Stability of mammalian lens phosphofructokinase

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Two interconvertible phosphofructokinase (PFK) forms were found in rat and human lenses; whereas only one predominant form was found in calf lens. PFK isolated from these lenses possessed a common property, i.e., pH-dependent cold (or acid) lability. The inactivation was prevented by including either adenosine triphosphate (ATP) or fructose-6-phosphate (fru-6-P) in the incubating media. The protective effect of ATP or fru-6-P was complete in rat or calf lenses. In human lens, although fru-6-P was fully protective, ATP protected only partially. The inactivation could be reversed by addition of fru-6-P, but not ATP, to the incubating media at 37.5° C. Lens organ culture studies showed that the depletion of lenticular ATP seemed to precipitate the loss of PFK.

Key words: phosphofructokinase, rat, calf, human lens, cold lability, interconvertible forms, reactivation, stability.

Although it has been observed that the activity of lens phosphofructokinase (PFK) is dependent on a number of cellular factors, such as pH, or the presence of organic or inorganic effectors, it is also important to note that the stability of this enzyme may also play a significant role in the regulation of glycolysis.

It is known that PFK is extremely unstable at acidic pH's; this inactivation process involved the dissociation of PFK molecules into inactive subunits, and is best described by the pH-dependent cold sensitivity behavior, which in effect is identical to the pH-dependent inactivation at higher temperatures and lower pH's. The inactivation can be prevented by addition of substances such as adenosine triphosphate (ATP), fructose-6-phosphate (fru-6-P), or related materials, depending on the PFK in question, to the incubating media.

In this paper, we attempt to compare PFK's isolated from lenses of rat, calf, and human beings on their resistance to cold treatment at acidic pH's. Also, lens organ cultures are employed to show the effect of ATP deprivation on PFK stability.

We hope to define the conditions for maintenance of PFK stability, since the loss of PFK may contribute to cataractogenesis by disruption of ATP formation in the lens.

Materials and methods

Isolation of the PFK's was carried out at 25° to 26° C. to avoid cold inactivation.
To study the presence of PFK forms, the lenses were homogenized in 0.05M Tris-Cl buffer (pH 7.05 or 8.2) containing 0.05M Na$_2$SO$_4$ and 0.005M ethylenediamine tetraacetic acid (EDTA) (this buffer will be referred to as the "starting buffer" throughout the paper) and centrifuged at 96,000 × g for 1 hour in a Beckman L2-65B ultracentrifuge. The supernatants were passed through diethylaminoethyl (DEAE)-cellulose column equilibrated with the starting buffer. At pH 8.2, rat lens PFK is predominantly in PFK-II form, and can be eluted with 0.15M Tris-Cl buffer; whereas PFK-I is eluted with the starting buffer at pH 7.05.1 The same elution schemes were applied to calf and human lens PFK isolation. Further purification of the enzymes was carried out by concentrating PFK-active fractions on an Amicon ultrafilter with Diaflo membrane no. XM-100A (Amicon, Lexington, Mass.) and then passed through either a Bio-Gel A-0.5m or a Bio-Gel A-1.5m column.

Since it was found that excessive lens proteins interfered with cold lability studies of calf and human lenses, the capsule-epithelia, after homogenization and centrifugation, were used as PFK source in these studies.

Rat lens organ cultures were performed as described previously.8 PFK activity (expressed as $\Delta A_{340\text{nm}} \times 10^3$/min/0.05 ml. of PFK solution) was assayed according to the methods described in an earlier paper.1 Protein was determined according to the method of Lowry and associates,9 and ATP was assayed fluorometrically with the procedures of Strehler and Totter.10 Albino rats were purchased from Charles River Breeding Laboratories, Wilmington, Mass. Calf eyes were delivered from a local slaughterhouse within 3 hours of the death of the animal. Human cataract lenses were collected in the operating rooms of the Massachusetts Eye and Ear Infirmary. The cataracts were classified according to the criteria of Pirie.11 Normal human lenses were excised from eye bank eyes immediately after the corneas were removed for keratoplasty. The lenses were used as soon as they arrived in the laboratory.

The organic compounds were products of Boehringer-Mannheim (New York). Bio-Gels were
Fraction No.

Fig. 2. DEAE-cellulose column chromatography of calf lens PFK. A, Enzyme solution prepared from homogenizing one calf lens in 1 ml. of starting buffer (pH 7.05) and the elution performed at pH 7.05 (see Materials and Methods). B, 2 ml. of PFK-active fraction, from a Bio-Gel A-0.5m column (1.5 by 28 cm.) equilibrated with starting buffer (pH 8.2), was applied to DEAE-cellulose column (1.5 by 27.5 cm.) and eluted at the same pH. Arrows indicate the start of the second buffer (i.e., 0.15M Tris-Cl buffer containing NaNO_3 and EDTA).

Results

Mammalian lens PFK forms. It has been established in rat lens that PFK exists in two interconvertible forms, namely, the stable alkaline PFK-II form and the acidic PFK-I form, which is relatively unstable. It appears that there are also two interconvertible PFK forms present in human lens. One of the PFK forms dominates at neutral pH (Fig. 1, A), whereas at alkaline pH, both forms are present (Fig. 1, C). These two forms can also be obtained by changing the pH of PFK collected at neutral pH and rechromatographing the enzyme at alkaline pH (Fig. 1, B).

Similar ion-exchange column chromatography of calf lens PFK shows that there is only one dominant form in the pH range tested (Fig. 2). The alkaline form is relatively insignificant compared to that of the rat or human lens PFK.

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Rat lens PFK. The enzyme was tested for its response to pH and temperature variations. It was found that cold inactivation started at temperature lower than 15.5° C. and at pH lower than 6.75. A 26 per cent loss in 1 hour was observed at pH 6.35 and 15.5° C. The sensitivity to cold increased as the temperature and pH were further lowered. Fig. 3, A shows that at 9.5° C., PFK incubated at pH 7.15 lost 12 per cent of its original activity in 1 hour; at pH 6.35, the loss was 34 per cent and increased to 77.5 per cent at 2° C. (Fig. 3, B).

However, in all cases, if the incubating media contained either ATP or fru-6-P at a final concentration of 5 mM, the cold inactivation was prevented. Incubation in
the presence of 5 mM adenosine diphosphate (ADP) retained 45 per cent of initial activity at pH 6.3 and 2°C. after 1 hour (i.e., the protection was incomplete); adenosine 5'-monophosphate (AMP), on the other hand, seemed to facilitate the inactivation (Table I).

At 25°C, the enzyme is stable at pH 6.2 (in the presence of sulfate and/or phosphate), but would lose its activity if the pH is further lowered.

Calf lens PFK. This enzyme is cold sensitive only at pH lower than 7.55. The extent of inactivation is independent of pH, however (Fig. 4).

Protection by either ATP or fru-6-P is complete at pH’s and temperatures as low as 6.2 and 2°C., respectively.

Human lens PFK. Like rat lens PFK, the human lens PFK is sensitive to pH changes in the incubating media in cold (not shown). Protection by fru-6-P, at 5 mM, is
Table I. Effect of various organic phosphates on the cold inactivation* of rat lens PFK

<table>
<thead>
<tr>
<th>Organic phosphate added†</th>
<th>PFK activity</th>
<th>Per cent of original activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Initial (1)</td>
<td>Final (2)</td>
</tr>
<tr>
<td>ATP</td>
<td>15.84</td>
<td>16.16</td>
</tr>
<tr>
<td>ADP</td>
<td>14.75</td>
<td>6.58</td>
</tr>
<tr>
<td>AMP</td>
<td>11.54</td>
<td>0.62</td>
</tr>
<tr>
<td>Fru-6-P</td>
<td>14.25</td>
<td>13.68</td>
</tr>
<tr>
<td>No addition</td>
<td>14.63</td>
<td>3.42</td>
</tr>
</tbody>
</table>

*Final pH 6.3, incubated at 2° C for 1 hour.
†Final concentration, 5 mM.

complete; whereas ATP, at up to 10 mM, protects only partially (Fig. 5). This is true for all types of normal and cataractous human lenses tested; the results are summarized in Table II. Note that the specific activity of PFK is drastically reduced in senile cataracts.

Reversal of PFK inactivation. Our earlier attempts at reactivating the cold- or acid-treated PFK had been unsuccessful.12 It gradually emerged that the reversal of PFK inactivation was dependent on the number of PFK protomers present. Fig. 6 shows that the extent of reversal is a function of protein (or protomer) concentration; the addition of nonspecific protein such as bovine serum albumin did not stimulate or accelerate the reactivation. Moreover, only fru-6-P was effective; ATP showed no activation power.

To show the presence of PFK protomers, 16 lenses were homogenized in 1 ml of pH 5.6 buffer to obtain PFK protomers. After passing through a Bio-Gel A 1.5-m column, the eluants were incubated with 5 mM fru-6-P at pH 7.95 and 37.5° C for 1 hour. PFK activity now appeared in both void volume (where the native PFK was eluted) and the end of the column chromatogram (Fig. 7); the latter is a result of the reformation of active PFK molecules.

Lens organ culture studies. The results obtained thus far suggest that ATP and fru-6-P play a central role in the stabilization of lens PFK. Physiologically, ATP is probably more important, since the intralenticular fru-6-P concentration is much lower than that of ATP (which is 2 to 3 mM in rat lens), and is strictly controlled by hexokinase. The depletion of ATP from the lens may then cause the destabilization and loss of PFK. The experimental "hypoglycemic" cataract of rat lens offers such an opportunity to examine the ATP effect.

Table III summarizes the results of rat
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Fig. 5. The human lens PFK preparations were adjusted to final pH of 6.35 to 6.4 at the conditions indicated, and PFK activity assayed periodically. The human lens in this case was obtained from an eye bank eye from a 23-year-old donor.

Table II. Protective effect of ATP and fru-6-P on cold inactivation of human lens PFK

<table>
<thead>
<tr>
<th>Type of lens</th>
<th>No addition</th>
<th>With 5 mM ATP</th>
<th>With 5 mM fru-6-P</th>
<th>No. of capsule-epithelium tested†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal, 23 years old‡</td>
<td>29</td>
<td>56.7</td>
<td>95.5</td>
<td>1</td>
</tr>
<tr>
<td>Normal, 64 years old</td>
<td>29.6</td>
<td>50.5</td>
<td>89.5</td>
<td>2</td>
</tr>
<tr>
<td>Cataract, Group II‡</td>
<td>15.9</td>
<td>45</td>
<td>85.6</td>
<td>3</td>
</tr>
<tr>
<td>Cataract, Group IV</td>
<td>33.9</td>
<td>58.5</td>
<td>78.9</td>
<td>2</td>
</tr>
</tbody>
</table>

*The PFK preparations were adjusted to pH 6.35 by addition of an equal volume of 0.1M phosphate buffer (with 0.05M NaSO₄ and 0.05M EDTA) containing 0 or 10 mM of ATP or fru-6-P. The mixtures were incubated at 2° C. for 1 hour, and the initial and final activities compared to zero-time activity (100%).
†Homogenized in the starting buffer (pH 8.2) (see Materials and Methods) at one capsule-epithelium per milliliter of buffer.

Table III. Summary of rat lens culture studies (mean ± S.D.); five to six lens pairs were used in each incubation

<table>
<thead>
<tr>
<th>Hours of incubation</th>
<th>mM glucose in medium</th>
<th>Change in lens wet weight (%)</th>
<th>ATP conc. (nmol/lens)</th>
<th>PFK activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Whole homogenate*</td>
<td>Soluble fraction†</td>
</tr>
<tr>
<td>0</td>
<td>—</td>
<td>—</td>
<td>58.6 ± 3.5</td>
<td>63.5 ± 7.5</td>
</tr>
<tr>
<td>5</td>
<td>12</td>
<td>0</td>
<td>71.5 ± 2.5</td>
<td>72.2 ± 9.7</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>−1.4</td>
<td>73.1 ± 5.5</td>
<td>54.2 ± 6.7</td>
</tr>
<tr>
<td>17</td>
<td>12</td>
<td>0</td>
<td>42.0 ± 6.3</td>
<td>54.2 ± 11.6</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>+15.0</td>
<td>53.3 ± 2.8</td>
<td>42.4 ± 4.4</td>
</tr>
<tr>
<td>44</td>
<td>12</td>
<td>0</td>
<td>0</td>
<td>36.3 ± 4.1</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>+25.4</td>
<td>44.1 ± 2.8</td>
<td>78.5 ± 9.6</td>
</tr>
</tbody>
</table>

°ΔA₃₄₀ nm. x 10⁴/min./0.05 ml. of homogenate.
†ΔA₃₄₀ nm. x 10⁴/min./mg. of soluble protein.
Fig. 6. The supernatants from 2 rat lenses homogenized in 2 ml. of 0.05M phosphate buffer (pH 6.2) containing 0.05M Na₂SO₄ and 0.005M EDTA were incubated at 2°C for 1.33 hours until 17.7 per cent of the original activity remained. The cold-treated supernatants were diluted to 75, 50, and 25 per cent (v/v), and then to each was added an equal volume of 0.1M Tris-Cl buffer (pH 8.6) containing 10mM fru-6-P. The mixtures were then incubated at 37.5°C, and PFK activity assayed. The final pH was 7.95, and the final protein concentrations were O, 4.16; □, 3.12; Δ, 2.17; and ▽, 1.15 mg./ml. No reactivation was observed if fru-6-P was omitted.

Discussion

It appears that there are at least two interconvertible forms of PFK present in rat and human lenses. The interconversion occurs at pH slightly lower than 7.4. The coexistence of the two forms may provide the basis for the pH dependence of kinetic properties such as ATP inhibition. The affinities of each form toward fru-6-P or ATP are different and are drastically influenced by changes in pH and temperature. The lack of significant pH-dependent molecular transformations of calf lens PFK may be the basis of its pH-insensitive kinetic properties as reported by Lou and Kinoshita.

At even lower pH (pH 5 to 6), the enzyme molecules undergo dissociation into inactive subunits, much like that of PFK's isolated from tissues other than the lens. Reaggregation of these subunits into active PFK molecules requires the presence of an effector at alkaline pH. In the case of lens PFK, the effector required appears to be fru-6-P, a substance whose availability is stringently controlled by hexokinase. A drop in pH may inactivate PFK and its inactivation may lead to accumulation of fru-6-P, which in turn may re activates PFK. It has to be noted that the reactivation process also depends on the concentration of PFK molecules. In old lenses, the relatively low PFK activity may preclude the inactivated enzyme from being restored in activity.

In all the lenses tested, either ATP or fru-6-P prevents PFK inactivation; however, between the two factors, ATP protection is probably more important because of the relative abundance of ATP and its long-term effect.

Rat lens PFK is quite well preserved by ATP, sulfate, and inorganic phosphate. The destabilization of PFK in vivo, however, can be induced under low ATP concentration and, more extensively, under high H⁺ concentration. The lens cultures showed that after 17 hours of glucose deprivation, although the steady-state concentration of ATP declined to zero, the glycolytic machinery after the step of hexokinase (which was mostly deactivated by this time) was comparable to that of normal lenses. In fact, only slight loss...
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Fig. 7. Sixteen rat lenses were homogenized in 1 ml. of 0.05M phosphate buffer (pH 5.6) containing 0.05M Na₂SO₄ and 0.005M EDTA. The supernate from centrifugation at 96,000 × g was loaded onto a Bio-Gel A-1.5m column (1.5 by 28 cm.) and eluted with the same buffer (A). After elution, 0.2 ml. was taken from each fraction and mixed with 0.2 ml. of 0.2M Tris-Cl buffer (pH 9.0) containing 10 mM fru-6-P and incubated at 37.5° C. for 1 hour (final pH 7.95). The enzyme activity was again determined (B). Re-chromatography of activated fractions 42 to 52 on the Bio-Gel A-1.5m column resulted in recovery of activity from slightly behind void volume, indicating reaggregation of PFK protomers during reactivation.

of PFK was observed at this time (Table III). After 44 hours of glucose deprivation (or 27 hours of ATP depletion), a significant loss of PFK was evident (Table III).

Human lens PFK, on the other hand, is incompletely protected by ATP (Table II). This seems to suggest that acidity may be more detrimental to the maintenance of PFK stability, and therefore its level, in human lens than in the rat lens. Human senile cataractous lenses do contain much less amount of PFK than normal lenses (Table II). Although other factors may also be involved, accumulation of aqueous H⁺ as a function of age¹³ or diabetic acidosis could possibly cause some loss of PFK. However, the extent of its loss and the consequences, e.g., disruption of anaerobic glycolysis, remain to be determined.

We wish to thank Mr. Frederick Schaefer for his skilled assistance in lens culture studies, Dr. Stephen Fricker for the use of his computer in statistical analysis (Table III), and the surgeons and nurses at the Massachusetts Eye and Ear Infirmary for assisting us in collecting human lenses.

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