Mechanism of inactivation of sheep liver cytoplasmic aldehyde dehydrogenase by disulfiram

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Stoichiometric amounts of [^{14}C]disulfiram react rapidly with sheep liver cytoplasmic aldehyde dehydrogenase to give loss of catalytic activity and incorporation of the expected amount of radioactivity. In a subsequent slower reaction the label is lost from the enzyme without re-emergence of enzymic activity. The results imply that *in vivo* disulfiram may act as an oxidation–reduction catalyst for the inactivation of aldehyde dehydrogenase.

A widely used method of limiting alcoholics' drinking is to give them disulfiram (tetraethylthiuram disulphide). This compound inactivates aldehyde dehydrogenase (EC 1.2.1.3) in the liver, with the result that high concentrations of acetaldehyde build up if ethanol is drunk. Acetaldehyde is probably the main cause of the unpleasant and, it is hoped, dissuasive symptoms that are experienced under these circumstances (Kitson, 1977). Accordingly, the mechanism of interaction between disulfiram and aldehyde dehydrogenase has been much investigated (Kitson, 1975, 1978, 1982).

Vallari & Pietruszko (1982) have shown that, if human liver cytoplasmic aldehyde dehydrogenase is treated with [^{14}C]disulfiram (at a stoichiometry equal to or less than two molecules of disulfiram per tetrameric enzyme molecule), left to stand for a considerable length of time (20 h at 25°C) and then subjected to exhaustive dialysis at 4°C, the resulting enzyme is inactive and carries no radioactive label. Furthermore, after this treatment the enzyme possesses fewer free thiol groups than it does initially. Treatment of the inactive enzyme with 2-mercaptoethanol leads to recovery of activity. They interpret these results in terms of the occurrence of reactions (A) and (B) as follows:

In reaction (A), disulfiram first combines with a thiol group to form a dithiocarbamate adduct, as suggested by Kitson (1975) on the basis of the general proposal of Neims *et al.* (1966). According to Vallari & Pietruszko (1982), the dithiocarbamate adduct then reacts with another suitably positioned enzyme thiol group to form a disulphide (reaction B). However, the authors leave certain important points unconsidered. What are the relative rates of reactions (A) and (B)? Is the inactivation of the enzyme consequent upon reaction (A), or is the enzymic activity lost only after reaction (B)? Is it necessary to incubate enzyme and modifier for 20 h, since it is well known that disulfiram causes inactivation of cytoplasmic aldehyde dehydrogenase very rapidly (Eckfeldt *et al.*, 1976; Hempel *et al.*, 1980; Kitson, 1982)? What happens with much shorter incubation times? In view of these unanswered questions, the authors' statement that [^{14}C]disulfiram reacts with aldehyde dehydrogenase 'with loss of catalytic activity and no incorporation of label' may be an oversimplification. In particular, their results do not rule out the possibility of the enzyme's reacting rapidly with disulfiram with concomitant loss of activity in accordance with reaction (A), followed later by the relatively slow

loss of label in accordance with reaction (B). The results presented below show that in fact this is exactly what does happen. Although the occurrence of reaction (B) is very interesting in its own right, as far as the inactivation of the enzyme is concerned it is not essential.

Experimental

Cytoplasmic aldehyde dehydrogenase from sheep liver, purified by the method of Dickinson *et al.* (1981), was dialysed before use against several changes of N_2 -saturated 50 mM-sodium phosphate buffer, pH 7.4, containing 0.3 mM-EDTA at 4°C. Disulfiram (tetraethylthiuram disulphide) labelled with ^{14}C was prepared as described before (Kitson, 1978); it had a specific radioactivity of 1.35×10^{12} c.p.m./mol in the counting system used.

Aldehyde dehydrogenase activity was determined spectrophotometrically at 25°C at 340 nm in 33 mM-sodium phosphate buffer, pH 7.4, containing 1 mM-NAD⁺ and 1 mM-acetaldehyde by using an Aminco DW-2a instrument. Protein concentrations were determined as before (Dickinson *et al.*, 1981). The M_r of the tetramer was taken to be 212 000 (MacGibbon *et al.*, 1979). Reaction mixtures consisted of 10 μ l of an ethanol solution of [^{14}C]-disulfiram added rapidly to 0.8 ml of enzyme solution. Immediately after mixing, a 0.5 ml sample was taken and applied to a column (11 cm $\times$ 0.8 cm) of Bio-Gel P-6. Alternatively, the reaction mixtures were incubated for various lengths of time at various temperatures (0, 25 and 37°C) before samples were passed down Bio-Gel. After incubation of the mixture at 0°C, the column was run at 4°C; after incubation of the mixture at 25°C or at 37°C, the column was run at room temperature. Elution of the columns was with N_2 -saturated 50 mM-phosphate buffer, pH 7.4, containing 0.3 mM-EDTA. Enzyme was collected in a volume of about 1 ml immediately after the void volume, and was well separated from small molecules (such as radioactive diethyldithiocarbamate). The enzyme could be separated from unbound radioactivity in this way within 4 min of mixing of enzyme and [^{14}C]-disulfiram. Before passage down the column a 50 μ l portion was taken from the reaction mixture to determine total radioactivity. After gel filtration duplicate 50 μ l portions of the enzyme solution were taken to determine protein-bound radioactivity. Radioactivity was determined as before (Kitson, 1978) by using a Beckman LS 8000 instrument.

Results and discussion

Immediately after the mixing of enzyme and [^{14}C]-disulfiram as described above, a 15 μ l sample

was taken for assay of enzymic activity. The percentage inactivation was found to be 65.2–66.4 for a disulfiram/enzyme tetramer molar ratio of 1.02 and 84–85 for ratio of 1.66 (with a different enzyme preparation). Such values are in the expected range; as discussed before (Kitson, 1978; Dickinson *et al.*, 1981), cytoplasmic aldehyde dehydrogenase probably requires 2 equivalents of disulfiram for complete inactivation [as also found by Vallari & Pietruszko (1982)], but the observed stoichiometry is often slightly less. This would arise if, on storage and dialysis before use, some of the enzyme were to become denatured; the protein concentration would then be an overestimate of the active enzyme concentration.

The results obtained on incubating enzyme and [^{14}C]-disulfiram under various conditions, followed by rapid separation of non-protein-bound radioactivity, are presented in Table 1. The last three columns in the Table compare the experimentally determined specific radioactivity of the protein with what would be expected assuming only reaction (A) to occur. The main observation is that within the first few minutes of mixing (at 25°C) the incorporation of radioactivity is clearly and reproducibly close to that predicted from reaction (A). (The average result of six experiments with various disulfiram/enzyme ratios was that over 81% of the predicted radioactivity was still bound to the enzyme at this point.) However, over the next hour the extent of incorporation of label falls; the last 10% or so apparently remains permanently attached to the protein. After 10 h incubation there is no re-emergence of the enzyme activity that was abolished by disulfiram treatment; this means that the loss of label is not due simply to a reversal of reaction (A), but is presumably due to reaction (B). Not surprisingly, the rate of loss of label is lower at 0°C and higher at 37°C. The presence of NAD⁺ does not appear to affect the rate of loss of label to any significant extent. Vallari & Pietruszko (1982) reported that there was zero radioactivity retained after 20 h at 25°C, but they presented no data for shorter incubation times. They stated that full catalytic activity could be recovered by adding 2-mercaptoethanol (20 mM) to the assay cuvette. A similar observation was made in the present work; the addition of dithiothreitol (4.3 mM) to an assay mixture of the modified enzyme (after 10 h incubation at 25°C) resulted in the gradual re-appearance (over a period of 20–30 min) of most of the activity that had been abolished by disulfiram treatment.

The results presented here allow a clearer understanding of the mechanism of inactivation of aldehyde dehydrogenase by disulfiram. The enzyme reacts rapidly with the modifier in accordance with reaction (A), accompanied by loss of activity. The

Table 1. Extent of incorporation of label from [^{14}C]disulfiram by aldehyde dehydrogenase after various incubation conditions

For experimental details see the text.

Initial enzyme concn. (μM)	Molar ratio of disulfiram to enzyme tetramer	Temperature of incubation ($^{\circ}\text{C}$)	Incubation period*	Specific radioactivity of eluted protein (c.p.m./mg)	Predicted specific radioactivity (on basis of reaction A) (c.p.m./mg)	Incorporation of radioactivity (% of predicted)
45.8	1.02	0	<1 min	2751	2822	97.5 ($n = 1$)
45.8	1.02	0	30 min	2583	2822	91.5 ($n = 2$)
49.9	0.55	25	<1 min	1362	1744	78.1 ($n = 1$)
45.8	1.02	25	<1 min	2533	2822	89.7 ($n = 3$)
49.9	1.11	25	<1 min	2656	3489	76.1 ($n = 1$)
49.9	1.66	25	<1 min	3409	5233	65.1 ($n = 1$)
45.8	1.02	25	10 min	1701	2822	60.3 ($n = 1$)
48.6	0.96	25	10 min	1460	2708	53.9 ($n = 1$)
48.6†	0.96	25	10 min	1332	2708	49.2 ($n = 2$)
45.8	1.02	25	30 min	711	2822	25.2 ($n = 1$)
45.8	1.02	25	1 h	430	2822	15.2 ($n = 1$)
45.8	1.02	25	3 h	360	2822	12.8 ($n = 1$)
45.8	1.02	25	10 h	433	2822	15.3 ($n = 2$)
49.9	1.66	25	20 h	570	5233	10.9 ($n = 3$)
48.6	0.96	37	10 min	258	2708	9.5 ($n = 1$)
48.6	0.96	37	1 h	263	2708	9.7 ($n = 1$)

* This is the period between mixing enzyme with disulfiram and application to the Bio-Gel column. Effectively all incubations were approx. 2–3 min longer, which is the time spent on the column.

† These incubations contained NAD^+ (2.8 mM).

inactivation is known to be extremely fast at 25°C , as shown by stopped-flow studies (Dickinson *et al.*, 1981), and even at 0 – 4°C reaction (A) is evidently complete within the short time necessary to run the Bio-Gel column. Subsequently reaction (B) occurs at a much lower rate, resulting in enzyme that is still inactive but now (largely) unlabelled. (The small amount of residual protein-bound radioactivity presumably means that some of the dithiocarbamate groups that become attached to the enzyme are inaccessible to displacement by other protein thiol groups.) In short, reaction (A) is a sufficient condition for inactivation of the enzyme, reaction (B) is unnecessary.

In previous studies (Kitson, 1978, 1982) the effect of disulfiram on cytoplasmic aldehyde dehydrogenase has been followed in one of two ways: usually by monitoring the rate of the enzyme-catalysed reaction when disulfiram is added to an assay at 25°C , or occasionally by pretreating the enzyme (kept on ice) with disulfiram and subsequently withdrawing samples for assay at 25°C . The results in Table 1 show that under both circumstances it is essentially the dithiocarbamate-modified form of the enzyme that is present in the assay mixture. Only if the enzyme is assayed at a higher temperature is reaction (B) rapid enough for much of the disulphide form of the enzyme to be produced during the time of the assay. However,

since both modified forms of the enzyme are inactive, this point may not be of crucial significance to kinetic studies.

Reaction (B) has implications for the action of disulfiram *in vivo*. On absorption into the bloodstream disulfiram is very largely if not completely reduced to diethyldithiocarbamate by reaction with reduced glutathione (Cobby *et al.*, 1977). Moreover, administration of diethyldithiocarbamate as such leads to inactivation of aldehyde dehydrogenase *in vivo* just as disulfiram does (Deitrich & Erwin, 1971), but diethyldithiocarbamate has little effect on the enzyme *in vitro* (Kitson, 1975, 1982). To explain these observations it has been proposed (Deitrich & Erwin, 1971; Kitson, 1977) that to some extent at least diethyldithiocarbamate can be re-oxidized to disulfiram, allowing a sufficient concentration of the latter to effect the inactivation. Since the enzyme has such a great affinity for disulfiram even in the presence of high concentrations of reduced glutathione (Kitson, 1981), the necessary concentration of disulfiram itself need not be high. Substances such as cytochrome *c*, methaemoglobin and xanthine oxidase have the capacity to oxidize diethyldithiocarbamate (DuBois *et al.*, 1961; Stromme, 1963; Fried, 1976). The present results show that reaction (B) is fairly rapid at 37°C ; this means that *in vivo* none of the modifier remains attached for long to the enzyme, and

Scheme 1. *Proposed interactions involving disulfiram and diethyldithiocarbamate in vivo*

Abbreviations: Enz, cytoplasmic aldehyde dehydrogenase; GSH and GSSG, the reduced and oxidized forms of glutathione.

therefore the cyclic scheme shown in Scheme 1 is suggested. In this, depending on the relative rates of re-oxidation and catabolism (Faiman *et al.*, 1978) of diethyldithiocarbamate, a single molecule of disulfiram has the potential to inactivate many enzyme molecules, acting in effect as a redox catalyst. Such a role for disulfiram could mean that the necessary daily maintenance dose of disulfiram (250 mg or less) is smaller than would be needed if some of it were being continually used up in a more straightforward pathway of protein modification [such as reaction (A) only].

It is implied by Vallari & Pietruszko (1982) that the occurrence of reaction (B) explains the fact that cytoplasmic aldehyde dehydrogenase, and not other enzymes with essential thiol groups, is specifically

modified *in vivo* by disulfiram. However, since reaction (A) precedes reaction (B) and is the cause of the inactivation, reaction (B) can have no bearing on this point. It must simply be that the sensitive thiol groups of aldehyde dehydrogenase are much more reactive towards physiological concentrations of disulfiram (according to reaction A) than are those of other enzymes.

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