**5-Keto-d-fructose**

V. PHOSPHORYLATION BY YEAST HEXOKINASE*

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**SUMMARY**

Yeast hexokinase was shown to catalyze the phosphorylation of 5-keto-d-fructose (D-threo-2,5-hexodiulose) in the presence of ATP. The product was identified as a monophosphate ester. A procedure for the preparation of this ester and some of its properties are described. Reduction of the monophosphate ester by the NADPH-5-keto-d-fructose reductase from *Gluconobacter cerinus* yielded fructose 1-phosphate.

5-Keto-d-fructose (D-threo-2,5-hexodiulose) is produced by several strains of *Acetobacter* growing on d-fructose (1-3). The reduction of this diketohexose to d-fructose has been shown to be catalyzed by a specific NADPH-linked reductase isolated from *Gluconobacter cerinus* cells (4, 5). Utilization of 5KF as a carbon source for the growth of *G. cerinus* could be attributed, at least in part, to this reaction. During a study of the utilization of 5KF by extracts of fructose-grown cells of *G. cerinus*, it became evident that the presence of a kinase activity resulted in the phosphorylation of this hexose with ATP. Subsequently, it was found that 5KF is a substrate for yeast hexokinase with formation of 5KF-1-phosphate. Some kinetic properties of this reaction system and the characterization of 5KF-P are presented in this paper. 5KF-P was found to be a substrate for the *G. cerinus* NADPH-5KF reductase (5) and the product of reduction was identified as fructose 1-P.

**METHODS**

Crystalline yeast hexokinase and other purified enzymes used in this work were purchased from Boehringer und Soehne, GmbH (Mannheim, Germany). 5KF was isolated as described before (3). The monocyclohexylamine salt of phosphoenolpyruvate was prepared by the method of Clark and Kirby (6). NADPH-5KF reductase was purified from *G. cerinus* extracts (5). Spectrophotometric and calorimetric analyses were carried out as previously described (4, 5).

**RESULTS AND DISCUSSION**

The phosphorylation of 5KF by yeast hexokinase was followed spectrophotometrically (Fig. 1). Compared to glucose and fructose, 5KF was found to have a slightly lower $K_m$ for the enzyme, although the $V_{max}$ was only 0.1 of that achieved with fructose (Fig. 1; Table I). In experiments in which the reaction was initiated by the successive addition of 5KF it was ascertained that 1 mole eq of ADP was liberated per mole of 5KF added. This result is compatible with the formation of a hexose monophosphate ester.

Preparation of Phosphate Ester—Synthesis of small quantities of 5KF-P could be accomplished by incubation of 5KF with stoichiometric amounts of ATP in the presence of yeast hexokinase. The product in this case could be isolated as described for other kinase systems (9, 10). However, a more convenient and less tedious procedure involves the synthesis and isolation of 5KF-P from incubation mixtures containing 5KF, yeast hexokinase, catalytic amounts of ATP, and a phosphoenolpyruvate kinase or creatine phosphate-creatine kinase coupling system. The following is a description of a typical experiment. Each 77.75 ml of reaction mixture contained (in millimoles): neutralized phosphoenolpyruvate, 3.98; Tris-HCl buffer (pH 7.4), 5.0; MgCl$_2$, 0.2; KCl, 4.0; ATP, 0.15; 5KF, 4.82; pyruvate kinase, 120 units; and hexokinase, 150 units. Incubation was carried out at 30°C and at various time intervals small aliquots were withdrawn for the estimation of pyruvic acid by the lactic dehydrogenase assay system. After 4 hours, 4.0 mmoles of pyruvate were produced and phosphoenolpyruvate could no longer be detected, indicating that the reaction had reached completion. The solution was passed through a column (20 x 2 cm) of Dowex 50-H$_{4}$-X8 followed by washing with 150 ml of water. The eluate was reduced in volume by lyophilization. The yellowish concentrate (20 ml) was repeatedly extracted with...
tion containing 4.15 mmoles of barium acetate were added, followed by 2 volumes of ethanol. The flocculent precipitate was collected by filtration, washed with 66% ethanol and then with absolute ethanol, and finally with ether and placed in a vacuum desiccator to dry: 1.46 g of a white material were obtained.

\[ \text{C}_4\text{H}_8\text{O}_4\text{P Ba·H}_2\text{O} \]

Calculated: C 17.52, H 2.69, P 7.52, Ba 33.40

Found: C 17.52, H 2.30, P 7.62, Ba 31.64

The molecular weight of the compound is 411.50.

**Characterization of 5KF-P**—The barium salt was dissolved in HCl at pH 2.0 and the barium was removed by the addition of a slight excess of Na₂SO₄ or by passage through a Dowex 50-Na⁺ column in order to obtain the sodium salt of 5KF-P. The concentration of ester in solution was determined by assay of total phosphate (11).

Oxidation of 5KF-P by sodium metaperiodate was carried out and compared to fructose-1-P treated under similar conditions (9). Phosphoglycolic acid was detected in these reaction mixtures as described by Chiu and Feingold (9), thus confirming that the phosphate group in 5KF was attached to a primary hydroxyl adjacent to a keto group.

5KF-P moved as a single spot on thin layer chromatograms (Table II). Compared to 5KF, the phosphate ester reacted with various sugar reagents as follows (on molar basis): 71% in the Somogyi-Nelson reagent (13); 75% in the resorcinol reagent (14); and 62% in the o-aminophenyl reaction (3).

As in the case with fructose-1-P, 5KF-P was easily hydrolyzed in acid. The hydrolysis in 1.0 M HCl at 98° of a 5.5 mM solution was followed by measuring the liberation of orthophosphate (11). A K value of 70 was found for this first order reaction (11) and 50% hydrolysis was actually observed at 5.5 min. Chromatographic analysis of these hydrolysates indicated the presence of some unidentified reducing spots in addition to the expected 5KF.

**5KF-P as Substrate for Various Enzymes**—The phosphate ester of 5KF was inactive either as a substrate or inhibitor when present at concentrations of up to 2 mM in the following standard spectrophotometric assay systems for yeast phosphoglucoisomerase (15), transaldolase (16), glucose-6-P dehydrogenase (15), muscle aldolase (17), and phosphoglucomutase (18).

5KF-P was found to be a substrate for the G. cerinus NADPH-5KF reductase (5). Under standard conditions of assay, a Kₜ of 4.2 × 10⁻⁴ M was determined, a value which corresponds closely to that found for 5KF (5). The maximal rate of reduction of 5KF-P, however, was only 0.16 of that observed for 5KF and the kinetics of NADPH oxidation in reaction mixtures containing both 5KF and 5KF-P was as expected for an enzyme reacting with competitive substrates (19, 20). The product of the enzymatic reduction of 5KF-P was evidently fructose-1-P since, for each mole of 5KF-P reduced by the NADPH-dependent G. cerinus reductase, stoichiometric amounts of subsequently added NADH were oxidized when the reaction mixture was further supplemented with excess aldolase, triose phosphate isomerase, and α-glycerolphosphate dehydrogenase (17, 21).

The reduction of 5KF to fructose as catalyzed by the NADPH-5KF reductase was considered as a major pathway for 5KF utilization by G. cerinus cells (5). The evidence presented in the present communication points to the possibility that the bacteria can also utilize 5KF via prior phosphorylation followed by reduction to fructose-1-P. Such a pathway would have the added advantage of preventing the accumulation of 5KF-P in the cell.
It is suggested that 5KF could serve as a useful analogue of fructose for trapping ATP in biological systems, particularly in cases in which a further reduction of 5KF-P to fructose phosphate does not occur.

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REFERENCES

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