehydrogenase, and the criteria as stated by Shaw (1970) to indicate interaction at the active site.

Experimental Procedures

Materials

All chemicals were reagent-grade. Propionaldehyde (J. T. Baker) was redistilled prior to use. Guanidine hydrochloride was ultrapure from Schwarz/Mann. HPLC[1]-grade methanol was from J. T. Baker, HPLC-grade sodium acetate was from Fisher, and trifluoroacetic acid was purchased from Pierce. Acetophenone, bovine serum albumin, *p*-nitrophenyl acetate, and NADH were from Sigma. Bromoacetophenone and chloroacetophenone (Aldrich) were twice recrystallized in MeOH–H_2O (8:2) prior to use. Other reagents included chloral hydrate (Matheson, Coleman and Bell), NAD (Boehringer Mannheim), and TPCK-treated trypsin (Worthington). [*carbonyl*^{14}C]Acetophenone (batch 1) was purchased from Amersham.

Methods

Enzyme Preparation. E1 and E2 isozymes of human liver aldehyde dehydrogenase were purified to homogeneity following the procedure of Hempel et al., (1982a). Homogeneity was confirmed by isoelectric focusing, starch gel electrophoresis, and specific activity. The enzymes were stored at −12 °C in well-nitrogenated 30 mM sodium phosphate, pH 6.0, containing 1 mM EDTA, 2% v/v 2-mercaptoethanol, and 25% glycerol. Prior to use, the enzymes were dialyzed in a Schleicher & Schuell apparatus against eight changes of nitrogen-saturated 30 mM sodium phosphate, pH 6.0, containing 1 mM EDTA to remove thiols and glycerol.

Nitrogen-saturated buffers were used in all experiments (except where otherwise stated) to prevent air oxidation of enzyme sulfhydryls. Buffers were first exhaustively evacuated on an aspirator at room temperature to remove dissolved air. Nitrogen was then continuously bubbled through the buffer while it was cooled to 4 °C to achieve saturation, and the buffer was then stored in a firmly capped container at 4 °C.

Determination of Enzyme Specific Activity. The standard assay system used to monitor aldehyde dehydrogenase activity was similar to that of Greenfield and Pietruszko (1977). Included in the assay mixture were 0.1 M sodium pyrophosphate, pH 9.0 (not nitrogenated), 500 μM NAD, 1 mM propionaldehyde, and 1 mM EDTA in a 3.0-mL total volume. Reactions were initiated by the addition of enzyme, and reaction rates were determined by taking tangents to steady-state portions of time progress curves. Time progress curves were measured on a Varian 635 recording spectrophotometer or a Gilford 252 updated Beckman DU spectrophotometer at 25 °C in 1-cm light path cuvettes by following the production of NADH at 340 nm and by using an extinction coefficient of 6.22 mM^{-1} cm^{-1}. Protein concentrations were determined with both 280-nm absorption (Greenfield & Pietruszko, 1977) and the procedure of Lowry et al. (1951) with bovine serum albumin as a standard.

Esterase activity was measured in nitrogen-saturated 30 mM sodium phosphate, pH 7.0, containing 1 mM EDTA by following the hydrolysis of *p*-nitrophenyl acetate to *p*-nitrophenol at 400 nm with an extinction coefficient of 9.45 mM^{-1} cm^{-1}. NAD-stimulated esterase activity was determined in the same system with 500 μM or 1 mM NAD. Reaction rates were determined by taking tangents to the steady-state portions of time-progress curves. Adjustments were made to account for the spontaneous hydrolysis of *p*-nitrophenyl acetate in the assay system. Stock solutions of *p*-nitrophenyl acetate were assayed by measuring the increase in absorbance at 400 nm following hydrolysis in 0.1 N sodium hydroxide with an extinction coefficient of 18 mM^{-1} cm^{-1} (Kezdy & Bender, 1962).

Steady-State Kinetics. Steady-state kinetics were performed in well-nitrogenated 30 mM sodium phosphate, pH 7.0, containing 1 mM EDTA, 1.5 mM NAD, and glycolaldehyde at concentrations of 12–250 μM. The data were graphed following the procedure of Lineweaver and Burk (1934).

Synthesis of [carbonyl-^{14}*C]Bromoacetophenone.* Since the studies required the synthesis of a radioactive α-haloacetophenone (which is not available commercially), due to the relative ease of bromination of the α-methyl group of acetophenone, bromoacetophenone was used. Radiolabeled bromoacetophenone was synthesized by incorporating [*carbonyl-*^{14}C]acetophenone (60 mCi/mmol) into the procedure of Rather and Reid (1919). Initially, the procedure was carried out on a large scale with cold material, which was then subjected to ^{1}H NMR to confirm both identity and purity. Synthesis of the labeled material proceeded as follows: 0.23 g of acetophenone (250 μCi of hot material diluted with cold acetophenone) was added to 0.34 g of glacial acetic acid and stirred continuously at room temperature (the glacial acetic acid was mixed with P_2O_5 and redistilled in a standard distillation apparatus prior to use to remove H_2O). To the stirring mixture one drop of bromine (Aldrich) was added. The resultant solution was allowed to stir until the reddish brown color disappeared, following which the remainder of the bromine (0.1 mL) was added dropwise. Following completion of the reaction (total disappearance of reddish brown color), the solution was diluted with 5 mL of distilled water and placed on ice for 1 h, during which time crystals formed. If necessary, a seed crystal of bromoacetophenone could be added to aid in the crystal formation. The crystals were then collected and recrystallized once in hot MeOH–H_2O (8:2), yielding 211 mg (55.4% yield) of white crystals with a specific activity of 6.8 × 10^5 cpm/μmol. The melting point of the crystals was found to be 48–50 °C, as compared to 50 °C reported in the *Merck Index* (1976).

Structural work required bromoacetophenone of a higher specific activity than that synthesized above. This was prepared by adding 11.5 mg of acetophenone (including 250 μCi of labeled acetophenone) to 20 mg of glacial acetic acid and reacting the mixture with 0.01 mL of bromine. The reaction mixture was then diluted in 1 mL of distilled water, and the resulting crystals were collected; however, to conserve material,

[1] Abbreviations: NAD, nicotinamide adenine dinucleotide (oxidized form); NADH, nicotinamide adenine dinucleotide (reduced form); TPCK, 1-(tosylamido)-2-phenylethyl chloromethyl ketone; HPLC, high-performance liquid chromatography; EDTA, ethylenediaminetetraacetic acid.

the recrystallization step was omitted. The [*carbonyl*-^{14}C]-bromoacetophenone produced had a specific activity of 7.6 × 10^6 cpm/μmol.

Inactivation Experiments. Experiments involving the inactivation of aldehyde dehydrogenase were performed in nitrogen-saturated 30 mM sodium phosphate, pH 7.0, containing 1 mM EDTA, at an enzyme concentration of 0.05 mg/mL. Incubations were initiated by the addition of bromoacetophenone into the enzyme-containing solution while vortexing. In the case of large incubations (i.e., to produce material for structural work), the enzyme was diluted into half of the total incubation volume, the bromoacetophenone was diluted in the other half, and the two were mixed together to initiate the incubation. Controls contained no bromoacetophenone. Incubations were performed for 16–20 h to ensure that all reactions went to completion. For time course experiments, protecting agents were added along with the enzyme, prior to determining the initial activity. Following determination of the initial activity, the inactivator was added to initiate the reaction. Aliquots were then withdrawn at various time intervals for determination of activity remaining in the standard pH 9.0 assay system.

Saturation kinetics (Kitz & Wilson, 1962) were performed with the E2 isozyme (0.05 mg/mL) and both chloroacetophenone and bromoacetophenone in nitrogen-saturated 30 mM sodium phosphate, pH 7.0, containing 1 mM EDTA at 0 °C in the presence and absence of 1 mM NAD, with enzyme activity being determined in the standard pH 9.0 assay system. Due to the high rate of inactivation by both compounds, only one time point (30 s) was used for determination of k_{app}. Results are presented as $1/k_{app}$ vs. $1/[I]$.

Stoichiometry of Incorporation. The stoichiometry of incorporation was measured by incubating different molar ratios of [*carbonyl*-^{14}C]bromoacetophenone with both isozymes (0.05 mg/mL) in nitrogen-saturated 30 mM sodium phosphate, pH 7.0, containing 1 mM EDTA and 1 mM NAD for 16 h with E1 and 20 h with E2 at 25 °C. The E1 isozyme was incubated with [*carbonyl*-^{14}C]bromoacetophenone (7.6 × 10^6 cpm/μmol) at stoichiometries of 1–8 equiv per tetramer while E2 was incubated with [*carbonyl*-^{14}C]bromoacetophenone (6.8 × 10^5 cpm/μmol) at stoichiometries of 2 and 4 equiv per tetramer. Following the incubation, the samples were concentrated in an Amicon Diaflo Ultrafiltration Apparatus, followed by further concentration and dialysis vs. six changes of nitrogen-saturated 30 mM sodium phosphate, pH 6.0, containing 1 mM EDTA in a Schleicher & Schuell apparatus. Samples were then centrifuged to remove precipitated protein, and the specific activity was determined at pH 9.0. Controls were carried through the same procedure except that bromoacetophenone was omitted. [*carbonyl*-^{14}C]Bromoacetophenone incorporated was then determined by counting an aliquot of the final protein solution with Biofluor scintillation fluid (New England Nuclear) and an Intertechnique SL30 liquid scintillation spectrometer.

Peptide Maps. Peptide maps were done on cyanogen bromide digests via polyacrylamide gel electrophoresis (PAGE) (Laemmli, 1970) or on tryptic digests via high-performance liquid chromatography on Waters HPLC system. Prior to analysis, the samples were carboxymethylated and digested with either cyanogen bromide or trypsin following the procedure of Hempel et al. (1984). The PAGE used a 16% (w/v) running and a 6% (w/v) stacking gel with the radiolabeled fragments visualized by autoradiography. The HPLC used a C_{18} μBondapak column (reversed phase, Waters) with a mobile phase of 0.1% v/v trifluoroacetic acid in water, and the column was developed with methanol, run in a linear 0–75% gradient. Runs were performed at a flow rate of 1.5 mL/min, and fractions were collected at 1-min intervals from which 0.5-mL aliquots were removed for scintillation counting.

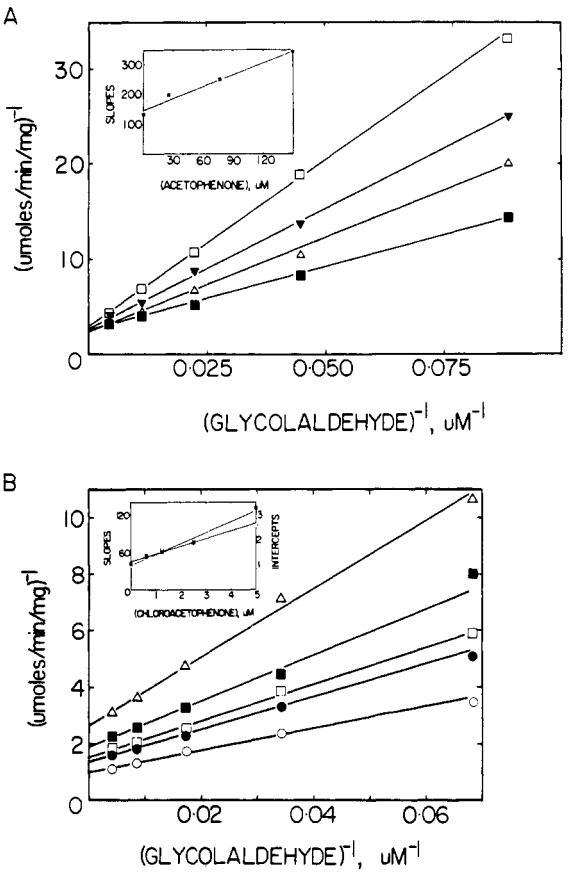

FIGURE 1: Kinetic studies of the dehydrogenase reaction catalyzed by the E2 isozyme with acetophenone and chloroacetophenone as dead-end inhibitors. In (A), the acetophenone levels are 0 (■), 26 (△), 77 (▼), and 150 μM (□) while in (B) the chloroacetophenone levels are 0 (○), 0.63 (●), 1.25 (□), 2.50 (■), and 5.00 μM (△). The inset of (A) is the slope replot yielding a K_i value of 110 μM for acetophenone. The inset to (B) is the slope (■) and intercept (□) replots yielding K_i values of 2.5 and 3.7 μM, respectively, for chloroacetophenone.

RESULTS

Steady-State Kinetics. Results for steady-state kinetics with the E2 isozyme vs. varied glycolaldehyde and with acetophenone as a dead-end inhibitor are presented in Figure 1A. Glycolaldehyde was used for this experiment instead of propionaldehyde due to its K_m for the E2 isozyme being 50 μM, vs. 1 μM for propionaldehyde. The pattern obtained was competitive, implicating binding of acetophenone to the same enzyme form as glycolaldehyde. A K_i (Table I) from the slope replot (see insert of Figure 1A) of 110 μM was determined.

Due to rapidity of covalent bond formation, steady-state kinetics with bromoacetophenone were impossible to perform. Steady-state kinetics were therefore performed vs. varied glycolaldehyde with chloroacetophenone as a dead-end inhibitor (see Figure 1B). Results gave a noncompetitive pattern. K_i values (Table I) for the slope and intercept replots (see insert of Figure 1B) were 2.5 and 3.7 μM, respectively.

Inactivation of Aldehyde Dehydrogenase by Bromoacetophenone. Inactivation of both the E1 and E2 isozymes with bromoacetophenone at a stoichiometry of 4 equiv per enzyme tetramer was followed vs. time at 0 °C. Results with E1 showed 93% of activity lost within 30 s while 58% of activity

Table I: Kinetic Constants for E2 Isozyme with Acetophenone, Chloroacetophenone, and Bromoacetophenone

inhibitor	method	result	K_i (μM)	k_3 (s^{-1})
acetophenone	steady state	competitive pattern (Figure 1A)	110	
chloroacetophenone	steady state	noncompetitive (Figure 1B)		
		slopes	2.5	
		intercepts	3.7	
	saturation kinetics (Figure 3A)			
	in the absence of NAD	y-axis intercept	8.5	0.035
	in the presence of NAD	y-axis intercept	3.8	0.016
bromoacetophenone	saturation kinetics (Figure 3B)			
	in the absence of NAD	y-axis intercept	2.1	0.068
	in the presence of NAD	y-axis intercept	4.3	0.084

was lost with E2 within the same time interval.

Both E1 and E2, at concentrations of 0.05 mg/mL (0.23 μM for the tetramer), were incubated with 1–4 equiv per tetramer of bromoacetophenone overnight at 25 °C. Results for the inactivation of E1, performed in the presence of 1 mM NAD, for both dehydrogenase and NAD-stimulated esterase activities can be seen in Figure 2A. Figure 2 shows results on the inactivation of E2, both in the absence of NAD (Figure 2B) and in the presence of 1 mM NAD (Figure 2C) for dehydrogenase, esterase, and NAD-stimulated esterase activities. These results show that the treatment of both isozymes for 16–20 h with 2 equiv of bromoacetophenone inactivates the majority of catalytic activity with total inactivation occurring in the presence of 3 equiv with E1 and 4 equiv with E2.

Stoichiometry of Incorporation Using [carbonyl-^{14}C]-Bromoacetophenone. ^{14}C-Labeled bromoacetophenone was next used to confirm the stoichiometry of incorporation vs. activity loss for both isozymes. Figure 2D shows the stoichiometry of incorporation of [*carbonyl*-^{14}C]bromoacetophenone vs. activity loss with the E1 isozyme, indicating the majority of activity to be lost upon incorporation of 2 equiv per tetramer while total inactivation occurs at 3–4 equiv per tetramer. Results from the incubation of E2 with 2 and 4 equiv of ^{14}C-labeled 2-bromoacetophenone in the presence of 1 mM NAD showed 1.96 molecules of bromoacetophenone bound per tetramer with 30% activity remaining and 3.34 molecules of bromoacetophenone bound per tetramer with only 1% activity remaining. These results correlated well with those in Figure 2A,B and confirmed that stoichiometric amounts of bromoacetophenone completely inactivate aldehyde dehydrogenase.

Attempts to Reverse Inactivation. Samples of E2 treated with 2 and 4 equiv of bromoacetophenone per tetramer in the absence of NAD (Figure 2B) were treated with 143 mM 2-mercaptoethanol. No reversal of the inactivation occurred upon addition of the 2-mercaptoethanol. Other inactivated samples were exhaustively dialyzed vs. six changes of 30 mM sodium phosphate, pH 6.0, containing 1 mM EDTA, and found to be still inactivated after dialysis. Furthermore, with both isozymes, when [*carbonyl*-^{14}C]bromoacetophenone was used, it was seen that the radiolabel was retained following denaturation in 6 M guanidine hydrochloride and exhaustive dialysis.

Saturation Kinetics. With the E2 isozyme, saturation kinetics (Kitz & Wilson, 1962) were performed to obtain more information about the specificity of the interaction with chloroacetophenone and bromoacetophenone. Results with both chloroacetophenone and bromoacetophenone (see panels A and B Figure 3, respectively) show the presence of distinct y-axis intercepts, suggesting that both compounds are recognized by the enzyme prior to any covalent interaction. From these results, K_i values of 8.5 and 3.8 μM and k_3 values (rate constant for covalent bond formation) of 0.035 and 0.016 s^{-1}

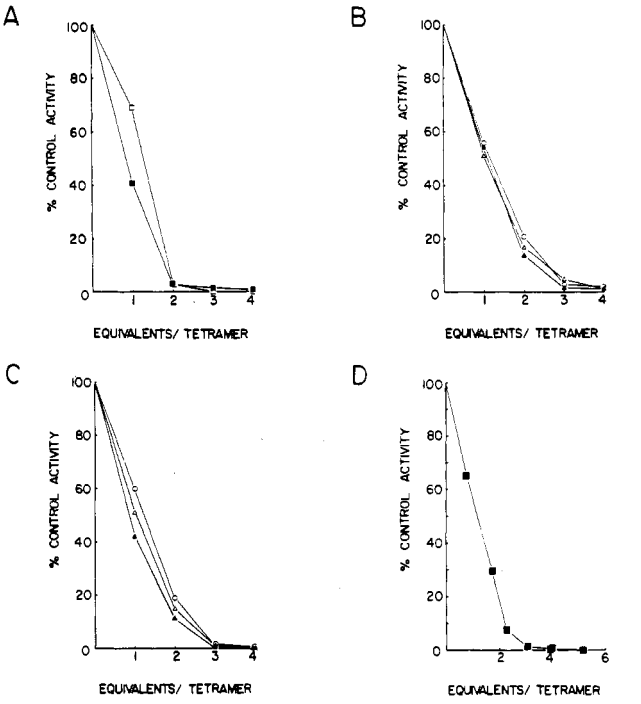

FIGURE 2: Inactivation of E1 and E2 isozymes with bromoacetophenone and the stoichiometry of incorporation of [*carbonyl*-^{14}C]-bromoacetophenone. (A) Comparison of dehydrogenase and NAD-stimulated esterase activity loss due to bromoacetophenone inactivation of the E1 isozyme. E1 (0.05 mg/mL) was incubated with stoichiometric amounts of bromoacetophenone (0–4 equiv per M_r 216 000) for 20 h at 25 °C in 30 mM sodium phosphate, pH 7.0, and 1 mM EDTA containing 1 mM NAD. Dehydrogenase activity (■) was determined in 0.1 M pyrophosphate, pH 9.0, and 1 mM EDTA containing 1 mM propanal and 500 μM NAD while NAD-stimulated esterase activity (□) was determined in 30 mM phosphate, pH 7.0, and 1 mM EDTA containing 200 μM p-nitrophenyl acetate and 500 μM NAD. (B) Comparison of dehydrogenase, esterase, and NAD-stimulated esterase activity loss due to bromoacetophenone inactivation of the E2 isozyme. E2 (0.05 mg/mL) was incubated with stoichiometric amounts of bromoacetophenone (0–4 equiv per M_r 216 000) in well-nitrogenated 30 mM phosphate, pH 7.0, containing 1 mM EDTA for 16 h at 25 °C. Activity remaining for the dehydrogenase reaction (○) was determined in 0.1 M pyrophosphate, pH 9.0, and 1 mM EDTA containing 1 mM propanal and 500 μM NAD while esterase activity (▲) was determined in 30 mM sodium phosphate, pH 7.0, and 1 mM EDTA containing 200 μM p-nitrophenyl acetate with 1 mM NAD added for the NAD-stimulated esterase activity (▲). (C) The same as (B) except the E2 isozyme was incubated with bromoacetophenone in the presence of 1 mM NAD. (D) Stoichiometry of incorporation of [*carbonyl*-^{14}C]bromoacetophenone vs. activity loss with the E1 isozyme in the presence of NAD (1.0 mM). See Methods for experimental procedure.

(Table I) were determined for chloroacetophenone in the presence and absence of 1 mM NAD, respectively. With bromoacetophenone in the presence of 1 mM NAD (Figure 3B), a K_i of 4.3 μM and a k_3 of 0.084 s^{-1} were determined, and in the absence of NAD, a K_i of 2.1 μM and a k_3 of 0.068 s^{-1} (not shown) were determined.

Table II: Distribution and Recovery of Radioactive Peaks Collected from Peptide Maps of 2-Bromoacetophenone-Modified Aldehyde Dehydrogenase

sample	% recovered of initial counts applied	% of total counts collected per peak[a]
E1 (Figure 5A)	96	11.5 (14–15), 3.8 (29–30), 14.4 (35–36), 46.8 (40–42), 15.8 (55–56)
E2 (Figure 5B)	87	7.6 (11), 7.0 (28–29), 5.7 (37–38), 42.3 (40–41), 6.5 (43–44)

[a] Numbers in parentheses indicate fractions pooled.

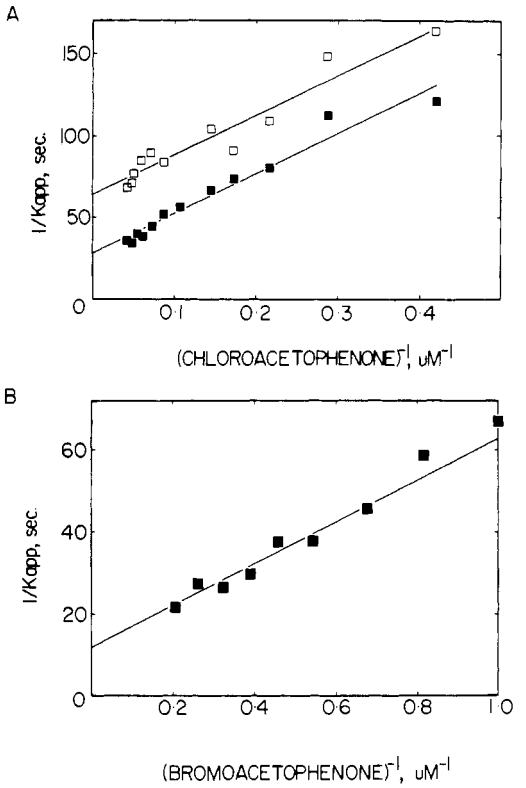

FIGURE 3: Saturation kinetics with the E2 isozyme and inhibitors chloroacetophenone and bromoacetophenone. The E2 isozyme (0.23 μM) was incubated with (A) chloroacetophenone (2.3–23.3 μM) in the absence (■) and presence (□) of 1 mM NAD and (B) bromoacetophenone (1.0–8.1 μM) in the presence of 1 mM NAD for 30 s at 0 °C. Values of K_{app} were determined by taking the natural log of the percent activity remaining at 30 s, with initial activity being determined prior to addition of inhibitor.

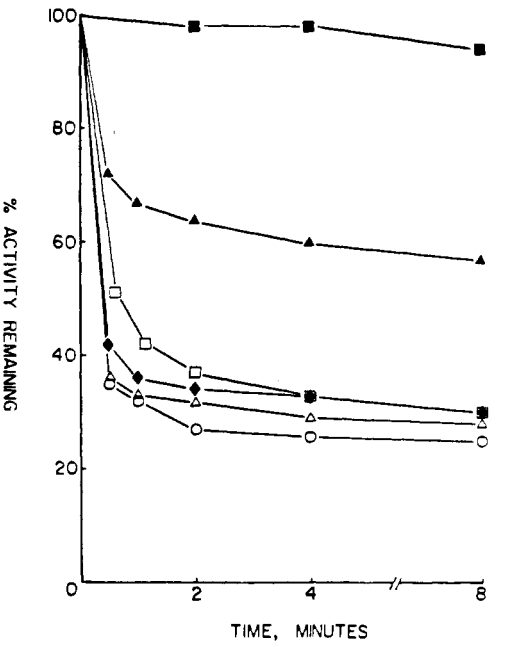

FIGURE 4: Effects of coenzyme and chloral hydrate on bromoacetophenone inactivation of the E2 isozyme. E2 (0.05 mg/mL) was inactivated by 0.92 μM bromoacetophenone (4 equiv per tetramer) either alone (♦) or in the presence of 1 mM chloral hydrate (□), 1 mM NAD (△), 1 mM NADH (○), and both 1 mM chloral and 1 mM NAD together (▲). Initial activity in the presence of protecting agents was determined prior to addition of bromoacetophenone. Control contained neither bromoacetophenone nor protective agents (■). Incubations were performed in nitrogenated 30 mM sodium phosphate, pH 7.0, containing 1 mM EDTA at 0 °C.

Protection by Substrates. Results on the protective effects of coenzyme and the substrate analogue chloral against bromoacetophenone inactivation can be seen in Figure 4. E2 (0.05 mg/mL) was incubated with 4 equiv per tetramer of bromoacetophenone, either alone or in the presence of 1 mM chloral hydrate, 1 mM NAD, 1 mM NADH, and both 1 mM chloral and 1 mM NAD together. As shown (Figure 4), coenzymes and chloral alone offered no protection against inactivation; however, the combination of chloral hydrate and NAD did offer partial protection. With both isozymes, however, total inactivation required incubations longer than that shown in Figure 4.

Specificity of Incorporation. Initial experiments to determine the specificity of incorporation involved cyanogen bromide digestion of [*carbonyl*-^{14}C]bromoacetophenone-labeled E1 isozyme followed by mapping via polyacrylamide gel electrophoresis (Hempel & Pietruszko, 1981). As a control, [^{14}C]iodoacetamide-labeled E1 was carried through the same procedure. Results revealed both bromoacetophenone and iodoacetamide to react with the same peptide, which corresponds to the B6 fragment in the published sequence of the E1 isozyme (Hempel et al., 1984).

Tryptic peptide maps of E1 modified with 2.3 equiv of [^{14}C]bromoacetophenone (Figure 5A) and of E2 modified with 1.3 equiv of [^{14}C]bromoacetophenone (Figure 5B) in the presence of 1 mM NAD were made to further determine the specificity of bromoacetophenone incorporation. Quantitation of the distribution of radioactivity in Figure 5 is presented in Table II. With the E1 isozyme, the specificity of incorporation was quite good, with one peak, in fractions 40–42, containing the largest amount of radioactivity. Results with the E2 isozyme also showed good specificity with one major peak, in fractions 40–41, containing the largest amount of radioactivity.

Attempt To Identify Tryptic Peptide and Type of Residue with Which Bromoacetophenone Reacts. Fractions containing the major radioactivity peak from HPLC peptide maps of E1 labeled with [*carbonyl*-^{14}C]bromoacetophenone (fractions 40–42 in Figure 5A) were pooled and rechromatographed on the same system with a shallower gradient. The radioactive peptide thus purified was then subjected to amino acid analysis. Important features in the composition include a high glycine content and a low histidine content, but the peptide could not be identified. The amino acid residue incorporating bromoacetophenone was also not identified, due to instability to acid hydrolysis. Work toward sequencing of the radioactive peptide is currently in progress.

Discussion

When designing an affinity reagent for an enzyme, one attempts to incorporate factors that mimic the enzymes substrate or transition-state analogue (Pauling, 1948) that will

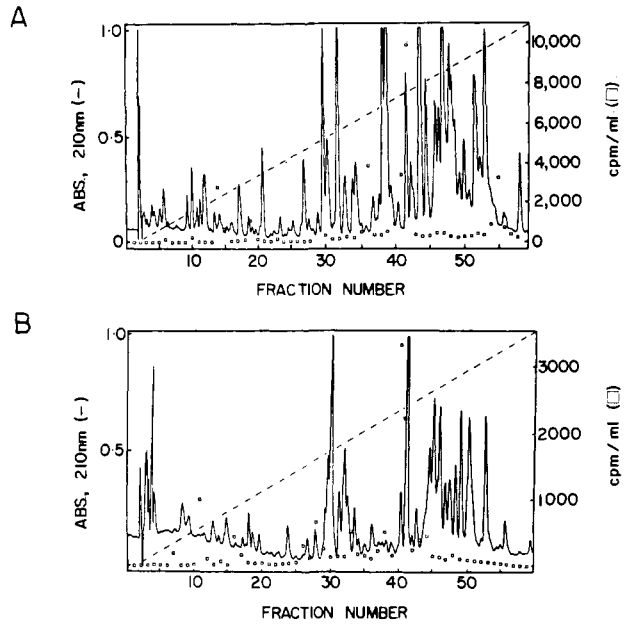

FIGURE 5: Peptide maps of bromoacetophenone-modified E1 (2.3 equiv per M_r 216 000) and E2 (1.3 equiv per M_r 216 000) via high-performance liquid chromatography. Modified E1 (A) and modified E2 (B), carboxymethylated and digested with trypsin, were each applied to a C_{18} μBondapak column initially in 0.1% trifluoroacetic acid. The column was then developed with a linear gradient (0–75%) of methanol (--) at a flow rate of 1.5 mL/min. Peptides were detected by absorbance at 210 nm (—), and radioactivity (□) was determined by scintillation counting of an aliquot (0.5 mL) from each fraction (1.5 mL).

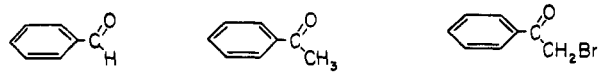

FIGURE 6: Chemical formula of benzaldehyde, acetophenone, and bromoacetophenone.

react covalently with the enzyme (Baker, 1967; Singer, 1967; Vallee & Riordan, 1969; Shaw, 1970). Following this line of reasoning, an affinity reagent for aldehyde dehydrogenase may contain a carbonyl group with the carbonyl proton of the aldehyde substituted with a reactive functional group. Furthermore, the compound should be nonpolar and not negatively charged, since acetic acid (Vallari & Pietruszko, 1981) and iodoacetic acid (Hempel & Pietruszko, 1981) failed to inhibit aldehyde dehydrogenase. Examination of the structure of bromoacetophenone (Figure 6) shows it to fulfill the above criteria; the benzene ring is nonpolar and uncharged, identical with that of benzaldehyde (K_m of benzaldehyde = 0.5 μM for both isozymes; R.C. Vallari, personal communication), and the side chain contains both a keto group and a reactive methylene group to which a bromine atom is bound. Bromoacetophenone, which can react with a variety of nucleophiles (Jones & Hysert, 1971a,b), has been used previously for chemical modification of chymotrypsin, where it modified methionine-192 (Lawson & Schramm, 1965; Lawson & Rao, 1980), carboxylesterase, where it modified a histidine residue (Willadsen et al., 1973), and pepsin (Erlanger et al., 1965).

Initial experiments with bromoacetophenone attempted to kinetically characterize the site of interaction with aldehyde dehydrogenase. However, the high rate at which bromoacetophenone inactivated aldehyde dehydrogenase (see Figure 4) precluded its use in steady-state kinetics; therefore, its analogues acetophenone and chloroacetophenone were used. Furthermore, due to the rapid inactivation of the E1 isozyme, the kinetic characterization was performed with the E2 isozyme, whose inactivation was slower (see Results).

Steady-state kinetics with acetophenone as a dead-end inhibitor (Figure 1A) show a competitive pattern vs. varied glycolaldehyde, indicating acetophenone to bind to the same enzyme form as the aldehyde, with a K_i value of 110 μM (Table I). Steady-state kinetics with chloroacetophenone gave dissociation constants ca. 2 orders of magnitude smaller than that with acetophenone, similar to the results seen with pyrazole, its halogenated derivatives, and alcohol dehydrogenase (Li & Theorell, 1969). However, both slope and intercept effects were obtained with chloroacetophenone (Figure 1B), with K_i values of 2.5 and 3.7 μM (Table I), respectively. Intercept effects can arise due to increased binding with slow dissociation such as described for halogenated pyrazole derivatives and alcohol dehydrogenase by Li and Theorell (1969). Because halogenated derivatives of acetophenone are very reactive, the possibility of irreversible inactivation of the enzyme accounting for the intercept effect cannot be excluded. However, the intercept effect observed here may also be real, as suggested by Figure 3A, where it is directly demonstrated that chloroacetophenone can react with the free enzyme. Realistically, the results should be interpreted as a combination of possibilities: chloroacetophenone binds to both the free enzyme and the enzyme–NAD binary complex (see Figure 3A), yielding both slope and intercept effects, while the enzyme is also being irreversibly inactivated during the assay, causing complications in interpretation of the intercept effects. Furthermore, interpretation of the data is complicated by the fact that the catalytic mechanism of the mitochondrial E2 isozyme is not strictly ordered (Sidhu & Blair, 1975). Thus, the slope effects vs. varied glycolaldehyde with acetophenone and chloroacetophenone indicate that these compounds bind to the same enzyme form as the aldehyde substrate, while the observed intercept effect (Figure 1B) may be due to binding of chloroacetophenone to the free enzyme or to irreversible inactivation or both.

Saturation kinetics with chloroacetophenone in the presence and absence of NAD (Figure 3A) gave y-axis intercepts indicating the inactivator to be recognized at a specific site on the enzyme surface prior to the covalent reaction. The slight decrease in the K_i value in the presence of NAD (Table I) may be due to an increase in binding of chloroacetophenone to the enzyme–NAD binary complex, as compared to binding to the free enzyme. However, since the situation is reversed with bromoacetophenone (see Results), the possibility of these values being identical within experimental error of the procedure employed cannot be excluded. The slight decrease in the K_2 value in the presence of NAD (Table I) may be due to an increase in binding of chloroacetophenone to the enzyme–NAD binary complex, as compared to binding to the free enzyme. However, since the situation is reversed with bromoacetophenone (see Results), the possibility of these values being identical within experimental error of the procedure employed cannot be excluded. Comparison of K_i values for chloroacetophenone from steady-state kinetics and from saturation kinetics (Table I) shows them to be numerically similar, suggesting that the same interaction is studied by steady-state and by saturation kinetics. Similarity of K_i values obtained by these two approaches suggests the involvement of the active site.

Comparison between bromoacetophenone (Figure 3B) and chloroacetophenone (Figure 3A) shows the two to have similar K_i values (Table I), but the values of k_3 are larger with bromoacetophenone than with chloroacetophenone. Thus, both

inactivators bind reversibly with similar dissociation constants, but bromoacetophenone reacts irreversibly much faster than chloroacetophenone, hence the difficulty in performing kinetic experiments with the former compound.

Further information as to the site of bromoacetophenone interaction was obtained by observing the protective effects of the substrate analogue chloral hydrate and coenzymes on inactivation of the E2 isozyme. Results (Figure 4) show partial protection against inactivation only by chloral and NAD together, indicating that the enzyme–coenzyme–aldehyde ternary complex is required for protection and thus again suggesting the active site as a likely participant in the interaction with bromoacetophenone. The lack of total protection is probably due to the low K_i of bromoacetophenone as compared to that for chloral (Hempel & Pietruszko, 1981).

Experiments were next conducted with bromoacetophenone to determine its effectiveness as an inactivator of aldehyde dehydrogenase. Figure 2A–C shows both E1 and E2 to be totally inactivated by 4 equiv of bromoacetophenone per tetramer, with the stoichiometry (see below) proportional to activity loss for the first two molecules incorporated. This stoichiometry was confirmed by the use of ^{14}C-labeled bromoacetophenone with both E1 (Figure 2D) and E2 isozymes. Furthermore, Figure 2A–C shows that the loss of dehydrogenase, esterase (with E2), and NAD-stimulated esterase activities occurs simultaneously for both isozymes. This would be expected with aldehyde dehydrogenase since the same enzyme nucleophile should be involved in the proposed covalent intermediate, according to the postulated mechanism (Jakoby, 1963).

Since denaturation and exhaustive dialysis of [*carbonyl*-^{14}C]bromoacetophenone-labeled aldehyde dehydrogenase failed to remove the radioactive label, it was concluded that bromoacetophenone inactivated the enzyme via formation of a covalent bond. Irreversible inactivation allowed the exact stoichiometry of [*carbonyl*-^{14}C]bromoacetophenone incorporation to be measured in relation to catalytic activity loss. With both E1 (Figure 2D) and E2 (see Results), a majority of catalytic activity was lost with ca. 2 equiv of [*carbonyl*-^{14}C]bromoacetophenone bound per tetramer; further incorporation, up to ca. 4 equiv, resulted in a total loss of activity of both isozymes.

Demonstration of bromoacetophenone's specificity of interaction with aldehyde dehydrogenase was accomplished by making peptide maps of CNBr and tryptic digests of both E1 and E2 inactivated with [*carbonyl*-^{14}C]bromoacetophenone. The CNBr maps gave one labeled peptide with both isozymes. The tryptic maps of both E1 and E2 (panels A and B of Figure 5, respectively) showed one peptide being the primary site of interaction in both cases. With E1, that peptide, in fractions 40–42, contained 46.8% of the total counts collected (Table II) while the primary peak with E2, in fractions 40–41, contained 42.3% of the total counts collected. Comparison of the two tryptic peptide maps show the primary labeled peptides to have similar elution times for both isozymes, suggesting that the two labeled peptides may be similar. Supporting this suggestion are the known homologies between the primary sequences of E1 (Hempel et al., 1984) and E2 (Hsu et al., 1985), which would be expected to be greatest in the region around the active site.

Since the amino acid sequence of human aldehyde dehydrogenase E1 and E2 isozymes is known, an attempt was made to identify the peptide reacting with bromoacetophenone. The labeled fragment was first identified by CNBr digestion and shown to be the same fragment with which iodoacetamide reacts, corresponding to fragment B6 of the E1 sequence by Hempel et al. (1984). Isolation and purification of the labeled tryptic peptide in E1 (see Figure 5A) were also done, and the resultant peptide was subjected to amino acid analysis. Interestingly, the low values of histidine and alanine, the high value of glycine, and the presence of lysine in the bromoacetophenone-labeled fragment indicated that this tryptic peptide was not the same as that which reacts with iodoacetamide (Hempel et al., 1982b).

The results presented in this paper fulfill the criteria of Shaw (1970) for a reagent reacting with catalytically important residues in the active site. First, the incorporation of bromoacetophenone was proportional to activity loss for the first 2 equiv incorporated. Second, a total loss of catalytic activity occurred upon incorporation of 4 equiv of the inactivator. Third, the combination of chloral and NAD gave protection against inactivation. And fourth, the incorporation appeared to be site-specific by both kinetics and peptide mapping. These results, along with the steady-state kinetics with acetophenone and chloroacetophenone and the structural similarity between bromoacetophenone and benzaldehyde, suggest that bromoacetophenone reacts with a residue in the active site of aldehyde dehydrogenase.

The molecule of aldehyde dehydrogenase is a tetramer with four identical subunits (Hempel et al., 1985; Hsu et al., 1985) and four coenzyme-binding sites per tetramer (MacGibbon et al., 1977). In view of this, the relationship between incorporation of bromoacetophenone and catalytic activity loss (Figure 2) is less than stoichiometric since the first two molecules essentially abolish activity. Such results may potentially be explained by "half of the sites" reactivity (Malhotra & Bernhard, 1968). This type of reactivity where only half of the active sites are participating in the reaction with a modifier is known to occur with glyceraldehyde-3-phosphate dehydrogenase when the inactivator β-(2-furyl)acryloyl phosphate is used (Malhotra & Bernhard, 1968). Evidence from coenzyme binding experiments also suggests that two molecules of NADH are bound differently to the molecule of aldehyde dehydrogenase (MacGibbon et al., 1977) than the other two molecules of NADH. Other chemical modifications experiments (Hempel & Pietruszko, 1981; Vallari & Pietruszko, 1982; Pietruszko et al., 1985), along with the results presented here, imply that a situation similar to that existing with glyceraldehyde-3-phosphate dehydrogenase and the inactivator β-(2-furyl)acryloyl phosphate exists with most of the reagents used to inactivate aldehyde dehydrogenase. Thus, asymmetric arrangement of the identical subunits within the molecule of aldehyde dehydrogenase is suggested.

Registry No. $BrCH_2COPh$, 70-11-1; AcPh, 98-86-2; $ClCH_2COPh$, 532-27-4; $BrCH_2{}^{14}COPh$, 103422-19-1; $CH_3{}^{14}COPh$, 5821-66-9; glycolaldehyde, 141-46-8; aldehyde dehydrogenase, 9028-86-8.

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Purification of Human Collagenases with a Hydroxamic Acid Affinity Column

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ABSTRACT: Human collagenase has been isolated from skin fibroblasts and rheumatoid synovium by using an affinity matrix, prepared by coupling Pro-Leu-Gly-NHOH to agarose. Following the methodology described herein, the skin enzyme was isolated in two steps in 76% yield and the synovial enzyme was purified in three steps in 71% yield. Importantly, each enzyme hydrolyzed collagen into $^3/_4$–$^1/_4$ cleavage fragments, indicating that a true collagenase had been isolated. The column was specific for the human enzyme since the collagenase from *Clostridium histolyticum* did not bind. The affinity ligand was designed according to the formalism proposed by Holmquist and Vallee [Holmquist, B., & Vallee, B. L. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 6216] that effective metalloenzyme inhibitors can be synthesized by coupling a suitable metal-coordinating group to a substrate analogue. In this case, the hydroxamic acid probably coordinates to the active-site metal and the Pro-Leu-Gly moiety is similar to the carboxyl side of the cleavage site of collagen, the enzyme's substrate. The IC_{50} for *N*-(benzyloxycarbonyl)-Pro-Leu-Gly-NHOH is 4×10^{-5} M for both enzymes. The affinity chromatographic procedures described here should aid in future studies on vertebrate collagenases.

Collagenase is a highly specific, neutral protease that cleaves undenatured collagen at a point three-fourths the distance from the amino-terminal end. There is increasing evidence that the enzyme plays a critical role in a variety of normal and pathogenic states, such as resorption of the postpartum uterus (Jeffrey & Gross, 1970), wound healing (Grillo & Gross, 1967), rheumatoid arthritis (Evanson et al., 1967; Werb et al., 1977), and tumor invasion (Liotta et al., 1983). Even though the importance of collagenase is now well recognized, there have been few attempts to regulate its activity because of the absence of structure–function studies that could be used for the rational design of inhibitors. These studies have not been performed for two reasons. First, the enzyme is syn-

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