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A METHOD FOR DETERMINING THE INTRACELLULAR DISTRIBUTION OF ENZYMES IN YEAST PROVIDES NO EVIDENCE FOR THE ASSOCIATION OF HEXOKINASE WITH MITOCHONDRIA

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A simple procedure based upon the principle discovered by Dürr et al. (Arch. Microbiol. (1975) 105, 319-327) was used to measure the intracellular distribution of enzymes in S. cerevisiae grown under both glucose repression and derepression. No substantial hexokinase activity was found to be associated with cellular organelles. The result does not support the hypotheses that reversible binding of hexokinase to mitochondria is important in regulation of glycolysis and cell growth.

A major portion of mammalian cell hexokinase is associated with mitochondria (1,2). In tumor cells, the amount of bound hexokinase is correlated with tumor growth rates and with differentiation of the cell (see ref. 3 for review). These findings have led to several theories in which the mitochondrial location of hexokinase represents an important factor in regulating aerobic glycolysis and cell growth (1-3).

The simple eucaryotic organism, Saccharomyces cerevisiae, would appear to be particularily suited for testing the role of hexokinase in regulation of glycolysis since these cells, like tumor cells, exhibit aerobic glycolysis. It is important, therefore, to know if hexokinase binding to mitochondria is also associated with glycolytic regulation in the yeast cell. Demonstration of hexokinase binding by subcellular fractiona-

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tion, as done with mammalian cells (4,5), can not be achieved with yeast since the mitochondria of these cells are highly ramified structures (6) which are likely to be damaged by even the mildest isolation procedures. Furthermore, early studies on the subcellular distribution of yeast hexokinase (7) were done under conditions (presence of EDTA) which release bound hexokinase due to chelation of Mg^{2+} (4). Thus, data showing the absence of bound hexokinase in yeast (7,8) can not be considered as conclusive.

In order to study the effects of varying metabolic states on hexokinase binding in intact yeast cells, we have developed a rapid and mild procedure based upon that of Dürr et al. (9) to selectively release soluble enzymes from protoplasts. By this method, no substantial amount of hexokinase was found to be associated with the cellular organelles in cells grown under two extreme conditions of glucose repression.

MATERIALS AND METHODS

A wild type strain of S. cerevisiae, DT XII, was grown at 30°C in Erlenmyer flasks filled to 0.1 volume with a semi-synthetic medium containing salts, peptone, yeast extract and glucose. Cells grown on 0.3% glucose were harvested at the late stationary phase (yield: 1.6×10^8 cells/ml) and cells grown on 10% glucose were harvested from the exponential phase (yield: 4×10^7 cells/ml).

Protoplasts were prepared from alkaline mercaptoethanol-pretreated cells (10) by digestion with crude snail gut enzyme or with lyticase (Sigma). The protoplasts were washed three times with 1.0 M sorbitol and were fractionated either immediately or after storage at 0° C for maximally 4 days. Storage did not effect the properties examined in this study.

The protoplasts were suspended in 1.0 M sorbitol buffered at pH 6.0 with 10 mM citrate buffer and then warmed to 30°C. DEAE dextran (Pharmacia), dissolved in 1.0 M sorbitol/10 mM citrate pH 6.0, was added to a final concentration of 0.05 to 2.5 pg/protoplast. The mixture, a total volume of 1 ml, was incubated in an Eppendorf tube at 30°C for 4 min and then centrifuged at 10,000 g for 20 sec. The supernatant was poured off and chilled to 0°C. The pellet was washed with 1 ml of cold sorbitol and then suspended in 1 ml of 1.0 M sorbitol containing 0.2% Triton X-100. Samples were frozen at -20°C.

Protease A (12), α-glucosidase (13) and the remaining enzymes (11), were measured in the supernatant and in the suspended pellet which had been thawed at 0°C and vortexed for 10 sec. Occasionally, the supernatants were frozen at -20°C for as long as 7 days before use. However none of the enzyme activities

were modified by this treatment. The sum of activities in the supernatant and pellet were compared with the activity measured in protoplasts suspended in 1.0 M sorbitol/0.2% Triton X-100. The recovery of all enzyme activities was essentially complete.

RESULTS AND DISCUSSION

In order to study the subcellular location of various yeast enzymes we have used DEAE-dextran, which was shown by Dürr et al. (9) to damage the plasma membrane of yeast protoplasts while leaving vacuole membranes relatively intact. Based upon this observation, Wiemken et al. (14) developed an ingenious method to assess the location of a number of yeast enzymes to vacuoles.

The method of Wiemken et al. (14) was simplified by replacing the elaborate gradient centrifugation step with a short spin-down of the protoplast pretreated with DEAE dextran. Fig. 1 shows a typical experiment for cells grown under conditions of derepression, in which > 90% of the hexokinase and of the soluble enzymes (alcohol dehydrogenase, α-glucosidase, glucose 6-phosphate dehydrogenase, aldolase and pyruvate kinase) remained in the supernatant after centrifugation of the

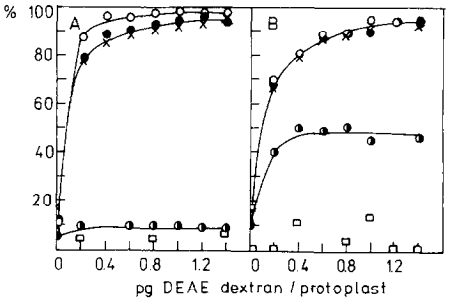

Fig. 1. Release of enzymes from protoplasts treated with DEAE dextran. Protoplasts of S. cerevisiae DT XII were exposed for 4 min. at 30°C to DEAE dextran at concentrations given at the abscissa, then centrifuged and enzyme activities were measured in supernatants and pellets. Ordinate: Per cent of activities in supernatant with respect to total activities.
O glucose-6-dehydrogenase (A) and hexokinase (B);
O alcohol dehydrogenase (A) and pyruvate kinase (B);
x α-glucosidase (A) and aldolase (B);
O citrate synthase (A) and malate dehydrogenase (B);
lactate dehydrogenase (A) and proteinase A (B).

treated protoplast. In contrast, less than 10% of the mitochondrial enzymes (citrate synthase and lactate dehydrogenase) and a vacuole enzyme (proteinase A (14)) were recovered in the soluble fraction. The remaining activity of the enzymes were recovered in the pellet. The distribution of malate dehydrogenase (Fig. 1) is consistant with a mixed cytosolic and mitochondrial location of the enzyme, as previously reported (15).

Release of hexokinase (Fig. 1) follows closely the release of two other glycolytic enzymes, pyruvate kinase and aldolase, suggesting that yeast hexokinase is not bound to mitochondria. This conclusion is supported by experiments in which no binding of purified yeast hexokinase to isolated yeast mitochondria was observed in vitro either in the absence or presence of 100 mM glucose and/or 10 mM $MgCl_2$ (not shown). Alcohol dehydrogenase, glucose 6-phosphate dehydrogenase and α-glucosidase were released at slightly lower concentrations of DEAE dextran than the former three glycolytic enzymes. The significance of this observation is not known. The experiment shown in Fig. 1 was done 3 times with essentially identical results.

Cells grown under conditions of severe catabolic repression (exponential phase of growth on 10% glucose) were also studied. Activities of lactate dehydrogenase, α-glucosidase and, interestingly, proteinase A were too low to measure under the conditions employed. Specific activities of other enzymes in the glucose-repressed and derepressed cells are shown in Table 1. Intact protoplast were difficult to prepare from these cells, and considerable enzyme activities were found in the supernatant even without DEAE treatment. The distribution of hexokinase followed closely that of alcohol dehydrogenase, pyruvate kinase, glucose 6-phosphate dehydrogenase and aldolase. More than 90% of the total activity of these enzymes was found in

Table 1. Specific activities of enzymes in protoplasts from glucose-repressed and derepressed cells

Enzyme	Derepressed cells	Repressed cells
Hexokinase	65	48
Glucose-6-phosphate dehydrogenase	5.3	3.2
Aldolase	1.6	1.6
Pyruvate kinase	152	263
Alcohol dehydrogenase	185	195
Malate dehydrogenase	105	14

Yeast cells were grown on 10% glucose to exponential phase (repressed cells) or on 0.3% glucose to stationary phase (derepressed cells). Activities are expressed in nmol NAD(P) changed/min/10^8 protoplast.

the supernatant fraction. Thus, even under conditions in which yeast cells are forced to use glucose as a major energy source, hexokinase is not apparently associated with subcellular structures.

Digitonin, which is used to fractionate mammalian cells (16,17), was also applied to yeast protoplasts. Digitonin forms complexes with cholesterol, the essential constituent of the mammalian cell plasma membrane, as well as with ergosterol, the sterol present in yeast plasma membranes (18). Surprisingly, attempts to apply the digitonin method to yeast cells were not successful. Even prolonged treatment of protoplasts (up to 20 min.), both at 0°C and 30°C, with high concentratons of digitonin (up to 0.2% with 5 x 10^8 protoplasts) failed to release all alcohol dehydrogenase activity unless the protoplasts were extensively vortexed. Only 6% of the total alcohol dehydrogenase activity of cells grown on glucose is due to the mitochondrial form of the enzyme (19). Digitonin fractionation of protoplasts also did not discriminate between the different cellular compartments. Citrate synthase (mitochondrial matrix) and succinate dehydrogenase (inner mitochondrial membrane) were

released into the supernatant to the same extent as alcohol dehydrogenase (cytosolic).

In summary, a mild procedure for measuring the intracellular distribution of yeast enzymes has been developed. Application of this method does not provide evidence for a substantial binding of hexokinase to mitochondria or other cellular organelles in S. cerevisiae under conditions of catabolic repression or derepression. However, the procedure would probably not discern hexokinase binding to the plasma membrane or to certain fragile cytoskeletal elements. Binding of small amounts of hexokinase to the plasma membrane is inferred from the proposed role of hexokinase II in glucose transport in S. cerevisiae (20). The present results do suggest, however, that a physical association of hexokinase cannot be invoked to support the contention that yeast cells preferentially use ATP generated by mitochondria (21). The findings are consistent with the conclusion drawn from other types of experiments (17,22) that reversible hexokinase binding to mitochondria plays little or no role in regulating glucose phosphorylation and glycolysis.

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