Glucose-induced inactivation of mitochondrial enzymes in the yeast *Saccharomyces cerevisiae*

Masaharu TAKEDA

Department of Biochemistry, The Waste Water Treatment Center, Yamagata University School of Medicine,
Zao-Iida, Yamagata, Japan 990–23

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1. Addition of glucose induced an inactivation of mitochondrial enzymes in the yeast *Saccharomyces cerevisiae* containing normal mitochondrial particles. 2. The glucose-induced inactivation of mitochondrial enzymes was inhibited by the presence of cycloheximide. 3. Pepstatin also inhibited the inactivation, but phenylmethanesulphonyl fluoride accelerated the inactivation. 4. The specific activities of fructose 1,6-bisphosphatase and cytoplasmic malate dehydrogenase were decreased on the exposure to glucose, as well as those of the mitochondrial enzymes. However, the glucose-induced inactivation of cytoplasmic enzymes was not inhibited by the presence of pepstatin. 5. The specific activities of hexokinase and phosphofructokinase, which are cytoplasmic enzymes were increased by the addition of glucose, and this effect was not affected by pepstatin. 6. Addition of glucose resulted in an increase in the synthesis of proteins of the mitochondria and the cytosol, and simultaneously in degradation of these mitochondrial and cytoplasmic proteins.

Glucose and its catabolites cause, not only repression of enzyme synthesis in some organisms (Spiegelman & Reiner, 1947; Neidhardt & Magasanik, 1956; Polakis *et al.*, 1964; Kim & Beattie, 1973; Perlman & Mahler, 1974), but also inactivation of certain cytoplasmic enzymes (Witt *et al.*, 1966; Molano & Gancedo, 1974; Brown *et al.*, 1975; Haarasilta & Oora, 1975; Neeff *et al.*, 1978; Frey & Röhm, 1979; Matern, 1979). Holzer (1976) proposed a proteolytic mechanism for this 'catabolite inactivation'. However, at present, little is known about the inactivation of the mitochondrial enzymes in any organism. The present investigation shows the results of addition of glucose on the mitochondrial enzymes and the regulatory mechanism for these enzymes in the yeast *Saccharomyces cerevisiae*.

Materials and methods

Materials

NADH (grade II), NADPH, NADP⁺, phosphoenolpyruvate, fructose 1,6-bisphosphate, cycloheximide, phenylmethanesulphonyl fluoride, glucose 6-phosphate dehydrogenase, phosphoglucose isomerase and pyruvate kinase were obtained from Boehringer Mannheim Corp., Mannheim, Germany. Bovine serum albumin, ATP (grade I),

pepstatin, 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyltetrazolium (grade I) and lactate dehydrogenase were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. Bactopeptone and yeast extract were obtained from Nakarai Chemicals, Kyoto, Japan. Zymolyase-60000 was purchased from Kirin Brewery Co., Takasaki, Japan. All other reagents used were commercial products (Waco Chemical Co., Osaka, Japan, Nakarai and Sigma). A diploid wild-type strain of *Saccharomyces cerevisiae* was kindly supplied by the Laboratory of Microbiology, Department of Agriculture, Tohoku University, Sendai, Japan. L-[4,5-³H]Leucine (40–60 Ci/mmol), L-[U-¹⁴C]leucine (> 300 mCi/mmol) and ACS II scintillant (complete 1,2,4-trimethylbenzene/xylene/surfactant-based liquid-scintillation 'cocktail') were obtained from The Radiochemical Centre (Amersham, Bucks., U.K.).

Growth of yeast

A diploid wild-type strain of *Saccharomyces cerevisiae* was grown in a liquid medium containing 0.5 g of Bactopeptone, 0.5 g of yeast extract, 4.53 g of disodium succinate hexahydrate, 0.1 g of (NH₄)₂SO₄, 0.1 g of KH₂PO₄, 50 mg of MgSO₄·7H₂O, 10 mg of NaCl, 10 mg of CaCl₂·2H₂O, 1.5 mg of FeCl₃·6H₂O and 0.8 g of glucose in 100 ml (medium B). The pH was adjusted

to 4.4 with HCl. The strain was maintained in a cold-room on an agar slant consisting of medium A, which was the same as medium B except for the amounts of glucose (5g) and disodium succinate hexahydrate (0.33g). Before the culture, the cells were initially transferred from the slant to 100ml of medium A. After 24h of the preculture, the cells were grown aerobically for various times at 30°C in Erlenmeyer flask shaken in an SS-8 Eyelashaker (50 strokes/min), and rapidly chilled in ice and centrifuged at 3000g for 10min. The pellet containing the cells was washed with cold distilled water, and weighed.

Preparation of spheroplasts

Washed yeast cells (2–3g wet wt.) were suspended in 2vol. of ice-cold buffer 1 (10mM-Tris/HCl buffer, pH 7.4, containing 1.3M-sorbitol, 0.1M-EDTA and 71μM-cycloheximide), and then EDTA (final concn. 4mM) was added. The yeast suspension was incubated for 40min at 30°C and centrifuged at 3000g for 10min. The pellet was resuspended in 2vol. of ice-cold buffer 1, and Zymolyase-60000 (1/1000th weight of the yeast cells) was added. After 90min of incubation at 30°C, spheroplasts were harvested and washed once with ice-cold buffer 1.

Preparation of mitochondria and cytosol

All steps were performed at 0–4°C. Preparation of mitochondria was by a modification of the method of Kim & Beattie (1973). Washed spheroplasts from yeast cells were broken in 10vol. of 0.25M-sucrose with a Potter–Elvehjem homogenizer (20 strokes). The homogenate was centrifuged at 1000g for 20min to remove unbroken cells and nuclei. The supernatant was centrifuged at 19000g for 20min. The mitochondria obtained were suspended in 0.25M-sucrose to a final protein concentration of approx. 5mg/ml. The post-mitochondrial supernatant was centrifuged at 100000g for 60min. The final supernatant fraction was used as cytosol.

Assays of enzyme activities

All spectrophotometric assays of enzyme activities, except those of cytochrome *c* oxidase and succinate dehydrogenase, were performed at 25°C in a Gilford 1240 spectrophotometer equipped with a recorder. The assay of cytochrome *c* oxidase activity was performed at 25°C in a Hitachi 200-20 spectrophotometer by the method of Orii & Okunuki (1965). Succinate dehydrogenase activity was assayed in the presence of 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyltetrazolium (Pennington, 1961). Malate dehydrogenase and glutamate dehydrogenase activities were determined from the decrease in the absorbance at 340nm caused by the

oxidation of NADH and NADPH respectively (Williamson & Corkey, 1969). Fructose 1,6-bisphosphatase activity was assayed by the method of Traniello *et al.* (1972). Hexokinase activity was determined from the increase in the absorbance at 340nm caused by the reduction of NADP⁺ (Williamson & Corkey, 1969). Phosphofructokinase activity was assayed by the method of Racker (1947). Enzymic activity is expressed in units (μmol of substrate converted/min), except that 1 unit of succinate dehydrogenase is defined as the amount of enzyme that increases A_{490} by 1.0 unit/min at 37°C. Specific activity is defined as units/mg of protein.

Determination of protein

Protein concentration was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

Determination of glucose

Residual glucose concentration in the medium was determined by the method of Saloman & Johnson (1959).

Measurement of radioactivity

Mitochondria or cytosol obtained from each sample of cells (50μl) was dissolved in 5ml of ACS II scintillant, and ³H and ¹⁴C radioactivities were determined with an LKB 1216 RackBeta liquid-scintillation counter. Counting efficiency for ³H was 30% and ¹⁴C counting efficiency was 80%. The observed radioactivities (c.p.m.) were corrected for quenching, to yield the d.p.m., by means of automatic external standardization.

Other growth medium

Medium C is the same as medium B except that glucose was omitted.

Presentation of data

In all experiments reported, each value is the mean (±s.d.) of results from duplicate or triplicate samples.

Results

Time course of changes in the specific activities of mitochondrial enzymes

When *Saccharomyces cerevisiae* was aerobically grown in the presence of 0.8% glucose in the medium (medium B) at 30°C, a characteristic growth curve was obtained. A lag period of several hours was observed before the exponential phase of growth started (Fig. 1). Stationary phase was reached after 17h of growth. Glucose utilization was completed at 8h, and the pH in the medium was constant for these experiments. The specific activities of mitochondrial enzymes (cytochrome *c*

Fig. 1. Time course of changes in the specific activities of the mitochondrial enzymes of yeast cells grown in medium B. Yeast cells were grown aerobically for various times at 30°C in medium B and the mitochondria were prepared as described in the Materials and methods section. The specific activities of cytochrome *c* oxidase (■), succinate dehydrogenase (●) and malate dehydrogenase (▲) were determined as indicated in the Materials and methods section. Cell growth in medium B was measured by reading the absorbance at 800nm (□). Residual glucose concentration in the medium (○) was determined as described in the Materials and methods section. The pH (Δ) was also measured.

oxidase, succinate dehydrogenase and mitochondrial malate dehydrogenase) increased rapidly after 17h and reached a plateau after 24h of growth (Fig. 1). Mitochondria were developed in the yeast cells grown for 24h (termed mitochondria-developed cells).

Effects of glucose on the specific activities of mitochondrial and cytoplasmic enzymes in the mitochondria-developed cells

The mitochondria-developed cells obtained (1g wet wt.) were transferred to 100ml of medium containing glucose or to 100ml of medium containing no glucose, with one of the inhibitors indicated, and then the mixtures were incubated aerobically for 3h at 30°C (with shaking at 50 strokes/min). The specific activities of the mitochondrial enzymes cytochrome *c* oxidase, succinate dehydrogenase and mitochondrial malate dehydrogenase were decreased by the exposure to glucose (Fig. 2a). The glucose-induced inactivation of the mitochondrial enzymes was inhibited by the presence of cycloheximide in the medium (Fig. 2a). Normally glucose utilization in the medium was completed at 3h after the transfer, but in the presence of cycloheximide the glucose utilization was 50% at that time (Fig. 3). Thus the prevention of the inactivation by the addition of cycloheximide together with glucose to the medium is interpreted as indicating, not only the inhibition of protein syn-

thesis, but also a change in the glucose-metabolizing system. Some preliminary experiments showed that the inactivation of the mitochondrial enzymes, observed in the cells grown for 17h (late exponential stage) or 48h (late stationary stage) in medium B, was not inhibited by cycloheximide. Therefore the effect of cycloheximide on the inactivation of the mitochondrial enzymes may be correlated to the stage of the cells.

Pepstatin, a potent inhibitor of yeast proteinase A (Saheki & Holzer, 1975; Betz & Weiser, 1976), inhibited the glucose-induced inactivation of the mitochondrial enzymes. However, in the presence of phenylmethanesulphonyl fluoride, a potent inhibitor of yeast proteinase B (Saheki & Holzer, 1975; Betz & Weiser, 1976), the decrease of the specific activities of the mitochondrial enzymes was accelerated (Fig. 2a). Thus the inhibition of the inactivation by pepstatin gives strong evidence that proteolysis is the mechanism of the inactivation (Scheme 1). The acceleration of the inactivation by phenylmethanesulphonyl fluoride suggests that the activation of the mitochondrial enzymes may be controlled by proteolysis (Scheme 1). Ulane & Cabib (1976) have reported that the yeast chitin synthase activating factor is identical with yeast proteinase B, and Saheki & Holzer (1974) have reported that tryptophan synthase is inactivated by yeast proteinases A and B. Our results suggest that the proteolytic regulatory mechanism applies to the mitochondrial enzymes also.

Fig. 2. Effects of glucose on the specific activities of mitochondrial (a) and cytoplasmic enzymes (b) in the mitochondria-developed yeast cells

Yeast cells were grown for 24 h in 100 ml of medium B as described in the Materials and methods section, then washed, transferred to medium (100 ml) and incubated aerobically for 3 h at 30°C (50 strokes/min). The transfer media used were medium B (■) with no inhibitor, with cycloheximide (CHI) (71 μ M), with chloramphenicol (CAP) (12 mM), with pepstatin (1.5 μ M) and with phenylmethanesulphonyl fluoride (PMSF) (5.0 mM), and medium C (□) with the same inhibitors just mentioned. Preparation of mitochondria and cytosol, enzyme assays and protein determination were as described in the Materials and methods section. The relative specific activities of the enzymes were calculated by taking the activity of the cells transferred to medium C as 100.

Scheme 1. Possible activation-inactivation mechanism of mitochondrial enzymes in the yeast *Saccharomyces cerevisiae*

Fig. 3. Effect of cycloheximide on glucose utilization by the yeast cells after transfer

Yeast cells were grown for 17 h (a) or 24 h (b) in medium B, then washed and transferred to medium B (○), medium B with cycloheximide (●) or medium B with pepstatin (■) as described in Fig. 2 legend. The residual glucose concentration in the medium at each time was determined as described in the Materials and methods section.

The specific activities of the cytoplasmic enzymes fructose 1,6-bisphosphatase (Fig. 2b) and cytoplasmic malate dehydrogenase (Table 1) were decreased by the exposure to glucose, as well as those of the mitochondrial enzymes. However, the glucose-induced inactivation of these enzymes was not inhibited by the presence of pepstatin in the medium (Fig. 2b). After the addition of glucose to acetate-grown cells of *Saccharomyces cerevisiae*, a rapid decrease in fructose 1,6-bisphosphatase (Molano & Gancedo, 1974) and cytoplasmic malate dehydrogenase (Witt *et al.*, 1966) activities was observed. Both in *Saccharomyces cerevisiae* cells grown on glucose and in those grown on acetate as carbon source, fructose 1,6-bisphosphatase and cytoplasmic malate dehydrogenase activities were rapidly decreased by the addition of glucose.

On the other hand, the specific activities of hexokinase, phosphofructokinase (Fig. 2b) and NADP-linked glutamate dehydrogenase (Table 1) were increased by the addition of glucose in the medium, and the effect was not affected by pepstatin. In the presence of cycloheximide, the specific activities of hexokinase and phosphofructokinase were slightly decreased (Fig. 2b). Both phosphofructokinase and hexokinase are key enzymes for glycolysis, and closely involved in the mechanism for regulation of glycolysis and respiration. Our results suggest that the glucose-induced inactivation of the

mitochondrial enzymes is not dependent on glycolysis.

Effects of cytochalasin B, colchicine, cyclic AMP and oligomycin

The effects of cytochalasin B and colchicine, both of which are known to act on microtubules or microfilaments, of cyclic AMP and of oligomycin, an inhibitor of energy coupling, were examined in the mitochondria-developed yeast cells. These substances had no significant effect on the glucose-induced inactivation of the mitochondrial enzymes (Table 1).

Incorporation *in vivo* of radioactive leucine by mitochondria or cytosol

The incorporation *in vivo* of radioactive leucine by mitochondria or cytosol was examined as follows. The mitochondria-developed yeast cells grown in the presence of [^{14}C]leucine were transferred directly to medium containing [^3H]leucine and cycloheximide or to medium containing [^3H]leucine and pepstatin. The results of this experiment, summarized in Table 2, indicated (a) that the residual radioactivities of [^{14}C]leucine in the mitochondria or cytosol were slightly decreased by the exposure to glucose, (b) that the net incorporation of [^3H]leucine by the mitochondria was 2.5-fold and that by the cytosol about 2.7-fold greater in the

Table 1. *Effects of cytochalasin B, colchicine, cyclic AMP and oligomycin on the specific activities of enzymes in the mitochondria-developed yeast cells*

Experimental details were as described in Fig. 2 legend. The transfer media used were medium B, medium C and medium B containing cytochalasin B (63 μ M), colchicine (0.1 mM), cyclic AMP (10 μ M) or oligomycin (400 μ g/100 ml).

Sample	Relative specific activity				
	Mitochondria			Cytosol	
	Cytochrome c oxidase	Succinate dehydrogenase	Malate dehydrogenase	Malate dehydrogenase	NADP-linked glutamate dehydrogenase
Medium B $\rightarrow$ medium C	100	100	100	100	100
Medium B $\rightarrow$ medium B	12 $\pm$ 3	23 $\pm$ 5	25 $\pm$ 5	80 $\pm$ 5	193 $\pm$ 21
Medium B $\rightarrow$ medium B + cytochalasin B	12 $\pm$ 5	26 $\pm$ 2	25 $\pm$ 4	183 $\pm$ 19	183 $\pm$ 20
Medium B $\rightarrow$ medium B + colchicine	10 $\pm$ 3	21 $\pm$ 5	20 $\pm$ 5	186 $\pm$ 21	164 $\pm$ 15
Medium B $\rightarrow$ medium B + oligomycin	11 $\pm$ 3	20 $\pm$ 4	26 $\pm$ 4	238 $\pm$ 13	161 $\pm$ 19
Medium B $\rightarrow$ medium B + cyclic AMP	13 $\pm$ 4	30 $\pm$ 5	22 $\pm$ 4	145 $\pm$ 20	203 $\pm$ 25

Table 2. *Effects of glucose, cycloheximide and pepstatin on the synthesis of mitochondrial and cytoplasmic proteins in yeast cells in vivo*

Yeast cells were grown in 100 ml of medium B supplemented with 10 μ Ci of L-[14 C]leucine. The cells were harvested at 24 h and washed twice with 50 ml of distilled water. The washed cells were transferred and incubated aerobically for 3 h in medium (100 ml) supplemented with 100 μ Ci of L-[3 H]leucine and one of the inhibitors indicated. Mitochondria and cytosol were obtained from each sample of cells, and radioactivity and protein were determined as described in the Materials and methods section. Other experimental details were as described in Fig. 2 legend.

Sample	$10^{-3} \times$ Radioactivity incorporated (d.p.m./mg of protein)					
	Mitochondria			Cytosol		
	14 C	3 H	3 H/ 14 C ratio	14 C	3 C	3 H/ 14 C ratio
Medium B $\rightarrow$ medium C	25 $\pm$ 1	80 $\pm$ 3	3.2	18 $\pm$ 0	66 $\pm$ 2	3.7
Medium B $\rightarrow$ medium C + cycloheximide	35 $\pm$ 0	77 $\pm$ 1	2.2	17 $\pm$ 0	50 $\pm$ 0	2.9
Medium B $\rightarrow$ medium C + pepstatin	24 $\pm$ 0	80 $\pm$ 0	3.3	16 $\pm$ 1	62 $\pm$ 4	3.9
Medium B $\rightarrow$ medium B	21 $\pm$ 0	170 $\pm$ 1	8.1	13 $\pm$ 1	140 $\pm$ 7	10.7
Medium B $\rightarrow$ medium B + cycloheximide	28 $\pm$ 1	60 $\pm$ 1	2.1	15 $\pm$ 1	37 $\pm$ 1	2.5
Medium B $\rightarrow$ medium B + pepstatin	16 $\pm$ 0	136 $\pm$ 0	8.5	13 $\pm$ 0	150 $\pm$ 5	11.5

presence of glucose and (c) that the net incorporation of [3 H]leucine into the mitochondria and cytosol was inhibited by cycloheximide, but not by pepstatin. Addition of glucose resulted in an increase in the synthesis of proteins of the mitochondria as well as those of the cytosol, and simultaneously in degradation of these mitochondrial and cytoplasmic proteins.

Discussion

The results of the present investigation show that the mitochondrial enzymes in the yeast *Saccharomyces cerevisiae*, as well as the cyto-

plasmic enzymes fructose 1,6-bisphosphatase (Molano & Gancedo, 1974), cytoplasmic malate dehydrogenase (Witt *et al.*, 1966; Neeff *et al.*, 1978), phosphoenolpyruvate carboxykinase (Haarasilta & Oura, 1975; Matern, 1979), α -isopropylmalate synthase (Brown *et al.*, 1975) and aminopeptidase I (Frey & Röhm, 1979), can be inactivated by glucose. Holzer (1976) proposed a hypothesis that glucose effects a change in the conformation or primary structure of these cytoplasmic enzymes in such a way that they become vulnerable to intracellular proteolytic degradation.

The inhibitor experiments of the present study indicate (a) that cycloheximide inhibits the glucose-

induced inactivation of the mitochondrial enzymes in the yeast *Saccharomyces cerevisiae*, (b) that pepstatin also inhibits the inactivation and (c) that phenylmethanesulphonyl fluoride apparently accelerates the inactivation (Fig. 2a). These findings provide strong evidence that proteolysis is the mechanism of the inactivation of the mitochondrial enzymes as well as those of the cytoplasmic enzymes. The acceleration of the inactivation by phenylmethanesulphonyl fluoride suggests that the precursors of the mitochondrial enzymes are converted into active forms by proteinase(s) in the yeast (Scheme 1). Proteinase I (phenylmethanesulphonyl fluoride-sensitive) converts the precursors of the mitochondrial enzymes into active forms. On the other hand, proteinase II (pepstatin-sensitive) degrades the active mitochondrial enzymes, and this inactivation process is greatly influenced by the presence of glucose.

Allan & Welman (1980) have reported that mitochondrial protein can be degraded by myocardial lysosomal enzymes *in vitro*. Also, Galkin *et al.* (1980) have reported that proteinases regulate the yeast respiratory system. Our results are entirely in accord with these findings.

Several intracellular proteolytic enzymes and proteinase inhibitors have been found in *Saccharomyces cerevisiae*. The ability to regulate proteinase and inhibitor concentrations independently might enable the cell to regulate proteolysis under different environmental conditions (Beck *et al.*, 1980; Murphy *et al.*, 1980). The results of the present investigation provide information that makes a contribution to an understanding of the regulatory mechanism of mitochondrial biogenesis *in vivo*.

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