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Properties of a cobalt-reactivated form of yeast alcohol dehydrogenase

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Abstract

Yeast alcohol dehydrogenase (Y-ADH) is a widely studied metal-enzyme for its well-known biotechnological importance. Although its structure has been extensively investigated, some topics still remain controversial (zinc content and role), and various attempts aiming at modifying its structure to improve its catalytic properties have been made. In this paper, a metal-substituted Y-ADH has been prepared in vitro, in which one Zn atom per molecule (only one of those directly involved in catalysis) has been substituted by one Co atom. The substitution was obtained through zinc removal by a chelating treatment (with Chelex 100) followed by cobalt insertion. The zinc content in the native enzyme was preliminarily evaluated (taking care to avoid contamination) to be 4.1 ± 0.1 g-at./molecule. After cobalt substitution, the ratio Zn:Co in the enzyme results to be 3:1. The active Co-Y-ADH has been compared with the native enzyme: it has lower specific activity (about 50%) and lower substrate affinity but greater thermo-resistance and a pH stability in a wider range than the native Y-ADH. A similar behavior, as far as cobalt content, thermo-resistance and pH stability are concerned, but greater specific activity and substrate affinity, were shown by an in vivo-substituted Co-Y-ADH obtained in a previous study.

Keywords: Modified Y-ADH; Zinc–cobalt substitution; Apoenzyme reactivation

1. Introduction

The biotechnological importance of yeast alcohol dehydrogenase (Y-ADH, EC 1.1.1.1) has long been established both for the food industry [1] and for the bioconversion of several organic wastes [2,3]. In particular, in the last few years, many attempts have been made to obtain ADHs with higher catalytic activity in order to improve a critical step in the conversion of different sugars (starch, lactose, etc.) into ethanol to be used as fuel [4].

Y-ADH is a tetramer of about 150 kDa [5–7], very similar in amino acid sequence to the dimeric ADH of mammalians, and the best studied of which is the horse liver ADH (HL-ADH). While the reaction mechanism of Y-ADH is at present well-characterized, different hypotheses have been reported on its quaternary structure and on its zinc content. In fact, in 1973, Cole-

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man and Weiner [8] reported a model in which zinc is necessary for quaternary structural integrity (structural zinc), not [9] excluding at the same time a possible catalytic role of the metal. In 1975, Veillon and Sytkowski [9] demonstrated, on the contrary, the presence of catalytically active, intrinsically bound, zinc atoms in a ratio of four zinc atoms per tetramer; pointing out, at the same time, ''adventitious'' zinc atoms not involved in the enzymatic activity. Two years later, Sytkowski [10] confirmed only the presence of four catalytically active zinc atoms.

In 1975, Branden et al. [5] referred to a model in which both structural and catalytic zinc atoms are present, the only difference being the ligands for the metal: for the structural zinc, four cysteines residues; and for the catalytic zinc, two cysteines, one histidine and one solvent molecule (H_2O or OH^-), with the last replaced by the alcoholic substrate during catalysis.

One year later, Klinman and Welsh [11] confirmed the hypothesis of 1.9 mol Zn/mol enzyme subunit, that is to say that each monomer contains one structural and one catalytic zinc. This model was proposed also in 1992 by Magonet et al. [12], which demonstrated the presence of eight zinc atoms per Y-ADH tetramer, i.e., two zinc atoms per subunit, according to HL-ADH.

Among the different techniques used to improve the catalytic properties of ADH, two are worth mentioning: amino acid substitution with site directed mutagenesis [13–16] and metal substitution [8,10,17–19]. Until now, only manganese and cobalt have been employed as possible zinc competitors due to their similarity in ion charge, dimension and coordination geometry.

In our laboratories, we have obtained, some years ago [20], a modified Y-ADH_1 by zinc–cobalt substitution in vivo, by growing *Saccharomyces cerevisiae* cells in zinc-depleted conditions and excess of cobalt (4 mM). This modified Y-ADH_1 exhibits a Co:Zn ratio of 1:3 and displays better catalytic activity, higher thermo-resistance and good stability in the alkaline range of pH, as compared to native Y-ADH.

In the present paper, we have tried to obtain Co-Y-ADH by in vitro substitution after a chelating treatment to remove zinc (for obtaining the apoenzyme) on a commercial Y-ADH produced in *S. cerevisiae*. On the Co-substituted enzyme, we determined the physical–chemical properties, specific activity and total metal content in order to compare these results with both the native Y-ADH molecule and the in-vivo-substituted enzyme.

2. Materials and methods

The determination of the zinc content and the preparation of the substituted enzyme have been difficult because of the interference due to the presence of zinc in water. As a consequence, it has been necessary to standardize the procedure for obtaining ultra-pure water and materials.

All materials have been previously purified with several washings with 1% HNO_3, 0.1 M EDTA, 1% HNO_3 and repeated rinsing with pure water [21] to eliminate organic and metallic compounds. The bidistilled water has been further on treated with purifying ELGASTAT (Elga, England), because of the analytical need to have zinc and cobalt contents less than 0.002 UA (to limit interference on blank). Native enzyme Y-ADH has been obtained from Boehringer Mannheim in lyophilized form and stored at 4°C. Its purity was verified by the SDS-PAGE (7.5% bisacrylamide) method. The chelating agent used (Chelex 100 resin 100–200 mesh, sodium form) has been obtained from BioRad and blown up in PBS 1 h before using it.

Phosphate buffer 0.15 M, pH = 7.6 (PBS) has been prepared using Suprapur reagents (sodium hydrogen phosphate, hydrochloric acid) obtained from Merck.

Kinetics assays to determine the specific activity have been carried out at 340 nm, by

means of Hitachi U-3200 spectrophotometer, using PVC cuvettes and continuously stirring, according to the procedure and the conditions previously described [20].

2.1. Inactivation and reactivation of the native enzyme

The native enzyme (Y-ADH) has been dissolved in PBS and treated with different amounts of Chelex 100 in vials (PVC, volume = 2.5 cm^3). Samples have been steady stirred (60 cpm), in a thermostatic cabinet to maintain the temperature at different fixed values. Because the competition between the enzyme and the chelating resin for metal binding is slow, the Chelex 100 has been replaced two times, as a function of the enzyme inactivation rate.

During this inactivation phase, samples and controls have been tested by measuring the specific activity as a function of treatment time (see Fig. 1). Corresponding to each point of the inactivation curve, the reversibility of the metal depletion process has been confirmed by incubating the sample with an excess of zinc chloride (Merck) for 1 h at room temperature and measuring the specific activity of the ''reactivated'' enzyme. At the same time, the metal depletion has been measured by atomic absorption spectroscopy in order to correlate the activity loss with the zinc depletion (see Fig. 1). Because further treatments allow a non-reactivable apoenzyme, the inactivation process has been stopped at the time corresponding to the achievement of a pseudo-apoenzyme form with the lower catalytic activity (10–20% of the native enzyme specific activity) but still reactivable with zinc chloride addition.

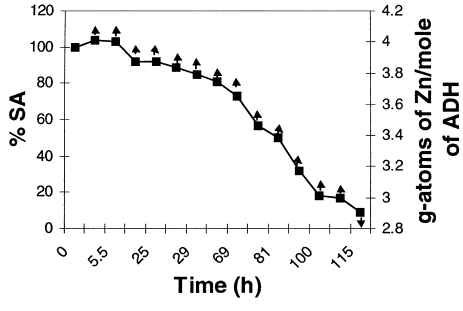

Fig. 1. Y-ADH inactivation by treatment with Chelex 100. Up arrow keys correspond to reversible denaturated forms (reactivation was obtained by means of zinc addition). Down arrow key corresponds to an irreversible denaturated form of the enzyme (no activation was observed by adding zinc). Operating conditions: ADH concentration = 0.4 mg/cm^3, Chelex 100 concentration = 0.05 mg/cm^3, temperature = 25°C, pH = 7.6.

The inactivation curves have been performed at different temperatures, by increasing the resin concentration and varying the ionic strength. No significant differences were observed in the temperature range 4–25°C and for resin concentration between 50 and 200 mg/cm^3. In the same way, significant variations of the inactivation process were produced by varying the ionic strength. Consequently, the following experiments have been carried out at 4°C, with Chelex 100 concentration of 50 mg/cm^3 with 0.15 M ionic strength.

The pseudo-apoenzyme reactivation curves have been obtained, after chelating resin filtration with 0.45-μm filters, incubating the samples with increasing concentrations of Co(II) chloride (Merck) and stirring for 1 h at room temperature. The same pseudo-apoenzyme reactivation has been carried out with increasing concentration of zinc chloride.

The evaluation of kinetic constants has been performed according to the Lineweaver–Burk methodology [22]. Structure differences have been investigated by means of affinity chromatography, and metal coordination in the active site has been studied by EPR and NMR techniques.

2.2. Atomic absorption spectroscopy

The zinc amount has been determined by means of flame atomization AAS, using a Perkin-Elmer 1100B spectrophotometer. Background has been corrected using a deuterium lamp.

The relative flow rate of gases (acetylene–air) has been optimized in order to maximize the analytical signal.

Before starting the analysis, a series of tests have been carried out to exclude the presence of contaminating zinc in samples and in reagents, as well as verifying the effectiveness of the washing method. It was found that there is a significant contamination (absorption values much larger than four times the background 0.001 UA) to both solvent and reagents.

The problem has been completely solved using Suprapur Merck reagents and properly purified water.

The cobalt amount has been evaluated by electrothermal AAS, using a Perkin-Elmer 5000 spectrophotometer and the same background correction. Even in the cobalt case, preliminary tests to put in evidence incidental contamination have been performed. However, they showed no significant contamination even if only analytical reagents are used.

To exclude the presence of matrix effects, the standard addition method has been used. The effectiveness of such a method has been verified in comparison with results obtained by calibration curves in 0.2% HNO_3. This allowed us to exclude, within the experimental errors, the matrix effects.

The amount of each metal has been determined on single samples with five repeated measurements.

2.3. NMR

The ^{1}H NMR spectra of native and Co-substituted enzymes, in HDO, have been obtained at 8°C on a 270-MHz instrument (GX270), equipped with an electromagnet and an external lock circuit granting a $\pm$1-Hz long-term stability. The residual signal of water (HDO) has been attenuated by means of pre-saturation at 4.8 ppm, the baricentre of this signal. Spectra have been accumulated for 7.2×10^4 scans (brief scan duration: 0.2 s) and the chemical shifts have been reported relative to Me_4Si (external standard).

2.4. EPR

EPR spectra have been recorded at 100 kHz frequency modulation and 9.4 GHz microwave irradiation with a Varian E9 spectrometer equipped with an Oxford Instruments ESR10 liquid helium cryostat. The measurements have been carried out at 5 K (accuracy of $\pm$0.5 K).

Samples have been pre-concentrated by super-filtration with a 30- to 3000–4000 rpm Amicon Centricon (MW > 10 kDa) centrifuge to a final concentration of 0.5 mM. The investigated range has been extended up to 10,000 G.

2.5. Affinity chromatography

Chromatograms have been obtained on a Progel-TSK Chelate-5PW column, treated with 10 mM sodium acetate (pH = 4.5) buffer and, then, loaded with zinc ions, according to the Supelco method. The gradient elution has been performed using a mobile phase of Tris–HCl buffer (pH = 8) and glycine (0.5 M) buffer. Detection was carried out at 280 nm (flow rate: 1 cm^3/min).

2.6. Stability

The behavior of Co-substituted enzyme as a function of temperature and pH has been evaluated in the range between 15°C and 50°C, and pH = 6–9.

3. Results

The commercial native enzyme showed a high purity degree (verified by SDS-PAGE) and a molecular mass of 149 000 Da.

The specific activity was 200 U/mg. This value did not change at different temperatures

(4–25°C), during 200 h and in a concentration until 10 mg/cm^3.

3.1. Metal content by atomic absorption spectroscopy

The experimental results, obtained by 10 repeated measures and by eliminating metal contamination, prove that, at the beginning, 4.1 ± 0.1 g-at. Zn/mol enzyme are present. The metal depletion during the inactivation process is reported in Fig. 1. The figure shows that the competitive equilibrium between the imminodiacetic functional groups of Chelex 100 and the amino acids of the enzymatic active site is slow but rather reproducible (RSD lower than 4.5).

During the decrease of specific activity until about 10–20%, the inactivation appears to be reversible (up arrow keys), on the contrary, when the specific activity is about 10–20%, the inactivation becomes irreversible (down arrow key). When specific activity is about 20%, the zinc content is of 3 g-at./mol protein. At the same time, the reactivation curves, obtained by adding cobalt after chelating resin filtration, show a maximum specific activity corresponding to the addition of 1 g-at. metal/mol protein (Fig. 2). Further metal addition produces an inhibitory effect. The reactivation curve carried out with zinc shown in the Fig. 2 confirms the results obtained with cobalt.

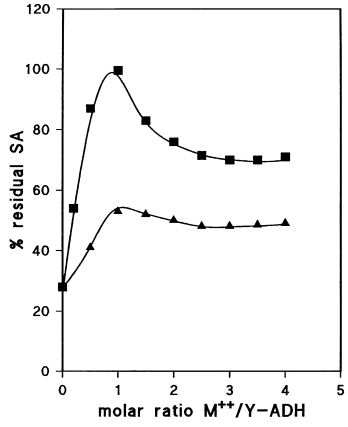

Fig. 2. Reactivation curve of apoenzyme by addition of cobalt (▲) to obtain the Co-substituted enzyme and by addition of zinc (■) to confirm that maximum of restored specific activity was obtained corresponding to the addition of 1 g-at. metal/mol protein. Operating conditions: ADH concentration = 0.4 mg/cm^3, temperature = 25°C, pH = 7.6.

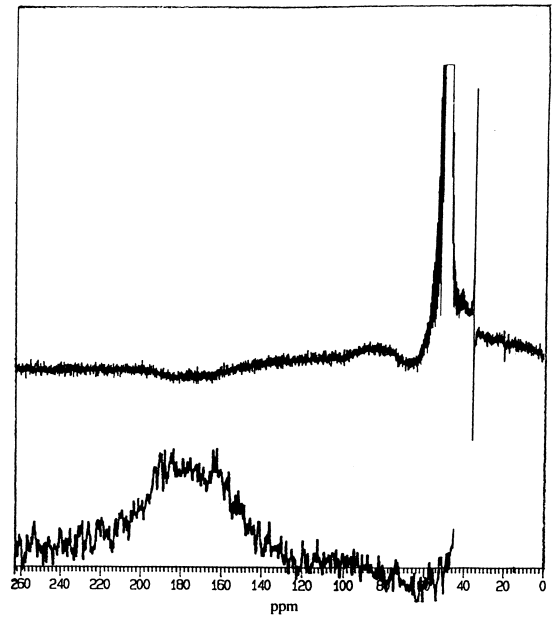

Fig. 3. ^{1}H NMR spectra of native Zn-ADH (upper spectrum) and substituted Co-ADH in HDO. The shifted signals due to the cobalt coordination are visible at about 180–190 ppm.

3.2. NMR

The analysis of the data obtained by NMR spectroscopy (Fig. 3) reveals a shift of signals concerning the amino acids near to the metal from the diamagnetic region (0–10 ppm) to 160–190 ppm, with respect to the data of native enzyme. Some distortions of the baseline of the spectrum are due to an acoustic ringing of carrier frequency and to a spike corresponding to the same carrier.

According to the literature [19], the shift can be attributed to the coordination of Co(II). Co(III), if present, would not cause signal shift

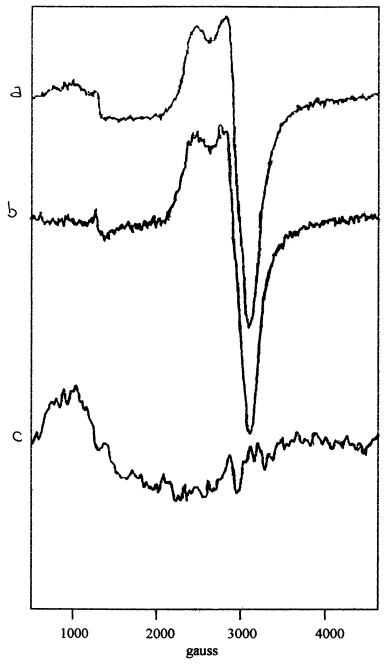

Fig. 4. First-derivative EPR absorption spectrum of Co-substituted ADH. The Co-ADH spectrum (curve c) was obtained by subtracting the signals of the quartz sample tube (curve b) to the whole spectrum (curve a). The g component at 5.6 at about 1000 G arises from the coordination of cobalt. Because of the low concentration of the substituted enzyme (0.5 mM) the hyperfine structure of ^{59}Co ($I = 7/2$) is not distinguished. No additional features were detected in scans to 10,000 G.

because it is diamagnetic and aqueous cobalt would produce only a very limited shift of the water signal. Furthermore, the comparison with Zn_2Co_2-HL-ADH NMR spectrum [19] shows that the number of shifted signals in our sample is lower. This finding might suggest a lower cobalt content.

3.3. EPR

Fig. 4 illustrates Co-substituted Y-ADH spectra, obtained by subtracting the quartz probe spectrum from that of the enzyme. The signal at about 1100 G ($g = 5.56$) can certainly be attributed to the presence of high spin ($S = 3/2$) Co(II) in the molecule, which is likely to be inserted in a geometrically distorted coordination, in analogy to that found by Makinen and Yim [23] for Co-HL-ADH. Because of the low metal concentration, it was not possible to put into evidence the hyperfine structure of Co ($I = 7/2$).

3.4. Stability and kinetic behavior

The obtained experimental results allow us to calculate that the native enzyme has a k_M for the oxidative reaction (conversion of ethanol into acetaldehyde) of 5.3×10^{-3} M ($T = 37$°C, pH = 7.6), while the Co-substituted enzyme has a k_M (oxidative reaction) of 1.2×10^{-2} M at the same value of temperature and pH, i.e., Co-Y-ADH shows lower affinity for ethanol than native Y-ADH. The other catalytic parameters, temperature and pH optima, are the same

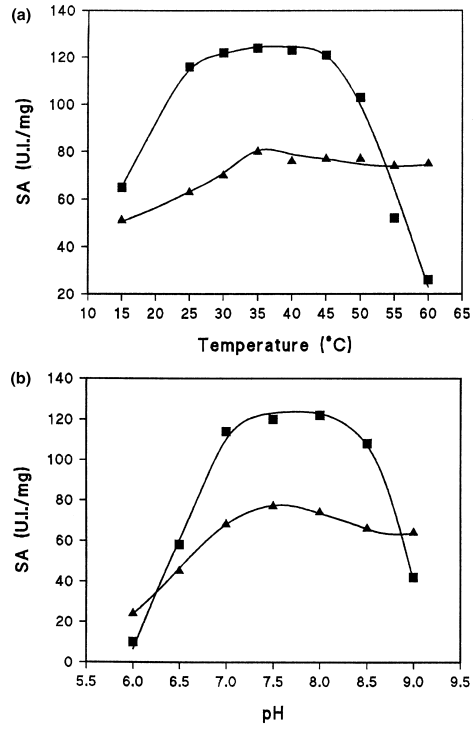

Fig. 5. Specific activity of native enzyme, Zn-ADH (■) and substituted enzyme, Co-ADH (▲) as a function of (a) the temperature (pH = 7.6) and (b) the pH (temperature = 25°C). ADH concentration = 0.4 mg/cm^3.

Table 1
Comparison among in vitro Co-substituted, in vivo Co-substituted and native ADH

	Present work[a]	Pergola et al. [20][b]	Native Y-ADH[b]
Kinetic parameters, k_M (ethanol) (M)	1.2×10^{-2}	8.4×10^{-3}	5.3×10^{-2}
Optimum temperature (°C)	35	35	35
Optimum pH	7.5	7.5	7.5
Thermo-resistance (°C)	> 50	> 60	> 35
pH stability	alkaline range	alkaline range	no

[a] In vitro Co-substituted.
[b] In vivo Co-substituted.

for the two enzymes (35°C (Fig. 5a) and 7.5 (Fig. 5b), respectively). On the contrary, when we compare the Co-Y-ADH behavior with that of the native enzyme, an important difference is shown in thermo-resistance and pH stability.

The obtained curves confirm the lower activity of $CoZn_3$-Y-ADH, in comparison with Zn_4-Y-ADH. However, the substituted enzyme shows pH and temperature resistance in a wider range (see Table 1).

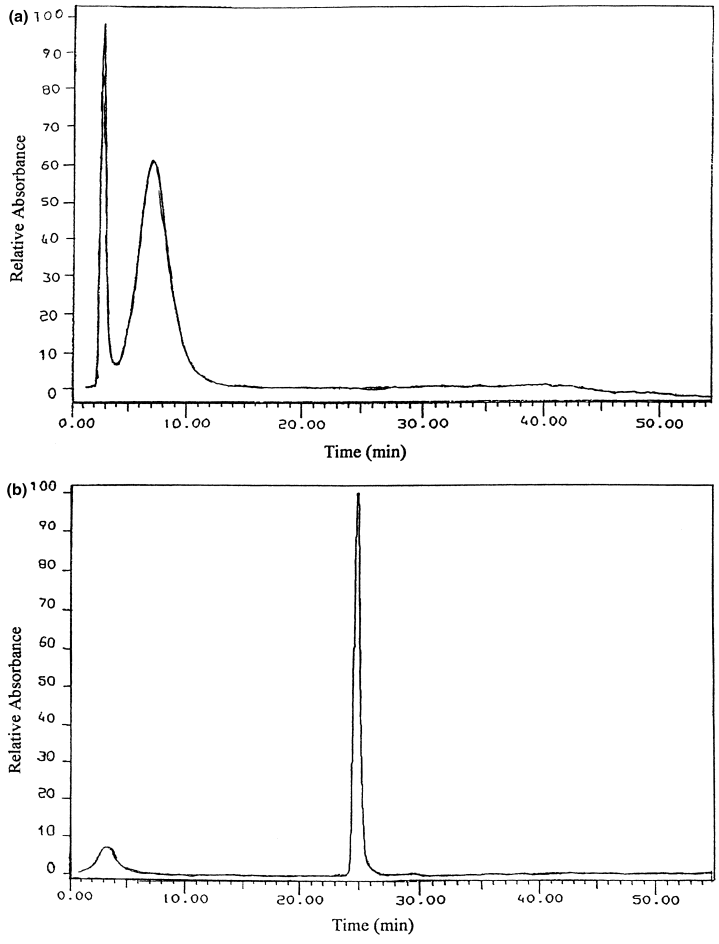

Fig. 6. Affinity chromatograms of native Zn (a)- and Co (b)-substituted enzyme. Enzyme concentration = 2.5 mg/cm^3. Integration time: 60.00 min, peak width: 10.00 min, peak sensitivity: 2.0%.

3.5. Affinity chromatography

The Y-ADH chromatogram consists of two peaks corresponding to the oxidative isoenzyme (Y-ADH_2) and the reductive isoenzyme (Y-ADH_1) (Fig. 6a). It is interesting to notice that in the case of Co-substituted enzyme (Fig. 6b), the signal of Y-ADH_1 isoform undergoes a remarkable shift (retention time of about 25 min rather than 7 min) and shows specific activity, while the signal of Y-ADH_2 remains at about 2 min.

4. Discussion

The zinc content of Y-ADH is still a subject of discussion. Our experimental results demonstrate that the zinc amount is 4 g-at./enzyme molecule, in agreement with the one suggested by Sytkowski [10], who also operated in conditions suitable to avoid contamination. The role of the four zinc atoms in the enzymatic activity (''structural zinc'' or ''catalytic zinc'') is not yet clear (for example, recently, Havlis and Studnickova [24] reported only the presence of catalytic zinc in Y-ADH). Our present results show that the removal of 1 g-at. Zn/enzyme molecule causes loss of the catalytic activity (see Fig. 1) and, in the same way, the re-insertion of 1 g-at. Zn completely restores the catalytic activity. Furthermore, the maximum activity for Co-substituted enzyme is observed when 1 g-at. metal/enzyme molecule (see Fig. 2) is introduced. Any attempt of further zinc removal from native Y-ADH produces an inactivated enzymatic form, which cannot be reactivated any more (see Fig. 1). This irreversible denaturing effect, probably at the level of the enzyme quaternary structure, might suggest that zinc in yeast ADH plays a ''structural'' role as well.

On the other hand, the tetramer subunit cohesion could be guaranteed either by zinc ions, or by other bivalent cations, such as Mg^{2+} and Ca^{2+}, as shown in Ref. [25]. This might justify the fact that the different preparations of the same enzyme, obtained from different cellular cultures, show different zinc contents, too (as reported by Sytkowski [10]); the cell, in zinc-depleted conditions, could substitute it with other cations more easily available in the sites in which it has the only function of keeping the subunits together. On the contrary, zinc or similar metals, like cobalt or manganese (in every case transition elements) [8,20], will always remain the only possible metal at the catalytic site, where the peculiarities of the type of chemical element play a fundamental role.

The cobalt substitution after the zinc removal produced enzymatic molecules in which the ratio of Zn:Co is 3:1, in strict analogy with what was obtained in the in vivo substitution, by growing *S. cerevisiae* cell in zinc-depleted conditions and excess of cobalt [20]. These results agree with those referred Curdel and Iwatsubo [17], who obtained an in vivo substitution of 32% cobalt in 1968, but are in contrast with both those of Coleman and Weiner [8], who obtained a total in vivo substitution with the use of manganese, and those of Sytkowski [10], who obtained a total substitution with cobalt by in vitro treatments. Other authors obtained a series of ''hybrids'' having different cobalt to zinc ratio, by means of dialysis [18] but using HL-ADH.

Our in vitro product has a lower specific activity and affinity for the catalytic reaction substrate than native Y-ADH. Even if these results are in contrast with what was observed for the in vivo-substituted enzyme, however, they may be explained if one considers that, in the case of the in vitro substitution, the metal is removed and substituted without possibility of modification in the amino acidic shape of the active site. Probably, the effect is a distortion in the ion co-ordination geometry, which causes a lower efficiency during the catalysis. Even if Co^{2+} ion displays strong analogies with Zn^{2+} — regarding dimensions (ionic radius according to Pauling: $Zn^{2+} = 0.74$ Å and $Co^{2+} = 0.72$ Å), co-ordination number, 4 [26], and characteristic of borderline behavior in the hard–soft classifi-

cation — the geometry of complexes and, mainly, the affinity to amino acidic ligands are different. In particular, cobalt usually shows weaker interactions than zinc with *S*- and *N*-ligands [27]. As a consequence, it probably ''adjusts itself'' worse than zinc to the cage of functional groups of amino acids present in the active site (2 S^- of *Cys* and 1 N of *His*).

In contrast, in the case of the in vivo-substituted enzyme, the protein can adapt, during the folding phase, the geometric structure of the active site, in such a way to conform itself to the characteristics of the available metallic ion. Another possibility is that the geometric structure remains unchanged, but the cobalt ion binds to different amino acidic residues present in the active site.

Despite these differences, it is worth noting that the thermo-resistance and pH stability are present in both the in-vivo- and in-vitro-substituted enzymes. Thermal inactivation occurs before noticeable conformational change of the whole molecule and it has been suggested that an enzyme active site may display more conformational flexibility and, as a consequence, a higher unfolding rate [28]. Cobalt is able to limit the flexibility of the active site, thus reducing the thermal denaturation.

The difference shown by in-vivo- and in-vitro-substituted enzymes can also be found among the various in-vitro-substituted enzymes because, as previously discussed, different preparation methods might allow different substitution products.

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