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PREPARATION OF *ESCHERICHIA COLI* PYRUVATE OXIDASE UTILIZING A THIAMINE PYROPHOSPHATE AFFINITY COLUMN

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Summary

An improved procedure is reported for the purification of *Escherichia coli* pyruvate oxidase (pyruvate:ferricytochrome b_1 oxidoreductase, EC 1.2.2.2), a peripheral membrane flavo-enzyme, which is much more reproducible and requires considerably less time than the original purification scheme. The key element in this protocol is a new Sepharose-based affinity resin designed for the isolation of thiamine pyrophosphate-requiring enzymes. The synthesis, partial characterization, and use of two such affinity resins is described. Pyruvate oxidase is a pure, homogeneous protein as it is eluted from the affinity resin. The enzyme is a tetramer with a subunit molecular weight of approx. 60 000. The subunits appear to be identical. The isoelectric point of pyruvate oxidase is 5.6.

Introduction

Pyruvate oxidase (pyruvate:cytochrome b_1 oxidoreductase, EC 1.2.2.2) is a peripheral membrane flavo-enzyme isolated from *Escherichia coli*. The enzyme catalyzes the oxidative decarboxylation of pyruvate to yield acetate plus CO_2, and the reduced flavo-enzyme is coupled to the *E. coli* membrane-bound cytochrome chain [1]. The enzyme will also reduce ferricyanide and 2,6-dichloroindophenol (DCIP), providing convenient spectrophotometric assays for activity. Although pyruvate oxidase can be isolated in a membrane-bound form, it can be easily released from the membrane by sonication. Once solubilized, the enzyme can be purified to homogeneity [2].

Pyruvate oxidase is a particularly interesting enzyme because a variety of lipids can increase its specific activity about 25-fold with ferricyanide as an electron acceptor. The lipids which serve as successful activators range from *E.*

coli membrane phospholipids to detergents such as sodium dodecyl sulfate. Some of the properties of the lipid-activated enzyme were recently reported by Cunningham and Hager [3,4]. In addition to lipid activation, the enzyme can be activated by proteolysis [1]. The protease-modified form of the oxidase has kinetic properties very similar to the enzyme activated by sodium dodecyl sulfate.

An *E. coli* W mutant (191-6) has been isolated which produces large quantities of pyruvate oxidase, and the enzyme has previously been purified to homogeneity using classical biochemical techniques. However, the original preparative scheme has been found to be unsatisfactory, both from the standpoint of the time required for the procedures and most especially from the standpoint of reliability. For this reason, an effort was made to improve the pyruvate oxidase preparative protocol. A novel feature of this work is the synthesis and successful use of affinity resins designed to take advantage of the natural strong affinity of the enzyme for one of its cofactors, thiamine pyrophosphate. Pyruvate oxidase has an absolute dependence on thiamine pyrophosphate for enzymatic activity; the K_m of thiamine pyrophosphate has previously been reported to be about 1 μM [3].

Materials and Methods

The following chemicals were used without further purification: Thiamine pyrophosphate, thiamine monophosphate, 1-ethyl-3(3-dimethylaminopropyl)-carbodiimide (Sigma); 1,6-diaminohexane (98%) and CNBr (97%) (Aldrich); electrophoretic grade acrylamide, ethanolamine, succinic anhydride, and *p*-nitrobenzoylazide (Eastman); P_2O_5 (Baker); 85% H_3PO_4 (Fisher); protamine sulfate (Nutritional Biochemicals). Other materials used were Sepharose 4B (Pharmacia), Dowex 50W-X8 ion-exchange resin (Bio-Rad), ampholytes for isoelectric focusing (LKB) and Protein Molecular Weight Marker Kit and ultrapure guanidine · HCl. (Schwarz/Mann). All other reagents used were analytical grade. All solutions were made with glass distilled deionized water.

Preparation of ethanolamine pyrophosphate. A polyphosphate mixture was prepared by heating 6.25 g of 85% H_3PO_4 with 7.40 g of P_2O_5 in an oil bath at 120° C for 15 min. 1.8 ml of ethanolamine was slowly added with stirring. After 35 min the reaction mixture was dissolved in 15 ml of cold absolute ethanol on ice and poured into 950 ml of cold ethanol which was being stirred rapidly. The fluffy white precipitate which resulted was collected by centrifugation and dissolved in 15 ml of water. This solution was applied to a Dowex 50W X8 column (H^+ form, 2.4 cm × 15 cm).

The ion-exchange column was eluted at 4° C with distilled water at a flow rate of 1 ml/min. 5-ml fractions were collected. The first ninhydrin-positive peak was pooled, neutralized with 5 M NaOH and then lyophilized. The solid remaining after lyophilization was dissolved in 5 ml of water and then stored at −20° C in the freezer.

Preparation of ethanolamine pyrophosphate-Sepharose. 1,6-Diaminohexane was coupled to 100 ml of washed Sepharose 4B according to the procedure of March et al. [5] except that the coupling reaction was performed for 3 h at room temperature rather than 20 h at 4° C [5]. The newly coupled aminohexyl-

Sepharose was then succinylated according to the procedure of Cuatrecasas [6].

Water-soluble 1-ethyl-3(3-dimethylaminopropyl)-carbodiimide was used to catalyze the reaction between 40 ml of the succinylaminohexyl-Sepharose and 4.8 mmol of ethanolamine pyrophosphate. The procedure was according to Cuatrecasas [6] except that the reaction suspension was shaken for 15 h at 4°C rather than at room temperature. After extensive washing, the gel was stored in water at 4°C.

Preparation of thiamine pyrophosphate-Sepharose. Aminohexyl-Sepharose (100 ml) was prepared as described above for the ethanolamine pyrophosphate affinity resin and used to prepare a *p*-aminobenzoyl derivative of Sepharose which was then diazotized according to the procedure of Cuatrecasas [6]. The diazobenzoyl-Sepharose was immediately reacted with thiamine pyrophosphate (15 g in 50 ml cold saturated sodium borate), and the pH was immediately adjusted to 8.6 with 5 M NaOH. The resulting reddish orange resin was stirred slowly for 8 h at 4°C. The gel was extensively washed and then stored in water at 4°C.

Determination of ligand capacity. Samples of the suspensions of packed gel in water were analyzed for acid-labile phosphate according to the method of Bartlett [7]. Corrections were always made for the small amount of inorganic phosphate which was present in the suspension.

Analysis of ethanolamine pyrophosphate reaction product. Samples of ethanolamine pyrophosphate were analyzed for acid-labile phosphate and for total phosphate according to the method of Bartlett [7].

Thin-layer chromatography was performed using fluorescent cellulose sheets (Eastman). The solvent system used was *n*-propanol/0.2 M potassium phosphate buffer, pH 4.9/water (60 : 20 : 20, v/v).

Thin-layer electrophoresis was run on cellulose acetate plates (2.5 cm × 7.5 cm) using a Millipore electrophoresis apparatus with 0.05 M sodium acetate buffer, 10 mM EDTA, at pH 5.0. Ethanolamine pyrophosphate was visualized by spraying the plate with either ninhydrin or Molybdenum Blue spray reagents.

Preparation of pyruvate oxidase. (a) Sonication: 300 g of frozen *E. coli* W. 191-6 were suspended in 600 ml of cold 0.1 M potassium phosphate buffer, pH 5.3, and sonicated in a stainless steel Rosette cell using a Branson Model W350 Sonifier at full power for 15 min. The sonicated suspension was centrifuged at 19 000 rev./min for 1 h at 4°C in a Beckman Model L5-50 ultracentrifuge. The pH of the supernatant was kept between 5.5 and 5.6.

(b) Protamine sulfate: To the supernatant from the sonication was added 7.5% by weight of glycerol to stabilize the pyruvate oxidase, 0.05 volume of saturated $(NH_4)_2SO_4$ and finally 0.40 volume of 2.5% protamine sulfate prepared in 0.1 M potassium phosphate buffer, pH 5.3. The protamine sulfate was added with rapid stirring at 0°C over a 1 h period. The precipitated material was separated by centrifugation for 15 min at 10 000 × g. The supernatant was then adjusted to pH 5.7 by the addition of solid $NaHCO_3$.

(c) 40% $(NH_4)_2SO_4$ fractionation: 19 g of solid $(NH_4)_2SO_4$ plus 0.1 g of $NaHCO_3$ were added for every 100 ml of the supernatant from the protamine sulfate step. This addition was made with stirring at 0°C over a 1.5 h period. The

suspension was centrifuged for 15 min at 10 000 × *g*.

(d) 70% $(NH_4)_2SO_4$ fractionation: 18 g of solid $(NH_4)_2SO_4$ were added for every 100 ml of supernatant from the 40% $(NH_4)_2SO_4$ fractionation. The addition was made over a 1.5 h period at 0°C, stirred for additional 3—4 h had centrifuged for 30 min at 10 000 × *g*. The precipitate was redissolved in 80 ml of 50 mM potassium phosphate buffer, pH 5.7, and dialyzed at 4°C against two 4-l volumes of the same buffer. After 10 h, the solution was clarified by centrifugation for 20 min at 16 000 × *g*.

(e) Heat step: The supernatant from the dialysis was heat denatured by being heated to 60°C in a 70°C water bath and immediately cooled to 0°C. The precipitated protein was removed by centrifugation for 30 min at 16 000 × *g*.

(f) DEAE-Sephadex column: 20% by weight of glycerol was added to the supernatant from the heat step in order to facilitate application of the enzyme solution to the DEAE-Sephadex column and also to stabilize the enzyme.

The heat step supernatant was applied to a 4 × 33-cm DEAE-Sephadex A-50 ion-exchange column which had been equilibrated with 0.1 M potassium phosphate buffer, 10% glycerol, pH 5.7. The enzyme solution was pumped at a rate of 40 ml/h; 8-ml fractions were collected. As soon as the enzyme had been applied, a linear gradient of 600 ml of 0.1 M potassium phosphate buffer, 10% glycerol, pH 5.7, plus 600 ml of 0.3 M potassium phosphate buffer, 10% glycerol, pH 5.3, was pumped onto the column. The column was run for 24 h with a flow rate of 40 ml/h. The fractions which contained the enzyme were pooled, precipitated with 70% $(NH_4)_2SO_4$ and redissolved in 0.1 M potassium phosphate buffer, 10% glycerol, pH 5.7.

(g) Thiamine pyrophosphate affinity column: The concentrated pool from the DEAE column was dialyzed against 3 l of 25 mM potassium phosphate buffer, 10 mM $MgCl_2$, 15% glycerol, pH 5.7, for 10 h at 4°C. The dialyzed enzyme solution was then applied to the thiamine pyrophosphate affinity column (2.5 × 13 cm) which had been previously equilibrated with the same buffer. After the enzyme solution had been applied to the column, it was allowed to equilibrate with the column for 15 min before elution was begun. The column was washed with the 25 mM potassium phosphate buffer, 10 mM $MgCl_2$, 15% glycerol, pH 5.7, at a flow rate of 40 ml/h. 4-ml fractions were collected. After one or two column volumes, the pyruvate oxidase was eluted off the column with 50 mM potassium phosphate buffer, 0.1 M KCl, 15% glycerol, pH 5.7. Those fractions which contained enzyme were pooled and concentrated with 70% $(NH_4)_2SO_4$. The precipitated protein was then redissolved in 0.1 M potassium phosphate buffer, 10% glycerol, pH 5.7. Any precipitate which did not redissolve was removed by centrifugation.

(h) Low ionic strength precipitation: The concentrated pool from the thiamine pyrophosphate affinity column was dialyzed twice against 500 ml of 10% glycerol for 1 h at 4°C, using a rapid dialysis apparatus (MRA Corporation, Boston, Mass.). The enzyme solution was finally dialyzed against 2 l of 10% glycerol for 10 h at 0°C. The resulting yellow precipitate was collected by centrifugation and redissolved in 0.1 M sodium phosphate buffer, 50% glycerol, pH 5.7.

(i) Storage conditions: Purified pyruvate oxidase has been found to be stable for several months when stored in 0.1 M sodium phosphate buffer, 50% glycer-

ol, pH 5.7 at −20° C, or with 20% glycerol at −7° C.

Tris-barbital gels. Tris-barbital gels were run according to the method of Williams and Reisfeld [8] except that the spacer gel was omitted. Also, the gels contained glycerol to stabilize the enzyme during electrophoresis.

Protein bands were visualized by staining with Coomassie Brilliant Blue R250 (0.05% solution in methanol/acetic acid/water (4.5 : 1.0 : 4.5, v/v). Pyruvate oxidase activity was detected by placing the gel in DCIP assay solution. Oxidase activity produced a clear band in the resulting blue gel.

Sodium dodecyl sulfate gel electrophoresis. Sodium dodecyl sulfate gels were run according to the method of Laemmli [9]. The gels were fixed in 12.5% trichloroacetic acid for 2 h and were then stained with Coomassie Brilliant Blue.

Isoelectric focusing gels. Isoelectric focusing gels were run according to the method of Catsimpoolas [10] with a few modifications. Pyruvate oxidase would not focus properly from the acidic end of the gel so the enzyme was applied to the basic end of a pre-formed gel as a solution in 25% glycerol-containing ampholytes. Then, 100 μl of 20% glycerol was carefully layered on top of the sample. Finally, 100 μl of 10% glycerol was layered on top of the 20% glycerol layer.

After extensive dialysis against 5% trichloroacetic acid to remove the ampholytes from the gels, the gels were stained with Coomassie Brilliant Blue.

8 M urea gels. Urea gels were prepared and run as described above for Tris-barbital gels except that the gels were 5.5% acylamide instead of 7.5%; the sample buffer and the gels contained 8 M urea.

DCIP reductase assay for pyruvate oxidase. DCIP reductase activity was measured spectrophotometrically by following the decrease in the absorbance at 600 nm at room temperature. Conditions were as described by Cunningham and Hager [3] with the following changes: 100 μmol of sodium phosphate buffer, pH 6.0, and 200 μmol of sodium pyruvate were used in place of reported amounts of the potassium salts. Also 0.02 μmol of sodium dodecyl sulfate was used to activate the enzyme. One unit of DCIP reductase activity is defined as a decrease in 0.001 absorbance unit per min. One decarboxylase (CO_2) unit equals about 136 DCIP reductase units.

Protein determinations. Protein concentrations were estimated by the Warburg-Christian method [11] so that the specific activities in this preparation scheme could be compared to the previously reported values in the literature [2]. After the low ionic strength precipitation, the pure oxidase concentration was determined from the $E^{1\%}_{280\,nm}$ of 11.9 [2]. All absorbance readings were made with a Zeiss PMQII spectrophotometer.

Flavin determination. The specific activity of pyruvate oxidase was also calculated on the basis of flavin content. Parameters used by Williams and Hager [2] were employed. It was assumed that the molar extinction coefficient at 438 nm of the enzyme-bound FAD is 14 600 $M^{-1} \cdot cm^{-1}$, and that one flavin is bound per subunit. The only flavoprotein in the purified preparation is pyruvate oxidase.

Gel-filtration chromatography in the presence of guanidine · HCl. The molecular weight of the pyruvate oxidase subunit was also determined using this technique. The samples were prepared and the column (0.9 × 23 cm) was run at room temperature according to Fish et al. [12] using Biogel A-15m (Bio-

Rad) with a flow rate of 5 ml/h. Samples were dialyzed after carboxymethylation prior to being loaded onto the column. All fractions were weighed.

Results

Affinity chromatography

(a) Ethanolamine pyrophosphate-Sepharose. The importance of the pyrophosphate moiety for thiamine pyrophosphate binding to pyruvate oxidase has long been realized. Both inorganic pyrophosphate and ethanolamine pyrophosphate were determined to be competitive inhibitors with respect to thiamine pyrophosphate with K_i values of 4.2 and 1.94 mM, respectively [13]. Previous work by Poludniak [13] suggested that ethanolamine pyrophosphate might be successfully used to make an affinity resin for pyruvate oxidase. This ligand was synthesized and linked through its amino group to a series of Sepharose derivatives, each with a different spacer arm. The ethanolamine pyrophosphate ligand was proven to be pure based on thin-layer chromatography and thin-layer electrophoresis. In both cases the ethanolamine pyrophosphate product was a single spot which gave positive results with both ninhydrin and molybdenum blue sprays. Upon 7-min hydrolysis of the ethanolamine pyrophosphate product a second spot appeared which had the same mobility as an ethanol-

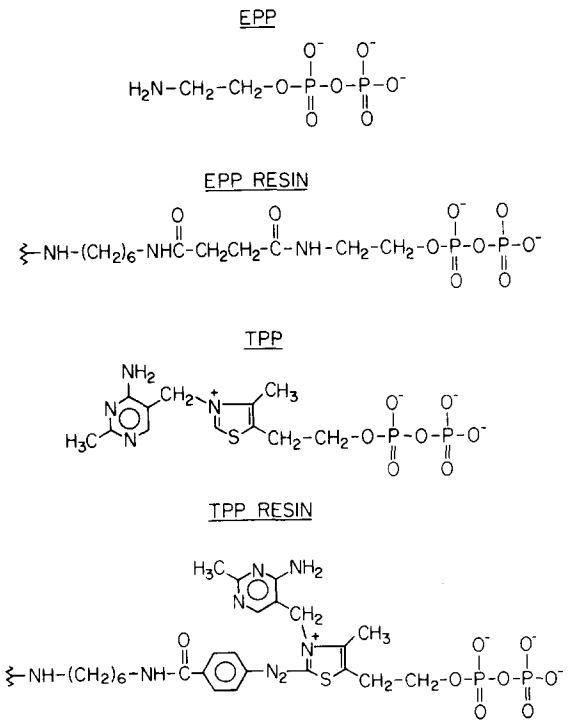


Fig. 1. Affinity ligands and suggested structures for the Sepharose derivatives used for affinity chromatography of pyruvate oxidase. EPP, ethanolamine pyrophosphate; TPP, thiamine pyrophosphate.

TABLE I

PHOSPHATE DETERMINATIONS OF ETHANOLAMINE PYROPHOSPHATE REACTION PRODUCT AND AFFINITY RESINS

Phosphate assays were performed as described in the text.

Sample	7-min phosphate	Total phosphate	Ratio of total phosphate to 7-min phosphate	Capacity (μmol ligand) / (ml wet packed resin)
Ethanolamine pyrophosphate	655 μmol/ml solution	1334 μmol/ml solution	2.01	—
Ethanolamine pyrophosphate-Sepharose	8.96 μmol/ml resin	16.88 μmol/ml resin	1.88	8.96
Thiamine pyrophosphate-Sepharose	6.00 μmol/ml resin	11.08 μmol/ml resin	1.85	6.00

amine monophosphate standard. The phosphate analysis of the ethanolamine pyrophosphate product is given in Table I.

One of the ethanolamine pyrophosphate-Sepharose derivatives is pictured in Fig. 1 and is typical of the more successful cases. Table I includes values for both the total phosphate and the 7-min acid-labile phosphate indicating that this resin contains a considerable amount of covalently bound pyrophosphate, usually about 8 μmol per ml of packed resin.

Unfortunately, under conditions (10 mM $MgCl_2$ in 50 mM phosphate buffer, pH 5.7) similar to those employed in the enzyme assay, this ethanolamine pyrophosphate-Sepharose resin showed no affinity for the enzyme (Fig. 2A). The enzyme employed in these tests had been purified through the DEAE column step and had a specific activity (600—900 CO_2 units/mg) which was 10—15% of the maximum which has been observed. This enzyme preparation also had virtually no endogenous thiamine pyrophosphate present as judged by the requirement for added thiamine pyrophosphate in the enzyme assay.

After considerable examination of the column buffer conditions, it was found that the enzyme bound to the resin quantitatively at lower ionic strength phosphate buffer (25 mM) at pH 5.3 in the presence of 10 mM $MgCl_2$. Contaminating protein elutes under these conditions, and the oxidase can be eluted after washing the column by increasing the pH or ionic strength, or both (Fig.

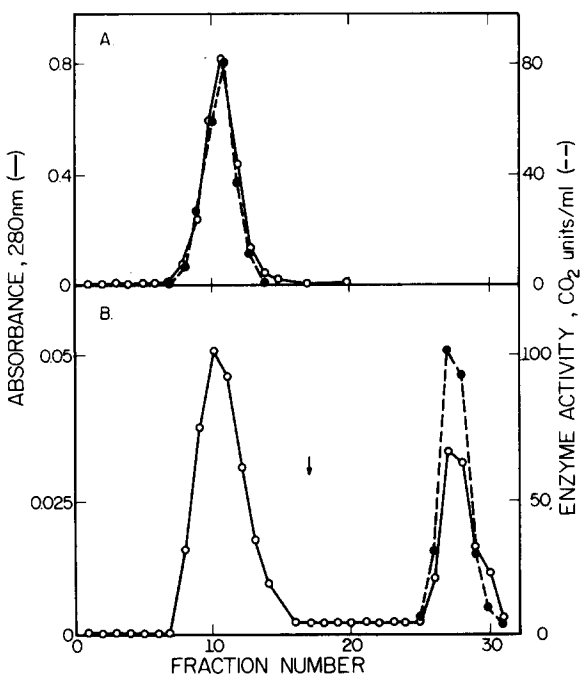


Fig. 2. Effect of ionic strength and pH on the binding of pyruvate oxidase to ethanolamine pyrophosphate-Sepharose. Two 10-ml columns of ethanolamine pyrophosphate-Sepharose were equilibrated with (A) 50 mM potassium phosphate buffer, 10 mM $MgCl_2$, pH 5.7; and (B) 25 mM potassium phosphate buffer, 10 mM $MgCl_2$, pH 5.3, respectively. Partially purified enzyme solution (0.5 ml) which had been dialyzed against the corresponding buffers was applied to each column. The columns were eluted with the starting buffer at a flow rate of 4 ml/h. 1-ml fractions were collected. At the arrow in B, 25 mM potassium phosphate buffer, 100 mM KCl, pH 5.3 was applied to the column.

2B). The enzyme was quantitatively recovered and was purified from 3- to 5-fold with a specific activity typically between 2500 and 3000 CO_2 units/mg.

Since the isoelectric point of pure pyruvate oxidase is about 5.6, it appears that the ethanolamine pyrophosphate-Sepharose is behaving as a cation-exchange resin. The affinity of pyruvate oxidase for a series of anionic resins including carboxymethyl-cellulose, carboxymethyl-Sephadex, and phosphocellulose, was determined as a function of both pH and ionic strength. In all cases, the same pattern of oxidase binding was found as in the ethanolamine pyrophosphate-Sepharose case, but considerable losses of enzyme activity were experienced when trying to elute the oxidase from the supports. That there may be some bio-specificity involved in the binding of the oxidase to the ethanolamine pyrophosphate-Sepharose is indicated in Fig. 3, where it is shown that 10 mM thiamine pyrophosphate will quantitatively elute the enzyme from the resin. However, this may represent in large part an ionic strength effect.

The ethanolamine pyrophosphate-Sepharose was not further characterized or used on a large scale due to difficulties caused by the requirement of working at pH 5.3. The oxidase proved to be very labile during all operations designed to reduce the pH to this point.

(b) Thiamine pyrophosphate-Sepharose. The use of a diazotized aryl amine linked to Sepharose to react with a potential biospecific ligand has been popular in recent years. In particular, these reactions are frequently used with the adenine moiety of molecules such as ADP, ATP and NAD^+ [14]. It was hoped that the active acidic position on the thiazole ring of thiamine pyrophosphate would react with the highly electrophilic diazotized aryl amine [15]. This appears to be the case, although the proposed structure of the thiamine pyrophosphate-Sepharose adduct in Fig. 1 has not been proven. The thiamine pyrophosphate-Sepharose has a deep red color typical of diazo dyes, and it is stable for many months. This color does not appear if the reaction is carried out in the absence of thiamine pyrophosphate; but if one substitutes thiamine, the resulting thiamine-Sepharose is deep red. These reactions also occur in solution, as between diazotized aniline and thiamine pyrophosphate or thiamine. Table I

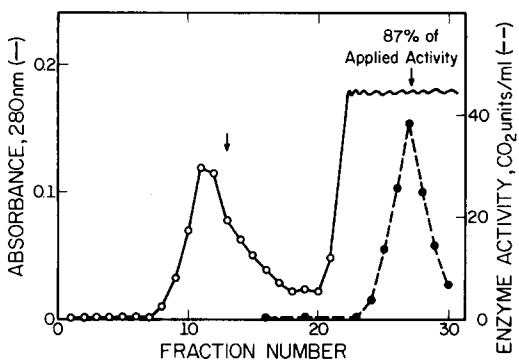


Fig. 3. Thiamine pyrophosphate elution of pyruvate oxidase from ethanolamine pyrophosphate-Sepharose. A 10 ml column of ethanolamine pyrophosphate-Sepharose was equilibrated and run as described above for Fig. 2B. At the arrow, 25 mM potassium phosphate buffer, 10 mM $MgCl_2$, 10 mM thiamine pyrophosphate, pH 5.3, was applied to the column.

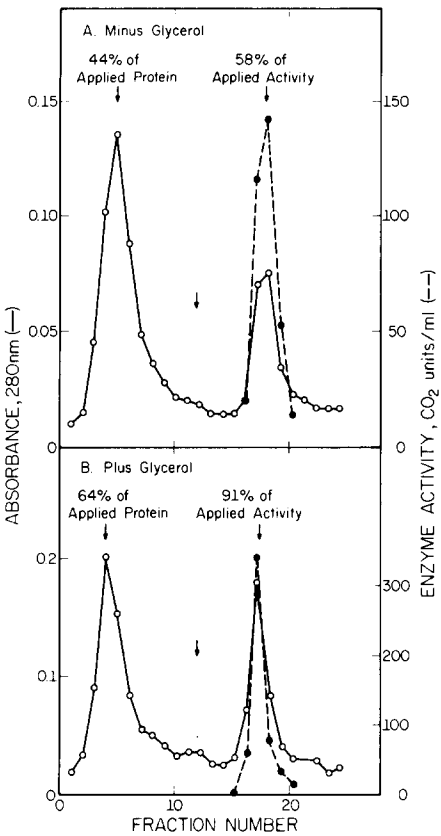


Fig. 4. Effect of glycerol on the stability and purification of pyruvate oxidase on thiamine pyrophosphate-Sepharose. Two columns (0.6 cm × 15 cm) of thiamine pyrophosphate-Sepharose were equilibrated with starting buffers: (A) 50 mM potassium phosphate buffer, 10 mM $MgCl_2$, pH 5.7; and (B), 50 mM potassium phosphate buffer, 10 mM $MgCl_2$, 10 % glycerol, pH 5.7. Partially purified enzyme solution (0.2 ml) which had been dialyzed against 50 mM potassium phosphate buffer, 10 mM $MgCl_2$, 10% glycerol, pH 5.7, was applied to each column. The columns were eluted with the starting buffers at a flow rate of 6 ml/h. 1-ml fractions were collected. At the arrows, the corresponding starting buffers plus 100 mM KCl were applied to the columns.

shows that, as in the case of ethanolamine pyrophosphate-Sepharose, the thiamine pyrophosphate-Sepharose has a very high capacity, about 6 μmol of pyrophosphate per ml of packed resin. Its great stability and the fact it can be made in less than 2 days make this resin particularly attractive.

Unfortunately, the thiamine pyrophosphate-Sepharose has a strong non-biospecific affinity for many proteins and considerable care was required to determine the optimum conditions for use. Non-biospecific binding, both hydrophobic and electrostatic, is now recognized as an important feature of many affinity resins [16,17]. The problem is probably exacerbated by the fact that all the synthetic chemical reactions occur on the Sepharose resin and no pure products are ever isolated. The proposed structure of the adduct (Fig. 1), even if correct, probably represents only a fraction of the many species present on the resin. These side products may contribute to the non-biospecific binding to proteins.

Both ionic strength and polarity-reducing agents have a large effect on the

binding of proteins to affinity resins [18]. This is certainly true for the binding of impure pyruvate oxidase preparations to thiamine pyrophosphate-Sepharose. Fig. 4 illustrates the effect of glycerol. Increasing the glycerol content of the buffer with which the column is equilibrated results in less protein binding to the column. In addition, omitting glycerol results in substantial losses of enzymatic activity. A 15% glycerol content was found to be optimal for use on the thiamine pyrophosphate-Sepharose column. At somewhat higher concentrations, the enzyme does not bind tightly to the column.

Pyruvate oxidase binds to thiamine pyrophosphate-Sepharose in buffers containing 10 mM $MgCl_2$, and 50 mM phosphate at pH 5.7. Under the same conditions, the enzyme shows no affinity for ethanolamine pyrophosphate-Sepharose (Fig. 2A), indicating a significant difference in the basis for the enzyme affinity for the two resins. However, as seen in Fig. 4, the oxidase is easily eluted from the thiamine pyrophosphate-Sepharose by increasing the ionic strength. The enzyme can also be eluted from the resin by including 10 mM thiamine pyrophosphate in the buffer in place of 0.1 M KCl (not shown). The specificity of the thiamine pyrophosphate-Sepharose is further seen from the much weaker binding of pyruvate oxidase to a thiamine-Sepharose column. Under the same conditions where the enzyme binds tightly to the thiamine pyrophosphate-Sepharose, it is retarded only slightly on thiamine-Sepharose. Thiamine is not competitive with thiamine pyrophosphate in binding to the oxidase and has no effect on enzymatic activity.

Enzyme preparation

Most of the steps in the enzyme preparation have been substantially altered. It was found that affinity chromatography was most effectively used with pyruvate oxidase which had already been partially purified. The comparative advantages of some of the modified steps in the enzyme purification are summarized below and results are presented in Table II.

(a) Sonication. The use of a high-power 350-W sonicator allows the extrac-

TABLE II

PURIFICATION OF PYRUVATE OXIDASE *

Step	Protein (mg)	Total activity **	Specific activity ** (units/mg protein)	Recovery (%)
Crude extract	—	405 600	—	100
Protamine sulfate precipitation	26 600	352 800	13	87.0
Ammonium sulfate fractionation	8 500	317 400	37	78
Heat step supernatant	4 500	268 800	59	66
Concentrated DEAE-Sephadex fractions	307	239 300	781	59
Concentrated affinity chromatography fractions	56	185 200	3325	46
Low ionic strength precipitation	29	151 000	5189	37

* Starting with 300 g *E. coli* cell paste.

** Enzyme activity is expressed in decarboxylase units [2].

tion of most of the enzyme in a single, relatively short step. It is important to maintain the temperature of the sample below 10° C during this step.

(b) Addition of glycerol. Poludniak [13] first showed that the pure enzyme is stabilized in 50% glycerol at −20° C. During much of the preparation glycerol has been added to the buffers to reduce the lability of the enzyme. For instance, the extreme loss of enzyme activity which was previously observed when the enzyme remained on the DEAE column for longer than 18 h is no longer seen [2].

(c) Protamine sulfate. If the nucleic acids are not effectively removed early in the preparation, all the subsequent steps are adversely affected. The use of nucleases was not satisfactory. By increasing the ionic strength with $(NH_4)_2SO_4$, and by decreasing the pH to below the isoelectric point of the enzyme (5.62), protamine sulfate could be used to rapidly precipitate the bulk of the nucleic acids without large losses in enzyme activity. If the protamine sulfate solution is added at pH 5.7 instead of pH 5.3, typically 50% of the enzyme activity is irreversibly lost.

(d) $(NH_4)_2SO_4$. This step is essentially as previously described except that glycerol is present in the buffers. Prior to the salting out, the pH of the enzyme solution is increased to pH 5.7. $NaHCO_3$ is included with the $(NH_4)_2SO_4$ to maintain this pH.

(e) Heat precipitation. This is performed essentially as previously described except it is done after dialysis rather than prior to the dialysis.

(f) DEAE column. The enzyme purity after the DEAE-Sephadex column is similar to that originally reported. A typical elution profile of this column is shown in Fig. 5.

(g) Thiamine pyrophosphate affinity chromatography. The thiamine pyrophosphate-Sepharose resin has been used successfully to replace the second protamine sulfate step and first crystallization in the original preparative protocol. A second heat precipitation has also been used at this point. In terms of reproducibility, specific activity, and yield of the resulting enzyme, the thiamine pyrophosphate affinity column is superior. The elution profile of a preparative scale thiamine pyrophosphate affinity column is shown in Fig. 6; Either free thiamine pyrophosphate (10 mM) or 0.1 M KCl can be used to elute the enzyme from the resin.

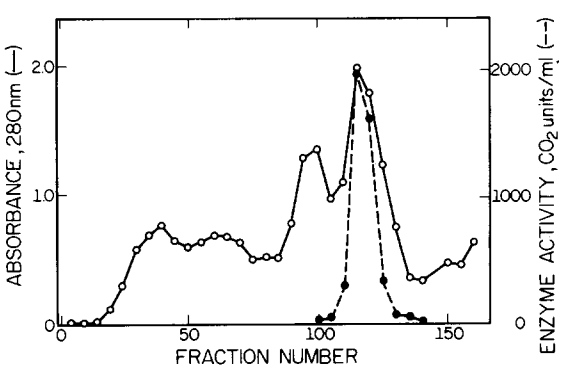


Fig. 5. DEAE-Sephadex elution pattern. The conditions for running the column are described in the text.

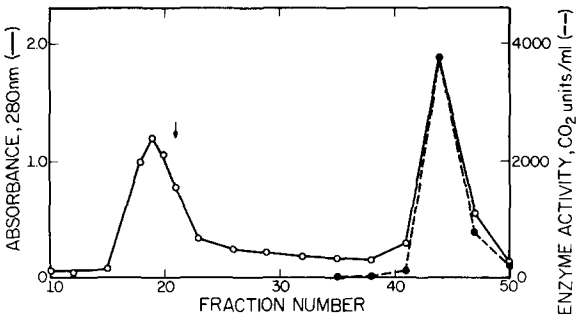


Fig. 6. Preparative scale thiamine pyrophosphate-Sepharose elution pattern. The column was run as described in text. At the arrow, 50 mM potassium phosphate buffer, 100 mM KCl, 15% glycerol, pH 5.7, was applied to the column.

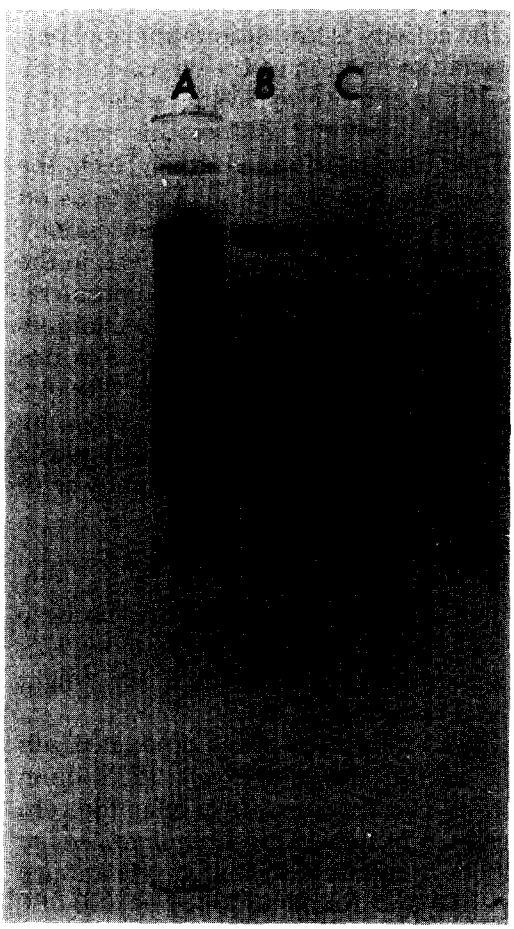


Fig. 7. Polyacrylamide gels showing purification obtained by thiamine pyrophosphate-Sepharose. Tris-barbital gels were run as described in the text. Gels B and C each contain about 10 μg of protein. A, concentrated pool from DEAE column; B, concentrated pool from thiamine pyrophosphate-Sepharose column; C, protein after low ionic strength precipitation.

TABLE III

SPECIFIC ACTIVITY BASED ON PROTEIN AND ON FLAVIN CONTENT

Data are averages of three preperations.

Step	$\frac{A_{280\ nm}}{A_{260\ nm}}$	$\frac{A_{280\ nm}}{A_{438\ nm}}$	Specific acitvity (CO_2 units/mg)	
			Based on protein	Based on flavin
Concentrated pool after affinity chromatography	1.34	9.28	3065	5667
After low ionic strength precipitation	1.24	5.63	5531	5789

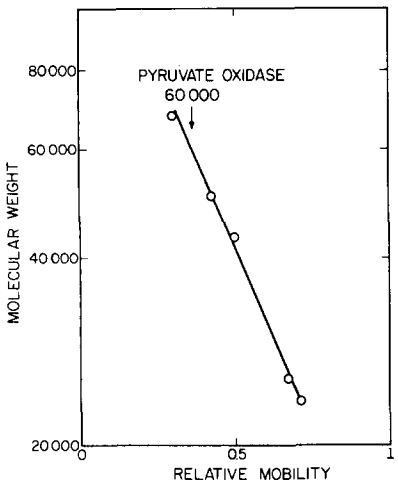
The enzyme eluted from this column has a specific activity between 3000 and 4000 CO_2 units/mg. Analysis by Tris-barbital acrylamide gels (Fig. 7) and sodium dodecyl sulfate acrylamide gels indicates that this material is always greater than 90% a single protein species. Absorbance studies summarized in Table III probably indicate why the specific activity is low despite the apparent purity of the enzyme preparation. A substantial fraction of the enzyme is deficient in FAD, i.e. the predominant impurity is inactive pyruvate oxidase. The simple addition of FAD to the assay buffer does not restore this activity.

(h) Low ionic strength precipitation. When this is performed slowly, as in the original purification protocol, pyruvate oxidase will crystallize. Variations on this technique are the only methods which have been found to successfully separate active from inactive pyruvate oxidase. The use of rapid dialysis in the presence of glycerol not only shortens the time required for this step considerably but also greatly increases its reproducibility. Using previous techniques large losses of enzyme activity frequently resulted during this step. For this reason, much of the recently reported work on pyruvate oxidase was performed on enzyme which had a low specific activity, below 3000 CO_2 units/mg [3,4]. The present procedure for low ionic strength precipitation usually yields oxidase with a specific activity of over 5000 CO_2 units/mg and usually with 80—90% recovery.

Enzyme characterization

The absorption characteristics of the purified pyruvate oxidase are the same as those reported by Williams and Hager [2]. The precipitated enzyme contains a full complement of FAD, using the same optical parameters and dry weight protein determinations used by Williams and Hager [2].

The protein is better than 95% pure based on optical scans of stained gels using the following techniques: (1) Tris/barbital acrylamide gels; (2) sodium dodecyl sulfate acrylamide gels; (3) micro-isoelectric focusing acrylamide gels; (4) Tris-barbital gels run in the presence of 8 M urea. The subunit molecular weight was determined using sodium dodecyl sulfate acrylamide gels and gel filtration in the presence of guanidine · HCl and found to be about 60 000 (Figs. 8 and 9). Also the protein tetramer molecular weight was determined by sedimentation velocity-diffusion experiments and was found to be about 254 000 (Russell, P., Raj, T. and Gennis, R.B., unpublished results). Williams and Hager [2] had previously calculated the subunit molecular weight to be



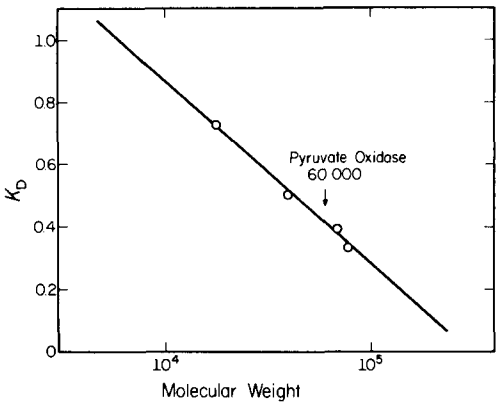


Fig. 8. Molecular weight determined by sodium dodecyl sulfate gel electrophoresis. See text for details. Pyruvate oxidase gave a single band at about 60 000. The following proteins were used to determine the standard molecular weight curve: bovine serum albumin (68 000); γ-globulin, H-Chain (50 000); ovalbumin (43 000); chymotrypsinogen (25 700) and γ-globulin, L-Chain (23 500).

Fig. 9. Gel filtration chromatography in the presence of 6 M guanidine · HCl. The column was run as described in the text. The following proteins were used to determine the standard curve: transferrin (76 000), bovine serum albumin (68 000), aldolase (40 000), and lysozyme (17 800). Blue dextran and [^{14}C]glucose were used to determine the excluded and included volumes of the column.

66 000 based on FAD content. The isoelectric point was determined to be 5.62 (Fig. 10). This value is consistent with the observed affinity of the enzyme for anionic and cationic resins.

Both the Tris-barbital gels and isoelectric focusing gels were run in the pre-

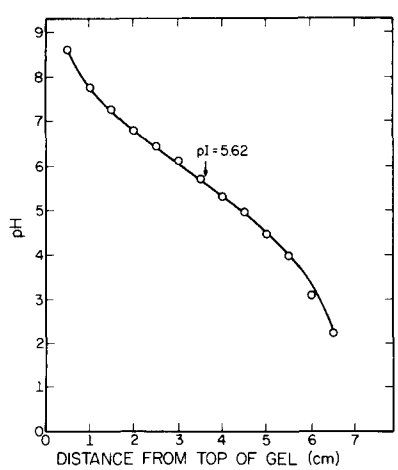


Fig. 10. Isoelectric focussing of pyruvate oxidase in polyacrylamide gels. Isoelectric focussing was performed as described in the text with 70% (pH 5—8) and 30% (pH 3—10) ampholyte mixtures to determine the pH gradient. Each of the points represents an average of nine values obtained by running nine gels simultaneously and determining each individual pH profile.

sence of glycerol to maintain enzyme activity. In both cases, enzyme activity could be clearly associated with the major protein band. This was performed by soaking the gels in assay buffer containing the dye DCIP. After about 30 min, each gel was stained blue with the exception of a narrow band which remained clear, indicating the presence of reductase activity corresponding to active enzyme. In the case of the isoelectric focusing gels, a yellow band is apparent at the location of the active enzyme, and staining for protein is not necessary to locate the oxidase.

Discussion

A preparative scheme for purifying pyruvate oxidase has been reported which takes less than half the time of the previously reported procedure, is much more reproducible, and produces enzyme with comparable purity and yield. The basic protein characterization has also been reported. This work confirms the earlier conclusions that pyruvate oxidase is a tetramer with one FAD bound per subunit. Furthermore, it is indicated that these subunits are probably identical. Urea acrylamide gels and sodium dodecyl sulfate acrylamide gels both indicate a single predominant subunit species. Such conclusions are relevant to any future research on the ligand and lipid binding properties of pyruvate oxidase.

The thiamine pyrophosphate-Sepharose is the first reported affinity resin based on thiamine pyrophosphate in which the pyrophosphate group is not used in linkage to the resin but is freely accessible for binding by protein. One other thiamine pyrophosphate-Sepharose affinity resin has been reported [19] in which the thiamine pyrophosphate seems to be linked to a diaminoethane arm through a phosphoamide linkage. Thus, this resin, though suitable for the purification of thiamine binding proteins, would probably not be useful for the purification of those thiamine pyrophosphate-requiring enzymes in which the pyrophosphate group is necessary for binding of the cofactor to the enzyme. The thiamine pyrophosphate-Sepharose and probably also the ethanolamine pyrophosphate-Sepharose can be used to purify pyruvate oxidase to near homogeneity. Although this research was motivated by the desire to study in detail the phenomenon of lipid activation of this peripheral membrane enzyme, it is hoped that the thiamine pyrophosphate affinity chromatography resins which resulted from this work will prove useful to others who are working with enzymes which require thiamine pyrophosphate as a cofactor.

The low specific activity of the homogeneous pyruvate oxidase eluted from the thiamine pyrophosphate affinity columns probably results from a portion of the enzyme missing FAD. Current work is directed at either avoiding the accumulation of flavin-deficient pyruvate oxidase or successfully replacing this missing cofactor.

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