ZINC BINDING CHARACTERISTICS OF THE SYNTHETIC PEPTIDE CORRESPONDING TO THE STRUCTURAL ZINC SITE OF HORSE LIVER ALCOHOL DEHYDROGENASE

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1. INTRODUCTION

Medium-chain dehydrogenases/reductases of the liver alcohol dehydrogenase type are zinc metalloenzymes (Vallee and Hoch, 1957; Åkeson, 1964; Drum et al., 1969), with two zinc atoms per subunit, one catalytic at the active site and one structural at a site influencing subunit interactions (Sytkowski and Vallee, 1976; Brändén et al., 1975). In horse liver alcohol dehydrogenase and in all other mammalian liver forms, the structural zinc atom is liganded by four closely spaced Cys residues, at positions 97, 100, 103, and 111 (Brändén et al., 1975; Vallee and Auld, 1990). The mechanism by which this zinc atom maintains its structural role is largely unknown. To probe the metal-binding characteristics of the structural zinc site of alcohol dehydrogenase, we have analyzed zinc-binding to a synthetic replica of the protein segment containing the four Cys residues and covering residues 93–115 of the parent molecule.

Previously we have shown this peptide to mimick the metal-binding properties of the intact enzyme in cobalt-substitution experiments (Bergman et al., 1992; Bergman et al., 1993; Gheorghe et al., 1995) and we have now continued this study and investigated experimental approaches to determine the zinc-binding constant. Titration of the peptide/metal complex with a metallochromic chelator is potentially a useful technique and we now report on tests with 4-(2-pyridylazo)resorcinol (cf. Hunt et al., 1985) to analyze the zinc site.
Figure 1. Amino acid sequence of the zinc-binding synthetic peptide corresponding to residues 93–115 of horse liver alcohol dehydrogenase. The four Cys ligands are underlined.

2. EXPERIMENTAL

Peptide synthesis was carried out with side-chain-protected tertiary butyloxycarbonyl amino acid derivatives. Final purification was by preparative reverse-phase HPLC and peptide integrity was checked by amino acid analysis and mass spectrometry.

In the zinc-binding studies, Hepes buffer (20 mM, pH 7.5) was used after pretreatment with diphenylthiocarbazone (dithizone) to remove traces of metal ions (Holmquist, 1988). The Cys-containing peptides were reduced with dithiothreitol (DTT) and stored at -70°C until used. Before zinc incubation the DTT was removed via exclusion chromatography on a Bio-Gel P4 column (BioRad) and the reduced peptides were collected under anaerobic conditions. Zinc was added to fractions and the excess unbound zinc was separated from the peptide/zinc complex via another exclusion chromatography (Bio-Gel P4). The zinc-binding stoichiometry was evaluated by atomic absorption spectrophotometry and amino acid analysis of the elution fractions. The metallochromic chelator 4-(2-pyridylazo)resorcinol (PAR) was tested for determination of the zinc-binding constant via extraction of zinc from the metal-saturated peptide by measuring the absorbance at 500 nm for the Zn(PAR)$_2$ complex.

3. RESULTS AND DISCUSSION

A zinc-binding peptide corresponding to the loop around the structural zinc atom of horse liver alcohol dehydrogenase was synthesized for metal affinity studies. The 23-residue peptide covers the protein segment between residues 93–115 with Cys zinc ligands at positions 97, 100, 103, and 111 in the protein (Figure 1).

Zinc was added to the reduced peptide followed by anaerobic exclusion chromatography and measurement of zinc-binding. As previously reported (Bergman et al., 1992), zinc is bound at a 1:1 zinc/peptide ratio which is also the stoichiometry found in the protein. To further characterize the zinc-binding properties of the peptide replica we needed a method to determine the zinc-binding affinity of the peptide. Metallochromatic chelators have been used to quantify zinc in biological fluids and for monitoring zinc release from proteins (Pollák and Kubán, 1979; Hunt et al., 1985). We tested the metallochromatic chelator PAR (Figure 2) as a competitor to the peptide for its bound zinc.

![Figure 2. Structure of 4-(2-pyridylazo)resorcinol (PAR). The oxygen at position 3 and the azo-nitrogen at position 4 of the resorcinol ring together with the nitrogen of the pyridyl moiety have been suggested to constitute a terdentate ligand to zinc with the three donor atoms in a plane (cf. Iwamoto, 1961).](image-url)