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Genetics

Catabolite inactivation of gluconeogenic enzymes in mutants of yeast deficient in proteinase B

(proteolysis/futile metabolic cycles)

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ABSTRACT Strains of *Saccharomyces cerevisiae* bearing nonsense mutations in the structural gene for proteinase B (EC 3.4.22.9) have been examined for the ability to make the transition from growth on acetate to growth on glucose and for the ability to inactivate three gluconeogenic enzymes during the transition because proteinase B has been proposed by others to be responsible for the inactivation of the three enzymes during the growth transition. The mutant strains make the growth transition normally. Catabolite inactivation of hexosediphosphatase (D-fructose-1,6-bisphosphate 1-phosphohydrolase, EC 3.1.3.11), malate dehydrogenase (L-malate:NAD$^+$ oxidoreductase, EC 1.1.1.37), and phospho*enol*pyruvate carboxykinase (ATP) [ATP:oxaloacetate carboxy-lyase (transphosphorylating), EC 4.1.1.49] occurred in *prb1* mutants with kinetics similar to those seen in wild-type strains. We infer that proteinase B activity is not essential for the process of catabolite inactivation.

Futile cycles might result if the enzymes in the glycolytic and gluconeogenic pathways were to operate simultaneously. For example, if the reactions catalyzed by 6-phosphofructokinase (ATP:D-fructose-6-phosphate 1-phosphotransferase, EC 2.7.1.11) and hexosediphosphatase (D-fructose-1,6-bisphosphate 1-phosphohydrolase, EC 3.1.3.11) were to proceed coincidentally, and at comparable rates, wasteful hydrolysis of ATP would result (Fig. 1). For this reason, the mechanism(s) that enables an organism to negotiate the transition from one mode of growth to the other has received much attention (1).

When glucose is added to a yeast culture that is using acetate as a source of carbon and energy, the gluconeogenic enzymes hexosediphosphatase, the cytoplasmic isozyme of malate dehydrogenase (L-malate:NAD$^+$ oxidoreductase, EC 1.1.1.37), and phospho*enol*pyruvate carboxykinase (ATP) [ATP:oxaloacetate carboxy-lyase (transphosphorylating), EC 4.1.1.49] are inactivated (2–5). This process has been termed catabolite inactivation. It has been suggested that catabolite inactivation prevents futile cycles that might be catalyzed by cofunctioning glycolytic and gluconeogenic enzymes (6). It has been proposed that catabolite inactivation involves proteolysis of the target enzymes, for disappearance of cytoplasmic malate dehydrogenase antigen accompanies the inactivation of the enzyme activity (7, 8). Proteinase B (EC 3.4.22.9) has been suggested as the enzyme responsible for this inactivation (6, 9, 10).

We have isolated strains bearing mutations in the structural gene for proteinase B (11, 12). We have reported that proteinase B is not essential for synthesis of the chitin containing septum during cell division (11, 12) contrary to what had been proposed (13). In this paper we report our investigation on the role of proteinase B in catabolite inactivation. The effect on growth of the addition of glucose to acetate-grown cultures of these mutants and the kinetics of inactivation of hexosediphosphatase, malate dehydrogenase, and phospho*enol*pyruvate carboxykinase during this growth transition are presented.

MATERIALS AND METHODS

Materials. Yeast extract, Bacto-peptone, and glucose were purchased from Difco. Manganous chloride, magnesium chloride, sodium acetate, and sodium bicarbonate were obtained from J. T. Baker. All other materials and enzymes were purchased from Sigma.

Strains. The wild-type strain used in this study was M16-14C, genotype *a leu1-1 ser1-171*. The mutant strain was isolated from M16-14C, bears an amber mutation in the structural gene for proteinase B, and is of genotype *a leu1-1 ser1-171 prb1-9*. The two strains are isogenic with the exception of the *prb1* mutation. The *prb1-1122* mutant was isolated in sl 26 (12), a derivative of X2180-1B and, aside from the *prb1-1122* mutation, is isogenic to X2180-1B carrying a *trp1* allele. The symbols *leu1, ser1,* and *trp1* refer to the requirements for the amino acids leucine, serine, and tryptophan, respectively; *prb1-9* is an amber mutation and *prb1-1122* an ochre mutation in the structural gene for proteinase B (11, 12).

Media. Minimal medium was prepared according to the recipe for Yeast Nitrogen Base without amino acids except that glucose was omitted (14). It was made 60 mM in sodium acetate. Leucine and serine were added to a concentration of 0.46 and 0.48 mM, respectively. YEPD medium consisted of 1% yeast extract, 2% Bacto-peptone, and 2% glucose.

Growth of Strains. For growth experiments, cells were inoculated in 5 ml of minimal medium and grown overnight at 30°C. On the following day an appropriate dilution of this inoculum was added to a side-arm flask containing the same medium and the culture was incubated in a temperature-controlled gyrotory shaker bath at 30°C. The turbidity of the cultures was monitored with a Klett–Summerson photoelectric colorimeter with a no. 54 filter. The cultures were made 2% in glucose by the addition of a 20% glucose solution that had been filter sterilized.

For inactivation experiments, cells were inoculated into Fernbach flasks containing 1.2 liters of YEPD medium and were grown to stationary phase at 30°C in a New Brunswick temperature-controlled gyrotory shaker. After 52 hr, the cells were harvested at 4°C by centrifugation in sterile canisters at 5000 × *g* in a Sorvall RC-5 refrigerated centrifuge. The cells were washed with sterile distilled water and repelleted under the same conditions. The cells were then resuspended in minimal medium containing leucine and serine but lacking a carbon source. Carbon starvation was accomplished by shaking the cells in this medium overnight at 30°C.

Inactivation of Gluconeogenic Enzymes. Cultures that had been starved for carbon were made 60 mM in sodium acetate.

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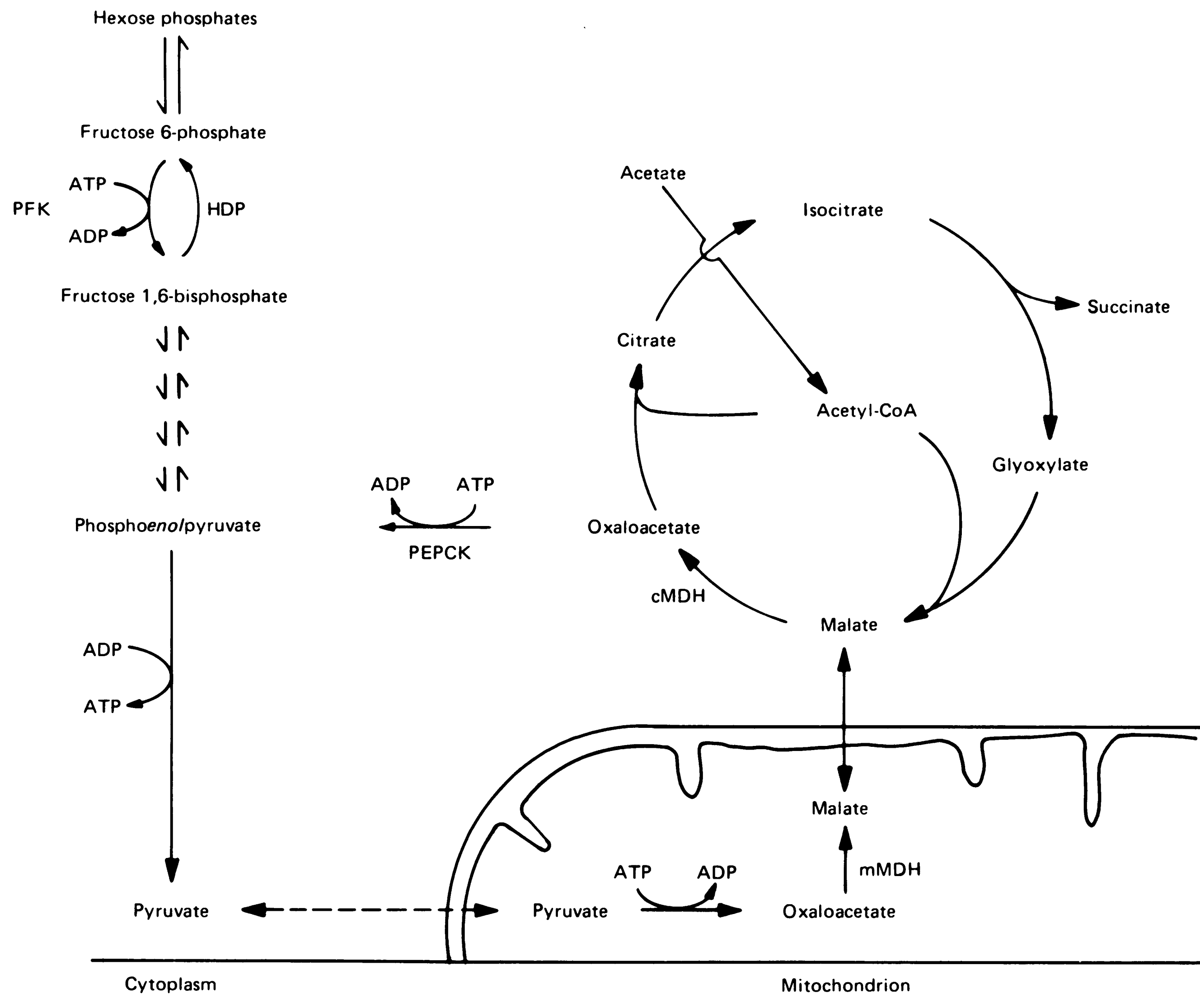

FIG. 1. Diagrammatic representation of glycolysis and gluconeogenesis illustrating potential futile cycles. Enzymes showing catabolite inactivation are HDP (hexosediphosphatase), PEPCK (phospho*enol*pyruvate carboxykinase), and cMDH (cytoplasmic malate dehydrogenase). Other abbreviations are PFK (phosphofructokinase) and mMDH (mitochondrial malate dehydrogenase).

Shaking at 30°C was continued. After 4 hr, the cultures were split and half of each original culture was made 2% in glucose. At intervals, 50-ml samples of the cultures were transferred to crushed ice and the cells were harvested at 4°C by centrifugation at 5000 × *g*. The pellets were washed once with distilled water, recentrifuged, and frozen until the following day.

Preparation of Cell Extracts. Cell extracts were prepared in 100 mM Tris·HCl (pH 7.6) according to Jones (15), except that titration with acetic acid was omitted.

Assay of Gluconeogenic Enzymes. Hexosediphosphatase was assayed according to Gancedo and Gancedo (16). Malate dehydrogenase was assayed by a modification of the method of Ochoa (17). The reaction mixture contained 5 mM $MgCl_2$, 5 mM dithiothreitol, 2.5 mM oxaloacetate, and 0.25 mM reduced 3-acetylpyridine adenine dinucleotide in 50 mM phosphate buffer (pH 7.5). Phospho*enol*pyruvate carboxykinase was assayed in the direction phospho*enol*pyruvate to oxaloacetate by a modification of the method of Cooper and Wood (18). The reaction mixture contained 2.5 mM $MnCl_2$, 5 mM dithiothreitol, 5 mM ADP, 5 mM phospho*enol*pyruvate, 2.5 mM $NaHCO_3$, 0.25 mM reduced 3-acetylpyridine adenine dinucleotide, and 84 units of porcine mitochondrial malate dehydrogenase.

Protein Determination. Protein concentrations of crude extracts were determined by the method of Lowry *et al.* (19), with bovine serum albumin as the standard.

Units. One unit of enzyme activity is that which catalyzes the conversion of 1.0 μmol of substrate to product per min at 30°C. Specific activities are given in units per mg of extract protein.

RESULTS

If catabolite inactivation of selected gluconeogenic enzymes by proteinase B must occur if futile cycles are to be prevented, then addition of glucose to cultures of proteinase B-deficient mutants growing on acetate should cause a reduced rate of growth or stasis. To test this expectation, wild-type and *prb1-9* mutant cells were grown in minimal medium with acetate as a source of carbon (Fig. 2). During the exponential phase of growth, the mutant and wild-type strains had doubling times of approximately 5 hr. Upon addition of glucose, a 1-hr growth lag occurred for both strains, after which growth resumed. Both the wild-type and mutant strains achieved doubling times of about 3 hr after the lag.

We therefore measured the rates of catabolite inactivation of the gluconeogenic enzymes hexosediphosphatase, cyto-

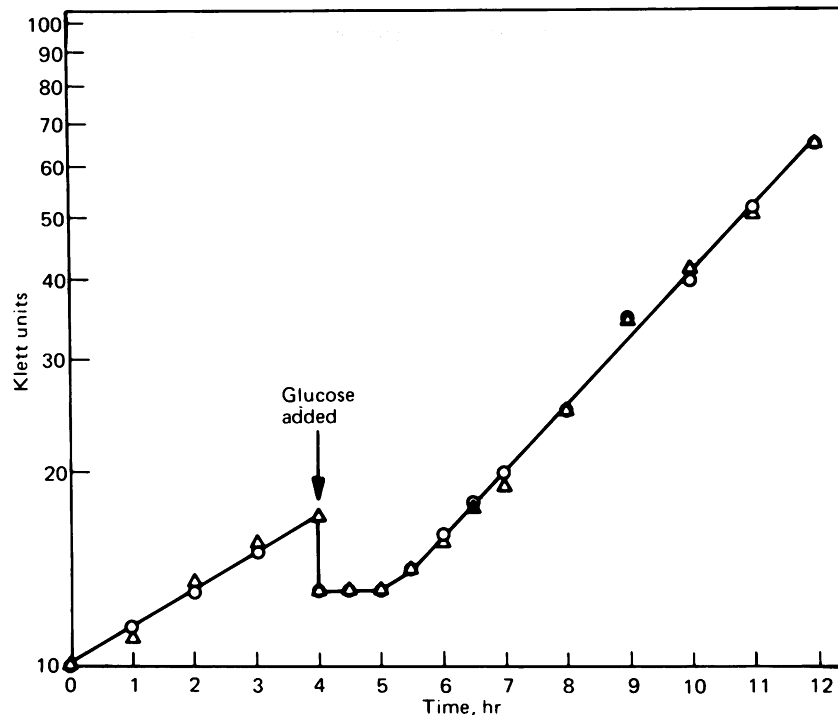

FIG. 2. Effect of glucose addition on growth of cells with acetate as a carbon source. The turbidity of exponential cultures of wild-type cells (△) and *prb1-9* mutant cells (○) growing on acetate was monitored before and after addition of glucose. The drop in turbidity upon the addition of the glucose solution was due to dilution.

plasmic malate dehydrogenase, and phospho*enol*pyruvate carboxykinase. The inactivation of hexosediphosphatase in the wild-type cell and *prb1-9* mutant is shown in Fig. 3. These data are replotted in Fig. 4 to allow the determination of half-lives. The half-lives of the hexosediphosphatase activity are 72 and 61 min for the mutant and wild-type strains, respectively. Data for the catabolite inactivation of malate dehydrogenase are presented in Fig. 5 for the two strains. It has been shown that cytoplasmic malate dehydrogenase is inactivated but the mitochondrial species is not subject to inactivation (5, 7). We have

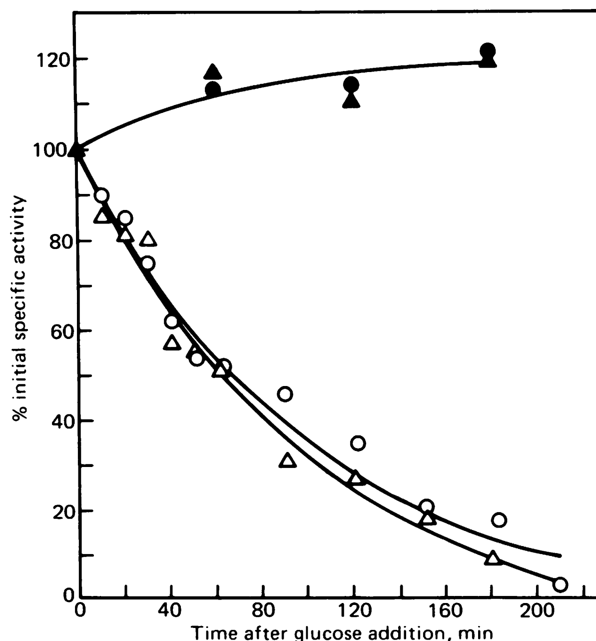

FIG. 3. Catabolite inactivation of hexosediphosphatase in wild-type and *prb1-9* mutant cells. Acetate-grown cultures of wild-type and mutant cells were split and half of each original culture was made 2% in glucose. The initial specific activities were 0.108 and 0.0804 unit/mg for the wild-type and mutant strains, respectively. ▲, Wild type, no addition; △, wild type with glucose; ●, mutant, no addition; ○, mutant with glucose.

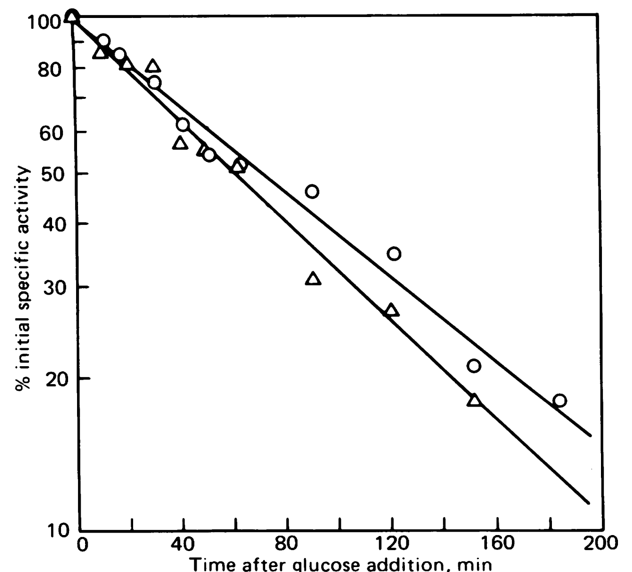

FIG. 4. Catabolite inactivation of hexosediphosphatase in wild-type and *prb1-9* mutant cells. Data in Fig. 2 were replotted on semilogarithmic scale for the determination of half-lives. The lines were determined by linear regression. The half-lives of the hexosediphosphatase activities were 61 min for the wild-type strain (△) and 72 min for the mutant strain (○).

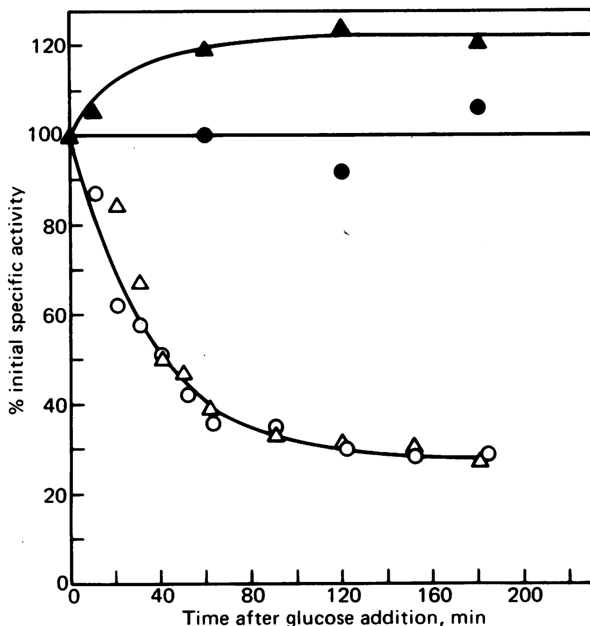

FIG. 5. Catabolite inactivation of malate dehydrogenase in wild-type and *prb1-9* mutant cells. Acetate-grown cultures were split and half of each original culture was made 2% in glucose. The initial specific activities were 0.0518 and 0.0643 unit/mg for the wild-type and mutant strains, respectively. ▲, Wild type, no addition; △, wild type with glucose; ●, mutant, no addition; ○, mutant with glucose.

assumed that the stable component of malate dehydrogenase activity corresponds to the mitochondrial species. To estimate the half-lives for cytoplasmic malate dehydrogenase activity in the two strains, we have subtracted the activity contributed

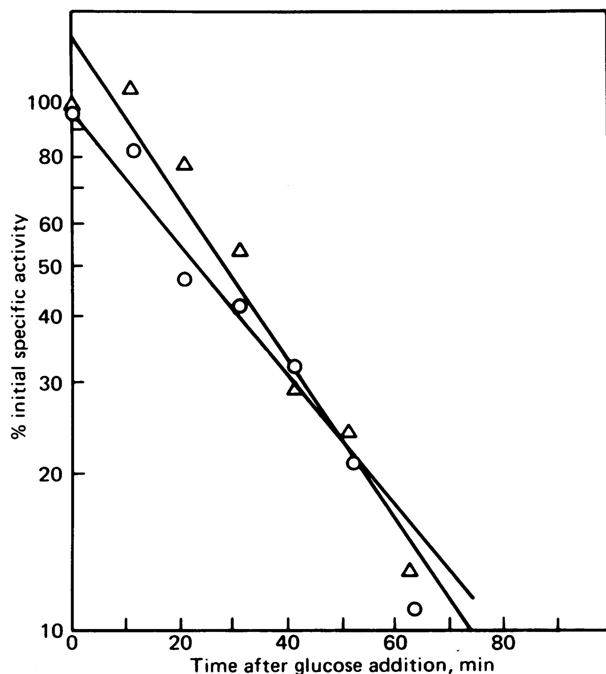

FIG. 6. Catabolite inactivation of cytoplasmic malate dehydrogenase in wild-type and *prb1-9* mutant cells. Data for Fig. 4 were replotted on a semilogarithmic scale after subtraction of the activity contributed by the stable species of malate dehydrogenase (30%). The lines were determined by linear regression. The half-lives of the cytoplasmic malate dehydrogenase activities were 20 min for the wild-type strain (△) and 23 min for the mutant strain (○).

by the stable species (30%) from all points and have replotted the data in Fig. 6. The half-lives of the cytoplasmic malate dehydrogenase activity are 23 and 20 min for the mutant and wild-type strains, respectively. It is clear that catabolite inactivation of hexosediphosphatase and cytoplasmic malate dehydrogenase activities occurred in the *prb1-9* mutant. There was an increase of approximately 15% in the half-lives of the two enzyme activities, but we do not consider this difference to be significant. Phospho*enol*pyruvate carboxykinase is also inactivated in the mutant and wild-type strains. Because inactivation in both strains occurred only after an initial increase in specific activity and because the subsequent loss of activity occurred slowly, it was not possible to determine reliable half-lives for this activity. For both strains, the half-life of phospho*enol*pyruvate carboxykinase was between 2 and 3 hr.

We have extended these observations to strains with the X2180-1B genetic background. A strain that bears an ochre mutation in the structural gene for proteinase B, *prb1-1122*, can negotiate the growth transition from acetate to glucose. Catabolite inactivation in this mutant strain of hexosediphosphatase and cytoplasmic malate dehydrogenase was indistinguishable from that observed in the corresponding wild-type parent. Phospho*enol*pyruvate carboxykinase was not assayed in these experiments.

DISCUSSION

Should the gluconeogenic and the glycolytic pathways function simultaneously and at comparable rates, wasteful hydrolysis of ATP could occur. One such potential futile cycle involves 6-phosphofructokinase and hexosediphosphatase and a second involves phospho*enol*pyruvate carboxykinase and cytoplasmic malate dehydrogenase (Fig. 1). During the transition from the gluconeogenic mode of growth to the glycolytic mode in yeast, it has been observed that the three aforementioned gluconeogenic enzymes are irreversibly inactivated (2–5). This inactivation, thought to prevent futile cycles, has been attributed to proteolysis (7, 8), with proteinase B being the responsible enzyme (6, 9, 10).

Our results with mutants bearing nonsense mutations in the proteinase B structural gene imply that proteinase B is not essential for the catabolite inactivation of these three gluconeogenic enzymes in *S. cerevisiae*. However, these data cannot be interpreted to mean that proteinase B does not participate in the inactivation of these enzymes. It is possible that proteinase B is but one of several enzymes responsible for catabolite inactivation *in vivo* and, in the absence of proteinase B, inactivation occurs through the agency of other proteolytic enzymes. It is also possible that in the absence of proteinase B activity an auxiliary mechanism catalyzes the inactivation. Our results provide no information on whether futile cycles can occur in yeast because catabolite inactivation of the three gluconeogenic enzymes took place in the *prb1* mutants.

It is possible, but unlikely, that the nonsense mutations we have recovered in *prb1* are a selected group of mutations that cause the production of nearly complete proteinase B molecules that possess biologically significant activities below our limit of detection but that are still capable of carrying out catabolite inactivation of the target enzyme. Of the 126 *prb1* mutations that we have obtained, 46 are amber or ochre mutations (unpublished data). This frequency is comparable to those observed for other structural genes (20–23). In addition, our assay for proteinase B is capable of detecting activities as low as 2% of those observed for the wild-type strain. We have never observed proteinase B activity in crude extracts made from mutants carrying nonsense mutations at *prb1*. However, final resolution of this question awaits the isolation of *prb1* deletions or the construction of a fine-structure map of the locus.

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