Effect of disulfiram on the pre-steady-state burst in the reactions of sheep liver cytoplasmic aldehyde dehydrogenase

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Stopped-flow spectrophotometric experiments show that modification by disulfiram not only lowers the steady-state rates but also decreases the size of bursts seen in both dehydrogenase and esterase reactions catalysed by sheep liver cytoplasmic aldehyde dehydrogenase. This observation is consistent with the proposal that a catalytically essential group is modified by disulfiram and that this group mediates both dehydrogenase and esterase activities.

INTRODUCTION

It has long been thought that the mechanism of action of mammalian aldehyde dehydrogenase may involve a catalytically essential enzymic thiol group (Feldman & Weiner, 1972). The sequence of steps in the postulated mechanism is (1) formation of a thiohemiacetal between the cysteine residue in question and the aldehyde substrate, (2) transfer of a hydride ion from the thiohemiacetal to NAD^+ , and (3) hydrolysis of the resulting enzymic thioester to regenerate the enzyme and liberate the carboxylate ion product. At present there is no direct conclusive evidence for this hypothesis; however, it is chemically reasonable, it is similar to the mechanism well established for certain other enzymes such as glyceraldehyde-3-phosphate dehydrogenase (Harris & Waters, 1976), and in particular it is consistent with the susceptibility of aldehyde dehydrogenase to thiol-modifying reagents. The classic such reagent in the case of aldehyde dehydrogenase is disulfiram [or Antabuse, $(\text{C}_2\text{H}_5)_2\text{NCS}-\text{SS}-\text{CSN}(\text{C}_2\text{H}_5)_2$], a compound used in anti-alcoholism therapy (Kitson, 1977). Disulfiram inactivates the cytoplasmic form of the enzyme *in vitro* almost instantaneously by labelling a particular reactive thiol group. It is possible that this group is the same as that selectively modified by iodo[^{14}C]acetamide, namely Cys-302 (Hempel *et al.*, 1985). Subsequently, and relatively slowly, the diethyldithiocarbamate label is displaced by a neighbouring enzymic thiol group, resulting in a cystine-containing enzyme species (Kitson, 1983).

It has been reported that the mitochondrial isoenzyme of horse liver aldehyde dehydrogenase has its activity greatly diminished by modification with disulfiram under conditions in which the amplitude of its pre-steady-state burst of NADH production is unaffected (Sanny & Weiner, 1987). This would mean that disulfiram does not modify the essential nucleophilic amino acid at the active site, since any such modification would necessarily lead to loss of the burst as well as loss of the subsequent steady-state activity. These results are at variance with an earlier publication (Kitson, 1982) concerning sheep liver cytoplasmic aldehyde dehydrogenase, in which it was found that the burst and the steady-state activity

declined more or less in tandem as a result of disulfiram modification.

The possible identity of the enzyme's catalytic and disulfiram-reactive residues is of sufficient importance to warrant further investigation. Accordingly, in this paper the results are presented of a series of experiments carried out by stopped-flow spectrophotometry into the effect of disulfiram on the size of the burst exhibited by aldehyde dehydrogenase. Both the burst of NADH in the enzyme's dehydrogenase reaction and the burst of *p*-nitrophenol seen in its action as an esterase were studied.

EXPERIMENTAL

Cytoplasmic aldehyde dehydrogenase from sheep liver was purified as previously described (Dickinson *et al.*, 1981). 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 11.3 (Hart & Dickinson, 1983). The M_r of the enzyme was taken to be 212000 (MacGibbon *et al.*, 1979). The apparatus used for the stopped-flow experiments was that described by Hart & Dickinson (1982). The effective cell path-length was 2.2 cm. For the dehydrogenase experiments syringe 1 contained the following, added rapidly in the order given: 2.4 ml of enzyme in 50 mM-sodium phosphate buffer, pH 7.4; 25 μl of disulfiram in ethanol (or, for the control experiments, ethanol alone); 0.1 ml of NAD^+ in 50 mM-sodium phosphate buffer, pH 7.4. After mixing, the concentrations in this syringe were: enzyme, 20 μM ; disulfiram, a range from 0 to 20 μM ; NAD^+ , 400 μM . Syringe 2 contained 400 μM - NAD^+ and 100 μM -propion-aldehyde in 50 mM-sodium phosphate buffer, pH 7.4. For the esterase experiments, syringe 1 contained 2.5 ml of 20 μM enzyme in 50 mM-sodium phosphate buffer, pH 7.4, and 25 μl of disulfiram (to give 0–20 μM) in ethanol. Syringe 2 contained 200 μM -*p*-nitrophenyl acetate in 50 mM-sodium phosphate buffer, pH 7.4, containing 2% (v/v) acetone. All experiments were carried out at 25 °C. Results were obtained and recorded as changes in transmittance with time (at 340 nm and 400 nm for the dehydrogenase and esterase experiments respectively)

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Fig. 1. Examples of stopped-flow spectrophotometric records showing the effect of disulfiram on the burst and the steady-state rate in the dehydrogenase and esterase reactions catalysed by cytoplasmic aldehyde dehydrogenase

(a) Dehydrogenase. The change in A_{340} was measured at 25 °C and pH 7.4 after mixing unmodified enzyme (20 μ M) and NAD⁺ (400 μ M) in one syringe with NAD⁺ (400 μ M) and propionaldehyde (100 μ M) in the other (●), and also with the use of enzyme (20 μ M) pre-mixed with disulfiram (20 μ M) in otherwise identical conditions (○). (b) Esterase. The change in A_{400} was measured at 25 °C and pH 7.4 after mixing unmodified enzyme (20 μ M) in one syringe with *p*-nitrophenyl acetate (200 μ M) in the other (●), and also with the use of enzyme (20 μ M) pre-mixed with disulfiram (10 μ M) in otherwise identical conditions (○).

and converted into absorbance units before the steady-state rate and the burst amplitude were measured. Steady-state rates were given by the slope of the linear portions of the resulting graphs (see, for example, Fig. 1) and the burst amplitude was assessed from the difference between the absorbance given by back-projecting the steady-state rate to zero time and the observed initial absorbance.

RESULTS AND DISCUSSION

There have been two previous investigations into the effect of disulfiram on the burst exhibited by aldehyde

Fig. 2. Correlation of the effect of disulfiram on the burst and the steady-state rate in the reactions of cytoplasmic aldehyde dehydrogenase

From the results of experiments such as those illustrated in Fig. 1, the burst size after modification with various amounts of disulfiram was calculated relative to the control value obtained with native enzyme and plotted against the corresponding value for the fractional remaining steady-state activity: ●, dehydrogenase activity; ■, esterase activity. Also shown for purposes of comparison are data taken from a previous study (Kitson, 1982) (○). The broken line is that expected for a simple 1:1 relationship between loss of burst and loss of steady-state activity.

dehydrogenase. The first (Kitson, 1982) used sheep liver cytoplasmic aldehyde dehydrogenase and a recording filter fluorimeter of the type described by Dalziel (1962). The recorded steady-state increase in fluorescence was projected backwards the few seconds to the time of mixing, giving an estimate of the amplitude of the burst of NADH production. The technique gave some variability in the results, but broadly speaking it was found that increasing amounts of disulfiram lower the burst of NADH fluorescence to much the same extent as they lower the steady-state rate of reaction. (Data from that report are included in Fig. 2.) Subsequently, Sanny & Weiner (1987) carried out similar experiments using the enzyme from horse liver mitochondria and a conventional Gilford spectrophotometer as described by Weiner *et al.* (1976). They found that the magnitude of the NADH burst did not decrease until approx. 75% of the steady-state activity had been abolished by disulfiram and concluded therefore that the cysteine residue modified by disulfiram is not catalytically essential.

A factor that might help to explain these conflicting results is the use of experimental techniques that do not allow the direct observation of the burst phase of the reaction. Thus the present work used the more appropriate technique of stopped-flow spectrophotometry; this gives an authentic record of the bursts over the time frame in which they occur. In Fig. 1(a) it is clearly seen that, with this method, the burst of NADH production and the steady-state rate are typically decreased in

magnitude to a similar extent by modification of the enzyme with stoichiometric amounts of disulfiram. The first-order rate constant for the burst is 14 s^{-1} , which is close to a previously published value (10 s^{-1}) determined under similar conditions, but at pH 7.0 rather than 7.4 (Hart & Dickinson, 1982). These workers found the burst size (in the absence of disulfiram) was equivalent to 0.86 molecule of NADH per tetrameric enzyme molecule; in the present work a value of 0.5 was found, but these values are dependent on the specific activity of the enzyme preparation used and whether any activity has been lost on storage, dialysis etc. Fig. 1(b) shows that the steady-state rate and the burst amplitude are also lowered in parallel when the enzyme's esterase activity is monitored. The burst rate constant is 15 s^{-1} , which agrees quite well with a value of $12 \pm 2\text{ s}^{-1}$ reported by MacGibbon *et al.* (1978), and the burst amplitude (in the absence of disulfiram) represents 0.4 molecule of *p*-nitrophenol produced per tetrameric enzyme molecule. Experiments such as those exemplified in Fig. 1 were repeated three or four times at several different disulfiram concentrations with good reproducibility. The averages of the relative burst size and of the remaining steady-state activity were taken for each disulfiram concentration and the data are plotted in Fig. 2, where it is seen that they lie close to the theoretical line expected if the disulfiram-sensitive group is indeed catalytically essential.

It is not clear why these results are different from those of Sanny & Weiner (1987). It seems improbable that a simple species difference (sheep versus horse) is responsible; a more likely cause is the use of enzymes from different subcellular locations. Mitochondrial and cytoplasmic aldehyde dehydrogenases differ substantially in amino acid sequence (Hempel *et al.*, 1985), and the mitochondrial isoenzyme used by Sanny & Weiner (1987) reacts very much more slowly with disulfiram than does the cytoplasmic isoenzyme used here. However, the latter appears to be only a superficial difference brought about by unfavourable steric interactions; closely related modifiers of smaller size such as methyl diethylthiocarbamoyl disulphide inactivate the mitochondrial isoenzyme very rapidly (MacKerell *et al.*, 1985).

The results given here are relevant to the debate over whether the dehydrogenase and esterase activities of aldehyde dehydrogenase occur at the same site or not (see Blackwell *et al.*, 1983; Duncan, 1985; Loomes & Kitson, 1986). It was known before that pre-modification of the cytoplasmic enzyme with disulfiram abolishes both types of steady-state activity (Kitson, 1982). It has now been shown that, in addition, both types of burst are

similarly diminished (Figs. 1 and 2). This provides another significant observation that is wholly consistent with the simpler 'common-site' model for the dehydrogenase and esterase activities. It seems unlikely that, in the alternative two-site model, disulfiram modification should simultaneously stop two different catalytic groups from bringing about their respective burst reactions.

In conclusion, it is clear that, for cytoplasmic aldehyde dehydrogenase at least, the effect of disulfiram on the burst is fully compatible with the modified group's being catalytically essential. Thus the conclusion of Sanny & Weiner (1987) that 'unequivocal evidence' has been found to show that disulfiram is not an active-site reagent is premature.

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