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PURIFICATION OF THE M-TYPE PHOSPHOGLYCEROMUTASE FROM RABBIT MUSCLE

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SUMMARY

A new method for the purification of M-type phosphoglyceromutase from rabbit muscle involving affinity chromatography on dye ligand media in the presence of 2,3-diphosphoglycerate 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 dodecyl sulphate and by Cellogel electrophoresis. Immunological techniques showed that the anti-M antibodies are specific and do not cross-react with the B isozyme.

INTRODUCTION

Phosphoglyceromutase (2,3-bisphospho-D-glycerate; 2-phospho-D-glycerate phosphotransferase; EC 2.7.5.3) is a glycolytic enzyme present in all mammalian tissues. Three isozymes have been described that result from dimeric combination of two subunits and differ in their electrophoretic mobility, thermostability and sensitivity to the sulphuryl groups[1–7]. The B-type isozyme is found in brain, liver, kidney and erythrocyte; the muscle contains almost exclusively the M-type. Both types are present in heart tissues together with the MB hybrid. In some cases, the presence of B-type and MB hybrid can be observed in rabbit muscle. Phosphoglyceromutase has previously been purified from rabbit muscle[8–10]. The authors used a long-winded technique with five steps of purification, including heat denaturation, ammonium sulphate precipitation and alcohol fractionations. They did not try to obtain antibodies. In this work, we have developed a simple method of isolating the rabbit muscle form of the enzyme (free of B and MB forms) by using three steps of chromatography, including dye ligand column chromatography; we have obtained specific antibodies that do not cross-react with rabbit B-type phosphoglyceromutase.

EXPERIMENTAL

Materials

All substrates and enzymes were purchased from Boehringer Mannheim Bio-

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chemicals. Buffer salts, 2-mercaptoethanol, poly(ethylene glycol) (mol.wt. 6000), sodium dodecyl sulphate and ethylenediaminetetraacetate (EDTA) were obtained from Merck. Acrylamide and bisacrylamide were supplied by Fluka. Cellogel was obtained from Chemetron. CM-Sephadex C-50 and DEAE-Sephacel were supplied by Pharmacia Fine Chemicals. Dyematrex red A was a product of Amicon (Lexington, MA, U.S.A.). Freund's complete and incomplete adjuvants were obtained from Gibco (Grand Island, NY, U.S.A.).

Enzyme assay

Phosphoglyceromutase activity was assayed as previously described[11]. The assay mixture contained, in a volume of 1 ml, 50 μmol of triethanolamine–hydrochloric acid buffer (pH 7.5), 10 μmol of magnesium chloride, 3 μmol of Na_2ATP, 0.2 μmol of reduced nicotinamide-adenine dinucleotide (NADH), 0.8 μmol of 2-phosphoglycerate, 0.08 μmol of 2,3-diphosphoglycerate, 3.3 U of glyceraldehyde phosphate dehydrogenase and 2 U of phosphoglycerate kinase.

Electrophoresis

Polyacrylamide gel electrophoresis was performed in the presence of sodium dodecyl sulphate (SDS) according to the method of Laemmli[12]. The proteins were stained with Coomassie blue R 250.

Cellogel electrophoresis was performed in 0.075 *M* Tris–citric acid–EDTA buffer (pH 8.0) at 220 V for 2 h according to methods previously described[3]. The staining mixture contained in a volume of 1 ml consisted of 50 μmol of triethanolamine–hydrochloric acid buffer (pH 7.5), 10 μmol of magnesium chloride, 5 μmol of glucose, 2 μmol of ADP, 0.8 μmol of 2,3-diphosphoglycerate, 2 μmol of 3-phosphoglycerate, 0.2 μmol of oxidized nicotinamide adenine dinucleotide phosphate (NADP), 2.2 U of enolase, 2.5 U of pyruvate kinase, 5.8 U of glucose-6-phosphate dehydrogenase, 4.7 U of hexokinase, 2.9 μmol of dimethylthiazol 2,5-diphenyltetrazolium bromide (MTT) and some crystals of phenazine methosulphate. A 1.5-ml volume of 3% liquid agarose was added to 5 ml of the staining mixture which had previously been heated to 45°C. The mixture was poured on the Cellogel strip. Phosphoglyceromutase activity was visualized as purple bands.

Immunological methods

Immunization. Two chickens were immunized by intramuscular injections of 1 ml of a mixture of 0.5 mg of the pure enzyme in 0.15 *M* sodium chloride and Freund's complete adjuvant. After two weeks, after another two weeks and after three months, the chickens were reinjected with the same amount of phosphoglyceromutase and Freund's incomplete adjuvant. After another three months, a last booster injection of 0.25 mg of enzyme and Freund's complete adjuvant was made. The best response was obtained at this time, the rate of antibodies being ten times higher than after the first booster injection for one chicken, and only twice for the second. Anti-M phosphoglyceromutase was tested by the double immunodiffusion technique according to Ouchterlony[13]. The antibodies were detected on Ouchterlony plates 5 days after the second injection for one chicken and 3 days after the third injection for the second. Immunoneutralization was performed according to the method previously described for biphosphoglyceromutase[14] and for B-type phos-

phoglyceromutase[15]. Before utilization, the chicken serum was heated at 56°C for 60 min to eliminate intrinsic serum phosphoglyceromutase activity. The pure enzyme and the tissue extracts were diluted to a phosphoglyceromutase concentration of 2.5 U/ml.

Purification procedure

All steps of purification were performed at 4°C except when otherwise specified. Ammonium sulphate precipitation was performed according to Petell[16] who used the method for purification of chicken breast muscle. Some modifications were made in the technique. Skeletal muscle obtained from the hind leggs of rabbits was rinsed with cold 0.9% sodium chloride. Tissue extracts (7.39) were prepared by homogenization with a mixer homogenizer for 2 min in 10 volumes of 10 m*M* Tris–HCl buffer (pH 7.5) containing 1 m*M* 2-mercaptoethanol and 1 m*M* EDTA (buffer A). Then, debris was removed by centrifugation at 20 000 *g* for 30 min. The supernatant was adjusted to 40% ammonium sulphate saturation at pH 5.1 with 1 *M* acetic acid, left to stand for 2 h under gentle stirring at room temperature and centrifuged at 20 000 *g* for 30 min. The precipitate was eliminated. Solid ammonium sulphate was added to the supernatant to obtain 75% saturation and the pH was adjusted to 7.5 with 1 *M* sodium hydroxide. The mixture was allowed to stand for 60 min, then it was centrifuged at 20 000 *g* for 30 min. The precipitate was dissolved in a minimum volume of buffer A and dialysed for 1 h at room temperature, then overnight against the buffer at 4°C.

DEAE-Sephacel chromatography. The concentrated sample was applied on a DEAE-Sephacel column (10 × 1.5 cm I.D.) equilibrated with buffer A. The column was then washed with the equilibrating buffer at its spontaneous flow-rate. Under these conditions, the M-type phosphoglyceromutase was not retained on the column. Fractions containing the enzyme were collected. The column was regenerated with 1 *M* potassium chloride and stored with 0.2% benzalkonium chloride in the equilibrating buffer.

CM-Sephadex C-50 chromatography. The eluate was directly applied on a CM-Sephadex column (18 × 2 cm I.D.) previously equilibrated in the same buffer A. The column was washed with the equilibrating buffer at a slow flow-rate (25 ml/min). The M isozyme of phosphoglyceromutase was not retained by the column and was spontaneously eluted. The column was regenerated with 1 *M* potassium chloride and stored with 0.02% natrium azide in buffer A.

Affinity chromatography. A cross-linked agarose Dyematrex red A column (2 × 1 cm I.D.) was regenerated with an 8 *M* urea solution at 25°C to eliminate excess dye. Then the column was washed at 25°C with 10 ml of buffer A and equilibrated at 4°C with 10 ml of the same buffer. The concentrated eluate from the CM-Sephadex containing 4.6 mg in a total volume of 0.5 ml was applied to the gel. The column was subsequently washed with 40 ml of buffer A containing 1 m*M* ADP to elute the remaining contaminant proteins possessing a nucleotide fold, and then with buffer A containing 0.02 *M* potassium chloride until no further absorbance was detected at 280 nm. Phosphoglyceromutase was eluted with the same buffer containing 2 m*M* 2,3-diphosphoglycerate. The column was regenerated with 10 ml of 1.5 *M* potassium chloride and washed with 20 ml of 10 m*M* Tris–HCl buffer (pH 7.5). This column was stored at +4°C in the same buffer containing 0.02% sodium azide, and may be re-used many times.

TABLE I

PURIFICATION PROCEDURE FOR RABBIT MUSCLE PHOSPHOGLYCEROMUTASE

Protein contents were measured by absorbance at 280 nm with bovine albumin as working standard.

Purification steps	*Total protein (mg)*	*Total activity (I.U.)*	*Specific activity (I.U./mg protein)*	*Yield (%)*	*Purification (-fold)*
Tissue extract	627	3764	6	–	–
Ammonium sulphate precipitation	158	3124	19.8	83	3.3
DEAE-Sephacel chromatography	76	2593	34	69	1.7
CM-Sephadex chromatography	4.6	1893	411	50	12
Dyematrex red A chromatography	3	1211	440	32	0.94

RESULTS AND DISCUSSION

The proposed method of purification of muscle phosphoglyceromutase involved four steps and enabled us to obtain 3 mg of purified enzyme. The results are summarized in Table I. The final yield was approximately 32% with a 73-fold increased specific activity. The purified M-type phosphoglyceromutase had a specific activity of *ca.* 440 U/mg. The first step of the purification procedure involved ammonium sulphate precipitation. This step was essential because it allowed the elimination of the major part of creatine kinase. About 99% of this enzyme was precipitated with 40% ammonium sulphate. The second step involved a DEAE-Sephacel column. The increase of the specific activity is only 1.7-fold but the yield is excellent, probably because at pH 7.5 the M-type phosphoglyceromutase did not bind to the DEAE ion-exchanger and was spontaneously eluted with the equilibrating buffer. Usually muscle contains almost exclusively M-type isozyme but sometimes traces of MB- and B-types are present and are completely retained on the Sephacel column, just like phosphoglucomutase which is eluted together with phosphoglyceromutase if it is still present in the affinity chromatography step. The most effective step was the CM-Sephadex C-50 column, which provided the greatest increase of specific activity as shown in Table I. CM-Sephadex did not bind the M-type phosphoglyceromutase but retained many proteins at pH 7.5. Complete elimination of two of them, phosphoglycerate kinase and phosphoglucose isomerase, is necessary at this point, because if present they are also eluted simultaneously with 2,3-diphosphoglycerate from the next Dyematrex column. The last step of purification was effected on a Dyematrex red A column and the yield was 64%. Before elution of phosphoglyceromutase, the equilibrating buffer containing 1 m*M* ADP eliminated some protein contaminants and especially triose phosphate isomerase. A wash with 20 m*M* potassium chloride in buffer A, a solution of ionic strength just below that required to elute phosphoglyceromutase, removed the remaining undesirable proteins. Elution of phosphoglyceromutase was obtained with 2 m*M* 2,3-diphosphoglycerate in the equilibrating buffer containing 20 m*M* potassium chloride. Identical results were ob-

tained with 2 m*M* 2-phosphoglycerate, but the elution profile was not so sharp. Instead of Dyematrex red A, we tried Dyematrex blue A which gave similar results but the yield was only 50%. Our attempt to avoid one of the steps preceding Dyematrex red A chromatography always failed, because 2,3-diphosphoglycerate is not a specific ligand for phosphoglyceromutase. It also eluted three weakly bound proteins (phosphoglucomutase, phosphoglucoisomerase and triose phosphate isomerase), along with two more tightly bound proteins (creatine kinase and phosphoglycerate kinase). The purity of the enzyme has been checked by electrophoresis in polyacrylamide gel in the presence of sodium dodecyl sulphate (Fig. 1). Only one band was visible when the purified phosphoglyceromutase was applied. In Fig. 2, the Cellogel electrophoretic pattern confirmed that the purified sample was totally free of the other B- and MB-types. Immunological methods confirmed the purity of the protein. As seen in Fig. 3, on Ouchterlony plates, a single precipitation line was observed between the anti-M phosphoglyceromutase serum and the two different preparations tested; rabbit muscle extract and purified M-type phosphoglyceromutase. No cross-reaction was obtained with the B isozyme from liver extract or red cell lysate. In Fig. 4, immunoneutralization curves of phosphoglyceromutase activity

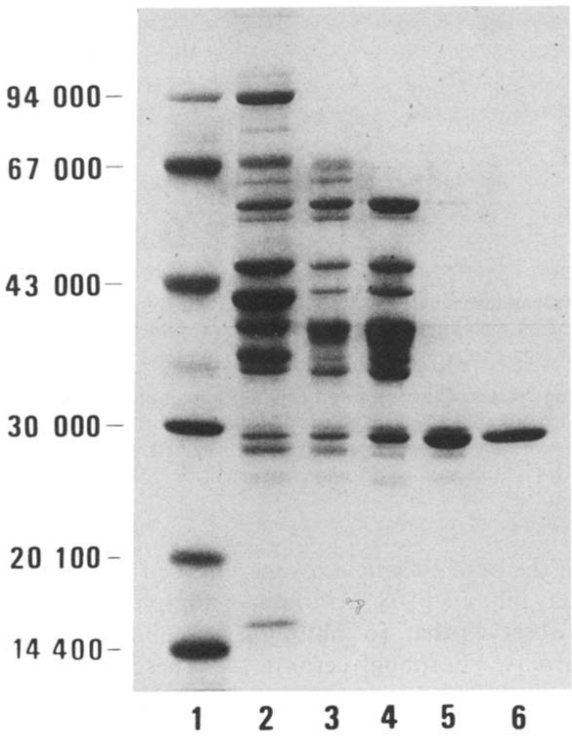

Fig. 1. SDS-polyacrylamide gel electrophoresis of phosphoglyceromutase at different steps of purification. (1) Phosphorylase *b* (mol.wt. 94 000), bovine serum albumin (mol.wt. 67 000), ovalbumin (mol.wt. 43 000), carbonic anhydrase (mol.wt. 30 000), soybean trypsin inhibitor (mol.wt. 20 100) and α-lactalbumin (mol.wt. 14 400); (2) muscle crude extract (80 μg of proteins); (3) fraction from 75% ammonium sulphate precipitation (50 μg); (4) fraction from DEAE-Sephacel (50 μg); (5) fraction from CM-Sephadex (10 μg); (6) pure phosphoglyceromutase (10 μg).

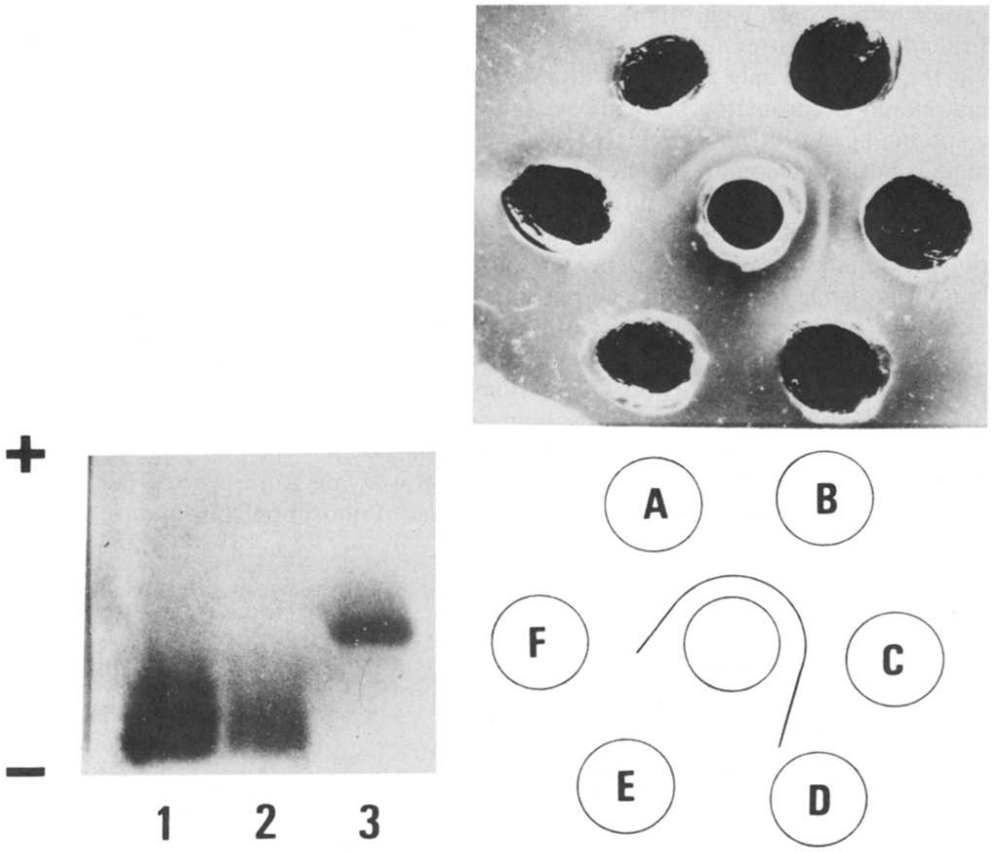

Fig. 2. Cellogel electrophoresis of phosphoglyceromutase activity. A 5-μl volume of each sample containing 1 U/ml of phosphoglyceromutase was applied on the strips. From left to right: (1) muscle extract; (2) purified M-type phosphoglyceromutase; (3) B-type phosphoglyceromutase from red blood cells[7].

Fig. 3. Double immunodiffusion on Ouchterlony plates. The centre well contained 20 μl of chicken antiserum directed against phosphoglyceromutase from rabbit. To each of the peripheral wells were added 20 μl of: pure M-type phosphoglyceromutase at two different concentrations (A, 10 μg). (B, 5 μg); muscle extract (C, 0.5 mg); haemolysate (D, 2 mg); B-type phosphoglyceromutase partially purified from haemolysate[7] (E, 120 μg); liver extract (F, 0.5 mg).

from rabbit muscle extract and from the pure enzyme are superimposed and it was found that 1 μl of antiserum completely inhibited 508 mU of phosphoglyceromutasse activity. On the contrary, it can be observed that no inhibition was obtained with the B-type from rabbit liver or erythrocyte phosphoglyceromutase. These results allow us to assume that the antibodies are completely specific of the M-type phosphoglyceromutase.

In conclusion, we have described a simple method to purify phosphoglyceromutase in the shortest time possible. Our method presents some advantages: first, the same equilibrating buffer is used during the three chromatography steps, so that it is not necessary to loose any time to dialyse; second, the three columns may be reused many times, which makes our purification procedure inexpensive; third, the

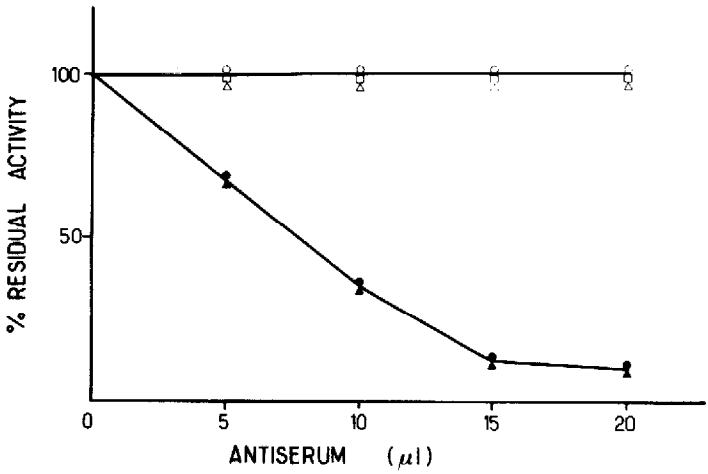

Fig. 4. Immunoneutralization of M-type phosphoglyceromutase. Chicken antiserum was 1/25 diluted. The samples consisted of: purified M-type phosphoglyceromutase (●), muscle extract (▲), liver extract (○), haemolysate (□), B type phosphoglyceromutase partially purified from haemolysate (△)[7]. The results are expressed as percentages of original activity remaining in the supernatant. The neutralized activity was calculated by extrapolation of the first portion of the curves.

enzyme is not retained on the first two columns and is eluted in the equilibrating buffer, which allows a final good yield to be obtained. The antibodies obtained from this purified preparation are specific and there is no cross-reaction between anti-M phosphoglyceromutase and the rabbit B-type isozyme, which affords additional arguments that the B and M subunits are encoded by two different genes as previously postulated[1,5,7,17].

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