SELECTION OF FRUCTOSE 6-PHOSPHATE KINASE MUTANTS IN ESCHERICHIA COLI

A. T. E. Morrissey and D. G. Fraenkel

Department of Bacteriology and Immunology
Harvard Medical School, Boston, Massachusetts

Received June 24, 1968

Fructose 6-phosphate kinase ("phosphofructokinase") is a complex enzyme which is thought to have a critical role in the metabolic control of carbohydrate metabolism (reviewed in Stadtman, 1966). The enzyme from many sources has been purified and characterized; these sources include Escherichia coli (Atkinson and Walton, 1964; Griffin, Houck, and Brand, 1967; Blangy, Buc, and Monod, 1968). Because of the relative ease of genetic and physiological experimentation with this organism, it would be useful to have E. coli mutants affected in this enzyme activity. In this report we describe their selection.

The immediate reason for searching for such mutants was that recent work suggested that fructose 6-phosphate was not a primary intermediate in fructose metabolism in E. coli. Evidence will be presented elsewhere for the functioning, in vitro of the pathway: Fructose → fructose 1-phosphate → fructose 1,6-diphosphate. (The first phosphorylation is catalyzed by the phosphoenolpyruvate phosphotransferase system discovered by Kundig, Ghosh, and Roseman (1964); the involvement of this system in fructose utilization was anticipated, since Tanaka et al. (1967) showed that a pleiotropic mutant which failed to grow on fructose (or other sugars) was lacking enzyme I of this system,

while a fructose-positive revertant had regained this enzyme. This reaction has recently also been demonstrated in Aerobacter aerogenes ((Hanson and Anderson, 1968). The second phosphorylation is catalyzed by a kinase, fructose 1-phosphate kinase, like that discovered in A. aerogenes by Hanson and Anderson (1966).) If this were the main pathway in vivo, then one might expect (Fig. 1) that a mutant lacking fructose 6-phosphate kinase might not be affected in growth on fructose. Using this as one criterion in the screening of pleiotropic carbohydrate mutants, we have found three mutants lacking fructose 6-phosphate kinase activity. We have recently learned that Dr. Anderson and his colleagues have used a similar rationale to select such mutants in A. aerogenes (J. Bacteriol., in press).

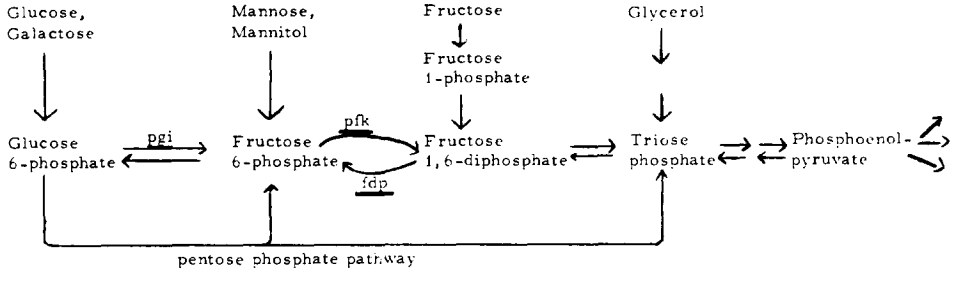

Fig. 1. Pathways of sugar metabolism in E. coli. Abbreviations are pgi, (a gene for) phosphoglucose isomerase, pfk, for fructose 6-phosphate kinase, and fdp, for fructose diphosphatase.

RESULTS

According to Fig. 1, the phenotype of a mutant lacking fructose 6-phosphate kinase would be normal growth on fructose, glycerol, and other gluconeogenic substances, but very slow growth, if any, on substances like glucose, glucose 6-phosphate, galactose, mannose, mannitol, or pentose, whose metabolism is via fructose 6-phosphate. Our primary selection used indicator plates to reveal mutants defective in glucose or mannitol utilization. These

isolates were screened by replica plating on a variety of carbon sources to allow us to recognize pleiotropic mutants and to distinguish potential fructose

Table 1

Growth on minimal plates [a,b]

Carbon source	Colony size (mm)						
	K10 (wild type)	AM1 (mutant)	AM1R3 (revertant)	AM2 (mutant)	AM2R1 (revertant)	AM3 (mutant)	AM3R1 (revertant)
D-Galactose	2.0	0.3	>2	<0.2	1.5	0.3	2.0
D-Glucose	>2	<0.2	>2	<0.2	>2	<0.2	>2
D-Glucose 6-P	2.1	0.7	-	0.3	1.0	0.7	>2
D-Mannitol	2.0	0.0	>2	0.0	1.6	0.0	>2
D-Xylose	1.0	0.3	-	-	-	-	-
L-Arabinose	1.5	0.3	-	-	-	-	-
D-Fructose	1.5	1.3	1.5	0.2-1.0	1.0	<0.2	1.3
D-Gluconate	1.5	1.5	-	-	-	-	-
Glycerol	1.3	1.1	1.3	0.9	0.6	1.2	1.2

[a] Mutant selection: Strain K10, a prototrophic Hfr, was grown to stationary phase in medium 63 (Sistrom, 1958) supplemented with 1% Bacto-tryptone (Difco). 0.1 ml of this culture was added to 0.9 ml 63 containing 4% ethyl methanesulfonate (Loveless and Howarth, 1959) at 37°. After 20 min (survival was ca. 100%) the cells were recovered by centrifugation, allowed to reach full turbidity in 63-Bacto-tryptone, and then subcultured to minimal medium containing 0.4% glycerol. The glycerol-grown cells were resuspended in minimal medium with 0.4% mannitol and after 2 doublings (Gorini and Kaufmann, 1960) treated with penicillin, 2000 u/ml. After 5 hr incubation at 37° dilutions of the lysed culture (survival was 0.02%) were spread on plates containing MacConkey agar base (Difco) and 1% glucose. AM1 was one of the apparent glucose-negative isolates. In another experiment mutagenesis of K10 was like the above but for 84 min, with 0.2% survival. No penicillin step was used; instead, after outgrowth in broth a portion of the mutagenized culture was spread on MacConkey-glucose plates, and AM2 was one of the glucose-negative clones appearing. Another portion was subcultured 1:100 in fructose minimal medium and then spread on MacConkey plates containing 1% mannitol; AM3 was an apparent mannitol-negative mutant. Spontaneous revertants were selected on minimal mannitol plates, and appeared with a frequency of ca. 10^{-8}.

[b] Growth: Stationary phase broth cultures were diluted, spread on minimal plates containing 0.4% of the indicated carbon source so as to give ca. 100 colonies/plate, and average colony size estimated after 48 hr incubation at 37°.

6-phosphate kinase mutants from known pleiotropic mutants - e.g. those lacking phosphoglucose isomerase (Fraenkel and Levisohn, 1967), and those lacking enzyme I of the phosphoenolpyruvate phosphotransferase system (Tanaka et al., 1967; Bourd et al., 1968; Fox and Wilson, 1968 (E. coli);

Table 2

Enzyme activities[a]

Enzyme	Strain						
	K10	AM1	AM1R3	AM2	AM2R1	AM3	AM3R1
Fructose 6-phosphate kinase	83[b]	9[c]	65	5[d]	62[e]	3[f]	75[g]
Phosphoglucose isomerase	710	710	-	384	-	466	-
Fructose diphosphatase	21	16	-	21	-	23	-

[a] Extracts were prepared from cells harvested from logarithmic growth in a broth containing medium 63, 1% Bacto-tryptone, and 0.4% glycerol. The cells were washed once in cold 0.9% NaCl, resuspended in (3 ml/g wet wt) 0.1 M tris-HCl, 0.02 M $MgCl_2$, pH 7.6, and treated 1 min/ml in an MSE ultrasonicator; the crude extract was centrifuged 17,000 x g 30 min and the pellets discarded. (In a few cases, instead of sonication the cells were disrupted in a French pressure cell at 15,000 lb/in^2 and the lysate treated with DNAase, 10 μg/ml, before centrifuging.) The fructose 6-phosphate kinase assay mixture (1 ml) contained 0.05 M tris-HCl, pH 8.5, 0.01 M $MgCl_2$, 1.3 mM fructose 6-phosphate (calcium salt), 1 mM ATP, 50 μg aldolase and 20 μg glycerol phosphate dehydrogenase-triose phosphate isomerase (Boehringer, rabbit muscle), 0.1 mM DPNH, 0.01 M NaCN (to eliminate DPNH oxidase activity), and 10-100 μg extract-protein. Fructose 6-phosphate kinase activity was calculated from the difference in the rate of DPNH oxidation between the complete assay and one lacking ATP (the minus ATP-control gives a substantial rate of DPNH oxidation, which is fructose 6-phosphate reductase activity not depending on the auxilliary enzymes). Assays for phosphoglucose isomerase, fructose diphosphatase, and protein were as described elsewhere (Fraenkel and Levisohn, 1967; Fraenkel et al., 1966). Reactions were followed with a Gilford 2000 spectrophotometer with the cell chamber at 25°, and are expressed as mμmoles/min/mg protein; for fructose 6-phosphate kinase the values are given as 1/2 the DPNH equivalents oxidized.

[b] average of separate experiments (97, 64, 94, 63, 86, 120, 56); [c] av. of 10, 11, 6; [d] av. of 4, 9, 4, 5, 3; [e] av. of 49, 76; [f] av. of 4, 4, 2; [g] av. of 79, 71.

Tanaka and Lin, 1968 (A. aerogenes); Simoni et al., 1967 (S. typhimurium)). A phosphoglucose isomerase mutant is readily distinguished from the other two types, as it grows normally on pentoses and on mannitol (unpublished). Recognizing fructose 6-phosphate kinase mutants among enzyme I mutants might be more difficult, however, because the growth phenotypes differ somewhat among the latter; nonetheless, as far as is known all share the inability to grow on fructose or mannitol but grow normally on glucose 6-phosphate.

About sixty primary isolates of apparent glucose or mannitol defectives were screened, and three of them, AM1, AM2, and AM3 had a growth phenotype like that expected for fructose 6-phosphate kinase mutants (Table 1). Spontaneous mannitol-positive revertants gave a wild-type growth response on the whole series of carbon sources (Table 1). Thus the mutant phenotype is probably caused by a single gene lesion.

Table 2 shows the results of in vitro enzyme assay of fructose 6-phosphate kinase, phosphoglucose isomerase, and fructose diphosphatase. The three mutants are deficient in phosphofructokinase, with 5-10% of the wild type level; they have normal activities of the other two enzymes. The revertants have regained wild type levels of fructose 6-phosphate kinase.

DISCUSSION

Three fructose 6-phosphate kinase mutants have been selected. Experiments are in progress on their further genetic and physiological characterization. It should be noted that some of their properties reported here are unexpected. One such finding is that not all three mutants grow normally on fructose, and thus the expectation that fructose 6-phosphate kinase should not be necessary for fructose metabolism is not confirmed. It is possible that the apparent abnormality in fructose growth of two of the isolates (AM2 and

AM3) is a secondary effect of the lesion, since several other mutants in pathways apparently unrelated to fructose metabolism fail to form colonies of fructose minimal plates (Fraenkel, in preparation). On the other hand, AM2 and AM3 may be less "leaky" than AM1 (Table 2), so the different responses to fructose may be an indication that fructose 6-phosphate kinase does have some role in normal fructose metabolism. Another characteristic of the present mutants which needs clarification is that their growth is not equally impaired on the various substances whose metabolism, according to Fig. 1, is restricted to triose phosphate formation by the pentose phosphate cycle; it is unexpected that mannitol negativity seems complete while small colonies appear on glucose 6-phosphate.

In view of these differences (between mutants and between substrates) it will be particularly interesting to learn whether these strains carry mutations in the structural gene for fructose 6-phosphate kinase. If they do, then these mutants, or their revertants, might contain a kinetically abnormal enzyme; such strains would be a useful probe for the further understanding of this enzyme function.

ACKNOWLEDGEMENTS

This work was supported by grants from the National Science Foundation (GB-7207) and the U.S. Public Health Service (GM-00177-09 and 5K03 GM07344).

REFERENCES

Atkinson, D. E. and Walton, G. M., J. Biol. Chem., 240, 757 (1965).
Blangy, D., Buc, H. and Monod, J., J. Mol. Biol., 31, 13 (1968).
Bourd, G. I., Andreeva, I. V., Shabolenko, V. P. and Gershanovitch, V. N., Molekuliarnaya Biologiya 2, 89 (1968).
Fox, C. F. and Wilson, G., Proc. Natl. Acad. Sci. U.S., 59, 988 (1968).
Fraenkel, D. G. and Levisohn, S. R., J. Bacteriol., 93, 1571 (1967).
Fraenkel, D.G., Pontremoli, S. and Horecker, B. L., Arch. Biochem. Biophys., 114, 4 (1966).
Gorini, L. and Kaufmann, H., Science, 131, 604 (1960).

Griffin, C. C., Houck, B. N., and Brand, L., Biochem. Biophys. Res. Comm., 27, 287 (1967).
Hanson, T. E. and Anderson, R. L., J. Biol. Chem., 241, 1644 (1966).
Hanson, T. E. and Anderson, R. L., Fed. Proc., 27, 644 (1968).
Kundig, W., Ghosh, S. and Roseman, S., Proc. Natl. Acad. Sci. U.S., 52, 1067 (1964).
Loveless, A. and Howarth, S., Nature, 184, 1780 (1959).
Simoni, R. D., Levinthal, M., Kundig, F. D., Kundig, W., Anderson, B., Hartman, P. E. and Roseman, S., Proc. Natl. Acad. Sci. U.S., 58, 1963 (1967).
Sistrom, W. R., Biochim. Biophys. Acta, 29, 579 (1958).
Stadtman, E. R., Advances in Enzymology, 28, 41 (1966).
Tanaka, S. and Lin, E. C. C., Proc. Natl. Acad. Sci. U.S., 57, 913 (1967).
Tanaka, S., Fraenkel, D. G. and Lin, E. C. C., Biochem. Biophys. Res. Comm., 27, 63 (1967).