ANOMERIC SPECIFICITY OF FRUCTOSE-6-PHOSPHATE KINASE (EC 2.7.1.11) FROM RABBIT MUSCLE

Bernd WURSTER and Benno HESS
Max-Planck-Institut für Ernährungsphysiologie, D-46 Dortmund, Rheinlanddamm 201, GFR

Received 31 October 1973
Original figures received 23 November 1973

1. Introduction

In aqueous solution D-fructose-6-phosphate exists as an equilibrium mixture of 20 ± 4% α-D-fructofuranose-6-phosphate and 80 ± 10% β-D-fructofuranose-6-phosphate [1]. The furanose configurations are equilibrated via the keto form of D-fructose-6-phosphate which is present in the equilibrium mixture at about 2.5% [2].

This multiplicity of configurations of D-fructose-6-phosphate prompts the questions whether enzymes catalyzing reactions of D-fructose-6-phosphate are specific with respect to one configuration or are non-specific, and whether there exist enzymes capable of catalyzing the anomerization of D-fructose-6-phosphate.

This paper describes stopped flow experiments which indicate, that most probably fructose-6-phosphate kinase specifically catalyzes the phosphorylation of β-D-fructofuranose-6-phosphate but not that of α-D-fructofuranose-6-phosphate. In addition it will be shown, that glucose-6-phosphate 1-epimerase from bakers' yeast, an enzyme that catalyzes the anomerization of D-glucose-6-phosphate [3–5], is not capable of catalyzing the anomerization of D-fructose-6-phosphate.

2. Materials and methods

All chemicals of p.a. grade were purchased from E. Merck AG., Darmstadt. Dithioerythritol was obtained from Serva, Heidelberg. D-fructose-6-phosphate, D-glucose-6-phosphate, phosphoenol-pyruvate, pyruvate, ATP, ADP, NADH, $NADP^+$, pyruvate kinase EC 2.7.1.40), lactate dehydrogenase (EC 1.1.1.27), both enzymes from rabbit muscle, and glucose-6-phosphate dehydrogenase (EC 1.1.1.49) from yeast as well as Precinorm S were bought from Boehringer Mannheim GmbH. Highly purified fructose-6-phosphate kinase from rabbit muscle [6] was a gift from Dr. H.W. Hofer, Konstanz. Glucose-6-phosphate 1-epimerase from bakers' yeast was prepared as described in ref. [5].

Protein was determined by the biuret reaction [7] using Precinorm S as a standard. Enzymes were freed from ammonium sulphate by dialysis. The determination of substrate concentrations and enzyme activities were performed at 25°C in standard buffer pH 7.6 (50 mM imidazole/HCl, 50 mM KCl, 8 mM $MgSO_4$, 1 mM dithiorythritol) using an Eppendorf photometer. With minor modifications substrates were assayed according to ref. [8]. Methods for enzyme activity determinations were taken from ref. [9]; modified conditions were: Fructose-6-phosphate kinase: 1 mM D-fructose-6-phosphate, 1 mM ATP, 1 mM phosphoenolpyruvate, 0.25 mM NADH, 10 U/ml pyruvate kinase and 10 U/ml lactate dehydrogenase. Pyruvate kinase: 1 mM phosphoenolpyruvate, 2 mM ADP, 0.25 mM NADH and 10 U/ml lactate dehydrogenase. Lactate dehydrogenase: 3 mM pyruvate and 0.25 mM NADH. Specific activities were: Fructose-6-phosphate kinase 180 U/mg, pyruvate kinase 350 U/mg, lactate dehydrogenase 430 U/mg.

The activity constant of glucose-6-phosphate 1-epimerase was determined in system 2 as described in ref. [3].

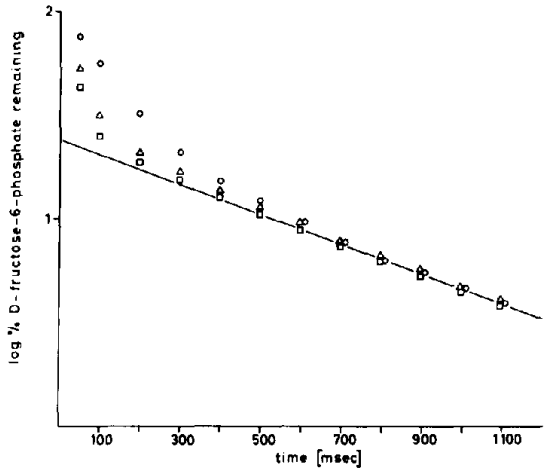


Fig. 2. Semilogarithmic plot of the progress curves of the fructose-6-phosphate kinase reaction at three different enzyme activities. 3500 U/ml pyruvate kinase, 5000 U/ml lactate dehydrogenase, 0.06 mM D-fructose-6-phosphate, 0.1 mM ATP, 1 mM phosphoenol-pyruvate and 0.25 mM NADH; (○) 60 U/ml, (△) 120 U/ml and (□) 240 U/ml fructose-6-phosphate kinase. Log % D-fructose-6-phosphate remaining is plotted against the time.

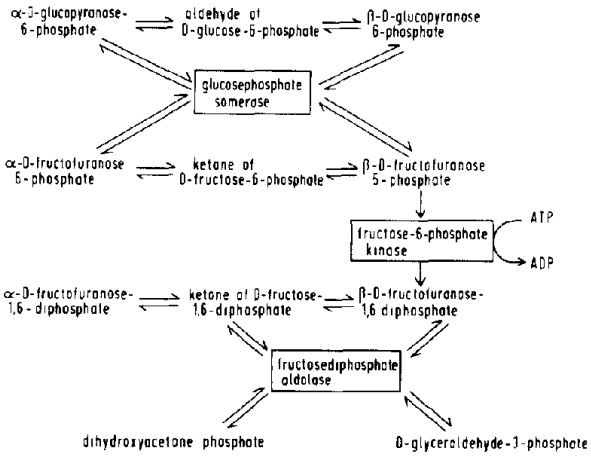
nose-6-phosphate as substrate. We cannot decide from our experiments whether the keto from of D-fructose-6-phosphate, present in the equilibrium mixture at 2.5% [2], is also a substrate of fructose-6-phosphate kinase. If the keto form of D-fructose-6-phosphate is not accepted by fructose-6-phosphate kinase, which seems likely, the slow reaction of first order observed in our experiments which is not influenced by the enzyme concentration, represents mainly the spontaneous anomerization of α- to β-D-fructofuranose-6-phosphate.

In order to test whether the spontaneous reaction is enhanched by glucose-6-phosphate 1-epimerase, which catalyzes the anomerization reaction of D-glucose-6-phosphate [3–5], this enzyme (activity constant 600 $min^{-1} \times ml^{-1}$) was added to the reaction mixture. Glucose-6-phosphate 1-epimerase did not cause an alteration of the progress curves, excluding a catalysis of the anomerization of D-fructose-6-phosphate.

4. Discussion

The experiments described above indicate, that most probably fructose-6-phosphate kinase specifically catalyzes the phosphorylation of β-D-fructofuranose-6-phosphate but not that of α-D-fructofuranose-6-phosphate. An earlier report that this enzyme only uses α-D-fructofuranose-6-phosphate as substrate [11] has been modified by these authors: They are now certain that fructose-6-phosphate kinase catalyzes the reaction of β-D-fructofuranose-6-phosphate, however with their rapid quench approach they cannot decide whether α-D-fructofuranose-6-phosphate is also a substrate (Dr. I.A. Rose, personal communication July 20, 1973).

Finally, we would like to point to the relevance of the anomeric specificity of fructose-6-phosphate kinase with respect to the anomeric specificity of the enzymes preceding and succeeding fructose-6-phosphate kinase in the glycolytic chain, glucosephosphate isomerase (EC 5.3.1.9) and fructose-diphosphate aldolase (EC 4.1.2.13), respectively. The results on the anomeric specificity of glucosephosphate isomerase, fructose-6-phosphate kinase and fructosediphosphate aldolase are summarized in the following scheme.



It could be shown that glucosephosphate isomerase nonspecifically catalyzes the isomeration of α- and β-D-glucopyranose-6-phosphate to α- and β-D-fructofuranose-6-phosphate [12, 13], however the α-anomers are the preferred substrates [12–14]. In addi-

For stopped flow experiments the equipments developed by Hess et al. [10] was used with the monochromator set at 366 nm. The flow system of the apparatus has a total dead time of 2 msec, flow velocity 5 m/sec, volume of the flow chamber 22 μl, d = 1 cm. Reactions were initiated by mixing equal volumes of a) fructose-6-phosphate kinase + pyruvate kinase + lactate dehydrogenase + ATP + phosphoenolpyruvate + NADH and b) fructose-6-phosphate kinase + pyruvate kinase + lactate dehydrogenase + D-fructose-6-phosphate + NADH in the respective drive syringes. Final conditions: Fructose-6-phosphate kinase 60, 120 and 240 U/ml, respectively, pyruvate kinase 3500 U/ml, lactate dehydrogenase 5000 U/ml, 0.06 mM D-fructose-6-phosphate, 0.1 mM ATP, 1 mM phosphoenolpyruvate and 0.25 mM NADH.

Activities of pyruvate kinase and lactate dehydrogenase were high compared with the velocity of the fructose-6-phosphate kinase reaction. Thus, the concentration of the intermediates ADP and pyruvate become negligibly small, and the oxidation of NADH in the lactate dehydrogenase reaction is a direct measure of the phosphorylation of D-fructose-6-phosphate in the fructose-6-phosphate kinase reaction.

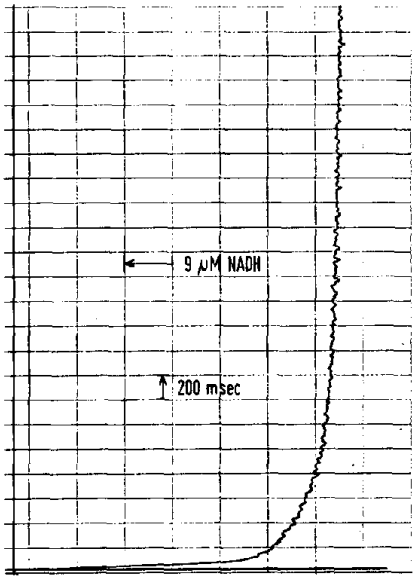


Fig. 1. Record of a stopped flow experiment in the fructose-6-phosphate kinase–pyruvate kinase–lactate dehydrogenase system. 240 U/ml fructose-6-phosphate kinase, 3500 U/ml pyruvate kinase, 5000 U/ml lactate dehydrogenase, 0.06 mM D-fructose-6-phosphate, 0.1 mM ATP, 1 mM phosphoenolpyruvate and 0.25 mM NADH. The reaction is recorded until it is complete. NADH concentration decreases from left to right.

3. Results

In order to analyse the catalytic activity of fructose-6-phosphate kinase towards the various configurations of D-fructose-6-phosphate reactions were followed starting from small concentrations of equilibrated D-fructose-6-phosphate in the presence of high enzyme activity.

Fig. 1 demonstrates a record of a stopped flow experiment in the fructose-6-phosphate kinase–pyruvate kinase–lactate dehydrogenase system. The course of the reaction is biphasic: a fast initial reaction is succeeded by a slower reaction. In fig. 2 the progress curves of three reactions of this type at three different activities of fructose-6-phosphate kinase are presented in a semilogarithmic plot. At increasing activity of fructose-6-phosphate kinase the fast initial reaction is accelerated, whereas the succeeding slower reaction of first order is not influenced by the enzyme activity. From these experiments it can be concluded that fructose-6-phosphate kinase in the first phase of the reaction rapidly reacts with one configuration of D-fructose-6-phosphate, whereas the second phase of the reaction is determined by the spontaneous conversion of another configuration into the one accepted by the enzyme.

From the slope of the regression line for this spontaneous reaction a first order velocity constant of 1.6 sec^{-1} is calculated. Extrapolating to zero time the intersection of the regression line with the ordinate reveals that approximately 76% of the equilibrium mixture of D-fructose-6-phosphate reacts in the fast initial reaction catalyzed by fructose-6-phosphate kinase, whereas 24% of D-fructose-6-phosphate can only be phosphorylated by the enzyme after a spontaneous alteration of configuration.

Comparison of these results with the results, obtained in NMR experiments, that in aqueous solution the equilibrium mixture of D-fructose-6-phosphate consists of 20 ± 4% α– and 80 ± 10% β-D-fructofuranose-6-phosphate [1] leads us to the conclusion that fructose-6-phosphate kinase accepts β-D-fructofura-

tion to the isomerization reaction this enzyme also catalyzes the anomerization reactions of α- to β-D-glucopyranose-6-phosphate [12, 13] and α- to β-D-fructofuranose 6-phosphate [13]. Thus, in vivo α- and β-D-fructofuranose-6-phosphate will be equilibrated. Recently we reported that fructosediphosphate aldolase accepts β-D-fructofuranose-1,6-diphosphate but not α-D-fructofuranose-1,6-diphosphate as substrate [15]. However, since this enzyme catalyzes, the cleavage of some ketosephosphates which can exist only in the open chain configuration [16–18], it seems likely that the keto form of D-fructose-1,6-diphosphate is also a substrate of fructosediphosphate aldolase.

Since fructose-6-phosphate kinase specifically catalyzes the phosphorylation of β-D-fructofuranose-6-phosphate, yielding β-D-fructofuranose-1,6-diphosphate, which can be accepted as substrate by frucose-diphosphate aldolase, enzyme-catalyzed anomerization of D-fructose-1,6-diphosphate does not seem to be necessary in the glycolytic pathway.

References

[1] Benkovic, S.J., Engle, J.L. and Mildvan, A.S. (1972) Biochem. Biophys. Res. Commun. 47, 852–858.
[2] Swenson, C.A. and Barker, R. (1971) Biochemistry 10, 3151–3154.
[3] Wurster, B. and Hess, B. (1972) FEBS Letters 23, 341–344.
[4] Wurster, B. and Hess, B. (1973) FEBS Letters 38,
[5] Wurster, B. and Hess, B. (1973) submitted to Z. Physiol. Chem.
[6] Hofer, H.W. and Pette, D. (1968) Z. Physiol. Chem. 349, 995–1012.
[7] Beisenherz, G., Boltze, H.J., Bücher, T., Czok, E., Garbade, K.H., Meyer-Arendt, E. and Pfleiderer, G. (1953) Z. Naturforsch. 8b, 555–577.
[8] Bergmeyer, H.U. (1971) in: Methoden d. Enzymat. Analyse, 2nd edn., Vol. 2, Verlag Chemie, Weinheim/Bergstr.
[9] Bücher, T., Luh, W. and Pette, D. (1964) in: Hoppe-Seyler/Thierfelder, Handbuch d. Physiol.- und Pathol.-Chemischen Analyse (Lang, K. and Lehnartz, E., eds.), 10th edn., Vol. VI/A, pp. 292–339, Springer Verlag, Berlin.
[10] Hess, B., Kleinhans, H. and Schlüter, H. (1970) Z. Physiol. Chem. 351, 515–531.
[11] Schray, K.J., Benkovic, S.J., Benkovic, P.A., Rose, I.A. and Mildvan, A.S. (1972) Federation Proc. 31, 419 Abs.
[12] Wurster, B. and Hess, B. (1973) Z. Physiol. Chem. 354, 407–420.
[13] Schray, K.J., Benkovic, S.J., Benkovic, P.A. and Rose, I.A. (1973) J. Biol. Chem. 248, 2219–2224.
[14] Salas, M., Viñuela, E. and Sols, A. (1965) J. Biol. Chem. 240, 561–568.
[15] Wurster, B. and Hess, B. (1973) submitted to Biochem. Biophys. Res. Commun.
[16] Lehninger, A.L., Sicé, J. and Jensen, E.V. (1955) Biochim. Biophys. Acta 17, 285–287.
[17] Rutter, W.J. (1961) in: The Enzymes (Boyer, P.D., Lardy, H. and Myrbäck, K. eds.) 2nd edn., Vol. 5, pp. 341–366, Academic Press New York.
[18] Mehler, A.H. and Cusic, M.E. (1967) Science 155, 1101–1103.