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Chemical modification of aldehyde dehydrogenase by a vinyl ketone analogue of an insect pheromone

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A major component of the sex pheromone from the tobacco budworm moth *Heliothis virescens* is a C_{16} straight-chain aldehyde with a single unsaturation at the eleventh position. The sex pheromones are inactivated when metabolized to their corresponding acids by insect aldehyde dehydrogenase. During this investigation it was demonstrated that the C_{16} aldehyde is a good substrate for human aldehyde dehydrogenase (EC 1.2.1.3) isoenzymes E1 and E2 with K_m and $k_{cat.}$ values at pH 7.0 of 2 μM and 0.4 μmol of NADH/min per mg and of 0.6 μM and 0.24 μmol of NADH/min per mg respectively. A vinyl ketone analogue of the pheromone inhibited insect pheromone metabolism; it also inactivated human aldehyde dehydrogenase. Total inactivation of both isoenzymes was achieved at stoichiometric (equal or less than the subunit number) concentrations of vinyl ketone, incorporating 2.1–2.6 molecules/molecule of enzyme. Substrate protection was observed in the presence of the parent aldehyde and 5′-AMP. Peptide maps of tryptic digests of the E2 isoenzyme modified with ^{3}H-labelled vinyl ketone showed that incorporation occurred into a single peptide peak. The labelled peptide of E2 isoenzyme was further purified on h.p.l.c. and sequenced. The label was incorporated into cysteine-302 in the primary structure of E2 isoenzyme, thus indicating that cysteine-302 is located in the aldehyde substrate area of the active site of aldehyde dehydrogenase. Affinity labelling of aldehyde dehydrogenase with vinyl ketones may prove to be of general utility in biochemical studies of these enzymes.

INTRODUCTION

Aldehyde dehydrogenase (EC 1.2.1.3) catalyses the dehydrogenation of aldehydes as well as the hydrolysis of esters. The chemical steps, reaction intermediates and catalytic groups utilized are believed to resemble those of glyceraldehyde-3-phosphate dehydrogenase and include a covalent intermediate (Buckley & Dunn, 1982; Dickinson, 1985). In the proposed mechanism, the carbonyl carbon atom of the aldehyde is attacked by the enzyme's nucleophile, which is thought to be a super-reactive thiol group. After hydride transfer to NAD^+, a thioester results that is hydrolysed to regenerate the enzyme and yield the acid product. The super-reactive cysteine residue was first identified in aldehyde dehydrogenase, before its primary structure was known, as a part of a 35-residue tryptic peptide (Hempel, 1981; Hempel & Pietruszko, 1981; Hempel *et al.*, 1982*a*) by employing the group-specific reagent iodoacetamide. When primary structures of human aldehyde dehydrogenase isoenzymes were established (Hempel *et al.*, 1984, 1985; Hsu *et al.*, 1985), it was found to occupy position 302 in a 500-amino acid-residue polypeptide chain. Iodoacetamide fulfilled all criteria for substrate-competitive active-site-directed reagents (Shaw, 1970) with the exception of total inactivation. Because of incomplete inactivation, several other attempts at identification of active-site residues have been made. These included use of coenzyme-based affinity reagents (von Bahr-Lindström *et al.*, 1985), which identified cysteine-369 and cysteine-302, and *N*-ethylmaleimide, which identified cysteine-49 and cysteine-162 (Tu & Weiner, 1988*a*,*b*). Our laboratory developed a substrate-based affinity reagent (MacKerell *et al.*, 1986*a*) that first identified glutamic acid-268 (Abriola *et al.*, 1987) and later cysteine-302 (Abriola *et al.*, 1990).

Female moths of the tobacco budworm *Heliothis virescens* emit a sex pheromone containing two long-chain aldehydes as the most abundant components of a complex blend. The structure of the major component, (*Z*)-hexadec-11-enal [compound (I) in Fig. 1], is metabolized to the carboxylic acid by aldehyde dehydrogenase and aldehyde oxidase enzymes occurring in the antennae of both male and female moths (Ding & Prestwich, 1986; Prestwich *et al.*, 1989; Tasayco J. & Prestwich, 1990*a*,*b*). Selective inhibition of aldehyde dehydrogenase activity can be achieved by using (*Z*)-hexadeca-1,11-dien-3-one [compound (II) in Fig. 1], a vinyl ketone analogue of this aldehyde (Ding & Prestwich, 1988; Prestwich *et al.*, 1989; Tasayco J. & Prestwich, 1990*a*,*b*). Fig. 1 illustrates the structures of the pheromone component, the reactive analogue and the proposed mechanism of reaction with cysteine thiol group. Conjugate (Michael) addition of cysteine to C-1 of the vinyl ketone would result in formation of a covalent adduct (IV). In this way the vinyl ketones function as thiol-specific reagents, since their reaction with oxygen-based or nitrogen-based nucleophiles is substantially slower. Ketones, in general, are structural analogues of aldehydes, and as such are known to function as inhibitors of aldehyde dehydrogenase; acetophenone, for example, is a substrate-competitive inhibitor (MacKerell *et al.*, 1986*a*). Thus the use of a vinyl ketone pheromone analogue to probe the structure of aldehyde dehydrogenase has been considered on three grounds: (*a*) its structural similarity to aldehyde substrate, (*b*) its specific reactivity with thiol groups and (*c*) the fact that it inhibits pheromone metabolism of the moth *Heliothis virescens*. In the present paper we show that the C_{16} *Heliothis virescens* pheromone [compound (I)] is a substrate and its vinyl ketone analogue [compounds (II) and (III)] is an inactivator of both isoenzymes of human aldehyde dehydrogenase and identify cysteine-302 as the residue with which the vinyl ketone reacts. These experiments also locate cysteine-302 at the substrate area of the active site of aldehyde dehydrogenase.

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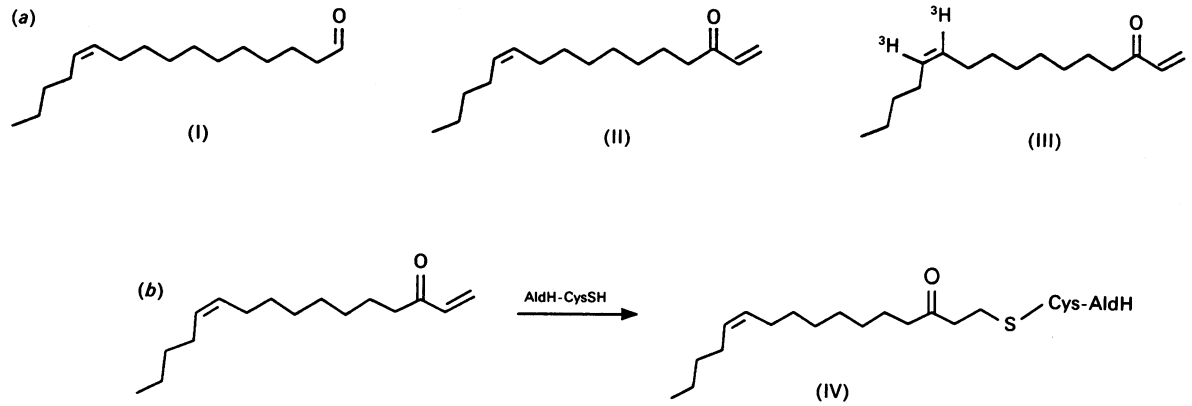

Fig. 1. (*a*) Compounds used in the present study and (*b*) proposed mechanism of interaction of compound (II) with human liver aldehyde dehydrogenase

Compound (I), (*Z*)-hexadec-11-enal; compound (II), (*Z*)-hexadeca-1,11-dien-3-one; compound (III), [11,12-$^3H_2$](*Z*)-hexadeca-1,11-dien-3-one; compound (IV), Michael addition product; AldH-CysSH, aldehyde dehydrogenase active-site thiol group.

EXPERIMENTAL

Reagents

H.p.l.c.-grade methanol, propan-2-ol and acetonitrile were from Fisher Scientific Co. (Fairlawn, NJ, U.S.A.). Propanal (redistilled before use), 2-mercaptoethanol, 5'-AMP, guanidinium chloride and iodoacetic acid (recrystallized from light petroleum) were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). NAD^+ (grade I) was from Boehringer Mannheim (Indianapolis, IN, U.S.A.). Trifluoroacetic acid was from Pierce Chemical Co. (Rockford, IL, U.S.A.). Trypsin (*N*-tosyl-L-phenylalanylchloromethane-treated) and chymotrypsin were from Worthington Corp. (Freehold, NJ, U.S.A.). Chloral hydrate was from Matheson, Coleman and Bell (Gibbstown, NJ, U.S.A.).

Enzyme preparation

E1 and E2 isoenzymes of human liver aldehyde dehydrogenase were purified to homogeneity by following the procedure of Hempel *et al.* (1982*b*). Homogeneity was confirmed by isoelectric focusing and specific activity. The enzymes were stored at 4 °C in N_2-saturated 30 mM-sodium phosphate buffer, pH 6.0, containing 1 mM-EDTA and 0.2% (v/v) 2-mercaptoethanol. For the E1 isoenzyme, to assure better stability NAD^+ (1 mg/ml) was also incorporated into the storage buffer. Before use the enzymes were dialysed against eight changes of N_2-saturated 30 mM-sodium phosphate buffer, pH 7.0, containing EDTA (1 mM) or passed through a Sephadex G-50 (fine grade) column (1.7 cm × 20 cm) to remove thiols and NAD^+. N_2-saturated buffers were used in all experiments to prevent air oxidation of enzyme thiol groups.

Determination of enzyme activity

Enzyme and protein assays were as previously described (Abriola *et al.*, 1990). K_m and $k_{cat.}$ values were determined from Lineweaver–Burk (1934) plots obtained from enzyme reaction progress curves by the method of Yun & Suelter (1977) at 25 °C. Reactions were carried out either in 30 mM-sodium phosphate buffer, pH 7.0, containing 1 mM-EDTA or in 100 mM-sodium pyrophosphate buffer, pH 9.0, containing 1 mM-EDTA. Reaction mixtures typically contained 3 ml of buffer, 500 μM-NAD^+ and 10–20 μM-aldehyde; a molar absorption coefficient of NADH of 6220 $M^{-1} \cdot cm^{-1}$ was used for calculation of reaction rates. Esterase activity was measured in 30 mM-sodium phosphate buffer, pH 7.0, containing 1 mM-EDTA by monitoring the hydrolysis of *p*-nitrophenyl acetate (150 μM) at 400 nm and 25 °C with the use of a molar absorption coefficient of 9450 $M^{-1} \cdot cm^{-1}$. Aldehydes were assayed enzymatically as previously described (MacKerell *et al.*, 1986*b*).

Synthesis and handling of vinyl ketones

(*Z*)-Hexadec-11-enal (I) and (*Z*)-hexadeca-1,11-dien-3-one (II) were synthesized as described by Ding & Prestwich (1988), and the (*Z*)-[11,12-$^3H_2$] hexadeca-1,11-dien-3-one (III) was described by Prestwich *et al.* (1989) and Tasayco J. & Prestwich (1990*b*). Unlabelled vinyl ketone and aldehyde were stored below −10 °C in hexane solution. Both were repurified by silica-gel chromatography (5% ethyl acetate/hexane eluent) when t.l.c. indicated a purity of less than 95%. Labelled vinyl ketone was prepared from a more stable radioactive precursor by a three-step procedure (Tasayco J. & Prestwich, 1990*a*) 2 days before required for experiments. The labelled vinyl ketone was stored in heptane/toluene (1:1, v/v) and used within 2 weeks. Purity was monitored by t.l.c. and fluorescence autoradiography with the use of En^{3}Hance (New England Nuclear, Boston, MA, U.S.A.) and Kodak XAR-5 film. Before use the labelled vinyl ketone (specific radioactivity 59 Ci/mmol) was diluted with unlabelled material: the solvent used for storage was removed by evaporation under N_2 and the vinyl ketone was redissolved in a known volume of ethanol. A portion was added to 5 ml of 40 μM unlabelled vinyl ketone in water, making the final specific radioactivity approx. 33.6 mCi/mmol (74000 c.p.m./nmol). The C_{16} vinyl ketone adhered strongly to glass and plastic; to eliminate this problem partially, glassware was made hydrophilic by soaking in ethanolic KOH.

Enzyme inactivation

Because of the extreme rapidity of inactivation, all reactions were carried out on ice in N_2-saturated 30 mM-sodium phosphate buffer, pH 7.0, containing 1 mM-EDTA at an enzyme concentration of 1.8–7.0 μM. To avoid local high concentration of inhibitor, the reaction mixtures were made up in two halves. One consisted of the enzyme with or without protecting agents and the other the vinyl ketone, each at twice the final concentration. Both were chilled to 4 °C on ice and mixed with vortexing before incubation on ice. Samples were withdrawn periodically and assayed for enzyme activity.

Measurement of pre-steady-state burst amplitude

By mixing the E2 isoenzyme (3.5–7.0 μM) with NAD^+ and

o-nitrobenzaldehyde at pH 5.5 in a 1 ml 1 cm-light-path cuvette at 25 °C the NADH produced could be easily measured on a Varian 635 spectrophotometer at 1:10 expanded scale. Since *o*-nitrobenzaldehyde also absorbed, the E2 isoenzyme was mixed first with *o*-nitrobenzaldehyde and the reaction was started by the addition of NAD^+. The burst amplitude was determined by difference between total absorbance (intersection of tangents to the burst and steady-state velocities) and that of *o*-nitrobenzaldehyde and NAD^+.

Direct labelling of the E2 isoenzyme

E2 (1.0 mg/ml, 4.6 μM) was incubated with 4 equivalents of (*Z*)-[11,12-$^3H_2$]hexadeca-1,11-dien-3-one (specific radioactivity 74000 c.p.m./nmol) in 30 mM-sodium phosphate buffer, pH 7.0, containing 1 mM-EDTA for 18 h at 4 °C in a total volume of 5 ml. The reaction was stopped by adding 2-mercaptoethanol (130 mM), which destroys unchanged vinyl ketone. After dialysis versus eight changes (100 ml each) of 30 mM-sodium phosphate buffer, pH 6.0, containing 1 mM-EDTA, stoichiometry was measured.

Differential labelling with vinyl ketone in the presence of chloral and NAD^+

The E2 isoenzyme (4.6 μM) was allowed to react with unlabelled vinyl ketone (40 μM) in the presence of chloral hydrate (50 mM) and NAD^+ (1.5 mM) for 3.5 min at 4 °C in a total volume of 10 ml. The reaction was stopped by the addition of 2-mercaptoethanol (130 mM). The enzyme was concentrated by vacuum dialysis and passed through a Sephadex G-50 (fine grade) column to separate it from reagents. After determination of activity, the enzyme was treated with tritiated vinyl ketone (16 μM) in a total volume of 9 ml and incubated for 18 h at 4 °C. The E1 isoenzyme (4.6 μM) was allowed to react with unlabelled vinyl ketone (20 μM) in the presence of chloral hydrate (10 mM) and NAD^+ (5 mM) for 5 min in 5 ml total volume at 4 °C. The reaction was stopped, and the enzyme was concentrated and separated from reagents in the same way as E2 isoenzyme, then treated with tritiated vinyl ketone (20 μM).

Stoichiometry of incorporation

After exhaustive dialysis, a sample of the enzyme was taken for protein determination by the Lowry *et al.* (1951) procedure, with BSA as standard. Another sample was used for counting incorporated radioactivity on an LKB 1219 Rackbeta liquid-scintillation counter. The extent of incorporation of radioactivity was calculated from specific radioactivity of labelling compound.

Peptide mapping and sequencing

The enzymes were reduced, carboxymethylated and digested with trypsin as described by Hempel *et al.* (1984). After purification by h.p.l.c. (Waters) the labelled material from the final column was applied to an Applied BioSystems model 477A protein sequencer; 40% of the amino acid phenylthiohydantoin derivatives produced by the Edman cleavage reaction were transferred automatically to an Applied Biosystems model 120 phenylthiohydantoin analyser and chromatographs were recorded on a Spectra Physics 4740 integrator. A reference chromatogram was obtained before the analysis with 25 pmol of each standard amino acid phenylthiohydantoin derivative. The limit of detection was 500 fmol. The other 60% was shunted to a fraction collector synchronized with the sequencing cycles and the radioactivity counted on an LKB 1219 Rackbeta liquid-scintillation counter with Biofluor (DuPont) scintillation cocktail to determine which cycle contained radioactivity.

RESULTS

(*Z*)-Hexadec-11-enal: an insect pheromone is a substrate for human aldehyde dehydrogenase

(*Z*)-Hexadec-11-enal was tested as a substrate for the E1 and E2 isoenzymes at pH 7.0 and 9.0. In Table 1 the K_m, $k_{cat.}$ constants and $k_{cat.}/K_m$ ratios are compared with those for propanal (which is a standard assay substrate for both isoenzymes) and for *o*-nitrobenzaldehyde (which is a poor substrate). In general, the E1 isoenzyme exhibited smaller $k_{cat.}$ and larger K_m values for a given substrate than did the E2 isoenzyme. This is even more apparent when the $k_{cat.}/K_m$ ratios are compared, showing that the E1 isoenzyme is a less efficient catalyst than the E2 isoenzyme. For the E1 isoenzyme the $k_{cat.}/K_m$ value for (*Z*)-hexadec-11-enal was larger than the corresponding one for propanal at both pH values, indicating that the pheromone was actually a better substrate. With the E2 isoenzyme the pheromone $k_{cat.}/K_m$ values were similar to those for propanal but much higher than for the poor substrate *o*-nitrobenzaldehyde. It can be seen from these data that (*Z*)-hexadec-11-enal is at least as good a substrate as propanal for both isoenzymes at pH 7.0 and 9.0.

Table 1. Comparison of K_m and $k_{cat.}$ values of the aldehyde dehydrogenase E1 and E2 isoenzymes with propanal, (*Z*)-hexadec-11-al and *o*-nitrobenzaldehyde as substrates

In the case of (*Z*)-hexadec-11-enal the reaction was started by the addition of aldehyde. With *o*-nitrobenzaldehyde its absorbance at 340 nm was large so the reaction was initiated by the addition of enzyme. Where appropriate, results are given as means ± S.E.M. for the numbers of determinations in parentheses. Abbreviation: N.D., not determined.

	pH 7.0			pH 9.0		
Substrate	K_m (μM)	$k_{cat.}$ (μmol of NADH/min per mg of protein)	$k_{cat.}/K_m$	K_m (μM)	$k_{cat.}$ (μmol of NADH/min per mg of protein)	$k_{cat.}/K_m$
E1 isoenzyme						
Propanal	4.80 ± 0.2 (2)	0.54 ± 0.04 (2)	0.11	11.3 ± 0.3 (2)	0.69 ± 0.1 (3)	0.06
(*Z*)-Hexadec-11-enal	2.05 ± 0.35 (2)	0.39 ± 0.07 (3)	0.19	1.0 (1)	0.29 ± 0.015 (4)	0.29
E2 isoenzyme						
Propanal	0.73 ± 0.13 (4)	0.25 ± 0.03 (3)	0.34	1.2 (1)	1.40 ± 0.2 (2)	1.17
(*Z*)-Hexadec-11-enal	0.58 ± 0.23 (2)	0.24 ± 0 (2)	0.41	1.07 ± 0.34 (2)	0.71 ± 0.11 (2)	0.66
o-Nitrobenzaldehyde	N.D.	N.D.	N.D.	1.6 ± 0 (2)	0.16 ± 0 (2)	0.1

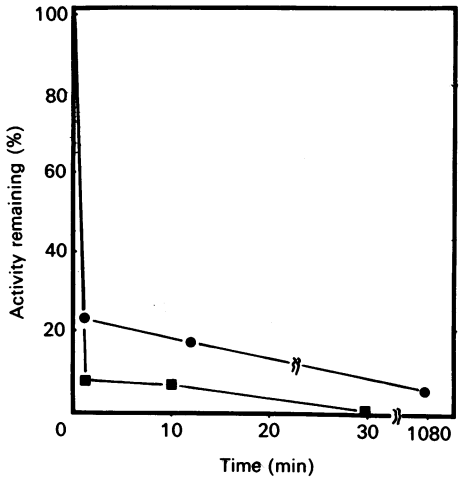

Fig. 2. Inactivation of the aldehyde dehydrogenase E1 and E2 isoenzymes by (*Z*)-hexadeca-1,11-dien-3-one

E1 isoenzyme (1.8 μM) was incubated at 4 °C with 3.6 μM-vinyl ketone (■); E2 (4.6 μM) was incubated at 4 °C with 18.4 μM-vinyl ketone (●). Different concentrations of vinyl ketone are used because of different susceptibilities of these isoenzymes towards inactivation.

Table 2. Protection by substrates and/or substrate analogues of the aldehyde dehydrogenase E1 and E2 isoenzymes against inactivation by the vinyl ketone

Incubation was at 4 °C; at the end of incubation the enzyme activity was measured by using standard assay system (see the Experimental section).

Protecting substrate or analogue	Concn. of vinyl ketone (μM)	Incubation time (min)	Activity remaining (%)
E1 isoenzyme (4.6 μM)			
Chloral (100 mM) + NAD^+ (0.75 mM)	2	2	89
None	2	2	36
(*Z*)-Hexadec-11-enal (0.059 mM) + 5′-AMP (10.0 mM)	3.7	2	30
None	3.7	2	7
E2 isoenzyme (4.6 μM)			
Chloral (100 mM) + NAD^+ (0.75 mM)	2	4	95
None	2	4	49
(*Z*)-Hexadec-11-enal (0.059 mM) + 5′-AMP (10.0 mM)	3.6	2	79
None	3.6	2	48

The vinyl ketone analogue of (*Z*)-hexadec-11-enal is an irreversible inhibitor of human aldehyde dehydrogenase

The vinyl ketone (*Z*)-hexadeca-1,11-dien-3-one [a structural analogue of the pheromone (*Z*)-hexadec-11-enal (Fig. 1)], when incubated at stoichiometric concentrations with human aldehyde dehydrogenase, produced rapid enzyme inactivation. The inactivation was so rapid even when incubations were done on ice (Fig. 2) that the reaction order could not be established. Enzyme activity was lost in two stages: a rapid step and a slow step for both E1 and E2 isoenzymes. By increasing the vinyl ketone concentration to 15 μM under the same conditions (not shown in Fig. 2), total inactivation of E1 was achieved in the first minute. For the E2 isoenzyme, even after 18 h the activity loss was incomplete. However, if enzyme concentration was lowered at similar concentrations of vinyl ketone (1.5 μM-E2 isoenzyme and 20 μM-vinyl ketone) 90% of the activity was lost in the first 3 min and a further 8% in the next 10 min. This inactivation was not reversible by dialysis or gel filtration, indicating covalent-bond formation. It was also found that the loss of esterase activity after incubation with the vinyl ketone parallelled that of dehydrogenase activity for both the E1 and E2 isoenzymes. Incubation of tritiated-vinyl ketone-modified E1 or E2 isoenzymes with 2-mercaptoethanol did not restore activity and no loss of label occurred.

Protection by substrates or substrate analogues

Two sets of substrate/substrate analogue pairs were tested for both the E1 and the E2 isoenzymes. Both are listed in Table 2, where their respective concentrations are shown. Protection occurred in both conditions with both isoenzymes.

Effect of modification of E2 isoenzyme by vinyl ketone on the burst amplitude

At high enzyme concentrations, at pH 5.5, a pre-steady-state burst was observed when the E2 isoenzyme was mixed with *o*-nitrobenzaldehyde and NAD^+. When 3.8 μM-E2 isoenzyme (specific activity 0.7 μmol/min per mg) was mixed with 100 μM-*o*-nitrobenzaldehyde and 525 μM-NAD^+, a burst of 3 μM-NADH (0.79 μmol/μmol of E2 isoenzyme) was observed. After incubation of 6.4 μM-E2 isoenzyme with 65 μM-vinyl ketone for 30 min, the enzyme activity decreased to 26% of control. After the reaction had been stopped with 2-mercaptoethanol (325 mM) and gel filtration at pH 5.5 to remove reactants, the modified enzyme was concentrated to 7.4 μM and then mixed with 100 μM-*o*-nitrobenzaldehyde and NAD^+. The burst was diminished to 0.56 μM-NADH (0.076 μmol/μmol of E2 isoenzyme), i.e. 10% of control. The control treated with 2-mercaptoethanol and subjected to gel filtration retained the full burst of 0.8 μmol/μmol of E2 isoenzyme.

Stoichiometry of incorporation

The stoichiometry of incorporation of tritiated vinyl ketone into the E2 isoenzyme was established by direct and differential labelling (see the Experimental section): the incorporation into the E1 isoenzyme was measured only after differential labelling. In conditions of direct labelling, incubation of E2 isoenzyme with 4 equivalents of tritiated vinyl ketone per tetramer resulted in inactivation of the enzyme to 11% of its original activity, with concomitant label incorporation of 2.5 mol/mol of enzyme. This experiment was repeated with a larger amount of enzyme (10 ml; 4.6 μM), and a similar stoichiometry (2.4 mol/mol of enzyme) was measured. Experiments involving differential labelling showed no loss and a slight loss (11%) of activity for E2 and E1 isoenzymes respectively after incubation with unlabelled vinyl ketone in the presence of NAD^+ and chloral. After reaction with tritiated vinyl ketone and dialysis, stoichiometries of 2.6 and 2.1 mol/mol of enzyme were measured for the E2 and E1 isoenzymes respectively.

Peptide mapping of the differentially labelled E2 isoenzyme

The dialysed vinyl ketone-modified enzyme was reduced with 20 mM-$NaBH_4$ [30 min at 4 °C followed by 15 min at 25 °C, then quenched with acetone (40 mM)]. The enzyme was alkylated with iodoacetic acid and digested with trypsin, and the products were freeze-dried. The first chromatographic step employed a Waters C_{18} μBondapak column [80 min, aq. 0.1% (v/v) trifluoroacetic acid to 100% (v/v) methanol]. Side fractions 64, 65

Table 3. Sequence analysis of vinyl ketone-labelled aldehyde dehydrogenase E2 isoenzyme

Labelled material sequenced	Amount applied* (pmol)	Amount of amino acid phenylthiohydantoin derivative in cycle 1† (pmol)	Composition	Sequence(s) found
Differentially labelled E2 fractions 64, 65 and 68	1670	826.0	(a) 48%	Ala-Val-Glu-Gln-Ala-His-Phe-Ala-Leu-Phe-Phe-Asn-Gln-Gly-Gln
		600.0	(b) 35%	Ser-Pro-Asn-Ile-Ile-Met-Ser-Asp-Ala-Asp-Met-Asp-Xaa-Ala-Val
		281.0	(c) 16%	Asp-Arg-Thr-Xaa-Leu-Ala-Ala-Leu-Glu-Thr-Leu-Asp
Differentially labelled E2 (fractions 66 and 67) digested with chymotrypsin	1300	400.0	(d) 53%	Ser-Pro-Asn-Ile-Ile-Met-Ser-Asp-Ala-Asp-Met-Asp-Xaa-Ala-Val-Glu-Gln
		200.0	(e) 27%	Ala-Val-Glu-Gln-Ala-His-Phe-Ala-Leu-Phe-Phe-Asn-Gln-Gly-Gln
		100.0	(f) 13%	Ala-Leu-Phe-Phe-Asn-Gln-Gly-Gln
		50.0	(g) 7%	Phe-Asn-Gln-Gly-Gln
Directly labelled E2	93	45.0	(h) 74%	Phe-Asn-Gln-Gly-Gln-Xaa-Xaa-Xaa-Ala-Gly-Ser-Arg
		16.2	(i) 26%	Ser-Pro-Asn-Ile-Ile-Xaa-Xaa-Asp-Ala-Asp-Xaa-Asp-Trp-Ala-Val-Glu-Gln

* Calculated from radioactivity.
† Calculated from absorbance of amino acid phenylthiohydantoin derivatives.

and 68 (Fig. 3*a*) were first repurified on a Supelco butyldimethyl column [40 min, aq. 0.1% (v/v) trifluoroacetic acid to 100% (v/v) acetonitrile]; front fractions 60–63 were also repurified in the same conditions. In both cases radioactivity was eluted at 34 min in a single peak. The results of sequencing showed three components (components a–c in Table 3). The largest component corresponded to residues 286–300, the second component corresponded to residues 273–287 and the third, smallest, component corresponded to residues 98–109 in the E2 isoenzyme. The Xaa residue in peptide (c) is tryptophan, which may have been altered by the $NaBH_4$ reduction. After tryptophan, the yield declined markedly and continued for only two additional cycles (only approx. 9 pmol in the last cycle). The alanine and valine in these two cycles had unusually low yields, suggesting that the disappearance of tryptophan had somehow rendered the remainder of the peptide unavailable for sequencing. The yields of both peptides approached background levels by residue 300. When the peptide is long, the complete sequence is difficult to obtain on automatic sequencer, where yields decrease considerably with each cycle. Frequently only the beginning of the peptide is sequenced before yields fall below detectable levels. In this instance the radioactivity remained bound to the sequencer membrane, indicating that the label-bearing amino acid occurred after residue 300. This information suggested that the peptide required further shortening in order to sequence the residues after residue 300.

Main fractions 66 and 67 (Fig. 3*a*) were repurified on a Waters C_{18} μBondapak column [80 min, 0.1% (v/v) trifluoroacetic acid to 100% (v/v) acetonitrile]. The radioactive material was subjected to chymotryptic hydrolysis [2.8% (w/w) chymotrypsin in 0.1 mM-ammonium bicarbonate buffer, pH 7.8, containing 0.1 mM-$CaCl_2$ for 3.5 h]. After being freeze-dried, the material was chromatographed on a Supelco butydimethyl column [40 min, aq. 0.1% (v/v) trifluoroacetic acid to 100% (v/v) acetonitrile]. The radioactive fractions were pooled, concentrated and sequenced. The sequences and yields shown in Table 3 (components d–g) indicate a mixture of four peptides that are all parts of a large tryptic peptide comprising residues 273–307; no other peptides were detected. The four peptides in order of abundance corresponded to residues 273–289, 286–300, 293–300 and 296–300. Sequencing cycles were counted for radioactivity. The background levels were high, making it impossible to assign radioactivity to any individual amino acid residue; the sequencer membrane also contained radioactivity.

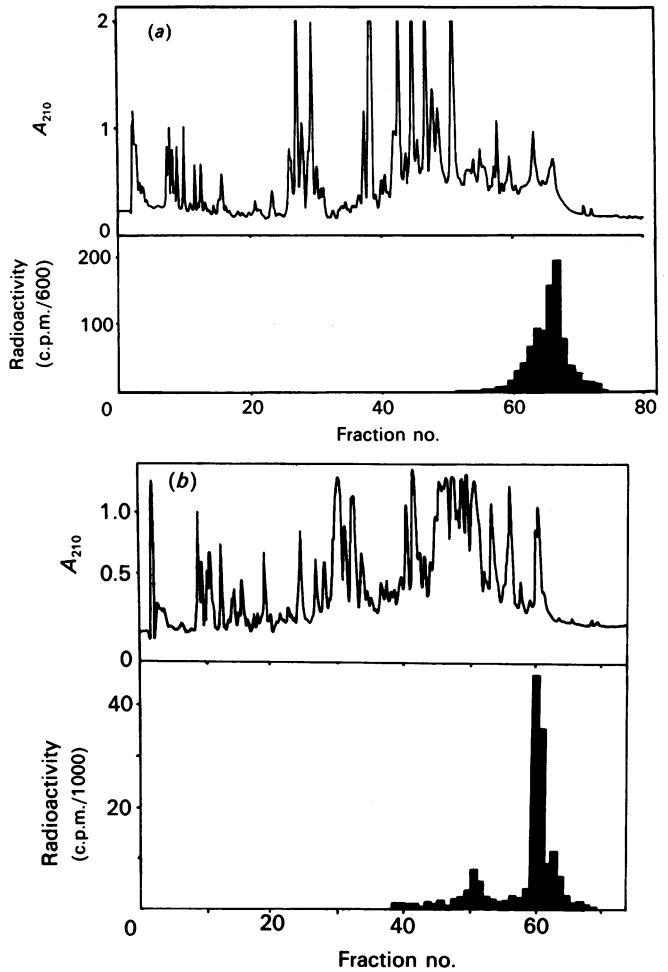

Fig. 3. Identification of the ^{3}H-labelled tryptic peptide from labelled aldehyde dehydrogenase E2 isoenzyme

H.p.l.c. was performed on a Waters C_{18} μBondapak column with a linear gradient of aq. 0.1% trifluoroacetic acid to 100% methanol at a flow rate of 1.5 ml/min. (*a*) Tryptic peptide map of differentially labelled E2 isoenzyme. The upper part of the Figure shows absorbance at 210 nm; the lower part shows the distribution of radioactivity. (*b*) Tryptic peptide map of directly labelled E2 isoenzyme. The upper part of the Figure shows absorbance at 210 nm; the lower part shows the distribution of radioactivity.

Table 4. Purification of vinyl ketone-labelled E2 peptide obtained by direct labelling

Abbreviations: TFA, trifluoroacetic acid; N.M., radioactivity not measured after concentration.

Step	Column used	Gradient conditions	Amount loaded (nmol)	Amount eluted (nmol)
1	Waters C_{18} μBondapak	80 min, aq. 0.1% TFA to 100% methanol	2.6	2.3
2	Waters C_{18} μBondapak	80 min, aq. 0.1% TFA to 100% acetonitrile	0.67	0.67
3	Supelco diphenyl	80 min, aq. 0.1% TFA to 100% acetonitrile	N.M.	0.48
4	Bakerbond (3 μm) C_{18}	80 min, aq. 0.1% TFA to 100% propan-2-ol	N.M.	0.152

Peptide mapping of directly labelled E2 isoenzyme

The labelled E2 isoenzyme was reduced with 2-mercaptoethanol, alkylated with iodoacetic acid and digested with trypsin. A portion was subjected to h.p.l.c. (Fig. 3*b*). The elution time of the labelled peptide (Fig. 3*b*) was shorter than when the enzyme was differentially labelled (compare Fig. 3*a* with Fig. 3*b*). The label was eluted in two components: a major one (fractions 53–70) comprising 80% of the total radioactivity and a minor one (fractions 39–52) that comprised 20%. The fractions with the highest radioactivity (61 and 62) were pooled, concentrated and purified via three additional chromatographic steps (Table 4). Because of the extremely hydrophobic nature of the vinyl ketone, approx. 39% of the material adhered to the sample tubes and pipette tip, leaving only 93 pmol to be sequenced. The amount of amino acid phenylthiohydantoin derivatives in the first sequencing cycle (45 pmol) approximated the amount of peptide applied (93 pmol) calculated from radioactivity. Results from sequencing showed two components (components h and i in Table 3); the major component comprised residues 296–307 in the primary structure of the E2 isoenzyme; the three Xaa residues correspond to cysteine-301, cysteine-302 and cysteine-303. When the material from the sequencing cycles was counted for radioactivity, cycle 7, corresponding to cysteine-302, was found to contain the label (Fig. 4). The impurity (component i in Table 3) is a part of the same tryptic peptide and contains a serine residue in cycle 7. This residue, however, cannot react with the vinyl ketone. Label in this portion has also been eliminated by prior sequence results (components b and d in Table 3).

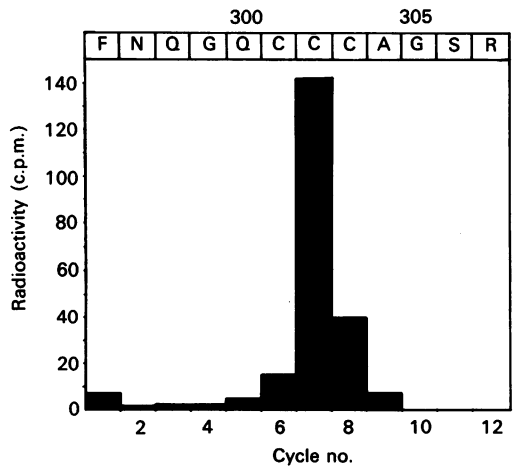

Fig. 4. Sequence analysis of the directly labelled E2 isoenzyme peptide

^{3}H radioactivities of the amino acid phenylthiohydantoin derivatives obtained from automated Edman degradation are shown on one axis and amino acid identity (one-letter code) and cycle number on the other.

DISCUSSION

To probe for a reactive cysteine residue in the active site of aldehyde dehydrogenase, a reagent was required that closely mimicked the structure of an aldehyde substrate and contained a reactive group that combined exclusively with thiol groups. Since the sex pheromone from *Heliothis virescens*, (*Z*)-hexadec-11-enal, was found to be an excellent substrate for both isoenzymes of aldehyde dehydrogenase (Table 1), (*Z*)-hexadeca-1,11-dien-3-one, a thiol-specific affinity reagent, was synthesized. This material had the same length C_{16} backbone and an unsaturation in position 11, making it nearly identical with the pheromone aldehyde. The only difference was a thiol-reactive vinyl ketone instead of an aldehyde. This gave a similar electron-withdrawing 'head' of the molecule and an identical hydrophobic tail [compare compounds (I) and (II) in Fig. 1].

The manner of inactivation by the vinyl ketone was tested in order to determine whether or not reaction was occurring at the active site. Inactivation occurred so rapidly and at very low stoichiometries, even at 4 °C, that saturation and steady-state kinetics could not be performed. When the stoichiometry of incorporation was measured with tritiated vinyl ketone [compound (III) in Fig. 1], it was found to be approx. 2 molecules of vinyl ketone per enzyme tetramer. This agreed well with a report (Ambroziak *et al.*, 1989) of the number of coenzyme-binding sites per molecule of aldehyde dehydrogenase. Substrates or substrate analogues (Table 2) protected the enzyme from reaction with the vinyl ketone, suggesting that the vinyl ketone could be binding in the same place as substrates. The amplitude of burst with *o*-nitrobenzaldehyde was decreased by 90% after the enzyme was partially inactivated. This decrease in amplitude indicated that there were fewer functional active sites in the enzyme after reaction with the vinyl ketone.

In order to obtain information about specificity of label incorporation and identify the labelled peptide, E2 isoenzyme was allowed to react with the vinyl ketone, with the use of the differential labelling method. After tryptic hydrolysis, the peptide map showed only one peak of radioactivity, eluted in fractions 60–68 (Fig. 3*a*). Sequence analysis (Table 3) showed that the broad peak consisted of a number of peptides, all of which were part of a 35-amino acid-residue tryptic peptide comprising residues 273–307. The only other component (component c in Table 3) comprised residues 98–109. After digestion with chymotrypsin in an attempt to shorten the peptide and to identify the labelled residue, a similar result was obtained. All four components (Table 3) were again parts of the tryptic peptide comprising residues 273–307. This peptide in E1 isoenzyme was first identified at the beginning of the last decade by chemical modification with iodoacetamide (Hempel & Pietruszko, 1981; Hempel *et al.*, 1982*a*). An analogous peptide in the E2 isoenzyme was established by cloning (Hsu *et al.*, 1985) and confirmed by amino acid sequence (Hempel *et al.*, 1985).

In order to identify the position of label, the material obtained by directly labelling the E2 isoenzyme (which showed the same stoichiometry as the differentially labelled E2 isoenzyme) was subjected to tryptic digestion and peptide mapping. The first chromatogram (Fig. 3*b*) showed two peaks, both migrating differently from the material obtained via differential labelling. The amino acid sequence of the main component (component h in Table 3), however, again showed a subcleavage of the same long tryptic peptide that was identified via differential labelling. The cleavage, however, appeared to be chymotryptic in origin, since it occurred between phenylalanine residues 295 and 296. Since different lots of trypsin were used for these two hydrolyses, the chymotryptic cleavage may have occurred as the result of the presence of residual chymotryptic activity in the *N*-tosyl-L-phenylalanylchloromethane-treated trypsin. This considerably shortened the peptide and allowed identification of the position of label, which corresponds to residue 302 in the intact E2 isoenzyme (Fig. 4).

Chemical modification of the tryptic peptide comprising residues 273–307 with the hydrophobic C_{16} vinyl ketone produced great alterations in its solubility and chromatographic properties. On a C_{18} h.p.l.c. column the vinyl ketone-modified peptide was eluted well after the same peptide modified by iodoacetamide, which was eluted at approx. 55 min. This may have been due to the hydrophobic interaction of the C_{16} structure of the vinyl ketone with the reverse-phase column. This property aided in purification by shifting the labelled peptide to the end of the chromatogram, thus separating it from most of the other material. The labelled peptide was eluted in a peak that was not gaussian; it also had a tendency to adhere strongly to glass, which resulted in losses during freeze-drying. Treatment of glassware with ethanolic KOH partially eliminated this problem.

The characteristics of the reaction of aldehyde dehydrogenase and the vinyl ketone fulfilled the criteria of Shaw (1970) for a reagent reacting with a residue in the active site. First, there was total activity loss when the enzyme was incubated with the vinyl ketones at low stoichiometries. Secondly, inactivation of the enzyme was protected against by incubation with substrates or substrate analogues. Thirdly, the inactivation stoichiometry was equivalent to the number of catalytic sites and the burst amplitude produced was significantly decreased when the enzyme was modified. Fourthly, the incorporation of vinyl ketone into aldehyde dehydrogenase was totally specific. The enzyme contains 36–44 thiol groups, of which only two are labelled. Since vinyl ketones can react with any thiol group, the structure of this inhibitor must closely mimic that of an aldehyde substrate to allow such specific reaction. It should be also noted that the reaction of the vinyl ketone with the enzyme abolished both dehydrogenase and esterase activities. Thus cysteine-302 is most probably localized at the substrate area of the active site of aldehyde dehydrogenase.

Up to the present time, ten aldehyde dehydrogenases have been sequenced (von Bahr-Lindström *et al.*, 1984; Hempel *et al.*, 1984, 1985; Pickett *et al.*, 1987; Johansson *et al.*, 1988; Jones *et al.*, 1988; Farres *et al.*, 1989; Kok *et al.*, 1989; Dunn *et al.*, 1989). Of all the cysteine residues present in human aldehyde dehydrogenase, only cysteine-302 is conserved. Cysteine-302 always aligns 28 residues to the *C*-terminal side of a totally conserved region that spans residues 265–274 in the numbering system of the human enzymes. This region, including glutamate-268, was previously identified in our laboratory by the use of bromoacetophenone (Abriola *et al.*, 1987). The peptide containing cysteine-302 is considerably less conserved than the one containing glutamate-268. Thus, at the present stage of investigation, both glutamate-268 and cysteine-302 are likely candidates for the catalytic residue of aldehyde dehydrogenase. The argument for glutamate stems from greater conservation of amino acid residues around it, the argument for cysteine is in its greater nucleophilicity. In the absence of concrete evidence, assuming that cysteine is the catalytic residue that forms a covalent intermediate with substrates, glutamate presumably functions in the ionization of this residue.

Aldehydes are degraded by oxidation to carboxylic acids in a variety of insect tissues (Ding & Prestwich, 1986), and it is postulated that the aldehyde dehydrogenase and aldehyde oxidase activities are important in pheromone clearance *in vivo* (Prestwich *et al.*, 1989). The development of the vinyl ketone in labelled form (Tasayco J. & Prestwich, 1990*b*) has enabled unambiguous identification of aldehyde dehydrogenases in the head and antennae of both the male and female tobacco budworm moth *Heliothis virescens*. In insects aldehydes dehydrogenase occurs in minute quantities that are difficult or almost impossible to detect and identify via direct isolation. These experiments therefore provide a basis for use of vinyl ketones as probes for aldehyde dehydrogenase. Affinity labelling of aldehyde dehydrogenase with vinyl ketones may prove to be of general utility in biochemical studies of these enzymes.

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