The EMBO Journal vol.3 no.2 pp.357–360, 1984

Sorbitol dehydrogenase is a zinc enzyme

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Communicated by H. Jörnvall

Evidence is given that tetrameric sorbitol dehydrogenase from sheep liver contains one zinc atom per subunit, most probably located at the active site, and no other specifically bound zinc or iron atom. In alcohol dehydrogenases that are structurally related to sorbitol dehydrogenase, more than one zinc atom per subunit can complicate investigations of zinc atom function. Therefore, sorbitol dehydrogenase will be particularly valuable for defining the precise roles of zinc in alcohol and polyol dehydrogenases, and for establishing correlations of structure and function with other important zinc-containing proteins.
Key words: alcohol dehydrogenase/ligand evolution/metalloprotein/sorbitol dehydrogenase/zinc enzyme

Introduction

The Enzyme Commission divides enzymes into six main classes, and each of these contains some zinc metalloproteins (Galdes and Vallee, 1983). Examples reported during the past few years include tRNA synthetase (Kisselev *et al.*, 1981) and the restriction enzyme *Eco*RI (Barton *et al.*, 1982). Certain enzymes, such as DNA polymerases (Slater *et al.*, 1971) and RNA polymerases (Lewis and Burgess, 1982) appear quite generally to contain zinc. One of the well characterized dehydrogenases, liver alcohol dehydrogenase, contains catalytic zinc (Brändén *et al.*, 1975) but this is not the case for most dehydrogenases (e.g. Barkman *et al.*, 1970; Branlant and Biellmann, 1980), not even for all alcohol dehydrogenases. The short chain alcohol dehydrogenase from *Drosophila* lacks the metal (Place *et al.*, 1980), a difference probably related to the evolution of two separate types of subunit for alcohol and polyol dehydrogenases (Jörnvall *et al.*, 1981).

In addition to liver alcohol dehydrogenase, soybean cinnamaldehyde reductase (EC 1.1.1.2) (Wyrambic and Grisebach, 1979), glycerol dehydrogenase from *Bacillus megaterium* (EC 1.1.1.6) (Scharschmidt, 1980), yeast alcohol dehydrogenase (EC 1.1.1.1) (Klinman and Welsh, 1976; Sytkowski, 1977; Dickinson and Berrieman, 1977) and alcohol dehydrogenase from *Leuconostoc mesenteroides* (Schneider-Bernlöhr *et al.*, 1981) are zinc enzymes. *Zymomonas mobilis* was recently reported possibly to contain a ferrous iron alcohol dehydrogenase (Scopes, 1983). Of the zinc-containing dehydrogenases, information on the primary structure is available only for several yeast and liver alcohol dehydrogenases (Brändén *et al.*, 1975; Jörnvall, 1977; von Bahr-Lindström *et al.*, 1978; Wills and Jörnvall, 1979a, 1979b; Bennetzen and Hall, 1982; Russell and Hall, 1983; Russell *et al.*, 1983), while information on the tertiary structure is available mainly for horse liver (Brändén and Eklund, 1980; Eklund *et al.*, 1982a, 1982b; Cedergren-Zeppezauer *et al.*, 1982; Plapp *et al.*, 1983; Schneider *et al.*, 1983) and, from comparisons, to some extent other mammalian liver and yeast alcohol dehydrogenases (Eklund *et al.*, 1976; Jörnvall *et al.*, 1978). Relationships between structure and function of zinc metalloproteins are of wide interest, and information on another zinc-requiring dehydrogenase would be valuable.

The inactivation of sheep liver sorbitol dehydrogenase (EC 1.1.1.14) by EDTA or 1,10-phenanthroline indicated a metal requirement for this enzyme (Jeffery *et al.*, 1981) and the amino acid sequence (Jeffery *et al.*, 1984) showed that marked similarities exist between parts of sorbitol dehydrogenase and the zinc-liganding active site regions of yeast and liver alcohol dehydrogenases, though with the possibility of differences in geometrical arrangement (Jörnvall *et al.*, 1984; H. Eklund, C.-I. Brändén, H. Jörnvall and J. Jeffery, in preparation). The presence of zinc in this sorbitol dehydrogenase, and the absence of iron are now reported.

Results

The zinc content found in sorbitol dehydrogenase preparations was between 0.6 and 0.7 atoms per subunit (Table I). Further dialysis for 36 h at 2°C against 0.1 mM dithiothreitol in 5 mM potassium phosphate, pH 7.4, lowered the value by only a few percent, confirming that the zinc is not loosely bound, unlike the 'adventitious' zinc of yeast alcohol dehydrogenase (cf. Sytkowski, 1977). The problems of establishing precise stoichiometry of zinc-containing dehydrogenases have been thoroughly discussed for alcohol dehydrogenases from horse liver (Drum *et al.*, 1969) and yeast (Sytkowski, 1977). In relation to such problems, the finding of 0.6–0.7 is fully compatible with a stoichiometry of one zinc atom per subunit (Table II).

Sorbitol dehydrogenase contains no iron (Table I), and chelating agents have an inhibitory effect (Jeffery *et al.*, 1981). The present findings are consistent with one zinc atom at the active site of sorbitol dehydrogenase, participating in catalysis as in liver alcohol dehydrogenase.

Table I. Zinc and iron contents of sorbitol dehydrogenase

Enzyme preparation	Found			Integer proposed
	I	II	III	
Zn atoms/subunit	0.65	0.67	0.61	1
Fe atoms/subunit	ND[a]	<0.004	ND[a]	0

[a]ND, not determined

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Table II. Zinc content of other dehydrogenases

Enzyme	Zinc atoms/subunit		Reference
	Found	Proposed[a]	
Glycerol dehydrogenase			
Bacillus megaterium	0.51 – 0.62	1	Scharschmidt, 1980
Cinnamaldehyde reductase			
Soybean	0.55 – 0.64[b]		Wyrambic and Grisebach, 1979
Alcohol dehydrogenase			
Horse liver			
	1.9 – 2.2[c]	2	Åkeson, 1964
	1.9 – 2.0[c]	2	Oppenheimer *et al.*, 1967
	1.6 – 2.1[d]		Drum *et al.*, 1969
Band 3[e]	1.7 – 2.2	2	Sandler and McKay, 1969
Band 4[f]	1.4 – 2.5	2	Sandler and McKay, 1969
	2	2	Eklund *et al.*, 1974
Chi isozymes	1.8		W.P. Dafeldecker and B.L. Vallee, personal communication
Human liver			
	1.8 – 2.1[g]		Lange *et al.*, 1976
Pi isozyme	1.8 – 2.0	2	Bosron *et al.*, 1979
Chi isozymes	1.8 – 2.1[h]	2	Parés and Vallee, 1981
Monkey liver			
Squirrel monkey	2.0[i]	2	Dafeldecker *et al.*, 1981a
	2.0[j]	2	Dafeldecker *et al.*, 1981a
Rhesus monkey	1.9 – 2.1[i]	2	Dafeldecker *et al.*, 1981b
	1.8[j]	2	Dafeldecker *et al.*, 1981b
Rat liver	1.8[k]	2	Arslanian *et al.*, 1971
Peanut kernels	0.51[l]		Swaisgood and Pattee, 1968
Saccharomyces cerevisiae	1.8 – 1.9	2	Klinman and Welsh, 1976
	0.9 – 1.1[d]	1	Sytkowski, 1977
	≥1.5	≥1.5	Dickinson and Berrieman, 1977
Drosophila melanogaster	–	0[m]	Schwartz and Jörnvall, 1976
	<0.01	0	Place *et al.*, 1980
Leuconostoc mesenteroides	1.9 – 2.1	2	Schneider-Bernlöhr *et al.*, 1981

[a]Empty spaces indicate analyses where definite assignments are not apparent from the reports. [b]Subunit size 34.5 – 40 K (uncertain). Values recalculated from reported 1.1 zinc atoms per 69 K. [c]Probably mixture of mainly EE and EE′ isozymes. [d]Reviews some earlier determinations. [e]In later nomenclature, EE isozyme. [f]In later nomenclature, EE′ isozyme. [g]Probably mixture of mainly pyrazole-sensitive isozymes. [h]Mixture of chi-1 and chi-2 isozymes. [i]Pyrazole-sensitive. [j]Pyrazole-insensitive. [k]Recalculated for subunit size 40 K from reported 2.94 μg of zinc per mg of protein. [l]Number of subunits not known. Value recalculated for 38 K from reported 1.5 zinc atoms per 112 K. [m]Suggestion based on amino acid sequence data.

Table III. Zinc-liganding residues in horse liver alcohol dehydrogenase, LADH (top), compared with residues at equivalent positions in the primary structures of yeast alcohol dehydrogenase, YADH (middle), and sheep liver sorbitol dehydrogenase, SDH (bottom)

Enzyme	Amino acid residues		Proposed total zinc atoms
	Active site zinc atom in LADH	Other zinc atom in LADH	
LADH	Cys-46 His-67 Cys-174	Cys-97 Cys-100 Cys-103 Cys-111	2
YADH	Cys-43 His-66 Cys-153	Cys-97 Cys-100 Cys-103 Cys-111	1 – 2[b]
SDH	Cys-43 His-67 Cys-162[a]	Arg-97 Asp-100 Cys-103 Ser-111	1

[a]The nature of the residue in SDH corresponding to Cys-174 in LADH is not fully established (cf., text) but is here given as Cys-162.
[b]Different reports (cf. Table II).

Discussion

Although the active-site zinc atom of horse liver alcohol dehydrogenase is known to be directly involved in catalysis (Brändén *et al.*, 1975), its exact roles are not yet fully established. It influences the kinetics and energetics of co-enzyme binding (Dietrich *et al.*, 1983; Zeppezauer, 1983), though it is not required *per se* for either the actual binding of co-enzyme (Maret *et al.*, 1979) or the accompanying gross conformational change (Schneider *et al.*, 1983). It serves as a Lewis acid in the activation of the substrate by direct (i.e., inner-sphere) coordination and, in addition, probably influences the proton translocation, which is believed to involve the water molecule that is a ligand of the zinc, the substrate molecule that becomes a zinc ligand, the hydroxyl groups of Ser-48, the nicotinamide ribose (2′) and the imidazole of His-51 (Brändén *et al.*, 1975; Schmidt *et al.*, 1979; Dunn *et al.*, 1982; Eklund *et al.*, 1982a; Cedergren-Zeppezauer *et al.*, 1982; Makinen *et al.*, 1983). The nature of the protein ligands (Table III) is important in providing a substrate-interacting metal centre with the necessary properties (cf. Kvassman *et al.*, 1981; Dietrich and Zeppezauer, 1982). In alcohol dehydrogenase, the ligands are one histidine and two cysteine residues. In sorbitol dehydrogenase, two of these (Cys-46 and His-67 in the numbering system of horse liver alcohol dehydrogenase) are identical (the corresponding alignment is well defined). The third ligand (corresponding to Cys-174 in horse liver alcohol dehydrogenase) is probably also identical although the alignment in this region is not unambiguous because of adjacent insertions/deletions (Jörnvall *et al.*, 1984; H. Eklund, C.-I. Brändén, H. Jörnvall and J. Jeffery, in preparation). The conservation of two or all of the zinc-liganding residues (Table III), together with the presence of serine and histidine residues that correspond to Ser-48 and His-51 (they are Ser-45 and His-48) (Jeffery *et al.*, 1984), supports the view that the essential zinc atom in sorbitol dehydrogenase can be assigned to the active site.

The other zinc atom in horse liver alcohol dehydrogenase has four cysteine residues as ligands (Table III), and in sorbitol dehydrogenase there is only one cysteine residue (Cys-103) available for alignment in the corresponding positions. The other three positions (Table III), then correspond to arginine, aspartic acid and serine residues, respectively, and there are no neighbouring cysteine or histidine residues that might serve as alternative ligands. The finding of 0.6 – 0.7 zinc atoms per subunit (i.e., not more than 1) in sorbitol dehydrogenase is consistent with the absence of the set of ligands that forms the binding site for the second zinc atom in the horse liver alcohol dehydrogenase subunit. Horse liver alcohol dehydrogenase has a dimeric subunit arrangement.

Yeast alcohol dehydrogenase and sheep liver sorbitol dehydrogenase both have tetrameric quaternary structures. The yeast enzyme subunit contains the seven residues corresponding to all the ligands of both zinc atoms in the subunit of the horse liver enzyme (Table III). Reports differ regarding the questions as to whether a site other than the active site is occupied by zinc in yeast alcohol dehydrogenase, and, if so, what the functional significance may be of such a stoichiometry (Table II). Experimental problems and difficulties in interpretation arising from the presence of two zinc atoms per subunit in horse liver alcohol dehydrogenase (Drum and Vallee, 1970; Maret *et al.*, 1979), and the uncertainties associated with the yeast enzyme, are avoided by the use of sorbitol dehydrogenase containing only one zinc atom per subunit. It is therefore suggested that sorbitol dehydrogenase is particularly suitable for mechanistic studies involving the catalytic metal and its protein environment.

Materials and methods

Sheep liver sorbitol dehydrogenase was obtained as described (Jeffery *et al.*, 1981). Double glass-distilled water low in metals was used throughout and all glassware was washed with nitric acid. Zinc and iron were determined using a Perkin Elmer Model HGA 76 carbon furnace fitted to a Perkin Elmer Model 460 atomic absorption spectrophotometer for most assays, but some zinc analyses were performed using an Instrumentation Laboratory Inc. Model 157 atomic absorption spectrophotometer. Calibration solutions of zinc nitrate and ferric chloride were Spectrosol standards for atomic absorption spectrophotometry (BDH Chemicals Ltd., Poole, UK). The method was checked using carbonic anhydrase, horse liver alcohol dehydrogenase, as well as yeast alcohol dehydrogenase, and gave values in agreement with published values for these zinc-containing proteins. Amounts of sorbitol dehydrogenase were determined by acid hydrolysis and amino acid analysis, making use of the known composition and subunit size (Jeffery *et al.*, 1981, 1984).

Acknowledgements

This work was supported by the University of Aberdeen and the Swedish Medical Research Council (13X-3532 and 13V-6471).

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Received on 20 October 1983