Properties of rat lens phosphofructokinase

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Two interconvertible forms of phosphofructokinase (PFK) have been eluted from a DEAE-cellulose column from the supernatant fraction of rat lens homogenates centrifuged at 96,000 x g for 1 hour at 0 to 4° C. The interconversion can be manipulated by a change in the pH of the extracting and eluting buffers. PFK-I is the dominant form at pH between 7.4 to 7.05, while PFK-II dominates at pH 7.4 to 8.2. PFK-II is believed to be the functional form; it is inhibited by high concentrations of ATP and the inhibitory effect is enhanced by more acidic pH. Fructose-6-phosphate counteracts ATP inhibition, but the most potent de-inhibitors are ADP and AMP. Among the inorganic ions tested, sulfate, phosphate, ammonium, and potassium also de-inhibit, whereas calcium further inhibits the enzyme. The behavior of PFK under physiologic conditions and the significance of the presence of two forms of PFK in the lens are discussed.

Key words: phosphofructokinase, rat lens, inhibition, de-inhibition, pH effect, interconversion of the enzymes, physiologic conditions, acidosis.

One of the regulatory steps of glycolysis in the lens involves phosphofructokinase (PFK).1 The activity of PFK, however, is dictated by various factors such as pH changes, influences of positive and negative effectors, and also by the interactions of these factors. The effectors may either be organic compounds (e.g., adenosine phosphates or fructose phosphates) or inorganic ions such as potassium, ammonium, sulfate, phosphate, or calcium. These effectors may stabilize, activate, or inhibit the PFK, or they may de-inhibit the inhibitory effect of high concentrations of ATP (for review, see Reference 2). Alternatively, some of the effectors may catalyze conformational changes of PFK,3 or affect the association-dissociation of the enzyme molecules.4 The cumulative effects of such effectors thereby determine the final PFK activity and influence the rate of anaerobic glycolysis.

In the lens, glycolysis provides more than 70 per cent of total ATP.5-6 To understand the role of PFK in the regulation of lens glycolysis, we have attempted to characterize the properties of lens PFK and measure the kinetics of PFK in the presence of positive or negative effectors. Some factors affecting PFK are fairly well defined in the lens.7 It is, therefore, possible to speculate on the behavior of PFK under physiologic conditions and thus on

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Fig. 1. Elution profiles of PFK from rat liver, heart, and lens on a DEAE-cellulose column (1.5 by 20 cm.). The tissues were homogenized in 0.02 M Tris-Cl buffer (pH 8.0) containing 0.05 M NaF, 1 mM ATP, and 1 mM dithiothreitol. Stepwise elutions with increasing strengths of Tris-Cl buffer were carried out. Determination of PFK activity was described in Materials and Methods.

Fig. 2. Rat lenses were homogenized in 0.05 M Tris-Cl (pH 8.2) containing 5 mM EDTA. After centrifugation, the supernatant was loaded onto a DEAE-cellulose column (1.5 by 18 cm.) and eluted with a linear gradient of 0 to 0.2 M Na₂SO₄ in the starting buffer.
its role in the control of glycolysis. We have also directed our attention to the implied significance of the presence of two interconvertible PFK forms in the lens.

Materials and methods
Albino rats (100 to 150 grams), purchased from Charles River Breeding Laboratories, Wilmington, Mass., were decapitated and from the enucleated globes the lenses were removed and homogenized in ice-cold buffer. The homogenates were centrifuged at 96,000 x g for 1 hour at 0 to 4 °C, in a Beckman L2-65B ultracentrifuge (all subsequent experiments were conducted at this temperature except where specifically mentioned). The supernatants were collected and passed through DEAE-cellulose columns (compositions of extracting and eluting buffers and also column constructions are described in the Results section). The PFK-active fractions were used for subsequent studies.

PFK assays were carried out at 26 °C in a medium containing various concentrations of ATP, fructose-6-phosphate (fru-6-P), and Mg²⁺ with 0.12 mM NADH, 5 μg of triosephosphate isomerase and glycerol phosphate dehydrogenase each, and 40 μg of aldolase in a final volume of 1 ml. To determine the PFK-active fractions of column chromatography, the concentration of ATP, fru-6-P, and Mg²⁺ was 0.1, 1.0, and 0.7 mM, respectively, with undesalted auxiliary enzymes in the media. The activity was indicated by a decrease in absorption at 340 nm on a Gilford automatic recording spectrophotometer (Model 2400-2). One PFK unit equals to A₃₄₀ × 10⁵ per minute per 0.05 ml PFK solution.

PFK and auxiliary enzymes were desalted on a Sephadex G-25 column to remove ammonium and sulfate ions which interfered with kinetic studies. The auxiliary enzymes and organic compounds were purchased from Boehringer (Mannheim, Germany). Inorganic compounds were products of Mallinckrodt (St. Louis, Mo.) or Fisher Scientific (Fair Lawn, N. J.). Sephadex and Sepharose gels were from Pharmacia (Framingham, N. J.) and DEAE-cellulose was a product of Brown Co. (North Stratford, N. H.).

Results
Identity of lens PFK. PFK has been classified into muscle and liver types. This is based on ion exchange column chromatography with electrophoretic and immunologic supporting evidence. We have compared the elution profiles of lens PFK from a DEAE-cellulose column to PFK prepared from heart and liver. Fig. 1 shows that lens PFK was eluted as a single peak with a small shoulder preceding it, and was probably closer to the muscle type than to the liver type.

Two forms of lens PFK. Two forms of PFK were found in the supernatant fraction (and also in the particulate fraction [unpublished observations]) of lens extracts. Fig. 2 shows that with a linear sulfate gradient on a DEAE-cellulose column, PFK-I was eluted at 20 mM sulfate, while PFK-II was eluted at 80 mM sulfate. The separation of the two forms can also be accomplished by stepwise elution with two different concentrations of Tris-Cl buffer containing the same sulfate and EDTA components (Fig. 3). Predominance of either PFK-I or PFK-II can be manipulated by a change of pH of the extracting and the eluting buffers (Fig. 3). PFK-I dominates at pH between 7.4 to 7.05, while...
Fig. 4. A, PFK-I, incubated with 5 mM ATP at room temperature for 15 minutes at pH 7.05 and then passed through a Sephadex G-25 column, retained 75% of its initial activity (which was 16, assayed at pH 8.2). The enzyme was assayed with 0.1 mM ATP, 0.2 mM Mg²⁺, and various concentrations of fru-6-P in the media at indicated pH values. B, PFK-I was desalted and stored at 0 to 4° C, pH 7.05, for 4 hours (initial activity, 23, assayed at pH 8.2). The enzyme was then assayed as in A.

Fig. 5. Ten rat lenses were prepared in 2 ml. 0.1 M Tris-Cl buffer with 0.1 M Na₂SO₄ and 5 mM EDTA at pH 7.05 or pH 8.2. Marker molecules: blue dextran, yeast hexokinase (molecular weight 96,600), and rabbit muscle aldolase (molecular weight 158,000) were prepared at pH 7.05. Sepharose 6B column (1.5 by 70 cm.) chromatography was carried out at pH 7.05 and pH 8.2.
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PFK-II dominates at 7.4 to 8.2. Rechromatography of PFK-I (eluted at pH 7.05), after the pH has been adjusted to 8.2, results in the appearance of PFK activity in the position of PFK-II from the DEAE-cellulose column equilibrated at pH 8.2. The two forms of PFK appear to be interconvertible.

Stability of lens PFK. At pH 8.2, sulfate was not required to stabilize PFK which remained unchanged in activity for at least 3 hours at 0 to 4°C. However, at pH 7.05, the enzyme lost 80 to 90 per cent of its original activity in 3 hours at 0 to 4°C if sulfate was removed. Adding sulfate to the assay media to a final concentration of 25 mM failed to reactivate the enzyme. However, incubation of the enzyme with 5 mM ATP at room temperature for 15 minutes followed by passing through a Sephadex G-25 column re-established PFK activity, stability, and pH sensitivity (Fig. 4, A). The desalted enzyme showed low affinity toward fru-6-P and the activity was not altered by changes in pH of the assay media (Fig. 4, B).

Molecular size of lens PFK. To determine if PFK-I might be a dissociated form of PFK-II, a Sepharose 6B column was used for this purpose. Fig. 5 shows that PFK eluted either at pH 7.05 or at pH 8.2 occupied the same position. The molecular weight was estimated to be 250,000 to 320,000 at either pH. This suggests that the behavior of PFK on DEAE-cellulose columns (Fig. 3) may involve a change in molecular conformation but not molecular association.

Kinetic studies. Lens PFK activity was affected profoundly by pH changes. Fig. 6 shows a Lineweaver-Burk plot of PFK activity under noninhibitory concentrations of ATP at three different pH values. The affinity for fru-6-P decreases with the decrease in pH. The pH effect was even more pronounced in examining the ATP inhibitory effect which appeared to be enhanced by more acidic pH values (Fig. 7).

De-inhibitors of lens PFK. Elevation of fru-6-P tended to counteract the ATP inhibitory effect (Fig. 8). The most potent de-inhibitors seemed to be ADP or AMP, 0.5 mM of either was sufficient to fully restore a 50 per cent inhibited PFK activity at pH 7.05 in the presence of 1 mM fru-6-P. Some inorganic ions, which are normally
Fig. 7. Effect of pH on PFK inhibition by ATP. Assay conditions: fru-6-P, 1 mM, and the concentration of Mg²⁺ was twice that of ATP concentration.

Fig. 8. Effect of fru-6-P in counteracting the ATP inhibition of PFK at pH 7.4 (A), and pH 7.05 (B).

Present in the lens, exhibited a similar de-inhibitory effect. Sulfate and phosphate are more effective than potassium or ammonium ions (Fig. 9). Calcium, however, further inhibited the enzyme and NaCl did not have any effect.

The sigmoidal response to fru-6-P under inhibitory ATP concentrations was not altered by the inorganic effectors (Fig. 10). The ions may act on the catalytic site of PFK molecule, but apparently do not affect the subunit interactions.
Fig. 9. Effect of inorganic ions on PFK activity in the presence of 1 mM fru-6-P at pH 7.5. The PFK was inhibited with 2 mM ATP to 36 per cent of its initial activity (5.5 units) or in testing inhibitory effect of calcum in addition to ATP inhibition, the PFK was inhibited to 58 per cent of its initial activity (6 units). Markers on the ordinate indicate the initial activity. The ions were included in the assay media to the indicated final concentrations.

Fig. 10. Effect of inorganic ions (final concentration: 25 mM) on sigmoidicity of PFK activity under inhibition by ATP. Concentrations of ATP were 2 mM at pH 7.5 (A) and 1 mM at pH 7.25 (B) Markers on the ordinate indicate the initial activity.

Discussion

Previous studies on calf lens PFK have indicated that PFK was inhibited by greater than 0.25 mM ATP and that the inhibition was independent of pH or levels of fru-6-P present. The rat lens PFK, however, showed sensitivity to pH changes (Figs. 4, 6) and to different levels of fru-6-P (Fig. 8). Similar observations have been noted on PFK from erythrocytes, muscle, liver, and other tissues. Although rat lens PFK-I did appear to be free from pH influences (Fig. 4, B), the functional form seemed to be PFK-II.

In the lens, ATP levels have been shown to differ topographically. Epithelial cells, having the highest metabolic activities, maintained the highest ATP level (up to 8 mM in rabbit lens, for example). On the average, rat lens contains 2 to 3 mM ATP. At pH 7.4, this ATP concentration alone is sufficient to cause total inhibition of PFK (Fig. 8, A), since the concentration of fru-6-P is less than 0.2 mM in the lens. The ATP inhibitory effect is most efficiently reversed by ADP, AMP, or Pi, signals of ATP deficiency. This is in agreement with the "adenylate energy charge"
concept, in which the PFK activity was proposed to being regulated by the ratio of ATP + \( \frac{1}{2} \)ADP to total adenylates. An increase of the ratio results in the decrease of PFK activity, while a decrease of the ratio activates PFK.

The presence of potassium (100 mM), ammonium (concentration presumably depends on degree of hypoxia), ADP (0.43 mM), AMP (0.17 mM), and Pi (4.2 mM) in the rat lens (calculated from data presented in Reference 7) will result in a minimal or basal level of PFK activity even if ATP is in great excess. This would be suggestive that there is always some phosphorylation of fru-6-P present in the lens. Although the turnover rate of ATP is unknown, the steady-state ATP concentration is always high in the lens, presumably due to extremely low levels of ATPase, the relatively low amount of "metabolic work" (e.g., active transport) done, low level of high-energy phosphate acceptors, and low activity of ATP-utilizing enzymes. It is conceivable that any ATP expenditure will be readily replenished through this basal PFK activity.

The role of sulfate in the lens is not clear. Sulfate ions are incorporated into glycoproteins and glycolipids in lens epithelium during lens development, but the effects of sulfate on PFK (e.g., stabilization and de-inhibition) are probably a simulation of Pi effect; the two ions have been shown to act on the same (catalytic) site on PFK.

The effect of calcium on PFK activity in the cataractous lens is not known. Moreover, the previously reported data on the calcium level in the cataractous lens are often contradictory (see References 21 and 22). Also, the amount of free and bound calcium in the lens remains to be determined. Only the free form would affect PFK activity.

The inter-conversion of PFK-I and PFK-II is pH-dependent. The distribution and the proportion or the equilibrium state of the two forms in vivo were not determined. Reports on rabbit muscle PFK, and guinea pig heart PFK have indicated that PFK underwent dissociation-association reaction following nonphysiologic pH variations (e.g., sudden decrease of pH from 8 to 6), in which the native PFK, a tetramer, dissociated into dimers and/or monomers at low pH, and re-aggregated in the presence of adenosine phosphates or fructose phosphates upon reversion of pH back to alkaline pH.

The smallest active PFK of the rat was found in jejunal mucosa with a molecular weight of 192,000 as determined by Sephadex G-200 gel filtration at pH 7.6. The molecular weight of rat liver PFK was determined to be 250,000 to 300,000 (at pH 8.0), which is probably also the size of lens PFK, since lens PFK was excluded from Sephadex G-200 (not shown) and was eluted in a Sepharose 6B column (Fig. 5).

It seems that lens PFK undergoes only conformational changes in the pH range of 7.05 to 7.4. Whether lens PFK dissociates at pH lower than 7.05 remains to be tested.

The conversion of PFK-II to PFK-I may occur during acidosis or when lactate dissipation is impaired. ATP-preconditioning then presents as a protective mechanism to maintain PFK activity throughout the crisis. When pH returns to normal (pH 7.4), PFK-I will be de-protonated and revert back to PFK-II, the functional form. In this respect, PFK-I may be considered as PFK reserve, a dormant form of PFK.

It appears that lens PFK is quite sensitive to pH variations. Little is known relative to the acid-base balance in the lens and aqueous humor and to the interaction of the two entities regarding lactate diffusion. The effect of H+ on PFK in vivo requires further investigation.

We are now in the process of verifying the role of PFK in the control of glycolysis with the use of organ culture of the lens.

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