MANNITOL ACETYL PHOSPHATE PHOSPHOTRANSFERASE OF ASPERGILLUS

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Received September 25, 1967

An Aspergillus strain that accumulates free mannitol in a glucose medium has been previously reported (Lee, 1967). This organism also grows in a medium with mannitol as the sole source of carbon. In studying the regulatory mechanism of mannitol metabolism it is of interest to find the enzyme that initiates the breakdown of mannitol. Soluble or particulate mannitol dehydrogenases, mannitol kinase, pyrophosphate phosphotransferase, and mannitol phosphoenopyruvate phosphotransferase could not be detected in cell-free extracts of this Aspergillus. There is, however, an active mannitol acetyl phosphate (Ac-P) phosphotransferase present.

$$\text{Mannitol} + \text{Acetyl Phosphate} \rightarrow \text{Mannitol 1-PO}_4 + \text{Acetate}$$

A specific enzyme assay was developed for mannitol 1-PO_4 (M1P). Mannitol is the specific substrate for this enzyme but either Ac-P or carbamyl phosphate can serve as phosphate donors.

Methods. The enzyme was extracted from an Aspergillus species (Upjohn collection No. 4177). Cultures were maintained by serial vegetative transfers. The mold was grown in shake flasks at 28° for 3-4 days in a modified Czapek-Dox mannitol medium on a reciprocal shaker at 100 cycles per minute. The medium contained mannitol 90 g, $MgSO_4$ 0.5 g, KCl 0.5 g, $ZnSO_4 \cdot 6H_2O$ 0.05 g, $CaCl_2 \cdot 2H_2O$ 0.05 g, and $FeCl_3 \cdot 6H_2O$ 0.01 g plus NH_4NO_3 3 g, K_2HPO_4 2 g, and KH_2PO_4 1 g (sterilized separately) per liter.

Enzyme extracts were obtained by rupturing the mycelium in pH 7.0 0.1M potassium phosphate buffer with 2.7 mM EDTA in a cold French Pressure-Cell (10-15,000 psi).

The enzyme was partially purified as follows. Interfering enzymes, MlP dehydrogenase and MlP phosphatase, in the crude extract were inactivated by lowering the pH to 5.0 with 2.5N acetic acid and the precipitate removed. The supernatant was dialyzed against 50% $(NH_4)_2SO_4$ (4°) and then $(NH_4)_2SO_4$ added to 75% saturation to remove more inactive proteins. Then the enzyme was precipitated at 100% saturation, centrifuged, and resuspended in pH 5.4 0.1M Kacetate buffer and kept frozen. Three enzyme preparations had specific activities of 0.48-0.61 units (all units expressed are μmole/min-mg protein).

MlP was assayed by adding 1.5 ml pH 9.1 buffer containing 1M hydrazine, 0.2M $KHCO_3$, and 5.4 mM EDTA, then 0.05-0.2 μmole MlP sample to be assayed and 2 μmole NAD were added separately and made up to 3 ml. The net change in O.D. at 340 mμ was measured after adding 0.05-0.1 units MlP dehydrogenase to the system, which was modeled after Wieland's (1965) glycerol assay. Repeated tests with MlP standard showed it to be accurate and reliable. It is specific for MlP and does not respond to glucose 6-phosphate, mannose 6-phosphate, sorbitol 6-phosphate, or α and β glycerol phosphate. MlP dehydrogenase was obtained from Aspergillus (Lee, 1967), dialyzed against buffer and precipitated by dialysis against 45-65% pH 7.0 $(NH_4)_2SO_4$. It is very stable when kept in a 70% $(NH_4)_2SO_4$ solution in the cold.

For further confirmation, MlP formed in an enzymatic reaction was absorbed and eluted from a Dow 1-formate column (Tanaka et al., 1967) and detected by paper chromatography (Stetten, 1965).

Results & Discussion. Repeated attempts to demonstrate the following enzymes in Aspergillus cell-free extracts were unsuccessful: Soluble mannitol dehydrogenase by the method of Edmundowicz & Wriston (1963); particulate mannitol dehydrogenase by the method of Arcus & Edson (1956);

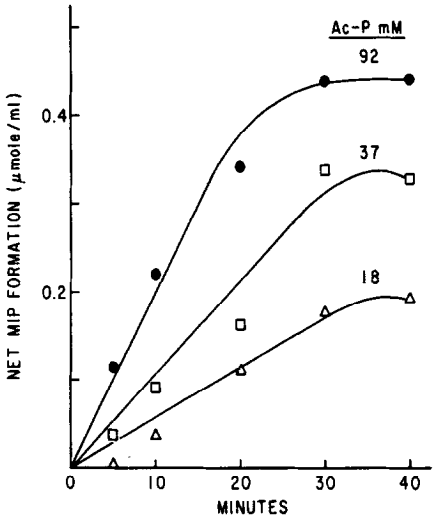

Fig. 1. Effect of Ac-P concentrations on mannitol Ac-P phosphotransferase activity. 2 ml of reaction system contains 0.1M K acetate pH 5.4, 0.6M mannitol, LiAc-P (by assay) as indicated and 0.04 mg enzyme protein at the start of the reaction, 30°. The reaction was stopped by adding 1 ml 10% TCA, chilled and centrifuged (boiling produced assay active substance). See text for MIP assay.

mannitol kinase by the method of Klungsoyr (1966); pyrophosphate phosphotransferase by the method of Stetten (1965); and mannitol phosphoenopyruvate phosphotransferase by the method of Tanaka et al. (1967). Most probably these enzymes do not exist in this Aspergillus strain.

Mannitol Ac-P phosphotransferase activity was repeatedly detected in cell-free extracts of Aspergillus. Its activity was proportional to enzyme and substrates concentrations (Fig. 1 & 2). The Km for Ac-P derived from Fig. 1 is roughly 0.1M and the Km for mannitol derived from Fig. 2 is about 2.5M. The enzyme preparation was not purified enough for precise enzyme kinetic studies, but the data show substrate affinities are weak and the rate is practically limited by mannitol solubility. High mannitol concentrations were necessary for activity and could not be replaced by a high salt (1.35M KCl) or a high glucose (0.4M) osmotic environment. The specificity of this enzyme is much narrower than the hexose Ac-P phosphotrans-

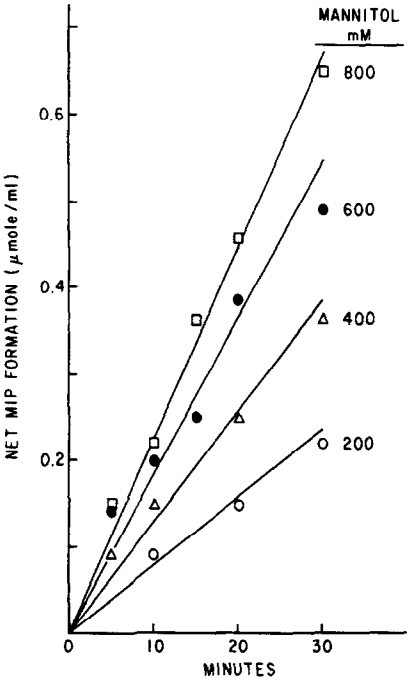

Fig. 2. Effect of mannitol concentration on mannitol Ac-P phosphotransferase activity. Conditions as in Fig. 1 with 92 mM LiAc-P and mannitol concentrations as indicated, pH 5.0, 37°.

ferase (Kamel & Anderson, 1966). Carbamyl phosphate was about as active as Ac-P, but $Na_4P_2O_7$, mannose-6-PO_4 or phosphoenopyruvate were inactive. Glucose and fructose (0.4M) were not phosphorylated and thus this enzyme could not be involved in hexose phosphorylation. Aspergillus cell-free extracts contain a very active hexokinase.

The phosphotransferase was active in a pH range of 4 to 6 and had a pH optimum of 5.0. Its activity decreased rapidly above pH 5.0 and it had no activity at pH 7.0. pH values below 4.0 were not tested. Enzyme activity was not affected by the addition of 5 mM $CaCl_2$, $MgCl_2$, EDTA, or 90 mM $(NH_4)_2SO_4$. Maleic buffer could replace acetate buffer but phosphate buffer decreased activity 60%. The enzyme was inhibited by MIP at much lower concentrations than the effective substrate levels (Fig. 3). Regulation by end-product could be significant in controlling mannitol oxidation.

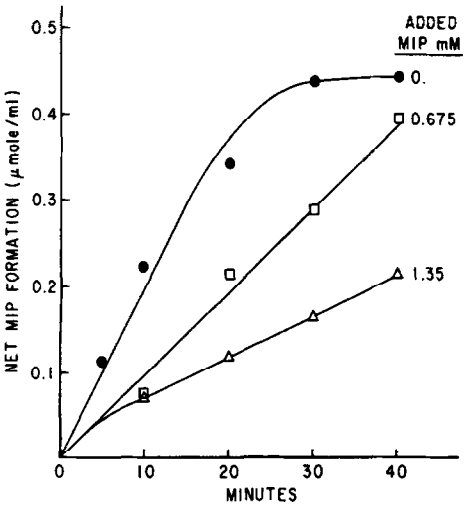

Fig. 3. End-product inhibition of mannitol Ac-P phosphotransferase. Conditions as in Fig. 1 (92 mM LiAc-P and 0.6M mannitol).

Acid phosphatase activity was detected in the enzyme preparation. This phosphatase differed from MIP phosphatase (Lee, 1967) in that it was active at pH 5.0, did not require $MgCl_2$, and it hydrolyzed many substrates other than MIP (glucose 6-PO_4, fructose 6-PO_4, mannose 6-PO_4, p-nitrophenylphosphate). The pyrophosphate phosphotransferase described by Stetten (1965) was also an acid phosphatase. Further purification is necessary to distinguish whether the acid phosphatase is a contaminant or another activity of the enzyme itself. One effect of this phosphatase activity was to reduce the measured rate of MIP synthesis.

A phosphotransferase was also isolated from another mannitol producing Aspergillus candidus NRRL 305 (Smiley et al., 1966) by the above methods. It had the same pH optimum and substrate specificity as the enzyme from Aspergillus (UC-4177). The enzymes from both strains are probably identical. The mannitol Ac-P phosphotransferase activity in the cell-free extracts of both strains (0.05 units) is enough to account for the mannitol oxidation rate by growing cells (3 mg/ml/day or $\pm$ 0.004 units). Although other mannitol catabolic enzymes could not be found, it should not be con-

cluded that mannitol Ac-P phosphotransferase accounts for mannitol phosphorylation solely on such negative evidence. If one could isolate a mannitol negative mutant of Aspergillus and the phosphotransferase activity was absent, then this would constitute a direct proof that mannitol was metabolized solely by this enzyme. Presently we are studying the relationship of mannitol catabolic and anabolic enzymes of Aspergillus to determine why free mannitol is accumulated by these strains.

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