Biochimica et Biophysica Acta, 986 (1989) 290–294
Elsevier

BBAMEM 74654

Mechanism of transport-associated phosphorylation of 2-deoxy-D-glucose in the yeast *Kluyveromyces marxianus*: characterization of the phosphoryl source

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(Received 22 March 1989)
(Revised manuscript received 13 June 1989)

Key words: Polyphosphate utilization; 2-Deoxy-D-glucose transport; Sugar phosphorylation; ATP; (*K. marxianus*)

The mechanism of 2-deoxy-D-glucose transport via the glucose/fructose carrier in *Kluyveromyces marxianus* has been studied by comparing the concentrations and specific activities of polyphosphate, orthophosphate, ATP and sugar phosphate in cells, pulse-labeled with [^{32}P]orthophosphate. During aerobic 2-deoxyglucose uptake, phosphate accumulates in the sugar phosphate pool, whereas both the orthophosphate- and the polyphosphate pool diminish. Comparison of the specific activities suggests that ATP is the primary phosphoryl donor. ATP is recycled at the expense of orthophosphate. Polyphosphate appears to replenish the orthophosphate pool and therefore has, at least mainly, an indirect role in sugar phosphorylation. Reduction of the ATP concentration results in a decrease of the 2-deoxyglucose transport rate. The uptake velocity can be increased again by raising the ATP level. These results corroborate the conclusion that ATP donates the phosphoryl group to the sugar.

Introduction

In *K. marxianus* cells, cultured on a synthetic medium with glucose as a carbon source, two different glucose-transport systems can be recognized, i.e., the glucose/fructose carrier and the glucose/galactose-proton symporter. The latter system is only present in the late exponential phase of growth [1], whereas the glucose/fructose carrier represents a constitutive system.

Like in the yeast *Saccharomyces cerevisiae*, the glucose/fructose carrier has been described as a phosphotransferase system [2,3]. Both under aerobic and anaerobic conditions, pulse-labeling experiments have shown that the glucose derivative 2-deoxyglucose (2-dGlc) enters the cell in the phosphorylated form. Under anaerobic conditions, at high external 2-dGlc concentrations, 2.5 μmol 2-dGlc-6-phosphate is formed, apparently at the expense of the peripherally localized polyphosphate [3]. During aerobic 2-dGlc transport, 2-dGlc-6-phosphate accumulates to a high level, with a concomitant decrease in the amounts of polyphosphate, orthophosphate and ATP [3].

Recently, a new polyphosphate isolation method has been described for *Propionibacterium shermanii* [4]. This extraction method, combined with the separation of the polyphosphates by gel electrophoresis [4–7], is applicable to yeast cells [8]. Besides the polyphosphates, also ATP and sugar phosphate were extracted and could be separated by gel electrophoresis. Using this extraction and separation method, the mechanism of aerobic 2-dGlc transport, via the glucose/fructose carrier, was studied in *K. marxianus*, by monitoring the specific activities of the orthophosphate, polyphosphate, ATP and sugar phosphate fractions in cells, pulse-labeled with $^{32}P_i$. The results are presented in this paper.

Materials and Methods

The yeast *K. marxianus* (CBS 397) was grown on a synthetic medium with 2% glucose as a carbon source as described previously [9]. To prevent activation of the glucose/galactose symporter, the yeast was cultured for 13 h [1].

The phosphate pools were selectively labeled by incubating a 10% (wet w/v) yeast suspension in 0.1 M Tris/maleate (pH 4.5) and 2% ethanol with 5 μCi $^{32}P_i$

Abbreviations: 2-dGlc, 2-deoxyglucose; $^{32}P_i$, ^{32}P-labeled inorganic orthophosphate.

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per ml suspension, for 40 min, under aerobic conditions, at 20°C. Subsequently, the external label was removed by washing the cells with ice-cold water. The cells were resuspended in 0.1 M Tris/maleate (pH 4.5) to a 10% (wet wt/v) suspension. To this suspension, 10 mM 2-dGlc and 2-[2,6-^{3}H]dGlc were added and aerobic uptake was measured. Samples were taken before the addition of 2-dGlc, and at 1.5, 20 and 40 min after addition of the substrate. The samples were washed with ice-cold water and the cells were collected by centrifugation, followed by extraction of polyphosphate, orthophosphate, ATP and sugar phosphate from the cells by the method of Clark et al. [4], as described for yeast in Ref. 8. Alternatively, the samples were filtered, washed and cells were extracted with ethanol. The ethanol was diluted to 40% and subsequently centrifugated, to remove insoluble material. In ethanol, the polyphosphates were precipitated and could, therefore, not be determined.

The phosphate-containing compounds were separated by polyacrylamide gel-electrophoresis with an acrylamide to bisacrylamide ratio of 20 : 1, as described by Wood and coworkers [5–7]. When the gels (37 × 20 × 0.03 cm) were loaded with samples containing 40% ethanol, 1/3 volume of formamide was added to increase the density of the samples and prevent floating (sample load: 5–10 μl; max. 13000 cpm ^{32}P per lane). Electrophoresis was performed until the marker xylene cyanol FF had migrated 5 cm (20% polyacrylamide gels), 6.5 cm (15% polyacrylamide gels) or 10 cm (10% polyacrylamide gels). To obtain autoradiograms, the gels were packed in plastic foil and exposed to X-ray film at −70°C. All the gels contained 5.25 M urea (unless indicated otherwise), to prevent cracking of the gel during thawing. This made it possible to cut the slice, containing a definite phosphate component, from the gel, after localization of its position with the aid of the autoradiogram.

For determination of the specific activity of polyphosphate, the cells were extracted according to the method of Ref. 8. The polyphosphates were precipitated by adding 0.25 vol. of saturated $BaCl_2$ and 0.25 vol. 3.5 M sodium acetate/acetic acid (pH 4.5) and a subsequent incubation for 15 h at 4°C. The precipitate was collected by centrifugation for 30 min at 8400 × *g* and washed with 75% ethanol. The label in the polyphosphate precipitate was determined by scintillation counting, using Picofluor 30 scintillation liquid (Packard). The amount of polyphosphate was determined after acid hydrolysis to orthophosphate as described by Lohmann and Langen [10], by mixing 2 ml of sample with 1 ml 2.5% ammonium molybdate in 2 M HCl and 200 μl 2 mM 1-amino-2-naphthol-4-sulfonic acid. After 30 min, the absorbance was measured at 700 nm.

The specific activity of orthophosphate was determined in an extract prepared according to the method of Ref. 8. Orthophosphate was separated from the other phosphates on a 15% polyacrylamide gel. The gel slice containing orthophosphate was counted in scintillation liquid. The orthophosphate concentration was determined in the same way as polyphosphate, but without hydrolysis at 100°C [10].

The amount of ^{32}P label in sugar phosphate was determined after extraction of the cells with ethanol. The supernatant was loaded on a 15% polyacrylamide gel. The gel slice containing sugar phosphate was counted in scintillation liquid. The concentration of sugar phosphate in the ethanol fraction was determined as described by Augustin and Hofmann [11].

To determine the specific activity of ATP, the same ethanol extract was used. The ATP concentration in the extract was assayed by the method of Addanki et al. [12], using FLE-50 Sigma Firefly lantern extract. Subsequently, ATP was separated from the other components, such as ADP and AMP, on a 10% polyacrylamide gel, containing 7 M urea, and the amount of label in the ATP band was counted in scintillation liquid.

The relationship between the ATP concentration in the cells and the uptake rate of 2-dGlc was determined by incubating a 10% (wet wt/v) yeast suspension in 0.1 M Tris/maleate (pH 4.5) for 5 min with various concentrations of antimycin A, at 25°C, under aerobic conditions. Subsequently, 2-dGlc transport was measured by adding a tracer amount of labeled sugar to the suspension. After 5 s of incubation, 5 ml of ice-cold water was added to 0.1 ml sample and the cells were isolated by filtration on cellulose-nitrate filters (0.45 μm pore, Schleicher and Schuell). The filters were washed with 5 ml ice-cold water, dried and the radioactivity was determined by scintillation counting. The blank in each experiment was determined by a 2-dGlc uptake experiment on ice. For ATP measurements, yeast cells were extracted with ethanol and ATP was assayed as described above.

[α-^{32}P]ADP was formed in the reaction:

$$[\alpha\text{-}^{32}\text{P}]\text{ATP} + \text{glucose} \rightarrow \text{glucose 6-phosphate} + [\alpha\text{-}^{32}\text{P}]\text{ADP}$$

using hexokinase (Boehringer). [α-^{32}P]AMP was formed in the reaction:

$$[\alpha\text{-}^{32}\text{P}]\text{ATP} \rightarrow [\alpha\text{-}^{32}\text{P}]\text{AMP} + \text{P2}$$

using phosphodiesterase I type VII (Sigma).

2-[2,6-^{3}H]dGlc, [^{32}P]orthophosphate and [α-^{32}P]ATP were purchased from Amersham International.

Results

In *K. marxianus*, cultured for 13 h on glucose, 2-dGlc is transported into the cells via a system having the characteristics of a phosphotransferase [1]. To study this

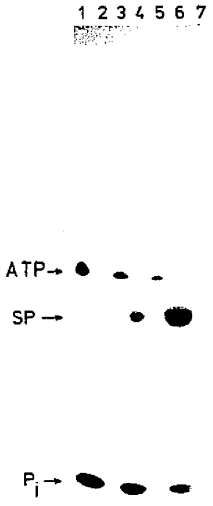

Fig. 1. Aerobic 2-dGlc uptake in cells, labeled for 40 min with $^{32}P_i$. The cells were extracted according to the method described by Clark et al. [4] in two subsequent steps. In the first step, cells were treated with TCA and acetone in order to isolate P_i and sugar phosphate. The resulting cell pellet was treated with EDTA and LiOH to extract P_n and ATP (see Ref. 8 for details). The components in the two fractions were separated on a 15% polyacrylamide gel, containing 5.25 M urea. Lanes 2, 4 and 6 are the TCA/acetone fractions of the extract, lanes 3, 5 and 7 are the EDTA/LiOH fractions. Lane 1: ATP; lanes 2 and 3: cells, before addition of 2-dGlc; lanes 4 and 5: cells, incubated for 1.5 min with 10 mM 2-dGlc; lanes 6 and 7: cells, incubated for 20 min with 10 mM 2-dGlc. SP, sugar phosphate.

transport mechanism, the cells were pulse-labeled with $^{32}P_i$ and used in an aerobic 2-dGlc uptake experiment. After extraction of the cells and subsequent separation of the phosphate pools by gel electrophoresis, the autoradiogram, shown in Fig. 1, gives the qualitative result of this experiment. This figure shows that, after the short period of labeling with $^{32}P_i$, no labeled polyphosphate is detected on the gel. After the addition of 2-dGlc, labeled sugar phosphate appears and the amount of label in orthophosphate and ATP diminishes.

To determine the role of ATP, polyphosphate and orthophosphate in sugar phosphorylation, the changes in their specific activities and concentrations were measured and compared to those of the sugar phosphate pool. Table I shows that at 1.5 min of incubation with 2-dGlc the sugar phosphate pool has the same specific activity as the orthophosphate pool before the addition of the sugar. As the specific activity of polyphosphate is much lower, these results suggest that polyphosphate does not directly donate the phosphate group to the sugar during transport-coupled phosphorylation. The most likely phosphoryl donor in this case will be ATP. However, the specific activity of ATP is about three times higher than the specific activity of orthophosphate. This indicates that all three phosphate groups of

TABLE I

Specific activities and concentrations of orthophosphate, polyphosphate, ATP and sugar phosphate during aerobic 2-dGlc uptake in K. marxianus

Cells were labeled with $^{32}P_i$ for 40 min. P_i, P_n, ATP and SP are: orthophosphate, polyphosphate, ATP and sugar phosphate, respectively. The specific activities are expressed in cpm/nmol, in case of polyphosphate in cpm/nmol orthophosphate units. The concentrations are expressed in μmol/g wet weight of yeast. The polyphosphate concentration is expressed in μmol orthophosphate units/g wet weight of yeast.

Incubation time with 1½ mM 2-dGlc (min)	Concentration				Specific activity			
	P_i	P_n	ATP	SP	P_i	P_n	ATP	SP
0	22.9	19.3	0.66	–	88.1	2.0	255	–
1.5	15.2	19.1	0.77	6.2	82.6	1.6	249	91.6
20	8.9	12.3	0.28	26.6	58.8	2.3	165	68.4
40	10.8	6.3	0.15	28.7	46.2	2.3	158	51.6

ATP must be labeled. This is confirmed by the following observations. Gel electrophoresis demonstrates that, during incubation with 2-dGlc, ATP is utilized and that, simultaneously, labeled AMP is formed (not shown). Further, as shown in Fig. 2, in ethanol extracts of cells pulse-labeled with $^{32}P_i$, ATP is converted to labeled AMP, after addition of hexokinase and glucose. This is probably due to the fact that ADP formed by the hexokinase reaction is transformed to AMP by adenylate kinase, which apparently has not been inactivated

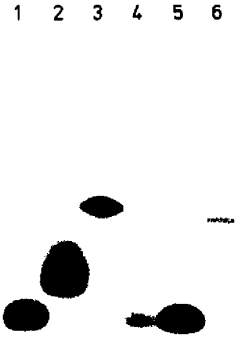

Fig. 2. Separation of ATP, ADP and AMP. Cells were labeled for 40 min with $^{32}P_i$ and extracted with ethanol. In this extract the labeled ATP was converted by addition of hexokinase and glucose. The components were separated on a 10% polyacrylamide gel, containing 7 M urea. Lanes 1 and 5: ATP; lane 2: ADP; lane 3: AMP; lane 4: ethanol extract of $^{32}P_i$-labeled cells; lane 6: ethanol extract, after addition of hexokinase and glucose.

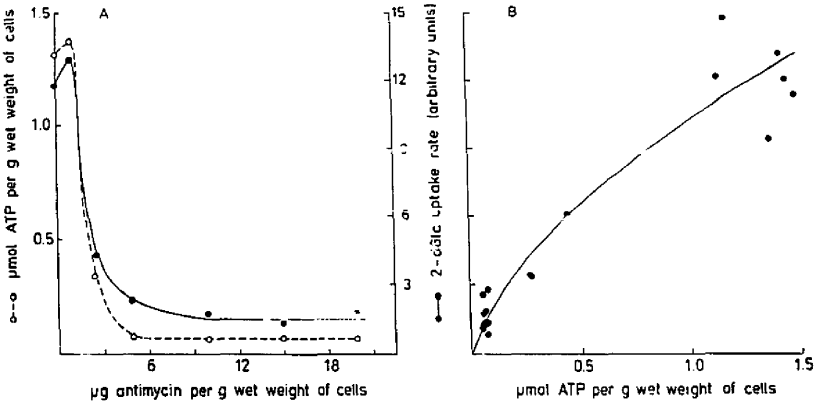

Fig. 3. (A) Relationship between the intracellular ATP concentration and the initial 2-dGlc uptake rate. Cells were incubated with different concentrations of antimycin A, for 5 min at 25°C. Subsequently, the transport velocity of a tracer amount of 2-dGlc and the ATP level were determined. (B) The initial uptake rate of 2-dGlc plotted against the cellular ATP concentration.

irreversibly in the ethanol extract. The presence of adenylate kinase in the assay mixture was confirmed by the fact that incubation of α-[^{32}P]ADP in the presence of an ethanol extract of yeast yielded ^{32}P-labeled ATP and AMP.

When ATP is the phosphoryl donor in transport-coupled phosphorylation of sugar, a correlation between the cellular ATP concentration and the 2-dGlc transport rate should be expected, similar to the results obtained with the yeast *S. cerevisiae* [13]. Fig. 3A shows that titrating yeast cells with antimycin A results in a gradual reduction of the ATP concentration, whereas the 2-dGlc transport rate changes in parallel with the ATP levels. When the ATP concentration is plotted against the 2-dGlc uptake rate, as shown in Fig. 3B, this clearly indicates a role of ATP in 2-dGlc transport.

In cells, treated with antimycin A, the oxygen consumption could be restored by incubating the cells with tetramethyl-*p*-phenylenediamine and ascorbate [14]. Table II shows that under these conditions both the ATP level and the 2-dGlc influx were increased. These data, again, indicate that ATP is involved in regulation of 2-dGlc transport.

TABLE II

Influence of tetramethyl-p-phenylenediamine/ascorbate on cellular ATP levels and 2-deoxyglucose influx

Antimycin-treated cells (20 μg/g wet weight of yeast) were incubated for 1 min with 12.5 mM tetramethyl-*p*-phenylenediamine (TMPD) and 25 mM ascorbate at 25°C. ATP levels and 2-dGlc were determined as described in Materials and Methods. In order not to influence cellular energetics, sugar transport was measured using tracer concentrations of 2-dGlc. Control: cells before addition of TMPD/ascorbate. ATP levels are expressed in μmol per g yeast, and 2-dGlc influx in arbitrary units.

Condition	ATP	2-dGlc influx
Control	0.045	2.6
TMPD/ascorbate	0.40	8.1

Discussion

In this paper the mechanism of 2-dGlc transport via the glucose/fructose carrier has been studied. Determination of the concentrations of orthophosphate, polyphosphate, ATP and sugar phosphate during 2-dGlc uptake shows that the increase of phosphoryl units in the 2-dGlc-6-phosphate pool is balanced by a decrease in the ATP-, orthophosphate- and the polyphosphate pool. Utilization of orthophosphate must occur via synthesis of ATP out of ADP and orthophosphate. The importance of this ATP synthesis for transport-coupled phosphorylation is illustrated by the reduction of the 2-dGlc transport rate due to a decrease of the ATP concentration (Fig. 3) and increase of the 2-dGlc uptake coupled to a rise in the ATP level. For the utilization of polyphosphates, three different pathways were described (for reviews, see Refs. 15–17).

(i) Direct phosphorylation of the sugar by polyphosphate, catalyzed by the enzyme polyphosphate-glucose phosphotransferase. However, this enzyme has not been found in yeast [15,17].

(ii) Conversion of polyphosphate to ATP by phosphorylation of ADP, catalyzed by the enzyme polyphosphate kinase [18,19], followed by sugar phosphorylation by ATP.

(iii) Breakdown of polyphosphate to orthophosphate. Subsequently, this orthophosphate may be used for sugar phosphorylation via ATP. Although the first two mechanisms cannot be excluded completely, the most likely mechanism is the latter. The reduction of the specific activity of the orthophosphate pool during the 2-dGlc uptake is apparently caused by replenishment of

orthophosphate by the much lower labeled polyphosphate (Table I). The specific activity of sugar phosphate, in turn, follows the specific activity of the orthophosphate pool, in accordance with the proposed mechanism. Since sugar phosphate is at the end of the chain of phosphorylations it will accumulate ^{32}P from ATP and P_i. This explains why in Table I the specific activity of sugar phosphate, at 20 and 40 min of incubation, is slightly higher than that of P_i. The specific activity of polyphosphate remains almost constant. Assuming that during pulse-labeling of cells with ^{32}P, polyphosphate is only labeled at the end groups, this suggests that polyphosphate breakdown is not random but rather chain after chain.

Summarizing, it can be concluded that in the yeast *K. marxianus*, sugar phosphorylation mainly proceeds with ATP as the phosphoryl donor. Polyphosphate is utilized in order to replenish the orthophosphate pool rather than being used directly as phosphoryl donor.

Finally, Table I shows that the specific activity of ATP is 3 times higher than the specific activity of the orthophosphate pool. This suggests that all the phosphate groups of ATP are labeled. Fast and high labeling of ATP with ^{32}P was found before in yeast cells [20] and in human erythrocytes [21], although a specific activity of ATP 3 times as high as the orthophosphate specific activity was not reported. The actual labeling of all three ATP phosphate groups in this yeast is confirmed by the presence of labeled AMP in the cells and the possibility to convert labeled ATP to labeled AMP in vitro.

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