Turkey Liver Xanthine Dehydrogenase: Further Observations on the Reaction with Arsenite

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The catalytically essential persulphide groups at the molybdenum centres of xanthine oxidase and xanthine dehydrogenase are essential to interaction with arsenite (Edmonson et al., 1972; Cleere et al., 1974) Inhibition of xanthine hydroxylation may result from arsenite forming a complex with the persulphides and vicinal thiol groups (Massey & Edmonson, 1970). The NADH–dichlorophenol-indophenol and NADH–trinitrobenzenesulphonate oxidoreductase activities of chicken liver xanthine are enhanced on incubation of the oxidized enzyme with arsenite (Rajagopalan & Handler, 1967). However, similar treatment of enzyme pre-reduced with NADH was found to inhibit the former activity. We wished to determine whether the effects of arsenite on NADH diphorase activity are restricted to the electron acceptors above and whether interaction with the inhibitor is confined to the persulphide groups. The experimental procedures used have been reported elsewhere (Cleere & Coughlan, 1975). Stopped-flow studies are described in the preceding paper (Ní Fhaoláin et al., 1976). The inactivation of oxidized and of reduced enzyme by arsenite was carried out by the methods described by Rajagopalan & Handler (1967).

Fig. 1(a) shows the time-course of inhibition (pseudo-first-order with respect to enzyme) of xanthine–NAD⁺ oxidoreductase activity accompanying incubation of oxidized enzyme with arsenite. In agreement with the findings of Rajagopalan & Handler (1967), such treatment increased the oxidation of NADH by dichlorophenol-indophenol. In complete contrast, however, with the report by these workers, we found that incubation of oxidized turkey enzyme with arsenite decreased its ability to catalyse the oxidation of NADH by trinitrobenzenesulphonate (Fig. 1a). The same trend was found in five separate experiments with different batches of the enzyme. These contrasting findings may reflect subtle differences in the environment of the persulphide groups in two otherwise-similar enzymes.

The rate of increase in NADH–dichlorophenol-indophenol oxidoreductase activity on arsenite treatment paralleled that of the decrease in activity with trinitrobenzenesulphonate as acceptor when enzyme samples at the same concentration and content of functional active sites were used. However, unlike the time-course of inhibition of xanthine–NAD⁺ oxidoreductase activity, neither followed first-order kinetics. This may reflect the fact that enzyme lacking completely the active-centre persulphide groups, and presumably incapable of reacting with arsenite, retains about 10% of the NADH–dichlorophenol-indophenol and NADH–trinitrobenzenesulphonate oxidoreductase activities of the fully functional enzyme (Ní Fhaoláin & Coughlan, 1976).

Fig. 1(b) confirms the inability of arsenite (incubated with oxidized enzyme) to alter either activity of the non-functional enzyme and shows that the extent of the increase in activity with dichlorophenol-indophenol, or of the decrease in activity with trinitrobenzenesulphonate as acceptor, is dependent on the content of functional active sites in the enzyme samples used.

In contrast with the above findings, incubation of the oxidized enzyme with arsenite had no effect whatsoever on the oxidation of NADH by Methylene Blue, by ferricyanide or by O₂. Moreover, stopped-flow studies at 450nm showed that the rate constants for the initial fast phase of reduction by NADH of native and arsenite-treated enzyme were not significantly different. These were 40.0s⁻¹ and 41.0s⁻¹ respectively.

Preliminary treatment of enzyme with xanthine or with NADH followed by exposure to arsenite did not affect the oxidation of NADH by Methylene Blue, ferricyanide or O₂, nor did it affect subsequent reduction of the enzyme by NADH. The rate constant in
Fig. 1. Effects of arsenite on various activities of turkey liver xanthine dehydrogenase

(a) Time-course of change in activity. Oxidized enzyme was incubated at 30°C in 0.05M-potassium phosphate buffer, pH 7.8, containing 0.1 mM-EDTA and 0.2 mM-sodium arsenite. Xanthine-dichlorophenol-indophenol (●), NADH-dichlorophenol-indophenol (○) and NADH-trinitrobenzenesulphonate (□) oxidoreductase activities at various times are expressed as a percentage of those of the untreated control.

(b) Relation between the content of functional active sites and the extent of change in activity accompanying arsenite treatment. Oxidized enzyme samples of different degrees of functionality (Cleere et al., 1974) were treated with arsenite as above. NADH-dichlorophenol-indophenol (○) and NADH-trinitrobenzenesulphonate oxidoreductase (□) activities are expressed as a percentage of the appropriate initial activity.

Table 1. Effects of pre-reduction on reactivity with arsenite

Enzyme (0.9 μM, 47.5% functional) was incubated anaerobically at 30°C in 0.05M-potassium phosphate buffer, pH 7.8, containing 0.1 mM-EDTA and 0.15 mM-xanthine (or 0.05 mM-NADH) for 2 min. Arsenite was then added to a final concn. of 1.0 mM and anaerobic incubation continued for 10 min before assay. Activities following such treatment are in each case expressed as a percentage of a control subjected to identical treatment except for the addition of arsenite.

<table>
<thead>
<tr>
<th>Enzyme sample</th>
<th>Pre-reducing agent</th>
<th>NADH-dichlorophenol-indophenol</th>
<th>NADH-trinitrobenzenesulphonate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native</td>
<td>None</td>
<td>157</td>
<td>45</td>
</tr>
<tr>
<td></td>
<td>Xanthine</td>
<td>94</td>
<td>36</td>
</tr>
<tr>
<td></td>
<td>NADH</td>
<td>46</td>
<td>29</td>
</tr>
<tr>
<td>Cyanide inactivated</td>
<td>None</td>
<td>97</td>
<td>101</td>
</tr>
<tr>
<td></td>
<td>NADH</td>
<td>91</td>
<td>97</td>
</tr>
</tbody>
</table>
this case was found to be \(42.0\text{s}^{-1}\) (see above). The effects on other NADH diaphorase activities of arsenite treatment of reduced enzyme are shown in Table 1. In direct contrast with its effects on oxidized enzyme (Fig. 1), is the marked inhibition of NADH–dichlorophenol-indophenol activity accompanying arsenite treatment of NADH-reduced enzyme. This could be due to the exposure on reduction of a second site with which arsenite may interact (Rajagopalan & Handler, 1967). However, in agreement with the data of Fig. 1(b), arsenite treatment of reduced-cyanide-inactivated enzyme failed to alter activity significantly (Table 1). Thus we suggest that pre-reduction merely alters the mode of binding of arsenite to the active-centre persulphide groups.

The effects of arsenite on the NADH diaphorase activity of oxidized or reduced enzyme clearly depend on the integrity of the molybdenum-centre persulphide groups and are restricted to activity with dichlorophenol-indophenol and trinitrobenzene sulphonate as electron acceptors. This, in our view, infers that these two acceptors also interact with the molybdenum centres of these enzymes.

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**Distribution Studies on Polyglutamate Forms of Folate**

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Three methods for the study of distribution of polyglutamate forms of folate in cells have emerged. One of these (Shin et al., 1972) separates intact folates according to their size on gel filtration, which is then followed by ion-exchange chromatography with standards. The disadvantages of this method reside in differential interaction of the various pteroyl derivatives with the gel, causing them to elute in a position different to that predicted by their size and to the complexity of the mixture to be separated. A different approach was taken by ourselves (Houlihan & Scott, 1972) where we converted all of the polyglutamate forms of folate present to the simpler p-aminobenzoyl-polyglutamates before separation, by using oxidative cleavage of the C-9–N-10 bond. Subsequently a similar method has been published by Baugh et al. (1974) using zinc-catalysed reductive cleavage of the C-9–N-10 bond in acid. Ideally both of these methods would result in a single p-aminobenzoylpolyglutamate species, differing only in the number of glutamate residues. However, the latter method converts all 5-formyltetrahydropteroylpolyglutamates [5-CHO-H\(_2\)PteGlu\(_{m}\)], 5,10-methyldiyinetetrahydropteroylpolyglutamates [5,10-CH=H\(_4\)PteGlu\(_{m}\)] and 10-formyltetrahydropteroylpolyglutamate [10-CHO-H\(_2\)PteGlu\(_{m}\)], not to p-aminobenzoylpolyglutamates, but to the corresponding N-methyl-p-aminobenzoyl derivative. Oxidative cleavage (Houlihan & Scott, 1972) results in some conversion of 10-formyltetrahydropteroylpolyglutamates into a corresponding formylaminobenzoylpolyglutamate. Although little of this derivative is formed in practice and thus no distortion of the folate-polyglutamate elution pattern occurs, it would clearly be better if it could be eliminated. Similarly in the reductive cleavage of Baugh et al. (1974), even though an unambiguous pattern is obtained it represents a disadvantage since even more of the methyl species is formed.