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THE INVOLVEMENT OF HIGH-ENERGY PHOSPHATE IN 2-DEOXY-D-GLUCOSE TRANSPORT IN *KLUYVEROMYCES MARXIANUS*

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Under aerobic conditions 2-deoxy-D-glucose was accumulated in *Kluyveromyces marxianus* mainly in a phosphorylated form. During sugar uptake both ATP, polyphosphate and orthophosphate levels decreased. Under anaerobic conditions considerably less sugar was taken up. The intracellular free sugar concentration did not exceed the medium concentration, whereas sugar phosphorylation leveled off at about 3 μmol/g yeast. In response to anaerobic 2-deoxy-D-glucose uptake only ATP and polyphosphate appeared to decrease. Within the experimental error sugar phosphorylation was counterbalanced by the polyphosphate decrease. Pulse labeling experiments revealed transport-associated phosphorylation under these anaerobic conditions. Further, kinetic studies on permeabilized cells showed that cytoplasmic ATP could not be the phosphoryl donor in this transport-associated phosphorylation. These results confirm and extend previous observations, indicating that polyphosphate plays a crucial role in 2-deoxy-D-glucose transport in *Kluyveromyces marxianus*.

Introduction

Previous studies have indicated that a phosphotransferase system is involved in the uptake of glucose and glucose derivatives in yeast [1–3]. Pulse-labeling experiments, designed to determine the temporal sequence of appearance of 2-deoxy-D-glucose in the intracellular free sugar and sugar-phosphate pools, respectively, revealed that in *Kluyveromyces marxianus* deoxyglucose first appears in the cell in the phosphorylated form [1]. Similar results have been presented by Meredith and Romano [2] and by Franzusoff and Cirillo [3] for *Saccharomyces cerevisiae*.

Based on circumstantial evidence a transport-associated phosphorylation has been proposed, with a peripherally localized polyphosphate fraction as phosphate donor [4]. Recently direct experimental evidence has been presented for the actual existence of a polyphosphate fraction, localized outside the plasma membrane of *Kluyveromyces marxianus* [5–7].

Other studies indicate the obligatory involvement of hexokinase in glucose phosphorylation in *Saccharomyces cerevisiae* [8,9] and thus support the suggestion of Franzusoff and Cirillo [3], that a plasma membrane-bound hexokinase could account for the experimentally established transport-associated phosphorylation.

To elucidate the transport system at the molecular level, it is essential to identify the ultimate phosphate donor in transport-associated phosphorylation. In the present study it will be shown that deoxyglucose uptake under aerobic conditions yielded a complex picture with respect to phosphate pool changes. Under anaerobic conditions, however, a functionally distinct polyphosphate fraction appeared to be involved in sugar phosphorylation.

Methods and Materials

Kluyveromyces marxianus (synonym: *Saccharomyces fragilis* CBS 397) was grown with glu-

cose as carbon source, as described before [10]. Transport studies were performed at 25°C on a 10% (wet weight/vol.) yeast suspension, buffered at pH 4.5 with 0.1 M Tris-maleate. Aerobic conditions were obtained by bubbling air through the suspension. Anaerobic incubations were carried out in a glass vessel, closed with a silicon stopper, under a constant stream of argon (containing less than 0.3 ppm oxygen). Addition were made from nitrogen-flushed solutions and samples were drawn with gastight Hamilton syringes through the narrow gas outlet.

Intracellular sugar concentrations were determined essentially as described before [1], utilizing ^{3}H-labeled deoxyglucose. Separation of phosphorylated and non-phosphorylated deoxyglucose derivatives in cellular extracts was done by Ba/Zn precipitation of sugar phosphates [11] and complete analysis of deoxyglucose derivatives in these extracts was performed by descending paper chromatography, as described in detail previously [1].

ATP was assayed by the method of Addanki et al. [12], utilizing Sigma FLE-50 firefly lantern extract. Phospho*enol*pyruvate was determined enzymatically, utilizing pyruvate kinase and lactate dehydrogenase. Orthophosphate and polyphosphate were measured as described before [7]. Chain length of cellular polyphosphates was determined by Sephadex gel filtration [7]. Hexokinase activity was measured on permeabilized cells [13].

Argon was obtained from AGA Gas BV and deoxyglucose (grade III) from Sigma. ^{3}H-labeled deoxyglucose was purchased from Amersham International.

Results

Transport of deoxyglucose under aerobic conditions

Uptake of deoxyglucose by *K. marxianus* appears to involve a system, in which the sugar is phosphorylated during transport [1]. Under aerobic conditions this yeast accumulated the sugar predominantly in the sugar-phosphate form (Fig. 1).

In studies, designed to identify the phosphoryl donor of the accumulated deoxyglucose phosphate, it appeared that both ATP, polyphosphate and orthophosphate decreased during sugar uptake (Fig. 2). ATP decreased rapidly to a low and

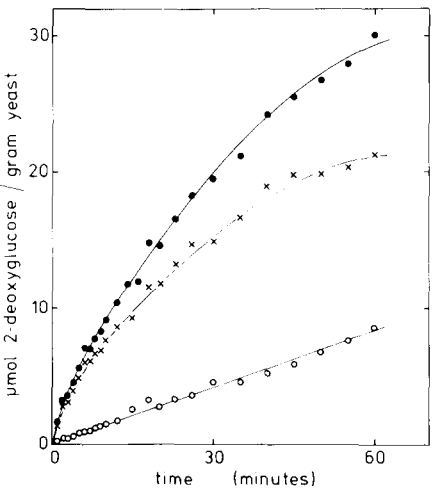

Fig. 1. 2-Deoxyglucose uptake by *K. marxianus* under aerobic conditions. Initial external deoxyglucose concentration: 10 mM. ●———●, total intracellular deoxyglucose; ×———×, deoxyglucose phosphate; ○———○, free deoxyglucose.

further constant level, whereas the other phosphate pools diminished more gradually. The decrease of polyphosphate during sugar uptake was caused by the disappearance of long-chain polyphosphate, as determined by sephadex gel filtration (data not shown).

Since these measurements under aerobic conditions did not allow identification of the primary phosphoryl donor, it was attempted to employ conditions, in which turnover between phosphate compounds would be blocked or diminished.

Transport of deoxyglucose under anaerobic conditions

Anaerobically, transport of deoxyglucose was greatly reduced as compared to aerobic uptake. Transport under anaerobic conditions was characterized by two phenomena: (a) a rapid intracellular appearance of deoxyglucose phosphate and (b) a much slower formation of a free, unphosphorylated deoxyglucose pool (Fig. 3). Paper chromatographic analysis of cellular extracts revealed that under these anaerobic conditions only deoxyglucose 6-phosphate and the free sugar were present inside the cells.

At high external deoxyglucose concentrations sugar phosphorylation leveled off at about 3

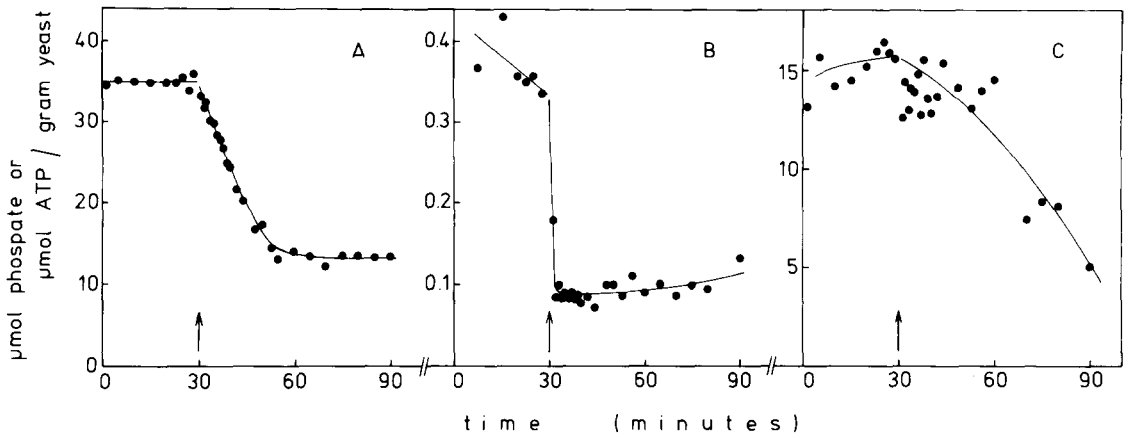

Fig. 2. Cellular contents of phosphate compounds during aerobic 2-deoxyglucose influx. Cells were incubated aerobically. After 30 min (arrow) 10 mM deoxyglucose was added. (A) P_i, (B) ATP and (C) polyphosphate (expressed in orthophosphate equivalents).

µmol/g yeast (Fig. 3). Apparently the phosphoryl donor can not be replenished under anaerobic conditions.

To identify the phosphate donor of this deoxyglucose phosphorylation under anaerobic conditions, ATP, phospho*enol*pyruvate, polyphosphate and orthophosphate concentrations were determined. Phospho*enol*pyruvate remained constant during deoxyglucose transport, at a level of 0.22–0.26 µmol/g yeast. The orthophosphate concentration increased slowly under anaerobic conditions (Fig. 4). This slow increase of the orthophosphate concentration was not influenced by deoxyglucose transport. The time-course of the polyphosphate and ATP levels during deoxyglucose uptake are shown in Figs. 5 and 6. ATP decreased about 0.1 µmol and polyphosphate about 1.6 µmol/g yeast. Within the experimental error these declines counterbalanced the accumulation of deoxyglucose phosphate (in this experiment about 2.4 µmol/g yeast).

In previous studies, under aerobic conditions, pulse-labeling experiments demonstrated unequivocally a transport-associated phosphorylation of

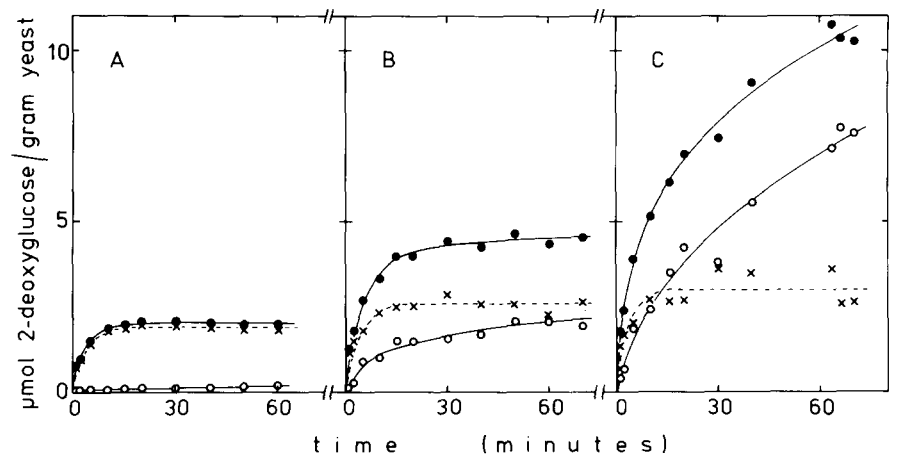

Fig. 3. 2-Deoxyglucose uptake by *K. marxianus* under anaerobic conditions. Initial extracellular deoxyglucose concentration: 1 mM (A), 10 mM (B) or 50 mM (C). ●———●, total intracellular deoxyglucose; ×------×, deoxyglucose phosphate; ○———○, free deoxyglucose.

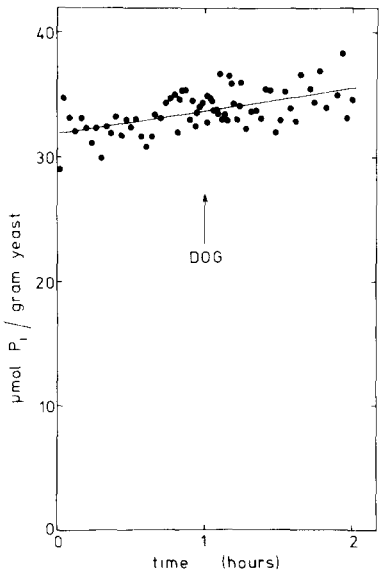

Fig. 4. Influence of anaerobic deoxyglucose (DOG) transport on the cellular orthophosphate content. Cells were incubated anaerobically. After 1 h 10 mM deoxyglucose was added. Identical results were obtained when instead of deoxyglucose an equivalent amount of water was added.

deoxyglucose in this yeast strain, followed by intracellular dephosphorylation of the sugar [1,14]. It seems a priori likely, that the same transport mechanism would be operative under anaerobic

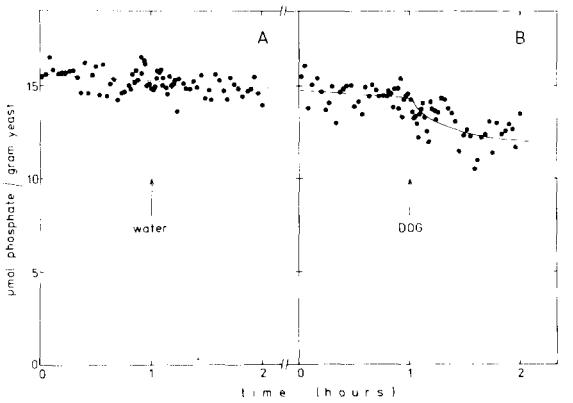

Fig. 5. Influence of anaerobic deoxyglucose (DOG) transport on the cellular polyphosphate content. Cells were incubated anaerobically. After 1 h 10 mM deoxyglucose (B) or, in the control, an equal amount of water (A) was added. Polyphosphate is expressed in orthophosphate equivalents. The dotted line is an extrapolation of the continuous line, drawn through the experimental points before deoxyglucose addition and calculated by means of the least-squares method.

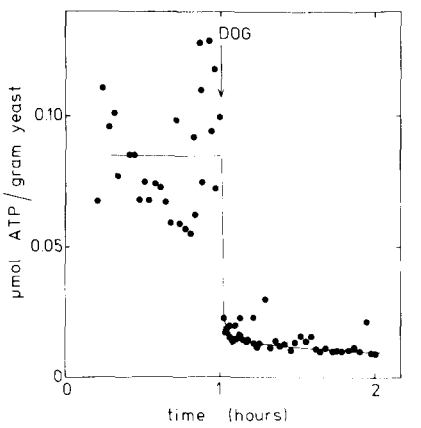

Fig. 6. Influence of anaerobic deoxyglucose (DOG) transport on the cellular ATP content. Cells were incubated anaerobically. After 1 h 10 mM deoxyglucose was added. Addition of water instead of a deoxyglucose solution did not result in an ATP decrease.

conditions. However, a complicating phenomenon under anaerobic conditions at high extracellular sugar concentrations is the continuing slow increase of the intracellular free sugar concentration (Fig. 3c) without concomitant transport-dependent

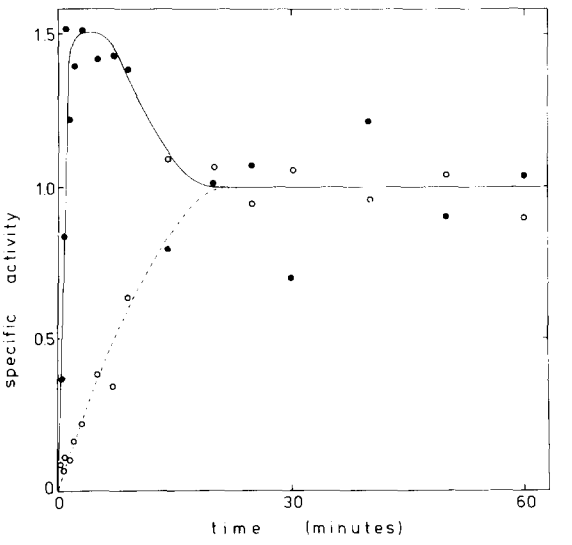

Fig. 7. Specific activity of intracellular deoxyglucose phosphate (●———●) and intracellular free deoxyglucose (○- - - - -○) after pulse labeling with deoxy[^{3}H]glucose, under anaerobic conditions. Cells were incubated with 50 mM deoxyglucose. After 48 min a trace amount carrier free deoxy[^{3}H]glucose was added.

TABLE I

HEXOKINASE ACTIVITY OF PERMEABILIZED *K. MARXIANUS* AT VARYING ATP AND DEOXYGLUCOSE CONCENTRATIONS

Deoxyglucose (mM)	ATP (mM)	Enzyme activity (μmol/g per min)
10	10	10.1
10	2	8.3
10	0.2	3.0
10	0.02	0.4
0.4	10	5.8
0.4	2	4.9
0.4	0.2	1.7
0.4	0.02	0.2

changes in the phosphate pools. This might reflect a shift of transport-associated phosphorylation to a non-phosphorylating transport mechanism, after depletion of the phosphate donor for deoxyglucose phosphorylation. Pulse-labeling experiments under these anaerobic conditions yielded the results, depicted in Fig. 7. In these experiments the yeast was incubated for 48 min with 50 mM unlabeled deoxyglucose, followed by addition of a trace amount of deoxy[^{3}H]glucose to the medium. The specific activity profiles, in particular the clear overshoot of the specific activity in the sugar-phosphate pool, demonstrate again transport-associated phosphorylation with subsequent intracellular dephosphorylation. It should be emphasized that although these results establish transport-associated phosphorylation as the predominant mechanism, they do not exclude the possibility of a coexisting minor transport component, proceeding via uptake of free sugar.

Finally the hexokinase activity of this yeast was studied in permeabilized cells. A V_m of 10.7 μmol/g yeast per min was found. The K_m with respect to deoxyglucose was 0.3 mM and with respect to MgATP 0.5 mM. Hexokinase-mediated deoxyglucose phosphorylation velocities at varying deoxyglucose and ATP concentrations are summarized in Table I.

Discussion

Experimental evidence indicates that a phosphotransferase system is involved in deoxyglucose transport in *K. marxianus*. This conclusion is mainly based on pulse-labeling experiments, described in detail previously [1,14]. Both under aerobic [1] and under anaerobic (Fig. 7) conditions, such pulse-labeling experiments show that the sugar enters the cell primarily as deoxyglucose phosphate. Transport-associated phosphorylation of deoxyglucose has also been demonstrated in *Saccharomyces cerevisiae* [2,3], suggesting a similar glucose transport system in this yeast species. To elucidate the mechanism of this transport system at the molecular level, it will be necessary to identify the ultimate phosphoryl donor in the transport-associated phosphotransferase step.

Under aerobic conditions this turned out to be impossible. Deoxyglucose phosphate accumulated to a high level, while simultaneously the levels of ATP, long-chain polyphosphate and orthophosphate decreased (Figs. 1 and 2). Since the amount of sugar phosphate formed during uptake exceeded each of the pools of high-energy phosphate compounds, it must be concluded that conversions between phosphate pools took place under these conditions. Thus, these results did not allow identification of a specific phosphoryl donor for deoxyglucose phosphorylation.

Under anaerobic conditions, however, the relationship between the various phosphate pools was more surveyable. The level of orthophosphate was not influenced by deoxyglucose-transport, indicating that no energy was available to convert orthophosphate into high-energy phosphates. The initial ATP level was very low under anaerobic conditions (about 0.1 μmol/g yeast, Fig. 6) and this could maximally account for 4% of the amount of deoxyglucose phosphate, accumulated in the cells. This means that deoxyglucose phosphorylation occurred essentially at the expense of polyphosphate, the only other phosphate pool that decreased during deoxyglucose uptake (Fig. 5).

Assuming phosphorylation via the hexokinase reaction, this would imply replenishment of ATP from polyphosphate. Although the enzyme ATP-polyphosphate phosphotransferase (EC 2.7.4.1), catalyzing the reaction: $ADP + P_n \rightleftarrows ATP + P_{n-1}$, has been detected in several microorganisms, including yeast, this enzyme activity has not yet been found in this yeast strain, utilizing standard methods [15]. Still assuming that this reaction plays

a crucial role in this context, it remains to be explained why only about 2 μmol polyphosphate/g yeast can be mobilized for ATP synthesis via this reaction, under anaerobic conditions. As the total cellular polyphosphate concentration amounts about 15 μmol/g yeast, this signifies a distinct compartmentation of polyphosphate, at least in a functional sense.

An apparent discrepancy concerns the velocity of transport-associated phosphorylation, as compared to the velocity of the hexokinase reaction. From the pulse-labeling experiments as depicted in Fig. 7 it can be calculated that deoxyglucose phosphorylation occurred at a velocity of about 6 μmol/g yeast per min. The cellular ATP content of 0.01 μmol/g yeast corresponds to a cytoplasmic concentration of about 0.02 mM. When saturation of the hexokinase is assumed with respect to deoxyglucose, the maximal phosphorylation capacity under pulse-labeling conditions would be about 0.4 μmol/g yeast per min, presuming that the reaction would rely on the cellular ATP pool (Table I). To explain transport-associated phosphorylaton still via this reaction, a rather extreme degree of compartmentation of ATP should be postulated. A very small compartment should then contain both the hexokinase and most of the cellular ATP.

Based on several lines of experimental evidence it has been suggested previously that polyphosphate, localized outside the plasma membrane, would be involved in the mechanism of transport-associated phosphorylation [4,16]. In recent experiments, designed to quantitate the amount of polyphosphate outside the plasma membrane of *K. marxianus*, an amount varying from 2.3 to 3 μmol/g yeast was found, utilizing different experimental approaches [5–7]. These values are in reasonable agreement with the maximal deoxyglucose phosphorylation under anaerobic conditions (Fig. 3) and the concomitant polyphosphate decrease (Fig. 5). It should be mentioned, however, that polyphosphate: hexose phosphotransferase activity has not yet been detected in this yeast strain, utilizing previously described methods [15].

In this context it is important that in recent investigations convincing evidence has been presented for a crucial role of hexokinase in the high affinity transport system for glucose and glucose derivatives in *Saccharomyces cerevisiae* [3,8,9]. The mentioned similarity between the glucose transport systems in this yeast species and in *K. marxianus* implies, that such a role of hexokinase is also conceivable in the latter. As discussed above, however, it is highly improbable that the hexokinase reaction would be able to account for the velocity of deoxyglucose phosphorylation, if this reaction would have to depend on the cellular ATP pool. The various observations can be reconciled by assuming that the transport system delivers both sugar and polyphosphate-derived high-energy phosphoryl groups directly to the kinase, rather than into a large cytoplasmic pool, in accordance with previous suggestions [16]. Such a mechanism would explain the compartmentation of both polyphosphate (with respect to ATP replenishment) and of ATP (with respect to the hexokinase reaction) in the sense as discussed above.

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