THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 248, No. 23, Issue of December 10, pp. 8174-8177, 1973
Printed in U.S.A.

The Substrate and Anomeric Specificity of Fructokinase*

(Received for publication, July 25, 1973)

FRANK M. RAUSHEL AND W. W. CLELAND

From the Department of Biochemistry, College of Agricultural and Life Sciences, University of Wisconsin-Madison, Madison, Wisconsin 53706

SUMMARY

Fructokinase from beef liver is shown to be specific for the β-furanose anomer of D-fructose (and the α anomer of L-sorbose) by showing that certain 2,5-anhydroalditols are substrates, while other 2,5-anhydro compounds, 2,6-anhydro-D-mannitol, and 2,6-anhydro-D-glucitol are not. K_m and V_{max} values relative to D-fructose at pH 7.5, 25°, 4 mM MgATP are 2,5-anhydro-D-mannitol (1.7 mM, 1.97); 2,5-anhydro-D-glucitol (5.9 mM, 1.33, phosphorylated in position 6); 2,5-anhydro-D-lyxitol (67 mM, 0.19); 2,5-anhydro-D-mannose (1.5 mM, 0.50). 2,5-Anhydro-D-xylitol inhibits (K_i, 40 mM), while 2,5-anhydro-L-iditol and cis 2,5-bis(hydroxymethyl)tetrahydrofuran are neither inhibitors nor substrates. Based on these results and other published data, the substrate specificity of fructokinase is for a tetrahydrofuran ring with β-D-(or α-L-) configuration at position 2, L-configuration at postion 3, and either D- or L-configuration at positions 4 and 5.

With yeast hexokinase the K_m and V_{max} values relative to D-fructose of several anhydro compounds at pH 7.5, 25°, 2 mM MgATP, are 2,5-anhydro-D-mannitol (6.3 mM, 0.52); 2,5-anhydro-D-glucitol (47 mM, 0.37); 2,5-anhydro-D-mannose (0.31 mM, 0.37).

Fructokinase (ATP:D-fructose 1-phosphatetransferase, EC 2.7.1.3) catalyzes the following reaction:

D-Fructose + MgATP → MgADP + D-fructose 1-phosphate (1)

Although there have been a number of investigations dealing with the purification and properties of this enzyme (1–4), little work has been done on its anomeric specificity. There have been reports that there is no difference in the rate of phosphorylation by fructokinase of a newly prepared solution of fructose and a solution where all anomeric forms have been allowed to equilibrate (1, 5). This would argue that fructokinase is relatively nonspecific for the anomeric form of its substrate. However, calculations show that under the conditions used by these workers the mutarotation rate of fructose (6, 7) is faster than the rate of phosphorylation. Nothing, therefore, can be concluded from these experiments.

* This work was supported by grants from National Science Foundation (GB 27407) and the National Institutes of Health (GM 18938).

Anhydro derivatives of sugar alcohols have recently been used to determine the anomeric specificity of phosphofructokinase[1, 2] (8). We have therefore prepared the four anhydrohexitols corresponding to the four anomers of D-fructose, and used them and a number of other similar compounds to determine the substrate and anomeric specificity of fructokinase.

EXPERIMENTAL PROCEDURE

Materials

Fructokinase was isolated from beef liver according to the procedure of Sanchez *et al.* (1). Adenosine triphosphate was purchased from P&L Biochemicals; pyruvate kinase, lactate dehydrogenase, hexokinase, and glucose-6-P dehydrogenase from Boehringer; NADH, NADP, phosphofructokinase, hexokinase, D-tagatose, L-sorbose, and fructose 1-phosphate from Sigma. 2,5-Anhydro-D-mannose (chitose) was synthesized by nitrous acid treatment of glucosamine, and 2,5-anhydro-D-mannitol by reduction of 2,5-anhydro-D-mannose with borohydride (9). 2,6-Anhydro-D-mannitol (styracitol, 1,5-anhydro-D-mannitol) was prepared by treatment of D-mannitol with HCl (10). 2,6-Anhydro-D-glucitol (1,5-anhydro-L-gulitol) was made according to the procedure of Ness and Fletcher (11). 2,5-Anhydro-D-glucitol was synthesized by anhydridization of 1,6 dibenzoylmannitol (12, 13). 2,5-Anhydro-D-xylitol and 2,5-anhydro-D-lyxitol were made according to the procedure of Defaye (14). 2,5-Anhydro-L-iditol was a gift from Dr. L. A. Hartman, ICI America, and cis 2,5-bis(hydroxymethyl)tetrahydrofuran from Dr. S. J. Benkovic, Pennsylvania State University.

Enzymatic Synthesis of 2,5-Anhydro-D-glucitol 6-Phosphate—The reaction mixture contained 12 mM ATP (pH 7.5), 10 mM 2,5-anhydro-D-glucitol, 10 mM $MgCl_2$, 10 mM triethanolamine-HCl (pH 7.5), and approximately 400 units of yeast hexokinase in a total volume of 5 ml. After allowing the mixture to react overnight, the pH was adjusted to 8.0 with dilute NH_3. The solution was applied to a column of Dowex-1-X8-borate and eluted from the column (0.6 × 30 cm) with a 200-ml gradient of ammonium tetraborate (0.1 to 0.4 M, pH 8.0) with a flow rate of 0.5 ml per min (15). Unreacted 2,5-anhydro-D-glucitol is eluted quickly from the column and ATP and ADP are not eluted under these conditions (15). Total phosphate determinations (16) were done on alternate fractions to determine those containing the 2,5-anhydro-D-glucitol 6-phosphate, and ammonium borate was removed under reduced pressure with methanol. The yield based on total phosphate was 92%.

[1] S. J. Benkovic, personal communication.
[2] J. Bar-Tana and W. W. Cleland, manuscript in preparation.

2,5-Anhydro-D-mannitol 1-phosphate was synthesized enzymatically with hexokinase, MgATP, and 2,5-anhydro-D-mannitol in a manner similar to that for 2,5-anhydro-D-glucitol 6-phosphate. The yield was 104%.

Periodate Oxidation of 2,5-Anhydro-D-mannitol 1-Phosphate and 2,5-Anhydro-D-glucitol 6-Phosphate—A 2-fold molar excess of sodium metaperiodate was used to oxidize 130 mM 2,5-anhydro-D-mannitol 1-phosphate and 23 mM 2,5-anhydro-D-glucitol 6-phosphate to their respective dialdehydes. The reaction was allowed to proceed overnight in the dark. Excess periodate was removed by the addition of ethylene glycol, and the optical rotation of each dialdehyde was determined using water as solvent.

A 20-fold excess of sodium borohydride over the amount of periodate used in the previous step was added to reduce the dialdehydes. The reaction was carried out at 0° with the pH being kept near 7 with the addition of sulfuric acid. After 2 hours the excess borohydride was destroyed by lowering the pH to 4.5 with sulfuric acid. Ammonium molybdate (final concentration, 15%, pH approximately 4.5) was used to enhance the optical rotations of the resulting alcohols.

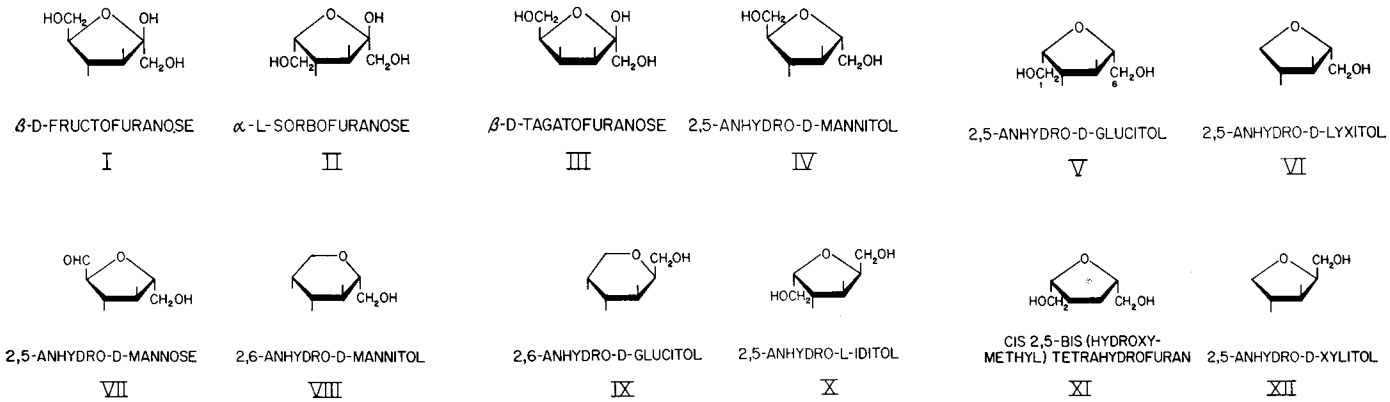
Enzyme Assay—Fructokinase activity was measured spectrophotometrically using the pyruvate kinase, lactate dehydrogenase coupling system. A Beckman DU monochrometer equipped with a Gilford 200 optical density converter and a 10-mv recorder was used to follow the reaction at 340 nm. Full scale sensitivity was 0.10 O.D. unit with a chart speed of 2 inches per min. All assays were done at 25°. Each 3-ml cuvette contained 33 mM triethanolamine-HCl (pH 7.5), 20 units of pyruvate kinase, 55 units of lactate dehydrogenase, 167 μM NADH, 1 mM phosphoenolpyruvate, 100 mM KCl, 4 mM ATP (pH 7.5), 5 mM $MgCl_2$, substrate, and enzyme. Hexokinase activity was followed as above except that 20 mM KCl, 2 mM ATP, and 3 mM $MgCl_2$ were used instead of the previously stated amounts. To follow the production of ATP in the reverse reaction of fructokinase a hexokinase, glucose-6-P dehydrogenase coupling system was used. Each 3-ml cuvette contained 0.5 mM ADP, 2 mM glucose, 50 mM piperazine-*N*,*N'*-bis(2-ethanesulfonic acid) (pH 7.0), 111 μM NADP, 1.5 mM $MgCl_2$, 6 units of hexokinase, 6 units of glucose-6-P dehydrogenase, substrate, and fructokinase. Fructokinase in all assays was added last using an adder-mixer.

Data Analysis—The initial velocity data were analyzed by the Fortran program of Cleland (17) which makes a least squares fit to the equation:

$$v = \frac{VA}{K + A} \qquad (2)$$

RESULTS

A total of twelve compounds were tested as substrates for fructokinase. Of the twelve only D-fructose (I), L-sorbose (II), D-tagatose (III), 2,5-anhydro-D-mannitol (IV), 2,5-anhydro-D-glucitol (V), 2,5-anhydro-D-lyxitol (VI), and 2,5-anhydro-D-mannose (VII) served as substrates. At concentrations of at least 20 mM, 2,6-anhydro-D-mannitol (VIII), 2,6-anhydro-D-glucitol (IX), 2,5-anhydro-L-iditol (X), and cis 2,5-bis(hydroxymethyl)tetrahydrofuran (XI) did not appear to be substrates nor did they inhibit the phosphorylation of fructose. 2,5-Anhydro-D-xylitol (XII) was not a substrate but it did act as an inhibitor with a K_i of 40 mM. The Michaelis constants and relative maximal velocities for those compounds serving as substrates for fructokinase appear in Table I.



Since some of the anhydro compounds that were used as substrates for fructokinase have not been well characterized as substrates for yeast hexokinase, the K_m values and relative maximal velocities were determined for these compounds with hexokinase. In particular, 2,5-anhydro-D-glucitol has been reported not to be a substrate, presumably because of its high K_m (18). Because of its greater availability, hexokinase is useful in preparing suitable quantities of these anhydro phosphates for use in studying the reverse reaction of fructokinase. The kinetic data appear in Table II.

Although it is very likely that hexokinase phosphorylates 2,5-anhydro-D-glucitol at carbon 6, since this compound is an analog of α-D-fructofuranose, there is the possibility that it might phosphorylate carbon 1. To determine which end is phosphorylated, 2,5-anhydro-D-glucitol phosphate and 2,5-anhydro-D-mannitol 1-phosphate were prepared with hexokinase as described under "Experimental Procedure." By cleavage with sodium periodate and reduction of the resulting dialdehyde with sodium borohydride the position of the phosphate on 2,5-anhydro-D-glucitol

TABLE I

Substrates for fructokinase at pH 7.5, 25°, 4 mM MgATP

Compound	K_m	Relative V_{max}
	mM	
D-Fructose	0.5 ± 0.1	100
L-Sorbose	0.4 ± 0.1	39
D-Tagatose	0.9 ± 0.1	103
2,5-Anhydro-D-mannitol	1.7 ± 0.2	197
2,5-Anhydro-D-glucitol	5.9 ± 0.3	133
2,5-Anhydro-D-lyxitol	67 ± 6	19
2,5-Anhydro-D-mannose	1.5 ± 0.2	50

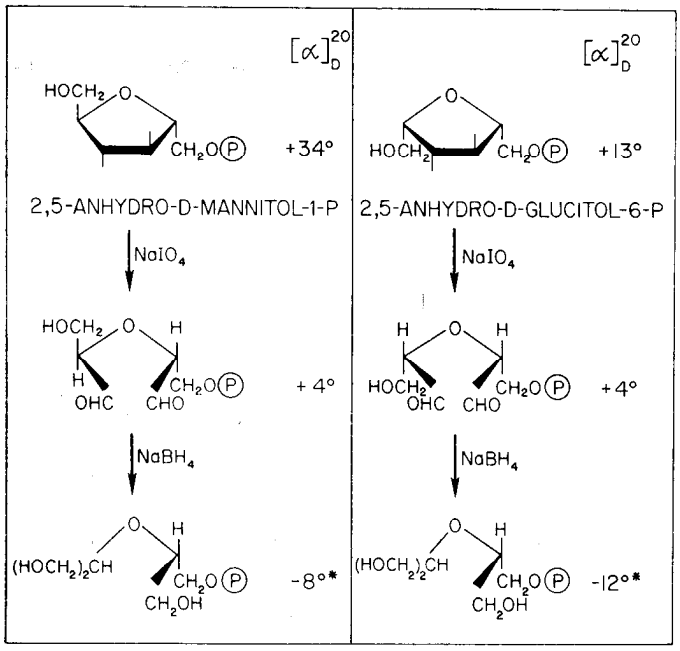
TABLE II

Substrates for hexokinase at pH 7.5, 25°, 2 mM MgATP

Compound	K_m	Relative V_{max}
	mM	
D-Glucose	0.086 ± 0.008	34
D-Fructose	0.98 ± 0.06	100
2,5-Anhydro-D-mannitol	6.3 ± 0.9	52
2,5-Anhydro-D-glucitol	47 ± 9	37
2,5-Anhydro-D-mannose	0.31 ± 0.07	37

TABLE III

Rotations of compounds formed during structural proof of 2,5-anhydroglucitol 6-phosphate



* Values in 15% molybdate

phosphate can be determined, since the resulting molecule contains only a single asymmetric carbon. If carbon 6 was phosphorylated, the final product from periodate and borohydride treatment should be the same as that from 2,5-anhydro-D-mannitol. If carbon 1 was phosphorylated, the enantiomer would be formed. The polarimetry data for these experiments appear in Table III. The specific rotations for the final products are small, and were observable only in the presence of 15% ammonium molybdate. Although the values are not exactly equal to each other they are within the experimental error, and since they are both negative it can be concluded that hexokinase phosphorylates 2,5-anhydro-D-glucitol at carbon 6 as expected.

2,5-Anhydro-D-glucitol 6-phosphate prepared enzymatically with hexokinase was then tested as a substrate for fructokinase in the reverse direction. At a concentration of 1.5 mM, it was a substrate and gave an initial velocity 30% that of a 2.0 mM solution of fructose 1-phosphate. This shows that in the forward direction 2,5-anhydro-D-glucitol acts as an analog of α-L-sorbofuranose, instead of α-D-fructofuranose, and is phosphorylated in position 6, rather than position 1.

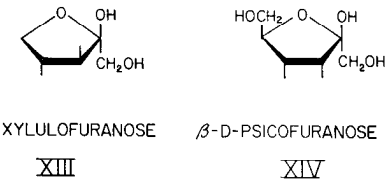
The products of the reaction of 2,5-anhydro-D-mannitol and of 2,5-anhydro-D-glucitol with fructokinase and MgATP were tested as substrates for phosphofructokinase. Experimental conditions were the same as before except that the concentrations of 2,5-anhydro-D-glucitol and 2,5-anhydro-D-mannitol were 100 μM. Each substrate was allowed to react with excess MgATP in the presence of fructokinase until the reaction was nearly complete. At this time phosphofructokinase was added to the cuvette, and the 2,5-anhydro-D-mannitol 1-phosphate was phosphorylated, while the product from 2,5-anhydro-D-glucitol was not.

These data agree with the results of previous workers who have found that 2,5-anhydro-D-mannitol-1-P (identical to 2,5-anhydro-D-mannitol-6-P) is a good substrate for phosphofructokinase, but that 2,5-anhydroglucitol-6-P is not used[1, 2] (8).

DISCUSSION

From the data presented above it appears that fructokinase is specific for the furanose ring form, since neither 2,6-anhydro-D-glucitol (IX) nor 2,6-anhydro-D-mannitol (VIII) is phosphorylated. Since 2,5-anhydro-D-mannitol (IV) and 2,5-anhydro-D-mannose (VII) are substrates, the enzyme is also specific for the β-D (or the α-L in the case of L-sorbose) anomer. 2,5-Anhydro-D-glucitol (V) is phosphorylated at carbon 6, which shows that it is acting as an analog of α-L-sorbofuranose (II) and not of α-D-fructofuranose. D-Xylulose (XIII) has been reported as a substrate for fructokinase (3). 2,5-Anhydro-D-lyxitol (VI), an analog of β-D-xylulofuranose, is a substrate, while 2,5-anhydro-D-xylitol (XII), an analog of the α form, is not a substrate, although it does inhibit. The fact that 2,5-anhydro-L-iditol (X) (an analog of β-L-sorbofuranose) is neither a substrate nor inhibitor, while 2,5-anhydro-D-xylitol (XII) inhibits, is evidence that the enzyme will not accept a —CH_2OH group in the β-L- or α-D-configuration at position 2, and that when 2,5-anhydro-D-xylitol combines it is rotated so that the —CH_2OH group occupies a position similar to carbon 6 of L-sorbose.

The position of the terminal —CH_2OH group is relatively unimportant since both D-fructose (I) and L-sorbose (II) are substrates. Since D-xylulose (XIII) is also a substrate this terminal —CH_2OH group can be absent from the substrate molecule, although there is a reduction in V_{max} and elevation of K_m (3). Activity of fructokinase with D-tagatose (III) shows that the hydroxyl at carbon 4 can be in either of its two possible positions, at least when positions 3 and 5 are similar to fructose. No activity with D-psicose (XIV) (3) indicates that the hydroxyl at



carbon 3 must be *trans* to the —CH_2OH group at carbon 2. In addition, the lack of activity by cis 2,5-bis(hydroxymethyl)-tetrahydrofuran shows that the hydroxyl groups at 3 and perhaps 4 are essential for activity, at least when position 5 resembles L-sorbose.

The anomeric hydroxyl at position 2 is not needed for activity, but appears to contribute to binding, since the K_m for D-fructose is considerably less than that of 2,5-anhydro-D-mannitol or 2,5-anhydro-D-mannose. Since in solution 31% of D-fructose is the active β furanose anomer (19), and the other anomers should not have any affinity for the enzyme, the 2-hydroxyl contributes a factor of 10 to the binding.

The specificity of fructokinase thus requires a tetrahydrofuran ring with β-D-(or α-L-) configuration at position 2, L-configura-

tion at position 3, and either D- or L-configuration at positions 4 and 5. The anomeric specificity for β-D-fructofuranose is identical with that found for phosphofructokinase[1, 2] (8), suggesting that the enzymes may have a common evolutionary ancestry, although phosphofructokinase will not phosphorylate L-sorbose 6-phosphate, and thus is specific for the D-configuration at position 5.

REFERENCES

1. Sánchez, J. J., González, N. S., and Pontis, H. G. (1971) *Biochim. Biophys. Acta* **227**, 67
2. Parks, R. E., Jr., Ben-Gershom, E., and Lardy, H. A. (1957) *J. Biol. Chem.* **227**, 231
3. Adelman, R. C., Ballard, F. J., and Weinhouse, S. (1967) *J. Biol. Chem.* **242**, 3360
4. Kuyper, C., and Hoenselaars, J. (1959) *Kominklike Ned. Akad. Wetensch. Proc. Ser. C* **62**, 484
5. Kuyper, C. (1959) *Kominlike Ned. Akad. Wetensch. Proc. Ser. B* **62**, 137
6. Isbell, H. S., and Pigman, W. (1969) in *Advances in Carbohydrate Chemistry and Biochemistry* (Wolfrom, M. L., and Tipson, R. S., eds) Vol. 24, p. 14, Academic Press, New York
7. Pigman, W., and Isbell, H. S. (1968) in *Advances in Carbohydrate Chemistry and Biochemistry* (Wolfrom, M. L., and Tipson, R. S., eds) Vol. 23, p. 11, Academic Press, New York
8. Koerner, A. W., Jr., Ashour, A. E., Voll, R. J., Younathan, E. S. (1973) *Fed. Proc.* **32**, 668
9. Bera, B. C., Foster, A. B., and Stacy, M. (1956) *J. Chem. Soc.* 4531
10. Fletcher, H. G., Jr. (1963) in *Methods in Carbohydrate Chemistry* (Whistler, R. L., and Wolfram, M. L., eds) Vol. II, p. 196, Academic Press, New York
11. Ness, R. K., and Fletcher, H. G., Jr. (1953) *J. Amer. Chem. Soc.* **75**, 2619
12. Hockett, R. C., Zief, M., and Goepp, R. M., Jr. (1946) *J. Amer. Chem. Soc.* **68**, 935
13. Brigl, P., and Grüner, H. (1933) *Ber.* **66**, 1945
14. Defaye, J. (1964) *Bull. Soc. Chim. Fr.* 2686
15. Lefebvre, M. J., González, N. S., and Pontis, H. G. (1964) *J. Chromatogr.* **15**, 495
16. Bartlett, G. R. (1959) *J. Biol. Chem.* **234**, 459
17. Cleland, W. W. (1967) *Advan. Enzymol.* **29**, 1
18. Burt, J. R. (1960) *Biochem. J.* **77**, 16p
19. Doddrell, D., and Allerhand, A., (1971) *J. Amer. Chem. Soc.* **93**, 2779

ARTICLE:

The Substrate and Anomeric Specificity of Fructokinase

Frank M. Raushel and W. W. Cleland

J. Biol. Chem. 1973, 248:8174-8177.