BIOSYNTHETIC INCORPORATION OF COBALT INTO YEAST ALCOHOL DEHYDROGENASE

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Many enzymes are metalloproteins. Numerous biochemical and biophysical studies have been done to collect information on the role of the metal in the enzymatically catalysed reaction. When it is possible to remove the metal reversibly and to obtain an inactive apoenzyme, the effect of the *in vitro* recombination of the apoenzyme, with one or several metals, can be studied; among other investigations, this has been done for bovine pancreatic carboxypeptidase [1–3], carbonic anhydrase [4–6], yeast D-LDH [7–11]; from a more biological point of view, a related problem is the study of *in vivo* effects of different metals on the synthesis of some metalloenzymes and on their properties; various questions arise, such as: is it possible to incorporate *in vivo* in a metalloenzyme a metal other than the one present in the molecule under natural conditions of synthesis? If so, it becomes of interest to study the modifications introduced by the substitution *in vivo* of the metallic cofactor of the enzyme during biosynthesis in cells.

This investigation can be carried out in the case of microorganisms for which the chemical composition (metal and salts) of growth media can be controlled, and when growth occurs in spite of deprivation or enrichment in some metals. Such a study has been performed for yeast D-LDH which is a zinc-flavoenzyme [7–11]; its inactive apoenzyme can be prepared [7], and has chelating properties [10]. When yeast was grown in synthetic media deprived or enriched with Zn, Co, Mn, it has been shown that, in the absence of zinc and in the presence of cobalt, yeast synthesizes *in vivo* a new D-LDH. The latter is different both from the Zn-D-LDH obtained in natural conditions and also from the Co-D-LDH reconstituted *in vitro* with cobalt and the apoenzyme of the natural D-LDH [9]. In the same way, other preliminary experiments done with yeast cultivated in zinc-deprived and cobalt-enriched media have suggested that cobalt is able to replace zinc *in vivo* in two other natural zinc-containing enzymes of yeast: namely D-lactic cytochrome c reductase, and alcohol dehydrogenase.

The present paper deals with experiments done on the last enzyme. It is known that yeast alcohol dehydrogenase (EC 1111), crystallized first by Negelen and Wulff in 1937 from brewer's yeast [12], later by Racker from baker's yeast, was identified as a zinc-enzyme by Vallée et al. [13–16]. They showed that one molecule of Y-ADH contains four atoms of zinc which cannot be exchanged with external zinc, as demonstrated by experiments with ^{65}Zn. The acid splitting of zinc atoms from the Y-ADH results in an irreversible loss of activity. The metal seems to play a role in the binding of the coenzyme to the enzyme and in the maintenance of protein configuration.

For our experiments, the yeast (strain: Yeast Foam) was grown anaerobically in a culture medium to which cobalt (specpure 10 mM) was added, for 48 hours at 30°. No special precautions were taken to eliminate completely the contaminating zinc in the medium in view of the overcharge of cobalt. 1 kg of yeast (wet weight) obtained from a culture of 80 litres was stored at −20°C. Y-ADH is quite stable in cells kept this way, even for a year. Y-ADH was prepared by the method of Racker [17] with a slight modification previously described [18]. The frozen yeast is thawed and suspended in the same volume of

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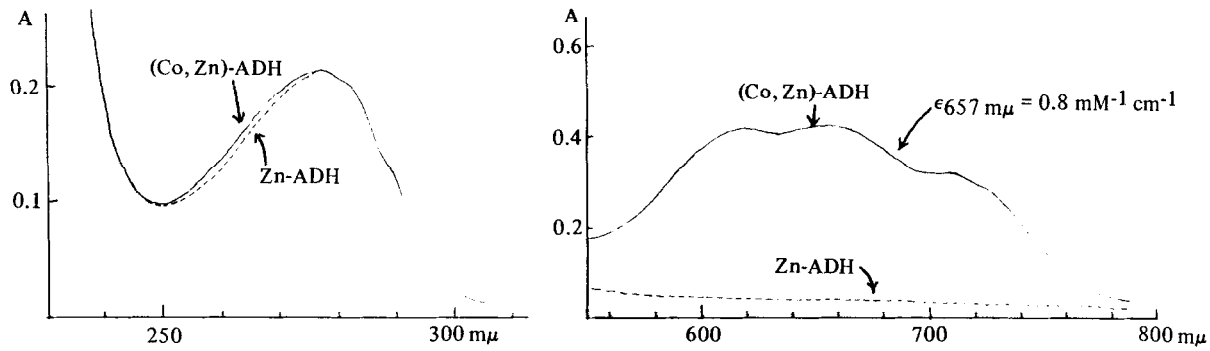

Fig. 1. Absorption spectrum of cobalt containing yeast alcohol dehydrogenase. (A) Visible and near-ultraviolet spectrum of a solution containing about 4.5 mg of three times recrystallized enzyme per ml. (B) Visible spectrum of a ten times more concentrated solution of the enzyme.

buffer (0.1 M phosphate, 1 mM EDTA pH 7.5), the suspension is allowed to autolyse at 38° for 1 hour. After elimination of cell debris by centrifugation at 0°, Y-ADH is precipitated from the extract with ammonium sulphate at 0.65 saturation. The precipitate was collected, dissolved in buffer and diluted to adjust the concentration of ammonium sulphate to 0.3 saturation. A heat denaturation is carried out at this step at 54° for 3–4 min. After elimination of the denatured proteins, the Y-ADH is precipitated with ammonium sulphate at 0.55 saturation. The precipitate is dissolved in a minimum volume of buffer. Dense green crystals appear immediately. It is important to underline that all the manipulations are made in a buffer containing EDTA 1 mM. Recrystallization is repeated three times. In the last steps of crystallization a spectroscopically pure ammonium sulphate (Johnson Matthey) is used to exclude contamination by other metals. The Y-ADH thus obtained is green. The yield of Y-ADH crystallized by this method is nearly 30% with respect to the total enzyme extracted. This yield is quite similar to the one obtained from the yeast grown in the same way but without added cobalt; in this latter case Y-ADH crystals are colorless.

Several arguments indicate that cobalt has been incorporated into the enzyme. The absorption spectrum of Y-ADH crystallized from Co-grown cells is shown in fig. 1. In the visible region, three maxima (or shoulders) were found respectively at 710, 670 and 620 mμ. An approximate value of the molar absorbancy coefficient of the band at 670 mμ is 1000 (calculated on the basis MW = 130000 as discussed below). This value is of the same order of magnitude as those given for some cobalt-containing proteins in this region of the spectrum [19]. In the near ultra-violet, two other absorption peaks were found as shoulders at 345 and 390 mμ in addition to the 278 mμ peak. The Y-ADH obtained from cells grown under normal conditions does not present any of these characteristic absorption maxima [20]. The metal content of the Co-grown Y-ADH was analyzed by three independent methods: by neutron activation * which gives the absolute amount of the metal in the protein, by emission spectroscopy ** and by atomic absorption ** which give only the relative amount of the two metals. All three methods have given perfectly concordant results (see table 1). The ratio of Co to Zn in Co-grown Y-ADH is very close to 1:2 (atom:atom), while no cobalt (< 0.01) can be detected in standard-grown (i.e. without added cobalt to the growth medium) Y-ADH. Anaerobically grown yeast has been used in both cases. The total amount of bound metal, (Co + Zn) or (Zn), was found to be 3.5 atoms/mole for Co-grown Y-ADH and 4.0 for the standard Y-ADH. These two values are considered not to differ significantly. The

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Table 1

Amounts of cobalt and zinc in cobalt-grown and in standard-grown yeast ADH.

	Protein concentration	Metal content determined by various methods			*Cobalt + Zinc* protein
		Co	Zn	Co/Zn	(atom/mole)
	(mg/ml)	(μg/ml)	(μg/ml)	(atom/atom)	
Standard-grown ADH	28	0 (1)	54 (1)	0/100 (1)	4 ± 0.4 (3)
			58	<1/100 (2)	
Cobalt-grown ADH	42	21 (1)	55 (1)	45/100 (1)	3.5 ± 0.4 (3)
	44	22	53	50/100 (2)	
		22			

Three times recrystallized preparations of both ADH's were analyzed for cobalt and zinc content by various methods:

(1) by thermal neutron activation followed by chromatographic separation and separate counting of the two activated metals.

(2) by emission spectroscopy and atomic absorption; the ratio given in the table is an average of both methods. Protein determinations were performed by spectrophotometric measurements for standard-grown Y-ADH ($E_{1mg/ml}$ = 1.26 at 280 mμ) and by biuret, after precipitation by TCA 5%, for cobalt-grown Y-ADH, using serum albumin as a standard. Molecular weight was taken as 130000 as discussed in the text.

(3) the cumulative errors of the determination protein and metal concentrations are lower than 10%.

molecular weight has been taken as 130000 (σ = 7000). This value has been determined on standard Y-ADH by approach-to-equilibrium method (in the Station Centrale d'Ultracentrifugation du C.N.R.S., under the direction of Dr. S.Filitti-Wurmser) following extrapolation to zero protein concentration of several separate determinations; it should be noted that it differs to some extent from previously published values for baker's yeast-ADH (150000 [20]). On a column of Sephadex G-100, Co-grown Y-ADH is eluted in exactly the same way as the standard-grown Y-ADH; the molecular weights have thus to be considered identical. It should also be noted that cobalt does not separate from the enzyme into the medium used for keeping solutions of Co-grown Y-ADH in spite of the presence of EDTA 1 mM in this medium. The binding of cobalt to the enzyme must therefore be very tight.

At present, we cannot distinguish between two possibilities: whether this preparation is a mixture of two enzymes, in proportion 1 : 2 (one enzyme containing only cobalt and one containing only zinc), or whether, statistically, one third of the metal binding sites are occupied by cobalt in all the Y-ADH molecules. No heterogeneity has been observed when the cobalt containing ADH preparation was treated by electrophoresis on acrylamide gel or by molecular sieving on Sephadex G-100 column. The single band was identical in position to the one corresponding to standard ADH. It must be stressed that the enzymic activity per molecule (measured by the reduction of NAD) of the cobalt containing Y-ADH is at least as high, if not higher, than that of the normal yeast Zn-ADH. Our values for the activity at the pure enzyme compare favorably with those published in the literature [17, 20]. It is therefore clear that, whatever is the distribution of Co among enzyme molecules, those containing Co must be enzymically active.

The bulk of data presented here and the accuracy of the method employed seem sufficient to establish that cobalt has been actually bound at the zinc sites and not as another supplementary site.

The possible synthesis of an enzyme, containing *in vivo* incorporated cobalt, opens a wide field for biophysical and biochemical studies of two homologous enzymes (ADH): one containing zinc and one containing cobalt. Obtaining a cobalt-containing ADH through the biosynthetic incorporation of metal should provide new possibilities for a study of the mechanism of enzyme action and of the regulation of

enzyme formation. This underlines the usefulness of a systematic approach in studying metalloenzymes by changing the nature of the metal during their biosynthesis [9, 11].

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