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Note

Purification of yeast alcohol dehydrogenase by Congo red affinity chromatography

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The successful purification of several dehydrogenases by dye ligand affinity chromatography has been described previously [1,2] and the design and application of biomimetic dyes in affinity chromatography has been discussed in detail [3,4]. However, the theoretical basis of the interactions of aromatic dye molecules with enzymes containing the dinucleotide fold is in part still undefined. Thus, a more empirical approach to finding dyes suitable for the affinity chromatography of alcohol dehydrogenase (E.C. 1.1.1.1) (ADH), a widely used enzyme in biochemistry and medicine, seemed to be justified and useful.

In addition to linking an adenyl-containing ligand to a Sepharose 4B matrix, different dyes were also tested as ligands in order to purify ADH. A comparison of different types of affinity chromatography showed purifications of 885-fold by Congo red (azo dye), 827-fold by toluidine blue (phenothiazine dye), 379-fold by safranin (phenazine dye) and 424-fold when AMP was covalently linked also to cyanogen bromide-activated Sepharose 4B via adipic dihydrazide as a spacer after periodate cleavage of the ribose ring. The ADH extracted from yeast with toluene–plasmolyse [5], precipitated with ammonium sulphate at 60% saturation and reprecipitated, then chromatographed on Congo red–Sepharose 4B, dialysed and crystallized, showed homogeneity of preparation when subjected to sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE).

EXPERIMENTAL AND RESULTS

Materials

Congo Red dye was obtained from Fluka (Neu-Ulm, F.R.G.) and Sepharose 4B from Pharmacia (Sweden). Toluidine blue and safranin dyes and all other chemicals (analytical-reagent grade) were purchased from Merck (F.R.G.). Yeast (*Saccharomyces cerevisiae*) was obtained from Uniferm (F.R.G.).

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Preparation of dye ligand–Sepharose 4B

A 20-ml volume of settled Sepharose 4B was activated with cyanogen bromide [6]. A 200-mg amount of the dye, e.g., Congo red, used as the ligand was dissolved in 60 ml of 0.05 mol/l phosphate buffer (pH 8.0) and coupled to the gel immediately after cyanogen bromide activation. The mixture of activated Sepharose and Congo red was gently stirred overnight at 4°C. The excess of the dye was removed by washing consecutively with 20 ml each of water, 0.2 mol/l acetic acid, 3 mol/l sodium chloride solution, 1% (w/v) Triton X-100 and ethanol water (1:1, w/v). This washing cycle removed the uncoupled ligand and ensured that no free ligand remained bound in any way to the immobilized material. The coupling efficiency was determined spectrophotometrically at 500 nm in a suspension of glycerol. For efficient adsorption a capacity of 5 μmol of Congo Red per ml of settled gel was found to be useful. The other ligands were attached using the same procedure.

Preparation of AMP ligand–Sepharose 4B

For AMP–Sepharose, adipic dihydrazide was used as a spacer, which linked the cyanogen bromide-activated Sepharose to the ribosyl part of adenosine monophosphate after periodate treatment [7].

In detail, 10 ml of cyanogen bromide-activated Sepharose, prepared as described above, were suspended in 15 ml of a 0.1 mol/l sodium carbonate solution containing 1.5 g of adipic dihydrazide and stirred overnight at 4°C. The spacer-linked Sepharose was washed with 200 ml of 0.2 mol/l sodium chloride solution and 1000 ml of distilled water and subsequently with 100 ml of 0.1 mol/l sodium acetate solution. Separately, 60 mg of AMP were dissolved in 0.7 ml of water and neutralized dropwise with 1 mol/l sodium hydroxide solution. After the addition of 18 mg of sodium

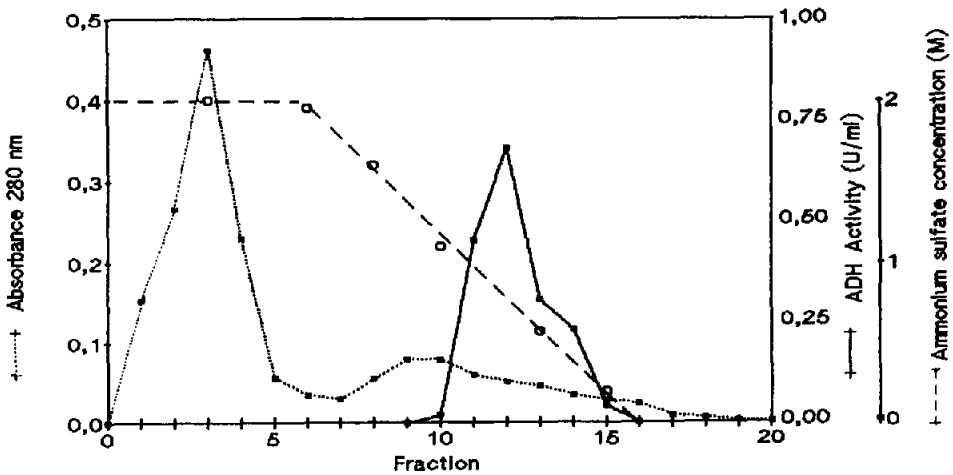

Fig. 1. Affinity chromatography of alcohol dehydrogenase from yeast extract on Congo red–Sepharose. A sample of yeast extract (12.1 U ADH) was applied to a column of Congo red–Sepharose equilibrated with 0.05 mol/l phosphate buffer (pH 8) containing 2 mol/l ammonium sulphate. Non-adsorbed protein was washed off with the same buffer and the enzyme was eluted with an ammonium sulphate gradient from 2 to 0 mol/l in 0.05 mol/l phosphate buffer (pH 8).

TABLE I

PURIFICATION OF ALCOHOL DEHYDROGENASE FROM BAKER'S YEAST

Fraction	Specific activity (U/mg)[a]	Yield (%)	Degree of purification (-fold)[b]
Yeast extract	0.15	100	1
Ammonium sulphate (60% saturated) precipitate	1.88	72	12.5
Ammonium sulphate, reprecipitated	7.92	66	53
Chromatographed on			
Congo red–Sepharose	132.8	11	885
Toluidine blue–Sepharose	124.0	10	827
Safranin–Sepharose	56.8	28	379
AMP–Sepharose	63.5	13	424

[a] 1 unit (U) = 1 μmol NADH formed/min. The enzyme activity was measured at 20°C in 15 mmol/l glycine pyrophosphate buffer (pH 8.8) containing 0.5 mol/l ethanol, 2 mmol/l NAD, 75 mmol/l semicarbazide and 1 mmol/l gluthathione, with a slightly modified method [9]. Protein (mg) was determined by the Lowry method [10] using bovine serum albumin as the standard.

[b] Increase in specific activity with respect to the start.

periodate the mixture was incubated at 0°C for 1 h. Finally, the spacer-linked Sepharose, suspended in 25 ml of 0,1 mol/l sodium acetate solution, was added to the periodate-treated nucleotide and the mixture was stirred at 4°C for 3h. Thereafter the material was collected and washed with 500 ml of 2 mol/l sodium chloride solution and 1000 ml of water.

Preparation of yeast extract

In accordance with the toluene–plasmolyse method [5], the yeast extract was prepared from 252 g of baker's yeast and precipitated with ammonium sulphate at 60% saturation. The precipitated material was redissolved and reprecipitated, then the reprecipitated material was dissolved again in 0.05 mol/l phosphate buffer (pH 8) containing 2 mol/l ammonium sulphate for the chromatographic step.

Affinity chromatography

The dye ligand–Sepharose 4B, prepared as described, was used in small Polyprep columns (Bio-Rad Labs, Richmond, CA, U.S.A.) and equilibrated with 2 mol/l ammonium sulphate in 0.05 mol/l phosphate buffer (pH 8). After equilibration, the ADH material was loaded on the column and incubated for 30 min. The high ammonium sulphate concentration increased the binding of ADH, mainly because of hydrophobic interactions. After washing with the equilibration buffer, a linearly decreasing ammonium sulphate gradient was used to elute the ADH. The elution rate was 30 ml/h and 3-ml fractions were collected (Fig. 1). A comparison of the efficiency of the dye ligand and AMP ligand chromatography is shown in Table I.

DISCUSSION

The binding of the dye or AMP ligand–Sepharose to the enzyme ADH is interpreted as mimicking by the ligands of the coenzyme NAD. The selectivity of ADH binding is limited because only the adenine subsite of NAD seems to be relevant for binding, as discussed previously [2], and the strength of affinity is mainly dependent on the hydrophobic interactions, as evidenced by the conditions of binding and elution of the enzyme.

The affinity chromatography using Congo red or Toluidine blue as the ligand led to a fairly high degree of ADH purification (about 850-fold). However, an even higher specific activity has been reported previously [8] using a recrystallized preparation of ADH from yeast after ethanol and ammonium sulphate precipitation, resulting in about a 40-fold purification. This discrepancy may be attributed to the high specific activity of the crude yeast-cell extract at the start of the preparation. However, the ADH preparation recrystallized after Congo red affinity chromatography also showed only a single band on SDS-PAGE and obviously no co-purification of proteolytic activities occurred, as the enzyme is stable at least 6 months.

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