Reactivation of apo horse liver alcohol dehydrogenase with the monovalent metal ion Ag(I)

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Each subunit of the liver alcohol dehydrogenase dimer contains one catalytic and one structural Zn(II) atom. Enzyme with the catalytic metal atoms selectively removed is inactive but can be partly reactivated in the presence of Ag(I) ions. Reactivation results from Ag(I) ions entering the empty metal-binding site in the catalytic center. The specific activity of this silver enzyme reached 24% of the native enzyme. Atomic absorption analysis gave equal amounts of Ag(I) and Zn(II), corresponding to one mole of each metal per monomer. Metal-directed affinity labelling using bromo-imidazolyl propionate showed that the properties of the silver-reactivated enzyme were distinct from those of the native enzyme.

Metallosubstitution in metalloenzymes has received increasing attention, particularly as enzymes with a catalytic metal ion often retain some activity when the metal in the native enzyme is replaced by another metal ion. Liver alcohol dehydrogenase is a dimer of 80000 Da with one structural and one catalytic zinc atom per subunit. To further clarify the role of the catalytic zinc atom, this has been substituted with several metals such as Co(II), Cd(II), Ni(II), Hg(II), and Pb(II) (1-4). In this work we show that activity can be restored to the dissolved apo-hybrid, apo(c)₂Zn(n)₂, with the monovalent metal ion Ag(I). (The native enzyme is written Zn(c)₂Zn(n)₂, where (c) and (n) denote the catalytic and non-catalytic metal sites.) Metal removed is designated by apo (4). The resulting Ag-hybrid, Ag(c)₂Zn(n)₂, which exhibits a specific activity 24% that of the native enzyme, has also been characterized by metal-directed affinity labelling and atomic absorption spectroscopy.

Materials and Methods

Crystalline horse liver alcohol dehydrogenase (EC 1.1.1.1) was from Boehringer-Mannheim. NAD⁺, N-tris(hydroxymethyl) methyl-2-aminoethanesulfonic acid (TES) and N-2-hydroxyethylpiperazine-propanesulfonic acid (EPPS) were from Sigma. AgNO₃ was from Merck. (R,S)-2-bromo-3-(5-imidazolyl)propionic acid (BIP) was from Sigma and 2,6-pyridinedicarboxylic acid from Fluka. All reagents were of the highest purity available.
The enzyme depleted of catalytic zinc ions, apo(c)$_2$Zn(n)$_2$, was prepared by dialyses against 2,6-pyridinedicarboxylic acid (5). Insertion of Ag(1) into the catalytic site was carried out by adding an aqueous solution of silver nitrate to apo(c)$_2$Zn(n)$_2$ dissolved in 25 mM TES/Na$^+$ buffer, pH 8.2, at 4°C. Reactivation was followed by removing aliquots of the protein/metal mixture for enzyme assay. All solutions were de-aerated and pre-equilibrated with nitrogen gas immediately before use. The results of the reactivations with Ag$^+$ are given as percentages of the specific activity of the native enzyme, which was 15ΔA$_{340}$.min$^{-1}$.mg$^{-1}$ when assayed as described previously (6).

Enzyme inactivations with BIP were performed at 23.5°C and pH 8.2 in 25 mM EPPS/Na$^+$ buffer, using an enzyme concentration of about 10 μM. Reaction was followed by withdrawing aliquots for enzyme assay. Inactivation data were processed using a DEC-10 computer and the program 'INAKT'.

Metal analyses were carried out using a Perkin-Elmer 5000 flame atomic absorption spectrophotometer.

**Results**

When crystalline apo(c)$_2$Zn(n)$_2$-enzyme was dissolved in 25 mM TES/Na$^+$ buffer, pH 8.2, at 4°C, the activity was less than 0.5% that of the native enzyme. In this apo-hybrid the metal content was 1.7-1.8 mole Zn per mole enzyme, while native enzyme had as expected a metal content of 3.9-4.1 mole Zn per mole enzyme (Table I).

Reactivation of apo(c)$_2$Zn(n)$_2$ with Ag(1) was performed by incubation with a 1.5-, 3-, and 5-fold excess of Ag$^+$ relative to active sites (Fig. 1). Even at the highest metal concentration no denaturation was detected within the first 8 h. The rate of reactivation did not change significantly with a 3- or 5-fold excess of Ag(1) relative to active sites, but was slower with a 1.5-fold excess. With all three concentrations the activity did not exceed 12% within the first 3 h, while maximum activity was first achieved after 36 h.

From these reactivation studies, a 3-fold excess of Ag$^+$ relative to active sites was chosen to make the Ag(c)$_2$Zn(n)$_2$-enzyme for further characterization. After 36 h reactivation, a light precipitate was removed by centrifugation and the resulting Ag-hybrid was dialyzed against 5 changes of 25 mM TES/Na$^+$ buffer, pH 8.2, to removed excess silver ions. The Ag-hybrid proved to be stable for days at 4°C and had a specific activity 24% that of the native enzyme.

Metal analyses gave 1.9-2.1 Ag and 1.9-2.1 Zn per mole enzyme, which indicates that the catalytic sites are fully occupied by Ag (Table I). The u.v.-spectrum of Ag(c)$_2$Zn(n)$_2$ was identical to that of the native enzyme, and thus the same molar extinction coefficient was used.

To further prove that the silver ion gave enzymatic activity, the Ag-hybrid was studied by metal-directed affinity labeling with BIP. This molecule inactivates the native enzyme by alkylating cysteine-46, one of the three protein ligands to the catalytic zinc atom (7). As with the native enzyme, reaction with the Ag-hybrid was first order.
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Fig. 1. Reactivation of apo(c)\textsubscript{2}Zn(n)\textsubscript{2}-enzyme (20 μN) with different Ag(I)-concentrations in 25 mM TES/Na\textsuperscript{+} buffer, pH 8.2, at 4°C. Excess of Ag(I) as indicated.

Table 1. Atomic absorption analyses and the enzymatic activities of the native, apo-, and silver-hybrid species

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>mol Zn/mol enzyme</th>
<th>mol Ag/mol enzyme</th>
<th>activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zn(c)\textsubscript{2}Zn(n)\textsubscript{2}</td>
<td>3.9-4.1</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>apo(c)\textsubscript{2}Zn(n)\textsubscript{2}</td>
<td>1.7-1.8</td>
<td>0</td>
<td>&lt;0.5</td>
</tr>
<tr>
<td>Ag(c)\textsubscript{2}Zn(n)\textsubscript{2}</td>
<td>1.9-2.1</td>
<td>1.9-2.1</td>
<td>24</td>
</tr>
</tbody>
</table>

with respect to enzyme (Fig. 2) and saturation kinetics were observed when the inactivator concentration was varied (Fig. 3). This is a result of inactivation following the mechanism

\[ E + BIP \overset{K_I}{\rightleftharpoons} E\text{-BIP} \overset{k_2}{\rightarrow} E' \]

where E is enzyme, E' inactive enzyme, \( K_I \) the dissociation constant for the reversible E-BIP complex, and \( k_2 \) the rate constant for inactivation (\( k_2 = \ln2/t_{\frac{1}{2}}\) minimum). Table 2 compares the different kinetic constants obtained for the native enzyme and the Ag-hybrid, Ag(c)\textsubscript{2}Zn(n)\textsubscript{2}.
Fig. 2. Semilog plots of inactivation of Ag(c)₂Zn(n)₂ with BIP in 25 mM EPPS/Na⁺ buffer, pH 8.2, at 23.5°C. BIP (mM) concentrations as indicated.

Fig. 3. Double-reciprocal plot for BIP inactivation of (●) native enzyme and (○) Ag(c)₂Zn(n)₂. k is the observed rate constant for each BIP concentration.
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Table 2. Kinetic data for BIP inactivation of the Ag(I)-hybrid and the native enzyme in 25 mM EPPS/Na+ buffer, pH 8.2, calculated using the computer program 'INAKT'.

K_I is the dissociation constant for the reversible E-BIP complex, t_{1/2(minimum)} the minimum halftime of inactivation, k_2 the rate constant for inactivation, and k_2/K_I the pseudobimolecular rate constant for inactivation.

<table>
<thead>
<tr>
<th></th>
<th>Zn(c)_2Zn(n)_2</th>
<th>Ag(c)_2Zn(n)_2</th>
</tr>
</thead>
<tbody>
<tr>
<td>K_I (mM)</td>
<td>0.680</td>
<td>1.82</td>
</tr>
<tr>
<td>k_2 (min⁻¹)</td>
<td>0.127</td>
<td>0.109</td>
</tr>
<tr>
<td>t_{1/2(minimum)} (min)</td>
<td>5.4</td>
<td>6.4</td>
</tr>
<tr>
<td>k_2/K_I (min⁻¹mM⁻¹)</td>
<td>0.189</td>
<td>0.060</td>
</tr>
</tbody>
</table>

Discussion

In this work we show that Ag(I)-ions can restore enzymatic activity to liver alcohol dehydrogenase depleted of metal ions in the catalytic center. Activity is regained as Ag(I) enters the empty metal-binding site in the catalytic center where in the native enzyme Zn is normally bound.

The silver hybrid enzyme Ag(c)_2Zn(n)_2 had a u.v. spectrum identical to that of the native zinc enzyme. Thus the spectrum could not be used to characterize the hybrid.

Zinc analyses of the native enzyme and the apo-hybrid, apo(c)_2Zn(n)_2, showed 3.9-4.1 and 1.7-1.8 mole of Zn per mole enzyme respectively and confirmed that dialyses against 2,6-pyridinedicarboxylic acid selectively removed the metal atom from the catalytic center (5).

As observed previously, the apo-hybrid was enzymatically inactive (less than 0.5% of the native enzyme). Atomic absorption analyses of the silver content of the enzyme species reactivated with silver showed equivalent amounts of Zn and Ag and indicated that Ag(c)_2Zn(n)_2 is formed and that the metal-binding site in the catalytic center is fully occupied with Ag(I)-ions.

To prove that metal substitution was in the catalytic center, metal-directed affinity labelling with the alkylating reagent BIP was carried out on the Ag-hybrid. Previously this reagent has been used to show that with native, Co(II)-, and Cd(II)-substituted liver alcohol dehydrogenase each metalloenzyme has distinctive properties (1). Comparison of the kinetic parameters for inactivation of the Ag(I)-hybrid with those of the native enzyme also showed that these two enzyme species had different properties (Table 2). While the values of k_2, which reflects the nucleophilicity of the metal-bound thiol of Cys-46, are similar, the value of K_I, which measures the stability of the reversible E-BIP complex, is higher in the case of the Ag(I)-hybrid. The lower affinity for the Ag-hybrid can reflect the imidazolyl ring of BIP binding weaker to the monovalent silver ion.
than to the divalent zinc ion. The pseudobimolecular rate constant $k_2/K_I$ differs for the two enzymes and indicates that BIP reacts about three times faster with the native enzyme when $[\text{BIP}] \ll K_I$.

Previously, substitution of several divalent metal ions [Co(II), Cd(II), Ni(II)] and monovalent Cu(I) for the native zinc ion in liver alcohol dehydrogenase have resulted in active enzymes (1-3,8). Recently we observed that the toxic metals Pb(II) and Hg(II) could also reactivate the enzyme (4). The present work is the first showing that Ag(I) can also replace Zn(II) in the catalytic center of liver alcohol dehydrogenase. This also appears to be the first silver enzyme reported to be enzymatically active.

**Acknowledgements**

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**References**