EVIDENCE THAT THE INDUCIBLE PHOSPHOENOLPYRUVATE:D-FRUCTOSE 1-PHOSPHOTRANSFERASE SYSTEM OF *AEROBACTER AEROGENES* DOES NOT REQUIRE "HPr"

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Summary: The constitutive, low-molecular-weight, soluble protein ("HPr") that is an indispensable component of most phosphoenolpyruvate (PEP):sugar phosphotransferase systems is shown not to be required for the inducible PEP:D-fructose 1-phosphotransferase system of *Aerobacter aerogenes*. Rather, this system has an absolute requirement for an inducible soluble protein previously termed a "K_m factor".

A constitutive, low-molecular-weight, soluble protein ("HPr") functions as a phosphoryl carrier between phosphoenolpyruvate (PEP) and sugars and hexitols in several species of bacteria (1,2). Phosphoryl transfer from PEP to HPr is catalyzed by a constitutive enzyme (enzyme I), whereas phosphoryl transfer from the resulting phospho-HPr to a sugar or hexitol is catalyzed by any of several membrane-bound, sugar-specific enzymes (enzymes II), some of which are inducible (3). HPr has been reported to be an absolute requirement for all of the PEP-dependent phosphotransferase systems so far described (1-5) except for that found in photosynthetic bacteria (6).

In *A. aerogenes*, D-fructose can be phosphorylated with PEP at carbon 1 by a constitutive system involving enzyme I, enzyme II, and HPr (7). However, growth of the organism on D-fructose induces another soluble protein (originally termed a "K_m factor") which, when incubated with enzyme II from D-fructose-grown cells, and enzyme I and HPr from cells grown on D-mannitol, D-fructose, or other substrates, decreases the K_m of the system for D-fructose (7). The present communication provides evidence that the "K_m factor" is

required intead of, rather than in addition to, HPr in the inducible PEP:D-fructose 1-phosphotransferase system of *A. aerogenes*.

MATERIALS AND METHODS

The bacterium used was *A. aerogenes* PRL-R3 or, for enzyme II^{fru} preparations, a "K_m factor"-deficient mutant (QQ17) (7). Procedures for cell growth and the preparation of cell-free extracts were described previously (7). Enzyme II active in D-fructose 1-phosphate formation (enzyme II^{fru}) was obtained from cells grown on glycerol (enzyme II^{fru}_{gol}) or D-fructose (enzyme II^{fru}_{fru}). Enzyme II^{fru} was obtained as the washed 100,000 x g pellet from crude cell-free extracts. Enzyme I, HPr, and "K_m factor", which were in the 100,000 x g supernatant solution, were separated from each other and purified about 60, 130, and 50 fold, respectively. Enzyme I was purified by ammonium sulfate precipitation (40 to 70% saturation) followed by DEAE-cellulose chromatography (0 to 0.4 M KCl gradient). HPr was purified by ammonium sulfate precipitation (40 to 70% saturation) followed by Sephadex G-75 chromatography and DEAE-cellulose chromatography (0 to 0.2 M KCl gradient). "K_m factor" was purified by ammonium sulfate precipitation (35 to 70% saturation), DEAE-cellulose chromatography (0 to 0.4 M KCl gradient), a second ammonium sulfate precipitation (45 to 65% saturation), Sephadex G-100 chromatography, and hydroxyapatite chromatography (0.005 to 0.07 M potassium phosphate gradient).

The rate of PEP-dependent D-fructose 1-phosphate formation from D-fructose was determined with an end-point assay (7). The reaction mixtures (0.2 ml volumes) contained: Tris-HCl buffer (8.0 µmoles, pH 7.5), 2-mercaptoethanol (0.57 µmole), PEP (1.0 µmole), $MgCl_2$ (1.0 µmole), D-fructose (0.02 or 18 µmoles), enzyme I (0.17 mg), enzyme II^{fru} (0.27 mg of enzyme II^{fru}_{gol} or 0.14 mg of enzyme II^{fru}_{fru}), HPr (0.12 mg), and "K_m factor" (0.11 mg). The tubes were incubated at 30°C for 10 minutes, after which the reactions were terminated by heating the tubes in a

95°C water bath for 7 minutes. The denatured protein was removed by centrifugation and samples of the supernatant solutions were analyzed for D-fructose 1-phosphate by the described assay for D-fructose 1-phosphate kinase (8), except that the buffer was glycylglycine-NaOH, and D-fructose 1-phosphate was limiting with D-fructose 1-phosphate kinase in excess. All reactions for which D-fructose 1-phosphate could not be detected are reported as < 0.2 nmole per minute per mg of enzyme II^{fru} (Table 1), as this reflects limitations of the sensitivity of the method.

D-Fructose 1,6-diphosphate was determined by the assay used for D-fructose 1-phosphate, except that D-fructose 1-phosphate kinase, $MgCl_2$, and ATP were omitted. D-Fructose 6-phosphate was determined spectrophotometrically by the use of phosphoglucose isomerase and glucose 6-phosphate dehydrogenase.

RESULTS

The results are summarized in Table 1. Consistent with all previous findings, constitutive activity (enzyme II^{fru}_{gol}) required HPr, and was detectable at 90 mM D-fructose but not at 0.1 mM D-fructose. "K_m factor" did not substitute for HPr when enzyme II^{fru}_{gol} was assayed, and also it did not activate the system at either 0.1 mM or 90 mM D-fructose.

Enzyme II^{fru}_{fru} activity also could be measured at 90 mM D-fructose in the presence of HPr (Table 1). In contrast with the enzyme II^{fru}_{gol} data, however, the further addition of "K_m factor" enhanced the activity at 90 mM D-fructose and allowed activity to be detected at 0.1 mM D-fructose. The new finding is that, in the presence of "K_m factor", HPr is not required for enzyme II^{fru}_{fru} activity either at 0.1 mM or 90 mM D-fructose (Table 1). This observation has been repeated many times with different preparations of the phosphotransferase components. All of the measured activities were dependent on PEP and enzymes I and II. The only D-fructose derivative that was formed in these reactions was D-fructose

TABLE I. Formation of D-Fructose 1-Phosphate from Fructose and PEP by Enzyme II^{fru} Supplemented with Various Combinations of Phosphotransferase Components*

Enzyme II	D-Fructose (mM)	Activity**			
		+ HPr		- HPr	
		+ K_m Factor	- K_m Factor	+ K_m Factor	- K_m Factor
II^{fru}_{fru}	0.1	3.3	< 0.2	2.7	< 0.2
	90.0	7.2	4.9	3.2	< 0.2
II^{fru}_{gol}	0.1	< 0.2	< 0.2	< 0.2	< 0.2
	90.0	7.2	9.0	< 0.2	< 0.2

*All reaction mixtures contained enzyme I and other additions as reported in this Table or in Materials and Methods.

**Activity is expressed as nmoles of D-fructose 1-phosphate formed per minute per mg of enzyme II^{fru}.

1-phosphate; D-fructose 6-phosphate and D-fructose 1,6-diphosphate were not detected.

DISCUSSION

From the data presented, it seems necessary to conclude that, in contrast to most other reported PEP-dependent phosphotransferase systems (1-5), the inducible PEP:D-fructose 1-phosphotransferase system of *A. aerogenes* does not require HPr. Rather, it has an absolute requirement for the inducible protein previously termed a "K_m factor". The possibility that the "K_m factor" functions like a *Staphylococcus aureus* factor III^{lac} (i.e., as a phosphoryl carrier protein between phospho-HPr and sugar) (9) seems unlikely because this would require the presence of HPr. Evidence that a functional level of HPr was not a contaminant of our enzyme II preparations was the observation

that activity at 90 mM D-fructose in the absence of added HPr was not detectable. Thus, the most reasonable interpretation of the data is that the "K_m factor" functions in place of HPr in the inducible system. It differs from HPr in its inducibility (7), its larger size (7), and its requirement for a D-fructose-induced enzyme II. That "K_m factor"-dependent activity was observed at 0.1 mM D-fructose with enzyme II^{fru}_{fru} but not with enzyme II^{fru}_{gol} indicates that a membrane-bound enzyme II with a high affinity for D-fructose is also induced by D-fructose and is present in D-fructose-grown cells in addition to the constitutive enzyme II^{fru}. The inducible and constitutive enzymes II^{fru} are distinguishable from each other by their different affinities for D-fructose, their differential induction, and their differential requirements for HPr and "K_m factor".

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REFERENCES

1. Kundig, W., Ghosh, S., and Roseman, S., Proc. Nat. Acad. Sci. U.S.A., 52, 1067-1074 (1964).
2. Anderson, B., Weigel, N., Kundig, W., and Roseman, S., J. Biol. Chem., 246, 7023-7032 (1971).
3. Kundig, W., and Roseman, S., J. Biol. Chem., 246, 1393-1406, 1407-1418 (1971).
4. Roseman, S., J. Gen. Physiol., 54, 138-184 (1969).
5. Kaback, H.R., Annu. Rev. Biochem., 39, 561-597 (1970).
6. Saier, M.H., Feucht, B.U., and Roseman, S., J. Biol. Chem., 246, 7819-7821 (1971).
7. Hanson, T.E., and Anderson, R.L., Proc. Nat. Acad. Sci. U.S.A., 61, 269-276 (1968).
8. Sapico, V., and Anderson, R.L., J. Biol. Chem., 245, 3252-3256 (1970).
9. Nakazawa, T., Simoni, R.D., Hays, J.B., and Roseman, S., Biochem. Biophys. Res. Commun., 42, 836-843 (1971).