Non-vectorial phosphorylation by the bacterial PEP-dependent phosphotransferase system is an artifact of spheroplast and membrane vesicle preparation procedures

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These data demonstrate that the phenomenon of non-vectorial phosphorylation [Saier, M.H. jr and Schmidt, M.R. (1981) J. Bacteriol. 145, 391–397] is an experimental artifact which arises during the conversion of whole cells to spheroplasts or membrane vesicles. It can be explained by a certain population of particles which are not properly sealed. There is no need to envoke two orientations of enzyme II in the membrane, one of which would have no physiological significance.

Enzyme II orientation *Vectorial phosphorylation* *Hexose transport* *Sealed versus leaky particle* *Phosphotransferase, PEP-dependent* *Experimental artifact*

1. INTRODUCTION

The bacterial phosphoenolpyruvate-dependent hexose transport systems are responsible for the transport of a large number of hexoses and hexitols. The sugar is phosphorylated during the transport step (vectorial phosphorylation) and appears on the inside as the sugar phosphate. In [1] Saier and Schmidt reported finding sugar phosphate on the outside of membrane vesicles as well as on the inside. It was suggested that there were two populations of enzyme II in the cytoplasmic membrane [1]; one population, oriented inward, was responsible for the vectorial phosphorylation; another population, oriented outward, was incapable of transport but could still phosphorylate, thus producing external sugar phosphate. Another explanation of the results in [1], which Saier and Schmidt rejected, is that a given population of their vesicles were leaky so that, after the sugar phosphate was formed in a vectorial manner it would leak back out of the vesicles appearing as external sugar phosphate. Since the correct assignment of the orientation of a membrane-bound enzyme is a critical element in unraveling its physical mechanism, we have re-examined the matter of vectorial and non-vectorial phosphorylation.

These data show clearly that non-vectorial phosphorylation does not occur to any substantial extent in whole cells but becomes more and more prominent as the cells are processed to the spheroplast and membrane vesicle stage. The increase in the extent of non-vectorial phosphorylation parallels an increase in the rate of NADH oxidation. Since NADH is oxidized at the inner side of the cytoplasmic membrane, these data demonstrate that a fraction of the particles become leaky during preparation. Therefore, there is no need to envoke two different orientations of enzyme II in the membrane to explain the occurance of external sugar phosphate. The external sugar phosphate arises from sugar which is phosphorylated in a vectorial manner and then leaks back to the exterior.

2. EXPERIMENTAL

2.1. *Bacterial strains*

Two strains were used in these studies, *E. coli* ML 308-225 ($i^-z^-y^+a^+$) which is constitutive for β-galactoside transport and *S. typhimurium* PP 1133 (*pts M416, trp B223*) which possesses the PTS enzyme II^{Glc} for transporting glucose and αMGlc but is deficient in the constitutive PTS enzyme II^{Man}, previously designated as the low affinity PTS for αMGlc transport.

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2.2. *Culture conditions*

The cells were cultured on mineral medium [2] enriched with 0.1% yeast extract (Difco). The carbon source was 0.5% glucose. Cells and spheroplasts were prepared as outlined in the flowchart (fig.1). Membrane vesicles were prepared as in [3].

2.3. *Transport experiments*

Accumulation of αMG was measured as in [4]. A stirred suspension of cells, spheroplasts, or membrane vesicles was brought to 30°C in a waterbath. Reagents were added as described in the text and figure legends. To measure uptake 100 μl aliquots were withdrawn from the incubation mixture at the stated times and pipetted directly onto moistened cellulose-nitrate filters (0.45 μm) under vacuum. The filters were rinsed immediately with 2.2 ml of the same buffer used in the incubation mixture and put into scintillation vials containing 8 ml Packard Emulsifier Scintillator 299. The surface coating of the filter dissolved after ~30–60 min and were then counted. Whole cells and spheroplasts maintain a $\Delta\tilde{\mu}H^+$ by oxidation of endogenous substrates. It is known that the transport and phosphorylation of αMGlc are inhibited by a $\Delta\tilde{\mu}H^+$ [5–7]. Therefore, measurements with whole cells and spheroplasts were done in the presence of 10 μM CCCP to dissipate the $\Delta\tilde{\mu}H^+$. The final alcohol concentration in the incubation mixtures was kept below 1%.

2.4. *Measurements of α methyl glucose phosphate*

External αMGlc phosphate was analyzed by collecting a portion of the filtrate from an uptake measurement and loading it on 1 ml columns of Dowex AG1 × 2. The columns were washed with 10 ml water twice. The radioactive αMGlc phosphate was subsequently eluted into scintillation vials with 12 ml 0.1 N HCl, mixed with Packard emulsifier scintillator and counted.

Total αMGlc phosphate was measured by adding a 100 μl sample of the incubation mixture to 10 μl 20% (v/v) Triton X-100, in disposable test tubes. The mixture was vortexed for 10 s, diluted with 1 ml water and loaded on 1 ml columns of Dowex AG 1 × 2. The tubes were rinsed with 1 ml water twice more and the rinses were added to the same columns. The columns were processed as above. When toluene was used at a final conc. of 1–2% instead of Triton X-100 to permeabilize the membranes the same results were obtained.

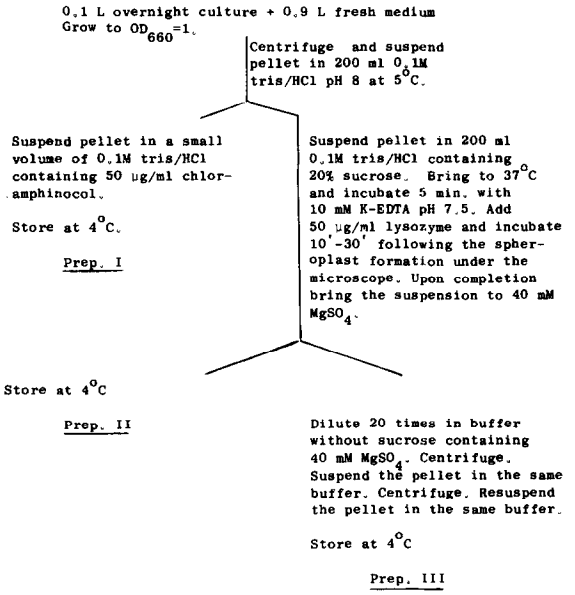

Fig.1. Flowchart for the preparation of cells and spheroplasts from either *E. coli* or *S. typhimurium*.

Internal αMGlc phosphate was calculated by subtracting total αMGlc-P from external αMGlc-P.

2.5. *Calculation of specific activities of uptake and phosphorylation*

Specific activities are given as nmol αMGlc transported or phosphorylated per min per mg of total protein in the assay mixture.

Protein concentration was determined as in [8].

NADH dehydrogenase activity was determined using dichlorophenolindophenol as the electron acceptor. The reactions were performed in Thurnberg cuvettes under a nitrogen atmosphere. Reaction mixtures (final vol. 1 ml) contained 50 mM potassium phosphate (pH 6.6), 10 mM magnesium sulfate, 0.1 mM dichlorophenolindophenol and the stated amount of membrane protein (see text). The reaction was started by addition of NADH (final conc. 0.1 mM). The extinction coefficient of oxidized dichlorophenolindophenol was taken to be 18.8 . $mmol^{-1}$. cm^{-1} at 600 nm [9].

α-[U-^{14}C]Methylglucopyranoside (279 mCi/mmol) was purchased from the Radiochemical

Center (Amersham). All other chemicals were of reagent grade.

3. RESULTS

3.1. *Comparison of external αMGlc-P with total αMGlc uptake in whole cells, spheroplasts and membrane vesicles.*

Fig.2 shows the level of αMGl accumulated in intact cells of *E. coli* ML 308-225 (prep. I, fig.1). High levels of αMGlc are accumulated*. Upon subtracting total αMGlc-P from external αMGlc-P we find that only 37% of the total accumulated sugar is phosphorylated. This agrees with the observation [10] that, after the αMGlc-P pool is partially dephosphorylated by a sugar phosphate phosphatase, an equilibrium is established between $\alpha MGlc_{in}$ and $\alpha MGlc\text{-}P_{in}$.

In contrast with the high levels of internal αMGlc the level of external αMGlc-P is very low in whole cell preparation. It never gets higher than ~5% of the total accumulated αMGlc. If we were to assume that the external αMGlc-P came from a population of leaky cells we could estimate what fraction of the total population were leaky by measuring the rate of external αMGlc-P formation before and after permeabilizing the intact cells with toluene. The results of such an experiment showed that after toluenization the rate of production of external αMGlc-P was 100-times as high as that in fig.2. Therefore the external αMGlc-P in fig.2 could have been produced if 1% of the cells were permeable.

When spheroplasts are prepared in and kept in 20% sucrose (prep. II, fig.1), their uptake and phosphorylation characteristics are somewhat different but not drastically different from whole cell preparations (fig.3A). The uptake activity is ~35% lower than that found in intact cells and ~70% of the internal αMGlc is phosphorylated. However, the levels of external αMGlc-P are elevated above those found in intact cells. Three times as much external αMGlc-P is found after 20 min in this

* We have employed low [αMGlc] (3–10 μM) here to compare these data with other studies involving changing the K_m of the carrier. (The K_m for αMGlc is 6 μM.) The plateaus reached in fig.2–4 are due to complete usage of αMGlc (transport and/or phosphorylation). When higher levels of αMGlc are used the production of external sugar-P is linear with time

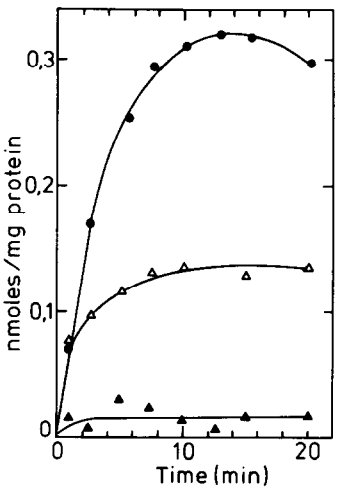

Fig.2. Plot of the accumulation of αMGlc and the production of total αMGlc-P and external αMGlc-P by intact cells of *E. coli* ML 308-225. The cells were diluted into Tris–HCl buffer (pH 8.0) and incubated at 30°C for 3 min. CCCP was added to final conc. 10 μM. After 1 min α-[^{14}C]MGlc was added to final conc. 3.5 μM. At the stated times 100 μl samples were removed and analyzed as stated in section 2. (•) αMGlc accumulation; (△) total αMGlc-P; (▲) external αMGlc-P.

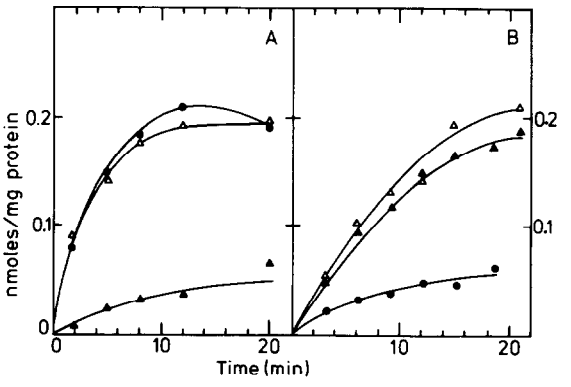

Fig.3. The accumulation of αMGlc and the production of total αMGlc-P and external αMGlc-P by spheroplasts of *E. coli* ML 308-225. (A) Spheroplasts prepared in and maintained in sucrose (prep.II, fig.1). Incubations were carried out at 30°C in 0.1 M Tris–HCl (pH 8.0) 20% (w/v) sucrose, 40 mM $MgSO_4$. After incubating 3 min at 30°C CCCP was added to 10 μM. After 1 min incubation α-[^{14}C]MGlc was added to final conc. 3.5 μM. (B) Spheroplasts in buffer (prep. III, fig.1). Incubations were carried out in 0.1 M Tris–HCl (pH 8) 40 mM $MgSO_4$. CCCP and αMGlc were added as stated in (A). (•) αMGlc accumulation; (△) total αMGlc-P; (▲) external αMGlc-P.

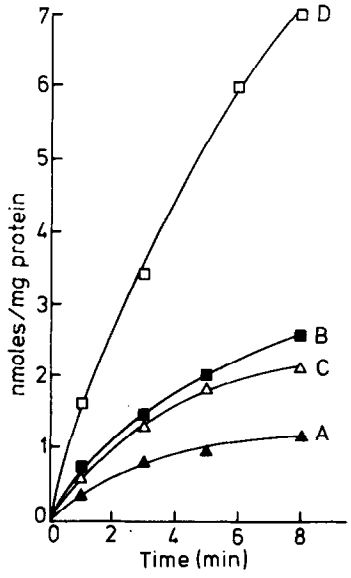

Fig.4. Accumulation of αMGlc in membrane vesicles of *E. coli* ML 308-225 and the production of αMGlc-P by these vesicles. The vesicles were prepared as in section 2. After preparation the vesicles were stored overnight at 5°C. Uptake and phosphorylation were measured the next day. As described in section 2 a single stock solution was used for the uptake and the corresponding total αMGlc-P determination (curves A,B or curves C,D). The solution for curves A,B contained 10 mM $MgSO_4$, 25 mM sodium phosphate (pH 6.6) membrane vesicles at final conc. 0.8 mg protein/ml and 10 μM αMGlc (spec. act. 184 mCi/mmol). The solution for curves C,D contained in addition to the above, 50 mM phosphoenolpyruvate (K^+ salt). All components except the sugar were mixed and incubated for 10 min at 30°C with stirring. αMGlc was added at $T = 0$ and incubation continued at 30°C. At the stated times aliquots were withdrawn and processed for uptake or total αMGlc-P as in section 2. Without added phosphoenolpyruvate (▲) αMGlc accumulated; (■) total αMGlc-P produced. With added phosphoenolpyruvate: (△) αMGlc accumulated; (□) total αMGlc-P produced.

Table 1

Preparation	NADH oxidation rate
I Intact cells	< 100 pmol . min^{-1} . mg $protein^{-1}$
II Spheroplasts in sucrose	1.98 nmol . min^{-1} . mg $protein^{-1}$
III Spheroplasts in buffer	5.53 nmol . min^{-1} . mg $protein^{-1}$

NADH oxidation was measured as the rate of production of reduced DCPIP as specified in section 2. The rates were corrected for a background rate in the absence of membrane of 1.33 nmol/min

preparation when compared with intact cells. Nevertheless the initial rate of production of external αMGlc-P is still only 5–10% of the initial rate of αMGlc accumulation.

When spheroplasts are prepared in 20% sucrose and subsequently washed several times in buffer with Mg^{2+} to remove the sucrose (prep. III, fig.1) the spheroplasts retain only 15–20% of the original uptake activity of whole cells (cf. fig.2, fig.3B). In addition, their external αMGlc-P levels have drastically increased. The rate of production of external αMGlc-P is 2–4-times as high as the rate of αMGlc accumulation.

Data similar to those in fig.2 and 3 for *E. coli* MI 308-225 preparations have also been obtained for *S. typhimurium* PP 1133 preparation (not shown).

Fig.4 shows that membrane vesicles give qualitatively the same data as found with washed spheroplasts. The total αMGlc-P production is higher than the levels of accumulation of αMGlc. Kaback has shown that freshly prepared vesicles contain a limited supply of endogenous precursors which can be converted to phosphoenolpyruvate and drive αMGlc accumulation [11]. Even under these sub-optimal conditions where the levels of αMGlc-P accumulation are low, there is still a significant excess of external αMGlc-P formed. When the vesicles are incubated with 50 mM phosphoenolpyruvate the levels of αMGlc accumulation increase but there is a disproportionately large increase in the level of external αMGlc-P.

3.2. *NADH dehydrogenase activity*

A straightforward explanation for the occurrence of external sugar phosphate is that a fraction of the spheroplasts or vesicles become leaky during the preparation procedures. Since NADH dehydrogenase is located at the inner side of the membrane [12] the rate of NADH oxidation can be used to test whether changes in permeability

have occurred. If all particles are impermeable no NADH oxidation should be detectable. However, an increase in permeability should be reflected in an increase in the rate of NADH oxidation. The NADH oxidation rate of intact cells and the two spheroplast preparations is given in table 1. There is clearly an increase in NADH oxidation which parallels the increase in levels of external αMGlc-P suggesting that at least some of the particles do become permeable during preparation.

4. DISCUSSION

We have shown that the nature of the handling is the critical factor in determining whether or not products of the PTS reaction will be found in the external medium. Freshly prepared intact cells which have been stored at 4°C produce very low levels of external αMGlc-P while accumulating αMGlc. However, any further processing leads to elevated levels of external sugar phosphates. Cells which have been stored at −20°C have high levels of external sugar phosphates comparable to those found with washed spheroplasts in buffer and with membrane vesicles (fig.3B,4). Substantial differences have even been found between spheroplast preparations. The gentlest method for preparing spheroplasts involves only lysozyme addition to EDTA-treated cells in 20% sucrose followed by addition of Mg^{2+} to stabilize the particles. The centrifuge steps, buffer changes and dilutions have been omitted. These spheroplasts show relatively limited increases in the external sugar phosphate levels over intact cells. However, when these particles are exchanged into buffers lacking sucrose or are converted to membrane vesicles by osmotic shock the resulting preparations produce much higher levels of external sugar phosphates. The most direct interpretation of these data is that such treatments lead to a given fraction of particles which are permeable to small molecules. Since most solute-transport systems do not involve a chemical change in the solute during transport this phenomenon would go undetected, because any molecule which has been transported and then diffused back into solution would not be distinguishable from one which was never transported. In the case of the phosphotransferase system, however, sugar is converted to sugar phosphate upon transport allowing us to detect this species if it shows up again outside the particles.

Saier and Schmidt [1] have reported observing the production of both external [^{14}C]mannitol-P and internal [^{14}C]mannitol-P using membrane vesicles where unlabeled mannitol-P was the phosphoryl group donor. The data is qualitatively the same as that shown above where phosphoenolpyruvate was the donor and αMGlc was the acceptor. However, these authors interpret the results differently. They suggest that the two processes are catalyzed by enzyme II oriented in two different directions in non-leaky vesicles: one population of enzyme II facing out and giving rise to the external sugar-P; another population of enzyme II facing in and giving rise to internal sugar-P. Our interpretation is that both processes are catalyzed by enzyme II oriented in one direction but that a small fraction of the vesicles or spheroplasts are leaky so that the internal sugar-P immediately returns to the exterior. This interpretation is supported by the data showing that the membranes producing more external sugar phosphate have higher rates of NADH oxidation. In [1], Saier and Schmidt claim that they could detect no NADH oxidation with their vesicles; however, they do not specify the detection method used nor the lower limit of detectability by their method. Another argument used to support their claim was that the non-vectorial process could also be observed in intact cells. We have shown that a small amount of external sugar-P is found with the intact cell preparation but that it can be attributed to 1% leaky cells. Saier and Schmidt [1] do not specify the levels of external sugar phosphate relative to accumulated sugar observed in their intact cell measurements.

Finally, the authors of [1] support their conclusion that the vectorial and non-vectorial processes are catalyzed by two different populations of enzyme II with different orientations by presenting data showing that the non-vectorial process is inhibited by the non-permeant SH reagent, PCMBS, while the vectorial process is only inhibited by lipophilic SH reagents. Obviously, however, when some of the particles are leaky, as we have shown, PCMBS can easily gain access to the interior, just as NADH does and can inhibit the enzyme II in those vesicles which are leaky and which give rise to external sugar P.

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