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AFFINITY CHROMATOGRAPHY STUDIES WITH THE PYRUVATE DEHYDROGENASE COMPLEX OF WILD-TYPE *ESCHERICHIA COLI*

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Summary

1. The lifetime of thiamine pyrophosphate-Sepharose 2B affinity matrices synthesized according to Matsuura et al. (Matsuura, A., Iwashina, A. and Nose, Y. (1973) Biochem. Biophys. Res. Commun. 51, 241—246) has been improved. The matrix interacts with bacterial pyruvate dehydrogenase complexes.

2. The synthesis of a stable thiochrome-Sepharose 2B matrix is described.

3. Both matrices bind the pyruvate dehydrogenase complex of *Escherichia coli* in a 50 mM phosphate buffer, pH 7.0. Elution is possible by an increase in ionic strength but not by the cofactor or metal · cofactor complexes.

4. The presence of Mg^{2+}, reduces the capacity of the affinity matrices but leads to higher specificity for the multienzyme complex.

5. The pyruvate dehydrogenase complex of *E. coli* has been successfully purified by combining a classical purification step with these affinity chromatography systems. The method is less suitable for large scale operation.

Introduction

The potential use of affinity chromatography in studying structural and regulatory mutants of enzymes has rarely been explored. Our interest is in the biochemical genetics of both prokaryotic and eukaryotic pyruvate dehydrogenase and we have been developing several affinity chromatography systems to be applied to the further characterization of mutants. As for prokaryotes, pyruvate dehydrogenase genetics has been mainly studied in *Escherichia coli* by both Henning et al. [1,2] and Guest et al. [3,4]. Structural mutants and deletions are known for all three components of the complex. The different cofactors involved in the overall reaction, as well as the protein-protein interactions

Abbreviation: Pipes, piperazine-*N*,*N*′-bis-(2-ethanesulphonic acid); SDS, sodium dodecyl sulphate.

among the different components, offer many possibilities to develop affinity systems. In this study, we report the use of thiamin pyrophosphate and thiochrome as affinity ligands for wild-type pyruvate dehydrogenase complex. Thiamine pyrophosphate affinity columns have been used to purify a thiamin-binding protein [5], thiamin pyrophosphokinase [6] and pyruvate oxidase [7].

Methods

Enzyme isolation and enzymatic assay. E. coli K 1-1 LR 8-13 (a pyruvate dehydrogenase-constitutive mutant) kindly given to us by Dr. U. Henning, was grown by the method of Vogel et al. [8]. Pyruvate dehydrogenase was partially purified as outlined previously [8]. Active fractions were sedimented by ultracentrifugation during 4 h at 140 000 $\times g$ at 4°C; the pellets were dissolved in 50 mM potassium phosphate (pH 7.0) and subsequently frozen (—20°C) in 0.5-ml aliquots at a protein concentration of 33 mg/ml. The overall activity was measured at 25°C [9]; the reaction was started by adding enzyme. The partially purified pyruvate dehydrogenase had a specific activity of 2.4 units which value slowly diminished during the course of this study.

Preparation of thiochrome. Thiochrome was prepared from thiamin as described by Nishimune et al. [10]. Paper chromatography on Whatman paper No. 1, using *n*-butanol/acetic acid/distilled water (4 : 1 : 5, v/v) as a solvent indicated a single product with a R_F value corresponding to thiochrome [11].

Preparation of affinity matrices. Thiamin pyrophosphate-Sepharose 2B (matrix I) was prepared as previously described [5]. Cyanogen bromide was dissolved in a small volume of dioxane and added. After washing the activated gel was reacted with 0.1 M ethylene diamine in 0.1 M bicarbonate buffer (pH 9.0). After washing the gel thiamin pyrophosphate was attached by a water-soluble carbodiimide condensation. The gel was extensively washed and stored in distilled water at pH 5.0—5.5.

Thiochrome-Sepharose 2B (matrix II) was prepared as follows. Sepharose-2B was activated using 1,4-butanediol-diglycylether according to Sandberg and Porath [13]. The activated matrix was reacted in the dark with 4 mM thiochrome at 4°C at pH 9.5 for 2 weeks. This resulted in a yellow, fluorescent matrix which was extensively washed before use. Coupling probably proceeds through the thiol anion, since no positive reaction was observed using Ellman's reagent [14]. This matrix was always kept in the dark and stored at —15°C in 50% glycerol.

SDS-polyacrylamide gel electrophoresis. Stock solutions were made according to Laemmli [15]. A standard gel of 10% acrylamide was used with a 3% stacking gel; a current of 3 mA/tube or 20 mA/slab was used and the gels were stained with Coomassie Brilliant Blue R. Samples were dialyzed against 10 mM sodium phosphate buffer (pH 7.0) to remove K^+.

Results

Thiamin pyrophosphate-Sepharose 2B (matrix I)

Stability. The stability of this affinity adsorbent is limited as indicated by a continuous release of ultraviolet-absorbing material. If stored at pH 7.0, 4°C in

30 mM potassium phosphate buffer the thiamin pyrophosphate coupled (approx. 40 μmol/ml) was released completely within 3 days. At pH 5.0, 20% of the bound ligand was detected in the supernatant over the same period; after 2 weeks, we noticed an approx. 35% loss. The matrix was therefore not used over prolonged periods and was always stored at pH 5.0, 4°C when not in use.

Biospecificity. Previous literature on this adsorbent suggests that the interactions are biospecific [5,6]. Since a number of thiamin pyrophosphate binding sites exists per multienzyme complex and since we use a high degree of activation of the matrix, this may well have led to multiple interactions. We, therefore, decided to verify biospecificity by testing a smaller thiamin pyrophosphate-dependent enzyme (yeast pyruvate decarboxylase). Apoenzyme was prepared according to Wittorf and Gubler [16]. A 50% reconstitution of the active complex was found by incubating the apoenzyme in 30 mM potassium phosphate buffer (pH 6.8), 2 mM $MgCl_2$ and 1 mM thiamin pyrophosphate [17,18]. Apoenzyme was then applied to a thiamin pyrophosphate-Sepharose 2B column under reconstituting conditions. The fractions were tested for enzyme activity by adding thiamin pyrophosphate. The enzyme mainly appeared in the void volume. However, 6% of the apoenzyme could be eluted by the addition of 1 mM thiamin pyrophosphate to the elution buffer, indicative of a biospecific effect. A likely explanation for the low affinity of pyruvate decarboxylase for this matrix can be found in the dissociation of the apoenzyme into monomers and the low affinity of this dissociated enzyme for the cofactor [17].

Pyruvate dehydrogenase. The affinity of partially purified pyruvate dehydrogenase was tested on a small scale in Pasteur pipettes. The enzyme was dialyzed against 30 mM potassium phosphate buffer (pH 7.0) 5 mM β-mercaptoethanol (or 2 mM dithiothreitol) and columns were equilibrated with the same buffer. Only a fraction of the activity appeared in the eluate. An almost quantitative elution (85—90%) of the total pyruvate dehydrogenase activity was possible by an increase in ionic strength. This was achieved either by the addition of NaCl or by a phosphate buffer gradient. No activity appeared upon the addition of 1 mM thiamin pyrophosphate to the 30 mM phosphate elution buffer either in the absence or presence of 5 mM Mg^{2+}.

Partial leakage was prevented by using a lower ionic strength to load the column. A phosphate buffer concentration of 3—10 mM gave no leakage. In Fig. 1 a 3—200 mM phosphate buffer gradient was used, leading to quantitative recovery of the activity. The electrophoretic analysis is shown as an insert. *

The elution profile of a purification of pyruvate dehydrogenase in the presence of $MgCl_2$ on a larger scale is shown in Fig. 2. Elution of the enzyme was achieved with a phosphate buffer gradient; magnesium phosphate precipitates were not formed in the enzymatically active fractions. The elution profile in the absence of $MgCl_2$ is essentially identical. The specific activity increased by a factor 5—6 to approx. 10—12 units. SDS-gel electrophoresis patterns show a high degree of purity of the complex (Fig. 3); in the absence of $MgCl_2$, minor bands between the transacetylase and lipoamide dehydrogenase component are present to a larger extent. Moreover, a fourth intensively stained band (molecu-

* The transacetylase of the enzyme used is partially degraded due to prolonged storage.

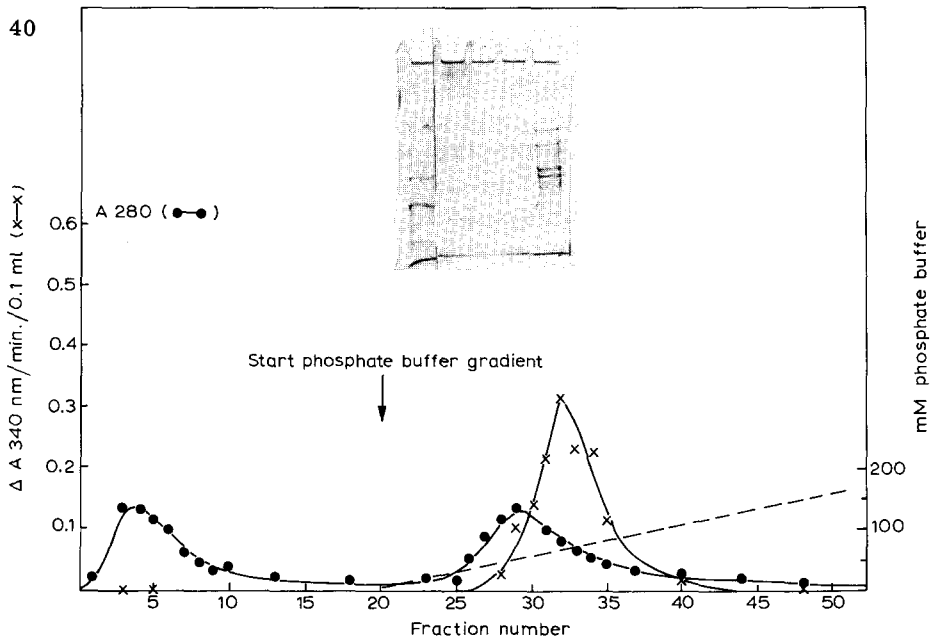

Fig. 1. Elution pattern of pyruvate dehydrogenase chromatography on thiamine pyrophosphate-Sepharose 2B (matrix I). 0.4 ml of a dialyzed enzyme sample (A_{280nm} = 5.8) was applied to a microcolumn (2 ml bed volume) and eluted with 3 mM potassium phosphate buffer containing 5 mM β-mercaptoethanol and 2.5 mM $MgCl_2$. Temperature 5°C. SDS electrophoresis patterns of pooled eluate and fractions 28, 31 and 32 are shown as an insert. At the right the crude extract is shown.

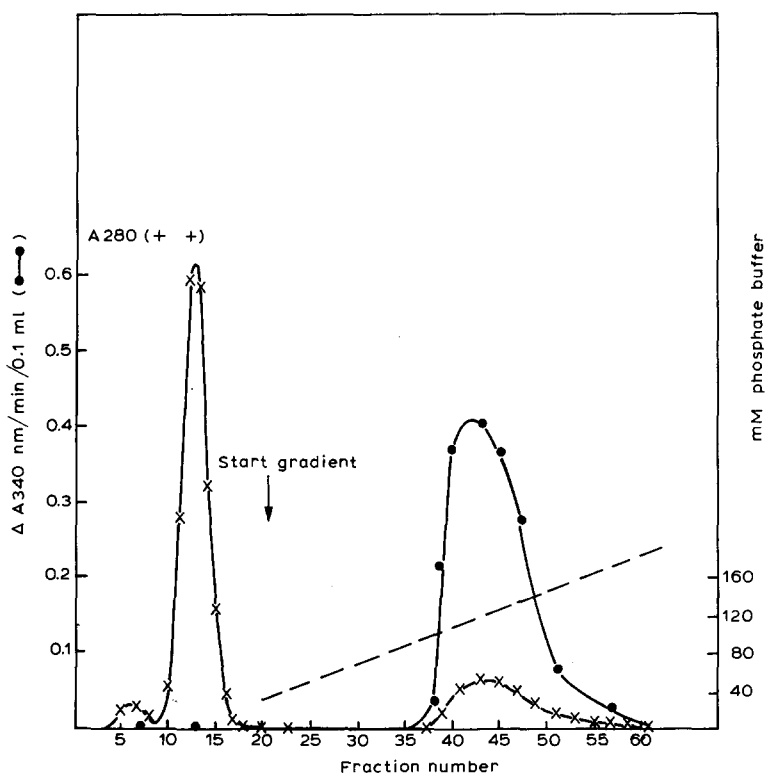

Fig. 2. Affinity chromatography of pyruvate dehydrogenase on thiamine pyrophosphate-Sepharose 2B (matrix I). 1 ml partially purified pyruvate dehydrogenase (25 mg protein/ml) in 30 mM potassium phosphate buffer was applied to a 5.5 X 2 cm column at 5°C which was equilibrated with the same buffer containing 10 mM $MgCl_2$. Fractions of 1.2 ml were collected.

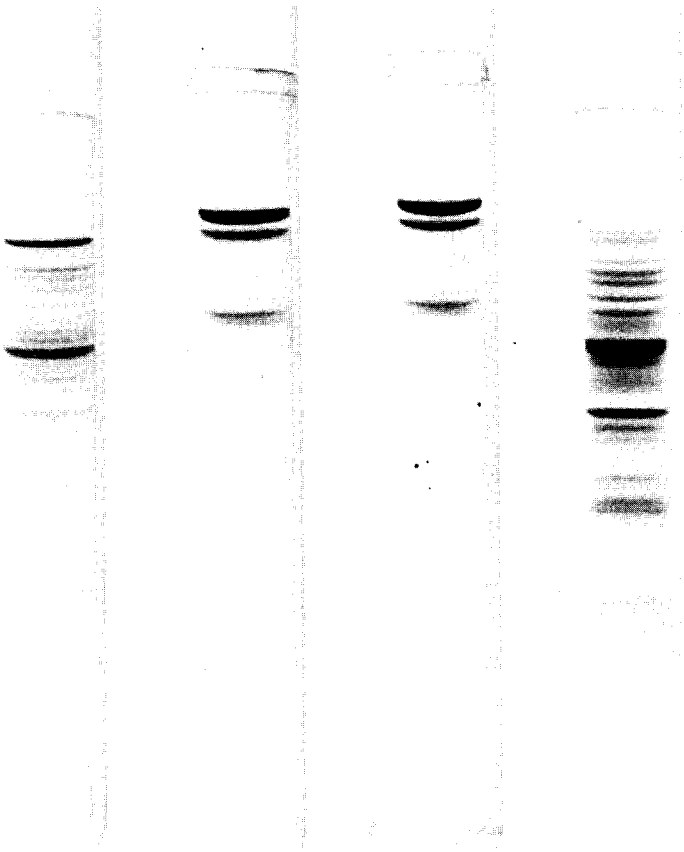

Fig. 3. SDS gel electrophoresis patterns of the purification described in Fig. 1. From left to right: starting material, fractions 47—49, fractions 44—46 and pooled eluate (fractions 10—17). The bands in the purified enzyme represent from top to bottom the pyruvate dehydrogenase, the transacetylase and the lipoamide dehydrogenase component.

lar weight 52 000) just beneath lipoamide dehydrogenase appears. These bands are mainly the result of transacetylase breakdown, as observed by others [19].

Effects of metal ions (Mg^{2+}/Mn^{2+}) and ionic strength. Both Mg^{2+} and Mn^{2+} influence the binding of thiamin pyrophosphate with the pyruvate dehydrogenase complex. The effect of these ions on affinity chromatography behaviour of pyruvate dehydrogenase complex was therefore studied in the case of matrix I. The influence of Mg^{2+} was determined in phosphate buffer and of Mn^{2+} in Pipes buffer. Elution with different $MgCl_2$ concentrations (0, 2.5 and 5 mM) in 30 mM phosphate buffer results in slightly different elution patterns. In the presence of Mg^{2+} less protein is adsorbed leading to a higher specific activity in the second fraction although a slight release of activity occurs (±15%). This can be prevented by using a lower ionic strength phosphate buffer, e.g. 10 mM. Total enzyme recovery is also better in the presence of Mg^{2+} viz. 70% (0 Mg^{2+}) and 90% (2.5 and 5 mM Mg^{2+}). In small scale experiments NaCl gradient

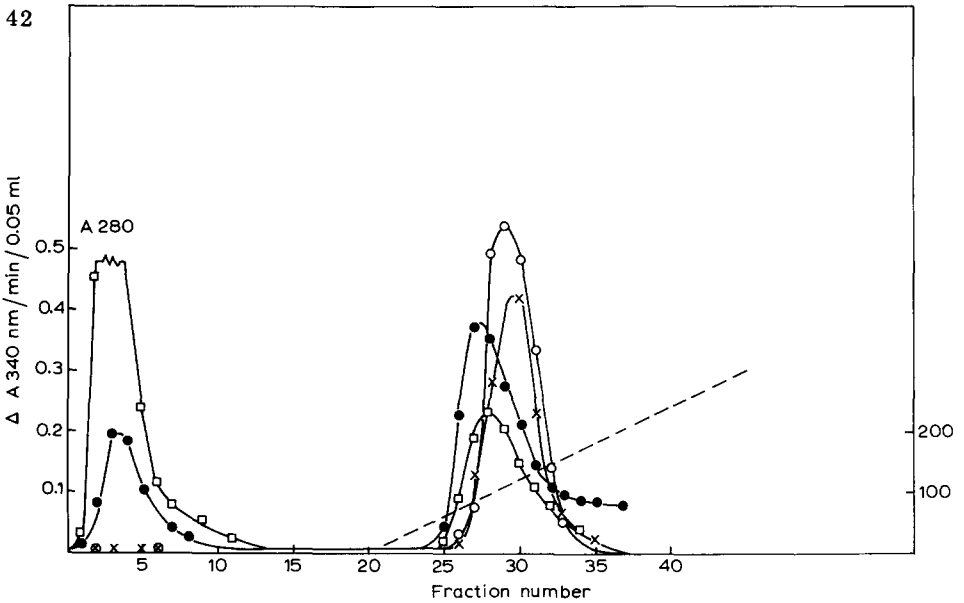

Fig. 4. Affinity chromatography of pyruvate dehydrogenase on matrix I. 0.4 ml of enzyme (A_{280nm} = 10.5) was applied to a microcolumn (1.5 ml bed volume at 5°C). The elution buffer contained 10 mM Pipes. pH 7.0, and 5 mM β-mercaptoethanol. Fraction volume 1.2 ml. Chromatography was performed in the absence and presence of $MnCl_2$ (2.5 mM). A_{280nm}: ●———●, without and □———□, with $MnCl_2$. Activity: X———X, without and ○———○, with $MnCl_2$.

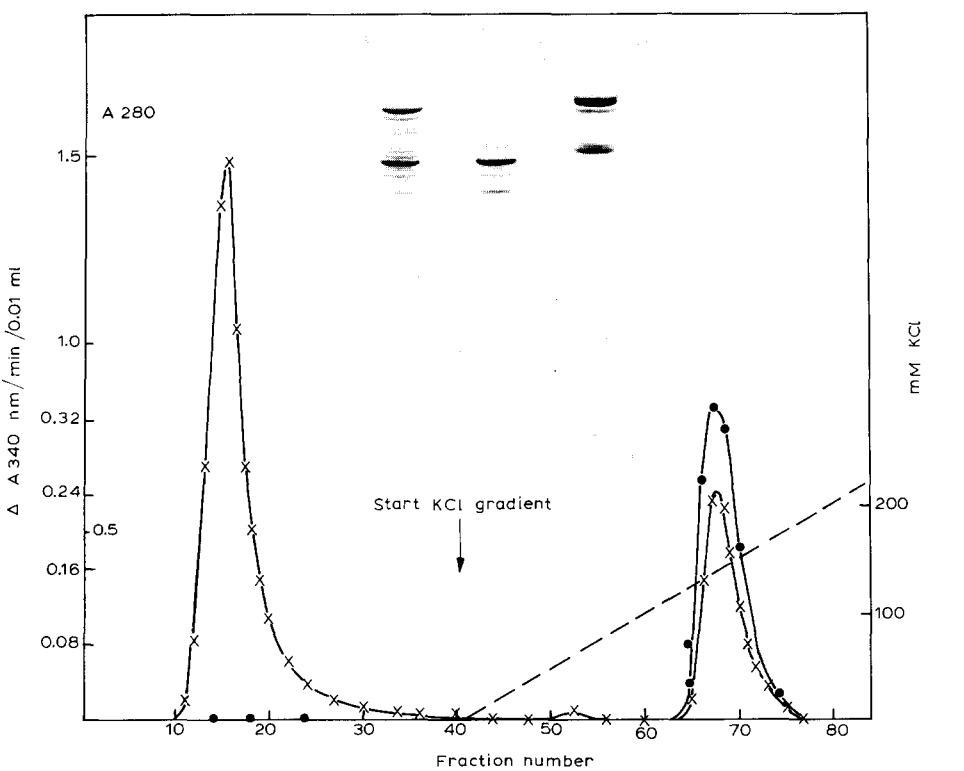

Fig. 5. Affinity chromatography of pyruvate dehydrogenase on thiochrome-Sepharose 2B (matrix II). 1.3 ml of a partially purified pyruvate dehydrogenase preparation (A_{280nm} = 10) were applied after dialysis against elution buffer to a column (7.5 × 2 cm). Elution occurred with a 10 mM Pipes buffer, pH 7.0, containing 5 mM $MgCl_2$. The insert shows SDS gel electrophoresis patterns of, from left to right: a, starting material; b, first eluate; c, fractions 65—72.

elution was applied. SDS gel electrophoresis indicates that some protein, corresponding with the pyruvate dehydrogenase component elutes before the transacetylase and lipoamide dehydrogenase components appear.

In Fig. 4 the effect of different Mn^{2+} concentrations on the elution pattern is shown using 10 mM Pipes buffer (pH 7.0) NaCl gradient elution was applied (0—0.3 M). Under all conditions enzyme activity and protein absorbance are not coincident. From the electrophoretic analysis it was seen that complexes containing transacetylase bands of lower molecular weight eluted more frontal.

Thiochrome-Sepharose 2B (matrix II). Although from analogy with pyruvate decarboxylase [20] one might expect thiochrome pyrophosphate to be a suitable ligand for the pyruvate dehydrogenase complex, we have first explored the possibility to use thiochrome itself. On a small scale in the absence of Mg^{2+} or at low concentrations of this ion (e.g. 0.1 mM), many proteins are bound to this matrix from a crude extract. However at 5 mM $MgCl_2$ the interaction gains in specificity although the capacity is drastically decreased leading to 60—70% of activity in the void volume. A typical larger scale purification is shown in Fig. 5, together with the electrophoretic data. On this scale no enzyme activity was lost in the void volume.

Discussion

Purification of the *E. coli* pyruvate dehydrogenase complex to an electrophoretically pure form starting from a partially purified preparation is possible using one affinity chromatography step with thiamin pyrophosphate as a ligand (matrix I). Due to instability of the 2-oxoglutarate dehydrogenase in *E. coli* and the relatively low concentration of the latter enzyme in the strain used [8], copurification of this multienzyme complex has not been observed. A disadvantage of this method for purification purposes is the instability of the ligand bonding although we have extended the life-span of the matrix considerably by storing at low pH (at 4°C or at —20°C in 50% glycerol).

However, this matrix is useful for some of our purposes viz. characterization of mutant enzymes if used in combination with another purification procedure (to be published). Biospecific interactions of this matrix were observed previously with other thiamin binding proteins [5,6] and with yeast pyruvate decarboxylase in the present study, though to a low extent. The pyruvate dehydrogenase complex however could not be eluted biospecifically by thiamin pyrophosphate in the absence or presence of Mg^{2+}. We suggest this to be due to multiple interactions of the complex with the matrix.

The transacetylase component of the complex is known to become degraded which leads to various products of lower molecular weight (cf. ref. 19). The matrix discriminates to some extent between complexes of different composition (viz. different ratio's of transacetylase breakdown products). These differences in elution behaviour also depend on the presence and nature of the bivalent ion in the elution buffer and (or) the buffer used. Matrix I has also been found to interact with the pyruvate dehydrogenase complex of various other prokaryotes (viz. *Bacillus subtilis*, *Pseudomonas fluorescens* and *Azotobacter vinelandii*). Our initial attempts to apply this matrix also to the complex of a eukaryote failed. The mammalian complex from pig heart was largely

inactivated, presumably due to dissociation since mixing the first eluate and the protein fraction obtained by a salt gradient restored the overall activity partially (40%).

Thiochrome-Sepharose 2B is a stable product. A disadvantage is that the specificity is low in the absence of Mg^{2+}, whereas the capacity is strongly reduced in the presence of Mg^{2+}. We have recently prepared several stable thiamine pyrophosphate derivatives essentially based upon the method developed by O'Brien et al. [7] but varying the length of the spacer arm. Our initial results are not very promising.

In our present studies we investigate the interactions of the various subcomplexes with the affinity adsorbents described in order to develop a tool to discriminate among various pyruvate dehydrogenase mutants, particularly those which lack one of the three different polypeptide components and those which have altered chains. Some of the latter mutants have already been largely purified by us this way.

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