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COENZYME BINDING CAPACITY OF YEAST ALCOHOL DEHYDROGENASE

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Summary

Commercial lyophilized preparations of yeast alcohol dehydrogenase from Boehringer G.m.b.H. (Mannheim, Germany) bind 2 mols of reduced coenzyme/144000 g of enzyme (1). After the purification by a DEAE-Sephadex column chromatography, the coenzyme binding capacity is raised to 4 mols of NADH/mol of enzyme. Commercial preparations and ionexchange-purified preparations are homogeneous on the ionexchange column chromatography and the disc gel electrophoresis, after reduction with thioglycolic acid. Ionexchange chromatography does not increase the -SH titer, zinc content and the specific activity of enzyme. It is suggested that ionexchange chromatography raises the NADH-binding capacity by removing some impurities present in commercial enzyme preparations.

Introduction

Yeast alcohol dehydrogenase is a tetrameric enzyme, composed of four seemingly identical polypeptide chains (2). Extensive investigations of Dickinson (3) indicated that the enzyme has only 2 coenzyme binding sites/tetramer; recently, the binding capacity found by Dickinson was questioned by Temler & Kägi (4) and by Karlović *et al.* (5), who have found 4 coenzyme binding sites/tetramer. We have systematically investigated commercial lyophilized preparations of Boehringer/Mannheim, and found that they invariably bind approx. 2 mols of NADH/mol of enzyme (1,6,7). In this communication we report the presence of two additional binding sites/tetramer of the commercial preparation, which are unmasked by the ionexchange column chromatography.

Materials and Methods

Lyophilized yeast alcohol dehydrogenase (EC 1.1.1.1) was purchased from Boehringer G.m.b.H. (Mannheim, Germany). Enzyme activity assay,

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determination of protein concentration and spectrophotometric measurements were performed as previously described (1,6,7); determination of coenzyme binding capacity by the fluorescent titration and the purification of commercial preparations by the DEAE-Sephadex column chromatography was performed as described elswhere (1). Disc gel electrophoresis was performed according to Brewer & Ashworth (8), and the reactivity of enzyme -SH groups was followed by the method of Ellman (9), in a manner described earlier (1).

Results and Discussion

Commercial preparations of lyophilized Boehringer yeast alcohol dehydrogenase are homogeneous on the DEAE-Sephadex column chromatography (1). Both commercial (1) and ionexchange-purified preparations show three distinct protein bands on disc gel electrophoresis, staining with amido-schwarz. After reduction by thioglycolic acid, both commercial (1) and ionexchange-purified preparations show a single band on disc gel electrophoresis, staining with amido-schwarz and the specific dehydrogenase-staining mixture (8). This indicates the homogeneity of enzyme, with -SH groups in variable states of oxidation, probably not exceeding the state of sulfenic acid since the reversal is obtained by thioglycolic acid.

Commercial preparations used in this work have had 20 - 24 free -SH groups/molecule, 8 zinc atoms/molecule and a specific activity of 380 U/mg at pH 9.0. Ionexchange chromatography did not change the -SH titer, zinc content and the specific activity of enzyme. Only the coenzyme binding capacity (Fig. 1), and the reactivity of -SH groups (Fig. 2) are changed by the column chromatography.

Commercial preparations of enzyme bind invariably approx. 2 mols of NADH/mol of enzyme (1), whereas ionexchange-purified preparations bind 4 mols of NADH/mol of enzyme (Fig. 1). Coenzyme binding capacity on the Figure 1 was determined by an appropriate linear extrapolation of $1/[NADH_{bound}]$ vs. $1/[NADH_{total}]$. Concentrations of enzyme-bound NADH were determined assuming that at each titration point, I =

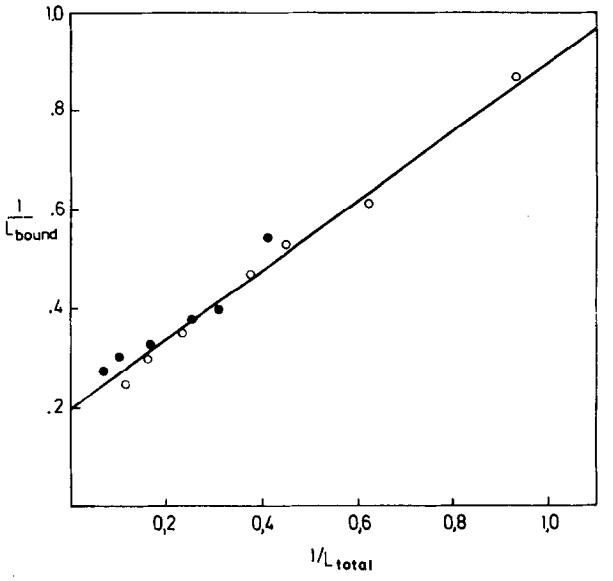

Fig. 1. Coenzyme binding capacity of purified yeast alcohol dehydrogenase, determined by the fluorescent titration. In the forward titration (presented on the figure), the enzyme (1.23 μM, mol. wt. 144000) was titrated with NADH (0 - 9.1 μM) in 0.1 M sodium phosphate buffer, pH 7.3 (with 0.5 mM EDTA and 0.5 M acetamide); final volume 3 ml. In the reverse titration (not shown), NADH (0.91 μM) was titrated with the enzyme (0 - 3.66 μM) in the same buffer. Different symbols represent different enzyme preparations. Coenzyme binding capacity (4.0 mols of NADH bound/mol of enzyme) was obtained by dividing the concentration of bound coenzyme at saturating NADH-concentrations ($[L_{bound}]$ = 5 μM) with the concentration of enzyme (1.23 μM).

$\alpha[NADH_{free}] + \beta[NADH_{bound}]$, where α and β are specific fluorescences of free and bound coenzyme, and I, the total fluorescence in a given titration point; β was determined in a reverse titration, by titrating the coenzyme with the enzyme. Thus the determination of $[NADH_{bound}]$, usually problematical in fluorescent titrations, was free of assumptions based on values easily determined with precision.

Ionexchange chromatography changes the reactivity of enzyme thiol groups towards 5,5'-dithiobis-(2-nitrobenzoic acid) (Fig. 2). Initially, 12 - 20 -SH groups/molecule of enzyme react with excess of 5,5'-dithiobis-(2-nitrobenzoate) at a uniform reaction rate, with k_2 = 25 $M^{-1}min^{-1}$ in the commercial and k_2 = 66 $M^{-1}min^{-1}$ in the ionexchange-purified pre-

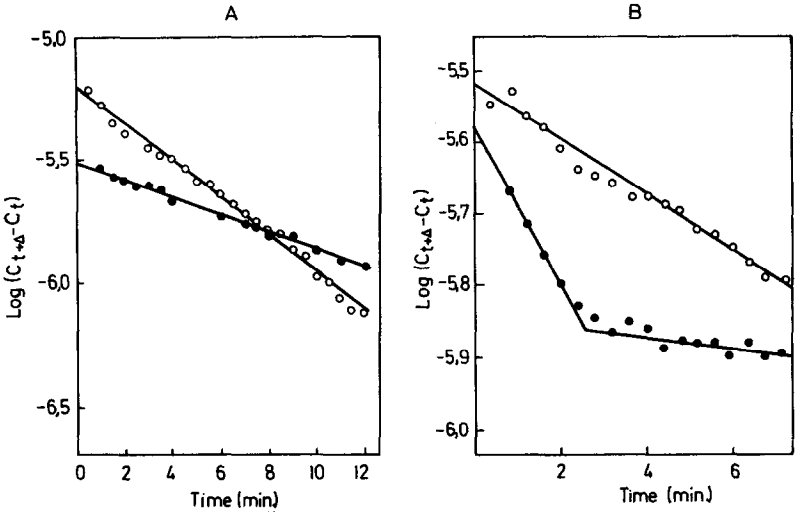

Fig. 2. Reactivity of enzyme -SH groups towards 5,5'-dithiobis-(2-nitrobenzoate).
A: Purified enzyme preparation (3.9 μM, mol. wt. 144000) was mixed with 5,5'-dithiobis-(2-nitrobenzoate) (3.25 mM) in 0.1 M Tris-HCl buffer, pH 8.6, in the presence (160 μM) (•) and in the absence of NADH (o); A_{412} was displayed on a recorder, and chart records were evaluated according to the method of Guggenheim (10).
B: Commercial preparation (5.25 μM) was mixed with 5,5'-dithiobis-(2-nitrobenzoate) (3.25 mM) in the same buffer, in the presence (160 μM) or absence of NADH, and A_{412} evaluated as above.

paration, respectively, at pH 8.6. In the commercial preparation, in the binary complex enzyme-NADH, reactivity of approx. 2 -SH groups/molecule towards 5,5'-dithiobis-(2-nitrobenzoate) is sensitized and enhanced 3-fold (k_2 = 72 $M^{-1}min^{-1}$), in comparison with the non-liganded enzyme (1). Ionexchange-purified enzyme is desensitized with respect to this phenomenon, and the selective sensitization by coenzyme is abolished; in the binary complex enzyme-NADH, the reactivity of all -SH groups is 2-fold lower (k_2 = 33 $M^{-1}min^{-1}$) in comparison with the non-liganded enzyme (Fig. 2).

We assume that the increase in coenzyme binding capacity arises from the removal of some impurity by the ionexchange chromatography. This impurity should represent less then 10% of the commercial material,

since larger impurities should have been detected either by the column chromatography or gel electrophoresis; the likely candidates are some nucleotide impurities or a protein stabilizer, added in small quantities to stabilize commercial preparations (11).

Our finding that the binding capacity of a nearly homogeneous preparation was raised from 2 to 4 mols of NADH/mol of enzyme, without the increase in specific activity, raises an intriguing question. This indicates that the enzyme may bind maximally 4 molecules of coenzyme, possessing at the same time only 2 catalitically active sites/tetramer. This property, ascribed usually to 'half-the-sites-active' enzymes, is substantiated by half-the-site reactivity of essential thiol groups (3) and tightly bound zinc atoms (7), reported for this enzyme. However, more experimental data are clearly needed to clarify the apparent half-the-sites reactivity of yeast alcohol dehydrogenase.

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