Journal of Chromatography, 285 (1984) 203–209
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 16,387

Note

Purification of human erythrocyte phosphoglyceromutase

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(Received October 7th, 1983)

Phosphoglyceromutase (E.C. 2.7.5.3) is a glycolytic enzyme present in all tissues. Two isozymes have been described in mammals, *i.e.*, the M type found in muscle and the B type in brain, liver and kidney. Both types are present in heart tissues together with a hybrid of the two types[1–5]. In red blood cells the electrophoretic pattern of phosphoglyceromutase activity is complex and three bands are detectable[2,4,6]; a major band migrates like the B type and two minor bands move anodically with respect to the major one. The faster of the two minor bands has been found to carry the phosphoglyceromutase activity of the trifunctional enzyme, bisphosphoglyceromutase (E.C. 2.7.5.4)[6–9] which also contains diphosphoglycerate phosphatase activity (E.C. 3.1.3.13). No information has been published about the intermediate band.

Sheibley and Hass[10] have described a procedure to obtain phosphoglyceromutase free from biphosphoglyceromutase but they did not provide evidence for the separation of the major from the intermediate band. In this work we have developed a simple method of isolating the main fraction of phosphoglyceromutase both from other proteins and from the minor fractions.

MATERIALS

The reagents used for the buffers, sodium dodecyl sulphate and ethylenediaminetetraacetate (EDTA) were obtained from Merck. All the substrates and purified enzymes were provided by Boehringer Mannheim; NADH was a product of Sigma. Acrylamide, bis-acrylamide and 2-mercaptoethanol were supplied by Fluka. CM-Sephadex C-50 and DEAE-Sephacel were obtained from Pharmacia Fine Chemicals. Dyematrex gel red A was a product of Amicon (Lexington, MA, U.S.A.). Purified bisphosphoglyceromutase was prepared as previously described[11].

METHODS

Enzyme assays

Bisphosphoglyceromutase, diphosphoglycerate phosphatase and phosphoglyceromutase activities were assayed as previously described[12]. The assay mixtures contained: for bisphosphoglyceromutase activity, in a volume of 1 ml, 50 μmol Tris–HCl buffer (pH 8.0), 1 μmol NAD, 7 μmol fructose-1,6-diphosphate, 7 μmol

0021-9673/84/$03.00

KH_2PO_4, 2 μmol 3-phosphoglycerate, 0.2 U aldolase, 0.8 U triose phosphate isomerase and 0.15 U glyceraldehyde phosphate dehydrogenase; for diphosphoglycerate phosphatase activity, 50 μmol triethanolamine–HCl buffer (pH 7.5), 10 μmol $MgCl_2$, 3 μmol Na_2ATP, 0.2 μmol NADH, 0.8 μmol 2,3-diphosphoglycerate, 3.3 U glyceraldehyde phosphate dehydrogenase, 2 U phosphoglycerate kinase and 1 μmol 2-phosphoglycolic acid; for phosphoglyceromutase activity, as for diphosphoglycerate phosphatase activity except that the 0.8 μmol 2,3-DPG was replaced by 0.08 μmol 2,3-DPG together with 0.8 μmol 2-phosphoglycerate and 2-phosphoglycolic acid was omitted.

Purification procedure

All the steps of purification were performed at 4°C except when specified otherwise.

Hemolysis and CM-Sephadex chromatography. The first steps were identical to those used for bisphosphoglyceromutase purification[11]. Briefly, after the red cells had been washed three times in 0.9% NaCl, frozen and thawed, the hemolysate diluted 1/1 in water was passed through a CM-Sephadex column which had previously been equilibrated with 5 m*M* Tris–HCl (pH 6.5). The first fractions free from the major portion of hemoglobin contained 90% phosphoglyceromutase. These fractions were pooled, centrifuged after addition of 1 m*M* 2-mercaptoethanol and 1 m*M* EDTA and concentrated.

Isolation of the major band of phosphoglyceromutase. The concentrated sample was applied on a DEAE-Sephacel column equilibrated with 10 m*M* Tris–HCl buffer (pH 7.5). This buffer and all the buffers in the subsequent steps contained 1 m*M* 2-mercaptoethanol and 1 m*M* EDTA. After the column had been washed with the same buffer containing 70 m*M* KCl, the major band of phosphoglyceromutase was eluted with 95 m*M* KCl added to the equilibration buffer. The minor phosphoglyceromutase band remaining on the column and bisphosphoglyceromutase were eluted together in the equilibration buffer containing 120 m*M* KCl. The elutes were concentrated and dialyzed against the equilibration buffer of the next column.

Affinity chromatography. An 8 *M* urea solution at 20°C was passed through a cross-linked agarose Dyematrex red A column (2 × 1 cm). The column was then washed and equilibrated in 10 m*M* Tris–HCl buffer (pH 7.5). It was then equilibrted at 4°C. The dialyzed preparation from the DEAE-Sephacel column containing 150 mg protein in a 0.5-ml volume was applied to the gel. The column was then washed with the equilibration buffer, at its spontaneous flow-rate (30 ml/h). Residual hemoglobin and many other non-adsorbed proteins were eluted in the first 10-ml fraction. After washing until no further absorbance was detected at 280 nm, phosphoglyceromutase was eluted with the same equilibration buffer containing 1 m*M* 2-phosphoglycerate. The column was regenerated by 1.5 *M* KCl and washed with 10 m*M* Tris–HCl buffer (pH 7.5). This column may be stored at 4°C in the same buffer containing 0.02% sodium azide, and may be reused many times.

DEAE-Sephadex chromatography. The concentrated and dialyzed eluate was applied on a DEAE-Sephadex column (20 × 1 cm) equilibrated in 50 m*M* potassium phosphate buffer (pH 6.4). The column was washed with 70 m*M* potassium phosphate buffer (pH 6.4) and phosphoglyceromutase was eluted by a 120 m*M* potassium phosphate buffer (pH 6.4). The fractions containing the enzyme were concentrated and kept at −80°C.

Electrophoresis. Cellogel electrophoresis was performed on Cellogel strips (Chemetron, Milan, Italy) as previously described[3], in 0.075 *M* Tris–EDTA/citric acid buffer (pH 8.0) at 220 V for 3 h. After electrophoresis, three strips were stained, two for phosphoglyceromutase activity and the third for protein detection with 0.5% Coomassie blue.

Polyacrylamide gel electrophoresis was performed in the presence of sodium dodecyl sulphate (SDS) according to Weber and Osborn[13]. Gels were stained with 0.5% Coomassie blue.

Immunological methods. Chicken antibodies against phosphoglyceromutase were obtained as follows. The chickens were immunized by intramuscular injections of 1 ml of a mixture of purified phosphoglyceromutase in 0.15 *M* NaCl and Freund's complete adjuvant. Two weeks later and again after another 2 weeks, the chickens were reinjected with the same amount of phosphoglyceromutase and Freund's incomplete adjuvant. At this time blood was withdrawn from the wing veins and collected in heparin. After centrifugation the plasma was removed and stored at −80°C.

Immunoelectrophoresis and double immunodiffusion were performed according to methods previously described for bisphosphoglyceromutase[11]. After immunodiffusion, the agarose plates were washed with 0.9% NaCl, then dried and stained with 0.5% Coomassie blue R 250.

Immunoneutralization was performed according to the technique previously described for bisphosphoglyceromutase[11]. Alternatively, either pure phosphoglyceromutase or purified bisphosphoglyceromutase was incubated with progressively increasing amounts of chicken phosphoglyceromutase antiserum. The serum was previously heated at 56°C and centrifuged to eliminate intrinsic phosphoglyceromutase.

RESULTS

In the initial chromatography, hemoglobin was eliminated from the preparation. Separation of the major band of phosphoglyceromutase was achieved after the second chromatographic step when this band was completely isolated from the other two. Fig. 1a shows the electrophoretic pattern of phosphoglyceromutase activity in the course of its purification. In the initial hemolysate and after its elution from the CM-Sephadex column, three bands of activity were detected (channel 2). The most anodic band displayed the activity of the trifunctional enzyme, bisphosphoglyceromutase. The other two bands (one major and one minor) represented phosphoglyceromutase. In this second step of purification the major band was eluted by 95 m*M* KCl (channel 1) and completely isolated from the other two which were eluted by 120 m*M* KCl buffer. The fractions containing the major band were pooled, concentrated and subjected to further purification. A single major band of specific phosphoglyceromutase activity was then detected. The purification procedure is summarized in Table I.

The final yield was 19%. The most effective step was the "Dyematrex red A" chromatography which provided the greatest increase of specific activity. The few remaining protein contaminants were eliminated in the following DEAE-Sephadex chromatography. After this last chromatographic step the major band of phosphoglyceromutase appeared homogeneous. On Cellogel electrophoresis of the native enzyme (Fig. 1b) and on SDS-polyacrylamide gel electrophoresis (Fig. 2) only a single

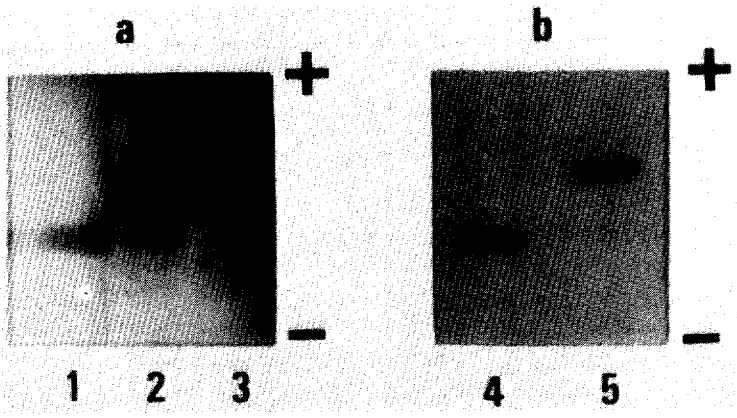


Fig. 1. Electrophoretic pattern of phosphoglyceromutase obtained as described in Materials and Methods. a, The strips were stained for phosphoglyceromutase activity. The mixture contained: 0.05 *M* triethanolamine–HCl buffer (pH 7.5); 1 m*M* $MgCl_2$; 0.8 m*M* 2-phosphoglycerate; 0.08 m*M* 2,3-diphosphoglycerate; 3 m*M* Na_2ATP; 0.25 m*M* NADH; 4.8 units phosphoglycerate kinase; 4.8 units phosphoglyceraldehydrogenase and 1% agarose. The enzymatic activity was visualized as dark bands on a fluorescent background with UV light at about 340 nm. A 5-μl volume of each sample containing 1 U/ml of phosphoglyceromutase was applied on the strips: 1, fractions of the second step of purification eluted by 95 m*M* KCl buffer; 2, fractions from the first step; 3, fractions of the second step eluted by 120 m*M* KCl buffer. b, The strips were stained for protein detection in 0.5% Coomassie blue R 250. Each sample (10 μl) contained 0.5 mg/ml of purified phosphoglyceromutase (4) or purified bisphosphoglyceromutase (5).

protein band was visible. The purified enzyme contained very slight diphosphoglycerate phosphatase activity (0.056 U/mg). No bisphosphoglyceromutase activity was detected.

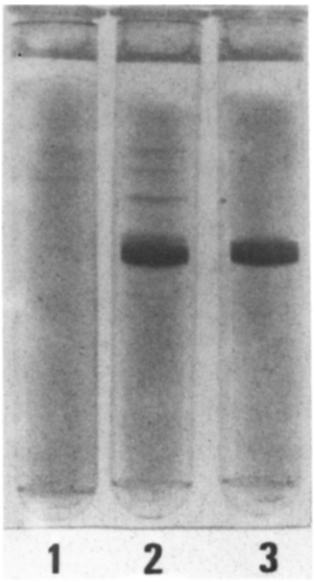
A concurrent loss of both phosphoglyceromutase and phosphatase activities was observed when the homogeneous preparation was heated at 55°C (Fig. 3). Specific anti-phosphoglyceromutase antibody produced a single arc of precipitation with a crude preparation of phosphoglyceromutase on Ouchterlony plates (Fig. 4a). However, a single line of precipitation was also obtained with pure bisphosphoglyceromutase and with a partially purified sample containing the two enzymes (Fig. 4a). No spur was detected between the arcs. On immunoelectrophoresis (Fig. 4b), phos-

TABLE I

PURIFICATION PROCEDURE FOR HUMAN ERYTHROCYTE PHOSPHOGLYCEROMUTASE

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

	Total protein (mg)	*Total activity (Units)*	*Specific activity (Units/mg)*	*Yield (%)*	*Purification (fold)*
Hemolysate	120,478	5542	0.046	–	–
CM-Sephadex chromatography	2610	4600	1.76	83	38
DEAE-Sephacel chromatography	172	2466	14.3	44	310
Dyematrex red A chromatography	3	1800	600	32	13,043
DEAE-Sephadex chromatography	1.63	1062	652	19	14,174



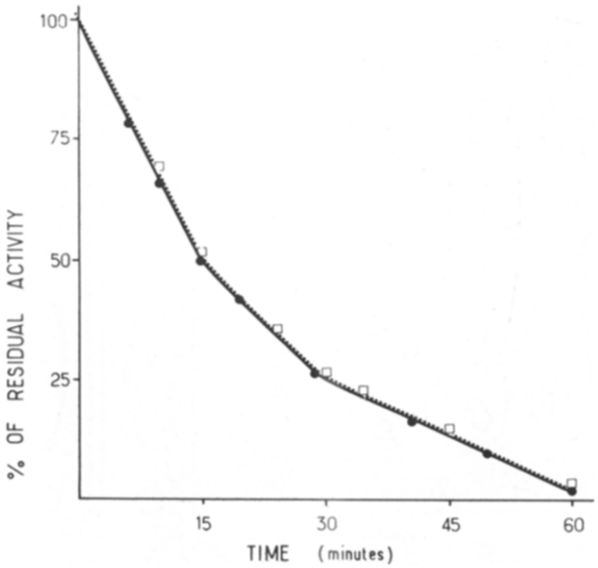


Fig. 2. SDS-polyacrylamide gel electrophoresis of phosphoglyceromutase from: 1, buffer without added protein; 2, fractions from Dyematrex red A; 3, fractions from DEAE-Sephadex. For 2 and 3, 50 μg of proteins were applied on the gels.

Fig. 3. Heat inactivation of phosphoglyceromutase (●——●) and diphosphoglycerate phosphatase (□-------□) activities. Purified phosphoglyceromutase in 0.010 *M* Tris–HCl buffer containing 1 m*M* EDTA and 1 m*M* 2-mercaptoethanol was incubated at 55°C. After 0, 15, 30 and 60 min the tubes were cooled and centrifuged. The supernatants were tested for phosphoglyceromutase and diphosphoglycerate phosphatase activities.

phoglyceromutase and bisphosphoglyceromutase react separately with the antiserum and a line of precipitation appeared at the level of each purified enzyme. On immunoneutralization tests (Fig. 5), phosphoglyceromutase activity was inhibited by the specific antiserum. By extrapolation of the linear portion of the curve, it was found that 50% inactivation of the enzyme was obtained with 8 μl of antiserum, while for bisphosphoglyceromutase about 40 μl antiserum were required for 50% inhibition.

DISCUSSION

We have developed a procedure for purification of phosphoglyceromutase from human red blood cells. This method is simpler than that described by Sheibley and Hass[10]. Only four steps are required, and all of the columns except that of DEAE-Sephadex can be reused many times. The Dyematrex red A column was used here for the first time in phosphoglyceromutase purification. This chromatography was extremely efficient, since an almost forty-fold increase in specific activity was achieved in one step, with a recovery yield of 72%. In the second step, the major band of phosphoglyceromutase was easily separated from the minor one and from bisphosphoglyceromutase. Residual activity of these enzymes was easily eliminated. The elimination of bisphosphoglyceromutase is of great importance, since this en-

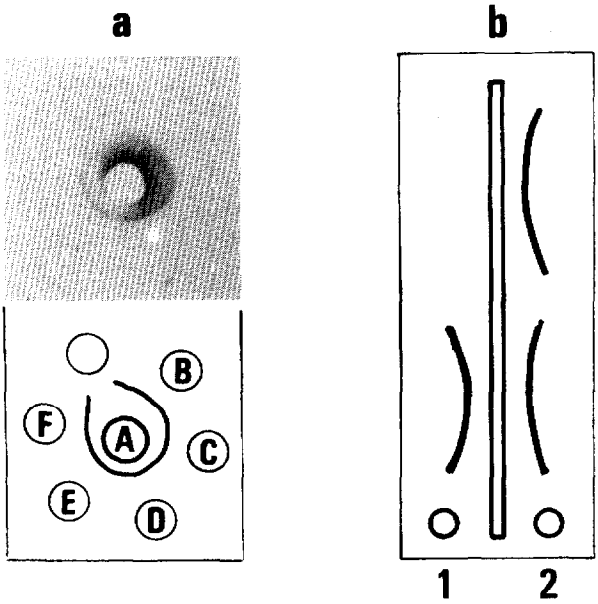


Fig. 4. a, Double immunodiffusion on Ouchterlony plates. The centre well (A) contained 20 μl of antiserum directed against phosphoglyceromutase. To each of the peripheral wells were added 20 μl of pure phosphoglyceromutase at two different concentrations (B, C), a mixture of phosphoglyceromutase and bisphosphoglyceromutase (D, E) and purified bisphosphoglyceromutase (F). b, Immunoelectrophoresis. A 10-μl volume of pure phosphoglyceromutase (1) or a mixture of pure phosphoglyceromutase and bisphosphoglyceromutase (2) was added to wells in a buffered agarose plate. The centre slot was filled with 60 μl antiserum. Electrophoresis was performed in a 0.0375 *M* Tris–EDTA/citric acid buffer (pH 8.0) at 150 V and 4°C for 2 h.

zyme also carries phosphoglyceromutase activity. Moreover the two enzymes are similar in molecular weight. Their separation can best be achieved by Cellogel electrophoresis and staining for phosphoglyceromutase activity (Fig. 1). By this means, we can also demonstrate the absence of the minor band of phosphoglyceromutase, which was not previously identified. The major band of phosphoglyceromutase has been completely purified, only one protein band being found in electrophoresis on

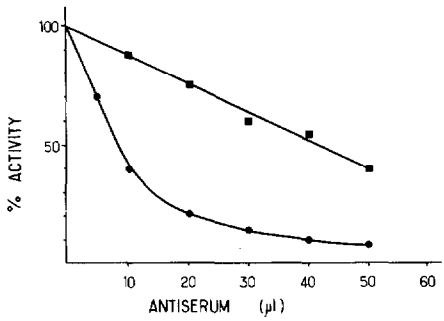


Fig. 5. Immunoneutralization. Chicken antiserum directed against phosphoglyceromutase was tested for neutralization of purified phosphoglyceromutase (●) and pure bisphosphoglyceromutase (■). The results are expressed as percentages of the residual activities in the supernatant.

Cellogel and in SDS-polyacrylamide gel. In addition, a single arc of precipitation was obtained on Ouchterlony plates when antibody against the purified enzyme reacted with a crude extract. No bisphosphoglyceromutase, but a very mild diphosphoglycerate phosphatase activity was found in the homogeneous preparation of phosphoglyceromutase. The thermostability study demonstrates the bifunctionality of the phosphoglyceromutase molecule. These results are in accord with the data obtained by Sheibley and Hass[10]. Clearly, the specific antibody obtained from phosphoglyceromutase also reacts with bisphosphoglyceromutase either by immunoneutralization or by double immunodiffusion. No spur was detected between the two arcs of precipitation obtained with phosphoglyceromutase and bisphosphoglyceromutase. These data suggest that the two enzymes possess common antigenic determinants. Similar results have been obtained by Hass *et al.*[14] with an anti-bisphosphoglyceromutase serum that reacted with phosphoglyceromutase. Such findings are not surprising since it has been demonstrated that the two enzymes possess a high degree of similarity in their structure, their catalytic activity and their immunologic characteristics[14]. Our results provide additional evidence for homology between the two enzymes.

ACKNOWLEDGEMENTS

This work was supported by the Institut National de la Santé et de la Recherche Médicale, by the Centre National de la Recherche Scientifique and by l'Université Paris Val de Marne. We wish to acknowledge Dr. Rose Schneider for reading the manuscript, and Mrs. A. M. Dulac and M. Segear for their technical assistance.

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