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Crystalline 3-Phosphoglycerate Kinase from Skeletal Muscle

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1. A procedure for preparing crystalline 3-phosphoglycerate kinase from rabbit or pig skeletal muscle is presented. 2. The preparation phosphorylates up to 975 μmoles of 3-phosphoglycerate/min./mg. at 30° and is not contaminated with myokinase. 3. The enzyme has an estimated molecular weight of 36500 $\pm$ 1000, and contains three residues each of tyrosine and tryptophan. 4. The preparation is suitable for use in the enzymic procedures for determining ATP, phosphocreatine and 3-phosphoglycerate.

The enzyme PGAK* has been crystallized from yeast (Bücher, 1947) and from erythrocytes (Hashimoto & Yoshikawa, 1962), but not from skeletal muscle, apart from a report (Czok & Bücher, 1960) that was not subsequently elaborated. Preparations of muscle PGAK have nevertheless been made with activities comparable with that of the yeast enzyme (Gosselin-Rey, 1963; Avramov & Repin, 1965) although still contaminated with other proteins, in particular myokinase (ATP–AMP phosphotransferase, EC 2.7.4.3). PGAK is widely used for determining ATP (Adam, 1963) and (with the addition of ADP and creatine kinase) phosphocreatine. For these purposes it must be substantially free of myokinase. The present paper describes a procedure leading to crystalline myokinase-free PGAK from rabbit or pig muscle in good yield, the specific activity of which is substantially higher than any reported previously.

MATERIALS AND METHODS

D-3-Phosphoglycerate (barium salt) and ATP (sodium salt) were obtained from Koch–Light Laboratories Ltd., Colnbrook, Bucks. NADH was obtained from Sigma Chemical Co., St Louis, Mo., U.S.A. Other chemicals used were A.R.-grade. Glyceraldehyde phosphate dehydrogenase was prepared as a by-product of the PGAK isolation procedure (see below).

Rabbits were immobilized with Myanesin (mephenesin) (British Drug Houses Ltd., Poole, Dorset) before being killed. Back and leg muscles were combined after the small redder muscles had been discarded. They were extracted by homogenizing in a Waring Blendor with 2 vol. of iso-osmotic (2%, v/v) glycerol containing 2mM-EDTA and 10mM-tris base (Scopes, 1966), and centrifuging at 10000*g* for 15min. Pig longissimus dorsi muscle was obtained soon after slaughter and extracted with the same medium but containing somewhat more tris (up to 25mM) to keep the pH of the homogenate above 6·5.

Column chromatography was carried out in polymethyl methacrylate columns of 8cm.^{2} cross-section (Wright Scientific Co. Ltd., Kenley, Croydon) and effluents were monitored at 280nm. CM-cellulose was in the microgranular form (Whatman CM32).

Phosphoglycerate kinase was assayed in the direction of 1,3-diphosphoglycerate formation, coupling with glyceraldehyde phosphate dehydrogenase (Bücher, 1955). The medium contained 10mM-D-3-phosphoglycerate, 4mM-ATP, 6mM-$MgSO_4$, 0·2mM-EDTA, 50mM-imidazole chloride buffer, final pH7·2, 0·2mM-NADH and approx. 20 μg. of glyceraldehyde phosphate dehydrogenase/ml. The reaction was followed at 340nm. at room temperature, and rates were converted into μmoles/min. at 30° by using the observed Q_{10} 1·8 for the range 20–30°.

Myokinase activity was measured in a solution containing 1mM-AMP, 1mM-ATP, 3mM-$MgSO_4$, 0·2mM-EDTA, 50mM-KCl, 50mM-tris chloride buffer, pH7·8, 0·2mM-NADH, 0·5mM-phosphoenolpyruvate, and pyruvate kinase and lactate dehydrogenase (heart type) each at 2 units/ml.

Protein was determined after dialysis by its extinction at 205nm. by using $E_{205}^{0.1\%}$ 32 (Goldfarb, Saidel & Mosovich, 1951). Purified PGAK was determined at 280nm. by using $E_{280}^{0.1\%}$ 0·57 (see below).

RESULTS

Isolation procedure. Since it is often useful to prepare several different enzymes from one extract, indications are given below of the enzymes which can be prepared from those fractions that otherwise would be discarded.

The extract (fraction I) obtained as described above had pH between 6·5 and 7·0. The pH was lowered to 5·4 with 5M-acetic acid, and the precipitate was removed by centrifugation. If this

* Abbreviation: PGAK, 3-phosphoglycerate kinase (ATP–D-glycerate 3-phosphotransferase, EC 2.7.1.31); this abbreviation is used rather than the commoner PGK, to be consistent with abbreviations for the enzymes of the whole glycolytic sequence (Scopes, 1966, 1968).

procedure was carried out in the cold, the precipitate could be used for the preparation of phosphofructokinase (Parmeggiani, Luft, Love & Krebs, 1966). To the supernatant was added 270g. of ammonium sulphate/l. (to 1·75M); the precipitate was collected by centrifugation and could be used for preparation of phosphorylase *b* (Fischer & Krebs, 1958). To the supernatant was added 100g. of ammonium sulphate/l. (to 2·35M); the precipitate could be used for preparing aldolase and α-glycerophosphate dehydrogenase (Beisenherz, Bücher & Garbade, 1955) from rabbit muscle, or lactate dehydrogenase (Jécsai, 1961) from pig muscle. The 2·35M-ammonium sulphate supernatant was then made 3·0M with respect to ammonium sulphate by adding 110g./l., and the precipitate (fraction II) was collected for PGAK preparation. The supernatant was made 3·2M with respect to ammonium sulphate, the pH was raised to 8·0 with 5M-ammonia and the preparation was left overnight in the cold. The last precipitate was collected and used for preparation of glyceraldehyde phosphate dehydrogenase, which crystallized readily from a turbid ammonium sulphate suspension, and was further purified by passage through a Sephadex G-100 column and several recrystallizations.

This ammonium sulphate fractionation procedure is similar to that described by Czok & Bücher (1960). It was carried out at room temperature, except that centrifuging was at 10°. Each fraction was allowed to equilibrate with the ammonium sulphate for at least 30min. before centrifuging. However, only slightly less PGAK was obtained by a much simpler fractionation procedure: the extract was adjusted to pH5·4, and ammonium sulphate was added to 2·35M (385g./l.) without any intermediate centrifugings; the precipitate was discarded and the 2·35–3·0M-ammonium sulphate fraction collected as above.

Fraction II was dissolved in 50mM-sodium acetate–1mM-EDTA buffer, pH5·4, containing 1·0M-ammonium sulphate and 5mM-3-phosphoglycerate. The volume was made to 20% of the volume of the original extract, and the pH, as measured directly with a glass electrode at room temperature, adjusted to 5·40. The flask containing the solution was then placed in water at about 65° and agitated continuously: heavy precipitation began as the temperature passed 35°. When the temperature reached 55°, the flask was transferred to a 55° bath and kept at that temperature (±0·5°) with continuous agitation for 10min. It was then cooled on ice, and the heavy residue of denatured protein was removed by centrifugation. This residue was extracted with half the previous volume of 1·2M-ammonium sulphate, and the extract was combined with the previous supernatant; both were filtered through glass wool to remove remaining flecks of denatured protein. This heat treatment resulted in a purification of nearly threefold, and most importantly removed at least 95% of the myokinase (the remaining 5% was lost in subsequent operations).

To the filtered solution was added 40g. of ammonium sulphate/100ml., and the precipitated protein (fraction III) was collected by centrifugation and dissolved in a small volume of 10mM-sodium EDTA, pH8·0. It was dialysed in the cold against two or more changes of 5l. of 0·2mM-EDTA adjusted to pH8·0 with tris. The stages to dialysis can be completed in 1 day, and the second day can be spent in preparing the two columns (see below) while dialysis continues.

CM-cellulose was equilibrated with a tris–cacodylate buffer (10mM - tris – 0·2mM - EDTA, adjusted to pH6·5 with 1M-cacodylic acid), and a column (8cm.^{2} × 30cm.) (suitable for fraction III from 500g. of muscle) was prepared and several hundred millilitres of buffer were passed through. Fraction III after dialysis was adjusted to pH6·5 with 1M-cacodylic acid, and a small precipitate was removed. It was then diluted to 25% of the volume of the original extract with tris–cacodylate buffer, and the solution was allowed to run into the column overnight in a room kept at 10°. The following day the column was brought out to room temperature and washed through with 300ml. of buffer. The non-adsorbed protein consisted mainly of serum albumin and triose phosphate isomerase; the latter could be crystallized after suitable treatment. A linear gradient in potassium chloride was applied to the column by using 500ml. of buffer and 500ml. of 0·15M-potassium chloride in a suitable mixing device. The flow rate was 120ml./hr.

The elution profile from the CM-cellulose column (rabbit preparation) is shown in Fig. 1. PGAK activity was associated with the central part of the main peak, eluted at about 50mM-potassium chloride. The first part of this peak consisted mainly of pyruvate kinase, which could be crystallized

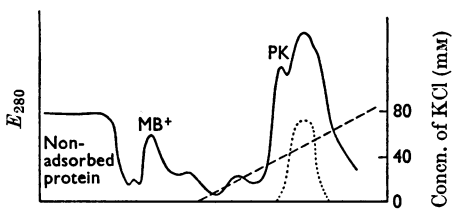

Fig. 1. Elution profile of protein (rabbit) from a CM-cellulose column. ——, E_{280}; ·····, PGAK activity; – – –, approximate concn. of KCl. Abbreviations: MB+, metmyoglobin; PK, pyruvate kinase.

after salting out. All tubes with significant PGAK activity were combined and made 1mM with respect to EDTA, and 60g. of ammonium sulphate was added/100ml. to salt out the protein. The precipitate was collected and dissolved in the minimum amount of a tris–potassium chloride buffer (20mM-tris–0·15M-potassium chloride–0·2mM-EDTA, pH8·0). It was centrifuged to remove a small residue, and then applied to a Sephadex G-100 column (8cm.^{2} × 55cm.) equilibrated at room temperature with the tris–potassium chloride buffer. The flow rate was 60–70ml./hr. PGAK was the most retarded protein, and separated clearly from the contaminants (Fig. 2). The trailing edge of the PGAK peak was discarded, as any remaining traces of myokinase would be eluted here.

The behaviour of the pig fractions on the columns was rather different. On the CM-cellulose the main peak eluted contained nearly pure PGAK, and only a small amount of contaminating protein was removed on the Sephadex column.

The enzyme from the Sephadex column was pure, judged by starch-gel electrophoresis (see below), and several preparations, both rabbit and pig, had specific activities in the range 920–975units/mg. The enzyme could be crystallized from ammonium sulphate solution after concentration by salting out. At pH7–8 fragile plates formed, later turning into clusters of needles (cf. Czok & Bücher, 1960). Some loss of specific activity occurred during crystallization. A summary of the purification of PGAK from rabbit muscle is given in Table 1.

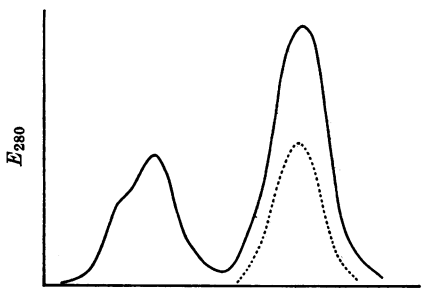

Fig. 2. Elution profile of protein (rabbit) from a Sephadex G-100 column. ——, E_{280}; ·····, PGAK activity.

Properties of the enzyme. The specific activity of the pre-crystalline fraction V is considerably higher than that reported for the yeast enzyme (Bücher, 1947), which would phosphorylate about 600μmoles of 3-phosphoglycerate/min. at 30° under the assay conditions described here. The activity of the crystalline preparation described by Czok & Bücher (1960) was 350units/mg. at 25° with unspecified substrate concentrations, but this was a purification of only 20-fold from the crude extract, suggesting that it was only about 50% pure, or else was partially inactivated. Gosselin-Rey (1963) obtained a 20-fold-purified preparation from chicken muscle with a specific activity of 475units/mg. at 37° (a chemical assay method), and Avramov & Repin (1965) prepared the enzyme from rabbit with a specific activity of only 240units/mg., but with suboptimum substrate concentrations and undefined temperature.

Both rabbit and pig enzymes had specific activities of up to 975units/mg. (based on freeze-dried dry weights) before crystallization, but lost some activity during crystallization. This was partly due to inactivation and denaturation occurring in the turbid suspension during crystallization. Very heavy losses are incurred at all stages of preparation if EDTA is not included in the solutions, suggesting that this inactivation is mainly due to heavy-metal impurities in the reagents; use of polypropylene apparatus where possible is also recommended.

Starch-gel electrophoresis of the preparations gave two bands with both rabbit and pig enzymes. Both of these were active when stained specifically for the enzyme (Scopes, 1968). Two active bands were not apparent in the stains of the crude extracts, and it may be that one represents a change brought about by the purification procedure. The pig enzyme bands run at a position suggesting that they have about half a negative charge more than rabbit enzyme bands.

Chromatography on Sephadex G-100, by comparison with myoglobin, chymotrypsinogen, ovalbumin and bovine serum albumin, has indicated a

Table 1. *Purification of phosphoglycerate kinase from* 500*g. of rabbit muscle*

Fraction	Volume (ml.)	Total protein (mg.)	Total activity (units)	Specific activity (units/mg. of protein)	Myokinase activity (units)
I: Iso-osmotic extract	940	29000	570000	20	365000
II: 2·35–3·0M-$(NH_4)_2SO_4$	190	7100	420000	59	175000
III: fraction II after heat treatment and dialysis	235	1950	302000	152	4200
IV: peak from CM-cellulose	88	405	282000	695	1200
V: peak from Sephadex G-100	61	265	258000	975	0
VI: crystals	14	205	155000	760	0

molecular weight for PGAK of about 38000. The extinction coefficient of a neutral solution at 280nm., based on freeze-dried dry weight (allowing for 2% moisture), is $E_{280}^{0.1\%}$ 0·57 (for both rabbit and pig enzymes). Determination of the tryptophan/tyrosine ratio spectrophotometrically, according to the equations of Goodwin & Morton (1946) or Beaven & Holiday (1952), on several different preparations has given values from 0·92 to 1·08 for both rabbit and pig enzymes. This indicates that tryptophan and tyrosine are present in equal amounts, and the molecular weight corresponding to one residue of each is 12100 ± 300. Thus it appears that muscle PGAK has a molecular weight of 36500 ± 1000 with three residues each of tryptophan and tyrosine.

DISCUSSION

It is evident that previous reported preparations of muscle phosphoglycerate kinase have either been contaminated with other proteins or partially inactivated, for specific activities were considerably lower than the 975units/mg. reported here, a purification of 50-fold from rabbit muscle extract. Losses due to inactivation by heavy-metal can be avoided by inclusion of EDTA at all stages of the preparation, and in the later stages by avoidance of glass apparatus. Some inactivation also occurs during crystallization with ammonium sulphate, and this is difficult to avoid if crystals are desired. The enzyme's molecular weight is apparently similar to that of the yeast enzyme (34000; Larsson-Raźnikiewicz & Malmström, 1961) and it has a low absorption at 280nm. due mainly to an unusually low tyrosine content.

The preparation is suitable for use in the enzymic methods of determination of ATP (Adam, 1963) and 3-phosphoglycerate (Czok & Eckert, 1963). However, traces of lactate dehydrogenase may remain in the final preparation and so upset measurements in the presence of pyruvate. The lactate dehydrogenase can be completely removed by repeating the Sephadex G-100 chromatography. The preparation has also been used successfully for the determination of ATP plus phosphocreatine, in which case some ADP must be added and all enzymes must be entirely free of myokinase. The heat treatment in mildly acid conditions described in the preparation procedure removes most of the myokinase, and gel filtration ensures that none exists in the final preparation. It is rather surprising that this heat treatment inactivates myokinase in view of the historic report that this enzyme resists boiling in 0·1M-hydrochloric acid (Colowick & Kalckar, 1943). PGAK is stabilized at pH 5·4 and 55° by ammonium sulphate, and further protected by the presence of 3-phosphoglycerate.

Bücher (1947) reported that the yeast enzyme catalysed the 'back' reaction (formation of ATP) some nine times as fast as the forward reaction. If this were so with the muscle enzyme it would possess one of the fastest turnovers known for any enzyme; however, preliminary experiments have indicated that the back reaction, with glyceraldehyde 3-phosphate and its dehydrogenase as substrate, is only about twice as fast; nevertheless a specific activity of 2000units/mg. is very high for a two-substrate–two-product reaction.

Part of this work was carried out by Mr J. G. Bowen while engaged on a sandwich course at Bath University of Technology.

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