Decrease in glycolytic flux in *Saccharomyces cerevisiae cdc35-1* cells at restrictive temperature correlates with a decrease in glucose transport

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The glycolytic flux was investigated in the thermosensitive *Saccharomyces cerevisiae* adenylate cyclase mutant *cdc35-1*. Directly after a shift to restrictive temperature, the specific CO₂ production rate increased from about 250 nmol min⁻¹ (mg protein)⁻¹ to more than 400 nmol min⁻¹ (mg protein)⁻¹, but then the CO₂ production gradually fell to about 70 nmol min⁻¹ (mg protein)⁻¹ after 5 h. O₂ consumption at restrictive temperature continued at more or less the same rate as at permissive temperature. The temperature shift in the mutant resulted in an increase in the estimated intracellular cAMP concentration from about 1.1 µM to 1.8 µM. This indicates that high cAMP levels are not sufficient for cell cycle progression and high glycolytic activity. The decrease in glycolytic activity at restrictive temperature was not paralleled by a similar decrease in the specific activity of any of the glycolytic enzymes, but correlated with a decrease in hexose transport. A drop in intracellular concentrations of the early metabolites of glycolysis further indicated a defect in transport at restrictive temperature. Our data suggest that glucose transport has a high control on glycolytic flux.

Keywords: glycolytic flux, glucose transport, *Saccharomyces cerevisiae*, yeast, cell cycle mutant

INTRODUCTION

The RAS/adenylate cyclase pathway in *Saccharomyces cerevisiae* plays a pivotal role in the control of cell growth and cell division (reviewed by Thorner, 1982; Matsumoto *et al.*, 1985; Tatchell, 1986; Broach, 1991). Components of the pathway which directly or indirectly regulate the adenylate cyclase activity include RAS, CDC25, IRA and CAP proteins (Toda *et al.*, 1985; Camonis *et al.*, 1986; Broek *et al.*, 1987; Tanaka *et al.*, 1989, 1990; Field *et al.*, 1990a; Fedor-Chaiken *et al.*, 1990). The gene for adenylate cyclase, *CYR1*, was found to be allelic to the earlier described *CDC35* gene (Casperson *et al.*, 1985; Kataoka

et al., 1985; Boutelet *et al.*, 1985). The original *cdc35-1* mutant was isolated as a thermosensitive cell division cycle mutant which, at restrictive temperature, arrested at the START point in the cell cycle (Hartwell, 1974; Reed, 1980). The mutant *cdc35*, together with *cdc25*, *cdc19* and *cdc33*, belongs to a group of *cdc* mutants which display class II START arrest. When class II START mutants are shifted to restrictive temperature, growth is inhibited and cells arrest as unbudded cells in the G1 phase (Reed, 1980). In contrast, class I START mutants (e.g. *cdc28*) continue to grow and some can form characteristic projections called 'shmoos' when they arrest at START at restrictive temperature (Reed, 1980).

It has been shown previously that the growth defect in START II mutants is correlated with a marked decrease in glycolytic flux (Oehlen *et al.*, 1993). In the present report, the mechanism of the decrease in glycolytic flux in *cdc35-1* was investigated in more detail. In *cdc35-1* cells the intracellular cAMP level rises after a shift to restrictive temperature. Since the glycolytic flux drops markedly in this mutant and cells arrest under restrictive conditions,

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Abbreviations: RQ, respiratory quotient; 3-PGA, 3-phosphoglycerate; 2,3-PGA, 2,3-bisphosphoglycerate; 2doG, 2-deoxyglucose.

this further indicates that cAMP levels *per se* are not a prime factor controlling glycolytic flux and that high levels of cAMP are not sufficient for cell cycle progression. The fall in glycolytic flux in *cdc35-1* cells correlates with a decrease in hexose transport. Our data suggest that hexose transport has a high control on glycolytic flux.

METHODS

Yeast strains and growth conditions. The haploid strains A364A (*MATa ade1 ade2 ura1 his7 lys2 tyr1 gal1*) and BR214-4a (*MATa cdc35-1 ade1 his7 trp1 ura1 arg*) of *Saccharomyces cerevisiae* were obtained from the Yeast Genetic Stock Center (Berkeley, CA, USA). The medium and growth conditions were as described previously (Oehlen *et al.*, 1993).

Determination of glycolytic flux and O₂ consumption. Gas production in the cultures was measured at the growth temperature in Warburg manometers using the direct method as described by Novak & Mitchison (1986). Manometer flasks (volume 15 ml) were filled with 3 ml culture and gas production was read every 15 min. After each reading, the manometers were opened, adjusted to zero and closed again. While most of the CO₂ produced by the cells is measured using this technique, CO₂ produced in the mitochondria as a result of tricarboxylic acid cycle activity is not detected. When glucose is fully oxidized in the mitochondria, using O₂ as the terminal electron acceptor, CO₂ production and O₂ consumption occur in equimolar amounts ($C_6H_{12}O_6 + 6O_2 \rightarrow 6CO_2 + 6H_2O$). Since the mitochondrially produced CO₂ thus equals O₂ consumption, specific total CO₂ production rates were calculated by adding the O₂ consumption rates to the gas production measured by direct Warburg techniques. The validity of this method has been shown by Lagunas (1979).

Respiratory activity was measured at the growth temperature in 1.4 ml Oxygraph vessels supplied with a Clark electrode. Fresh samples from cultures grown at permissive and restrictive temperature were taken every 15 min and oxygen consumption was recorded continuously. The respiratory quotient (RQ) was calculated by dividing the specific CO₂ production rates by the specific O₂ consumption rates (Lagunas, 1979). Specific CO₂ production and O₂ consumption rates were expressed in nmol min⁻¹ (mg total cellular protein)⁻¹. The cellular protein content was calculated from the optical density of the cultures, based on previous determinations that an OD₆₀₀ of 10 corresponds to 1 mg total cellular protein ml⁻¹. Alcohol concentrations were determined as described previously (Oehlen *et al.*, 1993).

Preparation of cell-free extracts and enzyme assays. The preparation of cell extracts by glass bead disruption was as described by De Koning *et al.* (1991). Enzyme activities were determined spectrophotometrically at 340 nm and 30 °C. All enzyme assays were performed in 50 mM Pipes buffer at pH 7.0 following protocols described previously (Oehlen *et al.*, 1993). Analyses were performed on a Cobas Bio automatic analyzer (Roche). Enzyme activities are expressed in U (mg protein)⁻¹ in the extract, where one unit is equivalent to the conversion of one μmol substrate min⁻¹. Protein was determined by means of the bicinchoninic acid reagent as described by the supplier (Sigma), using bovine serum albumin as a standard.

Determination of intermediates. For the determination of glycolytic intermediates, effectors of glycolysis and other metabolites, we used the extraction method described by De Koning & Van Dam (1992). In brief, samples (containing about 5 mg total cellular protein) were filtered on cellulose acetate

filters (diameter 48 mm, 1.2 μm pore-size, Schleicher and Schuell, Dassel, FRG) and quickly immersed in 3 ml methanol kept at -40 °C. The cells were resuspended and the filters removed. After addition of 5 ml cold (-40 °C) chloroform, the cells were stored at -80 °C until extraction. Cells were extracted with 2.2 ml MOPS buffer (4 mM, pH 7) at -35 °C by vigorous shaking for 1 h. The extracts were concentrated by means of a Speedvac vacuum concentrator (Savant Instr. Inc., Hicksville, NY, USA) to a final volume of about 500 μl.

Unless stated otherwise, the concentrations of the various metabolites were determined spectrophotometrically in 50 mM triethanolamine/HCl (TEA) buffer at pH 7.6. Measurements were done at 37 °C on a Cobas Bio automatic analyser, allowing handling of 24 samples simultaneously.

Determinations of glucose 6-phosphate, fructose 6-phosphate, fructose 1,6-bisphosphate, dihydroxyacetone phosphate, 3-phosphoglycerate (3-PGA), 2-phosphoglycerate, phosphoenolpyruvate, pyruvate, citrate, ATP, NADH, NAD⁺, ADP and AMP were essentially as described by Bergmeyer (1974).

Fructose 2,6-bisphosphate concentrations were determined by means of the kinetic assay described by Van Schaftingen & Hers (1983).

For the determination of 2,3-bisphosphoglycerate (2,3-PGA), a kinetic assay was used based on the ability of 2,3-PGA to stimulate 3-phosphoglycerate mutase. The assay mixture for this assay was composed as follows: 0.15 mM NADH, 0.6 mM ADP, 1 mM MgSO₄, 5 mM KCl, 2.5 mM 3-PGA, 5.5 U lactate dehydrogenase ml⁻¹, 0.8 U enolase ml⁻¹, 2 U pyruvate kinase ml⁻¹, 50 mM TEA buffered at pH 7.6. The conversion of 3-PGA (and NADH) was started by addition of 0.1 U 3-phosphoglycerate mutase ml⁻¹ and recorded at 340 nm on a Cobas Bio spectrophotometer. The degree of stimulation of this reaction by 2,3-PGA was measured and concentrations were calculated from a calibration curve in the range between 0 and 2 μM.

cAMP concentrations in the extracts were determined according to Tovey *et al.* (1974). This cAMP test is based on the competition of cAMP in the extract with [³H]cAMP for the regulatory subunit of bovine cAMP-dependent protein kinase.

For protein determination 10 ml 1 M NaOH was added to dried extracted cell material. To hydrolyse all proteins, this suspension was boiled for 15 min and subsequently cell debris were spun down. The protein content in the supernatant was determined by the Folin-Ciocalteu staining method, using bovine serum albumin as a standard. Cellular concentrations of the various metabolites were calculated as described previously (Oehlen *et al.*, 1993).

Hexose uptake measurements. Uptake experiments of radiolabelled sugars were performed essentially as described by Bisson & Fraenkel (1983a). Although we have recently reported an improvement on this method of hexose uptake (Walsh *et al.*, 1994), the results obtained with this 'classical' method are comparable under the conditions of the present experiments. Cells from *cdc35-1* cultures were collected by filtration on cellulose acetate filters (diameter 48 mm, 1.2 μm pore-size, Schleicher and Schuell) and washed with ice-cold potassium phosphate buffer (100 mM, pH 6.5). Cells were resuspended in phosphate buffer at 4 °C to a final concentration of 50 mg wet weight ml⁻¹ and kept on ice until they were used for uptake measurements.

For these measurements 50 μl of a suspension (preincubated aerobically at 30 °C for 5 min) was added to 12.5 μl solution of radiolabelled sugar. After 5 to 40 s of incubation at 30 °C,

Fig. 1. CO₂ production and O₂ consumption in *cdc35-1* cells after switching to restrictive temperature. *cdc35-1* cells were grown to exponential phase and then a portion of the culture was kept at permissive temperature (23 °C, ●) and one portion switched to restrictive temperature (36 °C, ○) at *t* = 0. Data are representative for three independent experiments. (a) Percentage of budding cells was determined by microscopic scoring of at least 100 cells. (b) Specific CO₂ production rates were determined by manometric techniques and are given in nmol min⁻¹ (mg protein)⁻¹. (c) Specific O₂ consumption rates in nmol min⁻¹ (mg protein)⁻¹ were determined with a Clark electrode. (d) Respiratory quotient = CO₂ production rate (data from panel b) divided by O₂ consumption rate (data from panel c).

uptake was terminated by adding 50 µl of the mixture of cells and substrate to 10 ml ice-cold phosphate buffer. The cells were immediately collected on glass-fibre filters (Whatman GF/F) and washed twice with 10 ml ice-cold phosphate buffer to remove external radiolabel. Radioactivity on filters was quantified by liquid scintillation counting. The blank in each experiment had labelled substrate and cells added separately to the cold buffer. These samples were further processed as described above.

Uptake of 2-deoxy-[³H]glucose (2doG) was determined at concentrations of 2 and 50 mM. Specific activity of the label was 0.5 µCi µmol⁻¹ (1 Ci = 37 GBq). At 2 mM of 2doG, uptake was linear in time for at least 30 s, whereas at 50 mM uptake quickly levelled off after about 5 s (data not shown). In order to obtain optimal incorporation we therefore used incubations of 30 s at the low 2doG concentrations and 5 s at the high concentration. Measurements for glucose and 6-deoxyglucose transport were as described for 2-doG with a similar specific activity of the radiolabelled sugar.

Transport activities were expressed in nmol min⁻¹ (mg total cellular protein)⁻¹. The cellular protein was determined by hydrolysing quantities of the concentrated cell suspensions in 1 M NaOH. Samples were boiled for 15 min and subsequently cell debris were spun down. The protein content in the supernatant was determined according to Lowry's method, using bovine serum albumin as a standard.

RESULTS

Glycolytic activity decreases in blocked *cdc35-1* cells

When exponential-phase BR214-4a cultures were transferred from permissive to restrictive temperature, cells stopped dividing and accumulated as unbudded cells (Fig. 1a). As expected, cells incubated at restrictive temperature for 6 h were arrested in G1 with unreplicated DNA (as determined by flow cytometry) and were approximately 40% bigger than cells under permissive conditions [cell size was determined as described previously (Van Doorn *et al.*, 1988); data not shown].

Metabolic fluxes for *cdc35-1* mutant cells were followed by

determining specific gas production rates (CO₂ production), specific O₂ consumption rates and alcohol concentration in the cultures. Specific CO₂ production rate at 23 °C was about 270 nmol min⁻¹ (mg protein)⁻¹, which is comparable to the CO₂ production in wild-type cells under these conditions (Oehlen *et al.*, 1993), and specific O₂ consumption rates about 20 nmol min⁻¹ (mg protein)⁻¹ throughout the duration of the experiment (Fig. 1b, c). The respiratory quotient was 13.5, which agrees with data reported by, for example, Beck & Von Meyenburg (1968) or Lagunas (1979) for wild-type cells grown in batch culture with excess glucose. When the *cdc35-1* cells were switched to 36 °C, the specific CO₂ production rate immediately increased, an effect which had been found previously for wild-type cells (Oehlen *et al.*, 1993). In contrast to wild-type cells, however, the CO₂ production in *cdc35-1* cells gradually fell from more than 400 nmol min⁻¹ (mg protein)⁻¹ just after the temperature-shift, to a level of about 70 nmol min⁻¹ (mg protein)⁻¹ after 6 h at restrictive temperature (Fig. 1b). As shown in Fig. 1c, specific O₂ consumption rates in *cdc35-1* cells were not affected in a similar way by the switch to restrictive temperature. With the exception of a short burst in O₂ consumption just after the shift to restrictive conditions, both at 23 °C and at 36 °C the specific O₂ consumption rates were about 20 nmol min⁻¹ (mg protein)⁻¹. As a result of the change in CO₂ production rates and the constant O₂ consumption rates, the RQ fell from 13.5 to about 3.5 after 5 h at 36 °C (Fig. 1d). As an alternative way of measuring glycolytic activity, ethanol concentrations were measured in cultures kept at permissive temperature and cultures switched to restrictive conditions. When cultures with a starting concentration of ethanol of about 6 mM were kept at permissive temperature, the ethanol concentration increased exponentially, as expected, and reached a level of 19 mM after 5.75 h of further incubation. When the same culture was shifted from 23 °C to the restrictive temperature of 36 °C, ethanol concentrations initially increased faster than in the culture at permissive temperature, but then, after

Fig. 2. Enzyme specific activities. The specific activities of selected glycolytic enzymes and fructose-1,6-bisphosphatase were determined for *S. cerevisiae cdc35-1* cells kept at permissive temperature (●) or switched to restrictive temperature (○) at $t = 0$. Data points represent the mean of two experiments. (a) Hexokinase, (b) phosphofructo-1-kinase, (c) glyceraldehyde-3-phosphate dehydrogenase, (d) pyruvate kinase, (e) pyruvate decarboxylase, (f) fructose-1,6-bisphosphatase. Note that fructose-1,6-bisphosphatase activities are multiplied by a factor of 1000 and are in the order of magnitude of $\text{mU (mg protein)}^{-1}$.

about 3 h at restrictive temperature, dropped below the concentrations measured for the culture at permissive temperature to reach a level of 10 mM after 5.75 h at restrictive temperature. This pattern of ethanol production was in qualitative and quantitative agreement with the observed changes in specific CO_2 production rates.

The decrease in glycolytic activity is not correlated with a similar change in the specific activity of glycolytic enzymes

To assess whether the decrease in glycolytic flux was correlated with or caused by a decrease in the activity of one of the glycolytic enzymes, the specific activity of various enzymes of the glycolytic pathway was measured. The specific activity of most of these enzymes was unaffected by the change in growth temperature and the largest effects observed were decreases of 20 to 40 % in the specific activity of glyceraldehyde-3-phosphate dehydrogenase, pyruvate kinase and pyruvate decarboxylase (Fig. 2). Virtually constant specific activities were measured at 23 °C and 36 °C for hexokinase and phosphofructokinase (Fig. 2) and glucose-6-phosphate isomerase [average activity about 4 U (mg protein)⁻¹], aldolase [3–4 U (mg protein)⁻¹], 3-phosphoglycerate kinase [10 U (mg protein)⁻¹], phosphoglycerate mutase [18 U (mg protein)⁻¹], enolase [4 U (mg protein)⁻¹] and alcohol dehydrogenase [6 U (mg protein)⁻¹] (data not

shown). As found previously for two *cdc25* mutants shifted to restrictive conditions (Oehlen *et al.*, 1993), none of the glycolytic enzymes of *cdc35-1* mutants showed a decrease which correlated with the prominent decrease in *in vivo* glycolytic flux.

The specific activity of the gluconeogenic enzyme fructose-1,6-bisphosphatase increased from about 2 mU (mg protein)⁻¹ at 23 °C to about 10 mU (mg protein)⁻¹ after 5 h at restrictive temperature (Fig. 2). Throughout the duration of the experiment, however, the specific activity of FBPase remained several orders of magnitude lower than the activities of glycolytic enzymes.

Levels of metabolites in blocked *cdc35-1* cells

In order to obtain further information about the molecular mechanisms underlying the observed decrease in glycolytic flux at restrictive temperature, the levels of several metabolites in *cdc35-1* cells at 23 °C and 36 °C were determined. The estimated cellular concentrations of most intermediates of glycolysis decreased after shifting *cdc35-1* cells from permissive to restrictive conditions (Table 1). Most prominent changes were observed for early intermediates of glycolysis, namely glucose 6-phosphate, fructose 6-phosphate, fructose 1,6-bisphosphate and for pyruvate. Changes in the concentrations of other metabolites were less pronounced and in some cases within the margin of experimental error. Concentrations

Table 1. Estimated intracellular concentrations of various metabolites

S. cerevisiae cdc35-1 cells were kept at a permissive temperature of 23 °C or were switched to the restrictive temperature of 36 °C. Metabolite concentrations were determined spectrophotometrically in neutral extracts. Data are the mean and standard error for samples taken at 2, 3 and 4 h after the temperature shift. Energy charge was calculated as $([ATP] + 0.5[ADP]) / ([ATP] + [ADP] + [AMP])$.

	Intracellular metabolite concentration (mM except where stated)	
	Permissive temperature	Restrictive temperature
Glucose 6-phosphate	2.93 ± 0.08	1.45 ± 0.10
Fructose 6-phosphate	0.44 ± 0.03	0.24 ± 0.03
Fructose 1,6-bisphosphate	3.25 ± 0.09	1.17 ± 0.25
Dihydroxyacetone phosphate	0.55 ± 0.03	0.41 ± 0.06
3-Phosphoglycerate	0.82 ± 0.02	0.56 ± 0.07
2-Phosphoglycerate	0.08 ± 0.02	0.04 ± 0.02
Phosphoenol pyruvate	0.29 ± 0.10	0.27 ± 0.05
Pyruvate	2.97 ± 0.08	1.89 ± 0.13
NAD ⁺	2.92 ± 0.10	3.18 ± 0.19
ATP	3.14 ± 0.20	2.33 ± 0.20
ADP	0.50 ± 0.15	0.35 ± 0.15
AMP	0.20 ± 0.10	0.20 ± 0.10
Energy charge	0.88	0.87
cAMP	1.12 ± 0.06	1.78 ± 0.21 µM
Fructose 2,6-bisphosphate	10.14 ± 0.73	7.21 ± 0.16 µM
2,3-Bisphospho-glycerate	243.68 ± 19.86	96.55 ± 3.75 µM
Citrate	1.84 ± 0.16	8.05 ± 3.29

of glyceraldehyde 3-phosphate were found to be too low to be reliably determined with the methods employed.

Although the concentrations of several intermediates of glycolysis were changed significantly by the temperature shift, and despite the observed decrease in *in vivo* glycolytic flux, cells were able to retain a high energetic status, as judged from the high energy charge in all samples taken at 23 °C or 36 °C (Table 1) (energy charge = $([ATP] + 0.5[ADP]) / ([ATP] + [ADP] + [AMP])$). No major changes were found in NAD⁺ levels (Table 1). NADH levels were too low to be reliably detected.

Because the *CDC35* gene encodes adenylate cyclase, and because cAMP and fructose-2,6-bisphosphate can regulate the activity of some glycolytic and gluconeogenic enzymes, cAMP and fructose-2,6-bisphosphate concentrations were determined. cAMP levels were found to rise after several hours at 36 °C, whereas fructose-2,6-bisphosphate concentrations decreased (Table 1). Similar observations have been reported earlier for *cdc25-1* (Oehlen *et al.*, 1993). Other potential effectors of glycolytic enzymes include 2,3-phosphoglycerate, which stimulates phosphoglycerate mutase activity, and citrate, which, in liver cells, can inhibit phosphofructokinase activity. Although the estimated cellular concentrations of 2,3-PGA decreased from more than 200 µM to less than 100 µM, the level remained far above the concentration necessary for half-maximal stimulation of phosphoglycerate mutase of 0.5–0.8 µM reported by

Sasaki *et al.* (1971). Citrate concentrations changed markedly in *cdc35-1* cells incubated at restrictive temperature: levels rose from less than 2 mM to more than 20 mM after 6 h at 36 °C (Table 1 and data not shown). Preliminary results did not show any effect of citrate in this concentration range on the specific activity of phosphofructokinase in *S. cerevisiae* cell extracts (data not shown). Thus, although prominent changes in 2,3-PGA and citrate levels were observed in *cdc35-1* cells at restrictive temperature, these changes are not likely to have a major effect on phosphoglycerate mutase and phosphofructokinase activities, respectively.

Hexose transport decreases in *cdc35-1* cells under restrictive conditions

The levels of some early intermediates of glycolysis were lower at 36 °C than at 23 °C. Possible explanations for this phenomenon include (a) a decrease in hexokinase activity, and (b) a decrease in glucose transport. Since there was no indication for lowered hexokinase activity at restrictive temperature (Fig. 2), the second possibility was pursued. Hexose transport was measured in *cdc35-1* cells grown at 23 °C or incubated at 36 °C for different time intervals. The procedures of sampling, preparation of a concentrated cell suspension in buffer, and aerobic preincubation at 30 °C did not affect ATP concentrations and the levels of various other metabolites including most intermediates of glycolysis (data not shown). Transport of

Fig. 3. Hexose transport in *cdc35-1* cells. 2-doG uptake was measured in *S. cerevisiae cdc35-1* cultures kept at permissive temperature (●) or shifted to 36 °C (○) at $t=0$. Uptake was determined in concentrated cell suspensions at 2-doG concentrations of (a) 2 mM or (b) 50 mM. Data are representative for two independent experiments.

the non-metabolizable glucose analogue 2-doG was measured at final sugar concentrations of 2 or 50 mM at 30 °C. 2-doG is transported by the same carrier as glucose, it can be phosphorylated at the 6-hydroxyl position, but is not further metabolized. Schuddemat *et al.* (1986, 1988) have reported a K_m value for 2-doG transport of 0.5 (1986 paper) or 1.25 mM (1988 paper) for wild-type cells. The *cdc35-1* mutant was found to have a higher K_m for both glucose (4.3 mM; Walsh *et al.*, 1994) and for 2-doG (approximately 5 mM; data not shown) at permissive temperature. The transport measured at 50 mM therefore reflects V_{max} conditions for sugar transport. As shown in Fig. 3(a, b) sugar transport markedly decreased in *cdc35-1* cells incubated at 36 °C. This decrease was observed both at the low and the high sugar concentration tested, although the decrease in transport at 50 mM appeared to be somewhat more pronounced than that at 2 mM. Similar observations were also made when radiolabelled glucose was used for the transport assays (data not shown). In a similar experiment with wild-type cells, glucose transport (measured at a final concentration of 100 mM) was approximately 1.5 times higher at 36 °C than at 23 °C throughout the experiment (data not shown). Also when transport of the non-phosphorylatable glucose analogue 6-deoxyglucose was measured, a marked change in sugar transport in *cdc35-1* cells could be observed at 36 °C. Transport of this sugar (measured at a final concentration of 100 mM) decreased from approximately 400 nmol min⁻¹ (mg protein)⁻¹ at 23 °C to about 60 nmol min⁻¹ (mg protein)⁻¹ after 5 h at the restrictive temperature. All these transport data taken together indicate that the

decrease in glycolytic activity in *cdc35-1* cells at restrictive temperature correlates with a defect in hexose transport activity.

DISCUSSION

Adenylate cyclase may have cAMP-independent functions

Since *cdc35-1* cells carry a thermosensitive mutation in the adenylate cyclase gene, cAMP levels might be expected to fall when *cdc35-1* carrying cells were shifted to restrictive temperature. Instead of a decrease, the intracellular cAMP levels were found to increase after a temperature shift, an effect that is usually observed for wild-type cells (Boutelet *et al.*, 1985; Camonis *et al.*, 1986; Oehlen *et al.*, 1993). At restrictive temperature, the *in vivo* catalytic activity of adenylate cyclase from *cdc35-1* cells may therefore be similar to that of wild-type cells. Sy & Tamai (1986) found that the *in vitro* adenylate cyclase activities of membranes prepared from *cdc35-1* cells grown at permissive temperature or incubated at restrictive temperature were the same. The enzyme, however, was altered in its dependency for Mn²⁺ and did not respond to guanine nucleotide stimulation. Based on these results it was suggested that the *cdc35-1* mutation was not in the catalytic site, but at a site where adenylate cyclase interacts with regulatory proteins. These *in vitro* properties of *cdc35-1* adenylate cyclase may help to explain the observed cAMP rise *in vivo* in *cdc35-1* at 36 °C.

In spite of the rise in cAMP levels *cdc35-1* cells arrest in the G1 phase of the cell cycle and the glycolytic flux of the cells at restrictive temperature gradually falls. Similar results have been reported earlier for *cdc25-1* carrying ts.321 cells (Oehlen *et al.*, 1993) and they indicate that high cAMP levels may be necessary but not sufficient for cell cycle progression and the maintenance of a high glycolytic flux. Interestingly, it appears that adenylate cyclase, at least in mutant *cdc35-1* cells, can exert cAMP-independent effects which lead to G1 arrest and a lowering of glycolytic flux. It remains to be established what the nature of these cAMP-independent pathways is and whether they are of importance for normal physiological regulation in cells with wild-type adenylate cyclase. It may be relevant to note that adenylate cyclase has been reported to be associated with at least two proteins (CAP and RAS), which, apart from their role in the regulation of adenylate cyclase activity, appear to be involved in cAMP-independent pathways (Field *et al.*, 1990b; Wigler *et al.*, 1988; Field *et al.*, 1990a; Fedor-Chaikin *et al.*, 1990; Gerst *et al.*, 1991; Vojtek *et al.*, 1991; Wang *et al.*, 1992). It is unclear, however, whether these CAP- and RAS-mediated cAMP-independent pathways can contribute to cell cycle arrest and a lowered glycolytic activity.

Hexose transport activity

When *cdc35-1* cells were incubated at restrictive temperature for 5 h hexose transport activity decreased significantly. Schuddemat *et al.* (1986, 1988) have reported monophasic transport kinetics for 2-doG transport with a

K_m of 0.5 or 1.25 mM for wild-type cells and we found for the *cdc35-1* mutant a higher K_m of about 5 mM for 2-doG transport. Our measurements at 50 mM are likely to closely reflect the V_{max} for hexose transport. Although the number of glucose transporter molecules was not directly quantified, the decrease in overall V_{max} indicates that the total number of glucose transporters gradually falls in *cdc35-1* cells at restrictive temperature. A possible explanation for this decrease may lie in the fact that protein synthesis in *cdc35-1* mutant cells is much diminished. The half-life for glucose transporter molecules is relatively short compared to the half-lives of other proteins (Lagunas *et al.*, 1982; López & Gancedo, 1979). Inhibition of protein synthesis therefore could result in a decrease in the steady-state amount of glucose transporter molecules (mg total cellular protein)⁻¹ (Lagunas *et al.*, 1982). Iida & Yahara (1984) showed that the total protein synthesis rate in *cdc35-1* cells drops significantly when cells are incubated at restrictive temperature. This decrease in overall protein synthesis rate may contribute to a decrease in the number of glucose transporter molecules per mg protein, but it is unclear if the kinetics of the decrease in protein synthesis activity can fully explain the kinetics of the decrease in hexose transport activity. It is possible that additional, more specific mechanisms for the decrease in glucose transport exist.

Regulation of glycolytic flux

The gradual fall in glycolytic flux in *S. cerevisiae cdc35-1* cells at restrictive temperature is not paralleled by a similar fall in the specific activity of any of the glycolytic enzymes, but is correlated with a decrease in hexose transport. Thus, at least in this experimental setting, glucose transport is likely to be an important factor in controlling glycolytic flux. A flux-controlling role for glucose transport was earlier suggested from experiments of Lagunas *et al.* (1982), who observed a correlation between a decrease in flux and a decrease in glucose transport when cells were starved for nitrogen. In addition to the correlation between flux and transport measurements in arrested *cdc35-1* cells, other data indicate the importance of transport in controlling glycolytic flux. The specific activities of glycolytic enzymes and the distribution of glycolytic intermediates in *cdc35-1* cells at restrictive temperature do not point to any other step in glycolysis which might be inhibited. There were, for example, no indications for restrictions of phosphofructokinase or phosphoglycerate mutase activities, which might result from an increase in citrate levels and a decrease in 2,3-PGA levels respectively. Also the changes in cAMP and fructose 2,6-bisphosphate levels did not seem to affect PFK or other enzymes of glycolysis. The observation that the V_{max} for hexose transport was similar to that of the *in vivo* glycolytic flux could be regarded as another argument in favour of a prominent role for hexose transport in controlling glycolytic flux. It should be noted, however, that our observation that the decrease in flux during the first hour at restrictive temperature is not paralleled by a decrease in transport,

suggests that, at least during the first hour, other factors may be involved in the control of glycolytic flux.

Under permissive conditions, most of the glucose that enters the cell is converted to ethanol/CO₂ and only 2–3% is oxidized in the mitochondria to CO₂ and water. The decrease in fermentative ethanol/CO₂ production in *cdc35-1* cells at restrictive temperature is not paralleled by a decrease in O₂ consumption. This distribution between respiration and fermentative ethanol/CO₂ production, indicated by the respiratory quotient, resembles – as in many other aspects – the fall in RQ when yeast cells are starved for their nitrogen source (Lagunas *et al.*, 1982). Käppeli (1986) has proposed that ethanol production in *S. cerevisiae* is the result of an overflow metabolism at the level of pyruvate. According to this hypothesis, reduction of the input into the glycolytic pathway and reduced levels of pyruvate would in the first place affect fermentative ethanol/CO₂ production. The fall in RQ, both in *cdc35-1* cells at restrictive temperature and in nitrogen starved cells, is consistent with this ‘overflow’ hypothesis.

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