THE ZINC CONTENT OF YEAST ALCOHOL DEHYDROGENASE[1]

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Received April 8,1976

Summary: Analyses for zinc in high specific activity preparations of yeast alcohol dehydrogenase (YADH) indicate a metal content of 1.8-1.9 moles of zinc per mole of enzyme subunit. This zinc content is observed for YADH prepared from Bakers yeast by recrystallization from Am_2SO_4 containing 1 mM EDTA, followed by chromatography on DE-52 and Sephadex-G-200. YADH obtained from Boehringer-Mannheim is characterized by a variable specific activity: preparations with Sp. Ac. = 380-400 U/mg contain 1.8-1.9 moles of zinc per mole of subunit. Dialysis of YADH against EDTA (pH 8.5, 25°, under N_2) reduces the specific activity and zinc content in an approximately linear fashion down to a Sp. Ac. = 150 U/mg, consistent with the preferential loss of a single, weakly bound zinc per subunit which is essential for catalytic activity. Dialysis of YADH against 1 mM $ZnCl_2$ (pH 6.5-8.5, 25°, under N_2) does not lead to an increase in the zinc content of the enzyme, indicating that under these conditions zinc does not bind adventitiously to YADH. Dialysis against 50 mM $CoSO_4$ (pH 5.5, 25°, under N2, 60-90 hr) leads to an exchange of $\sim$ 40% of the enzyme-bound zinc by cobalt. Our preparations of YADH are consistently characterized by a zinc content of $\sim$ 2 per subunit and we are unable to reduce the zinc content of YADH by dialysis against EDTA without a concomitant loss in enzyme activity, in contrast to reports of one zinc per subunit [Veillon, C. and Sytkowski, A.J., BBRC 67:1499 (1975); Vallee, B.L. and Hoch, F.L., Proc. Nat. Acad. Sci. USA 41:327 (1955)]. The findings reported here, together with the observed structural similarities between YADH and horse liver alcohol dehydrogenase [Jornvall, H., Woenckhaus, C. and Johnscher, G., Eur. J. Biochem. 53:71 (1975)], suggest a role for zinc at both a structural and catalytic site in YADH.

Introduction: The 2.4 Å crystal structure of horse liver alcohol dehydrogenase (HLADH) a dimer of 40,000 molecular weight subunits containing two zinc atoms per subunit, indicates a dual role for zinc: one zinc atom is at the catalytic site and is liganded to protein by two cysteines and a histidine; a second zinc, 18 Å from the catalytic site, is liganded to four cysteines (1). Yeast alcohol dehydrogenase (YADH) has been reported in the literature to be a tetramer comprised of four 37,500 molecular weight subunits, and to contain 1-1.25 zinc atoms per subunit (2,3,4). The role of zinc, structural vs. catalytic, in the yeast enzyme has been the subject of some speculation, especially in light of the reported

[1] This work is supported by USPHS Grants GM-20627, CA-06927, RR-05539; by NSF Grant BO-40663; and also by an appropriation from the Commonwealth of Pennsylvania.

structural homologies between yeast and horse liver alcohol dehydrogenase (5). Recently, Veillon and Sytkowski have reported that YADH obtained commercially contains zinc in excess of one per subunit, which can be removed by dialysis without a loss in enzyme activity; enzyme prepared from Baker's yeast is reported to contain one zinc atom per subunit (4). The findings reported here differ in two important respects from those of Veillon and Sytkowski: in the first instance, high specific activity YADH prepared from Baker's yeast contains 1.8-1.9 moles zinc per subunit; secondly, dialysis against EDTA is found to give rise to a concomitant loss of both enzyme activity and zinc content.

Experimental: YADH was either purchased from Boehringer-Mannheim as an ammonium sulfate suspension or prepared from Baker's active-dried yeast (P-L Biochemicals). Dialysis tubing was purchased from A. H. Thomas and was boiled in 0.1 M EDTA (pH 9) and stored in 0.01 M EDTA (pH 9). Chelex 100 (Bio-Rad) was washed with 1 N HCl, deionized water, and titrated to the desired pH with 0.1 N KOH. Sephadex G-25 and G-200 and DE-52 cellulose were purchased from Pharmacia and Whatman, respectively. The standards used for metal analyses were obtained from Fisher. All other reagents were obtained commercially and were reagent grade. Deionized water and chelexed buffers were used in these studies. Determination of pH was carried out on a Radiometer (Type TTlC) equipped with an expanded scale attachment. Enzyme was assayed spectrophotometrically by following the appearance of NADH at 340 nm on a Cary 118B spectrophotometer. Unless otherwise indicated, the stock enzyme assay solution contained PPi buffer (40 mM KPPi, 140 mM glycine, 5 mM KCl), 0.9 mM NAD and 0.8 M ethanol, pH 8.5. Enzyme assayed at pH 8.8 had a higher specific activity by a factor of 1.17. Protein concentration was measured either by absorbance at 280 nm, $\varepsilon = 1.26$ for a 0.1% solution, or by the method of Lowry et al. (6).

Metal Analyses. Metal analyses were carried out by the method of flame ionization on a Techtron atomic absorption spectrophotometer. All materials used for these analyses were prepared and stored in plastic containers. Standard curves for zinc were determined in the range of 0-10 μM zinc, and were

normally linear up to 8 μM zinc. Standard curves for cobalt were determined in the range of 0-80 μM. Each metal determination was carried out at least in duplicate. As a control, the zinc content of bovine carbonic anhydrase (Boehringer) was measured, and found to be 1.0 ± 0.1 (7).

Preparation of yeast alcohol dehydrogenase from Baker's yeast. The procedure of Hvidt and Kagi was followed with minor modification (8). In our best preparation, the yield of protein from a kg of yeast was 1000 mg. After three recrystallizations from ammonium sulfate in the presence of 1 mM EDTA, Sp. Ac. = 284 U/mg. Five hundred mg of desalted YADH was added to a DE-52 column, 2.5 x 35 cm. The column was washed with 200 ml of 5 mM KPi, pH = 7, followed by 1200 ml of a linear gradient, 5-100 mM KPi. The protein, which eluted at 42 mM of KPi, was characterized by a Sp. Ac. = 400 U/mg. Further chromatography on G-200 was attempted: 100 mg was added to a column, 2.5 x 30 cm, equilibrated with 5 mM KPi, pH = 7; the void volume of this column was 55 ml. Protein eluted at 95 ml, Sp. Ac. = 380 U/mg.

Results and Discussion: The specific activity of YADH used for these studies falls within the range of 300-400 U/mg, comparable to the specific activity of 420 U/mg reported by Dickenson for highly purified preparations of enzyme (9). Analysis for zinc in enzyme either prepared from Baker's yeast or obtained commercially indicates a metal content of 1.8-1.9 moles of zinc per mole of enzyme subunit, Table I. In addition to zinc, YADH was assayed for copper, cobalt, and magnesium. Copper and cobalt were absent whereas magnesium was found in one preparation of enzyme from Baker's yeast (0.11 moles/mole of enzyme subunit); the presence of small amounts of magnesium was previously attributed to a contaminant resulting from the 100-fold excess of magnesium over zinc in the crude extract of yeast (2). The elution profile of YADH (Sp. Ac. = 382 U/mg, Zn/subunit = 1.89) from a DE-52 cellulose column is illustrated in Fig. 1; protein and activity are seen to co-chromatograph. The specific activity and zinc content across the peak are 349 ± 36 and 1.80 ± .12, respectively.

Wallenfels and Sund reported that YADH recrystallized in the presence of

Table I

Zinc Content of Yeast Alcohol Dehydrogenase[a]

Sample	Specific Activity	moles Zn/mole enzyme subunit
Boehringer-Mannheim	382	1.89
Prep I	412	1.84
Prep II	387	1.77

[a] Specific activity and zinc determined as described in the Experimental Section.

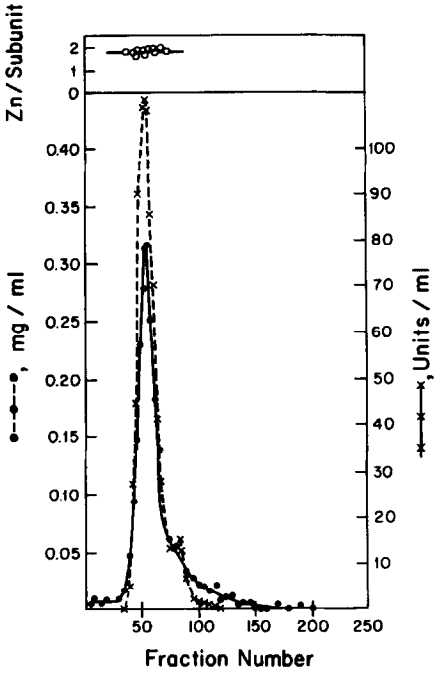

FIG. 1. Yeast alcohol dehydrogenase (12.5 mg) was eluted from a DE-52 column, 0.8 x 37 cm, with 300 ml of a linear gradient of 5-100 mM KPi, pH 7; the peak tube of protein eluted at 32 mM KPi. The column effluent was assayed for activity, X-X-X; protein, •-•-•; and zinc content, O-O-O, as described in the Experimental Section.

zinc may contain up to 30 moles of zinc per mole of enzyme; the specific activity of this high zinc enzyme was found to be slightly lower than enzyme containing 1.25 moles of zinc per mole of subunit (3). We find that dialysis against 1 mM $ZnCl_2$ (pH 6.5-8.5, 25°, under N_2) does not lead to an increase in the zinc content of YADH, indicating that under these conditions zinc does not bind adventitiously to enzyme. Several experiments have been carried out to demonstrate that the enzyme-bound zinc observed in these studies is due to a specific, activity-related, binding of zinc to YADH. Some loss of zinc is observed for YADH which has been stirred with Chelex, passed through a Chelex column or dialyzed in the presence of Chelex or EDTA at pH 6.5-8.5. However, in all cases, the loss of zinc is paralleled by a loss in specific activity. The relationship between specific activity and total zinc content was investigated in some detail by dialyzing YADH against EDTA at pH 8.5, 25°, under N_2. Under these conditions, the half time for the loss of enzyme activity is found to be $\sim$ 5 hr. The zinc content of enzyme dialyzed from 0-8 hr was determined, and is plotted as a function of specific activity in Fig. 2. Down to a specific activity of 150 U/mg, the loss of zinc and activity occur in an approximately linear fashion, consistent with the preferential loss of a single zinc per subunit which is necessary for catalytic activity. The curvature observed below 150 U/mg most likely results from the subsequent loss of zinc from inactive subunits. The lowest specific activity obtained, 120 U/mg, is one-fourth the value obtained by extrapolation to 2 moles of zinc per mole of enzyme subunit, 480 U/mg.

Various efforts have been made by other investigators to grow yeast in media depleted of zinc and enriched in either cobalt or manganese (10,11,12). A manganese enriched enzyme has been reported to be unstable, and has not been purified to homogeneity (10,12). A cobalt YADH was reported by Curdel and Iwatsubo (11) to contain 4 moles of M^{2+} (Zn + Co) per mole of enzyme; the specific activity of this enzyme preparation was reported to be similar to low values in the literature (13,14). In Table II the results of dialyses of YADH

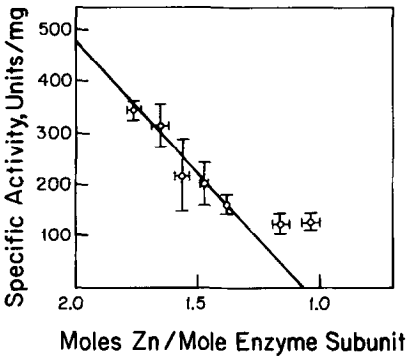

FIG. 2. The relationship between specific activity and zinc content for enzyme dialyzed 0-8 hrs against a 200-fold volume excess of chelexed PPi buffer which contained 0.2 mM dithiothreitol and 1 mM EDTA, pH 8.5, 25°, under N_2. At long times, e.g. 8 hr of dialysis, precipitated protein represented $\sim$ 40% of the total protein, and was removed by centrifugation prior to analysis of the enzyme for activity and zinc content. Each data point represents the average of 2-7 enzyme samples (a total of 30 determinations) assayed for activity and zinc, both before and after chelexing of the protein, as described in the Experimental Section.

Table II

Replacement of Zinc by Cobalt in Yeast Alcohol Dehydrogenase[a]

Buffer Components	Time of Dialysis, hr	% Protein Recovered	Specific Activity, U/mg	Moles M^{2+}/ mole enzyme subunit		
				Zn	Co	Zn + Co
50 mM $CoSO_4$	60	68	247	1.40	0.26	1.66
50 mM Am_2SO_4	60	70	360	1.76	ND	1.76
50 mM $CoSO_4$	99	55	302	1.40	0.36	1.76
50 mM $CoSO_4$, 100 mM NaCl	88	49	280	0.95	0.60	1.55
50 mM $CoSO_4$, 100 mM NaCl	94	53	278	1.07	0.73	1.80

[a] Replacement of zinc by cobalt was achieved by dialyzing $\sim$ 10 mg/ml of YADH under a stream of N_2 against 10 mM NaAc, pH 5.2, 0.2 mM dithiothreitol, and either 50 mM $CoSO_4$ alone or with 100 mM NaCl. Following dialysis excess metal was removed by gel filtration on Sephadex G-25. Peak protein fractions were analyzed for activity and metal content both before and after chelexing.

against $CoSO_4$ are summarized. At long times of dialysis in the presence of NaCl, ∿ 40% of the total metal is replaced by cobalt. These enzyme preparations are green in color, have absorption spectra in the 550-750 nm range similar to that observed by Curdel and Iwatsubo and are characterized by a specific activity about 80% that of native YADH. Further studies on this hybrid enzyme are in progress.

The experiments described in this paper indicate that high specific activity preparations of YADH are characterized by a ratio of zinc to enzyme subunit close to 2.0. Enzyme is relatively unstable, losing zinc and activity upon storage and dialysis. Early reports of 1-1.25 moles of zinc per subunit were likely the result of the low specific activity of the protein preparations used for metal analysis. Although Veillon and Sytkowski have recently reported that high specific activity preparations of YADH contain only one zinc per subunit (4), we find that YADH purified from Baker's yeast under carefully controlled conditions contains 2 moles of zinc per subunit, and that dialysis to remove "adventitious" zinc reduces the activity of YADH to one-fourth the value of fully active enzyme. Our findings, together with the observed structural homologies between yeast and horse liver alcohol dehydrogenase (5), suggest a role for zinc at both a structural and active site in YADH.

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