CRYSTALLIZATION OF RABBIT MUSCLE PHOSPHOFRUCTOKINASE*

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In the past few years several independent reports have indicated that phosphofructokinase (PFK) plays an important role in the regulation of glycolysis in different mammalian tissues (1, 2, 3, 4, 5), as well as in schistosomes (6) and yeast (7). Experiments in vitro have further supported the possibility that the complicated kinetic properties of PFK are controlled by physiological levels of substrate, ATP and a number of other intracellular metabolites (8, 9, 10, 11, 12, 13, 14, 15). These observations were based largely on experiments carried out with crude or partially purified preparations, and the importance of having a pure, homogeneous and stable enzyme for a better understanding of kinetic and physico-chemical properties became apparent. Recently it was observed that a fractionation procedure developed for the purification of rabbit skeletal muscle phosphorylase b kinase (16) also could be applied to PFK (17, 18). Further steps have now led to the isolation of the latter enzyme in a stable, crystalline form. Previous attempts to purify this enzyme from rabbit muscle resulted in only partial purification (19, 20) or yielded highly purified but unstable preparations (21, 22).

EXPERIMENTAL AND DISCUSSION

The initial steps for the purification of PFK are the same as those that have been developed for phosphorylase b kinase up to the 40,000 rpm

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pellet (16) and, therefore, they will be described only briefly. Skeletal muscle from adult rabbit is homogenized in 4 mM EDTA at 2° and centrifuged. The pH of the supernatant solution (muscle extract) is adjusted to 6.15, and the precipitate which forms is dissolved in 50 mM glycerol-P - 2 mM EDTA buffer, pH 7.2. Two differential centrifugation steps follow, the first one at 30,000 rpm (78,000 xg) for 1 1/2 hours and the second one at 40,000 rpm (105,000 xg) for 3 hours. In the last step PFK and phosphorylase b kinase are both concentrated in the pellet which is dissolved in 50 mM glycerol-P - 2 mM EDTA buffer, pH 7.2, to give a protein concentration of 30-40 mg/ml. The separation of kinase from PFK and the further purification of the latter enzyme is carried out as follows:

The above solution (40,000 rpm pellet) is heated at 60° for 2 minutes to destroy phosphorylase b kinase; it is then cooled and centrifuged. (Except where noted, all steps are carried out at 2°.) PFK is then precipitated from the supernatant between 40 and 50% saturation with $(NH_4)_2SO_4$. The protein is collected by centrifugation and dissolved in a small volume of glycerol-P - 2 mM EDTA buffer pH 7.2, to give a final protein concentration of 2 to 3%. The resulting solution is dialyzed against 36% saturated $(NH_4)_2SO_4$ in 50 mM glycerol-P - 2 mM EDTA buffer in the presence of 4 mM ATP at pH 7.0. After 1 or 2 weeks crystallization starts, at which time the $(NH_4)_2SO_4$ concentration is slowly increased to 40% over a 4-day period. The crystallization procedure can be accelerated by seeding; the process is also hastened by warming the solution to 25° and recooling. The crystals usually occur as plates or bipyramides (Fig. 1), but in a few preparations rods have been observed. For recrystallization, the crystals are dissolved in 50 mM glycerol-P - 2 mM EDTA buffer, pH 7.2, at 30° and the above procedure is repeated.

By this method PFK has been crystallized in fourteen successive preparations. Attempts to crystallize the enzyme in the absence of ATP or in the presence of other cofactors have been unsuccessful. To remove ATP from crystalline PFK, the protein is first dialyzed for a few hours

against several changes of 50 mM glycerol-P - 2 mM EDTA buffer, pH 7.2, and then passed through a small charcoal-cellulose column. The treated enzyme slowly becomes turbid when stored, but in the presence of ATP aggregation is delayed for at least two weeks; activity is maintained on storage with or without ATP. Suspensions of crystalline PFK stored in ammonium sulphate are stable for at least several weeks. The following table shows the average results obtained in four preparations of PFK starting with 1 kg of muscle:

Purification of PFK from Rabbit Skeletal Muscle

Fraction	Total Activity	Specific Activity	Purification
	Units	Units/mg	X
Extract	35,100	0.9	
Acid Precipitate	21,300	6.3	7
30,000 rpm Supernatant	13,200	18.6	21
40,000 rpm Pellet	7,000	36.8	41
Heated 40,000 rpm Pellet	6,715	96.2	104
$(NH_4)_2SO_4$ Precipitate	5,300	121	134
First Crystals	4,600	139	154
Second Crystals	4,400	145	161

PFK activity was assayed by determining the amount of fructose-1,6-di-P formed in a reaction mixture at pH 8.2 and 26°, containing 50 mM glycylglycine - 1 mM EDTA buffer, 1 mM fructose-6-P, 1 mM ATP, 6 mM $MgCl_2$, 4 mM NH_4^+, 10 mM cysteine, 0.5 mM DPNH, and 0.01% albumin. The auxiliary enzymes, aldolase, α-glycero-P dehydrogenase, and triose-P isomerase (all from Boehringer) were present in excess. The rate of fructose diphosphate formation was followed spectrophotometrically at 340 mμ as DPNH disappearance (23). Activity is expressed as μmoles fructose diphosphate formed per minute.

On electrophoresis, crystalline PFK migrates as a single symmetric boundary in the presence of ATP (Fig. 2). In the absence of nucleotide, some turbidity often develops in the cell and noticeable asymmetry of the peak is present.

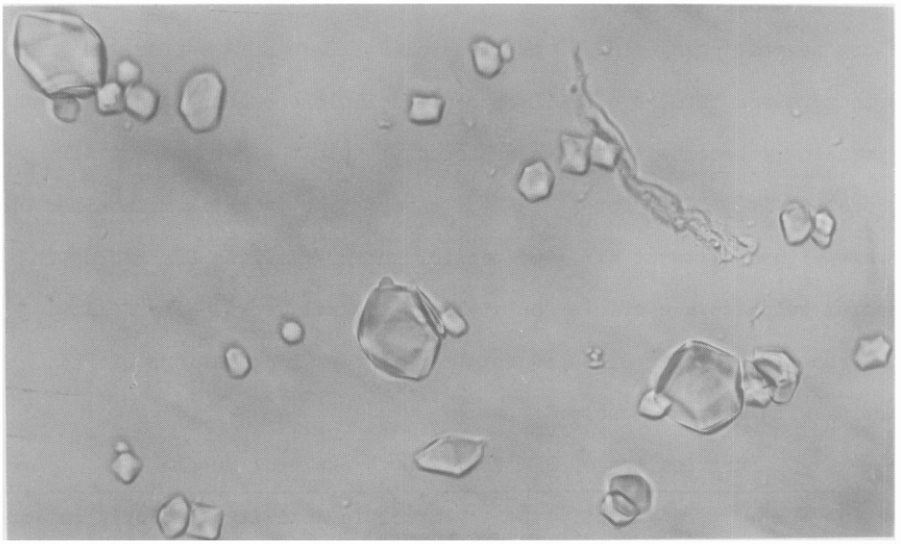

Fig. 1. Crystals of rabbit muscle phosphofructokinase, approx. 400x.

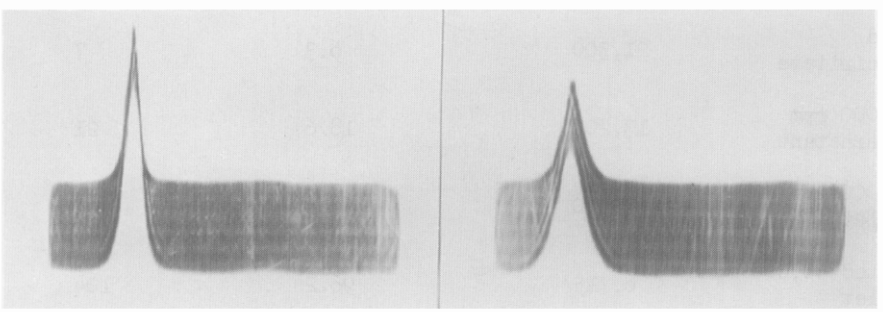

Fig. 2. Electrophoretic pattern (ascending limb left and descending limb right) of 3x crystallized rabbit muscle PFK at pH 7.2 in 50 mM glycerol-P - 2 mM EDTA buffer containing 10 mM ATP. Migration from right to left.

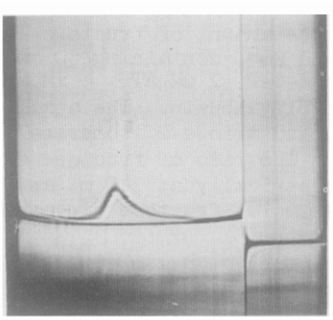

Fig. 3. Ultracentrifuge pattern of 3x crystallized PFK in 50 mM glycerol-P - 2 mM EDTA buffer, pH 7.2, after 40 minutes at a speed of 39,460 rpm. Bar angle 70°. Protein concentration 0.6%. Sedimentation from right to left.

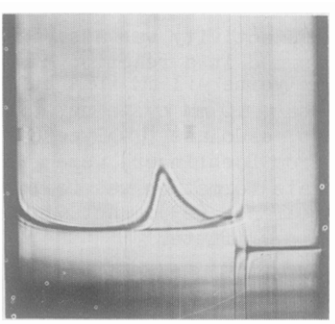

Fig. 4. Identical conditions as in Fig. 3 with the addition of 10 mM ATP. Protein concentration 0.7%.

In the ultracentrifuge the enzyme shows an asymmetric peak with a trailing edge (Fig. 3 and 4). The latter feature is more pronounced in the presence (Fig. 4) than in the absence of ATP (Fig. 3). In the presence of ATP, $s_{20,w}$ for the main peak is 25S, while in the absence of ATP it increases to 40S. This behavior of PFK, together with the changes in sedimentation observed under various conditions in sucrose-gradient centrifugation (24, 25), suggests that the enzyme is involved in a complicated associating-dissociating system. Evidence presented here indicates that ATP affects this system, as might be expected for a compound interacting with a regulatory site in PFK. It is not yet known whether these properties are specific for ATP or if they are related to the action of this nucleotide on the kinetic behavior of PFK.

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