ON THE PHOSPHORYLATION OF SUGARS IN *ACHOLEPLASMA LAIDLAWII*

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1. Introduction

The phosphoenolpyruvate: hexose phosphotransferase system (PEP-PTS) first described by Kunding et al. [1], has been shown to play an important role in the transport of many hexoses across bacterial membranes and to trap these substances as their phosphate esters. The physiological significance of this system has been established by intensive genetic and biochemical analysis in a number of bacteria – *Escherichia coli* [2, 3], *Staphylococcus aureus* [4], and *Bacillus subtilis* [5].

Recently, Romano et al. [6] reported that PEP-PTS is physiologically important in facultative anaerobes. We previously reported that *Acholeplasma laidlawii* possessed a specific glucose transport system [7] and the present investigation was done to determine whether PEP-PTS plays a role in the utilization of some hexoses by *A. laidlawii,* which is a representative facultative anaerobe.

2. Materials and methods

A. laidlawii (obtained from Köller, Jena, DDR) was grown and harvested as has been reported [7]. Proteins were determined by the method of Lowry et al. [8]. Cell-free extracts were prepared by suspending washed cell suspensions in a mixture containing: 0.01 M Tris-HCl buffer, pH 7.6, 0.1 mM $MgCl_2$, 0.1 mM EDTA and 0.001 M dithiothreitol. The suspension was frozen at $-70°$ and thawed at room temperature. Freeze-thawing was repeated 3 times and then the suspension was triturated with quartz powder. The resulting lysate was centrifuged for 10 min at 20 000 *g* and the supernatant used as the cell-free extract. The reaction mixture contained: $MgCl_2$ (0.5 mM), NaF (0.01 M), dithiothreitol (2 mM), Tris-HCl buffer (0.1 M, pH 7.6), PEP (0.05 M), D-[^{14}C]glucose (0.05 mM, Amersham, England) and cell-free extract (0.25 mg protein). After incubation at 37° the reaction was stopped by the addition of an equal volume of unlabelled glucose (0.2 M) and mixture immersed in ice. The amount of sugar phosphates was determined by the column chromatographic method of Winkler [9]. In some experiments separation of sugar phosphates was carried out by a modification of the Somogyi procedure described by Fomina and Titova [10]. For the intracellular determination of the accumulation of sugar phosphates, cells of *A. laidlawii* were allowed to take up sugars (D-[^{14}C]glucose, 0.012 mM or D-[^{14}]fructose, 0.075 mM) under standard assay conditions [7]; the washed filter membranes bearing the cells were transferred immediately to the tubes containing 2 ml cold water and shaken in a boiling water bath for 15 min to extract the sugars. The extract was centrifuged for 10 min at 14000 *g*, the supernatant was used for analyses of sugar phosphates. The activity of glucokinase (ATP:D-glucose-6-phosphotransferase, EC 2.7.1.2.) was measured by the method of Anderson and Kamel [11].

The radioactivity was counted in a liquid scintillation spectrometer Multi Mat (Intertechnique, France) in Bray's solution [12].

3. Results and discussion

Glucose was phosphorylated by cell-free extracts

Table 1
Specific activity of phosphorylation[a] and reactions for glucose in cell-free extracts of *A. laidlawii*[b].

N	Phosphate donor	Concentration (M)	Phosphorylation activity
1	–	–	0.040
2	PEP	4×10^{-2}	0.048
3	ATP	5×10^{-2}	0.336
4	–"–	1×10^{-2}	0.640
5	–"–	1×10^{-3}	0.480
6	–"–	1×10^{-4}	0.146
7	ATP + PEP	1×10^{-3} + 4×10^{-2}	0.490
8	ADP + PEP	1×10^{-3} + 4×10^{-2}	0.190

[a] Specific activity: nmoles phosphorylated glucose × min^{-1} × (mg extract protein)$^{-1}$.

[b] Reaction mixture as described in Materials and methods.

of *A. laidlawii* in the presence of ATP (table 1). ATP could not be replaced by PEP as phosphate donor. The activity shown by the combination of ADP and PEP is presumably the result of ATP formation from PEP and ADP.

Omission of NaF from the reaction mixture did not influence the amount of glucose phosphorylation when ATP was used as phosphate donor.

When extracts were exposed to glucose, the rate of phosphorylation increased linearly with time for 20 min and then remained relatively constant (fig. 1). Exposure of extracts to increasing concentrations of sugar resulted in saturation kinetics (fig. 2). The Michaelis constant (K_m) calculated from these experiments was about 1.3×10^{-4} M and V_{max}, about 5.5 μmoles per min per mg of protein. The apparent V_{max} for glucose uptake by whole cells is 0.5 [7], about 11 times lower than the V_{max} for glucose phosphorylation, suggesting a transport step between extracellular and intracellular glucose. Interestingly, the K_m's for both processes were similar (1.3×10^{-4} M and 4.8×10^{-4} M).

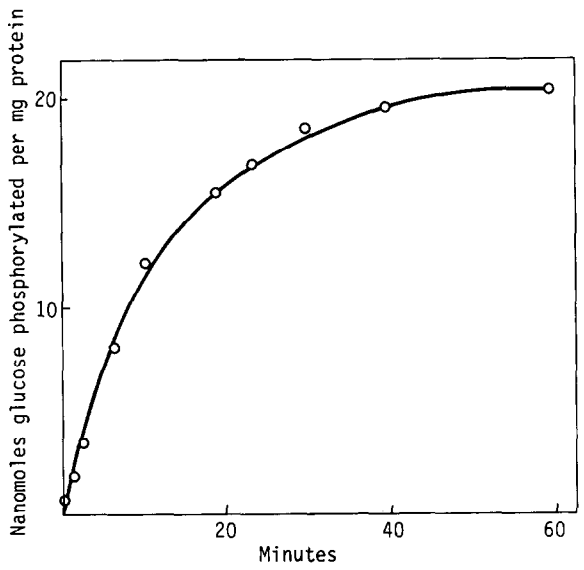

Fig. 1. Rate of glucose phosphorylation by cell-free extracts of *A. laidlawii*. Incubation mixture as described in Materials and methods.

Under these conditions other carbohydrates (fructose, α-methylglucoside, mannose, galactose and 2-deoxyglucose) were not converted into sugar phosphates by extracts when ATP or PEP were added

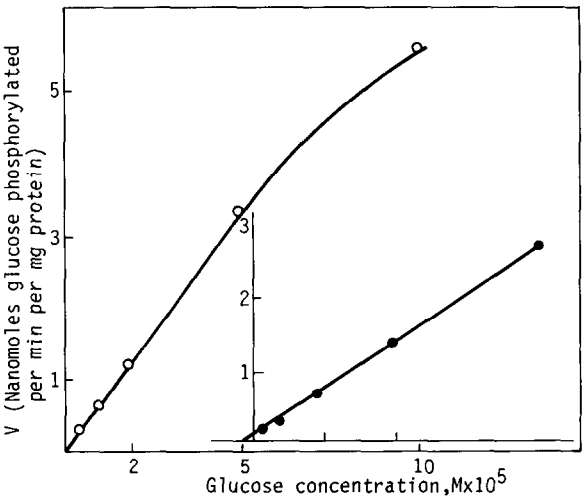

Fig. 2. Rate of glucose phosphorylation as a function of glucose concentration by cell-free extracts of *A. laidlawii*. Inset: Lineweaver – Burk plot.

Table 2
Accumulation[a] of sugar phosphates by *A. laidlawii* cells[b].

Incubation time (min)	Cells grown as glucose	Fructose
0	0.10 (0.22)[c]	0.12 (0.17)
10	0.40 (0.55)	0.50 (0.39)
30	0.56 (0.88)	0.60 (0.50)
60	0.80 (1.20)	0.76 (0.65)

[a] Accumulation in nmoles × (mg cell protein)$^{-1}$.
[b] Reaction mixture as described in Materials and methods.
[c] Determination of sugar phosphates by the Somogyi method are given in parentheses.

(using both methods of determining phosphorylation). None of these carbohydrates, at up to 100 times the concentration, inhibited glucose phosphorylation. Table 2 shows typical results on the sugar phosphates recovered from the cells previously incubated with glucose or fructose. The specific activity of glucokinase was the same in extracts from cells grown either on or without glucose (0.049 μmoles glucose-6-phosphate/min/mg protein). Other carbohydrates (2-deoxyglucose, fructose, galactose, α-methylglucoside) did not compete with glucose for glucokinase, thus demonstrating the high specificity of this enzyme.

Fructose was not phosphorylated by extracts from fructose-grown cells at the expense of ATP or PEP, but there was fructose phosphorylation in the cells (table 2). Fructokinase (ATP: D-fructose 6-phosphotransferase EC 2.7.1.4.) activity was not detected in extracts from fructose-grown cells. Therefore, the mechanism operating in fructose utilization (phosphorylation or oxidation followed by phosphorylation) remains unknown.

These experiments have shown that the PEP-PTS system could not be detected in cell-free extracts of *A. laidlawii* and, therefore, it appears to play no role in the transport and/or phosphorylation of carbohydrates by this organism. Our data contradict those of Romano [6] about the universality of this system in facultative anaerobes.

After this work was completed, Van Demark and Plackett reported that PEP-PTS could be detected in *Mycoplasma* Strain. Y. [13].

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