Reaction between sheep liver mitochondrial aldehyde dehydrogenase and various thiol-modifying reagents

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Sheep liver mitochondrial aldehyde dehydrogenase reacts with 2,2'-dithiodipyridine and 4,4'-dithiodipyridine in a two-step process: an initial rapid labelling reaction is followed by slow displacement of the thiopyridone moiety. With the 4,4'-isomer the first step results in an activated form of the enzyme, which then loses activity simultaneously with loss of the label (as has been shown to occur with the cytoplasmic enzyme). With 2,2'-dithiodipyridine, however, neither of the two steps of the reaction has any effect on the enzymic activity, showing that the mitochondrial enzyme possesses two cysteine residues that must be more accessible or reactive (to this reagent at least) than the postulated catalytically essential residue. The symmetrical reagent 5,5'-dithiobis-(1-methyltetrazole) activates mitochondrial aldehyde dehydrogenase approximately 4-fold, whereas the smaller related compound methyl 1-methyltetrazol-5-yl disulphide is a potent inactivator. These results support the involvement of mixed methyl disulphides in causing unpleasant physiological responses to ethanol after the ingestion of certain antibiotics.

INTRODUCTION

A considerable amount of work has been done on the interaction of cytoplasmic aldehyde dehydrogenase with thiol-modifying reagents (Kitson, 1984, 1987a; Kitson & Loomes, 1985). These studies grew from an interest in the use of disulfiram (tetraethylthiuram disulphide) in alcoholism therapy (Kitson, 1977) and led on to the examination of various other disulphides such as 2,2'-dithiodipyridine and 4,4'-dithiodipyridine. It was proposed that, with the latter reagents, the resulting modified cysteine residue acts as an intramolecular inactivator of the enzyme by reacting with a second, possibly catalytically essential, cysteine residue (Kitson & Loomes, 1985). Later, experiments with compounds such as 5,5'-dithiobis-(1-methyltetrazole) were performed to try and elucidate how certain cephalosporin antibiotics bring about the inactivation of aldehyde dehydrogenase *in vivo* (Kitson, 1987b). The reason why this work concentrated on the cytoplasmic form of the enzyme is that from early times it has been known to be far more sensitive to disulfiram *in vitro* than is the mitochondrial isoenzyme (Kitson, 1975). However, most of the oxidation of acetaldehyde during alcohol metabolism occurs in the mitochondria (Parilla *et al.*, 1974), and inactivation of mitochondrial aldehyde dehydrogenase must thus be important in producing the physiological responses to alcohol after administration of such things as disulfiram or 1-methyltetrazol-5-ylthio-containing antibiotics. The purpose of the present paper, therefore, is to help redress the balance in what is known about the two forms of aldehyde dehydrogenase, specifically by examining the effect on the mitochondrial form of 2,2'-dithiodipyridine, 4,4'-dithiodipyridine, 5,5'-dithiobis-(1-methyltetrazole) and methyl 1-methyltetrazol-5-yl disulphide.

EXPERIMENTAL

5,5'-Dithiobis-(1-methyltetrazole) and methyl 1-methyltetrazol-5-yl disulphide were prepared as before (Kitson, 1986). Sheep liver mitochondrial aldehyde

dehydrogenase was prepared by the method of Hart & Dickinson (1977) as modified by Allanson & Dickinson (1984). Before use it was thoroughly dialysed against 50 mM-sodium phosphate buffer, pH 7.4, containing 0.3 mM-EDTA. Protein concentrations were calculated by using an $A_{280}^{1\%}$ value of 9.7 (Allanson & Dickinson, 1984). The M_r of the enzyme was taken to be 212000 (MacGibbon *et al.*, 1979). In modification experiments, the enzyme in 35 mM-sodium phosphate buffer, pH 7.4, containing 1 mM-NAD⁺ was mixed at 25 °C with 15 μ l of an ethanol solution of the appropriate modifier. After 10 min acetaldehyde was added to give 1 mM in a final volume of 3 ml and the production of NADH was monitored at 340 nm. In some cases enzyme and modifier were mixed in the absence of NAD⁺, which was then added, along with acetaldehyde, 10 min later. The release of 4-thiopyridine from labelled enzyme was investigated as follows. Duplicate enzyme solutions (14 μ M) in 0.7 ml of 50 mM-sodium phosphate buffer, pH 7.4, containing NAD⁺ (1.6 mM) were mixed at 25 °C with 15 μ l of an ethanol solution of 4,4'-dithiodipyridine (126 μ M final concentration). After 5–10 min the reaction mixtures were passed down Bio-Gel P-6 columns (11 cm $\times$ 0.8 cm) equilibrated with 50 mM-sodium phosphate buffer, pH 7.4, at room temperature. A volume of 1 ml was collected immediately after the void volume. This solution was then left at 25 °C and was periodically observed at 324 nm (the $\lambda_{\max}$ of 4-thiopyridone). Small samples were taken at intervals for the determination of enzymic activity at pH 7.4 with the use of 1 mM-NAD⁺ and 1 mM-acetaldehyde. A similar experiment was carried out with enzyme (19 μ M) and 2,2'-dithiodipyridine (140 μ M), and the release of 2-thiopyridone was monitored at 342 nm.

RESULTS AND DISCUSSION

Effects of 2,2'-dithiodipyridine and 4,4'-dithiodipyridine

Fig. 1 shows the results of modifying sheep liver mitochondrial aldehyde dehydrogenase with 2,2'-dithiodipyridine, isolating the modified enzyme, and incubating it at 25 °C and pH 7.4 while periodically the enzymic

Fig. 1. Displacement of 2-thiopyridone from mitochondrial aldehyde dehydrogenase after labelling by 2,2'-dithiodipyridine

Enzyme ($19\ \mu\text{M}$) was treated with 2,2'-dithiodipyridine ($140\ \mu\text{M}$) in the presence of NAD^+ ($1\ \text{mM}$) and modified enzyme was isolated as described in the Experimental section. The release of 2-thiopyridone from the labelled enzyme at 25°C and pH 7.4 was monitored at 342 nm ($\circ$ and $\bullet$). The enzymic activity was also assayed ($\square$ and $\blacksquare$), expressed as a percentage of a control that had been treated in exactly the same way except for the omission of 2,2'-dithiodipyridine. On one occasion, 4,4'-dithiodipyridine (a 9-fold excess over the enzyme concentration) was added to the assay of the modified enzyme with the result shown ($\triangle$).

activity and the production of 2-thiopyridone are monitored. Similar experiments with 4,4'-dithiodipyridine are illustrated in Fig. 2. Clearly, the site of initial labelling in both cases is sufficiently close to a second thiol group for the label to be slowly displaced off, just as previously been found with the cytoplasmic enzyme (Kitson & Loomes, 1985). However, the results with the mitochondrial and cytoplasmic enzymes show significant differences. First, modification with 2,2'-dithiodipyridine (unlike the case of the 4,4'-isomer) does not activate the mitochondrial enzyme at all, whereas both reagents achieve a similar extent of activation of the cytoplasmic form. Secondly, as the cystine-containing form of the mitochondrial isoenzyme is produced, the activity stays constant in the case of 2,2'-dithiodipyridine but declines progressively with 4,4'-dithiodipyridine. (After 27 h the activity is down to 40% of the control.) In previous work with the cytoplasmic enzyme both reagents gave a pronounced loss of activity as the label was displaced, and these results were interpreted in terms of the displacing group's being a possibly catalytically essential cysteine residue (Kitson, 1984; Kitson & Loomes, 1985). The same explanation could apply to the situation with the mitochondrial enzyme and 4,4'-dithiodipyridine. Clearly, however, the lack of inactivation of the mitochondrial enzyme by 2,2'-dithiodipyridine precludes either thiol group involved in cystine formation in this case from having any essential role in enzymic catalysis.

Fig. 2. Displacement of 4-thiopyridone from mitochondrial aldehyde dehydrogenase after labelling with 4,4'-dithiodipyridine

Conditions were as for Fig. 1, except that enzyme, modifier and NAD^+ concentrations were $14\ \mu\text{M}$, $126\ \mu\text{M}$ and $1.6\ \text{mM}$ respectively, and absorbance was monitored at 324 nm ($\circ$ and $\bullet$). Activity was also assayed ($\square$ and $\blacksquare$).

It seems surprising to conclude that reagents as similar as 2,2'-dithiodipyridine and 4,4'-dithiodipyridine might react with different enzymic thiol groups, and yet such a situation is known to occur with the cytoplasmic enzyme and the related modifiers methyl diethylthiocarbamoyl disulphide and methyl pyrid-2-yl disulphide (Kitson & Loomes, 1985). A further observation that suggests that different groups are involved is that mitochondrial enzyme pre-modified by 2,2'-dithiodipyridine is still strongly activated by the 4,4'-isomer (Fig. 1). Sheep liver aldehyde dehydrogenase has not been sequenced, but the horse and human mitochondrial enzymes have seven and nine cysteine residues respectively per subunit (Johansson *et al.*, 1988). Six of these are common to the horse and human cytoplasmic enzyme sequences also, and include two that are contiguous (301 and 302) and three that have been implicated as having possible functional significance (49, 162 and 302) (Tu & Weiner, 1988a,b; von Bahr-Lindström *et al.*, 1984). On the basis of the variety of different effects that various thiol-modifying reagents can have on the cytoplasmic and mitochondrial forms of aldehyde dehydrogenase, it is possible that several of the cysteine residues are closely positioned in the enzyme's tertiary structure (perhaps in a substrate-binding pocket) and that subtle steric factors control precisely where a particular reagent reacts. This could explain the results referred to above and below and also such observations as the different site of labelling by two NAD^+ analogues that are identical but for a single $-\text{CH}_2-$ unit (von Bahr-Lindström *et al.*, 1985). It might also have a bearing on the controversy over whether the esterase activity of aldehyde dehydrogenase occurs at the enzyme's normal dehydrogenase active site (Motion *et al.*, 1988; Loomes & Kitson, 1986). A possible explanation of the conflicting results is that both activities occur at essentially the same location, but that different thiol groups are involved in the actual mechanism of reaction.

Effects of 1-methyltetrazol-5-ylthio-containing modifiers

Antibiotics that possess a 1-methyltetrazol-5-ylthio side chain (such as moxalactam, cefoperazone and cefa-

Fig. 3. Effects of various thiol-group modifiers on the activity of mitochondrial aldehyde dehydrogenase

Enzyme ($0.083 \mu\text{M}$) in the presence of NAD^+ (1 mM) was mixed with 5,5'-dithiobis-(1-methyltetrazole) (■), 4,4'-dithiodipyridine (○), 2,2'-dithiodipyridine (●) or disulfiram (△) at pH 7.4 and 25°C . Alternatively, 5,5'-dithiobis-(1-methyltetrazole) was mixed with enzyme first and then NAD^+ was added later (□). Activity was measured after the addition of acetaldehyde (1 mM).

Fig. 4. Effect of methyl 1-methyltetrazol-5-yl disulphide on mitochondrial aldehyde dehydrogenase

Enzyme ($0.083 \mu\text{M}$) in the presence (●) or absence (○) of NAD^+ (1 mM) was mixed with various concentrations of methyl 1-methyltetrazol-5-yl disulphide and subsequently assayed for activity. For comparison, the effect of methyl diethylthiocarbamoyl disulphide is also shown (□).

mandole) have the potential to cause an unpleasant 'anti-alcohol' reaction much like that brought about by disulfiram and cyanamide (drugs used in alcoholism therapy) (Kitson, 1987b). On the basis of the results of modification experiments with sheep liver cytoplasmic aldehyde dehydrogenase it was proposed that such antibiotics release the side chain *in vivo* and this is then oxidized to the reactive disulphide 5,5'-dithiobis-(1-methyltetrazole) (Kitson, 1986). Alternatively, by analogy with the hypothesis of MacKerell *et al.* (1985)

concerning the metabolism of disulfiram, it was suggested that the reactive species in the antibiotic-alcohol reactions might be the mixed disulphide methyl 1-methyltetrazol-5-yl disulphide (Kitson, 1986, 1987b). For any physiological effect to be manifested it is important that the mitochondrial form of aldehyde dehydrogenase becomes inactivated, as this is the isoenzyme primarily responsible for the oxidation of acetaldehyde under normal conditions (Parilla *et al.*, 1974). In Fig. 3 we see that, rather than lowering the activity of the mitochondrial enzyme (as does happen in the cytoplasmic case), 5,5'-dithiobis-(1-methyltetrazole) causes a very pronounced activation. On the other hand, the corresponding mixed methyl disulphide inactivates the mitochondrial enzyme strongly (Fig. 4), as it does the cytoplasmic form (Kitson, 1986). If inactivation is indeed consequent on the reaction of a catalytically essential group, then obviously it is more freely accessible in the cytoplasmic case where the bulky symmetrical modifiers [disulfiram and 5,5'-dithiobis-(1-methyltetrazole)] are effective. Only the smaller methyl-containing disulphides can successfully reach the critical group belonging to the mitochondrial enzyme. Clearly these results support the suggestion that *in vivo* anti-alcohol reactions depend on the co-oxidation of methanethiol (or possibly some other small thiol) with diethylthiocarbamate (derived from disulfiram) or 1-methyltetrazole-5-thiol (derived from moxalactam etc.). It seems quite illogical to propose (as has recently been done; Yourick & Gaiman, 1987) that methyl diethylthiocarbamate is the actual metabolite responsible for the disulfiram-ethanol reaction. This chemically rather unreactive compound has no effect whatsoever on aldehyde dehydrogenase *in vitro* (Kitson, 1976) and neither does the analogous species 1-methyl-5-methylthiotetrazole, although both compounds are effective in producing anti-alcohol reactions *in vivo* (Yourick & Faiman, 1987; Fromtling & Gadebusch, 1983). It appears more likely that both species are demethylated and then co-oxidized with methanethiol and that the corresponding mixed methyl disulphides are the true modifiers of aldehyde dehydrogenase *in vivo*.

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