Journal of Chromatography, 258 (1983) 213–222
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 15,519

PURIFICATION OF HUMAN ERYTHROCYTE PHOSPHOGLYCERATE KINASE BY DYE LIGAND AFFINITY CHROMATOGRAPHY

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(First received September 16th, 1982; revised manuscript received November 13th, 1982)

SUMMARY

A new method for the purification of human erythrocyte phosphoglycerate-kinase involving affinity chromatography on dye-ligand media (Red A), in the presence of 3-phosphoglycerate and ATP, is described. The method is rapid and technically simple. The purity of the enzyme was verified by electrophoresis in polyacrylamide gel in the presence of sodium dodecylsulphate, by amino acid analysis and by immunoprecipitation in Ouchterlony plates. Peptide mapping of tryptic digests of the purified enzyme was performed and the immunoneutralization of the enzyme activity evaluated with rabbit antibodies.

INTRODUCTION

Human erythrocyte phosphoglycerate kinase (PKG: adenosine-3-phospho-D-glycerate-1-phosphotransferase, E.C. 2.7.2.3) has already been purified by various workers[1–4]. The importance of this ATP-producing enzyme and the existence of variants in a number of patients justify the search for new purification procedures that would permit comparative, analytical and immunological studies. With erythrocyte PGK, the presence of haemoglobin renders the procedure laborious, and in most methods numerous chromatographic steps are needed to complete the whole purification.

Our aim was to develop a relatively simple method of purification that is not time consuming and at the same time would permit the preparation of an enzyme that attained the commonly accepted criteria of purity.

EXPERIMENTAL

The reagents used for the buffers were obtained from Merck (Darmstadt, G.F.R.), Sigma (St. Louis, MO, U.S.A.) and Fluka (Buchs, Switzerland). 3-Phosphoglycerate, AMP, ATP and glyceraldehyde-3-phosphate dehydrogenase were provided by Boehringer (Mannheim, G.F.R.); NADH was a product of Sigma. Acryl-

0021-9673/83/0000–0000/$03.00

amide and bisacrylamide were supplied by Fluka. CM-Sephadex C-50 was obtained from Pharmacia (Uppsala, Sweden). Dyematrex Gel Red A was a product of Amicon (Lexington, MA, U.S.A.) and Freund's complete and incomplete adjuvant were obtained from GIBC (Grand Island, NY, U.S.A.).

Concentrations were performed on Diaflo ultrafiltration membranes (PM 10) in ultrafiltration cells obtained from Amicon.

Electrophoresis in polyacrylamide gel in the presence of sodium dodecylsulphate was carried out according to Weber and Osborn[5]. Gels were stained with Coomassie Blue R-250 after fixation in 15% acetic acid.

Protein contents were measured by the method of Lowry *et al.*[6] with bovine serum albumin as the working standard. Haemoglobin was determined by Drabkin's method[7].

Phosphoglycerate kinase activity was assayed at 25°C by monitoring the decrease in absorbance at 334 nm in an Eppendorf 1101M photometer. The backward reaction, *i.e.*, conversion of 3-phosphoglycerate to 1,3-diphosphoglycerate, was determined as described by Bücher[8]. The reaction mixture contained 50 m*M* triethanolamine, 0.25 m*M* EDTA at pH 7.5, 10 m*M* magnesium chloride, 3 m*M* ATP, 2 m*M* 3-phosphoglycerate, 0.3 m*M* NADH and an excess of glyceraldehyde-3-phosphate dehydrogenase.

One enzyme unit is defined as the activity catalysing the utilization of 1 μmole of 3-phosphoglycerate per minute. Results are expressed in international units (IU).

The materials and methods used to elaborate the peptide map (*i.e.*, aminoethylation, tryptic hydrolysis, analytical fingerprints and specific staining), and for amino acid analysis on a Biotronik LC 6001 amino acid analyser, have been described previously[9].

Rabbit antibodies to PGK were prepared as follows: each rabbit (Blanc de Bouscat, 2.5 kg) was injected intramuscularly with a mixture containing 1.5 mg of purified enzyme in 2 ml mixed with 2 ml of Freund's complete adjuvant. For the subsequent injections, at intervals of 10 days, 1 mg of enzyme was injected in the same way, but with incomplete adjuvant. Before being bled, each rabbit had received a total of 4.5 mg of enzyme.

Immunoneutralization

Before utilization, all the rabbit sera were heated at 56°C for 60 min. The haemolysate and the partially purified or purified enzyme were diluted to a specific enzyme concentration and mixed with various dilutions, on the one hand of anti-PGK rabbit serum and on the other of normal rabbit serum. The mixtures were incubated at room temperature for 60 min and the residual enzymatic activity was assayed in the whole mixtures.

Immunodiffusion

Anti-PGK serum was tested by immunodiffusion according to the method of Ouchterlony[10].

RESULTS

Purification procedure

The complete purification procedure is summarized in Table I and the different steps are described below.

TABLE I

DIFFERENT STEPS IN THE PURIFICATION PROCEDURE FOR HUMAN ERYTHROCYTE PHOSPHO GLYCERATE KINASE

Fraction	*Total volume (ml)*	*Protein (mg)*	*Adenylate kinase activity (IU)*	*Phosphoglycerate kinase activity*		*Yield (%)*
				Total units	*Specific activity (IU/mg)*	
Haemosylate	675	$67.5 \cdot 10^3$	14,850	8000	0.12	100
Supernatant of ethanol–chloroform treatment	530	$1 \cdot 10^3$	910	4750	4.75	59
Precipitate with ethanol dissolved in buffer and dialysed	82	820	145	4260	5.2	53
Eluate from CM-Sephadex	59	19.5	0	2770	142	35
Eluate from Dyematrex gel (Red A)	5	5.3	0	2330	440	29

Elimination of haemoglobin from the haemolysate by ethanol–chloroform precipitation. Washed human erythrocytes (225 ml) were mixed with 450 ml of cold haemolysing solution (for 450 ml: 445.5 ml of water, 4.5 ml of 0.27 *M* EDTA and 225 μl of 14 *M* β-mercaptoethanol). The haemolysate contained 131 IU of PGK per gram of haemoglobin. The method used for the elimination of haemoglobin was that described by Yoshida and Watanabe[2], adapted to our conditions. A 225-ml volume of ethanol–chloroform (2:1) cooled to -60°C was added to 675 ml of ice-cold haemolysate previously adjusted to pH 7.3 with 1 *M* potassium hydroxide solution. The mixture was stirred for 20 min in an ice-bath and then centrifuged (15,000 *g*) for 20 min at 4°C. The haemoglobin precipitate was discarded. The precipitation of PGK from the light orange supernatant was effected at -15°C in an ice — salt bath: to 1 volume of supernatant were added 2.5 volumes of ethanol cooled to -25°C. After stirring for 20 min, the precipitate was collected by centrifugation at 15,000 *g* for 15 min. The pink precipitate was dissolved in 60 ml of a buffer containing 10 m*M* Tris–HCl (pH 8.0), 1 m*M* magnesium chloride, 1 m*M* EDTA and 1 m*M* β-mercaptoethanol.

After stirring for 30 min, the undissolved fraction was eliminated by centrifugation at 5000 rpm for 15 min. The orange supernatant was dialysed overnight in boiled dialysis bags at 4°C against the next buffer: 5 m*M* Tris–HCl at pH 6.4 and 1 m*M* EDTA.

Carboxymethyl-Sephadex C-50 column chromatography. The dialysed supernatant (82 ml) was concentrated to approximately 8 ml in an ultrafiltration cell. The concentrated solution was then loaded on to a CM-Sephadex C-50 column (35 × 2.5 cm I.D.) previously equilibrated at 4°C with the dialysis buffer containing 5 m*M* Tris–HCl at pH 6.4 and 1 m*M* EDTA. The flow-rate was 50 ml/h. The non-haeme proteins were eliminated by two successive buffers (Fig. 1): buffer A (100 ml of equi-

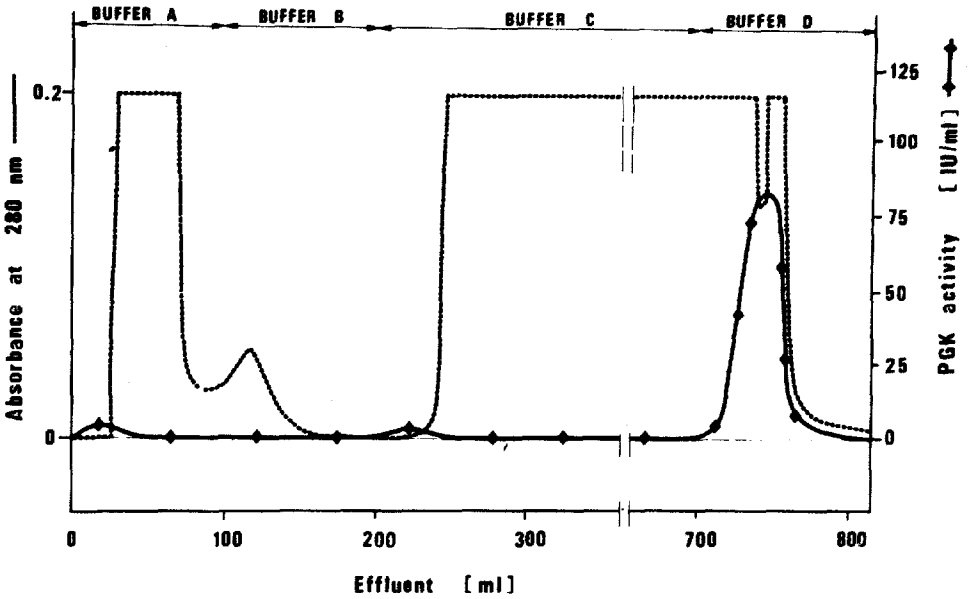

Fig. 1. Elution pattern from CM-Sephadex column. The column (35 × 2.5 cm I.D.) was equilibrated with 5 m*M* Tris–HCl buffer at pH 6.4 and PGK was eluted with 5 m*M* Tris–HCl (pH 8.0), 1 m*M* EDTA, 1 m*M* β-mercaptoethanol, 25 m*M* KCl and 4 m*M* 3-phosphoglycerate. ◆——◆, Phosphoglycerate kinase activity.

librium buffer containing 1 m*M* β-mercaptoetanol) and then buffer B (100 ml of equilibrium buffer containing 1 m*M* β-mercaptoethanol and 25 m*M* potassium chloride).

Once this step had been completed, no further protein was eluted from the column, and buffer C (100 ml of equilibrium buffer containing 1 m*M* β-mercaptoethanol, 25 m*M* potassium chloride and 1 m*M* AMP) was added. This elution, which washes away 5% of PGK, was left overnight at a reduced flow-rate (30 ml/h). After washing with buffer C, PGK was eluted with buffer D [5 m*M* Tris–HCl (pH 8.0), 1 m*M* EDTA, 1 m*M* β-mercaptoethanol, 25 m*M* potassium chloride and 4 m*M* 3-phosphoglycerate] at a flow-rate of 50 ml/h.

The presence of both AMP and haemoglobin in the column effluent makes it impossible to follow the protein absorbance at 280 nm, and consequently the elution pattern of the PGK. For this reason, fractions of 5 ml per tube were collected and tested for PGK activity. The major PGK fraction was eluted in approximately 60 ml. The effluent pool, which still remained orange, was then concentrated in an ultrafiltration cell to a final volume of less than 1 ml (500–800 μl) and dialysed overnight at 4°C in a boiled dialysis bag (1 cm diameter) against the following buffer: Tris–HCl (pH 8.0) 1 m*M* magnesium chloride, 1 m*M* EDTA and 1 m*M* β-mercaptoethanol.

Dyematrex (Red A) gel column chromatography. The concentrated dialysed preparation was loaded on to a cross-linked agarose Dyematrex Red A column (2 × 1 cm) equilibrated at 4°C with the dialysis buffer (Tris–HCl).

The column was washed with 50 ml of equilibrium buffer at its spontaneous flow-rate (30 ml/h). In the first 10 ml of eluate, a small amount of PGK may be eluted with the major part of haemoglobin and the unadsorbed proteins.

After washing, PGK was eluted with 10 ml of the equilibrium buffer containing 2 m*M* 3-phosphoglycerate and 1 m*M* ATP. Fractions of 1 ml per tube were collected and tested for PGK activity (Fig. 2). As for the CM-Sephadex column, the presence of ATP makes it impossible to follow the elution of the enzyme by monitoring absorbance at 280 nm. Tubes containing the highest enzymatic activity (about 5 ml) were pooled. These represent the purified PGK. Under the above conditions, 5.3 mg of purified enzyme were obtained, with a specific activity of 440 (3666-fold purification). The yield of enzyme protein of the Dyematrex column was 84%.

Criteria of purity

The purity of the enzyme was tested by electrophoretic, analytical and immunological methods.

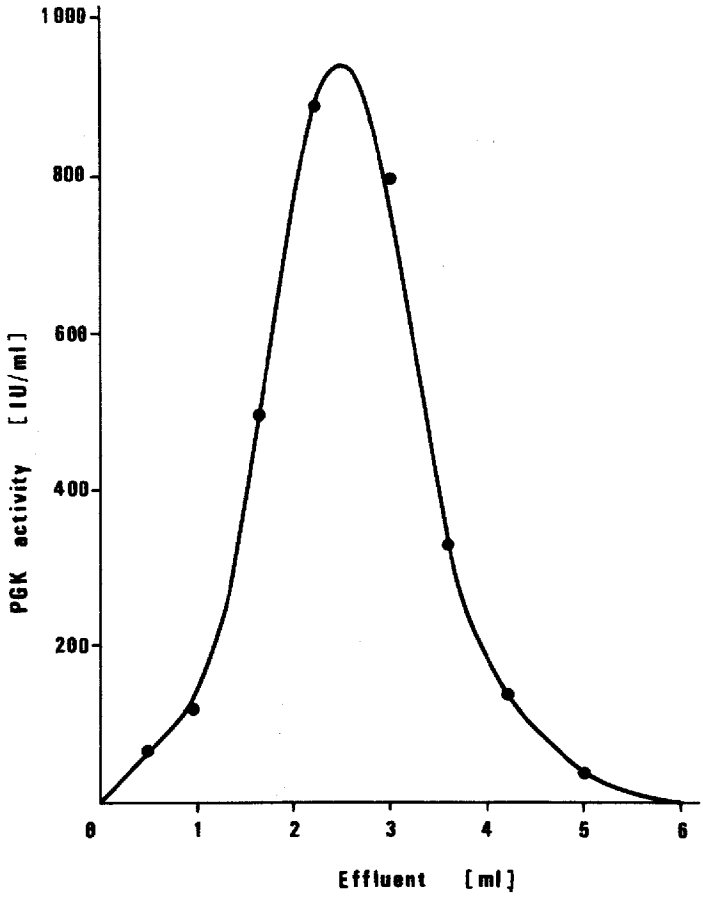

Fig. 2. Affinity elution pattern of phosphoglycerate kinase on Dyematrex Red A column (2 × 1 cm I.D.). The column was equilibrated with 10 m*M* Tris—HCl (pH 8.0), 1 m*M* EDTA and 1 m*M* β-mercaptoethanol buffer. The PGK was eluted with the equilibrium buffer containing 2 m*M* 3-phosphoglycerate and 1 m*M* ATP.

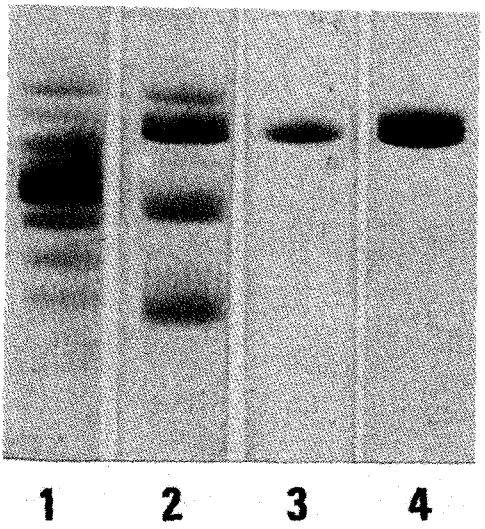

Fig. 3. Sodium dodecylsulphate polyacrylamide gel electrophoresis of phosphoglycerate kinase at each of the three streps of purification. (1) Ethanol-precipitated extract, 120 μg of protein; (2) partially purified PGK after elution on CM-Sephadex column, 100 μg of protein; (3) and (4) purified PGK after elution on Dyematrex gel. In (3) the sample contained 15 μg and in (4) 50 μg of protein. The gels were stained with Coomassie Blue R-250.

Sodium dodecylsulphate gel electrophoresis. Fig. 3. illustrates the various steps of the enzyme purification. Only one band was visible in the gel containing the purified PGK, even when a large amount of sample was applied (50 μg).

Double immunodiffusion (Ouchterlony). As shown in Fig. 4, a single precipitation line was observed between the anti-PGK serum and the different tested preparations: haemolysate (well 2), partially purified PGK after elution on CM-Sephadex (well 3) and the purified PGK (well 1).

Amino acid analysis. A 100-μg amount of purified PGK was dialysed against 1% formic acid, lyophilized and hydrolysed. The hydrolysate was analysed for its amino acid composition on a Biotronik LC 6001 amino acid analyser. The values

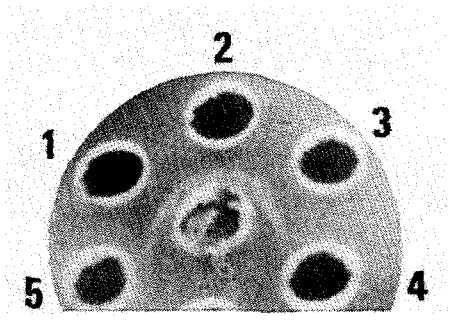

Fig. 4. Double immunodiffusion of antiserum to purified phosphoglycerate kinase and various preparations. Centre well: rabbit antiserum to purified PGK. (1) Purified PGK (5 μg); (2) human haemolysate (2 mg); (3) partially purified PGK after elution on CM-Sephadex (25 μg); (4) and (5) buffer.

TABLE II

AMINO ACID COMPOSITION OF HUMAN ERYTHROCYTE PHOSPHOGLYCERATE KINASE

Not determined: proline and tryptophan.

Amino acid	*Number of amino acid residues per molecule*	
Aspartic acid	48	(48)**
Threonine	15	(17)
Serine	25	(24)
Glutamic acid	33	(33)
Glycine	43	(40)
Alanine	41	(41)
Cysteine	7	(7)
Valine*	31	(39)
Methionine	11	(13)
Isoleucine	15	(19)
Leucine	38	(38)
Tyrosine	4	(4)
Phenylalanine	15	(16)
Histidine	5	(5)
Lysine	41	(43)
Arginine	10	(11)
Total	382	(398)

* Values after hydrolysis for 20 h.

** Yoshida and co-workers' values from the amino acid sequence in ref. 11.

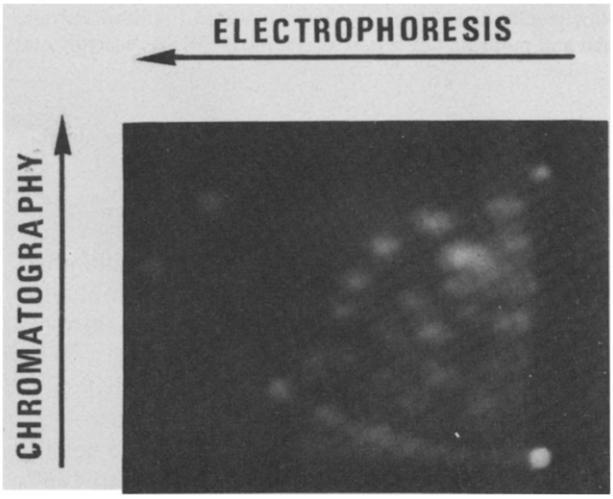

Fig. 5. Fluorescamine staining of the peptide map of tryptic digests (100 μg) of aminoethylated erythrocyte phosphoglycerate kinase on silica gel G-1500. First dimension: electrophoresis for 3 h at 260 V and 15 mA in pyridine–acetic acid–water (1:10:90), pH 3.5. Second dimension: chromatography for 5 h in pyridine–acetic acid–butanol–water (32:8:40:21).

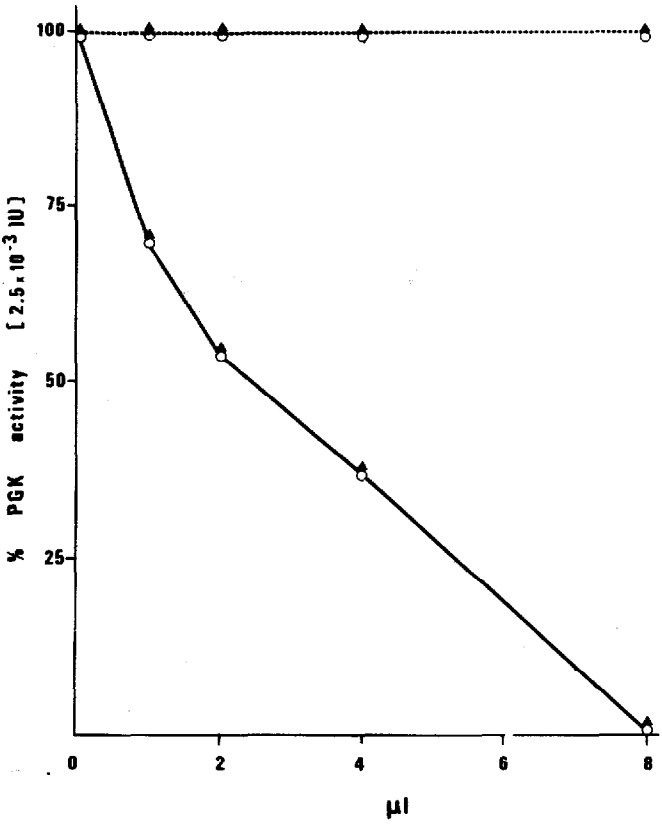

Fig. 6. Immunoneutralization of phosphoglycerate kinase from the haemolysate and purified enzyme. ——, Normal rabbit serum; ———, rabbit anti-purified PGK serum. ○, Purified PGK; ▲, haemolysate.

obtained for the number of amino acid residues per molecule (Table II) are similar to those obtained by Yoshida and co-workers[2,11,12].

Peptide mapping of purified PGK

A 3.5-mg amount of purified PGK was dialysed against water and submitted to the successive treatments for peptide mapping. Fig. 5 shows the pattern obtained from 100 µg of tryptic hydrolysate after staining with fluorescamine. About 30 spots can be readily distinguished.

Immunoneutralization of PGK from haemolysate and purified enzyme

As can be seen in Fig. 6, the haemolysate and purified enzyme were equally neutralized by the anti-PGK rabbit serum. Use of 8 µl of antiserum led to 100% inhibition of enzyme activity with both the haemolysate and the purified preparation. No inhibition of the enzymatic activity occurred with normal rabbit serum when mixed with either the haemolysate or purified PGK.

DISCUSSION

The proposed method of purification of erythrocyte PGK involves three steps and enabled us to obtain more than 5 mg of purified enzyme in 3.5 days. The purification is 3666-fold and the yield is approximately 30%.

Affinity chromatography[4,13–16] or affinity elution chromatography[17,18] have already been used for enzyme purification. More recently, Lowe *et al.*[19] isolated yeast PGK using high-performance liquid affinity chromatography (HPLAC) on Cibracron Blue F3G-A silica. This adsorbent appears to be particularly efficient in protein purification, as it specifically binds a wide range of proteins. However, with erythrocyte PGK, the method that provides the best yield, *i.e.*, an affinity chromatography[4], involves first the preparation of the adsorption material, which is time consuming and sometimes difficult to effect efficiently. Our method has a yield inferior to that obtained by affinity chromatography, but one of its advantages is that no preliminary material need be prepared. Further, it is perfectly adequate for the rapid and economical preparation of PGK.

The first step in the purification procedure involves the precipitation of haemoglobin by ethanol–chloroform treatment. For this step, we followed Yoshida and Watanabe's method[2]; however, during the numerous preparations that we have performed, we have been unable to achieve the high yield that they reported. Although this step is drastic, it is nevertheless essential, as it allows the elimination of the major part of adenylate kinase, as shown in Table I. About 99% of this enzyme was eliminated after ethanol precipitation.

Adenylate kinase and PGK have very similar ligands, given that adenylate kinase catalyses the reaction AMP + ATP $\rightleftharpoons$ 2 ADP. The last step in the purification procedure involves elution with ATP and at this point no more adenylate kinase should remain on the column. It is for this reason that CM-Sephadex chromatography involves elution of the remaining adenylate kinase, and other proteins (probably other kinases), in the presence of AMP. Of course, PGK was also eluted simultaneously, but this amounted to no more than 5% of the total.

After elution from the CM-Sephadex column, the partially purified PGK (Fig. 3) had a specific activity of 140.

The last step of the purification was effected on a Dyematrex (Red A) column and, as shown in Table I, the yield was 84%. This ready-made column is very convenient to use, and given its limited dimensions (2 × 1 cm I.D.), PGK can be rapidly eluted, and in a small volume. The rapidity of elution of the enzyme is probably one explanation for the excellent yield from the Dyematrex column. Before using this small column, we used a larger one (18 × 2.5 cm I.D.) with the same gel. Under these conditions, the yield was only 30%. Once regenerated, the column can be used again a large number of times without any loss in its adsorption capacity. As shown in Table I, about 20 mg of protein can be loaded on to the column, but this amount can be larger.

In most erythrocyte purification procedures, the main problem is to eliminate haemoglobin completely at the beginning of the procedure. In our method, the complete elimination of haemoglobin is not necessary, as the partially purified PGK eluted from the CM-Sephadex column still contains some of it. The major part of this remaining haemoglobin was eliminated during the washing of the Dyematrex

column, and the small amount that remained adsorbed on the column did not perturb the elution of the PGK.

The enzyme is eluted in a total volume of 5 ml, at a concentration of usually 1 mg/ml; its specific activity is usually about 440. In the presence of its two ligands the purified PGK eluted from the Dyematrex column can be maintained in solution at −80°C for several months.

The enzyme is pure, as shown by electrophoresis in polyacrylamide gel in the presence of sodium dodecylsulphate (Fig. 3) and double immunodiffusion in gelose (Fig. 4), and its amino acid composition is indistinguishable from that established by Yoshida and co-workers (Table II).

The entire procedure for peptide mapping (Fig. 5) was carried out on two different preparations of enzyme; prior to treatment, the purity of the enzyme was verified by amino acid analysis. The two patterns obtained were essentially identical.

As can be seen in Fig. 6, no difference could be observed between immunoneutralization of the haemolysate or of purified PGK by the specific rabbit antiserum. With both preparations, immunoneutralization was complete. This result confirms those obtained by Cottreau *et al.*[17] with granulocyte PGK, but contrasts with the results of Okonkwo *et al.*[3], who found that the haemolysate could not be completely neutralized by anti-PGK antibodies.

In conclusion, the method of purification of human erythrocyte described here allows one to obtain in the shortest possible time an enzyme that allows comparative, analytical and immunological studies.

ACKNOWLEDGEMENTS

This work was supported by the Institut National de la Santé et de la Recherche Médicale, CRL No. 82 1053, by the Centre National de la Recherche Scientifique and by l'Université Paris Val-de-Marne. The authors thank Dr. J. Chapman for careful assistance in reviewing the manuscript.

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