REACTIVATION OF APO LIVER ALCOHOL DEHYDROGENASE WITH LEAD, MERCURY AND CADMIUM

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1. Introduction

In metalloenzymes substitution of metal with other metal ions is often used to study the enzyme and the role of the metal. Few reports show substitution with the heavy metals lead and mercury, although cadmium is known to replace zinc in enzymes. Lead and mercury are highly toxic elements and often inhibit enzymes.

Liver alcohol dehydrogenase is a dimer of 80 000 M_r with one structural and one catalytic zinc atom per subunit. It is possible to substitute either one or both zinc atoms by different methods [1–4]. Within each liver alcohol dehydrogenase molecule, there are 2 subunits, 2 active sites and 4 zinc atoms, 2 catalytic (c) and 2 structural or non catalytic (n). The native enzyme is consequently written $Zn(c)_2Zn(n)_2$. Removal of metal is designated by apo. Thus apo-$(c)_2Zn(n)_2$ represents enzyme with the catalytic zinc atoms removed. It is often most convenient to make the 'hybrid-enzyme', $Me(c)_2Zn(n)_2$, from the 'apo-hybrid', $apo(c)_2Zn(n)_2$, since this is more stable than the fully depleted enzyme, $apo(c)_2apo(n)_2$.

Here, we show that activity can be restored to dissolved apo liver alcohol dehydrogenase, $apo(c)_2Zn(n)_2$, with the heavy metals cadmium, mercury and lead.

2. Materials and methods

Crystalline horse liver alcohol dehydrogenase (EC 1.1.1.1) was purchased from Boehringer-Mannheim, NAD^+ and *N*-tris-(hydroxymethyl)-methyl-2-aminoethane sulfonic acid (TES) from Sigma. $ZnCl_2$ and $HgCl_2$ were from Merck, $CdCl_2$ from BDH and $PbCl_2$ from Baker, all as analytical reagents. 2,6-Pyridinedicarboxylic acid (DPA) was from Fluka and *tert*-butanol from Merck.

The enzyme depleted of catalytic zinc ions, $apo(c)_2Zn(n)_2$, was prepared as in [1] by dialysis of crystalline enzyme against 25 mM TES/Na^+ (pH 7.0) containing 10 mM DPA and 25% (v/v) *tert*-butanol until the specific activity was <0.2%. The chelating agent was removed by subsequent dialysis against 25 mM TES/Na^+ (pH 7.0) with 25% (v/v) *tert*-butanol.

The results of the reactivations are given as percentages of the specific activity of the native enzyme, which was 15 $\Delta A_{340} \cdot min^{-1} \cdot mg^{-1}$, when assayed as in [5]. The reactivation experiments were performed with dissolved $apo(c)_2Zn(n)_2$ in 25 mM TES/Na^+ pH 8.2 at 4°C by adding aqueous solutions of the metallochlorides. Reactivation was followed by removing aliquots of the protein/metal mixture for enzyme assay. All work was performed under nitrogen, and all solutions were deaerated and pre-equilibrated with nitrogen gas immediately before use. Absorption spectra were scanned on a Cary 219, double-beam recording spectrophotometer using 1 ml quartz cuvettes with a 10 mm lightpath.

3. Results

Reactivation of $apo(c)_2Zn(n)_2$ to native enzyme with Zn(II), was so fast that when using a 1.5-fold excess over active sites, the activity of the native enzyme was completely restored within 5 min. However, with equivalent amounts, reactivation was rapid during the first 5 min before declining to give a maximum activity of 78% after 45 min (fig.1a). Reactivation of dissolved $apo(c)_2Zn(n)_2$ at pH 6.9 has been reported [1].

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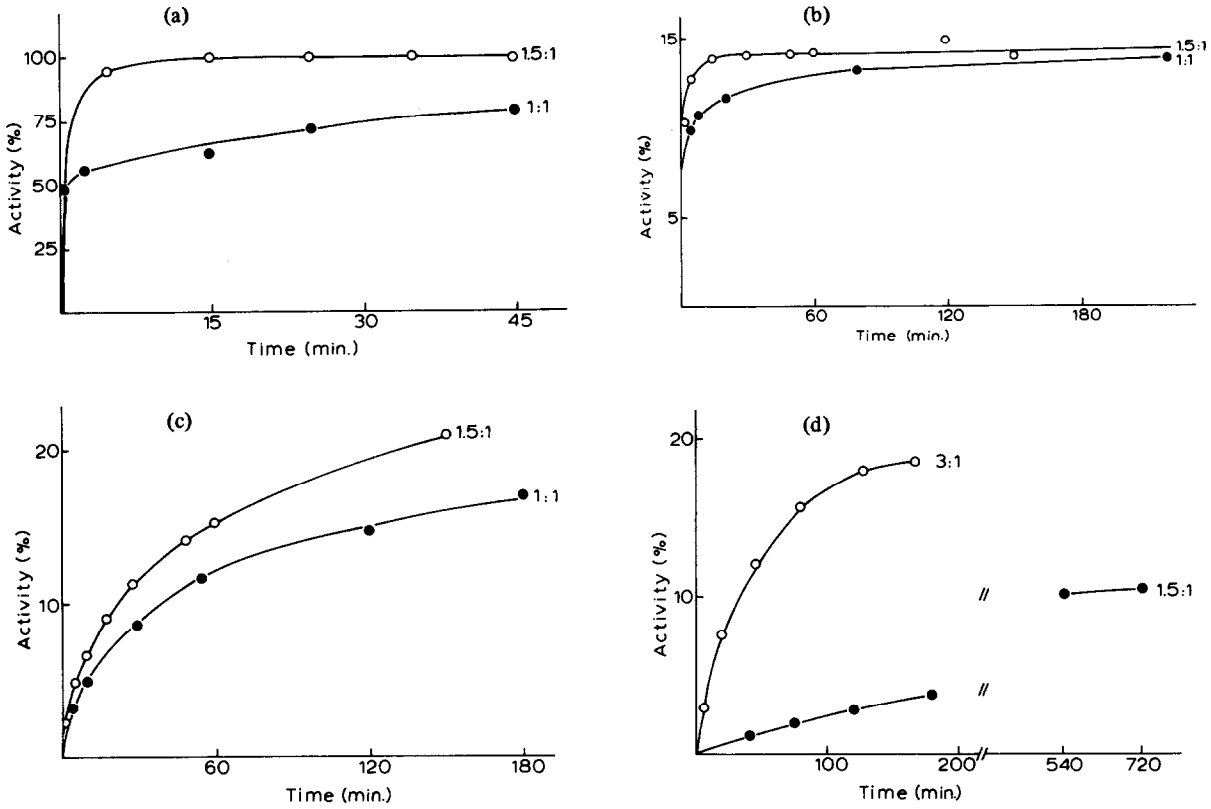

Fig.1. Reactivation of apo(c)$_2$Zn(n)$_2$ (20 μM) in 25 mM TES/Na$^+$ pH 8.2 and 4°C, with different metals (amounts relative to active sites): (a) Zn(II); (b) Cd(II); (c) Hg(II); (d) Pb(II).

Reactivation with Cd(II) ions was slower than with Zn(II) ions, but faster than with Hg(II) ions. The rate of reactivation was observed to increase with increasing metal concentration, but the maximum activity did not exceed 14%. With a 1.5-fold excess of Cd(II) ions relative to active sites, maximum activity resulted after 15 min, while a lower metal concentration gave the same maximum activity after 3–4 h (fig.1b). The Cd(II)-reactivated enzyme was stable for days under the present conditions.

Reactivation with Hg(II) ions proved to be complicated since some of the dissolved apo(c)$_2$Zn(n)$_2$ precipitated in the presence of mercury. Some precipitation occurred even when the amount of Hg(II) incubated, was equivalent to active sites, and denaturation increased with increasing metal ion concentration. After 24 h the precipitate was removed by centrifugation, and a stable soluble enzyme with a total activity 25% of the native enzyme resulted. When reactivation was followed for 3 h, a hyperbolic curve

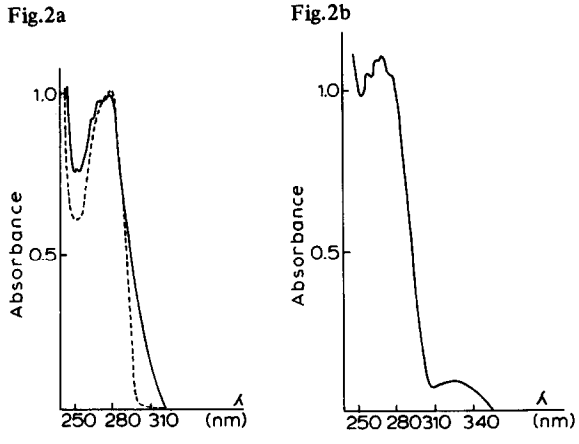

Fig.2a. UV-spectrum of Hg-hybrid (——) compared to native enzyme (– – –) taken 3 h after addition of a 1.5-fold excess of Hg(II) relative to active sites. Apo(c)$_2$Zn(n)$_2$ was 27 μM.

Fig.2b. UV-spectrum of Pb-hybrid taken 3 h after addition of a 1.5-fold excess of Pb(II) relative to active sites. Apo(c)$_2$-Zn(n)$_2$ was 27 μM.

resulted (fig.1c). The spectrum of the mercury reactivated enzyme is shown (fig.2a). No denaturation was detectable within the first 2 h with either concentration presented. More than a 3-fold excess, relative to active sites gave immediate denaturation.

Pb(II) showed a different pattern to the IIB transition metals, in that a 3-fold excess of metal was required to achieve detectable reactivation within 3 h (fig.1d). The rate of reactivation with concentrations less than presented here was negligible, while a >4-fold excess relative to active sites gave denaturation during the time required for reactivation. After 24 h the precipitate was removed by centrifugation and a soluble enzyme resulted. This was stable for days and had a total activity of ~20% that of the native enzyme. The UV-spectrum is shown in fig.2b.

4. Discussion

These experiments show that the heavy metals cadmium, mercury and lead are effective in restoring activity to apoliver alcohol dehydrogenase. Reactivation with these metals results from their entry into the active center of the soluble apo(c)$_2$Zn(n)$_2$. This work is the first showing insertion of Hg(II) and Pb(II) into the active center of liver alcohol dehydrogenase, and that the resulting species exhibit enzymatic activity. A possible Pb(II)-enzyme has been mentioned [6].

With mercury and lead, reactivation reached, respectively, 25% and 20% of the native enzyme, but the specific activities of the Hg(c)$_2$Zn(n)$_2$ and Pb(c)$_2$Zn(n)$_2$ are no doubt higher due to precipitation in the reactivation experiments. The UV-spectrum of the mercury and lead enzymes confirm that these really are the hybrids with mercury or lead in the active center. The UV-spectrum taken 3 h after addition of equivalent amounts of Hg(II) relative to active sites, had a maximum at 280 nm and a minimum at 258 nm. The UV-spectrum of the Pb(II)-hybrid taken 3 h after addition of a 3-fold excess of Pb(II) ions relative to active sites, had a minor absorption band around 330 nm and a blueshift of the 280 nm band to 268 nm. Both these spectra thus show differences from that of the native enzyme.

Reactivation experiments performed with Zn(II) and Cd(II) confirm that the conditions used for reactivation in this work, are suitable for insertion of a metal into the empty metal-binding site in the active center of liver alcohol dehydrogenase. As expected, reactivation with Zn(II) totally restored activity, while Cd(II) in agreement with other reports, gave an enzyme with 14% activity [7,8].

Cadmium, mercury and lead are known as highly toxic elements, a property attributed to their high binding-constants with sulfur and nitrogen in biomolecules. The stability constants with thiolate have been reported to follow the order Hg > Pb > Cd > Zn [9]. The enzyme denaturation observed upon exposure to the IIB metals and lead is considered to result from the metal binding to sites other than the metal-binding site in the catalytic center. The order of thiolate affinity explains the greater denaturation with mercury and lead. It also agrees with the finding that once formed, the Hg(II), Pb(II) and Cd(II) enzymes are fully stable enzymes like the native Zn(II) enzyme.

The observed order for the rate of reactivation; Zn > Cd > Hg > Pb is, however, different from the order of the binding constants. Thus parameters other than just affinity for cysteine residues are involved. The rate of reactivation probably also depends on factors such as pH, metal ion concentration, solvation of the salts, and affinity for the active center. However, metal-complex formation with the buffer used here is negligible [9].

The maximum activity recovered for the different enzyme species studied in this work follows the order Zn > Hg > Pb > Cd. This order of activity is difficult to explain. It is notable that it neither follows the order of the stability constants nor that of the ionic radii. It seems likely that optimum assay conditions differ for the 4 enzymes, so that other assay conditions could change the observed order of activity.

Mercury has been inserted into the active center of carbonic anhydrase and carboxypeptidase, with the latter having activity [10–12]. With alkaline phosphatase a reactivation pattern similar to that observed here, is seen with lead and mercury [13–15]. The present work shows that Hg(II) and Pb(II) ions can enter the active center of apo-liver alcohol dehydrogenase restoring enzymatic activity. It is interesting that with this liver enzyme excess lead and mercury which are toxic destroy activity, but stoichiometric trace amounts give biological activity. Further characterization of these enzyme species is in progress.

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