THIOL GROUPS OF LIVER ALCOHOL DEHYDROGENASE. ACCESSIBILITY TO GENERAL THIOL REAGENTS AND TO POTENTIAL AFFINITY-LABELS

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1. Introduction

Horse-liver alcohol dehydrogenase (EC 1.1.1.1) contains one thiol group per polypeptide chain which is reactive towards iodoacetate [1,2], at position 46 in the sequence [3]. However, mercurials [4,5] readily react with all 14 thiol groups per polypeptide chain [3], or 28 per molecule [6]; while maleimides [4], iodine [7] and silver ions [6] react with an intermediate number of thiol groups.

In this communication, we show that (i) iodoacetamide alkylates several thiol groups per subunit; (ii) 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) reacts with all thiol groups probably accompanied by denaturation, and (iii) the potential affinity-label 3-(2-bromoacetyl)-pyridine (bromoacetyl-pyridine) inactivates the enzyme rapidly giving approximately 1–1.3 residues of modified cysteine per subunit, but protection experiments failed to confirm the formation of a reversible enzyme–inhibitor complex; 2-chloroethanol and 2-iodoethanol are strong reversible inhibitors but very weak irreversible inhibitors.

2. Materials and methods

The source, assay and purity of horse-liver alcohol dehydrogenase, iodoacetic acid and iodoacetamide were as described previously [8,9]. Bromoacetyl-pyridine hydrobromide was a generous gift from Dr D. K. Apps. Other chemicals were of reagent grade. Amino acid analysis was performed, after hydrolysis of protein for 24 hr at 105°C in 5.5 N HCl, using a Locarte amino acid analyser [10]. Unless otherwise stated all experiments were performed at 23.5°C.

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3. Results and discussion

3.1. Reaction with iodoacetamide

In yeast alcohol dehydrogenase, one thiol per subunit reacts rapidly with iodoacetamide [11], but a preliminary investigation suggested that iodoacetamide reacted with several thiols in the liver enzyme [12]. The results in fig. 1 confirm this. The increasing negative slope of the thiol-content with time suggests that after one or two thiols have been alkylated, others become more reactive. The slight increase in slope for the inactivation at pH 6.2 suggests that non-essential thiols may be reacting first, and increasing the reactivity of essential ones which react later accompanied by inactivation. Alternatively, if one subunit is inactivated it may promote lability in the other subunit. Iodoacetamide, like iodoacetate, may inactivate primarily by reacting with cysteine-46, but recent results with yeast alcohol dehydrogenase at pH 6.5 indicate that iodoacetamide reacts with either of two thiol groups [13], and it is possible that a second reactive thiol group might be present in the liver enzyme too.

Unlike iodoacetate, no 'saturation-effect' was observed at high concentrations of iodoacetamide

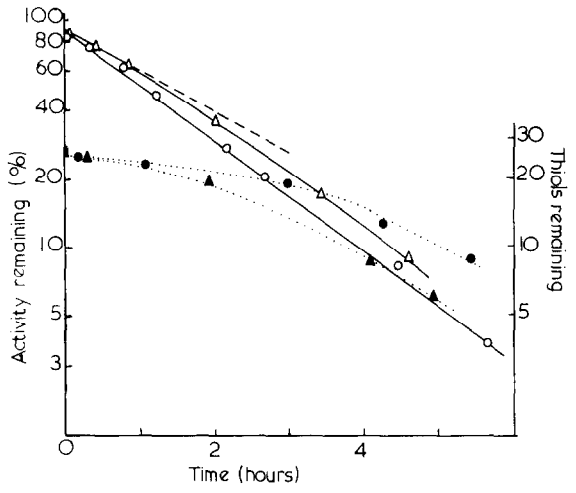

Fig. 1. Reaction of liver alcohol dehydrogenase with iodoacetamide (semi-log graph). Iodoacetamide, 13.3 mM; enzyme 4 μM; at 23.5°C. Circles, pH 7.4 (40 mM sodium phosphate buffer); triangles, pH 6.2 (80 mM sodium phosphate buffer). Open symbols and continuous lines, catalytic activity; filled symbols and dotted lines, thiol content per molecule (measured on withdrawn aliquots with DTNB [18], in 4 M urea + 2.5 M guanidine hydrochloride). The 'activity remaining' extrapolates to less than 100% due to reversible inhibition of the enzyme assay by iodoacetamide [14].

[8]. Amides, including iodoacetamide [14,15] do form reversible complexes with liver alcohol dehydrogenase, but it appears that this does not find expression in the inactivation-kinetics. It would be of interest to know whether carboxamidomethyl enzyme, like carboxymethyl enzyme [16], still retains some catalytic activity. Iodoacetamide appears to be less reactive than iodoacetate towards cysteine-46 (ref. [17]), but relatively more reactive with other thiol residues, hence the lack of selectivity for cysteine-46.

3.2. Reaction with DTNB

DTNB reacted much more slowly with liver alcohol dehydrogenase than with non-protein thiol groups, or protein thiol groups in the presence of 5 M guanidine HCl. The half-time was 5 hr, compared with a few seconds, when measured in 35 mM sodium phosphate buffer, pH 8, with 160 μM DTNB. The rate of reaction of the enzyme thiol groups was increased 10-fold by 1.0 M guanidine HCl (1.0 M NaCl having no effect), suggesting that conformational rigidity may normally prevent access of DTNB to thiol groups.

Measurement of the catalytic activity and of the thiol groups reacted with DTNB (by the increase in absorbance at 412 nm [18] showed that the catalytic activity remaining was directly proportional to the content of intact thiol groups remaining. This stoichiometry was maintained during the accelerated inactivation given by 1.0 M guanidine HCl. DTNB probably reacts with one thiol initially (which may not always be the same thiol), followed by a rapid series of reactions, probably including conformational changes, resulting in an inactive molecule with all the thiol groups modified. After partial inactivation (to 30–35% initial activity) with DTNB (0.3 mM), dithiothreitol was added (1.3 mM) and, after correcting for some inhibition of the enzyme assay by the dithiothreitol, a slow partial recovery of activity was observed: after one hour approximately half of the inactive enzyme had been reactivated.

As with iodoacetate [8,9], increasing the concentration of DTNB did not proportionately increase the rate-constant of inactivation. At pH 7.4 (in 40 mM sodium phosphate buffer), concentrations of DTNB of 0.67, 1.33 and 3.33 mM gave first-order inactiva-

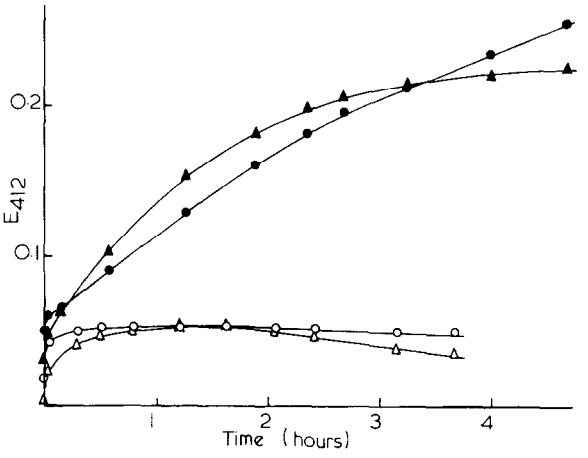

Fig. 2. Reaction of native and carboxymethyl alcohol dehydrogenase with DTNB, and effect of imidazole. In 40 mM sodium phosphate buffer (pH 7.4) at 23.5°C, with 0.33 mM DTNB. Circles, native enzyme (0.75 μM); triangles, carboxymethyl enzyme (0.75 μM). Open symbols, in the presence of imidazole (base form 26 mM); filled symbols, without imidazole.

tions with half-times of 76, 71 and 71 minutes respectively. This suggests the formation of a reversible enzyme–DTNB complex. However, the slowness of the reaction and the non-selective thiol reactivity indicate that the reversible complex may protect the enzyme from external alkylation by other DTNB molecules, rather than act as an intermediate for an affinity-labelling reaction by enzyme-bound DTNB [9].

Imidazole promotes the reaction of iodoacetate or iodoacetamide with the enzyme [9,17]; in contrast, it protected the enzyme from DTNB (fig. 2). AMP and orthophenanthroline also protected the enzyme from DTNB. At pH 9.6 (in 0.1 M glycine buffer) liver alcohol dehydrogenase was inactivated by DTNB three times faster than at pH 7.4, while iodoacetate shows less reactivity at higher pH [8,17]. Carboxymethyl liver alcohol dehydrogenase, where cysteine-46 has been alkylated with iodoacetate [1,7, 16], and imidazole also protects the carboxymethyl enzyme from DTNB. Thus DTNB reacts very similarly with native enzyme and with enzyme where cysteine-46 is blocked. It seems clear that cysteine-46, although uniquely reactive with iodoacetate, does not play any significant role in the reaction of the enzyme with DTNB.

3.3. Reaction with 2-chloroethanol, 2-iodoethanol and 2-(3 bromoacetyl)-pyridine

Both chloroethanol and iodoethanol were found to be very weak irreversible inhibitors, with or without added NAD^+ or NADH. Chloroethanol (50 mM) produced 27% inhibition after 90 min (in 40 mM sodium phosphate buffer, pH 7.4) which was prevented almost completely by imidazole (base form 30 mM). However, both reagents are strong reversible inhibitors of the enzyme. Iodoethanol gave mixed non-competitive–uncompetitive inhibition with respect to ethanol at pH 10, with inhibitor constants of 250 μM (slopes) and 490 μM (intercepts) determined from double-reciprocal plots.

Bromoacetyl-pyridine, by analogy with other nitrogen bases such as imidazole and pyridine [19,9], might bind reversibly to liver alcohol dehydrogenase, as well as irreversibly by alkylation. It was found to be a powerful inactivator of the enzyme, 0.67 mM reagent inactivating with a half-time of 3.6 minutes (0.5 μM enzyme in 40 mM sodium phosphate buffer, pH 7.4). If a non-covalent complex was first formed, then imidazole and other competitive ligands should displace the bromoacetyl-pyridine and protect the enzyme. However, no significant protection was given by imidazole or by decanoate, although AMP and orthophenanthroline did protect. While it is possible that imidazole both displaces bromoacetyl-pyridine and promotes the alkylation of a thiol group as with iodoacetate and iodoacetamide [9,17], it seems unlikely that alkylation requires the prior formation of a reversible complex.

Amino acid analysis indicated a stoichiometry of 1–1.3 modified cysteine residues per inactivated subunit, as compared with derivatised *N*-acetyl-cysteine [20]; unlike lactate dehydrogenase [20], no modified histidine was found, although it is possible that this might have been eluted with the arginine peak and therefore not detected. However, the active centres of lactate and alcohol dehydrogenases appear to be somewhat different [21,22].

In yeast alcohol dehydrogenase, butyl cyanate (a substrate analogue) and iodoacetate have been found to react with different thiol groups [13,23]. 2-Bromo, 2-phenylacetaldehyde also reacts with a thiol group in the active centre [24], but was inactive against liver alcohol dehydrogenase. Thus with the possible exception of iodoacetate [8], affinity labelling of liver alcohol dehydrogenase by substrate analogues has been unsuccessful to date. Coenzyme analogues have proved more rewarding [25–27], which may be explained by the positioning of cysteine-46 near the binding-site of the coenzyme rather than of the substrate [22].

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