Genetic Evidence for a Role of Hexokinase Isozyme PII in Carbon Catabolite Repression in *Saccharomyces cerevisiae*

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A mutant of *Saccharomyces cerevisiae* that was selected for resistance to carbon catabolite repression also had reduced hexokinase activity. Hexokinase isoenzymes were purified from mutant and wild type cells. The specific glucokinase and hexokinase isozyme PII were present at normal levels in mutant and wild type, but no hexokinase isozyme PII activity was detected in the mutant. Staining for enzyme activity after electrophoresis of crude extracts also indicated that hexokinase PII was absent in the mutant. Mutant and wild type segregants gained by tetrad analysis were investigated electrophoretically. Staining for enzyme activity confirmed that catalytically inactive hexokinase PII and the defect in carbon catabolite repression always co-segregated. The results support the hypothesis that hexokinase PII might mediate carbon catabolite repression.

In the yeast *Saccharomyces cerevisiae* enormous differences in the activities of certain enzymes have been reported between cells growing on glucose and on nonfermentable carbon sources, such as ethanol or acetate. Such differences were observed for some enzymes of the tricarboxylic acid cycle and glyoxylate cycle (Polakis and Bartley, 1965), respiratory enzymes (Polakis et al., 1965), gluconeogenic enzymes (Gancedo et al., 1965; Witt et al., 1965; Gancedo and Schwerzmann, 1976), α-glucosidases (Wijk and van Ouwehand, 1969), and invertase (β-fructofuranosidase) (Gascón et al., 1968). This phenomenon has been called carbon catabolite repression in analogy to the situation defined in bacteria by Magasanik (1961). A very effective selection system for isolating mutants resistant to carbon catabolite repression was described by Zimmerman and Scheel (1977). Three mutant classes were identified (Zimmerman and Scheel, 1977; Entian and Zimmerman, 1980). (a) *hex1* mutants were no longer repressible by glucose for invertase, maltase, malate dehydrogenase, and respiratory enzymes. Hexokinase activity was strongly decreased (Entian et al., 1977). (b) *hex2* mutants had a similar defect in carbon catabolite repression, but their hexokinase activity was strongly elevated (Entian and Zimmerman, 1980). Elevation of hexokinase activity could be attributed to increased hexokinase PII synthesis (Entian, 1981). Additionally, the maltose uptake system was disregulated in *hex2* mutants, which led to a strong inhibition of cell metabolism by maltose (Entian, 1980). (c) The third class of mutants, called *cat80*, was defective in repression of invertase, maltase, and malate dehydrogenase, but the hexokinase activity was like wild type (Entian and Zimmerman, 1980).

According to the hypothesis of Magasanik (1961), carbon catabolite repression is triggered by an accumulation of catabolic derivatives of hexoses. However, determination of such metabolites during growth on hexoses in these catabolite repression-resistant mutants did not indicate such a simple situation (Entian and Zimmerman, 1980). The abnormal hexokinase activities in *hex1* and *hex2* mutants suggested an important role of hexokinase in carbon catabolite repression.

There are three enzymes that can phosphorylate d-glucose in *S. cerevisiae*. One is specific for glucose and is called glucokinase (Maitra, 1970; Ramel et al., 1971). The others, called hexokinase isozymes PII and PII, are nonspecific and can phosphorylate glucose, fructose, and mannose. PII and PII can be distinguished by their different ratios of fructose over glucose phosphorylation. P F/G for PII is about 3 and for PII is about 1.2 (for a review, see Colowick, 1973). Two active forms of hexokinase PII (called HK B and HK C) are reported in the literature. HK C is converted to HK B by high ionic strength at pH values below 5 (Ramel et al., 1971). To clarify the role of hexokinases during carbon catabolite repression, hexokinase isoenzymes in a *hex1* mutant were investigated. The results showed that *hex1* mutants are specifically defective in hexokinase isozyme PII.

EXPERIMENTAL PROCEDURES

The strains were cat2.3-2A (α his4 MAL2-1 MAL3-1 CAT1-1 HK1), and cat2.1-2A-18 α his4 MAL2-1 MAL3-1 CAT1-1 HK1.

* corresponds to the mating type; and *his4* causes nutritional requirement for histidine. MAL2-1 causes largely constitutive, but glucose-repressible synthesis of maltase (Zimmermann and Paffen, 1974) which allows for an induced synthesis of maltase. However, such is the structural gene of invertase (Grossmann and Zimmermann, 1979). cat2-1 allows rapid derepression of invertase. Both malase and invertase are induced by maltose (Zimmermann et al., 1977). cat2-1 allows rapid derepression of various enzymes after growth on glucose (Zimmermann et al., 1977). *hex1* causes non-repressible maltase, invertase and malate dehydrogenase (Zimmermann and Scheel, 1977; Entian and Zimmermann, 1980).

1 The abbreviations used are: Q F/G, ratio of fructose over glucose phosphorylating activity; PMSF, phenylmethylsulfonyl fluoride; MTT, 3-(4,5-dimethylthiazolyl)-2,5-diphenyl tetrazoliumbromide; EDTA, ethylenediaminetetraacetate.

2 Portions of this paper (including "Experimental Procedures." Figs 5 and 6, and Table III) are presented in miniprint as prepared by the authors. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 3060 Rockville Pike, Bethesda, MD 20814. Request Document No. 81M-1988, cite the authors, and include a check or money order for $4.00 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.
Enzyme and Protein Determination

Hexokinase activity with glucose as substrate was tested according to \( \text{ref.}\). When fructose was used as a substrate, 0.5 U/ml glucose-6-phosphate dehydrogenase was added. Maltase and invertase were assayed according to \( \text{ref.}\). Specific activity is defined at 280 nm.

Purification Procedure

Buffers used: K-Po4 buffer pH 6.5, sucrose buffer, 1 M EDTA, pH 6.5.

Cells were grown in 5 l flasks, containing about 1.5 l of culture media, on a rotating shaker at 115 rpm (Lab shaker, Braun-Melsungen, F.R.G.). Components of the medium were yeast extract, 24 maltose, peptone (both Difco) and 4% D-glucose. 20 ml K-Po4 buffer were added to the suspension for cooling.

Washed twice with 100 ml K-Po4 buffer and frozen until use. Washed weight was about 280 g.

Cell Disruption

220 g of cells (wet weight) were suspended in 100 ml K-Po4 buffer. 720 ml suspension was cooled at high speed in a laboratory blender (Karing 1 BAW-GL, Braun-Melsungen) for 10 min. The temperature did not exceed 25°C.

Ammonium Sulfate Chromatography According to King (1972)

Gels were stained for protein with Coomassie blue.

Electrophoretic Procedures

Discontinuous gel electrophoresis was carried out in the Ultras Phor apparatus (Lorch, F.R.G.). Using the system of O'Farrell (1974). Gels were stained for protein with Coomassie blue.

Hexokinase activity was detected by the formazan procedure (Thorne et al., 1963). A method developed by Frohlich and Zittin (unpublished results) was used. Gels were cut to slices of about 1 mm. Each slice was incubated with 0.5 ml hexokinase assay mixture for 10 to 45 min, depending on enzyme activity. Afterwards 0.5 ml formazan mixture (100 mg MTT, 5-dimethyl-thiazolyl-2,5-diphenyl tetrazoliumbromide (Serva) and 17.5 mg phenazine methosulphate (Serva) per 100 ml K-Po4 buffer) was added. The solution was immediately transferred to another vessel. The enzymatic reaction stopped immediately, because hexokinase remained in the gel. The dye reaction needed about 30 sec and was stable for at least 10 min. Extinction was measured at 578 nm.

Ammonium Sulfate Chromatography

To inhibit proteases (Easterby and Rosemeyer, 1972), a 40 mM solution of PMSF (phenylmethylsulfonylfluoride) (Sigma) in absolute ethanol was slowly added to give a final concentration of 0.2 ml, nuclei acids were precipitated by streptomycin sulfate (Serva, Heidelberg) at a final concentration of 1%. After 20 min stirring, the suspension was centrifuged (30 min, 27 000 g).

DE 52 Chromatography

Active fractions were pooled and diluted 1:10 to allow direct addition onto a DE 52 column (Whatman, 2 cm, bed height 15 cm). In contrast to Barnard (1975), the DEAE cellulose was equilibrated with succinate buffer, pH 6.3, because hexokinase PI did not bind satisfactorily at pH 5.8, when the diluted pool was used. A slightly concave pH gradient was used for elution (120 ml pH 6.3 to 4.6, flow rate 30 ml/h). Active fractions of isoenzymes PI and PII were pooled and purified simultaneously. The pools were carefully titrated to pH 6.3 using NaOH and passed onto another DE 52 column (diameter 1 cm, bed height 10 cm, equilibrated with succinate buffer). A 200 mM salt gradient (0-0.2 M KC1) was used for elution (fraction volume 1 ml, flow rate 20 ml/h). Active fractions were pooled, diluted 1:10 and finally passed onto a similar column. Hexokinase activity was eluted by a pH-gradient (200 ml, pH 6.3 to 4.6, fraction volume 1 ml, flow rate 20 ml/h). Profiles of protein and hexokinase activity were identical (Fig. 5 and 6, see also Table 3).

RESULTS

Comparative Purification of Hexokinases from an \textit{hexl} Mutant and Wild Type Cells

Ammonium Sulfate Chromatography—For ammonium sulfate chromatography (King, 1972), proteins, which had been salted out by high ammonium sulfate concentrations, were adsorbed to Celite and packed into a column (see under "Experimental Procedures"). The proteins were eluted by a retrogade ammonium sulfate gradient. Glucokinase, which precipitates below 50% ammonium sulfate saturation, and hexokinases, which precipitate above 50% ammonium sulfate, were completely separated. As shown in Fig. 1a, the hexokinase isozymes PI and PII were also partly separated in extracts of wild type cells. Glucokinase was eluted at 45% saturation, hexokinase PI at 60% saturation, and hexokinase PII at about 53% saturation. In the \textit{hexl} mutant (Fig. 1b), glucokinase was eluted at 43% saturation, similar to results with the wild type.
Yeast Hexokinase PII and Carbon Catabolite Repression

For further purification the hexokinase PII fractions were pooled from the wild type and the hex1 mutant, respectively. These pools were placed on a DEAE-Sephacel ion exchanger and eluted with a linear salt gradient (0-0.2 M KCl). This fast chromatography did not separate the isoenzymes, but yielded a good purification (see Table III).

**Separation of Hexokinases Isozymes PII and PII**—Wild type isoenzymes PII and PII were separated by elution with a pH gradient on DEAE-cellulose according to Barnard, 1975, with modifications (see under “Experimental Procedures”).

PII was eluted at pH 5.5 and PII at pH 5.1 (Fig. 2a). In the extracts. However, the elution profile of the hexokinases differed from wild type. Q F/G ratios and ammonium sulfate concentration of resolution indicated that only isozyme PII was present in the hex1 mutant.

**Fig. 2. Elution profile of the hexokinases after DE52 cellulose chromatography.** ○○, glucose activity; △△, fructose activity; ■■, pH. a, wild type; b, hex1 mutant.

**Fig. 3. Disc electrophoresis pattern of the purified hexokinases PII and PII of wild type.**

**Fig. 4. Hexokinase activity after disc electrophoresis.** ○○, glucose activity; △△, fructose activity. a, crude extract, wild type; b, purified PII, wild type; c, purified PII, wild type; d, crude extract, hex1 mutant; e, purified PII, hex1 mutant.
Yeast Hexokinase PII and Carbon Catabolite Repression

The separated isoenzymes were further purified using two smaller DEAE-cellulose columns, which were eluted with a salt gradient and a pH gradient, respectively. In the *hex1* mutant and wild type preparations, isoenzyme PII had the same elution behavior. No additional enzyme was detected in *hex1* mutant. Isoenzyme PII from wild type cells was purified similarly. These purification procedures yielded single bands for hexokinases PII and PIII on polyacrylamide disc electrophoresis (Fig. 3).

Depending on the purification conditions, hexokinases PII and PIII can change their electrophoretic behavior. Proteases in crude extracts, which can modify PII and PIII without loss of catalytic activity, are responsible for these changes (see Colowick, 1973). Only direct comparison by electrophoresis between crude extract enzymes and purified enzymes could detect those proteolytic modifications during enzyme preparation. Direct staining of gels for hexokinase activity was unsatisfactory, especially with fructose as substrate, because the two indicator enzymes needed caused extensive diffusion

**Table I**

Genetic analysis of the *hex1* mutant

Co-segregation of a carbon catabolite repression defect and the absence of hexokinase PII

| Segregant | Specific activity on 4% glucose (milliunits/mg) | Hexokinase isozymes detected by electrophoresis |
|-----------|-----------------------------------------------|-----------------------------------------------|
| A         | 32.6 806                                     | PI no PII                                     |
| B         | 46.8 1605                                    | PI no PII                                     |
| C         | 0.2  3.2                                     | PI PII                                        |
| D         | 0.4  7.1                                     | PI PII                                        |
| E         | 1.2  6.8                                     | PI PII                                        |
| F         | 37.1 1118                                    | PI no PII                                     |
| G         | 41.5 1248                                    | PI no PII                                     |
| H         | 0.4  3.1                                     | PI PII                                        |
| I         | 45.7 876                                     | PI no PII                                     |
| J         | 0.9  4.1                                     | PI PII                                        |
| K         | 1.4  5.5                                     | PI PII                                        |
| L         | 39.0 1408                                    | PI no PII                                     |

* Italics indicates that high values for maltose and invertase were always accompanied with a loss of PII activity.

**Table II**

\( K_m \) values of hexokinase PII isozymes purified from wild type and an *hex1* mutant

| Substrate | Co-substrate | cat2.3-2A (wild-type) | cat2.3-2A/18 (hex1 mutant) |
|-----------|--------------|-----------------------|---------------------------|
| Glucose   | ATP          | 0.11                  | 0.11                      |
| Fructose  | ATP          | 1.16                  | 1.18                      |
| ATP       | Glucose      | 0.20                  | 0.20                      |
| ATP       | Fructose     | 0.10                  | 0.10                      |

**hex1** mutant, only isoenzyme PI was eluted at pH 5.5 (Fig. 2b). No further activity was resolved, even after the addition of 1 M KCl to elution buffer. This indicated that no additional activity was retained on the column. Recovery of enzyme activity was 93% in the *hex1* mutant. These results clearly indicated that isoenzyme PII was not present in the *hex1* mutant.
of bands. Instead, the gels were cut into 1 mm slices, and the enzyme activity was assayed in each slice. No differences in electrophoretic mobility between crude extract enzymes and purified enzymes PI and PII were detected in wild type cells. The positions of hexokinase activity and the positions of protein bands after purification were identical (Fig. 4, a, b, and c). hexl crude extracts had only PI activity. Purified hexokinase PII from the hexl mutant and the PI of mutant crude extracts had the same electrophoretic mobility as hexokinase PI of wild type (Fig. 4, b, d, and e). This also indicated that hexokinase PII, if present at all, was not catalytically active in the hexl mutant.

Co-segregation of the Catabolite Repression Defect and Loss of PII Activity in hexl Mutants

After an appropriate cross, diploid cells were obtained that contained mutant allele hexl and wild type allele HEXL. After sporulation and tetrad analysis, segregation of mutant allele hexl was followed. All of the segregants of three tetrads were investigated by electrophoresis and staining for hexokinase activity. All of the segregants that were defective in carbon catabolite repression also had no hexokinase PII activity (Table I), which confirms that a defect in carbon catabolite repression and the loss of hexokinase PII activity are directly associated.

Substrate Saturation Kinetics of Hexokinase PI from the hexl Mutant and Wild Type Cells

As shown in Table II, Kᵢ values for glucose, fructose, and ATP as substrates were the same for hexokinase PI from the hexl mutant and the wild type cells. This gave further evidence that decreased hexokinase activity in the hexl mutant was not due to alterations in the properties of hexokinase PI.

DISCUSSION

The results described here show that the decreased hexokinase activity in hexl mutants is attributable to an absent or catalytically inactive hexokinase PII. The activity and kinetic behavior of hexokinase PI was not affected. About 500 spontaneous mutants, which are allelic to the hexl mutant have so far been isolated according to the very efficient selection system of Zimmermann and Scheel (1977). At least 50 of them were also tested for hexokinase activity. In all cases hexokinase activity was additionally reduced. Since hexl mutant was isolated, about 100 tetrads have been investigated. In all cases low hexokinase activity and defect in carbon catabolite repression co-segregated. All efforts to identify alterations in the concentrations of glycolytic derivatives of glucose in carbon catabolite repression mutants have been unsuccessful (Entian et al., 1977; Entian and Zimmermann, 1980). This made the hypothesis of Magasanik (1961) that carbon catabolite repression might be triggered by an accumulation of glycolytic derivatives of hexoses unlikely. On the other hand, the properties of the hexl mutant and the central role of hexokinase in glycolysis give evidence that hexokinase PII might trigger carbon catabolite repression. We suggest that hexokinase PII is a bifunctional enzyme, having a catalytic and a regulatory function. As proposed by Zimmermann,² we propose a role for hexokinase PII as the "recognition site" of carbon catabolite repression. Such a role would require that the enzyme also be able to respond in some way to the availability of hexoses or their derivatives.

Indeed, this proposed regulatory function might be associated with some known effects of hexoses and glycolytic intermediates on hexokinase PII (for a review, see Colowick, 1973). The addition of glucose leads to dissociation of the enzyme into catalytically active monomers, a phenomenon which was observed for hexokinase PII (Derechin et al., 1972) and hexokinase PI (Eastery and Rosemeyer, 1972).

The presence of ATP induces association of hexokinase PII (Derechin et al., 1974). X-ray structure analysis by Steitz et al. (1977) gave evidence for an allosteric activator site for ATP which lies between the subunits. At low ATP concentrations a pronounced activation by 3-phosphoglycerate and phosphate has been reported (Kosow and Rose, 1971). This, however, was probably an artifact resulting from chelation of inhibitory aluminium present in ATP preparations (Viola et al., 1980). Furthermore, two conformations of hexokinase PII have been described (see Barnard, 1975). Possibly one of these properties of hexokinase PII might be involved in the sequence of events that bring about carbon catabolite repression.

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² F. K. Zimmermann, personal communication.
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