INACTIVATION OF CRYSTALLINE YEAST ALCOHOL DEHYDROGENASE BY ENZYME-BOUND PROTEOLYTIC ACTIVITY

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1. Introduction

Stability of yeast alcohol dehydrogenase has often been the subject of investigation. Especially for correct interpretation of coenzyme binding studies it has been necessary to know the contamination of the preparation with denatured enzyme.

Several authors found with different techniques a maximum of 2.7–5.2 molecules bound coenzyme/enzyme molecule, indicating a binding stoichiometry of 1 coenzyme/subunit [1–4]. Subsequent binding studies suggest only 2 NAD^+ binding sites/tetramer protein [5–7]. The finding of < 4 bound coenzyme molecules/yeast ADH does not correspond with the latest data of tertiary and quarternary structure of dehydrogenases [8] and has been interpreted in terms of negative cooperativity [3,4]. On the other hand, a contamination of the preparation with inactive protein was discussed again [6].

Rapid inactivation of yeast ADH preparations as observed earlier was interpreted as due to heavy metal inactivation [9–12], SH-groups oxidation [10,13,14] or enzyme dissociation [14]. Our experiments to determine the molecular weight of the ADH subunit after purification of the enzyme by affinity chromatography have shown, that in presence of SDS time-dependent changes in the electrophoretic pattern occur. This behaviour of yeast ADH is similar to that of hexokinase reported in [15]. The action of a protease which is tightly bound to the enzyme is postulated in [15].

The existence of proteases in ADH preparations could produce the rapid decrease of enzymatic activity and the appearence of denatured products.

Abbreviations: ADH, alcohol dehydrogenase (alcohol: NAD^+ oxidoreductase EC 1.1.1.1); HCl-Hb, acid-denatured hemoglobin; SDS, sodium dodecyl sulphate

2. Materials and methods

Crystalline alcohol dehydrogenase was purified from dried baker's yeast [3,16]; a freeze-dried enzyme, essentially prepared in a similar manner was supplied by Boehringer (Mannheim). Both preparations show similar kinetic and molecular properties [17]. Substances for electrophoresis and chromatography were purchased from Serva, Heidelberg. All other chemicals were analytical grade reagents of the highest grade available.

Proteolytic activity was measured by the hydrolysis of HCl-Hb at pH 3 by the method in [18] and by the hydrolysis of azocasein at pH 7 by the method in [19]. The assay mixtures were incubated at 30°C over 20 h. The activity of yeast ADH was measured with ethanol and NAD^+ as substrates [17]. Protein was monitored as A_{280}. Analytical polyacrylamide gel electrophoresis was performed in 8% gels (w/v Cyanogum 41) over 3 h at 10°C with different buffer systems [20,21]. The gels were stained for protein by the method in [22].

SDS–polyacrylamide gel electrophoresis and estimation of molecular weights were performed according to [23] in a 7% (w/v Cyagonum 41) gel. Incubation of yeast ADH was done in a solution containing

1% (w/v) SDS and 1% (w/v) 2-mercaptoethanol at 23°C for different times.

Preparative polyacrylamide gel electrophoresis was carried out with a 7% (w/v Cyanogum 41) gel and a buffer system described in [20]. The apparatus was according to [24].

3. Results

Figure 1 shows the changes in the structure of yeast ADH during incubation in 1% SDS. The original subunit of ADH [25] is degraded to fragments with mol. wt 22 000 and 15 000. This is probably a result of the action of a protease, indicated by the ability of the preparation to cleave usual protease substrates, e.g., hemoglobin or azocasein. Figure 2A shows a clear dependence of the rate of proteolysis of HCl-Hb from the quantity of yeast ADH in the incubation

Fig.1. SDS–polyacrylamide gel electrophoretograms of yeast ADH. Incubation of ADH (0.4 mg/ml) was done in 1% SDS and 1% 2-mercaptoethanol at 23°C for different times: A, 1 day; B, 5 days; C, 9 days; D, 20 days. In each case 12 μg protein was applied. Separation was carried out for 7 h at 6 mA/tube and 23°C.

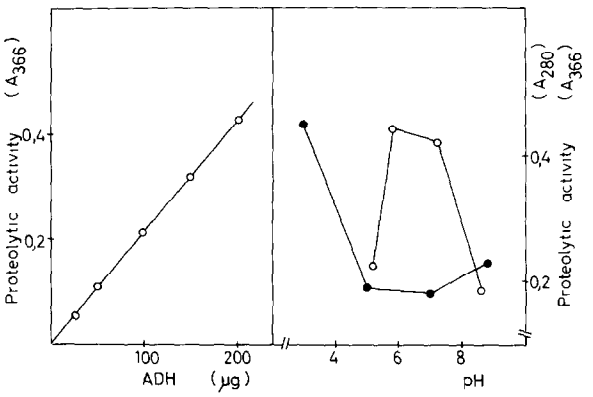

Fig.2. Estimation of proteolytic activity in yeast ADH preparations. (A) Dependence of the hydrolyzing activity with respect to azocasein from the concentration of yeast ADH in the incubation mixture. (B) pH-dependence of the proteolytic activity with respect to HCl-Hb (•) and azocasein (○). Yeast ADH was dissolved in a buffered solution of 1.6% azocasein (200 μg enzyme) or 1.5% HCl-Hb (50 μg enzyme) and incubated at 30°C for 20 h. The reaction was stopped by adding 1 ml 5% trichloroacetic acid in the case of azocasein and 1 ml 10% trichloroacetic acid in the case of hemoglobin to 1.2 ml and 1.5 ml, respectively, of the reaction mixture.

mixture. Alcohol dehydrogenase itself is accepted as substrate only in the presence of denaturing agents. The pH dependence of proteolytic activity against the two different substrates (fig.2B) points to the presence of two proteases in the ADH preparation, which are probably identical with protease A and protease B from yeast [26–28]. This assumption is supported by inhibition experiments (table 1). Typical inhibitors of protease B (*p*-chloromercuribenzoate, phenylmethylsulfonyl fluoride) decrease only the proteolytic activity of the ADH preparation against azocasein, at pH 7. The remaining activity is probably caused by protease A, which is not inhibited by these agents. Heating of ADH preparations to 95°C destroys the proteolytic activity against both of the substrates.

The proteolytic activity cannot be separated from ADH by fractionated ammonium sulphate precipitation or ion-exchange chromatography. On the other hand, a separation of the proteases during electrophoresis can be observed (fig.3). Under such conditions alcohol dehydrogenase is separated into two

Table 1
Inhibition of the proteolytic activity in yeast ADH preparations

	Activity (%)	
	Azocasein hydrolysis at pH 7.2	HCl-Hb hydrolysis at pH 3.0
p-Chloromercuribenzoate	57	96
Phenylmethylsulfonyl fluoride	67	92
95°C	1.8	0

Yeast ADH (1 mg/ml) was incubated (1 h, 23°C) without protease substrate in 0.025 M potassium phosphate buffer (pH 6) containing 1.7 mM phenylmethylsulfonyl fluoride or in 0.05 M Tris/HCl buffer pH 7 containing 1 mM *p*-chloromercuribenzoate. In the experiment at 95°C the enzyme was incubated 5 min in 0.025 M potassium phosphate buffer (pH 6). In each case samples (50 μg enzyme) were taken and proteolytic activity was estimated as in section 2

components (fig.4A). The proteolytic activity is located in the inactive lower molecular weight portion of the enzyme. The active ADH-part of the electrophoretogram is free of protease (fig.4A,B).

On the basis of these results we have attempted to

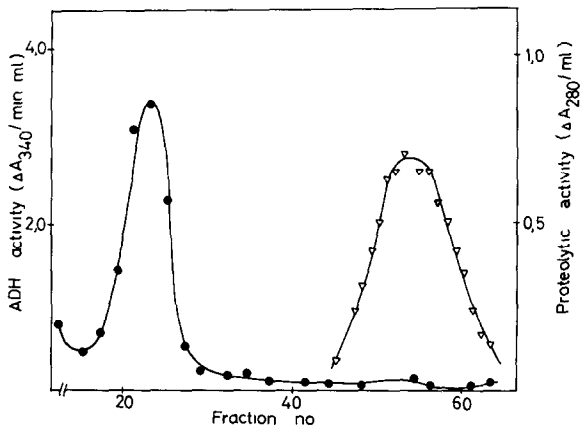

Fig.3. Preparative polyacrylamide gel electrophoresis of yeast ADH. Yeast ADH (30 mg) was dissolved in 1 ml 0.06 M Tris/HCl buffer pH 6.7 and applied to the gel column (15 ml). Electrophoresis was carried out for 50 h at 10 mA. Elution was done with 5 mM Tris/glycine buffer (pH 8.3) at 4°C and a flow rate of 15 ml/h. The volume of the collected fractions was 5 ml. (•) Proteolytic activity. (v) Activity of alcohol dehydrogenase.

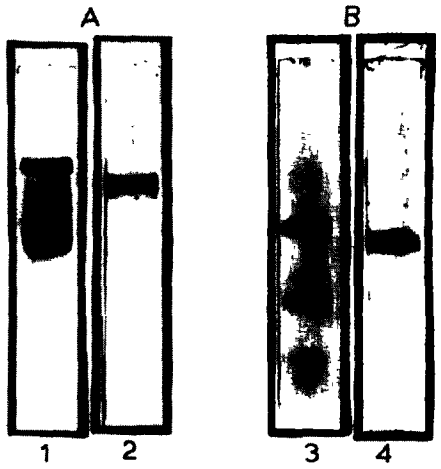

Fig.4. Separation of proteolytic activity from alcohol dehydrogenase by preparative polyacrylamide gel electrophoresis. (A) Analytical polyacrylamide gel electrophoresis of ADH preparations: (1) before preparative electrophoresis; (2) after preparative electrophoresis. Electrophoresis was carried out for 3 h at 4 mA/tube according to [20]. (B) SDS–polyacrylamide gel electrophoresis of ADH preparations: (3) before preparative electrophoresis; (4) after preparative electrophoresis. The two ADH preparations (12 μg each) were subjected to each gel after incubation in 1% SDS for 5 days.

separate the proteases from ADH on a preparative scale by gel chromatography at high ionic strength. Figure 5 demonstrates the separation of the proteolytic from the ADH activity on Sephadex G-200 in presence of 2 M NaCl. This leads to an increased stability of alcohol dehydrogenase. No such separation occurs in the absence of NaCl. In solution the activity of protease-free ADH was constant for at least 24 h at 25°C. The activity of the original preparation is diminished under the same conditions to 60%.

4. Discussion

The existence of an enzyme-bound protease was postulated in [15] for hexokinase. Our results show that the behaviour of yeast ADH is similar to that of hexokinase, suggesting the presence of an enzyme-bound protease too. An important indication for the action of such a proteases is the formation of ADH fragments, if the enzyme preparation is incubated

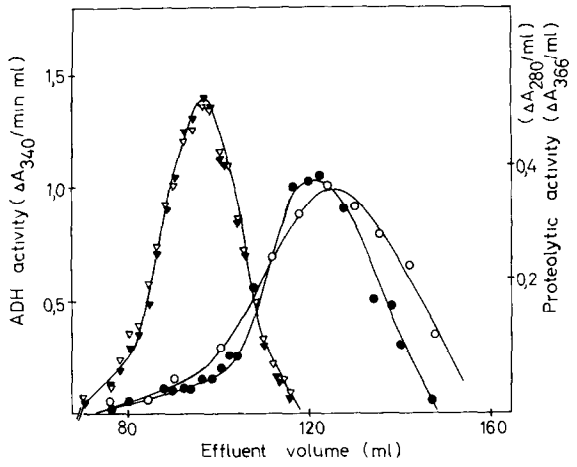

Fig.5. Separation of proteolytic activity from alcohol dehydrogenase by chromatography on Sephadex G-200 in 0.067 M potassium phosphate (pH 8), containing 2 M NaCl. In each case 10 mg ADH was applied to a column (38 × 2.6 cm) containing 210 ml Sephadex G-200. Elution was done at 4°C with a flow rate of 40 ml/h. The volume of the collected fractions was 2.0 ml. (▽) ADH activity; (▼) ADH activity after 20 h storage at 23°C; (●) proteolytic activity against azocasein, at pH 7; (○) proteolytic activity against HCl-Hb at pH 3.

with SDS. The fact, that an enzyme is active in presence of SDS is unusual, but some enzymes, especially proteases are known to retain their activity under such conditions [29–31].

Since the ADH preparations hydrolyze hemoglobin and azocasein also in absence of denaturants one can assume, that SDS leads to unfolding of the ADH molecule, yielding a favourable configuration of peptide backbone for the proteolytic attack. A similar behaviour was reported in [32] for tryptophanase. The susceptibility to proteolytic digestion depends on the aggregation state of this enzyme. Treatment with chaotropic ions caused the formation of the enzyme dimer, which is digestible by proteases. Contrary to this, the tetrameric form, stabilized by structure making anions, resists the proteolytic attack.

In absence of SDS probably other factors, e.g., high dilution of ADH or long storage time, are able to cause small conformational changes. Under such conditions the effect of proteases can be demonstrated only by a decrease in enzyme activity but not by different molecular forms.

The binding of proteases to yeast ADH is very tight. ADH preparations of the highest grade of purity available, (Lyophilisat of Boehringer, Mannheim; ADH purified by hydrophobic chromatography [33] – both with spec. act. 400 U/mg) possess a detectable proteolytic activity. The ability of yeast ADH to bind amino acids or peptides was already shown in 1963 [34]. It is possible, that the binding of proteases is realized on the same basis. On the other hand, protein species with mol. wt 20 000 in one preparation of yeast ADH were observed [14] and the existence of a peptidase was postulated [14]. Immunochemical investigations [35] support the same hypothesis.

All of these results are in agreement with our experiments and our interpretations of data. Kinetic studies, especially with protease-free enzyme will show, whether protease-induced changes of ADH are reflected in functional properties, too.

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References

[1] Hayes, J. E. and Velick, S. F. (1954) J. Biol. Chem. 207, 255.
[2] Pfleiderer, G. and Auricchio, F. (1964) Biochem. Biophys. Res. Commun. 16, 53.
[3] Dickinson, F. M. (1970) Biochem. J. 120, 821.
[4] Yamada, T. and Yamato, M. (1973) J. Biochem. (Tokyo) 74, 971.
[5] Dickinson, F. M. (1974) Eur. J Biochem. 41, 31
[6] Karlovic, D., Amiguet, P., Bonner, F. J. and Luisi, P. F (1976) Eur. J. Biochem. 66, 277.
[7] Lescovac, V and Pavkov-Pericin, D. (1974) Biochem. J. 145, 581.
[8] Brandèn, C.-I., Jornvall, H., Eklund, H. and Furugren, B. (1975) The Enzymes 11A, 104.
[9] Snodgrass, P. J., Vallee, B. L. and Hoch, F. L. (1960) J. Biol. Chem. 235, 504.
[10] Wallenfels, K. and Sund, H. (1957) Biochem. Z. 329, 17.
[11] Kaplan, N. O. and Ciotti, M. M. (1956) J. Biol. Chem 221, 823.
[12] Wallenfels, K. and Arens, A. (1960) Biochem. Z. 332, 217.
[13] Wallenfels, K. and Müller-Hill, B. (1964) Biochem. Z. 339, 352.

[14] Bühner, M. and Sund, H. (1969) Eur. J. Biochem. 11, 73.
[15] Rustum, Y. M., Massaro, E. J. and Barnard, E. A. (1971) Biochemistry 10, 3509.
[16] Racker, E. (1950) J. Biol. Chem. 184, 313.
[17] Schöpp, W. and Aurich, H. (1976) Biochem. J. 157, 15.
[18] Schlamowitz, M. and Peterson, L. U. (1959) J. Biol. Chem. 234, 3137.
[19] Langer, L., Wakil, A., Zimmermann, M., Ansorge, S., Bohley, P., Kirschke, H. and Wiederanders, B. (1973) Acta Biol. Med. Germ. 31, 1.
[20] Davis, B. J. (1964) Ann. NY Acad. Sci. 121, 404.
[21] Williams, D. E. and Reisfeld, R. A. (1964) Ann. NY Acad. Sci. 121, 373.
[22] Diezel, W., Kopperschläger, G. and Hofmann, E. (1972) Analyt. Biochem. 48, 617.
[23] Weber, K. and Osborn, M. (1969) J. Biol. Chem. 244, 4406.
[24] Diezel, W., Kopperschläger, G. and Hofmann, E. (1970) Acta Biol. Med. Germ. 24, 721.
[25] Hersh, R. T. (1962) Biochim. Biophys. Acta 58, 353.
[26] Hata, T., Hayashi, R. and Doi, E. C. (1967) Agr. Biol. Chem. 31, 150.
[27] Lenney, J. F. and Dalbec, J. M. (1967) Arch. Biochem. Biophys. 120, 42.
[28] Saheki, T. and Holzer, H. (1974) Eur. J. Biochem. 42, 621.
[29] Nelson, C. A. (1971) J. Biol. Chem. 246, 3895.
[30] Porter, W. H. and Preston, J. L. (1975) Analyt. Biochem. 66, 69.
[31] Hilz, H., Wiegers, U. and Adamitz, P. (1975) Eur. J. Biochem. 56, 103.
[32] Raibaud, O. and Goldberg, M. E. (1976) J. Biol. Chem. 251, 2820.
[33] Schöpp, W., Grunow, M., Tauchert, H. and Aurich, H. (1976) FEBS Lett. 68, 198.
[34] Arens, A., Sund, H. and Wallenfels, K. (1963) Biochem. Z. 337, 1.
[35] Linke, R., Wachsmuth, E. D. and Pfleiderer, G. (1970) Immunochemistry 7, 99.