Identification of a catalytically essential nucleophilic residue in sheep liver cytoplasmic aldehyde dehydrogenase

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Sheep liver cytoplasmic aldehyde dehydrogenase was labelled by reaction with the substrate p-nitrophenyl di[^{14}C]methylcarbamate. After tryptic digestion and peptide fractionation the labelled residue was identified as Cys-302. This is the first unequivocal identification of the essential enzymic nucleophile in the esterase activity of aldehyde dehydrogenase. By implication, Cys-302 is probably also the residue that is acylated by aldehyde substrates and the first residue that is modified by disulfiram.

INTRODUCTION

Certain reactive amino acid residues in mammalian aldehyde dehydrogenase have already been identified. For example, Cys-302 is specifically modified by iodoacetamide (Hempel *et al.*, 1985) and by a coenzyme analogue, 5-{[3-(bromoacetyl)-pyridinio]pentyl}diphosphoadenosine (von Bahr-Lindström *et al.*, 1985). Pre-modification of the enzyme by disulfiram (a well-known inactivator of aldehyde dehydrogenase; Kitson, 1987) stops the reaction of iodoacetamide at Cys-302. Recently an active-site-directed inactivator in the form of a vinyl ketone has also been shown to modify Cys-302 (Tasayco & Prestwich, 1990). A closely similar coenzyme analogue to the one above, with a butyl spacer arm rather than pentyl, labels not Cys-302 but some other (and so far unidentified) cysteine residue (von Bahr-Lindström *et al.*, 1985). Work with N-ethylmaleimide has implicated both Cys-49 and Cys-162 as possibly having important roles in the actions of aldehyde dehydrogenase (Tu & Weiner, 1988a,b). Finally, bromoacetophenone has been shown to modify not a thiol or amino group as might have been expected, but the less nucleophilic carboxylate side chain of Glu-268 (Abriola *et al.*, 1987). More significant results than those mentioned above are likely to be obtained if a genuine substrate can be used to label the enzyme, for example by trapping an acyl-enzyme intermediate. This approach has been taken with NN-dimethylaminocinnamaldehyde; inconclusive evidence indicates that Ser-74 may be the group that becomes acylated by this substrate (Loomes *et al.*, 1990). However, sequence analysis of various aldehyde dehydrogenases shows that Ser-74 is not a strictly conserved residue and is therefore unlikely to have a catalytic role. (For references concerning the sequence of aldehyde dehydrogenases, see end of the Discussion section.) The present work utilized p-nitrophenyl (PNP) dimethylcarbamate. As described previously (Kitson, 1989b,c), this relatively unreactive compound acts as a substrate for cytoplasmic aldehyde dehydrogenase in an analogous way to the more familiar esterase substrates such as PNP acetate, although in the case of PNP dimethylcarbamate the rate of deacylation is so slow that effectively the enzyme becomes inactivated. There can be little doubt, therefore, that the amino acid residue (Cys-302) identified as described below is truly the essential nucleophile involved in the esterase activity of the enzyme.

EXPERIMENTAL

Materials

The source of routine chemicals was as previously (Kitson,

1989a). Di[^{14}C]methylamine hydrochloride (56 Ci/mol) was purchased from Amersham International, Amersham, Bucks., U.K. Bovine pancreatic trypsin (treated with N-tosyl-L-phenyl-alanylchloromethane) was from Sigma (London) Chemical Co., London S.W.6, U.K.

Enzyme

Cytoplasmic aldehyde dehydrogenase from sheep liver was purified and assayed as previously described (Dickinson *et al.*, 1981).

Synthesis of PNP di[^{14}C]methylcarbamate

Dimethylamine hydrochloride (7.8 mg) was placed in a small Quickfit glass tube and an aqueous solution of di[^{14}C]methyl-amine hydrochloride (0.37 mg, 250 μCi) was added. The solvent was evaporated in a gentle air stream. The last traces of water were removed by standing the tube in an oven at 110 °C. After cooling, but before the hygroscopic salt had had chance to absorb atmospheric moisture, it was dissolved in 1 ml of dry chloroform and cooled in ice. Triethylamine (27 μl) was added, followed by a solution of PNP chloroformate (19.3 mg) in 1 ml of dry chloroform. The reaction mixture was then left at room temperature overnight. The chloroform solution was extracted with 5 × 2 ml of water, by using a vortex mixer. As much water as possible was removed by Pasteur pipette, and 2 ml of acetone was then added to give a homogeneous solution. The solvents were evaporated in a stream of air. The addition and evaporation of acetone were repeated. The dry product was recrystallized *in situ* from 0.3 ml of ethanol. The mother liquor and washings were removed by a Pasteur pipette with a finely drawn-out tip [yield: 16.4 mg (82%)]. Specific radioactivity by the counting method used was 5.42×10^{12} c.p.m./mol. The same procedure, but using non-radioactive starting material, was shown previously to give authentic PNP dimethylcarbamate (Kitson, 1989b).

Liquid-scintillation counting

This was performed as described by Kitson (1978).

Isolation of the active-site peptide

[Some of the procedures used below were based on those of Hempel *et al.* (1982).] A 2 ml sample of enzyme (0.11 mM) in 50 mM-sodium phosphate buffer, pH 7.4, containing 0.3 mM-EDTA, was mixed with 0.1 ml of PNP di[^{14}C]methylcarbamate in ethanol (to give a concentration of modifier of 0.5 mM) and

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Abbreviation used: PNP, p-nitrophenyl.

left at 30 °C for 2.5 h and then overnight at room temperature. The resulting yellow solution was passed down a column (25 cm × 0.9 cm) of Bio-Gel P-6 equilibrated with the same buffer, and the first 3 ml of eluate immediately after the void volume was collected. To this was added 200 mM-Tris buffer, pH 8.05, containing 0.5 mM-EDTA, and sufficient solid guanidinium chloride to give a concentration of 6 M in a final volume of 25 ml. The solution was placed in a boiling-water bath for 1–2 min and then cooled to 37 °C. 2-Mercaptoethanol (5 μl) was added, followed by a further 5 μl after 5 min, and the solution was left at 25 °C for 1 h. Iodoacetamide (34 mg, a 1.2-fold excess over the total thiol content of the solution) was then added and the mixture was left at room temperature for 1 h. 2-Mercapto-ethanol (10 μl) was added to react with excess iodoacetamide, and the solution was dialysed against four changes of 500 ml of 0.1 mM-NH_4HCO_3 in the cold. After dialysis, trypsin (1.33 mg) was added and the solution was left at 37 °C for 30–40 h and then freeze-dried. The residue was dissolved in 4 ml of 0.1 M-formic acid and the solution was filtered through glass wool before application to a column (140 cm × 1.6 cm) of Sephadex G-50-50. Fractions (approx. 6.5 ml) were collected by elution with 0.1 M-formic acid. The A_{280} was measured and 20 μl samples were taken for counting of radioactivity.

The peak radioactive fraction from the Sephadex column was freeze-dried and the residue was dissolved in a few ml of 20 mM-sodium acetate buffer, pH 3.65, containing 2 M-urea. This solution was applied to a column (35 cm × 2 cm) of CM-cellulose (Whatman CM-52) equilibrated in the same medium. The column was eluted with a gradient of 400 ml of this medium and 400 ml of an otherwise identical medium containing 140 mM-NaCl. Fractions (approx. 9 ml) were collected, the A_{226} was measured, and 50 μl samples were taken for counting radioactivity. The peak radioactive fractions from the CM-cellulose column were dialysed against three changes of 2 litres of 1 mM-HCl and then freeze-dried. Alternatively (since dialysis led to some loss of radioactivity, presumably owing to leakage of the peptide through the dialysis membrane), the material in the peak fractions was dialysed only briefly against 4 litres of 0.1 M-formic acid, freeze-dried, redissolved in 4 ml of 0.1 M-formic acid, and eluted a second time from the Sephadex column described above (in order to remove residual urea, NaCl etc.). Finally, the peak radioactive fractions were freeze-dried. Subsequently, the separation was repeated by using first ion-exchange chromatography (as described above) and then gel filtration on a Pharmacia f.p.l.c. system using a Superose 6 HR 10/30 column eluted with 0.1 M-formic acid.

Peptide sequencing

Peptides were sequenced on an Applied Biosystems model 470A protein sequencer with an associated Applied Biosystems reversed-phase h.p.l.c. system 120A. A portion of the effluent from each cycle was collected and a sample was assayed for radioactivity.

RESULTS AND DISCUSSION

Aldehyde dehydrogenase was labelled by PNP di[^{14}C]methyl-carbamate and then separated from excess reagent as described above. The amount of bound radioactivity showed that 1.17 mol of di[^{14}C]methylcarbamate residues was incorporated into the tetrameric enzyme molecule, in excellent agreement with the value estimated by measuring the release of p-nitrophenoxide (Kitson, 1989b). Less than full-site reactivity is characteristic of

aldehyde dehydrogenase (Hempel & Jörnvall, 1989). The modified enzyme was digested by trypsin, and, after gel filtration of the digest, the radioactive peptide was purified by ion-exchange chromatography as shown in Fig. 1. A repetition of the whole procedure gave a profile virtually identical with that shown in Fig. 1. Sequence analysis of the single radioactively labelled peptide gave the result:

His-Gln-Gly-Gln-Cys-Cys-Ile-Ala-Ala-Ser-(Arg)

Both the cysteine residues gave a peak corresponding to carbox-amidomethylcysteine (which is eluted from the sequenator's chromatography slightly after the derivative of glutamic acid). We do not know where to expect a peak for cysteine carrying the $-CON(CH_3)_2$ group, and could not unequivocally identify one among the small background peaks. This is not surprising, in view of aldehyde dehydrogenase's lack of full-site reactivity; if a cysteine carries the label from PNP dimethylcarbamate, then only about 25 % of it should be in this form, and the rest would appear as carboxamidomethylcysteine. Serine was the last identifiable residue during sequencing of the peptide; the terminal arginine is assumed by comparison with the published sequence of the cytoplasmic form of human aldehyde dehydrogenase (Hempel et al., 1984). In fact our peptide is identical with residues 297–306 of the human enzyme. The result was somewhat unexpected in that the preceding residue (296) of published aldehyde dehydrogenase sequences is not lysine or arginine, but tyrosine (and residue 295 is phenylalanine). It is possible that cleavage occurred in this very hydrophobic region because of the presence of residual traces of chymotrypsin in the trypsin used for digestion, even though it was treated with N-tosylphenyl-alanylchloromethane. This treatment (according to the Sigma Chemical Co. catalogue) decreases the chymotrypsin, which is usually present in trypsin preparations, to less than 0.1 N-benzoyl-L-tyrosine ethyl ester units/mg, but the level is not claimed to be zero.

During the identification of the peptide shown above, samples of the effluent from each cycle of operation of the Sequenator were monitored for radioactivity. Initially, the result was dis-
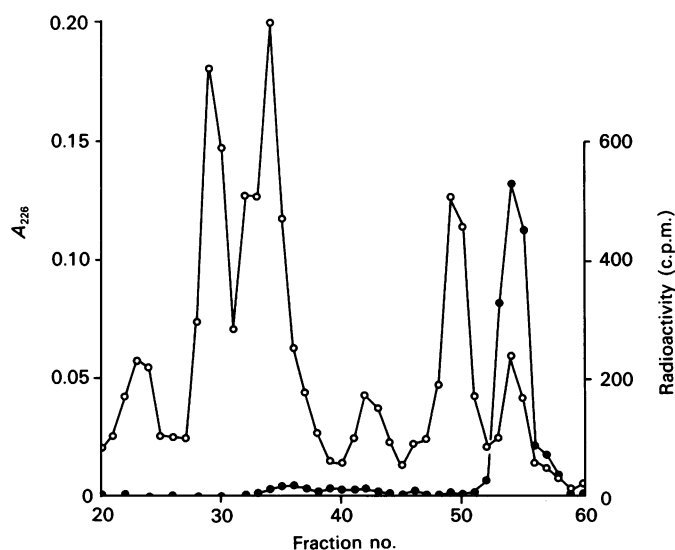

Fig. 1. Ion-exchange elution profile of tryptic peptides of aldehyde dehydrogenase labelled by PNP di[^{14}C]methylcarbamate

The tryptic digest of labelled enzyme was subjected to gel filtration and then the radioactive peptide was purified by elution from a column of CM-cellulose as described in the Experimental section. ○, A_{226}; ●, radioactivity.
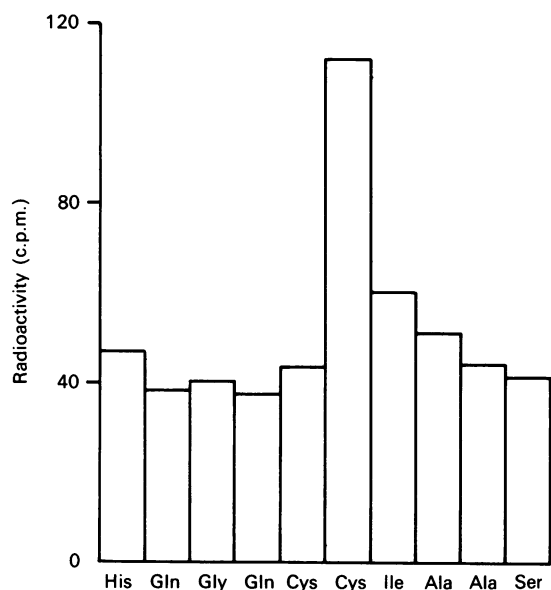
Fig. 2. Location of bound radioactivity within the peptide fragment of aldehyde dehydrogenase that is labelled by PNP di[^{14}C]methylcarbamate

Samples of the effluent from each cycle of operation of the sequenator were monitored for radioactivity.

appointing; radioactivity was found over the whole peptide, reaching peaks in residues Gly-299, Gln-300 and Cys-302. It is of course difficult to imagine the dimethylcarbamoyl label truly being associated with the first two of these residues. Possibly there is a tendency for the label to become detached during the chemical processes involved in the Sequenator. The labelling, digestion and isolation procedures were repeated for a third time, using a fresh enzyme preparation, except that the order of carrying out the gel-filtration and ion-exchange chromatography steps was reversed and Superose was used in place of Sephadex. The former gives a better resolution, and the order of steps was changed so that the gel filtration would concomitantly remove urea and salts left from the ion-exchange step. This time the CM-cellulose column again gave a peak corresponding exactly to the radioactive one shown in Fig. 1, but also gave two more radioactive peaks earlier in the elution profile. Neither of these extra peaks gave any meaningful result on sequence analysis, appearing to consist of mixtures of very small peptides containing mainly lysine and hydrophobic residues. However, after gel filtration, the identity of the peptide in the original peak was reconfirmed, and now the radioactive label was found to be cleanly associated with Cys-302, as shown in Fig. 2. We found that a better radioactivity profile was obtained if only a relatively small sample of material was applied to the Sequenator. The recovery of radioactivity in Fig. 2 was approximately quantitative, taking into account the proportion that goes to waste. We feel confident in stating, therefore, that the thiol group of Cys-302 is the nucleophile that becomes acylated by PNP dimethylcarbamate (and by other ester substrates such as the widely used PNP acetate).

There has long been disagreement as to whether ester and aldehyde substrates interact with aldehyde dehydrogenase at the same active site or not (Blackwell et al., 1983; Duncan, 1985; Loomes & Kitson, 1986; Motion et al., 1988). The esterase and dehydrogenase reactions may occur at completely different enzymic sites, or at the same active site utilizing the same

catalytic enzymic groups, or conceivably at the same general binding site but with different enzymic residues participating in the mechanisms of the two activities. The following points concerning PNP dimethylcarbamate (taken from Kitson, 1989a,b) suggest that the first of these three possibilities is unlikely: (1) PNP dimethylcarbamate is a competitive inhibitor of the dehydrogenation of D-glyceraldehyde or propionaldehyde; (2) the presence of propionaldehyde or chloral hydrate (a competitive inhibitor of the dehydrogenase activity) slows the reaction of PNP dimethylcarbamate with the enzyme; (3) pre-modification of aldehyde dehydrogenase by disulfiram largely abolishes both the dehydrogenase activity of the enzyme and its action on PNP dimethylcarbamate; (4) modification of aldehyde dehydrogenase by PNP dimethylcarbamate profoundly affects the fluorescence of enzyme-bound NADH and the rate of dissociation of NADH; these results are consistent with the site of modification being the dehydrogenase active site; (5) high propionaldehyde concentration affects the fluorescence of enzyme-bound NADH, but not when the enzyme is pre-modified by PNP dimethylcarbamate, suggesting that the aldehyde substrate cannot bind to the modified enzyme. Clearly, all these points are most simply explained if the esterase and dehydrogenase reactions are mediated by the same catalytic nucleophile. The present work has identified this group as Cys-302 in the case of a particular ester substrate; we suggest therefore that it is also this group that is acylated by aldehyde substrates. Our conclusion is supported by the modification studies referred to in the Introduction and by the fact that, of all the cysteine residues in aldehyde dehydrogenase (the human cytoplasmic isoenzyme, for example, having 11 of them per subunit), only Cys-302 is strictly conserved in all the sequences published to date (Hempel et al., 1984, 1985; von Bahr-Lindström et al., 1984; Pickett et al., 1987; Johansson et al., 1988; Jones et al., 1988; Kok et al., 1989; Guan & Weiner, 1990).

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