Jacob, G. S., Brown, R. D., & Koenig, S. H. (1980) *Biochemistry 19*, 3754–3765.
Kanamori, K., & Roberts, J. D. (1983) *Biochemistry 22*, 2658–2664.
Kannan, K. K., Petef, M., Fridborg, H., Cid-Dresdner, H., & Lovgren, S. (1977) *FEBS Lett. 73*, 115–119.
Khalifah, R. G. (1971) *J. Biol. Chem. 246*, 2561–2573.
Khalifah, R. G. (1977) *Biochemistry 16*, 2236–2240.
Khalifah, R. G. (1980) in *Biophysics and Physiology of Carbon Dioxide* (Bauer, C., Gros, G., & Bartels, H., Eds.) pp 206–215, Springer-Verlag, Berlin.
Khalifah, R. G., Strader, D. J., Bryant, S. H., & Gibson, S. M. (1977) *Biochemistry 16*, 2241–2247.
Khalifah, R. G., Rogers, J. I., & Mukherjee, J. (1987) *Biochemistry* (in press).
Lindskog, S. (1982) *Adv. Inorg. Biochem. 4*, 115–170.
Lindskog, S. (1983) in *Zinc Enzymes, Metal Ions in Biology* (Spiro, T. G., Ed.) pp 78–121, Wiley-Interscience, New York.
Lindskog, S., & Thorslund, A. (1968) *Eur. J. Biochem. 3*, 453–460.
Lindskog, S., & Coleman, J. E. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70*, 2505–2508.
Lindskog, S. Henderson, L E., Kannan, K. K., Liljas, A., Nyman, P. O., & Strandberg, B. (1971) *Enzymes (3rd Ed.) 5*, 587–665.
Maren, T. H. (1967) *Physiol. Rev. 47*, 595–781.
Pocker, Y., & Stone, J. T. (1968) *Biochemistry 7*, 2936–2945.
Pocker, Y., & Sarkanen, S. (1978) *Adv. Enzymol. Relat. Areas Mol. Biol. 47*, 149–274.
Sigel, H., & Martin, R. B. (1982) *Chem. Rev. 82*, 385–426.
Simonsson, I., Jonsson, B.-H., & Lindskog, S. (1982) *Biochem. Biophys. Res. Commun. 108*, 1406–1412.
Stewart, R. (1985) in *The Proton: Applications to Organic Chemistry*, Academic, New York.
Sundberg, R. J., & Martin, R. B. (1974) *Chem. Rev. 74*, 471–517.
Taylor, P. W., & Burgen, A. S. V. (1971) *Biochemistry 10*, 3859–3866.
Taylor, P. W., King, R. W., & Burgen, A. S. V. (1970) *Biochemistry 9*, 3894–3902.
Taylor, P. W., Feeney, J., & Burgen, A. S. V. (1971) *Biochemistry 10*, 3866–3875.
Tibell, L., Forsman, C., Simonsson, I., & Lindskog, S. (1985) *Biochim. Biophys. Acta 829*, 202–208.
Verpoorte, J. A., Mehta, S., & Edsall, J. T. (1967) *J. Biol. Chem. 242*, 4221–4229.
Whitney, P. L. (1970) *Eur. J. Biochem. 16*, 126–135.
Whitney, P. L. (1973) *J. Biol. Chem. 248*, 2785–2789.
Whitney, P. L., & Brandt, H. (1976) *J. Biol. Chem. 251*, 3862–3867.
Whitney, P. L., Folsch, G., Nyman, P. O., & Malmstrom, B. G. (1967) *J. Biol. Chem. 242*, 4206–4211.
Wolpert, H. R., Strader, C. D., & Khalifah, R. G. (1977) *Biochemistry 16*, 5717–5721.

Active Site of Human Liver Aldehyde Dehydrogenase†

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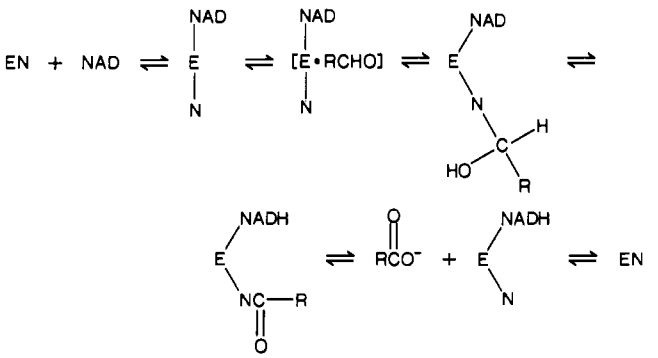
ABSTRACT: Bromoacetophenone (2-bromo-1-phenylethanone) functions as an affinity reagent for human aldehyde dehydrogenase (EC 1.2.1.3) and has been found specifically to label a unique tryptic peptide in the enzyme. Amino-terminal sequence analysis of the labeled peptide after purification by two different procedures revealed the following sequence: Val-Thr-Leu-Glu-Leu-Gly-Gly-Lys. Radioactivity was found to be associated with the glutamate residue, which was identified as Glu-268 by reference to the known amino acid sequence. This paper constitutes the first identification of an active site of aldehyde dehydrogenase.

Aldehyde dehydrogenase (EC 1.2.1.3) is the enzyme involved in metabolism of administered ethanol. It is a tetramer of M_r 216 000, which occurs in two isozymes (both homotetramers, one mitochondrial and the other cytoplasmic). It has broad substrate specificity and catalyzes an irreversible conversion of aldehydes to acids, employing NAD as coenzyme. The chemical steps, reaction intermediates, and catalytic groups utilized by aldehyde dehydrogenase in the oxidation of aldehydes remain virtually unknown but are believed to closely resemble those determined for glyceraldehyde-3-phosphate dehydrogenase. Jacoby (1963) and Weiner (1979) have proposed the general scheme:



In this scheme, the carbonyl carbon of the aldehyde is attacked by the enzyme (E) nucleophile (N), which by analogy with glyceraldehyde-3-phosphate dehydrogenase (Harris & Waters,

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1976) is believed to be a superreactive SH group. Upon removal of the hydride to NAD, a thio ester results that then undergoes hydrolysis to form an acid product. Enzymes that follow this model also exhibit esterase activity; an attack by the enzyme on the ester directly leads to the formation of the acyl intermediate.

Although aldehyde dehydrogenase from human liver has been fully sequenced both via protein sequence (Hempel et al., 1984, 1985) and via DNA (Hsu et al., 1985), and X-ray crystallography studies are in progress (Hempel et al., 1985), the active site of the enzyme has never been identified for any aldehyde dehydrogenase from any species. The primary structure of aldehyde dehydrogenase is so distinct from that of other dehydrogenases that even the coenzyme binding area cannot be identified from this structure. The results presented in this paper thus constitute the first identification of the active site.

In order to obtain an improved understanding of the aldehyde dehydrogenase mechanism, we have attempted the development of affinity and suicide inactivators. In our previous paper (MacKerell et al., 1986) we showed that bromoacetophenone (2-bromo-1-phenylethanone) is an affinity reagent for the mitochondrial and cytosolic isozymes of human aldehyde dehydrogenase. It totally inactivated both isozymes, fulfilling all criteria for an active site directed reagent. The specifically labeled tryptic peptide from both isozymes appeared to be the same by preliminary chromatographic identification. Bromoacetophenone was specifically designed to react with the catalytic residue (residue N in the above scheme) that forms a covalent intermediate with aldehyde substrates and would be involved in ester hydrolysis. In the mechanism presented above, it would be expected to react with the cysteine sulfhydryl or a hydroxyl of threonine or serine; in this paper we show that bromoacetophenone reacts with a glutamate residue. A glutamate residue is known to be part of the active sites of many enzymes, and its presence in the catalytic site of aldehyde dehydrogenase was not totally unexpected. Derivatization of glutamate by bromoacetophenone was however an unexpected outcome of this investigation. There is, therefore, the possibility (at present speculative) that glutamate is in fact the catalytic residue.

MATERIALS AND METHODS

Materials

Enzyme Preparation. E1 and E2 isozymes of human liver aldehyde dehydrogenase were purified to homogeneity following the procedure of Hempel et al. (1982). Homogeneity was confirmed by isoelectric focusing, starch gel electrophoresis, and specific activity. The enzymes were stored at −10 °C in nitrogenated 30 mM sodium phosphate, pH 6.0, containing 1 mM EDTA,[1] 2% (v/v) 2-mercaptoethanol, and 25% glycerol. Prior to use, the enzymes were dialyzed against eight changes of nitrogen-saturated 30 mM sodium phosphate, pH 6.0, containing 1 mM EDTA to remove thiols and glycerol. The standard assay system used to monitor aldehyde dehydrogenase activity was as previously described (MacKerell et al., 1986). Protein concentrations were determined both by 280-nm absorption and by the procedure of Lowry et al. (1951) with bovine serum albumin as a standard.

Synthesis of [carbonyl-^{14}C]Bromoacetophenone. Structural work requires bromoacetophenone of a high specific activity. This was prepared by adding 11.5 mg of labeled acetophenone (250 μCi) 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 (MacKerell et al., 1986). The [*carbonyl*-^{14}C]bromoacetophenone produced had a specific activity of 7.6×10^6 cpm/μmol.

Active Site Labeling. Experiments involving active site labeling 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 and a bromoacetophenone to enzyme molar ratio of 2:1. Incubations were initiated by the addition of bromoacetophenone into the enzyme-containing solution. 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 on ice to initiate the incubation. Following mixing, the temperature was allowed to rise to 25 °C. Incubations were continued for 16–20 h until all reactions went to completion.

Reduction, Alkylation, and Tryptic Digestion. After modification of the enzyme with bromoacetophenone, the reaction mixture was concentrated to a small volume and then dialyzed into 30 mM sodium phosphate buffer (pH 6.0) containing 1.0 mM EDTA and 1.0 mg/mL NAD^+ to remove 2-mercaptoethanol. Guanidine hydrochloride, EDTA, and Trizma base were added to bring the solution to 6 M guanidine hydrochloride, 0.024 M Tris-HCl, and 0.002 M EDTA, pH 8.0 (enzyme concentration was 2.0 mg/mL). A solution of 0.5 M sodium borohydride was prepared freshly for reduction of the modified enzyme. The enzyme solution was made 40 mM in sodium borohydride at 0 °C, with constant stirring, using an aliquot of the stock solution. The reaction was continued for 30 min at 0 °C and then for 15 min at room temperature. Acetone was added to a final concentration of 40 mM to quench excess reagent at the end of this incubation period.

After reduction, the enzyme was carboxymethylated by addition of enough iodoacetic acid to provide a 20% excess of reagent over SH groups contributed by the enzyme. The reaction was allowed to continue for 1.0 h at room temperature in dim light, with the pH maintained at 8.0 by the addition of Tris. At the end of this period, excess reagent was scavenged by the addition of 2-mercaptoethanol. The carboxymethylated protein was then dialyzed vs. 0.1 M ammonium bicarbonate to remove reagents and prepare for trypsin digestion.

Tryptic hydrolysis was carried out by addition of 1.5% (w/w) TPCK-trypsin to the enzyme solution, which was maintained at 37 °C throughout the hydrolysis. After 12 h, another 1.5% of trypsin was added, and an additional 1% of trypsin was added at 24 h. The reaction was allowed to proceed for a total of 48 h at 37 °C. After this period, the tryptic digest was lyophilized and redissolved 3 times in water to remove ammonium bicarbonate. In preparation for peptide mapping, the remaining residue was dissolved in 0.1% TFA and filtered through a 0.5-μm filter to remove fine particulates.

Purification of Labeled Peptide

Procedure 1. The tryptic digest (14 mg of E1 protein) was dissolved in formic acid–acetic acid (7:3) solvent and applied to a column of Sephadex G-50 (1.5 × 200 cm). Elution was carried out with acetic acid–water (3:7 v/v) and was monitored by absorbance at 280 nm, employing a Beckman DB-GT double-beam spectrophotometer and a flow cell with a 1.0-cm light path. Fractions from the G-50 elution (center of the broad peak) that contained the labeled peptide were subjected

[1] Abbreviations: EDTA, ethylenediaminetetraacetic acid; HPLC, high-pressure liquid chromatography; PTH, phenylthiohydantoin; TFA, trifluoroacetic acid; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

first to HPLC in a system of TFA and methanol (the initial mobile phase consisting of 0.1% TFA, with a gradient to 100% methanol over 80 min; the flow rate was 1.5 mL/min). Material recovered between 40 and 41 min on this gradient was collected and again chromatographed in the same system. Following this, the radioactive material was chromatographed by HPLC with a system in which 2-propanol replaced methanol as the organic mobile-phase component, and a final chromatography substituted acetonitrile for methanol.

Procedure 2. Purification of the radioactively labeled peptide involved a six-step HPLC procedure. (1) At first, the complete tryptic digest was chromatographed with a gradient from 0.1% aqueous TFA to 100% methanol on a C18 μBondapak column. (2) Labeled fractions eluting approximately at 50% methanol were taken for further purification, which involved rechromatography on the same C18 column in a system of TFA and acetonitrile in the initial and final mobile phases, respectively. (3) The third step involved another rechromatography in the same system except that the gradient of acetonitrile was much shallower. (4) The next step involved chromatography on a Supelco diphenyl reverse-phase column, with a gradient of TFA and acetonitrile. (5) This was followed by chromatography on μBondapak C18 column with 2-propanol in place of acetonitrile for elution from the column, and finally, (6) the diphenyl column was used again with a very shallow gradient of TFA into acetonitrile. Labeled material was taken from each HPLC step, and in most cases, the volumes of the fractions selected were reduced almost to dryness before rechromatography.

Amino-Terminal Sequence Analysis. Fractions from HPLC that contained ^{14}C radioactivity were combined, and the volume was reduced to between 60 and 120 μL in polypropylene conical centrifuge tubes, on a Savant Speed-Vac concentrator. Sequences were determined on an Applied Biosystems Model 470A protein sequencer similar to that described by Hewick et al. (1981), and identification of phenylthiohydantoin (PTH) amino acid derivatives was carried out by reversed-phase HPLC on an Applied Biosystems Model 120A PTH analyzer. Approximately 40% of the sample from each cycle was injected into the column. The remainder was quantitatively recovered in the fraction collector by alteration of the flow path between the sequencer and the PTH analyzer and by modification of the programming steps. The fractions collected after each sequencing step were subsequently transferred to scintillation vials, and 9 mL of Hydrofluor (National Diagnostics) was added to each for determination of radioactivity by scintillation counting (10 min per vial).

RESULTS

Identification of Labeled Peptide by HPLC. When E1 (the cytoplasmic isozyme of human liver aldehyde dehydrogenase) is modified with [*carbonyl*-^{14}C]bromoacetophenone, followed by reduction with 2-mercaptoethanol, alkylation with iodoacetic acid, and extensive trypsin digestion, the peptide that incorporates most of the radioactivity elutes at about 40–41 min in the HPLC gradient system utilized for peptide mapping (Figure 1). The major radioactively labeled peptide that results upon modification of the E2 isozyme elutes in the same fractions. Although fractions 40–41 constitute the major radioactive component (up to 50–70% incorporated, with the E1 isozyme), minor components can be seen in fractions 14, 19–20, 36, and 55 of Figure 1. The work reported here is concerned with isolation and identification of the peptide in fractions 40–41.

Purification and Identification of the Labeled Peptide. (*A*) *Purification by Procedure 1.* Initial attempts at purification

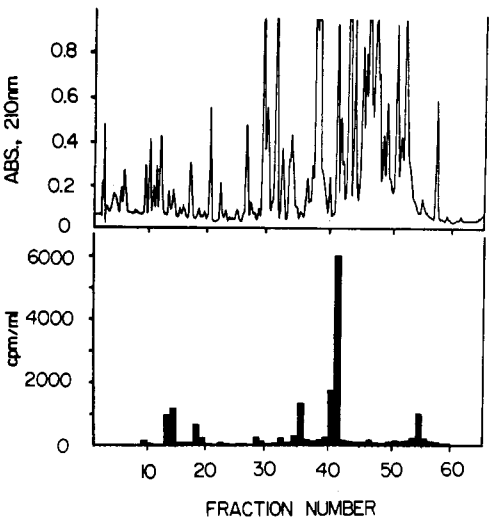

FIGURE 1: Identification of the ^{14}C-labeled tryptic peptide from aldehyde dehydrogenase after HPLC on C18 μBondapak, with an 80-min linear gradient from 0 to 100% MeOH (solvent A = 0.1% aqueous TFA; solvent B = 100% MeOH; flow rate = 1.5 mL/min). The upper part of the figure shows absorbance at 210 nm; the lower part shows the distribution of radioactivity.

involved repeated chromatography on a Waters μBondapak C18 column with a mobile-phase gradient system containing TFA and methanol, varying only the shape of the gradient. An attempt to identify the peptide directly following this procedure, by means of amino acid composition analysis, yielded inconclusive results. An attempt to identify the modified residue also failed due to decomposition during acid hydrolysis. In further work, the modified enzyme was reduced with sodium borohydride, since a reduction step was thought to stabilize most of the possible products of reaction of bromoacetophenone with amino acid side-chain groups.

(*B*) *Sequencing the Peptide Isolated by Procedure 1.* Equimolar amounts of Val and Ile appeared in cycle 1, followed by Gln and Thr in cycle 2; cycle 3 produced Tyr and Leu, again in equimolar amounts. Cycle 4 yielded Thr and Glu with two unknown peaks in addition. When the products of this sequencing cycle were examined for radioactivity, they were found to contain 3400 cpm, and the next few cycles showed diminishing ^{14}C radioactivity. These results are consistent with a lag in chemical cleavage that is expected in the Edman sequences. Reference to the sequence of human liver aldehyde dehydrogenase E1 (Hempel et al., 1984) showed that we were sequencing two peptides simultaneously: tryptic fragments T2 (residues 17–21 from the amino terminus) and T19 (residues 265–272).

(*C*) *Purification by Procedure 2.* The E1 isozyme (8.8 mg) was freshly labeled with [^{14}C]bromoacetophenone, reduced with sodium borohydride, carboxymethylated with iodoacetic acid, and hydrolyzed with trypsin. In this procedure, the gel filtration step was omitted, and a purification scheme entirely involving HPLC separations on μBondapak C18 and Supelco diphenyl columns, with methanol, 2-propanol, and acetonitrile as organic solvents, was used. The degree of purity was assessed at three stages during the purification by subjecting a small portion of the peptide(s) to sequence analysis (Table I). After the last step, sequencing showed that T19 had been separated from all other peptide contaminants (Table I), and all ^{14}C radioactivity was associated exclusively with the T19 peptide.

(*D*) *Sequencing and Counting of Radioactivity.* Material obtained at each cycle of sequencing was counted for radioactive material, and it was found that cycle 4 contained most

Table I: Purification of Radiolabeled Tryptic Peptide by Procedure 2

step no.	column used	gradient conditions	peptides in sample[a]	amount found (pmol)[b]
1	Waters C18 μBondapak	A = 0.1% TFA; B = 100% MeOH; 0–100% B in 80.0 min	*c*	
2	Waters C18 μBondapak	A = 0.1% TFA; B = 70% CH_3CN in 0.08% TFA; 0–100% B in 80.0 min	*c*	
3	Waters C18 μBondapak	A = 0.1% TFA; B = 70% CH_3CN in 0.08% TFA;	T5	800
		0–27% B in 5.0 min,	T9	4000
		27% B–55.0% B in 15 min, and 55.0% B–100% B in 60.0 min	T19	300
4	Supelco diphenyl	A = 0.1% TFA; B = 70% CH_3CN in 0.1% TFA;	T5	<1
		0–27% B in 5.0 min,	T9	700
		27% B–55.0% B in 65.0 min, and 55% B–100% B in 10 min	T19	120
5	Waters C18 μBondapak	A = 0.1% TFA; B = 60% 2-propanol in 0.1% TFA; 0–100% B in 80.0 min	*c*	
6	Supelco diphenyl	A = 0.1% TFA; B = 70% CH_3CN in 0.1% TFA;	T5	<1
		0–27.0% B in 5.0 min,	T9	<1
		27.0% B–35% B in 65 min, and 35.0% B–100% B in 10.0 min	T19	40[d]

[a] Tryptic peptides enumerated according to the assignment of Hempel et al. (1984). [b] Determined as the extrapolated initial yield of PTH-amino acid derivative for each peptide. [c] No sequence analysis this stage. [d] All ^{14}C radioactivity was found to be associated exclusively with peptide T19.

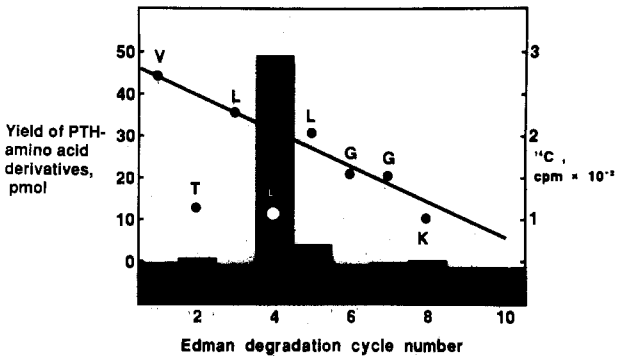

FIGURE 2: Radiolabeled sequence analysis of tryptic peptide T19 (VTLELGGK) and ^{14}C radioactivity of the PTH-amino acid derivatives after each cycle of Edman degradation. Yields of amino acids were estimated from the amount of PTH-amino acid, and no correction was made for PTH-dehydro-α-aminoisobutyric acid in cycle 2, or for other reaction products. The unexpectedly low yield of glutamic acid (E) in cycle 4 was accompanied by the appearance of two unknown peaks (see Figure 3). Near quantitative recovery of ^{14}C radioactivity was obtained in this cycle.

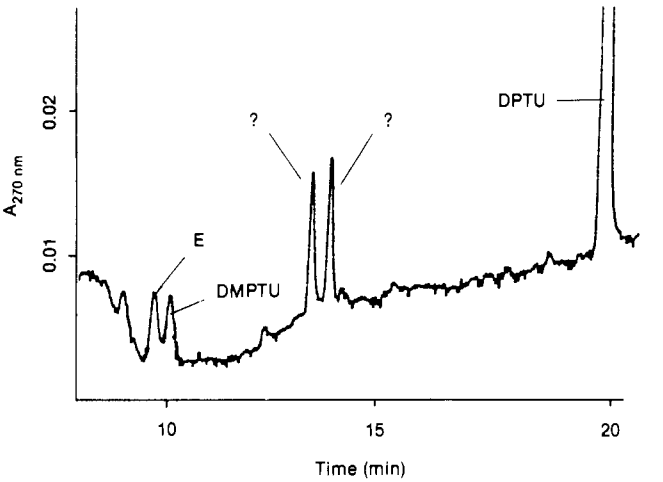

FIGURE 3: PTH-amino acid analysis of the fourth cycle of Edman degradation of peptide T19, as described under Materials and Methods. The two peaks marked by question marks appeared only in this cycle, and no other peaks corresponding to PTH-amino acids were present. E denotes glutamic acid; DMPTU, *N,N*-dimethyl-*N'*-phenylthiourea; DPTU, *N,N'*-diphenylthiourea.

of the ^{14}C, corresponding to incorporation of bromoacetophenone at the glutamate residue of T19 (Figure 2). At the same time, the yield of PTH-derivatized glutamic acid was much lower than expected on the basis of yields in other cycles. Furthermore, the chromatograph of PTH derivatives detected in cycle 4 showed the appearance of two unusual compounds, which do not have retention times corresponding to any of the standard PTH-amino acids (Figure 3). All of the sequence analyses performed during this purification, and in prior attempts to purify the T19 tryptic peptide, show these two unusual species in cycle 4, and not in any of the other cycles. These new peaks, therefore, are thought to represent products arising from the reaction of bromoacetophenone with the glutamate residue and subsequent reduction with borohydride.

Discussion

This paper demonstrates that bromoacetophenone specifically reacts with Glu-268 of the primary structure of the E1 isozyme of human aldehyde dehydrogenase. The peptide containing this residue has been purified from tryptic digests by two different procedures and has been identified by its amino acid sequence as tryptic peptide T19 of the E1 isozyme molecule (Hempel et al., 1984). In both cases the label was determined to be in position 4 of the peptide, which corresponds to glutamate-268.

Bromoacetophenone was initially designed (MacKerell et al., 1986) for use as a probe to locate the catalytic residue of aldehyde dehydrogenase [i.e., the residue (N) that forms a covalent intermediate with aldehyde substrates; see above]. Structurally, the compound resembles the substrates benzaldehyde, with the aldehyde group replaced by a keto group to allow alignment with the catalytic residue. In its behavior as an enzyme inactivator, it fulfills all criteria (Shaw, 1970) for an active site directed reagent. It was expected that bromoacetophenone, with its reactive α-halo keto group, would react irreversibly with the catalytic residue, which was thought to be an –SH or an –OH group (N, see above). Such a reaction, however, would presumably occur only if the bromine part of the bromoacetophenone molecule had sufficient rotational freedom after binding to the enzyme. If such rotational freedom were limited, only a nucleophile in close proximity to the reactive bromine would react. In each case, however, an active site residue would be expected to be derivatized.

Halo ketones (such as bromoacetophenone) are capable of reacting with any protein nucleophile that is in proximity of binding (Means & Feeney, 1971). These reactions are well recognized and have been described for the sulfhydryl, hydroxyl, and amino groups of various amino acid residues of proteins. In certain special conditions, halo ketones are also capable of reacting with carboxyl groups; such a reaction has in fact been demonstrated to occur with *p*-bromophenacyl bromide and an aspartyl carboxyl located at the active site of pepsin (Erlanger et al., 1965, 1966), with formation of an ester derivative. Such reactions also occur in model systems in the presence of crown ether catalysts (Durst et al., 1975), which

provide a hydrophobic environment and possibly also orient the reactive molecules appropriately. In the absence of crown ethers, bromoacetophenone does not react with carboxyl groups, even in anhydrous conditions. During this investigation, bromination of acetophenone was routinely accomplished in anhydrous glacial acetic acid (see above). In pancreatic ribonuclease, two active site imidazole groups are several orders of magnitude more reactive with bromoacetate than is histidine under similar conditions (Heinrickson et al., 1965; Takahashi et al., 1967). Thus, the reactivity of Glu-268 with bromoacetophenone per se suggests an unusual environment for this residue.

The presence of a negatively charged group at the active site of aldehyde dehydrogenase was previously suggested by kinetic studies of dehydrogenation of acetaldehyde by aldehyde dehydrogenase (Feldman & Weiner, 1972; Eckfeldt et al., 1976; Vallari & Pietruszko, 1981). It has been demonstrated with several enzymes from several species that acetic acid formed in the reaction did not function as a product inhibitor, even at high concentrations. Furthermore, we had previously shown (Hempel & Pietruszko, 1981) that human cytoplasmic and mitochondrial aldehyde dehydrogenases could not be inactivated with iodoacetic acid while being readily inactivated by iodoacetamide. The presence of a negatively charged residue at the active site of aldehyde dehydrogenase is also likely to be responsible for the unidirectionality and irreversibility of the reaction catalyzed, since the product (carboxylic acid) has a negative charge, whereas the substrate (aldehyde) does not.

Our previous studies with iodoacetamide (Hempel & Pietruszko, 1981) identified Cys-302 as the superreactive residue of the aldehyde dehydrogenase molecule. Its location close to the coenzyme binding site was later established by another group of investigators, who employed coenzyme-based affinity reagents (Bahr-Lindstrom et al., 1985). However, location of this residue in the active site is uncertain because of inability to totally abolish the catalytic activity of aldehyde dehydrogenase. Another group of investigators sequencing the genes of naturally occurring variants in Oriental populations found differences in residue 459 and suggested that this area of the molecule was likely to be a part of the active site (Yoshida et al., 1984). Thus, the extremely well-conserved area around residue 268 has been the totally unexpected outcome of this investigation.

Up to the present time, three aldehyde dehydrogenases have been sequenced. These include human mitochondrial aldehyde dehydrogenase (Hempel et al., 1985) and horse cytoplasmic aldehyde dehydrogenase (Bahr-Lindstrom et al., 1984), in addition to the human cytoplasmic isozyme (Hempel et al., 1984). The sequence homology between the cytoplasmic isozymes from man and from the horse is much greater than that between the human mitochondrial and human cytoplasmic enzymes, and this suggests that a gene duplication occurred at some early time in the evolution of these isozymes. It is, therefore, interesting to note that all of these enzymes share the same sequence between residues 265 and 272, which is consistent with an important functional role for this area of the molecule.

Although location of glutamate-268 at the active site of aldehyde dehydrogenase appears to be certain, the precise function of this residue in catalysis will remain for some time a subject for speculation. By analogy with the mechanisms for serine proteases and glyceraldehyde-3-phosphate dehydrogenase, it has been realized that more than one residue at the active site may be catalytically essential. Previous studies of aldehyde dehydrogenase showed that groups with pK = 7.0 and 9.2 (suspected to be His and Tyr, respectively) were involved in the catalytic function (Takahashi et al., 1981). This led to the postulation of a charge-relay system involving the above amino acid residues and a sulfhydryl of cysteine. Since pK values of residues located at the active site are often greatly altered, it is possible that pK = 7.0 belongs to glutamate rather than histidine. This would be consistent with the fact that attempts to modify His in aldehyde dehydrogenase failed to completely abolish catalytic activity (Weiner et al., 1985), suggesting that His is less than essential for the catalytic function.

The following additional possibility has been considered: the hydroxyl group of the carboxylate function of the glutamate side chain may be the catalytic residue (N) that forms a covalent intermediate with aldehyde substrates. This has presented considerable difficulties since no model reactions of aldehydes with carboxyl groups are known. Such reactions, however, are not impossible in specific environments such as encountered here. Reaction of an aldehyde with a carboxylate hydroxyl followed by a hydride transfer would produce an anhydride rather than an ester as a covalent intermediate. Aldehyde dehydrogenase also catalyzes an esterase reaction; the same intermediate and the same catalytic residue are believed to participate in both dehydrogenase and esterase reactions. The esterase reaction is also catalyzed by a variety of proteolytic enzymes whose mechanisms include covalent intermediates. A glutamate residue has been found to be involved in ester hydrolysis of carboxypeptidase A for which an acyl intermediate mechanism via an anhydride rather than an ester has been proposed (Makinen et al., 1976). Thus, the possibility of Glu-268 being the catalytic residue (N) of aldehyde dehydrogenase cannot be excluded.

Registry No. EC 1.2.1.3, 9028-86-8; Glu, 56-86-0; [*carbonyl*-^{14}C]bromoacetophenone, 103422-19-1; ^{14}C-labeled acetophenone, 5821-66-9.

REFERENCES

Bahr-Lindstrom, H., Hempel, J., & Jornvall, H. (1984) *Eur. J. Biochem. 141*, 37–42.

Bahr-Lindstrom, H., Jeck, R., Woenckhaus, C., Sohn, S., Hempel, J., & Jornvall, H. (1985) *Biochemistry 24*, 5847–5851.

Durst, H. D., Milano, M., Kikta, E. J., Jr., Connelly, S. A., & Grushka, E. (1975) *Anal. Chem. 47*, 1797–1801.

Eckfeldt, J., Mope, L., Takio, K., & Yonetani, T. (1976) *J. Biol. Chem. 251*, 236–240.

Erlanger, B. F., Vratsanos, S. M., Wassermann, N., & Cooper, A. G. (1965) *J. Biol. Chem. 240*, PC3447–PC3448.

Erlanger, B. F., Vratsanos, S. M., Wassermann, N., & Cooper, A. G. (1966) *Biochem. Biophys. Res. Commun. 23*, 243–245.

Feldman, R. J., & Weiner, H. (1972) *J. Biol. Chem. 247*, 260–266.

Harris, J. I., & Waters, M. (1976) *Enzymes (3rd Ed.) 13*, 1–40.

Heinrickson, R. L., Stein, W. H., Crestfield, A. M., & Moore, S. (1965) *J. Biol. Chem. 240*, 2921–2934.

Hempel, J. D., & Pietruszko, R. (1981) *J. Biol. Chem. 256*, 10889–10896.

Hempel, J. D., Reed, D. M., & Pietruszko, R. (1982) *Alcohol.: Clin. Exp. Res. 6*, 417–425.

Hempel, J., Bahr-Lindstrom, H., & Jornvall, H. (1984) *Eur. J. Biochem. 141*, 21–35.

Hempel, J., Kaiser, R., & Jornvall, H. (1985) *Eur. J. Biochem. 153*, 13–28.

Hewick, R. M., Hunkapillar, M. W., Hood, L. E., & Dreyer, W. J. (1981) *J. Biol. Chem. 256*, 7990–7997.
Hsu, L. C., Tani, K., Fujiyoshi, T., Kurachi, K., & Yoshida, A. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 3771–3775.
Jacoby, W. B. (1963) *Enzymes, 2nd Ed. 7*, 201–221.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.
MacKerell, A. D., Jr., MacWright, R. S., & Pietruszko, R. (1986) *Biochemistry 25*, 5182–5189.
Makinen, M. W., Yamamura, K., & Kaiser, E. T. (976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 3882–3886.
Means, G. E., & Feeney, R. E. (1971) *Chemical Modification of Proteins*, Holden-Day, San Francisco.
Shaw, E. (1970) *Enzymes (3rd Ed.) 1*, 91–146.
Takahashi, K., Stein, W. H., & Moore, S. (1967) *J. Biol. Chem. 242*, 4682–4690.
Takahashi, K., Weiner, H., & Filmer, D. (1981) *Biochemistry 20*, 6225–6230.
Vallari, R. C., & Pietruszko, R. (1981) *Arch. Biochem. Biophys. 212*, 9–19.
Weiner, H. (1979) in *Biochemistry and Pharmacology of Ethanol* (Majchrowicz, E., & Noble, E. P., Eds.) pp 107–124, Plenum, New York.
Weiner, H., Lin, F.-P., & Sanny, C. G. (1985) *Enzymology of Carbonyl Metabolism*, Vol. 2, pp 57–70, Liss, New York.
Yoshida, A., Huang, I.-Y., & Ikawa, M. (1984) *Proc. Natl. Adac. Sci. U.S.A. 81*, 258–261.

Kinetics of Leukotriene A_4 Synthesis by 5-Lipoxygenase from Rat Polymorphonuclear Leukocytes

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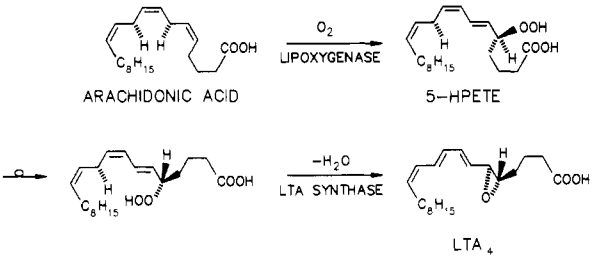
ABSTRACT: When arachidonic acid is added to lysates of rat polymorphonuclear leukocytes, it is oxidized to (5*S*)-hydroperoxy-6(*E*),8(*Z*),11(*Z*),14(*Z*)-eicosatetraenoic acid (5-HPETE). The 5-HPETE then partitions between reduction to the 5-hydroxyeicosanoid and conversion to leukotriene A_4 (LTA_4). Both steps in the formation of LTA_4 are catalyzed by the enzyme 5-lipoxygenase. When [^{3}H]arachidonic acid and unlabeled 5-HPETE were incubated together with 5-lipoxygenase, approximately 20% of the arachidonic acid oxidized at low enzyme concentrations was converted to LTA_4 without reduction of the specific radioactivity of the LTA_4 by the unlabeled 5-HPETE. A significant fraction of the [^{3}H]-5-HPETE intermediate that is formed from arachidonic acid must therefore be converted directly to LTA_4 without dissociation of the intermediate from the enzyme. This result predicts that even in the presence of high levels of peroxidase activity, which will trap any free 5-HPETE by reduction, the minimum efficiency of conversion of 5-HPETE to LTA_4 will be approximately 20%, and this prediction was confirmed. 5-HPETE was found to be a competitive substrate relative to arachidonic acid, so that it is likely that the two substrates share a common active site.

Leukotrienes are potent biologically active metabolites of arachidonic acid that play a role in inflammation and immediate hypersensitivity (Samuelsson, 1983; Hammarström et al., 1979). The first step in the biosynthetic pathway for leukotrienes is the oxidation of arachidonic acid to 5-HPETE[1] catalyzed by 5-lipoxygenase (Scheme I). The 5-HPETE then partitions between LTA_4 synthesis, catalyzed by LTA synthase, and reduction to 5-HETE (Samuelsson, 1983; Borgeat & Samuelsson, 1979; Skoog et al., 1986b). LTA_4 is the precursor to all of the biologically active leukotrienes, so that the synthesis of the leukotrienes depends critically on the partitioning of 5-HPETE between reduction and LTA_4 synthesis.

Scheme I



Both LTA synthase and 5-lipoxygenase activities have now been shown to be expressed by a single enzyme.[2] The two activities have been shown to copurify from four different sources: potatoes (Shimizu et al., 1984), human leukocytes (Rouzer et al., 1986), porcine leukocytes (Ueda et al., 1986), and murine mast cells (Shimizu et al., 1986). The control of the synthesis of LTA_4 is, therefore, a function of the kinetics

[1] Abbreviations: LTA_4, leukotriene A_4, (5*S*)-*trans*-5,6-oxido-7(*E*),9-(*E*),11(*Z*),14(*Z*)-eicosatetraenoic acid; LTB_4, (5*S*,12*R*)-5,12-dihydroxy-6(*Z*),8(*E*),10(*E*),14(*Z*)-eicosatetraenoic acid; 5-HPETE, 5-hydroperoxy-6(*E*),8(*Z*),11(*Z*),14(*Z*)-eicosatetraenoic acid, 5*S* unless otherwise specified; 5-HETE, (5*S*)-5-hydroxy-6,8,11,14-eicosatetraenoic acid; 5,12-diHETEs, 6(*E*)-LTB_4 and 12-*epi*-6(*E*)-LTB_4; 5,6-diHETEs, (5*S*,6*RS*)-5,6-dihydroxy-7(*E*),9(*E*),11(*Z*),14(*Z*)-eicosatetraenoic acid; PMN, polymorphonuclear leukocytes; 5-LO, 5-lipoxygenase; HPLC, high-pressure liquid chromatography; EDTA, ethylenediaminetetraacetic acid.

[2] 5-Lipoxygenase will be used in this work to refer to the enzyme with both activities, which will be differentiated as the "lipoxygenase" and "LTA synthase" activities.

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