Inactivation of horse liver mitochondrial aldehyde dehydrogenase by disulfiram

Evidence that disulfiram is not an active-site-directed reagent

Charles G. SANNY* and Henry WEINER*†‡

*Department of Biochemistry, The Oklahoma College of Osteopathic Medicine and Surgery, Tulsa, OK 74107, U.S.A., and

†Department of Biochemistry, Purdue University, West Lafayette, IN 47907, U.S.A.

The inhibition of mitochondrial (pI 5) horse liver aldehyde dehydrogenase by disulfiram (tetraethylthiuram disulphide) was investigated to determine if the drug was an active-site-directed inhibitor. Stoichiometry of inhibition was determined by using an analogue, [³⁵S]tetramethylthiuram disulphide. A 50% loss of the dehydrogenase activity was observed when only one site per tetrameric enzyme was modified, and complete inactivation was not obtained even after seven sites per tetramer were modified. Modification of only two sites accounted for a loss of 75% of the initial catalytic activity. The number of functioning active sites per tetrameric enzyme, as determined by the magnitude of the pre-steady-state burst of NADH formation, did not decrease until approx. 75% of the catalytic activity was lost. These data indicate that disulfiram does not modify the essential nucleophilic amino acid at the active site of the enzyme. The data support an inactivation mechanism involving the formation of a mixed disulphide with a non-essential cysteine residue, resulting in a lowered specific activity of the enzyme.

INTRODUCTION

Disulfiram (tetraethylthiuram disulphide) is a drug commonly given to alcoholics to deter them from drinking ethanol. The drug is an inhibitor *in vivo* of aldehyde dehydrogenase (aldehyde: NAD⁺ oxidoreductase, EC 1.2.1.3; ALDH). The inactivation of ALDH *in vivo* results in an increase in blood acetaldehyde after the ingestion of ethanol, and is followed by the manifestation of the physiological disulfiram–ethanol reaction (Kitson, 1977; Faiman, 1979).

Numerous studies *in vivo* and *in vitro* revealed that the drug is an irreversible covalent inhibitor of ALDH. It was demonstrated that the number of thiol groups decreased in human (Vallari & Pietruszko, 1982) and sheep (Kitson, 1983) liver ALDH after the enzyme had been treated with disulfiram. It was originally proposed that the modified cysteine residue was at the active site of the enzyme (Deitrich & Erwin, 1971). More recent studies with human (Vallari & Pietruszko, 1982) and sheep (Kitson, 1983) cytosol ALDH revealed that a thiol group from a second cysteine residue can displace the bound inhibitor, resulting in an internal disulphide bond being formed.

Extensive studies *in vitro* on the inhibition of the enzyme isolated from sheep liver cytosol and mitochondria have been performed. The cytosol enzyme retained 10% of its initial activity (Kitson, 1978), whereas the mitochondrial enzyme retained 50% (Hart & Dickinson, 1977). Thus it appears that disulfiram may not be an active-site-directed reagent. Further, with sheep (Dickinson *et al.*, 1981; Kitson, 1982) and human (Vallari & Pietruszko, 1982) liver enzyme, maximal, but not total,

inhibition is achieved when only two mol of disulfiram have been bound per mol of tetrameric enzyme.

To determine whether or not the compound was attacking the active site, we chose a different approach. It has been shown with horse liver ALDH (Weiner *et al.*, 1976) that there is a pre-steady-state burst of NADH formation. The magnitude of the burst represents the number of functioning active sites (Takahashi & Weiner, 1980). Thus, if the reagent did not destroy the active-site residue, the modified enzyme would still have its full complement of active sites, even though its catalytic-centre activity is lowered. In the present study we report data to show that the disulfiram-labelled horse liver enzyme, which had its $V_{\max}$ decreased to less than 70% of the control value, still had its full complements of active sites. Thus unequivocal evidence has been found to show that, as suspected, disulfiram is not an active-site reagent.

EXPERIMENTAL

Materials

Tetraethylthiuram disulphide (disulfiram) and tetramethylthiuram disulphide (recrystallized twice from acetone before use) and dithiothreitol were from Sigma Chemical Co. Tetramethylthiuram [³⁵S]disulphide (18.5 mCi/mmol) was from Amersham/Searle Corp. Propionaldehyde was obtained from Eastman Organic Chemicals; *p*-nitrophenyl acetate was from Aldrich. NAD⁺ was purchased from P-L Biochemicals. Other routine reagents were obtained from the Biochemistry Department storeroom. Horse livers were provided by

Abbreviation used: ALDH, aldehyde dehydrogenase.

‡ To whom reprint requests should be addressed, at Purdue University.

the Purdue University School of Veterinary Medicine. Deionized distilled water was used in all buffers.

Preparation of ALDH

The pI 5 isoenzyme of horse liver was purified by the procedure of Feldman & Weiner (1972a) with pH 6.0 and pH 5.5 DEAE-cellulose ion-exchange column-chromatography steps substituted for the pH 6.8 and pH 6.0 DEAE-cellulose steps used in the original procedure. For both chromatographic steps, a linear salt gradient from 10 to 100 mM-sodium phosphate, at the appropriate pH, was used to elute the proteins from the column. The specific activity and concentration of the enzyme were determined as previously reported (Feldman & Weiner, 1972a).

Inactivation of ALDH by disulfiram

Purified ALDH was passed through a Pharmacia K 9/30 column packed with Sephadex G-25 (coarse grade) equilibrated with 50 mM-sodium phosphate buffer, pH 7.5, to remove the mercaptan used to protect the enzyme from oxidation during storage. Inactivation of ALDH by disulfiram was monitored as a function of time at 25 °C in this buffer containing 10% (v/v) ethanol and various concentrations of disulfiram. At different times a sample of the reaction mixture was removed and then assayed for aldehyde dehydrogenase activity in 50 mM-sodium phosphate buffer, pH 7.5, containing 0.4 mM-NAD⁺ and 0.14 mM-propionaldehyde. The activity of the remaining active enzyme was monitored by determining the change in A_{340} . Esterase activity was measured at 400 nm in 50 mM-sodium phosphate, pH 8.0, with *p*-nitrophenyl acetate as substrate.

Stoichiometry of thiuram binding

ALDH was allowed to react with various amounts of tetramethylthiuram [³⁵S]disulphide at 25 °C in 50 mM-sodium phosphate buffer, pH 7.5, containing 10% (v/v) ethanol. As a function of time, a sample was removed and assayed for dehydrogenase activity in 50 mM-sodium phosphate buffer containing 1 mM-NAD⁺ and 1 mM-propionaldehyde. Simultaneously, a sample was subjected to gel filtration through Sephadex G-25 equilibrated with 10% ethanol in phosphate buffer to separate protein from reactants. Equal volumes of the fraction containing the protein peak were analysed either for protein, by the procedure of Lowry *et al.* (1951), or for radioactivity. Dimethylthiuram also was separated from the unchanged tetramethyl compound on the Sephadex column. A peak was verified as being the dimethyl compound by t.l.c. on silica gel, with benzene/acetone (9:1, v/v) as the solvent.

Radioactivity was counted in 3 ml of fluor (Tank & Weiner, 1979) with a Beckman LS-100C liquid scintillation counter. Counting efficiency with a wide window was 75%. The total radioactivity associated with protein was essentially equal to that found for the [³⁵S]dimethyl compound. This observation suggests that the mode of binding to tetramethylthiuram disulphide to the horse liver mitochondrial enzyme is through a mixed disulphide bond, analogous to the mechanism proposed by Neims *et al.* (1966), and shown to occur by Kitson (1982). The number of sites modified was calculated from the specific radioactivity associated with the modified enzyme, assuming the following inactivation scheme:

where ESH represents reactive cysteine residues of the enzyme, R is the dimethylamine group, and n is the stoichiometry.

Pre-steady-state burst studies of inactivated enzyme

The pre-steady-state burst magnitude of NADH formation in the dehydrogenase reaction was determined as described by Weiner *et al.* (1976), with a Gilford spectrophotometer. Enzyme was incubated in the presence and absence of disulfiram at pH 7.5 in 50 mM-phosphate buffer containing 10% (v/v) ethanol. After a period of time, concentrated NAD⁺ and aldehyde were added to the cuvette. The rate of NADH production during the steady-state phase after the initial burst phase was measured to determine enzyme activity.

RESULTS

Stoichiometry of thiuram disulphide inhibition

Owing to availability, it was necessary to use the [³⁵S]tetramethyl analogue of disulfiram for the inactivation studies. The inactivation of the enzyme by this derivative appeared to mimic that by the tetramethyl compound. The amount of inhibitor bound to the enzyme and the amount of remaining catalytic activity are presented in Fig. 1.

The data reveal that approx. 50% of the activity was lost when only 1 mol of thiuram binds per mol of tetrameric enzyme. Furthermore, complete inactivation

Fig. 1. Stoichiometry of [³⁵S]thiuram binding to ALDH

Incubations were performed with various concentrations of tetramethylthiuram [³⁵S]disulphide in 50 mM-phosphate, pH 7.5, containing 10% ethanol at 25 °C for different time periods. Inactivated enzyme was chromatographed on Sephadex G-25 (38 cm × 2 cm), with 100 mM-phosphate/10% ethanol as the elution buffer. The protein peak was divided into three portions. One portion was used to determine the catalytic activity in a $V_{\max}$ assay. The second portion was used to determine protein concentration. The last portion was used to determine radioactivity. Insert: it was assumed that 22% of the initial velocity was the final amount of activity remaining (A_1). The data were plotted with A being the remaining activity and A_0 (original activity) as 100%. A reasonable extrapolation to two sites modified per molecule of ALDH could be made by using the data corresponding to the linear portion of the original curve (100 to 40% activity).

was not obtained even after 7 mol of inhibitor was covalently bound per mol of enzyme. It appears that approx. 10–25% of the activity remained after the initial two sites were modified. The insert in Fig. 1 shows a replot of the data on the assumption that 22% activity remained in the modified enzyme (A_1). Hence, there was essentially a linear loss of catalytic activity as the first two sites were modified.

Non-active-site modification of ALDH by disulfiram

It was not possible to determine by sequence analysis if the disulfiram analogue was labelling the catalytically essential residues, since the amino acids at the active site of mitochondrial ALDH are not known. If disulfiram was labelling the catalytic residues, then that molecule of enzyme would be totally inactive. If the inhibitor was modifying a non-essential amino acid, then the catalytic-centre activity of the modified enzyme could decrease, but the number of active sites would remain unaltered. To determine if the disulfiram modification affected the number of active sites, the magnitude of the pre-steady-state burst of NADH formation, which is proportional to

Fig. 2. Active-site titration of ALDH after disulfiram inactivation

The magnitude of the pre-steady-state burst of NADH is equal to the number of mol of active sites remaining per mol of enzyme. The steady-state velocity was the percentage of activity remaining. The enzyme was incubated at pH 7.5 in 50 mM-phosphate buffer containing 10% ethanol. After a period of time, concentrated NAD^+ and aldehyde were added to the cuvette to initiate the reaction, which was monitored with a Gilford spectrophotometer as previously described (Weiner *et al.*, 1976). The control incubation in the presence of disulfiram was performed to determine the magnitude of the burst in the absence of inhibitor ($\square$). Essentially no loss in the number of active sites occurred until only some 25–30% of the catalytic activity remained. Modification of the two reactive sites caused the loss of approx. 78% of the initial activity (Fig. 1).

active-site concentration, was correlated with the specific activity of the enzyme remaining after modification.

The data presented in Fig. 2 clearly reveal that the magnitude of the pre-steady-state burst does not decrease until approx. 75% of the catalytic activity is lost. This shows that disulfiram does not react initially with an essential amino acid at the active site of the enzyme.

Rate of inactivation of ALDH by disulfiram

Inactivation of ALDH by disulfiram produced a biphasic inactivation curve when the log of per cent activity remaining versus time was plotted. The slower second phase is presented in Fig. 3. The inactivation rate was dependent on disulfiram concentration. A similar biphasic inactivation curve was obtained when the tetramethyl derivative was employed. The presence of NAD^+ decreased both the rapid and the slow disulfiram inactivation. Enzyme incubated with 100 μM -disulfiram in the presence of 370 μM - NAD^+ retained 70% of its catalytic activity after 20 min, compared with 10% in the absence of NAD^+ . The presence of both NAD^+ and aldehyde afforded just slightly better protection. Aldehyde alone was not effective at protecting the enzyme against inactivation.

Esterase activity was more resistant than was the dehydrogenase activity to inactivation by disulfiram, as shown in Fig. 3. The presence of NAD^+ (370 μM) afforded only slight protection against inactivation (results not shown). There is some controversy as to whether or not the dehydrogenase reaction utilizes the same active site as does the esterase reaction (e.g. Duncan, 1985). In either case the catalytic residue

Fig. 3. Inactivation of ALDH by thiurams

Incubations were performed at pH 7.5 in 50 mM-phosphate containing 10% ethanol with disulfiram ($\bullet$, 100 μM ; $\blacksquare$, 25 μM) or the tetramethyl derivative ($\circ$, 25 μM). At the indicated times a portion of the reaction mixture was diluted into assay medium, and the activity remaining at that time was determined from the steady-state slope of the curve of the increase in A_{340} versus time. To show the protection by NAD^+ , 370 μM - NAD^+ was added to an incubation in the presence of 100 μM -disulfiram ($\square$). The broken line represents esterase activity after incubation with 100 μM -disulfiram ($\blacktriangle$). Inactivation in the presence of 100 μM -disulfiram and both 400 μM - NAD^+ and 140 μM -propionaldehyde was monitored by measuring the change in A_{340} ($\triangle$).

involved in acyl-enzyme intermediate formation does not appear to be the primary target for the initial covalent binding of disulfiram to the enzyme.

Inactivation rates in the presence of Mg^{2+} ions

It was shown previously (Takahashi & Weiner, 1980) that millimolar Mg^{2+} ions caused an increase in enzymic activity. Rates of disulfiram inhibition were performed in the presence or absence of the ions. The rates of inactivation were the same (results not shown). No stoichiometry experiments were performed, owing to lack of label.

DISCUSSION

Though it has been known for many years that disulfiram inactivates ALDH *in vitro*, the detailed aspects of the inactivation have not been well characterized. It was proposed that the compound covalently binds to the active site of the enzyme (Deitrich & Erwin, 1971). The data from the present study clearly show that disulfiram is not an active-site-directed reagent with the horse mitochondrial enzyme.

It was possible to achieve a high degree of inactivation of the mitochondrial enzyme only when many disulfiram molecules were attached to the enzyme. However, most (approx. 80%) of the enzyme activity, but not the total, was lost when only 2 mol of disulfiram was bound per mol of tetrameric enzyme. Most significant was the observation that this modified enzyme still had its full complement of active sites, as determined by the magnitude of the pre-steady-state burst. The magnitude of the burst is independent of the specific activity of the enzyme; hence, if active sites were destroyed by the inhibitor, a proportional decrease in burst magnitude would have been observed. This titration of the number of functioning active sites remaining in the chemically modified enzyme is analogous to what has been performed with proteolytic enzymes (Ray & Koshland, 1963) and with this ALDH (Takahashi & Weiner, 1980; Takahashi *et al.*, 1981). The value of such a titration is that the results are independent of the catalytic activity of the enzyme. Hence, it differentiates between chemical modification at the active site, which totally inactivates the enzyme, and modification at a non-active-site residue, which only decreases specific activity.

It was demonstrated with human (Vallari & Pietruszko, 1982) and then with sheep (Kitson, 1982) cytosolic ALDH that the bound diethyldithiocarbamate could be displaced by a second cysteine residue, forming an internal disulphide bond in the enzyme. This reaction did not occur with the horse mitochondrial enzyme under the conditions of the experiment. We found 2 mol of dimethyldithiocarbamate bound per mol of tetrameric ALDH and an equal amount of free dimethyldithiocarbamate, proving that the bound reagent was not displaced from the enzyme. These results are similar to those found with the sheep enzyme (Kitson, 1982) if proper reaction conditions are employed.

Since the ^{35}S label remained in the modified enzyme, it was possible to calculate the number of sites modified. The correlation between catalytic activity remaining and the number of sites modified showed that, as the first 1–1.5 sites were modified, a linear decrease in catalytic activity occurred. It was found that approx. 2 critical sites were modified per tetrameric enzyme. The sheep

enzymes were shown also to be inactivated by just 2 mol of disulfiram binding per mol of tetrameric enzyme (Hart & Dickinson, 1977; Kitson, 1978; Dickinson *et al.*, 1981). Why just 2 mol of disulfiram initially bind per mol of tetrameric enzyme is not known. It may somehow be related to the fact that the enzyme functions with half-of-the-site reactivity when in the tetrameric state (Weiner *et al.*, 1976; Takahashi & Weiner, 1980; Takahashi *et al.*, 1981). Steric hindrance may also play a role in the stoichiometry of disulfiram inactivation (Mackerell *et al.*, 1985).

Finding that disulfiram is not attacking an essential thiol residue does not rule out a thiol group being at the active site. Hart & Dickinson (1977) observed complete inactivation of sheep mitochondrial ALDH by 5,5'-dithiobis-(2-nitrobenzoate) with modification of four thiol residues per tetramer. Thus far, however, no one has truly identified the active site of ALDH, though the sequences for the human cytosolic and human mitochondrial enzymes are known (von Bahr-Lindström *et al.*, 1984; Hemple *et al.*, 1984, 1985). All evidence, kinetic (Weiner *et al.*, 1976; Feldman & Weiner, 1972b) and spectroscopic (Buckley & Dunn, 1982), leads one to conclude that aldehyde oxidation proceeds through an acyl intermediate. The acylated residue could be serine rather than cysteine. More recent spectroscopic data, however, make it appear that the residue is indeed cysteine (Dickinson, 1985; Dunn & Buckley, 1985).

Finding that disulfiram is not an active-site inhibitor of ALDH explains why experiments *in vivo* do not produce fully inhibited enzymes. Studies *in vitro* with human (Greenfield & Pietruszko, 1977), bovine (Sugimoto *et al.*, 1976), sheep (Allanson & Dickinson, 1984; Dickinson *et al.*, 1981; Kitson, 1975) and horse liver enzyme (Eckfeldt *et al.*, 1976) showed that the cytosol enzymes were more susceptible to inhibition by the drug than were the mitochondrial enzymes. The mitochondria, however, are the major site of acetaldehyde oxidation, at least in the rat (Svanas & Weiner, 1985; Weiner, 1979) and monkey (Havre *et al.*, 1976). Thus the person taking the drug may still have some limited capacity to oxidize aldehydes and may be afforded minimal protection against their toxic effects.

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