Hexokinase from *Aspergillus parasiticus*

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Preliminary reports (1–3) have indicated that galactosamine may arise biosynthetically from glucose by means of an epimerase type reaction which converts uridine diphosphoacetyl glucosamine to uridine diphosphoacetyl galactosamine. The latter compound probably represents the form in which galactosamine residues are transferred to polysaccharide chains. Direct phosphorylation of galactosamine to form galactosamine 1-phosphate has been reported to be catalyzed by extracts of liver or of galactose-adapted yeast (4). Galactosamine 6-phosphate has been prepared synthetically and shown to be a substrate for a purified acetylase which forms N-acetyl hexosamine 6-phosphates from the respective hexosamine phosphates and acetyl-CoA (5). The present study was undertaken to clarify the mechanism of galactosamine kinase activity so that routine controls included a cuvette containing no enzyme at this stage is stable for at least 30 days when stored at 0-° and centrifugations for 10 minutes at 2,400 × g. *Aspergillus parasiticus* 1018 was grown with vigorous aeration on a sucrose-salts medium from spore inocula (17). Mycelia were harvested after 40 hours of growth, washed exhaustively with water, and extracted in portions of 2 g by grinding in a Waring Blendor for 3 minutes with 3 volumes of cold 0.15 M KCl containing 5 × 10⁻⁴ M glutathione. The resulting suspension was centrifuged at 18,000 × g for 10 minutes and the supernatant solution designated crude extract. Generally, 200 g of mycelia have been processed in this manner and the combined residues extracted with an additional 100 ml of KCl-glutathione.

The crude extract was treated with 0.1 volume of 2% protamine sulfate and after standing for 10 minutes in an ice bath, the precipitate was removed by centrifugation and discarded. The supernatant solution was brought to 0.50 ammonium sulfate saturation by addition of the solid salt and the resulting precipitate removed by centrifugation and discarded in 20 ml of ice water. It is essential that the purification be carried to this step as promptly as possible since the crude mold extract contains materials, probably proteases, which rapidly inactivate the kinase. The solution resulting from the above was made 0.05 M in potassium acetate and acetone, precooled to −10°, was added with efficient stirring (to maintain temperatures below 0°) until 30% concentration was reached. The precipitate formed was removed by centrifugation and dissolved in 0.1 M phosphate buffer, pH 7.5. Additional acetone fractions were obtained at 38, 46, and 55% acetone concentration. The majority of recoverable activity was found in the 38 to 46% fraction, although significant quantities of enzyme may be present in either of the other two fractions.

The dissolved acetone precipitate was treated with an equal volume of alumina gel C γ (18) (18 mg of solids per ml) and after 15 minutes, the gel was removed by centrifugation and discarded. The supernatant solution was treated with sufficient solid ammonium sulfate to reach 60% saturation, any precipitate that formed was discarded, and the ammonium sulfate concentration raised to 80% saturation. The resulting precipitate was harvested and dissolved in the minimal volume of water. The enzyme at this stage is stable for at least 30 days when stored.
RESULTS AND DISCUSSION

The substrate specificities of the crude and purified enzyme fractions are listed in Table II. As may be seen, galactose and galactosamine are phosphorylated at approximately 80% of the rate of the corresponding sugars with the D-glucose configuration. This is in marked contrast to the comparable activities reported for brain or yeast hexokinase as is the relatively slower rate for D-fructose.

TABLE I
Purification of Aspergillus hexokinase

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Total units</th>
<th>Recovery %</th>
<th>Specific activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>1,000</td>
<td></td>
<td>0.8</td>
</tr>
<tr>
<td>Protamine supernatant</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ammonium sulfate I (50 to 70% saturation)</td>
<td>895</td>
<td>90</td>
<td>7.1</td>
</tr>
<tr>
<td>Acetone, 38 to 46%</td>
<td>440</td>
<td>44</td>
<td>65</td>
</tr>
<tr>
<td>Alumina C γ supernatant</td>
<td>425</td>
<td>43</td>
<td>80</td>
</tr>
<tr>
<td>Ammonium sulfate II (60 to 80% saturation)</td>
<td>330</td>
<td>33</td>
<td>180</td>
</tr>
</tbody>
</table>

* One unit represents an optical density change at 340 μm of 1.00 per minute.
† Micromoles of substrate phosphorylated per mg of protein per hour; D-galactosamine was used as substrate throughout the purification procedure. For assay details, see text.

TABLE II
Substrate specificities of crude and purified kinase. Relative rates based on D-glucose = 1.00

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Rate</th>
<th>Yeast (19)</th>
<th>Brain (30, 31)</th>
</tr>
</thead>
<tbody>
<tr>
<td>D-Glucose*</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>D-Galactose</td>
<td>0.86</td>
<td>0.88</td>
<td>0.00</td>
</tr>
<tr>
<td>D-Glucoseamine</td>
<td>0.59</td>
<td>0.55</td>
<td>0.75</td>
</tr>
<tr>
<td>D-Galactosamine</td>
<td>0.40</td>
<td>0.41</td>
<td>0.00</td>
</tr>
<tr>
<td>D-Mannose</td>
<td>0.67</td>
<td>0.64</td>
<td>0.50</td>
</tr>
<tr>
<td>D-Fructose</td>
<td>0.36</td>
<td>0.28</td>
<td>2.0</td>
</tr>
<tr>
<td>L-Sorbose</td>
<td>0.02</td>
<td>0.02</td>
<td>0.00</td>
</tr>
<tr>
<td>L-Arabinose</td>
<td>0.08</td>
<td>0.04</td>
<td>0.00</td>
</tr>
</tbody>
</table>

* Rates are maximal initial velocities; for details of assay method, see text.

FIG. 1. Absorption spectra of iodate (X—X), periodate (O—O), and glycolaldehyde phosphate (■—■) after reaction with diphenylamine reagent (11). Concentration is 2 × 10−4 M in each case.

Fig. 2. Variation of kinase activities with pH. Phosphate buffer, 0.1 M, was used except at pH 8.5 and 9.0 where glycine buffer was employed. Curves A and B represent glucose and galactose as substrates, respectively; Curves C and D represent glucosamine and galactosamine as substrates, respectively.

FIG. 3. Variation of activity with substrate concentration. Data plotted according to Lineweaver and Burk (26). Curve A, D-glucose (Km = 1.6 × 10−4); Curve B, D-galactose (Km = 2.1 × 10−4); Curve C, D-glucoseamine (Km = 3.4 × 10−4); Curve D, D-galactosamine (Km = 4.3 × 10−4).
Since the only previous reports of galactose or galactosamine kinase activity indicated that phosphorylation occurred in the 1-position, identification of the products of kinase action as terminal or 6-phosphates was necessary. The mechanism of periodate oxidation of sugar phosphates has not been completely clarified and it is not certain whether the major products arise from the oxidation of the ring or open chain forms of the sugar (22-26). Glycolaldehyde phosphate can only arise from periodate oxidation of terminal aldehyde phosphates but direct measurement of glycolaldehyde content on aliquots of periodate reaction mixtures is not possible because of the strong interference of both periodate and iodate ions (Fig. 1). A comparison of the ADP and the glycolaldehyde phosphate assays is presented in Table III. Confirmatory evidence for the position of the phosphate group in the hexosamine phosphates was afforded by their conversion to acetylglycosamine 6-phosphates by a partially purified fungal acetylase.

The comparable activities towards several substrates in the crude and purified enzyme preparations indicate that the phosphorylations are being carried out by a single enzyme. Attempts to resolve activities based on partial inactivation through heat denaturation or dialysis were unsuccessful. A comparison of the pH activity curves for the purified enzyme with glucose, galactose, glucosamine, or galactosamine as substrates is presented in Fig. 2. The action pattern of this enzyme may be related to the occurrence of glycosidically bound forms of sugars with the D-galacto configuration either as cell wall constituents or metabolic products of this organism.

SUMMARY

1. A hexokinase of broad specificity has been purified approximately 200-fold from extracts of *Aspergillus parasiticus*.

2. This enzyme phosphorylates D-galactose and D-galactosamine to yield the respective 6-phosphates. Corresponding sugars with the D-glucos configuration are phosphorylated at a rate only 20% faster than those with the D-galacto configuration.

3. The comparable activities during purification, the marked difference in action pattern from either brain or yeast hexokinase, and the pH activity relationships indicate that the phosphorylations are catalyzed by a single enzyme.

REFERENCES

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