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RABBIT MUSCLE PHOSPHOGLUCOMUTASE IS A MONOMER

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SUMMARY

A procedure which avoids the use of heat or precipitation with salt at acid pH has been developed for the isolation of phosphoglucomutase from rabbit muscle. Contrary to earlier reports, the enzyme obtained by this procedure as well as by published techniques is a single polypeptide chain. This is true of phosphorylated as well as dephosphorylated proteins.

Several reports have described the isolation and characterization of phosphoglucomutase[1] from diverse sources (1). Most of the work has been conducted with the enzyme from rabbit muscle. A comprehensive review has summarized our knowledge of this enzyme till 1972 (2). Najjar isolated this enzyme from rabbit muscle in crystalline form. The procedure involved fractionation of water extracts of muscle using heat steps at pH 5.0 followed by a series of precipitations with ammonium sulfate (3). Subsequent modifications involved chromatography on carboxymethylated resins (4,5) and final molecular sieving by Sephadex G-100 column (4). The final preparation was homogeneous as judged by a variety of conventional techniques. The molecular weight of this enzyme was found to be about 65000 (5). Until recently, this protein was considered to be a single polypeptide chain. This conclusion was based on (i) the finger print maps of the tryptic digest (4), (ii) a single metal binding site (2), (iii), one serine capable of being phosphorylated covalently (6),

[1]Abbreviations used: Phosphoglucomutase, PGM; Sodium dodysyl sulfate, SDS; Glucose-1-P, glucose-1-phosphate; glucose-1,6P_2, glucose 1,6 diphosphate; glucose-6-P-dehydrogenase, glucose-6-phosphate dehydrogenase.

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and (iv) one N-terminal amino acid residue, lysine (2). Finally, the protein sedimented as a single 65000 molecular weight species in the presence of denaturing agents known to be capable of dissociating proteins into subunits (7). These studies were conducted with a phosphoenzyme, a form in which it is isolated from rabbit muscle. Subsequent reports showed (8) that the phosphoenzyme was remarkably stable against denaturing agents. However, the dephosphoenzyme was dissociated into two subunits of identical molecular weight, 32000, but non-identical in sequence since they had non-identical N-terminal residues, lysine and valine. Furthermore, the kinetic studies suggested that the phosphate binding site and metal binding sites resided on two separate chains (8,9). It is noteworthy that even the dephosphoenzyme dissociated into subunits only after incubation in sodium dodycyl sulfate for several days.

Our attempt to test regulatory properties of PGM have been unsuccessful (unpublished observation). Indeed, of all the compounds tested, only citrate inhibited this enzyme from E. coli, magnesium reversed the inhibition only partially (10). Duckworth and Sanwal subsequently confirmed these observations and showed rabbit muscle PGM to be inhibited by a variety of other nucleotides (8). Hormones such as insulin, epinephrine also seem to increase the activity of this enzyme in vivo (1,11,12). This regulation, at least in part, was due to the conversion of a less active form of the enzyme, the E-Zn complex, to a fully active form, the E-Mg complex.

Regulatory and chemical properties of enzymes are often altered by the methods of isolation (13). Our failure to observe any siginficant regulatory properties of rabbit muscle PGM isolated by earlier procedures and the controversy concerning its subunit structure prompted the studies described below.

Materials and Methods

Materials: Fresh frozen rabbit muscle (Pel-Freeze) was used. Glucose-1-P, glucose 1-6 P_2, glucose-6-P dehydrogenase from yeast, protein standards, sodium dodycyl sulfate (SDS) were purchased from sigma. Sephadex G-100 was purchased from Pharmacia, DEAE-cellulose (DE-50) was purchased from Whatman.

Methods: Phosphoglucomutase activity was determined as described previously (1). Polyacrylamide gel electrophoresis of the native as well as SDS

treated enzyme was performed as described by Davis (14) and Weber and Osborn (15) respectively. Denaturation in SDS was performed as described by Pringle (16). Impurities in SDS known to interfere with accurate molecular weight determinations (17) were removed by recrystallizing SDS from ethanol. Dephospho PGM was prepared as described by Ray and Peck (2).

Results and Discussion

Isolation of PGM without heat or pH precipitation step: Unless otherwise indicated, all steps were conducted at 4°C. One kilogram of fresh rabbit muscle (Pel-Freeze) was minced in a meat grinder, extracted with 2 volumes of water, filtered through cheese cloth and residue re-extracted with one volume of water. The pooled extract was fractionated with solid ammonium sulfate. Most of the PGM activity precipitated between ammonium sulfate saturations of 0.45 and 0.7. This precipitate was collected by centrifugation dissolved in water and dialyzed against 50 volumes of 0.001M phosphate pH 7.0 for 24 hours, with a buffer change every four hours. A small amount of precipitate formed was centrifuged and discarded. The supernatant was diluted with the same buffer to a final protein concentration of 5 to 8 mg per ml and adjusted to pH 8.0 with 0.1N ammonium hydroxide. Some precipitate formed was removed and the supernatant applied to a DEAE cellulose column (5cm x 70cm) previously equilibrated with the 1 mM phosphate pH 7.8 containing 0.1 mM EDTA. The column was washed with 1 liter of the same buffer and eluted with a gradient. The mixing chamber contained 2 l of 1mM phosphate buffer, pH 7.8, and the reservoir contained 2 l of the same buffer containing 0.2 M NaCl. Fractions of 14 ml each collected were assayed for protein and activity. The activity eluted in a major peak and a shoulder (Fig. 1). Fractions containing major peak which represented 80% of the total activity were pooled, concentrated with ammonium sulfate (0.75 saturation), centrifuged and supernatant discarded. The precipitate was dissolved in water, dialysed for 24 hours against phosphate buffer, 1 mM in Na^+.[2] Some inactive precipitate formed was centrifuged and supernatant diluted with the buffer to protein con-

[2]A stock buffer, Na_2HPO_4-HCl, pH 7.0, 0.1M with respect to Na^+ was prepared. Whenever required, this was diluted as desired and used without further adjustment of pH.

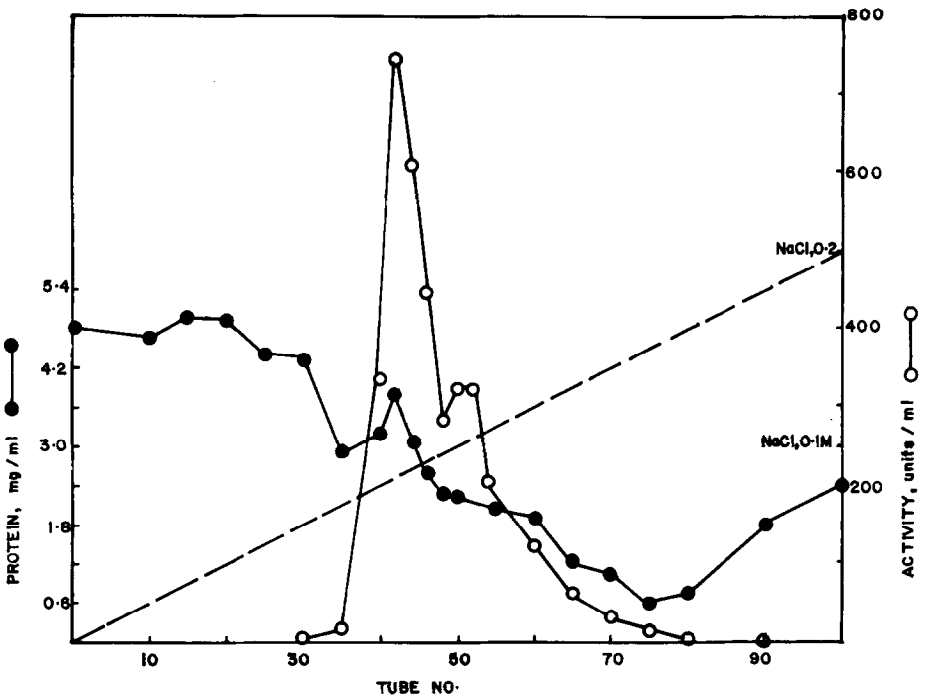


Fig. 1 DEAE-cellulose chromatography of rabbit muscle PGM: ●, proteins, ○, activity; ---, NaCl gradient. Note that the major peak eluted first whereas on CM cellulose column it elutes after the minor peak (ref. 5).

centration of 5 mg/ml and applied to a CM-sephadex column (2.5cm x 50cm) previously equilibrated with the same buffer. The column was washed with 500 ml of 1 mM Na^+ buffer and eluted with a gradient (1) with a mixing chamber and reservoir containing 1 mM Na^+ and 33 mM Na^+ respectively. Each chamber contained 0.1 mM EDTA in addition. Ten ml fractions were collected. Aliquots were assayed for protein and activity. Fractions containing PGM activity were pooled, protein concentrated by ammonium sulfate precipitation (0.75 saturation), centrifuged, the precipitate dissolved in 0.05 M PO_4 buffer pH 7.0 containing 0.1mM EDTA and 1mM Mg^{++} and applied to a Sephadex G-100 column (2.5 x 100 cm) and eluted with the same buffer. One ml fraction collected were assayed for protein and activity. The final preparation had a specific activity of 1488, about twice that obtained with the enzyme prepared by procedures including heat steps (1). Unless otherwise indicated, these were frozen in liquid nitrogen at a

Table 1

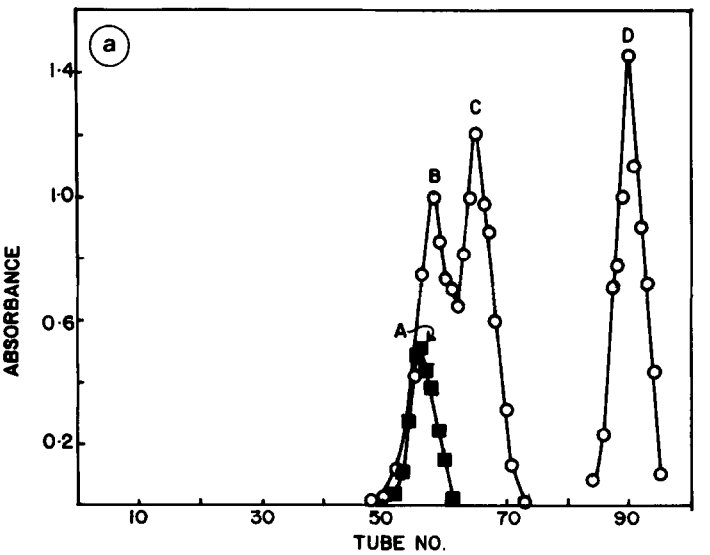
Summary of Purification Procedure

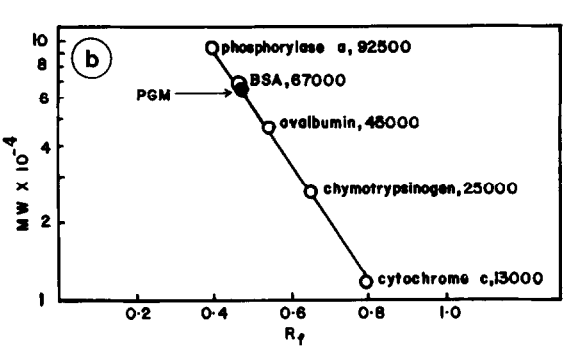
Step	Prot. (mg)	Units	Sp. Activity	% Yield
Extract	67,500	120,000	1.78	100
Ammonium Sulfate Precipitate 0.5-0.7 saturation, dialysis	7,450	98,500	13.2	81.5
DEAE-cellulose chromatography	367	55,000	150	45.8
CM Sephadex chromatography	81	42,000	520	35.0
Sephadex G100 chromatography	21.4	32,000	1488	26.8

protein concentration of about 5-8 mg/ml. All the studies described below were performed with this preparation. This procedure is summarized in Table 1.

Fig. 2a and 2b show that PGM as isolated has a molecular weight of about 64,500 when judged by gel filtration technique or by SDS electrophoresis. Since this enzyme is isolated in a phosphorylated form, similar experiments were repeated with a dephospho enzyme. Here again only one protein band corresponding to a molecular weight of about 64,500 was observed. Thus PGM isolated by the "no-heat" procedure is a monomer. In contrast Duckworth and Sanwal (8,9) reported a molecular weight of about 63000 for the native enzyme and of 32000 for the SDS treated enzyme. These data were obtained with enzyme isolated by procedures using heat step and incubated with SDS at room temperature for several days. Thus the differences in observed subunit could be due to different procedures of isolation and/or of denaturation in SDS. This possibility was investigated by repeating the molecular weight determinations with enzyme isolated by the procedure including the heat step (4). The results (Fig. 2c) show that PGM isolated by either procedure is a monomer.

Rabbit muscle phosphoglucomutase is most stable at pH 7.0 in phosphate buffer and in liquid nitrogen. Storage at -10° or as a precipitate in 0.65 saturated ammonium sulfate resulted in the inactivation (17). Such preparations





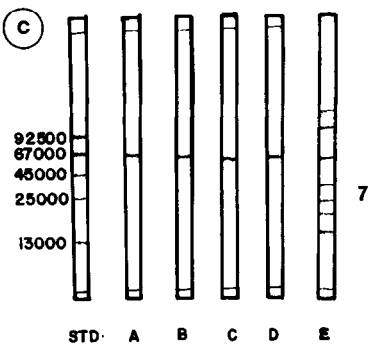


Fig. 2a Molecular weight determination by gel filtration in 6M guanidine hydrochloride and 2-mercaptoethanol as described by Fish et al. (21). A, 4.8 mg of carboxymethylated PGM; B, 10 mg aldolase (subunit MW, 40,000); C, BSA; D, hemoglobin (subunit MW, 15,500). Turbidity was measured as absorbance at 450nm.

Fig. 2b Polyacridamide gel electrophoresis in sodium dodysyl sulfate. All protein samples were processed as described by Pringle (16) and electrophoresis carried out as described by Davis (14). The PGM sample used in Fig. 2a was analysed. R_f = mobility of protein/mobility of marker dye.

Fig. 2c Schematic representation of polyacrylamide gel electrophoresis of carboxymethylated PGM in sodium dodysyl sulfate. A, PGM isolated without the heat step; B, PGM isolated with the heat step; C, PGM isolated without heat step and then dephosphorylated; D, PGM isolated with heat step and then dephosphorylated; E, PGM isolated with heat step and stored for 30 days at 4° in 0.01M phosphate, pH 7.0. The bottom line in each column shows the position of the marker dye. STD-standards as in Fig. 2b.

upon electrophoresis in SDS resolved into several peptides (Fig. 2c). In a separate experiment such samples were treated with dansyl chloride and the acid hydrolysates analysed by thin layer chromatography (19). Several fluorescent spots were observed. These results were very reproducible for a given sample, but varied from one preparation to another and with the conditions of the storage of the native enzyme. The appearance of multiple bands may be attributed to the presence of a contaminating protease(s) and heavier bands to polymerization. Although the existance of such contaminating proteolytic enzymes was not detected, the possibility appears very likely since Pringle has shown that such comtaminating proteases in "pure" muscle hexokinase preparations are active at room temperature even in the presence of SDS and produce artificial subunits (16). Finally, the amino acid composition of enzyme isolated by the two procedures was very similar (data not shown).

It may therefore conclude that (1) rabbit muscle PGM isolated by the procedures including the heat step or by the procedure described here is a monomer and (2) confirms the recent observations made with phosphoenzyme (20) and extends it to the dephospho enzyme preparations isolated by either procedure. The higher specific activity observed here must be attributed to the milder techniques employed.

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