Biochimica et Biophysica Acta, 321 (1973) 503–511

BBA 67024

HUMAN ERYTHROCYTE PHOSPHOGLYCERATE KINASE: PURIFICATION, PROPERTIES, AND INTERACTION WITH ITS ANTIBODY

P. O. OKONKWO[a], A. ASKARI[a] AND L. KORNGOLD[b]

[a]*Department of Pharmacology, Cornell University Medical College, New York, N.Y. 10021 (U.S.A.) and* [b]*Hospital for Special Surgery, New York, N.Y. 10021 (U.S.A.)*

(Received May 21st, 1973)

SUMMARY

Crystalline human erythrocyte phosphoglycerate kinase (ATP: 3-phospho-D-glycerate 1-phosphotransferase, EC 2.7.2.3) was prepared by the modification of the previously described methods, and its purity was ascertained by several means. Some chemical, physical, and kinetic properties of this preparation were compared with those of other highly purified phosphoglycerate kinase preparations from erythrocytes and other tissues.

Precipitating antibodies to the crystalline erythrocyte enzyme and the purified yeast phosphoglycerate kinase were prepared. Specific reaction of each antibody with its respective enzyme was demonstrated by double-diffusion tests. The activity of each purified enzyme was completely inhibited by its antibody.

Varying concentrations of the antibody to the erythrocyte enzyme were added to erythrocyte hemolyzates; and phosphoglycerate kinase activity and lactate production, from fructose 1,6-diphosphate, were measured. Under these conditions, complete inhibition of phosphoglycerate kinase activity by the antibody was not obtained. Lactate production was not affected until 30% of the phosphoglycerate kinase activity was neutralized. Thereafter, the rate of lactate formation decreased with increasing inhibition of the enzyme activity.

INTRODUCTION

In recent years the works of several laboratories have established that the rate of ATP generation by the glycolytic pathway of human erythrocytes is regulated by the activity of the Na^+,K^+-pump of the cell membrane[1]. The mechanism of this control is not clear, but certain studies[2,3] have pointed to a possible interaction between the Na^+,K^+-activated ATPase and a key step in the glycolytic sequence; namely, the reaction catalyzed by phosphoglycerate kinase (ATP:3-phospho-D-glycerate 1-phosphotransferase, EC 2.7.2.3). In the course of our studies (to be published elsewhere) on the mechanism of such an interaction, the desirability of having

an antibody to erythrocyte phosphoglycerate kinase became apparent. The purification of this enzyme has been reported before[4,5], but we found no reported studies on the antibody to the enzyme. Therefore, here we present the results of our work on the preparation of an antibody to erythrocyte phosphoglycerate kinase, and its interaction with the enzyme. For comparison, the antibody to yeast phosphoglycerate kinase was also prepared and studied. Since we encountered some difficulties in preparing the erythrocyte enzyme by the previously published procedures, we are also presenting a modified method for its purification. Brief comparison of the properties of our enzyme preparation with the reported properties of other preparations, as well as some hitherto unreported properties of the erythrocyte enzyme are also described.

MATERIALS AND METHODS

Crystalline yeast phosphoglycerate kinase, all substrates, DEAE-cellulose, and the reagents and enzymes used for the assay of lactate were obtained from Sigma Chemical Company (St. Louis, Mo.). The purified phosphoglycerate kinase preparations from various muscles were generous gifts of Dr R. K. Scopes, Agricultural Research Council, Langford, Great Britain. All common chemicals were of Reagent Grade quality.

Phosphoglycerate kinase activity was assayed by the backward reaction (conversion of 3-phosphoglycerate to 1,3-diphosphoglycerate) as described by Bücher[6]. The reaction mixture contained 90 mM triethanolamine–HCl (pH 7.8), 2 mM ATP, 8 mM $MgCl_2$, 5 mM 3-phosphoglycerate, 0.3 mM NADH, and excess of glyceraldehyde 3-phosphate dehydrogenase. The decrease of absorbance at 366 nm was determined at 25 °C. One enzyme unit is defined as the activity catalyzing the utilization of 1 μmole of 3-phosphoglycerate per min.

Experiments on lactate production from fructose- 1,6-diphosphate by erythrocyte hemolyzates were done under the conditions described by Parker and Hoffman[2]. Lactate was determined by the enzymic method[7], and protein was measured by the procedure of Lowry *et al.*[8]. Preparation of agar plates, and immunodiffusion were done by methods previously described[9]. Amino acid analysis was done by standard methods on a sample of enzyme which was dialyzed, dried and subsequently hydrolyzed in 6 M HCl at 100 °C for 22 h. Ultracentrifugation experiments were performed on a solution of the enzyme (5 mg/ml) in 0.2 M NaCl, 1 mM EDTA, 0.05 M phosphate (pH 7.0). The analysis was done in a Spinco Model E centrifuge at the rotor speed of 59 700 rev./min at 21.8 °C. Gel electrophoresis was carried out in the Buchler apparatus (Fort Lee, N.J.). The gels contained 7% acrylamide and 0.4% agarose. The buffers (pH 9.5, 8, 6.6) were those described by Davis[10]. Electrophoresis on cellulose acetate strips was done in a Buchler universal electrophoresis cell (Fort Lee, N.J.). The buffers used were 0.05 M barbital–NaOH (pH 8.6), and 0.35 M β-alanine–acetic acid (pH 4.6). A voltage gradient of 20–25 V/cm for 60 min was applied. Starch gel electrophoresis was done at pH 8.3 according to Smithies[11].

Preparation of the erythrocyte enzyme

The major purification steps were the same as those reported by Hashimoto

and Yoshikawa[5]. One step (precipitation with ethanol) was eliminated, and replaced with $(NH_4)_2SO_4$ precipitation. A variety of procedural modifications were also introduced as described below. Human red cells were obtained from out-dated blood-bank blood by centrifugation, and washed three times each time with 4 vol. of 0.9% NaCl. Unless otherwise stated, all subsequent operations were done at 0–4 °C. Hemolysis of red cells, and removal of hemoglobin were performed according to the procedure of Armstrong *et al.*[12]. The hemoglobin-free solution was then dialyzed against 100 vol. of 0.1 mM EDTA (pH 7.3) for 24 h. Solid $(NH_4)_2SO_4$, in small portions, was added to the dialyzate to obtain 80% saturation. This was done over a period of 2 h at room temperature. During this procedure small quantities of solid Tris were also added to maintain the solution at neutrality. The final precipitate was recovered by centrifugation, dissolved in minimum required amount of water, and dialyzed against 120 vol. of deionized water for 24 h. A suspension of aged calcium phosphate gel (300 mg/ml) was prepared[13]. The dialyzate from the previous step was adjusted to pH 6 by the addition of a few drops of 0.1 M sodium acetate–acetic acid (pH 5.8), mixed with the calcium phosphate gel (40 ml of gel suspension per 100 ml of solution), and stirred for 15 min. After centrifugation, the supernatant was discarded and the gel was washed three times, each time with an equal volume of the above acetate buffer solution. The washed gel was then mixed with 0.3 vol. of 10 mM sodium pyrophosphate, stirred for 10 min, and centrifuged. The supernatant was retained, and the gel was washed in the same manner until no phosphoglycerate kinase activity could be detected in the supernatant. Usually 5–6 washings were sufficient. A polypropylene column (4 cm × 10 cm) packed with DEAE-cellulose was equilibrated with a solution containing 5 mM sodium pyrophosphate and 1 mM disodium EDTA. The combined washings (100 ml) from the previous step were passed through the column, and the column was washed with the above pyrophosphate–EDTA solution. Fractions (10 ml each) were collected and tested for phosphoglycerate kinase activity and protein. Those containing the highest specific activities (usually Fractions 10–15) were pooled and concentrated by ultrafiltration (Amicon ultrafilter apparatus, Amicon Corp., Lexington, Mass.). To the concentrated protein solution (5 mg/ml) solid $(NH_4)_2SO_4$ was added to obtain 60% saturation. The precipitate was discarded. $(NH_4)_2SO_4$ was again added to the solution to obtain 75% saturation. The solution was allowed to stand at room temperature for 2 h, and was subsequently left at 4 °C for several days. Needle-like crystals grew to elongated rods, which were harvested and stored at 4 °C as a suspension in 3 M $(NH_4)_2SO_4$, 1 mM sodium pyrophosphate, and 1 mM EDTA.

Preparation of antibodies

Enzyme preparations were freed of $(NH_4)_2SO_4$ by exhaustive dialysis against 0.9% NaCl. 1 ml of enzyme solution, containing 2 mg of protein, was mixed with 3 ml of Freund's complete adjuvant and injected intramuscularly into rabbits. The injection was repeated weekly for 3 weeks, after which 2 weeks elapsed before a booster injection was given. Each rabbit received 8 mg of enzyme protein through the immunization schedule. Rabbits were bled before the start of immunization procedure by the puncture of the marginal ear veins to obtain preimmune sera. After the booster injection, blood was collected by the same procedure. Serum was obtained by

TABLE I

PURIFICATION OF HUMAN ERYTHROCYTE PHOSPHOGLYCERATE KINASE

Summary of a typical purification procedure as described in Materials and Methods.

Fraction	*Specific activity (units/mg protein)*	*Recovery (%)*
Hemolyzate	0.2	100
Dialyzed hemoglobin-free fraction	21	85
Sediment after the first $(NH_4)_2SO_4$ addition	50	80
Eluate from calcium phosphate gel	290	55
Eluate from DEAE-cellulose	387	40
Supernatant from second $(NH_4)_2SO_4$ fractionation	490	28
Crystals	580	20

centrifugation of the blood, and stored at −70 °C. γ-Globulins were isolated by the standard techniques of $(NH_4)_2SO_4$ fractionation.

RESULTS AND DISCUSSION

Crystalline erythrocyte enzyme

Table I shows the summary of a typical purification procedure. The specific activity of the crystalline enzyme (Fig. 1), obtained in five different purification experiments, was within the range of 500 to 750. In view of some confusing statements in the literature regarding the activities of the various preparations of this

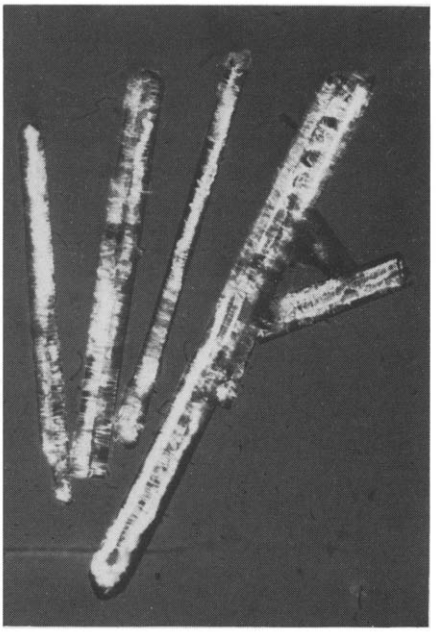

Fig. 1. Photomicrograph of crystalline erythrocyte phosphoglycerate kinase. Magnification × 1200.

enzyme, it is appropriate to compare the specific activity of our preparation with those reported for other crystalline preparations. Hashimoto and Yoshikawa[4] were the first to report the crystallization of human erythrocyte phosphoglycerate kinase. In a subsequent paper[5] the same investigators established the presence of impurities in the crystalline preparation, and reported an improved purification procedure which resulted in the removal of the contaminating proteins. Their second crystalline preparation had a specific activity of 300 (by the same definitions of enzyme unit and specific activity as we have used in this report). Thus, the modification of their purification procedure introduced by us seems to have improved the specific activity of the enzyme approximately 2-fold. More recently, Yoshida and Watanabe[14], using an entirely different method of purification, reported the isolation of a crystalline enzyme with the specific activity of 650–700. These investigators, apparently unaware of the second report of Hashimoto and Yoshikawa[5], claimed that their preparation was about twenty times as active as the previously reported preparations. From the discussion presented here, it is obvious that the second preparation of Hashimoto and Yoshikawa[5] must have been a highly active and essentially pure enzyme, and that the subsequent preparations (both ours and that of Yoshida and Watanabe) are only slight improvements.

Purity and properties of the erythrocyte enzyme

The enzyme sedimented in the ultracentrifuge as a single peak. In acrylamide gel electrophoresis, cellulose acetate electrophoresis, and starch gel electrophoresis, it migrated as a single sharp band, or a single zone, under all tested conditions. Homogeneity of the enzyme was further established by the immunological means as described in a subsequent section. The amino acid composition of the enzyme was determined, and found to be essentially the same as reported before[14].

The enzyme has an absolute specificity for 3-phosphoglycerate as phosphate acceptor. Various nucleosidetriphosphates were tested as substrates. Their relative reactivities (Table II) were similar to those reported for the enzymes obtained from skeletal muscle and yeast[15]. The enzyme required Mg^{2+} for activity. Ca^{2+} could replace Mg^{2+}, but under the standard conditions of assay the maximum activity obtained in the presence of 8–10 mM Ca^{2+} was about 30% of the maximum activity obtained with Mg^{2+}. When Ca^{2+} was added in the presence of Mg^{2+}, partial inhibition

TABLE II

NUCLEOSIDETRIPHOSPHATE SPECIFICITY OF ERYTHROCYTE PHOSPHOGLYCERATE KINASE

The various nucleotides were substituted for ATP under the standard conditions of assay described in Materials and Methods. All activities are expressed as % of the activity obtained with ATP.

Nucleotide	*Enzyme activity (% of control)*
ATP	100
ITP	64
GTP	60
UTP	0
CTP	0
TTP	0

of activity was observed (Table III). The K_m values for all substrates were determined and found to be essentially the same as reported before[5].

The crystalline enzyme was rather unstable. The dried crystals, or dilute aqueous solutions lost all activity within a few weeks. The best storage conditions were those described in Materials and Methods. The enzyme suspension kept at 4 °C retained more than 75% of its activity after 18 months.

TABLE III

EFFECTS OF Mg^{2+} AND Ca^{2+} ON ERYTHROCYTE PHOSPHOGLYCERATE KINASE

Activities with indicated concentrations of cations were determined under the standard conditions of assay described in Materials and Methods, and are expressed as % of the activity obtained with Mg^{2+}.

Added divalent cations	*Enzyme activity (% of control)*
8 mM Mg^{2+}	100
8 mM Ca^{2+}	30
8 mM Mg^{2+} + 8 mM Ca^{2+}	54
None	8

Interactions of enzymes with antibodies

All rabbits developed precipitating antibodies to the immunizing preparations. Fig. 2, typical plates of double-diffusion tests, shows the specific reactions of antisera to yeast phosphoglycerate kinase and human erythrocyte phosphoglycerate kinase with their respective enzymes. In similar experiments, antiserum to the erythrocyte enzyme was also tested against various dilutions of erythrocyte hemo-

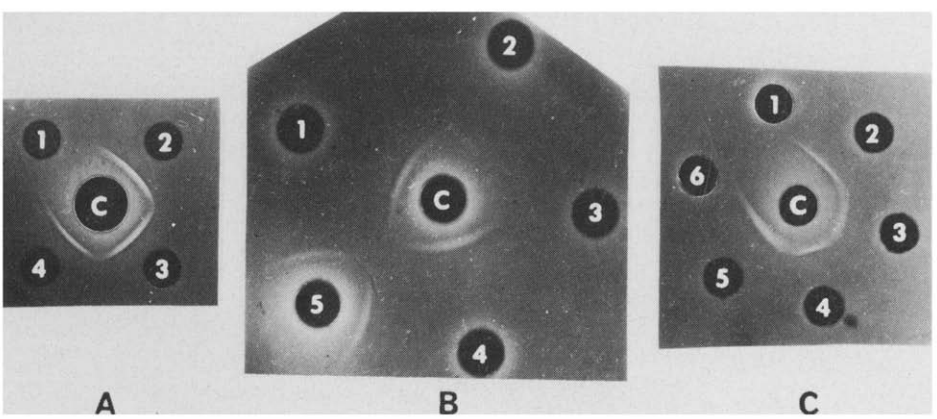

Fig. 2. Immunodiffusion analysis of the reactions of antisera to human erythrocyte phosphoglycerate kinase and yeast phosphoglycerate kinase with their respective enzymes. (A) Center well contained antiserum (10 μl) to the erythrocyte enzyme. Wells 2, 3 and 4 contained the purified erythrocyte enzyme (4 μg). Well 1 contained the buffer solution in which the enzyme was dissolved. (B) Center well and Well 5 contained antiserum to the erythrocyte enzyme. The erythrocyte enzyme was added to Wells 1 and 4. Well 2 contained control serum, and Well 3 the yeast enzyme. (C) Center well contained antiserum to the yeast enzyme. Wells 2, 3, 4 and 5 contained 0.25 μg, 0.5 μg, 1 μg, and 2 μg, respectively, of the yeast enzyme. Wells 1 and 6 each contained 2 μg of the erythrocyte enzyme.

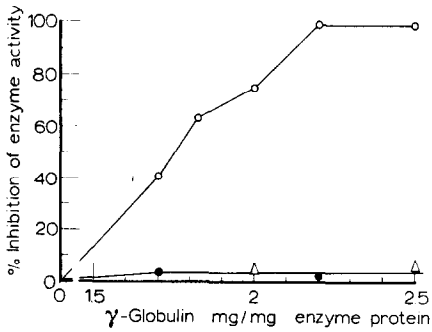

Fig. 3. Effects of varying concentrations of antibody to erythrocyte phosphoglycerate kinase on the activity of the purified enzyme. Aliquots of enzyme solution, each containing 0.1 mg of enzyme protein, were incubated at 37 °C for 1 h, with different amounts of γ-globulins obtained from antiserum to the erythrocyte enzyme (○), antiserum to the yeast enzyme (●), and control serum (△). The solutions were then left at 4 °C for 24 h. After thorough mixing, an aliquot of each sample was tested for enzyme activity by the standard procedure described in Materials and Methods.

lyzates. In all cases single precipitation arcs identical to those obtained against the purified enzyme were observed.

Fig. 3 and Table IV show the effects of the antisera to the yeast and the erythrocyte enzymes on the catalytic activities of these enzymes. It is evident that complete inhibition of activity of each enzyme may be obtained in the presence of its specific antibody. In similar experimnets when antibody and enzyme were preincubated in the presence of various substrates, and subsequently tested, no evidence for the protective effects of substrates against the enzyme–antibody interaction was obtained.

Both antisera were also tested against crystalline phosphoglycerate kinase of bovine, equine, and porcine muscles. No cross reactivities were apparent.

Effects of antiserum to the erythrocyte enzyme on the phosphoglycerate kinase activity and lactate production in hemolyzates

Hemolyzates of human erythrocytes were preincubated with various concentrations of antibody to phosphoglycerate kinase. Aliquots were then tested for

TABLE IV

INHIBITION OF YEAST PHOSPHOGLYCERATE KINASE BY ITS SPECIFIC ANTIBODY

Crystalline enzyme, 0.12 mg in 3 ml of 0.1 M Tris–HNO_3 (pH 7.6), was mixed with 0.5 ml of antiserum to yeast enzyme. Similar mixtures with control serum, antiserum to the erythrocyte enzyme, and 0.9% NaCl, were also made. The solutions were incubated at 37 °C for 1 h, and were subsequently left at 4 °C for 24 h. After thorough mixing, aliquots were tested for enzyme activity by the standard procedure described in Materials and Methods. Results are expressed as % of the activity of the control enzyme that was mixed with normal saline.

Yeast enzyme incubated with:	*Enzyme activity (% of control)*
0.9% NaCl	100
Control serum	94
Antiserum to yeast enzyme	6
Antiserum to erythrocyte enzyme	98

phosphoglycerate kinase activity, and for their ability to produce lactate when fortified with appropriate substrates and cofactors. From the results of Fig. 4 the following points are evident: (1) The phosphoglycerate kinase activity of the whole hemolyzate is not completely inhibited by the antibody. This is in contrast to the effect of antibody on the activity of the purified enzyme (Fig. 3), and suggests either that the enzyme–antibody interaction is modified by factors present in the hemolyzate, or that a portion of enzyme in the hemolyzate is protected from interac-

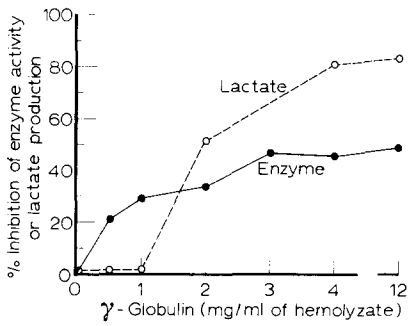

Fig. 4. Effects of varying concentrations of antibody to erythrocyte phosphoglycerate kinase on phosphoglycerate kinase activity and lactate production in erythrocyte hemolyzates. Washed erythrocytes were hemolyzed in 4 vol. of 0.1 mM EDTA. The mixture was frozen and thawed 3 times. Various amounts of γ-globulin from antiserum and preimmune serum were added to 1 ml portions of the hemolyzate. The mixtures were incubated as described in legend to Fig. 3. Phosphoglycerate kinase activity of each mixture was determined as described in Materials and Methods. A separate aliquot of each mixture was also incubated as described in Materials and Methods, and the rate of lactate formation from fructose-1,6-diphosphate was measured. Control experiments were done with a hemolyzate which was similarly incubated in the absence of γ-globulin. Effects of the antibody on the enzyme activity and lactate formation are expressed as % of the control values. Enzyme activity and lactate formation in hemolyzates which were incubated with γ-globulin from preimmune serum were not significantly different from the control values.

tion with antibody. (2) Inhibitory effect of antibody on lactate formation is not evident until about 30% of phosphoglycerate kinase is neutralized. This was not totally unexpected. Studies on the relative levels of the enzymes of glycolytic pathway in human erythrocytes have shown that phosphoglycerate kinase is one of the enzymes that is present in large excess[16]. What is perhaps somewhat surprising is that the excess enzyme is no more than 30%. From the comparison of phosphoglycerate kinase activity with the activities of the enzymes beyond phosphofructokinase[16], one would have suspected a larger excess of phosphoglycerate kinase than the experimental data suggest. These experiments emphasize again the well-recognized dangers of drawing conclusions regarding rate-limiting steps of a metabolic pathway, on the basis of profile analysis of enzyme activities in the presence of excess substrates.

ACKNOWLEDGMENT

This work was supported by U.S. Public Health Service Research Grant HL-10884 from the National Heart and Lung Institute, and by U.S. Public Health Training Grant GM-99.

REFERENCES

1 Whittam, R. and Wheeler, K. P. (1970) *Annu. Rev. Physiol.* 32, 21–60
2 Parker, J. C. and Hoffman, J. F. (1967) *J. Gen. Physiol.* 50, 893–916
3 Minakami, S. and Yoshikawa, H. (1966) *J. Biochem. Tokyo* 59, 145–150
4 Hashimoto, T. and Yoshikawa, H. (1962) *Biochim. Biophys. Acta* 65, 355–357
5 Hashimoto, T. and Yoshikawa, H. (1964) *J. Biochem. Tokyo* 56, 279–284
6 Bücher, T. (1947) *Biochim. Biophys. Acta* 1, 292–314
7 Hohorst, H. J. (1965) in *Methods of Enzymic Analysis* (Bergmeyer, H., ed.), pp. 266–270, Academic Press, New York
8 Lowry, O. H., Rosenbrough, N. J., Farr, A. L. and Randall, R. J. (1951) *J. Biol. Chem.* 193, 265–275
9 Korngold, L. (1957) *Ann. N.Y. Acad. Sci.* 69, 681–697
10 Davis, B. J. (1964) *Ann. N.Y. Acad. Sci.* 121, 404–427
11 Smithies, O. (1959) *Biochem. J.* 71, 585–587
12 Armstrong, J. M., Myers, D. V., Verpoorte, J. A. and Edsall, J. T. (1966) *J. Biol. Chem.* 241, 5137–5149
13 Keilin, D. and Hartree, E. F. (1938) *Proc. Royal Soc. London* 124B, 397–405
14 Yoshida, A. and Watanabe, S. (1972) *J. Biol. Chem.* 247, 440–445
15 Krietsch, W. K. G. and Bücher, T. (1970) *Eur. J. Biochem.* 17, 568–580
16 Yoshikawa, H. and Minakami, S. (1968) *Folia Haemt.* 89, 357–375