IDENTIFICATION OF A MANNOKINASE IN *Escherichia coli*

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E. coli grows at a similar rate on glucose, mannose, and fructose as carbon and energy sources. The metabolism of these sugars seems to be initiated by phosphorylated intermediates. The enzyme(s) required for these phosphorylations have posed challenging problems (Asensio, 1960; Fraenkel, Falcoz-Kelly and Horecker, 1964; Wood, 1966). The main difficulty, even in the light of recent findings, has been to account for the phosphorylation of mannose and fructose. In this communication the discovery of a constitutive mannokinase in *E. coli* is reported. This new enzyme is ATP-dependent, can also readily phosphorylate fructose, and could be the long searched phosphorylative system for these sugars.

Materials and Methods

The strains 3300, FR-1 and GN-2 used in this work are *E. coli*-K12, and were kindly provided by Dr. B. L. Horecker. Strain FR-1, a derivative of 3300, has lost the glucokinase but is still able to grow fairly well on glucose; strain GN-2 is similar to FR-1 but has additionally lost the capacity to grow on glucose, mannose and fructose (2).

The bacteria were usually grown overnight in a nutrient broth medium, in a rotary shaker. Extracts were obtained by grinding washed cells with alumina, at 4° C, suspending the homogenates in 20 mM phosphate - 1 mM EDTA, pH 7.0 (3 vol. per gram of fresh cells) and centrifuging at 10,000 x g for 15 min. The supernatant ("crude extract") was used, unless stated otherwise.

Enzyme activities were estimated by either sugar disappearance (Somogyi, 1945), or spectrophotometrically (see Table I), at pH 7.0.

Results and Discussion

Crude extracts of FR-1 cells were assayed (4) for their ability to phosphorylate glucose, mannose, and fructose, with a variety of phosphoryl donors. Nearly similar values were obtained with glucose and mannose when ATP, PEP, and acetyl-P were used. Sugar concentration usually was 4 mM. Phosphoryl compounds were used at 6 mM concentration.

The most relevant results were those obtained with ATP and the three hexoses, particularly considering the fact that strain FR-1 lacks glucokinase. Activities with the ATP system (mμmoles/min/mg protein) were as follows: glucose, 5.9, mannose, 9.0, and fructose, 5.8 [1,2]. These results raised the question as to whether they corresponded to a new kinase, obviously different from glucokinase, or might be due to some enzyme already described in bacteria (see later). This problem was approached by trying to eliminate the several known systems, which was achieved by the following simple treatments: 1) Heating the crude extracts (60^{o}-5 min) destroyed the activity of the PEP-phosphotransferase system, while activities with acetyl-P and ATP remained practically unchanged. 2) By centrifugation of the crude extracts at high speed (150,000 x g for 2 hr.) a supernatant fraction was obtained which lacked PEP-dependent and had lost most of the acetyl-P-dependent activities (in some experiments the acetyl-P residual activity was lost after freezing and thawing), while retaining the activity with ATP. 3) Heating intact cells (60^{o}-5 min) in the presence of 4 mM EDTA, before the preparation of the extracts, led to the loss of both the PEP and acetyl-P dependent activities, with little change in the activity with ATP.

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[1] Approximately 100% higher values were obtained when the spectrophotometric method (see Table I) was used with fructose and glucose.

[2] No appreciable activity was found with the following sugars: 2-deoxyglucose (as determined by a modification of the Waravdekar and Saslaw method (6)), N-acetylmannosamine and N-acetylglucosamine (estimated as Reissig et al. (7)), and galactose (4).

Cross-inhibition experiments with glucose, mannose, and fructose, suggested that a single enzyme was involved for the phosphorylation of the three hexoses with ATP as donor. This hypothesis was supported by the specificity and kinetic results shown in Table I. From these results the existence of an enzyme to our knowledge hitherto undescribed can be inferred. The most striking property is its requirement for the hydroxyl group in position C-2, which renders glucose a relatively poor substrate, while lyxose has greater affinity for the enzyme than xylose. In addition to ATP, ITP and to a lesser extent other trinucleotides can act as phosphoryl donors. With fructose as substrate, a Km for ATP of 0.5 mM was obtained.

Table I. Michaelis' constants of *E. coli* (FR-1) mannokinase

Compound	Km (or Ki) mM
Mannose	0.1
Fructose	0.3
Glucose	7
2-Deoxyglucose	10
Lyxose	3
Xylose	50
N-Acetylmannosamine	50
N-Acetylglucosamine	70

The Km for fructose and glucose were determined spectrophotometrically. The assay mixture contained TPN (0.3 mM), ATP-Mg (3 mM), phosphate, pH 7.0 (40 mM), excess of glucose-6-phosphate dehydrogenase. In the case of fructose, an excess of phosphoglucose isomerase was also added. Fructose or glucose were added at appropriate concentrations. To avoid interference by N-acetylglucosamine kinase (Asensio and Ruiz-Amil, 1966) the activity on glucose was estimated in the presence of 0.4 mM N-acetyl-glucosamine. The affinity constant for mannose was obtained from the competitive inhibition by this sugar of either fructose or glucose phosphorylation. Ki values for the other compounds were estimated with the same spectrophotometric system (fructose as substrate) and the inhibitors added as appropriate. In most cases crude extracts of FR-1 (see Methods) were used. In some cases, activities were also measured by using heated (60°-5 min) crude extracts, and the values obtained were similar. Assays were carried out at room temperature.

With such specificity, the trivial name of "mannokinase" seems appropriate for the new kinase and clearly distinguishes it from other known hexose phosphotransferases of the trinucleotide-dependent group, as hexokinase (Sols et al., 1958), bacterial glucokinase (1), and plant fructokinase (Medina and Sols, 1956).

The mannokinase activity in extracts of FR-1 is independent of the carbon source present in the media. The optimal pH for activity is in the 7-8 range. The thermostability of the enzyme is of particular interest when compared to E. coli glucokinase, which is completely inactivated at 60° in 5 min (1). There is no direct evidence on the phosphoryl group position of the primary product, although indirect evidence indicates it is the 6-phosphate since the reaction products can be coupled with glucose-6-phosphate dehydrogenase without apparent lag when glucose is the substrate, and with phosphoglucose isomerase (plus the same dehydrogenase) when fructose is used.

Several constitutive phosphotransferases have been characterized in E. coli. The more relevant are the glucokinase, able to phosphorylate glucose, but not mannose and fructose (1), and the PEP-hexose phosphotransferase, a complex system involving several proteins, with great affinity ("enzyme II") for glucose (Km 0.4 mM), smaller for mannose (Km 2 mM) and no detectable activity on fructose (Kundig, Ghosh and Roseman, 1964). An acetyl-P hexose phosphotransferase previously found in Aerobacter aerogenes (Kamel and Anderson, 1964, 1965) is also present in strain FR-1, and has been studied in some detail; its Michaelis' constants* for glucose, mannose and fructose are (mM) 6, 18, and 100, respectively. When comparing these data with those corresponding to mannokinase (see Table I) it appears that only this enzyme has a high affinity for both mannose and fructose. Thus, the function of the mannokinase is presumably

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* The assays were carried out by using 0.2 M glycil-glycine buffer, pH 7.5. Similar results were obtained with TRIS and imidazol, but not with phosphate buffer, wherein the activities dropped about 90%.

related to the utilization of mannose and fructose by E. coli, therefore solving the problem of the "missing kinases" as it was recently referred to by Wood (3). Nevertheless, more evidence would be required of the actual involvement of this enzyme, particularly since the maximal rate of phosphorylation observed in extracts is considerably smaller than the rate of utilization of mannose and fructose by intact cells.

It has been recently reported (Tanaka, Fraenkel and Lin, 1967; Asensio and Sebastián, 1967) that strain GN-2, unable to grow on the common hexoses (see Materials), lacks the PEP phosphotransferase system. The suggestion of Tanaka et al. (13) is that this system could be the previously postulated "hexokinase" (Fraenkel et al., 2). However, it should be emphasized (see above) that since the PEP-dependent system has no detectable activity on fructose, it does not fulfil the requirements of the expected "hexokinase". An alternative hypothesis could be to assume that strain GN-2 is impaired in the permeation mechanism for hexoses. In fact, Kundig et al. (1966) have recently presented evidence whereby the function of the PEP-hexose phosphotransferase might be located at the permease level, which is in agreement with the tentative conclusion drawn in a former study carried out on a similar mutant (MM-6) (Asensio, Avigad and Horecker, 1963). Thus, the function of such a permeation factor would be a prior requirement for the utilization of common hexoses, so that its impairment would determine cripticity if the corresponding kinases were present in the cells. In fact, strain GN-2 showed similar mannokinase activity than strains FR-1 and 3300 ("wild type"). Further studies on the mannokinase and its physiological role are in progress.

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REFERENCES

1. Asensio, C., R. esp. Fisiol., 16, Suppl. II, 121 (1960).
2. Fraenkel, D.G., Falcoz-Kelly, F. and Horecker, B.L., Proc. Natl. Acad. Sci. U.S., 52, 1207 (1964).

3. Wood, W.A., Ann. Rev. Biochem., 35, 521 (1966).
4. Somogyi, M., J. Biol. Chem., 160, 69 (1945).
5. Asensio, C. and Ruiz-Amil, M., in Methods in Enzymology (S.P. Colowick and N.O. Kaplan, eds.) vol. 9, p. 421. New York, Academic Press, 1966.
6. Waravdekar, V.S. and Saslaw, L.D., Biochim. Biophys. Acta, 24, 439 (1957).
7. Reissig, J.L., Strominger, J.L. and Leloir, L.F., J. Biol. Chem., 217, 959 (1955).
8. Sols, A., DeiaFuente, G., Villar-Palasí, C. and Asensio, C., Biochim. Biophys. Acta, 30, 92 (1958).
9. Medina, A. and Sols, A., Biochim. Biophys. Acta, 19, 378 (1956).
10. Kundig, W., Ghosh, S. and Roseman, S., Proc. Natl. Acad. Sci. U.S., 52, 1067 (1964).
11. Kamel, M.Y. and Anderson, R.L., J. Biol. Chem., 239, PC3607, (1964).
12. Kamel, M.Y. and Anderson, R.L., Federation Proc., 24, 422 (1965).
13. Tanaka, S., Fraenkel, D.G. and Lin, E.C.C., Biochem. Biophys. Res. Commun., 27, 63 (1967).
14. Asensio, C. and Sebastián, J., IV Meeting FEBS. Abstracts Communs. Oslo 1967, in press.
15. Kundig, W., Kundig, F.D., Anderson, B. and Roseman, S., J. Biol. Chem., 241, 3243 (1966).
16. Asensio, C., Avigad, G. and Horecker, B.L., Arch. Biochem. Biophys., 103, 299 (1963).