Case 18
Purification of Phosphofructokinase 1-C

Focus concept
The purification of the C isozyme of PFK-1 is presented and the kinetic properties of the purified enzyme are examined.

Prerequisites
- Protein purification techniques.
- Enzyme kinetics and inhibition.
- The glycolytic pathway.

Background
In this case, Foe and Kemp purified the isozyme phosphofructokinase-1 C (PFK-1 C) from brain tissue. There are three isozymes of PFK-1 and they are designated A, B, and C. The A isozyme (Mr = 84,000 dal) is found in the muscle and the brain; the B isozyme (Mr = 80,000 dal) is found in the liver and the brain; and the C isozyme (Mr = 86,000) is found in the brain. Because the brain contains all three isozymes and there isn’t a location where the C isozyme is found exclusively, the enzyme has been difficult to purify. In this case, the investigators purified the desired enzyme to homogeneity, and also presented ample evidence that the C isozyme is distinct from the A and B isozymes. The availability of a pure C preparation means that antibodies can be generated which can be used to detect the isozyme. Since the levels of PFK-1 isozymes have been shown to change during malignant transformation of cells, the availability of a C antibody might be a valuable diagnostic tool.

Questions
1. To accomplish the purification, rabbit brain tissue was homogenized and centrifuged to remove insoluble material. Next, the soluble preparation was loaded on top of an ATP-Sepharose column. This is an affinity column in which ATP is covalently linked to a polysaccharide bead. The sample is loaded on top of the column, washed with a low-salt buffer, followed by a wash with a high salt buffer. What is the rationale for using this procedure? Draw a diagram of the expected elution profile.

2. Next, the fractions containing PFK-1 activity were applied to a DEAE-Sephadex (anion exchange) column. The column was equilibrated with a pH = 8.2 buffer. The column was eluted with a salt (ammonium sulfate) gradient and the results are shown in Figure 18.1. Using the elution profile as well as the results from SDS-PAGE analysis shown in Figure 18.2, identify which isozyme is found in each of the two peaks. How might the amino acid composition of PFK-1 B differ from that of PFK-1 A and C based on the manner of elution from the DEAE column?
Figure 18.1: Purification of PFK-1C by DEAE Sephadex (anion exchange) chromatography (based on Foe and Kemp, 1985).

Figure 18.2: SDS-PAGE analysis of the PFK-1C purification. Lane 1: Molecular weight standards. Lane 2: Eluant from the ATP-Sepharose affinity column. Lane 3: Pooled fractions 16-26 from the DEAE-Sephadex column. Lane 4: Pooled fractions 55-60 from the DEAE-Sephadex column. Lane 5: Fractions 30-35 from the CM-52 column (elution profile shown in Figure 18.3). Lane 6: Fractions 41-50 from the CM-52 column (based on Foe and Kemp, 1985).
3. Next, the investigators took Fractions 16-26 from the DEAE-Sephadex column, pooled them, and adjusted the pH to 5.0. This preparation was then loaded onto a CM-52 (cation exchange) chromatography column and eluted with a pH gradient. Fractions 30-35 were collected and pooled, as were Fractions 41-50. Identify the peaks in the chromatogram in Figure 18.3, using information in the SDS-PAGE gel. Based on their elution from the cation exchange column, how might the amino acid compositions of these two proteins differ?

4. Write the reaction catalyzed by PFK-1.

5. There are several allosteric effectors that influence the activity of PFK-1 in the cell. What are they? List both activators and inhibitors of the enzyme.

6. The investigators next carried out kinetic studies using their newly purified PFK-1C isozyme. They studied the catalytic behavior of the enzyme in the presence of the metabolites AMP, inorganic phosphate (P_i) and fructose-2,6-bisphosphate (F-2,6-BP). The results are shown in Figure 18.4. Additional information concerning the three isozymes’ response to allosteric effectors is presented in Tables 18.1 and 18.2.
   a. Compare the ability of PFK-1C to catalyze the phosphorylation of fructose-6-phosphate in the absence of, and in the presence of AMP, F-2,6-BP or P_i. How do these allosteric effectors influence the velocity of the reaction?
   b. Evaluate whether the investigators have shown that PFK-1 C is different from the PFK-1 A and PFK-1 B isozymes. Speculate why there might be functional differences among the isozymes.
Table 18.1: Relative potency of the allosteric effector citrate on PFK isozymes. The concentration given is the micromolar concentration of citrate required to inhibit 50% of the enzyme activity.

<table>
<thead>
<tr>
<th>Isozyme</th>
<th>Citrate, µM</th>
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<tbody>
<tr>
<td>A</td>
<td>100</td>
</tr>
<tr>
<td>B</td>
<td>&gt; 2000</td>
</tr>
<tr>
<td>C</td>
<td>750</td>
</tr>
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Table 18.2: Relative potency of allosteric effectors on PFK isozymes. Numbers given are the micromolar concentrations of each effector required to achieve 50% of the maximal velocity.

<table>
<thead>
<tr>
<th>Isozyme</th>
<th>Phosphate</th>
<th>AMP</th>
<th>Fructose-2,6-BP</th>
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<tbody>
<tr>
<td>A</td>
<td>80 µM</td>
<td>10 µM</td>
<td>0.05 µM</td>
</tr>
<tr>
<td>B</td>
<td>200 µM</td>
<td>10 µM</td>
<td>0.05 µM</td>
</tr>
<tr>
<td>C</td>
<td>350 µM</td>
<td>75 µM</td>
<td>4.5 µM</td>
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Reference