ETHANOLAMINE, A USEFUL LIGAND FOR LARGE SCALE PURIFICATION OF BACTERIAL PYRUVATE DEHYDROGENASE COMPLEX BY AFFINITY CHROMATOGRAPHY

Jaap VISSER, Walter VAN DONGEN and Marijke STRATING

Dept. of Genetics, Agricultural University, Gen. Foulkesweg 53, Wageningen, The Netherlands

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1. Introduction

In our laboratory we are interested in the biochemical genetic characterization of pyruvate dehydrogenase complex mutants both in prokaryotes (*Escherichia coli*) and eukaryotes (*Aspergillus nidulans*). Moreover, evolutionary aspects of this multienzyme complex have our interest. Although various purification procedures exist [1–3], our work would benefit from suitable affinity chromatography systems. Apart from the advantages for the purification of mutant enzymes, such systems may provide us with a refined tool to establish the nature of certain mutations. We have investigated the possibility of applying the various cofactors involved in the overall reaction. Thiamin pyrophosphate has been found to be a useful ligand [4]; NAD^+ and AMP derivatives are still under investigation.

Besides general ligand affinity chromatography also the possibility to use hydrophobic chromatography in isolating the enzyme has been explored. This approach seems therefore worthwhile as hydrophobic interactions contribute largely to the formation of the stable and highly ordered structure of the pyruvate dehydrogenase complex. Dihydrolipoamide transacetylase is known to play a central role in this association [5]. It was found before that lipoamide dehydrogenase, one of the constituent enzymes of the complex, has indeed affinity for hydrocarbon-coated Sepharoses [6]. We have tested hydrophobic matrices of various length (C_3–C_5) based on both alkyl and ω-amino alkyl residues with the pyruvate dehydrogenase complex of *E. coli*. All these matrices demonstrated strong affinity for the multienzyme complex [13]. Introduction of an ω-carboxyl moiety leads to complete loss of affinity. The hydrophobic chromatography approach was then not further considered as it turned out to be impossible to elute both overall activity and lipoamide dehydrogenase activity under mild conditions. In this paper the effect of introducing hydroxyl groups in the alkyl chains will be considered.

Abbreviations: TPP, thiamin pyrophosphate; PMSF, phenylmethylsulfonyl fluoride; DTT, dithiothreitol

2. Methods

2.1. *Enzyme source and isolation*

E. coli K1-1 LR 8-13, a pyruvate dehydrogenase complex constitutive mutant was grown according to [2]. The crude extract was prepared as described [2] using minor modifications. The cells (60–80 g bacterial wet wt) were broken with an X-press (LKB). Then 250 ml extraction buffer was added. The 0.1 M potassium phosphate extraction buffer, pH 7.0, contained in addition to DNAase and RNAase (5–10 μg/ml), 3 mM magnesium chloride, 0.1 mM EDTA, 2 mM TPP, 2 mM dithiothreitol or 5 mM β-mercaptoethanol and 50 μM PMSF. The centrifugation step was followed by dialysis against 50 mM potassium phosphate buffer, pH 7.0, which contained all components mentioned above except for DNAase, RNAase and TPP. Operating temperature in all steps was 4°C. Mg^{2+} is added since the enzyme preferenti-

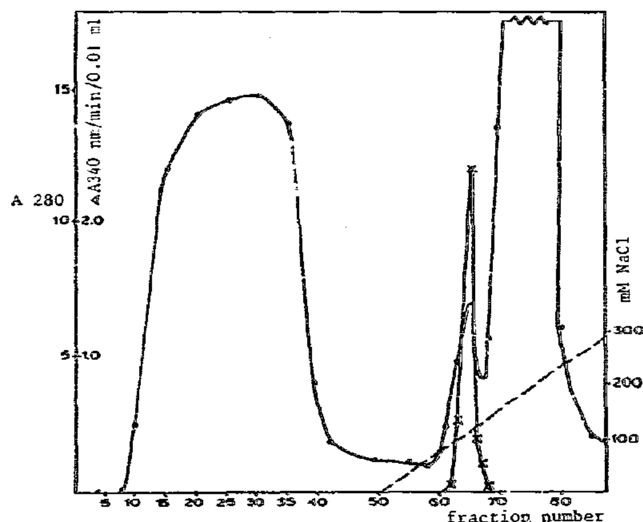


Fig.1. Affinity chromatography of pyruvate dehydrogenase complex on ethanol–Sepharose 2B (column dimensions 2.5 × 30 cm) Crude extract was prepared from 60 g bacteria as described in section 2. Fractions of 15 ml were collected. A_{280} (●–●) and enzyme activity (X–X) are shown.

ally binds metal-TPP whereas EDTA is added in order to inactivate metal requiring proteases.

2.2. *Enzyme activity*

Pyruvate dehydrogenase complex overall activity was measured according to [7] at 25°C.

2.3. *Matrix preparation*

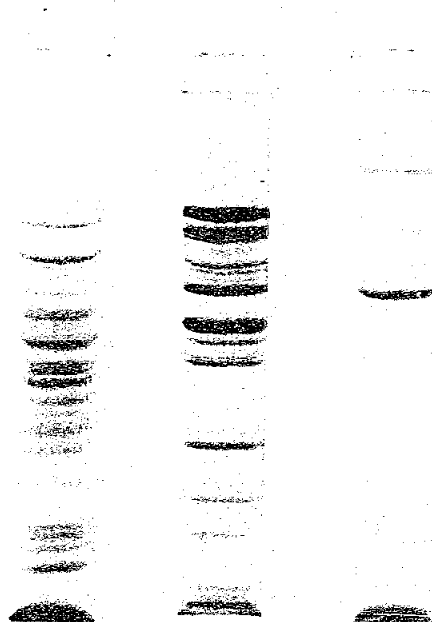
Sepharose-2B was activated with 1,4 butanediol diglycydylether according to [8]. The activated matrix was reacted with 0.1 M ethanolamine in 0.2 M bicarbonate buffer at pH 9.5 during 24 h at room temperature.

2.4. *SDS–polyacrylamide gel electrophoresis*

Stock solutions were made according to [9]. Routinely a standard gel percentage of 10% was used with a 3% stacking gel. A current of 3 mA/tube or 20 mA/slab was given. Staining occurred with Coomassie brilliant blue R. Samples were dialyzed against 10 mM sodium phosphate buffer pH 7.0 to remove potassium ions.

3. Results and discussion

When trying to prepare a thiochrome–Sepharose matrix starting with epoxy-activated Sepharose we made an interesting discovery. Blocking the excess of reactive groups was done with ethanolamine as suggested by Pharmacia Fine Chemicals AB [10]. In a control experiment no thiochrome and only ethanolamine was coupled. The latter material turned out to be a very effective affinity adsorbent for pyruvate dehydrogenase from *E. coli*, and this interaction with the ligand is completely reversible. An increase in ionic strength elutes the enzyme quantitatively. CNBr-activated Sepharose can also be used. For stability reasons the epoxy-derivatized [11] Sepharose was used. We have applied this principle for large scale purifications (60–80 g bacterial wet wt). The initial



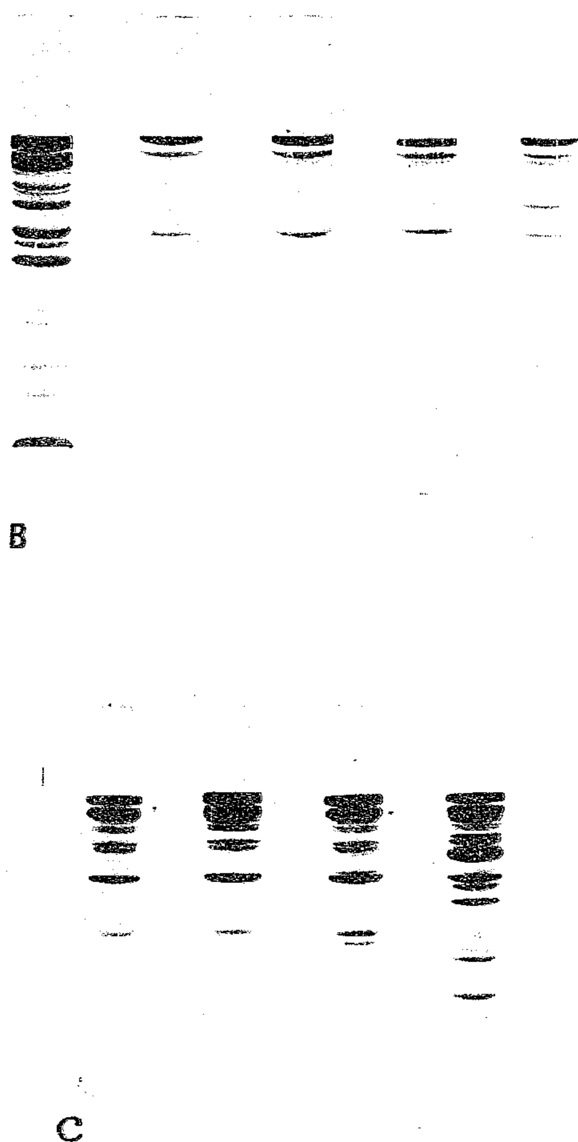


Fig.2. SDS–polyacrylamide disc gel electrophoresis patterns of different stages of pyruvate dehydrogenase complex purification. Protein, 10–50 µg, is applied/gel. (2.A) From left to right: pooled eluate (fraction 10–40); fraction 65, fraction 70; (2.B) Biogel chromatography in the presence of TPP from left to right: starting material; 3rd, 7th, 10th and 14th fraction upon appearance of activity; (2.C) Biogel chromatography in the absence of TPP 3rd, 5th, 11th and 13th fraction upon appearance of activity.

purification steps are given in the methods. The affinity matrix is used after the dialysis step (2.5 × 35 cm column). Results are shown in fig.1 whereas the electrophoretic analysis on SDS gels is given in fig.2.

The active fractions contain, besides the three characteristic components and some low molecular weight contaminants, breakdown products of the transacetylase component (cf. [12]). The purification in this step on the basis of enzyme activity is approx. 80-fold whereas on the basis of protein a 40-fold purification is obtained. This discrepancy is due to the fact that all our activity measurements, also those with the crude extract, have been performed aerobically. NADH oxidase activity in the crude extract is considerable, leading to an underestimation of the activity present. The active fractions were precipitated with ammonium sulfate (65%). Further purification was obtained by Biogel A-50-M chromatography (column dimensions 2.5 × 90 cm) applying 60–100 mg protein at a time after dialysis against the equilibration buffer. The gel was equilibrated with 0.1 M potassium phosphate buffer pH 7.0, 5% glycerol (v/v), 4 mM DTT, 3 mM magnesium chloride and 2 mM TPP. Elution was carried out in the same buffer. Under the conditions used, the intact complex separates well from those molecules containing the characteristic transacetylase breakdown products and from low molecular weight contaminants. The presence of TPP is crucial; otherwise breakdown products are observed in all fractions as shown in fig.2. Separation between intact complex molecules and those containing partially degraded transacetylase on molecular weight basis in the presence of high TPP concentrations raises interesting questions like, is TPP just protecting against breakdown or does its effect involve re-equilibration of the different subunits?

We should like to point out that the use of ethanolamine to block excess of reactive groups may well lead to complications in other affinity chromatography systems. Particularly since in the absence of Mg^{2+} more proteins are adsorbed from a crude extract, which is shown in fig.3. Moreover, the amount of protein adsorbed varies with the bacterial species used. In the case of *Pseudomonas fluorescens*, e.g., a considerable amount of adsorption takes place even in the presence of Mg^{2+}. In small scale experiments we have also investigated the effect of an increase in chain length. In all cases tested (C_2–C_5) the results

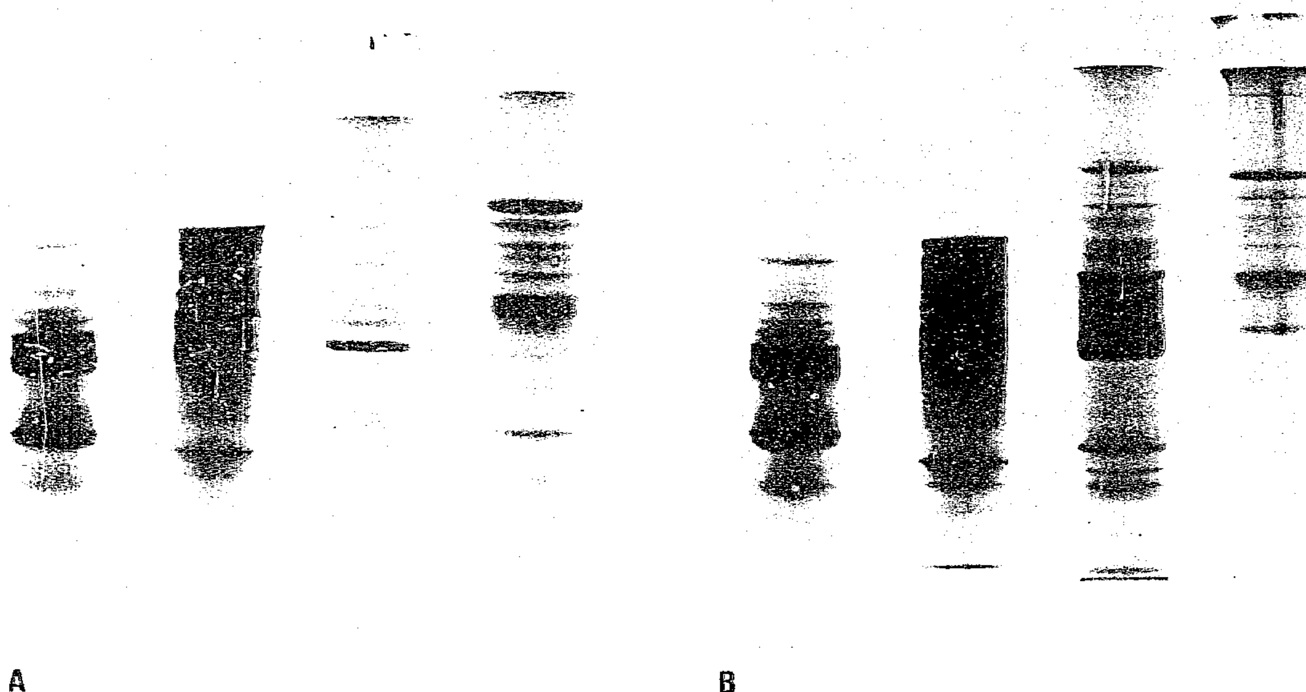


Fig.3. Affinity chromatography of pyruvate dehydrogenase complex on ethanol–Sepharose and propanol–Sepharose in the absence and presence of Mg^{2+} in the elution buffer. Experiments were performed using 1.5 ml gel/column. Elution of the enzyme occurred by applying 0.3 M NaCl to the 50 mM phosphate elution buffer. SDS–gel electrophoresis patterns show from left to right: (3.A) Ethanol–Sepharose 2B. From left to centre, Mg^{2+} absent: initial eluate; active fraction. From centre to right, Mg^{2+} (2.5 mM) present: initial eluate, active fraction. (3.B) Propanol–Sepharose 2B idem.

were very similar. Pyruvate dehydrogenase complex activity could be eluted quantitatively at the same molarity (0.12 M) applying a phosphate buffer gradient. However, as expected, at higher chain lengths (C_4, C_5) total protein was not recovered quantitatively. Moreover, some proteins which hardly adsorb on the C_2–Sepharose contaminate the enzyme using higher chain lengths. The potentials of these adsorbents have not been rigourously tested though, as no gradient elution has been applied which might improve the results (cf. fig.1). Some results are shown in fig.3.

We have tested whether the affinity chromatography step is applicable to mutant pyruvate dehydrogenase complex enzymes from *E. coli*. Purification of 4 different structural mutants thus far leads to a similar or even better state of purity as in the case of wild type in a similar stage of the procedure. We have also applied this method succesfully to other prokaryotes viz *Azotobacter vinelandii, Bacillus subtilis, Bacillus polymyxa* and *Pseudomonas fluorescens*. In some cases though, other ionic strength conditions are required for adsorption and desorption (to be published elsewhere).

The method presented here is certainly very valuable as it is mild and avoids both protamine sulfate precipitation and isoelectric fractionation. We have combined affinity chromatography with a molecular sieve procedure as the next step. It might be advantageous in order to scale up the complete purification to investigate whether calcium phosphate gel–cellulose chromatography can be used instead of the molecular sieving step.

We have not yet determined whether a particular individual component of the enzyme complex is required or that binding depends on the presence of all components. These points will be elucidated by

dissociating the enzyme complex and by studying appropriate mutant enzymes. We have tested some other matrices to establish the structural requirements a ligand has to possess in order to be effective. Coupling of β-mercaptoethanol and of glycerol both lead to products without affinity. The combination of a positively charged nitrogen and an alcohol side chain seems to be necessary.

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