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Correlation of loss of activity of human aldehyde dehydrogenase with reaction of bromoacetophenone with glutamic acid-268 and cysteine-302 residues

Partial-sites reactivity of aldehyde dehydrogenase

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Bromoacetophenone (2-bromo-1-phenylethanone) has been characterized as an affinity reagent for human aldehyde dehydrogenase (EC 1.2.1.3) [MacKerell, MacWright & Pietruszko (1986) Biochemistry **25**, 5182–5189], and has been shown to react specifically with the Glu-268 residue [Abriola, Fields, Stein, MacKerell & Pietruszko (1987) Biochemistry **26**, 5679–5684] with an apparent inactivation stoichiometry of two molecules of bromoacetophenone per molecule of enzyme. The specificity of bromoacetophenone for reaction with Glu-268, however, is not absolute, owing to the extreme reactivity of this reagent. When bromo[^{14}C]acetophenone was used to label the human cytoplasmic E1 isoenzyme radioactively and tryptic fragmentation was carried out, peptides besides that containing Glu-268 were found to have reacted with reagent. These peptides were purified by h.p.l.c. and analysed by sequencing and scintillation counting to quantify radioactive label in the material from each cycle of sequencing. Reaction of bromoacetophenone with the aldehyde dehydrogenase molecule during enzyme activity loss occurs with two residues, Glu-268 and Cys-302. The activity loss, however, appears to be proportional to incorporation of label at Glu-268. The large part of incorporation of label at Cys-302 occurs after the activity loss is essentially complete. With both Glu-268 and Cys-302, however, the incorporation of label stops after one molecule of bromoacetophenone has reacted with each residue. Reaction with other residues continues after activity loss is complete.

INTRODUCTION

Aldehyde dehydrogenase (EC 1.2.1.3) is a homotetrameric enzyme consisting of four identical subunits. In the human liver two isoenzymes have been identified: one cytoplasmic (E1) and the other mitochondrial (E2) (Greenfield & Pietruszko, 1977). Comparison of the subunits of the two isoenzymes shows the primary structures to have 68% positional identity (Hempel *et al.*, 1984, 1985). The subunits are large (500 amino acid residues) and do not resemble any of the known dehydrogenases; even the coenzyme-binding area (Rossmann fold; Rao & Rossmann, 1973), known to be present in dehydrogenases, cannot be identified from the primary structure.

Studies of various dehydrogenases (Boyer, 1975) show that the subunit number corresponds to the stoichiometry of coenzyme binding and to the number of active sites per molecule. Even with glyceraldehyde-3-phosphate dehydrogenase, which showed 'half-of-the-sites reactivity' (MacQuarrie & Bernhard, 1971), the presence of four coenzyme-binding sites could be unequivocally established (Taylor *et al.*, 1948). With the human cytoplasmic E1 isoenzyme only two coenzyme-binding sites were detected (Ambroziak *et al.*, 1989), and two sites were also detected in the corresponding enzymes from horse and sheep liver (Eckfeldt & Yonetani, 1976; Dickinson *et al.*, 1981; Hart & Dickinson, 1983); thus the number of functioning active sites appears to be half the number of subunits.

Early attempts at identification of the active site established the presence of a reactive cysteine residue in both the cytoplasmic E1 and the mitochondrial E2 isoenzymes (Hempel & Pietruszko, 1981). However, failure to abolish activity completely precluded its definite assignment to the active site. Thus the location of the active site remained unknown until bromoacetophenone (2-bromo-1-phenylethanone) was carefully characterized as an affinity reagent (MacKerell *et al.*, 1986) and used for chemical modification, where it was shown to react with Glu-268 (Abriola *et al.*, 1987). During chemical modification, the overall stoichiometry of inactivation was also half the number of subunits. Investigation of the specificity of the bromo[^{14}C]acetophenone reaction showed one major radioactive peptide during the h.p.l.c. gradient used for peptide mapping. This peptide was identified (Abriola *et al.*, 1987) by purification, amino acid sequencing and comparison of the sequence obtained with the total sequence of the E1 isoenzyme (Hempel *et al.*, 1984). It was found to consist of amino acid residues Val-265–Lys-272, with Glu-268 being the amino acid residue with which bromoacetophenone reacted. However, preliminary calculation of the radioactivity incorporated into the Glu-268-containing peptide showed that reaction of only one molecule of bromo-[^{14}C]acetophenone per molecule corresponded to total

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loss of activity (Pietruszko & MacKerell, 1987). Other fractions containing the second equivalent of bromo-[^{14}C]acetophenone might have been either products of further or partial cleavage of the same peptide or other peptides representing the reaction of bromoacetophenone with other residues on the enzyme. Identification of these other fractions would lead to further information about the exact stoichiometry of inactivation and/or identify other active-site residues.

MATERIALS AND METHODS

Materials

All chemicals were reagent-grade. Propionaldehyde (J. T. Baker or Aldrich Chemical Co.) was redistilled before use. Guanidinium chloride (ultrapure) was from Schwarz/Mann or Sigma Chemical Co. H.p.l.c.-grade methanol was from J. T. Baker or Fisher Scientific Co. Other h.p.l.c. solvents (propan-2-ol and acetonitrile) were from Fisher Scientific Co., and trifluoroacetic acid was purchased from Pierce Chemical Co. Bovine serum albumin was from Sigma Chemical Co., NAD^+ was from Boehringer Mannheim, and trypsin [(1-tosylamino)-2-phenylethyl chloromethyl ketone ('TPCK')-treated] was from Worthington Corp. [*carbonyl*-^{14}C]Acetophenone was purchased from Amersham Corp.

Methods

Enzyme preparation. The E1 isoenzyme of human liver aldehyde dehydrogenase was purified to homogeneity by following the procedure of Hempel *et al.* (1982*a*). Homogeneity was confirmed by isoelectric focusing, starch-gel electrophoresis and specific activity. The enzyme was stored at −20 °C in N_2-saturated 30 mM-sodium phosphate buffer, pH 6.0, containing 1 mM-EDTA, 2% (v/v) 2-mercaptoethanol and 25% (v/v) glycerol. Before use, the enzyme was dialysed against eight changes of N_2-saturated 30 mM-sodium phosphate buffer, pH 6.0, containing 1 mM-EDTA to remove thiols and glycerol. N_2-saturated buffers were used in all experiments (except where otherwise stated) to prevent air oxidation of enzyme thiol groups. To achieve N_2 saturation, buffers were first exhaustively evacuated on an aspirator at room temperature to remove dissolved air. N_2 was then continuously bubbled through the buffer while it was cooled to 4 °C, and the buffer was stored under N_2 in a firmly capped container at 4 °C.

Determination of enzyme specific activity. Included in the standard assay mixture were 0.1 M-sodium pyrophosphate buffer, pH 9.0, 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 monitoring the production of NADH at 340 nm and by using an absorption coefficient of 6.22 $mM^{-1} \cdot cm^{-1}$. Protein concentrations were determined by both 280 nm absorption (Greenfield & Pietruszko, 1977) and the procedure of Lowry *et al.* (1951), with bovine serum albumin as a standard.

Synthesis of bromo[*carbonyl*-^{14}C]acetophenone. Bromoacetophenone was synthesized by the method of Rather & Reid as described by MacKerell *et al.* (1986). The bromo[*carbonyl*-^{14}C]acetophenone of low specific radioactivity (6.82×10^5 c.p.m./μmol) was first synthesized. The compound was recrystallized and dried, and its specific radioactivity was determined by weighing and by scintillation counting. The bromo[*carbonyl*-^{14}C]acetophenone of higher specific radioactivity (7.5×10^6 c.p.m./μmol) was synthesized from only 11.5 mg of starting material and could not be recrystallized and weighed. Its specific radioactivity was therefore determined by comparison of stoichiometry of inactivation of cytoplasmic aldehyde dehydrogenase E1 with bromo[*carbonyl*-^{14}C]acetophenone of specific radioactivity 6.82 ± 10^5 c.p.m./μmol. At the total stoichiometry of reaction of less than 1.0 molecule per molecule of enzyme the incorporation of bromoacetophenone is quantitative and activity loss is proportional to bromoacetophenone incorporated.

Inactivation experiments. Experiments involving the inactivation of aldehyde dehydrogenase were performed at room temperature in N_2-saturated 30 mM-sodium phosphate buffer, pH 7.0, containing 1 mM-EDTA, at an enzyme concentration of 0.05 mg/ml. The enzyme was diluted into half of the total incubation volume, the bromoacetophenone was diluted into 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.

Stoichiometry of the reaction. The stoichiometry of the reaction was measured by incubating different molar ratios of bromo[*carbonyl*-^{14}C]acetophenone with the E1 isoenzyme (0.05 mg/ml) in N_2-saturated 30 mM-sodium phosphate buffer, pH 7.0, containing 1 mM-EDTA and 1 mM-NAD^+ for 16 h. After the incubation, the samples were concentrated in an Amicon Diaflo ultrafiltration apparatus, followed by further concentration and dialysis in a Schleicher and Schuell apparatus against six changes of N_2-saturated 30 mM-sodium phosphate buffer, pH 6.0, containing 1 mM-EDTA. Samples were then centrifuged to remove precipitated protein, and the specific radioactivity was determined. Controls were carried out through the same procedure except that bromoacetophenone was omitted. Bromo[*carbonyl*-^{14}C]acetophenone incorporation was determined by counting the radioactivity of a sample of the final protein solution with Biofluor scintillation cocktail (New England Nuclear) and an Intertechnique SL30 liquid-scintillation spectrometer.

Tryptic digestion and peptide mapping. Before analysis, the samples were carboxymethylated and digested with trypsin (Hempel *et al.*, 1982*b*). Peptide maps of tryptic digests were obtained via h.p.l.c.; the h.p.l.c. used a C_{18} μBondapak column (reverse-phase; Waters Associates) with a mobile phase of 0.1% (v/v) trifluoroacetic acid in water and the column was eluted with methanol, run in a linear 0–75% (v/v) or 0–100% (v/v) gradient, over a period of 80 min. All other purification steps were run at a flow rate of 1.0 ml/min. All references to fraction numbers (i.e. 55, 35, 40 and 15) are based on this initial chromatography for identification purposes.

Calculation of specific incorporation of reagent. The following calculation was used:

$$\text{mol of reagent incorporated into a specific fraction} = \frac{\text{c.p.m. recovered in that fraction}}{\text{total c.p.m. recovered}} \times \text{overall stoichiometry}$$

Overall stoichiometry is determined before tryptic digestion and is expressed as mol of reagent per mol of enzyme.

Purification of fraction 55 peptide. Following the first chromatography, with methanol as an organic eluent on a Waters C_{18} μBondapak column, the radiolabelled peptide eluted in fraction 55 was re-loaded on to the same column and re-run with a gradient of 0.1% trifluoracetic acid to 100% (v/v) acetonitrile in 50.0 min. The third step was again on the Waters C_{18} μBondapak column with a linear gradient of 0.1% trifluoroacetic acid to 100% (v/v) propan-2-ol in 60.0 min. These runs were followed up by two gradients on a Supelco LC-3DP (diphenyl-bonded) column, the first one with acetonitrile as an organic modifier [0–100% (v/v) in 80.0 min] and the second with methanol [0–100% (v/v) in 80.0 min]. This was followed by two additional h.p.l.c. steps: the sixth step was on a Supelco LC-304 (butyldimethyl-bonded) column, with a linear gradient of 0.1% trifluoroacetic acid to 100% (v/v) methanol in 80.0 min, and the seventh was on the Waters C_{18} μBondapak column, with a linear gradient of 0.1% trifluoroacetic acid to 100% (v/v) acetonitrile in 80.0 min. A modification of the first three h.p.l.c. steps was employed to purify this peptide on a second occasion. The first column and gradient used was the same as in previous attempts at purification [Waters C_{18} μBondapak column, 0.1% trifluoroacetic acid to 100% (v/v) methanol in 80.0 min). This step was then followed by a rechromatography on the same column with a linear gradient of 0.1% trifluoroacetic acid to 38% (v/v) methanol in 25 min, and then a curved transition from 38% to 100% (v/v) methanol during the period from 25 to 80 min. The final step in this purification involved using the same Waters C_{18} μBondapak column and a linear gradient of 0.1% trifluoroacetic acid to 60% (v/v) propan-2-ol in 80.0 min.

Purification of fraction 35 peptide. The initial chromatography on a Waters C_{18} μBondapak column was followed by rechromatography on the same column with a linear gradient of 0.1% trifluoroacetic acid to 50% (v/v) propan-2-ol in 80.0 min, and then a third chromatography on this column with a linear gradient of 0.1% trifluoroacetic acid to 50% (v/v) acetonitrile in 80.0 min. At this point, two radioactive peaks were obtained. The major component was purified by the following additional steps: first, a Supelco LC-304 column was used with a linear gradient of 0.1% trifluoroacetic acid to 45% (v/v) acetonitrile in 80.0 min, and the last step used the same column with a slightly shallower linear gradient [0.1% trifluoroacetic acid to 40% (v/v) acetonitrile in 40.0 min]. The minor component of fraction 35 was purified in two steps: the first involved the use of a Supelco LC-3DP column and a linear gradient of 0.1% trifluoroacetic acid to 45% (v/v) propan-2-ol in 80.0 min, and the second was on a Supelco LC-304 column with a linear gradient of 0.1% trifluoroacetic acid to 40% (v/v) acetonitrile in 80.0 min.

Purification of fraction 15. Chromatography on a Waters C_{18} μBondapak column was followed by a linear gradient of 0.1% trifluoroacetic acid to 25% (v/v) methanol in 50.0 min. The third step was a rechromatography on the same column with a linear gradient of 0.1% trifluoroacetic acid to 5% (v/v) acetonitrile in 40.0 min, and this was followed by a linear gradient of 0.1% trifluoroacetic acid to 10% (v/v) acetonitrile in 40.0 min on a BakerBond 5 μ C_{18} column. The remaining material was subjected to the following additional steps. In the fifth step, a Supelco LC-304 column was used with a 15 min initial isocratic phase of 0.1% trifluoroacetic acid, followed by a shallow linear gradient of 0.1% trifluoroacetic acid to 10% (v/v) acetonitrile from 15 to 30 min. Finally, since it was found that the radiolabelled material was eluting during the isocratic portion of this gradient, a fifth step was carried out on the Supelco LC-304 column with only 0.1% trifluoroacetic acid as mobile phase. Subsequent purification of fraction 15 involved isocratic separations on Supelco LC-3DP and LC-304 columns. An initial gradient elution from a Waters C_{18} μBondapak column, however, was used as described above for initial identification.

Other methods. Mass spectrometry was carried out at Rutgers University (desorption-chemical-ionization spectrometry) or by fast-atom bombardment, at the Massachusetts Institute of Technology Mass Spectrometry Facility.

N-Terminal sequence analysis was carried out either at the Center for Advanced Biotechnology at Rutgers University or at the New York Blood Center Laboratory of Microchemistry. The sample was sequenced on an Applied Biosystems 470A gas-phase sequencer with a 120A on-line amino acid phenylthiohydantoin analyser and a 900A data-control analysis module. The chromatograms were recorded and the sample results were calculated by using the applied Biosystems 475A report generator.

Samples to be subjected to amino acid analysis were hydrolysed by the methods described in Meltzer *et al.* (1987), and analysis was carried out by post-column formation of derivatives with *o*-phthalaldehyde (Roth, 1970, 1976).

RESULTS

Reaction of bromoacetophenone with the EI isoenzyme

The E1 isoenzyme with a specific activity of 0.6 μmol/min per mg was used in these experiments. This is the highest specific activity available from our enzyme preparations. Titration of this enzyme with NAD^+ or NADH (Ambroziak *et al.*, 1989) shows the presence of two coenzyme-binding sites per molecule; enzyme with lower specific activity yields less than two coenzyme-binding sites. Whether the specific activity shown here represents the maximal specific activity attainable by this enzyme cannot be answered at present. Total incorporation of bromo[^{14}C]acetophenone into the E1 isoenzyme has been correlated with activity loss. After

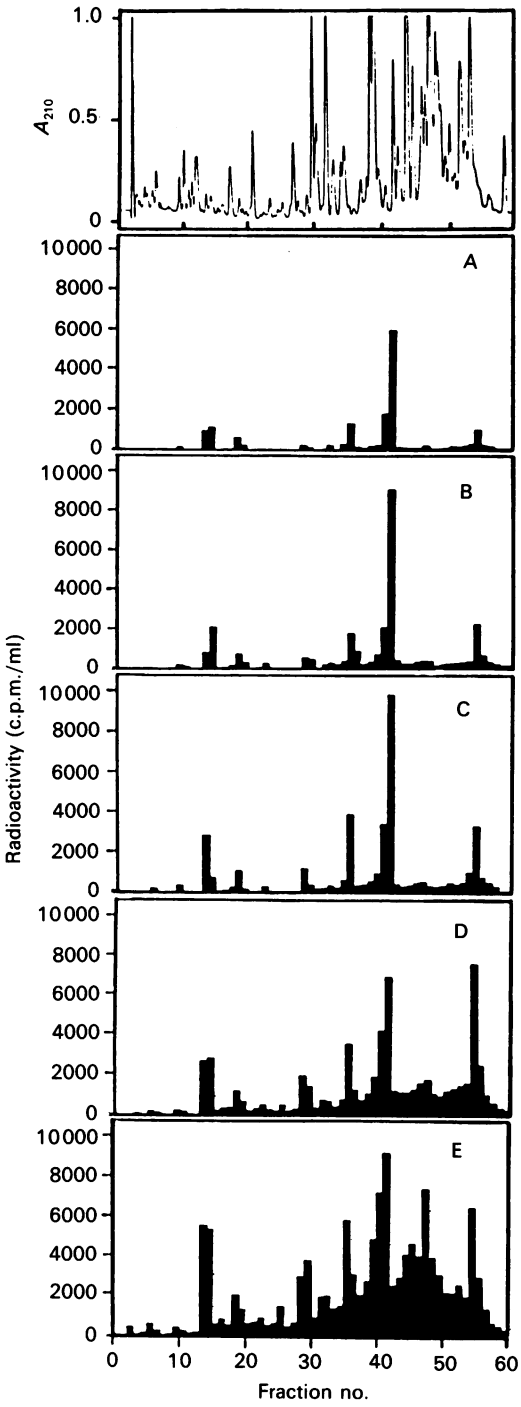

Fig. 1. H.p.l.c. peptide mapping of bromo[^{14}C]acetophenone-modified E1 isoenzyme at increasing modification stoichiometries

E1 enzyme, modified as described in the Materials and methods section with increasing amounts of bromo-[^{14}C]acetophenone, was reduced and carboxymethylated, digested with trypsin and applied to a Waters C_{18} μBondapak column for h.p.l.c. The initial solvent used was 0.1% trifluoroacetic acid and elution was carried out with a linear gradient of 0.1% trifluoroacetic acid to 75% methanol at a flow rate of 1.5 ml/min. Peptides were detected by the absorbance at 210 nm (upper panel shows a representative elution absorbance profile; this profile was identical for all samples chromatographed). Peptides were also detected according to radioactivity detected in fractions from samples with overall stoichiometry of 0.77 (panel A), with overall stoichiometry of 1.76 (panel B), with overall stoichiometry of 2.28 (panel C), with overall stoichiometry of 3.90 (panel D) and with overall stoichiometry of 5.18 (panel E). Scintillation counting was carried out on a 0.5 ml sample of each fraction (1.5 ml).

incubation with bromo[^{14}C]acetophenone and dialysis, the amount of reagent bound per molecule was determined, as well as the remaining enzymic activity. Almost complete loss of activity occurs when about two molecules of bromoacetophenone have reacted per molecule of enzyme, and complete activity loss occurs at higher stoichiometries. The M_r of bromoacetophenone-modified enzyme was determined by gradient gel electrophoresis and was found to be identical with that of the native control, showing that no dissociation occurred during chemical modification with bromoacetophenone.

Specificity of the reaction

Specificity of the reaction was examined after the modified enzyme was fragmented with trypsin, and the resulting mixture of tryptic peptides was separated by h.p.l.c. on a reverse-phase column with a gradient of 0.1% trifluoroacetic acid to 100% methanol (Fig. 1). From data shown in Fig. 1 (panels A–C) it can be seen that at low stoichiometries (where activity loss occurs), the incorporation of bromo[^{14}C]acetophenone is fairly specific in that most of the label is found in fractions 40–42, which were found to contain a peptide with Glu-268 being the reactive residue (Abriola *et al.*, 1987). The incorporation is, however, not completely specific and becomes less specific with increasing stoichiometry (Fig. 1, panels D and E). At low stoichiometries incorporation of radioactive label consistently occurs into fractions 14–15, 35–37 and 54–56, in addition to the main peak. Since these could conceivably have represented partial or other cleavage products containing the same reactive residue as the main peak peptide eluted in fractions 40–42, they were selected for identification via amino acid sequence analysis. There is also radioactivity incorporation into fractions 19–20 and fractions 29–30, which is shown consistently in Fig. 1 (panels A–E). However, radioactivity incorporation into these fractions at low stoichiometries represented less than 5% of total incorporation and could not be reproduced in subsequent experiments. Lack of reproducibility may be due to use of different lots of trypsin, which could produce different cleavage products.

Identification of the reactive residues

Fraction 55 peptide. The first attempt at purification of this peptide achieved complete separation from other, non-radioactive, species, but the low level of radioactivity detected in material from the cycles during sequencing only tentatively established the reactive residue as that in the 30th cycle. There was also a discrepancy in quantification by specific radioactivity and 254 nm absorbance of phenylthiohydantoin derivatives (Table 1), which necessitated a second attempt. The second attempt at purification confirmed this assignment, and comparison with the primary structure allowed determination that Cys-302 was the reactive amino acid in this tryptic peptide (Table 1). The results of sequencing of the labelled peptide in fraction 55 obtained from second purification are shown in Fig. 2. The upper part of the

Table 1. Results of sequence analysis of peptide fractions obtained by tryptic digestion of bromo[^{14}C]acetophenone-labelled E1 isoenzyme

The quantity of peptide loaded on to the sequencer was determined from the radioactivity (c.p.m.) and the specific radioactivity of the radiolabelled reagent. In each case, the amount of peptide found during sequencing was established according to the amino acid phenylthiohydantoin derivative present in the highest amount during the first several cycles of sequencing (this was not always the derivative present in the first cycle, since some of the amino acid phenylthiohydantoin derivatives are of characteristically low yield).

Fraction no.	Amount of peptide loaded on to sequencer (pmol)	Amount of peptide detected (in cycle with high yield) (pmol)	Results	
			Purity (%)	Identity of labelled peptide
15	1070	5.0	–	None identified*
35A (major component	400	370	99.0	Tyr-His-Gln-Gly-Gln-CMCys-?†-?-Ala-Ala (corresponding to Tyr-296–Ala-305 of E1 enzyme); radioactive label in cycle 7 corresponding to Cys-302
35B (minor component)	1000	630	50.0	His-Gln-Gly-Gln-CMCys-CMCys†-Ile-Ala-Ala-Ser (corresponding to His-297–Ser-306 of E1 enzyme); radioactive label in cycle 6 corresponding to Cys-302; also present: Pro-334–Lys-352
42	50	40	95.0	Val-Thr-Leu-Glu-Leu-Gly-Gly-Lys (corresponding to Val-265–Lys-272 of E1 enzyme) radioactive label in cycle 4, corresponding to Glu-268 (Abriola *et al.*, 1987)
55	190	40	90.4	Ser-Pro-CMCys-Ile-Val-Leu-Ala-Asp-Ala-Asp-Leu-Asp-Asn-Ala-Val-Glu-Phe-Ala-His-His-Gly-Val-Phe-Tyr-His-Gln-Gly-Gln-CMCys-CMCys†-Ile-Ala-Ala-Ser-Arg (corresponding to Ser-273–Arg-307 of E1 enzyme); radioactive label in cycle 30, corresponding to Cys-302
55	6670	8880	80.0	Same as previously obtained; also present: Glu-210–Lys-240 (at approx. 20% level); radioactive label in cycle 30, corresponding to Cys-302

* Mass spectrometry and amino acid composition analysis were also carried out on purified material from this fraction. Upon amino acid analysis, no amino acids were found in amounts higher than background (analysis was done according to the procedures cited in the Materials and methods section). Further indication that no peptide material was present in this fraction came from mass-spectrometry analysis, where the largest ion found was of m/z 311.

† During sequencing of several of these samples, the appearance of some *S*-carboxymethylcysteine (CMCys) was noted. This is presumably the result of alkylation of cysteine residues that had not reacted with radiolabelled reagent, and the yield of these cycles is noticeably diminished (as shown in Fig. 2).

Figure shows the yield of each amino acid phenylthiohydantoin derivative (in nmol) versus cycle number. The lower part displays the amount of radioactivity present in the material from each cycle. The cycle containing the largest amount of radioactivity was cycle 30; there was, however, a prolonged 'lag' period extending after cycle 30 through several subsequent cycles, during which significant amounts of radioactivity were present. There was also a significant amount of 'preview' radioactivity appearing in the cycle corresponding to Cys-301. The percentage of total radioactivity recovered in this cycle, however, is comparable with that found during sequencing of the peptide labelled by iodoacetamide, a cysteine-specific reagent studied in previous work (Hempel *et al.*, 1982*b*), and shown to react primarily with Cys-302. The appearance of some amino acid phenylthiohydantoin material in the cycle previous to the primary cycle of occurrence, or 'preview', is common during sequencing.

Fraction 35 peptide. Material from fractions eluted at approx. 35 min was resolved into two distinct components, each of which was then purified separately by different schemes (described above). Sequencing of the major component of this fraction showed that the labelled peptide was a shorter version of the same peptide that occurred in fraction 55, beginning at Tyr-296 rather than at Ser-274 (Table 1). Independent purification of the minor component led to a double sequence, containing one peptide that was an even further shortened portion of the fraction 55 peptide, starting at His-297. Thus both fraction 35 peptides have been identified as further cleavage products of the fraction 55 peptide, with the label appearing with Cys-302 in each case (Table 1). A prolonged lag period extending after the cycle containing Cys-302 was also seen during sequencing of the shorter peptides of fraction 35, and may be due to poor cleavage of this cysteine phenylthiohydantoin derivative by the Edman procedure.

Fraction 15. Sequencing of material originally in fractions 14–15 from the first h.p.l.c. chromatography was carried out on approx. 1 nmol of labelled derivative, but no clear sequence was obtained corresponding to this quantity, and no radioactivity was detected either in material from any of the cycles or on the filter disc of the sequencer. A different sample of fraction 15 was then purified by isocratic separations on two columns and

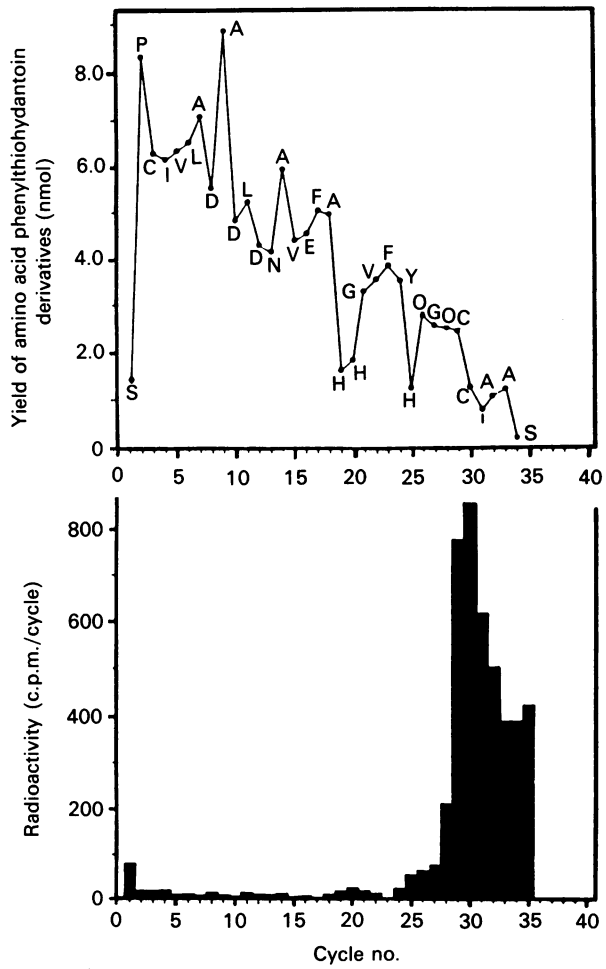

Fig. 2. Sequencing of fraction 55 peptide

During automated *N*-terminal sequencing of the purified radiolabelled peptide originally eluted in fraction 55 on h.p.l.c., a portion of the material obtained after each cycle was used to determine the yield of each amino acid phenylthiohydantoin derivative (upper panel). Scintillation counting of the remainder of the cycle material allowed calculation of total radioactivity (c.p.m.) recovered per cycle (lower panel).

subjected to amino acid composition analysis. None of the amino acids commonly occurring in proteins was detected above the background level. Steps were taken to purify remaining material further on an additional h.p.l.c. column, and both desorption-chemical-ionization and fast-atom-bombardment mass spectrometry were carried out on three separate occasions, giving inconclusive results each time (footnote to Table 1). Since the radioactive species in fractions 14–15 has no identifiable amino acid it appears to be derived from the reagent. The exact nature of the derivative could not be determined.

Recovery of radioactivity from ^{14}C-labelled bromoacetophenone

Recovery of radioactivity from bromo[^{14}C]acetophenone after chromatography on the first h.p.l.c. column (Waters C_{18} μBondapak) is shown in Table 2. The results demonstrate that overall recovery of material loaded on to the h.p.l.c. column is good; peptides labelled with the radioactive reagent that appear in Fig. 1 quantitatively account for all radioactive material present before the h.p.l.c. The percentage of radioactivity incorporated at Glu-268, however, is only 51 % of the total recovered label in the case of lowest stoichiometry. Since recovery was almost complete, the low percentage of label incorporated at Glu-268 cannot be explained by a loss of material. As the stoichiometry of incorporation increases, the percentage due to incorporation at Glu-268 falls from 51 % to a much lower level (Table 2 and Fig. 1). In the case of reagent incorporated at Cys-302 (as represented by peptides eluted in fractions 35 and 55), the percentage of label increases up to a stoichiometry of total bromoacetophenone incorporation of 3.90.

Correlation of activity loss with incorporation of label at Glu-268, Cys-302 and fraction 15

In Table 3 the total stoichiometry of incorporation into the E1 tetramer is shown along with the activity loss and molecules of reagent incorporated at Glu-268, Cys-302 and fractions 14–15. These results show that the activity loss correlates well with incorporation of radioactivity at Glu-268. Reagent incorporation at fractions 14–15 and Cys-302 also increases with increasing pre-fragmentation stoichiometry, but these ratios do not correlate well with activity loss. The greatest incorporation of label at Cys-302 occurs after the activity loss is essentially complete. Label incorporation into the non-specific fractions and fractions 14–15 increases with the increase of the stoichiometry of total incorporation. However, label incorporation at either Glu-268 or Cys-302 stops once the stoichiometry of approximately 1 per molecule is reached.

DISCUSSION

Bromoacetophenone is a bromomethyl ketone, a highly reactive compound which can form a covalent bond with any nucleophile in its vicinity. Its structure resembles that of benzaldehyde, a substrate for the enzyme, allowing it to bind specifically to the substrate-binding site of the aldehyde dehydrogenase molecule. It does not normally react with a carboxy group, unless it is positioned in an extremely hydrophobic environment. Thus the reaction of bromoacetophenone with Glu-268 further supports the active-site location of that residue. In view of the above, bromoacetophenone could be confidently used as a probe for stoichiometry of inactivation of aldehyde dehydrogenase. H.p.l.c. for peptide mapping makes it possible to examine the stoichiometry of chemical modification from the 'inside' of the molecule by determination of the incorporation into specific residues.

The results of identification of the consistently occurring major radioactive peaks in the peptide maps (Fig. 1) of the enzyme after reaction with bromo-[^{14}C]acetophenone are shown in Table 1. These results show that no partial or different cleavage products of the peptide containing Glu-268 exist in detectable amounts. The only consistently occurring peaks besides the major one that incorporate bromo[^{14}C]acetophenone are the tryptic fragment comprising residues 273–307 or smaller pieces of the same peptide. These peptides (fractions 35–36 and fractions 54–55) incorporate most of the second equivalent of bromo[^{14}C]acetophenone. That the reagent is incorporated at Cys-302 suggests that this residue is probably localized in close proximity to Glu-

Table 2. Recovery of radioactivity from bromo[^{14}C]acetophenone-labelled E1 isoenzyme after tryptic digestion and separation of the fragments by h.p.l.c.

The amount (mol) of reagent incorporated per mol of E1 isoenzyme, or the overall stoichiometry of incorporation, was determined before tryptic digestion as detailed in the Materials and methods section. The overall percentage recovery was calculated by totalling the radioactivities (c.p.m.) in all fractions collected from the h.p.l.c. and determining what fraction of the radioactivity initially loaded on to the h.p.l.c. columns that this represented. The percentages of the total radioactivity in specific fractions was calculated by dividing the radioactivity in fractions 40–42 (for Glu-268) by the total radioactivity recovered in all fractions, or, in the case of calculations for Cys-302, by dividing the radioactivity in fractions 35 and 55 by the total radioactivity. The results of calculations in the last two columns here are from similar treatment of data from fractions 14–15 and all fractions except those at 15, 35, 40–42 and 55.

Bromoacetophenone incorporated into E1 isoenzyme (mol/mol)	Overall recovery of radioactivity on h.p.l.c. (%)	Radioactivity recovered in Glu-268 (% of total)	Radioactivity recovered in Cys-302 (% of total)	Radioactivity recovered in fractions 14–15 (% of total)	Radioactivity recovered in other fractions (% of total)
0.77	97.0	51.4	18.7	13.1	16.8
1.76	109.0	44.0	21.9	10.4	23.7
2.28	110.0	39.5	26.2	9.6	24.7
3.90	108.0	21.3	27.8	8.7	42.2
5.18	113.0	16.9	17.2	8.6	57.3

Table 3. Correlation of incorporation of bromo[^{14}C]acetophenone into E1 isoenzyme with loss of activity

The amount of reagent (mol/mol of enzyme) incorporated into specific fractions was calculated by multiplying the fraction of radioactivity recovered in these fractions (see Table 2) by the stoichiometry of incorporation as determined before tryptic fragmentation (see the Materials and methods section). Variability in the amount of reagent required to produce a loss of a given amount of enzyme activity has been thoroughly discussed in MacKerell *et al.* (1986).

Bromoacetophenone incorporated into E1 isoenzyme (mol/mol)	Activity loss (% of control)	Bromoacetophenone incorporated into Glu-268 (mol/mol)	Bromoacetophenone incorporated into Cys-302 (mol/mol)	Bromoacetophenone incorporated into fractions 14–15 (mol/mol)	Bromoacetophenone incorporated into other fractions (mol/mol)
0.77	35.0	0.40	0.14	0.10	0.13
1.76	72.0	0.77	0.39	0.18	0.42
2.28	92.5	0.90	0.60	0.22	0.56
3.90	98.5	0.83	1.08	0.34	1.65
5.18	100.0	0.88	0.89	0.45	2.97

268. This residue was previously identified in our laboratory as a super-reactive cysteine residue (Hempel & Pietruszko, 1981; Hempel *et al.*, 1982*b*), but owing to lack of total inactivation its location at the active site could not be established. Thus the results of current experiments demonstrate that Cys-302 is also located at the active site of aldehyde dehydrogenase.

The incorporation of label from bromo-[^{14}C]acetophenone during activity loss occurs primarily at two residues: Glu-268 and Cys-302. The results, presented in Table 3, demonstrate that there is a limit to the total incorporation at either of these two residues; this limit is one molecule of bromoacetophenone per tetramer of E1 enzyme per either residue. Incorporation into the consistently occurring fractions 14–15 increases with the increase in overall incorporation stoichiometry, but the amount of total radioactivity incorporated into this fraction does not reach a plateau as with Cys-302 or Glu-268. The same is true about the incorporation of label into other fractions (Table 3). Since the material in fractions 14–15 appears to be derived from the reagent (see the Results section), the possibility was considered that it might have formed during the reaction with Glu-268. However, the fact that its amount increases after the reaction with Glu-268 is complete argues against it. Also, incorporation into this fraction at low total incorporation stoichiometries, where activity loss occurs, is small in comparison to that of Glu-268 and Cys-302.

In view of the above, it was of interest to see whether activity loss with bromoacetophenone correlated better with incorporation at Glu-268 or Cys-302. Results shown in Table 3 (when plotted) demonstrate that activity loss correlates in a linear fashion with incorporation at Glu-268 as compared with incorporation at Cys-302 (curvilinear correlation). At low total incorporation stoichiometries, the incorporation of bromoacetophenone at Cys-302 relative to that at Glu-268 is small (Fig. 1 and Table 3), making it impossible to ascertain whether it contributes to total activity loss. However, the fact that greatest incorporation at Cys-302 occurs at a total stoichiometry of 3.9, after activity loss is essentially complete, suggests that incorporation of bromo[^{14}C]-

acetophenone at Cys-302 occurs after that at Glu-268, and probably does not contribute to total activity loss. Results on the chemical modification of aldehyde dehydrogenase are clearly different from those of other dehydrogenases in that incorporation of only one molecule of reactant into a specific residue is required to abolish catalytic activity. Since Cys-302 is the only other residue that reacts with bromoacetophenone in a manner similar to Glu-268, the question arises whether that cysteine residue is located on the same or on a different subunit from the modified Glu-268. At the present time this cannot be answered with certainty, since both possibilities are likely.

It is surprising, however, that during activity loss bromoacetophenone does not react with other residues besides Glu-268 and Cys-302. Any residues of catalytic importance would be expected to be in close proximity to the reactive group of bromoacetophenone. It is possible, then, that the residue that forms a covalent intermediate with aldehyde substrates is unreactive with this reagent, or alternatively that either Glu-268 or Cys-302 is that residue. Supporting an essential catalytic role for Glu-268 in aldehyde dehydrogenase are results from several other amino acid sequences of aldehyde dehydrogenases from prokaryotes and eukaryotes that have been published in recent years (Hempel *et al.*, 1984, 1985; Pickett *et al.*, 1987; Johansson *et al.*, 1988; Jones *et al.*, 1988; Kok *et al.*, 1989; Farres *et al.*, 1988; Dunn *et al.*, 1989). One of these sequences was of a more distant relative (aldehyde dehydrogenase, EC 1.2.1.5) from rat hepatoma (Jones *et al.*, 1988), which showed only approx. 30% sequence identity with aldehyde dehydrogenase (EC 1.2.1.3). This amino acid sequence was more informative in that it was found to lack all the cysteine residues of other mammalian enzymes except for Cys-302. However, the amino acid residues around this residue were not highly conserved and in no way resembled the degree of conservation seen around active-site serine or cysteine residues in the serine proteinases and cysteine proteinases (Dayhoff *et al.*, 1972). The degree of conservation around Cys-302 most closely approximated that found around the aspartic acid residue of the serine proteinases, when the conservation of sequences surrounding the three critical residues in the charge-relay mechanism (serine, histidine and aspartate) was examined. On the other hand, the conservation around Glu-268 was almost absolute in that the sequence Val-Thr-Leu-Glu-Leu-Gly-Gly-Lys appeared in almost all aldehyde dehydrogenases so far sequenced. The only exception is the bovine enzyme (Farres *et al.*, 1989), where Leu-269 has been substituted by isoleucine.

The experiments reported here were designed to understand better the 'half-of-the-sites reactivity' of aldehyde dehydrogenase (Pietruszko & MacKerell, 1987). The term 'half-of-the-sites reactivity' has been applied to a variety of enzymes (glyceraldehyde-3-phosphate dehydrogenase, in particular; Harris & Waters, 1976), and refers to the fact that at any given time only half of the potentially active sites are catalytically active. It also refers to the fact that with some chemical modifiers the stoichiometry of inactivation is less than the number of active sites. The molecule of aldehyde dehydrogenase consists of four identical subunits and contains four identical sequences containing Glu-268. Using increasing amounts of bromoacetophenone, we attempted to determine whether specific incorporation would occur at the other potential active sites after activity loss was complete. The surprising result of this work was that reaction of reagent with only one glutamate residue occurs, with an almost total activity loss. Since it is unlikely that incorporation of 0.25 equivalent of bromoacetophenone would occur into each subunit with concomitant total activity loss, the incorporation of bromoacetophenone at Glu-268 residues of the three other subunits of the aldehyde dehydrogenase molecule most probably does not occur. It does occur at a single cysteine residue that may be located on the same or on a different subunit. This suggests a high degree of asymmetry in the tetrameric enzyme and an overall structure in which one active site is unique and different from other sites in the molecule.

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