METABOLIC CONTROL OF β -GLUCOSIDASE SYNTHESIS IN YEAST

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ABSTRACT

MACQUILLAN, ANTHONY M. (University of Wisconsin, Madison) AND HARLYN O. HALVORSON. Metabolic control of β -glucosidase synthesis in yeast. *J. Bacteriol.* **84**:23-30. 1962—The hybrid *Saccharomyces fragilis* $\times$ *S. dobzhanskii* produced a constitutive β -glucosidase when grown in succinate synthetic medium. Upon addition of β -glucosides, thio- β -glucosides, or low concentrations of glucose, a further induction of enzyme synthesis was observed. Studies with other sugars revealed some specificity in response to hexose induction. Phenyl-thio- β -D-glucoside did not affect constitutive synthesis nor induction by glucosides, thio-glucosides, or glucose. Repression of β -glucosidase synthesis is brought about by high concentrations of glucose and other carbon compounds. Preinduction does not confer resistance to catabolic repression of enzyme synthesis; this leads to the conclusion that two sites of control for β -glucosidase synthesis are present in yeast. Multiplicity of control is further suggested from: (i) the properties of the inducing system; (ii) semiconstitutive nature of enzyme synthesis; (iii) the repression of constitutive synthesis by glucose; (iv) the elevated derepressed rates of enzyme synthesis after glucose inhibition; and (v) the selection of a family of low constitutive mutants with variable inducibility.

We previously reported (MacQuillan, Winderman, and Halvorson, 1960) that the constitutive synthesis of β -glucosidase in the yeast hybrid *Saccharomyces fragilis* $\times$ *S. dobzhanskii* is subject to metabolic control. Enzyme synthesis was inhibited by a variety of carbon sources including glucose, acetate, and lactate. These findings, as well as an inductive response of enzyme synthesis to low levels of glucose, suggested that at least two distinct sites must be

involved in regulation of β -glucosidase synthesis. The present experiments were undertaken to further clarify the nature and specificity of the metabolic controls operative in induction and repression and to provide a background for genetic studies on these control mechanisms. The induction (Duerksen and Halvorson, 1959) and the isolation and characterization of a β -glucosidase in *Rhodotorula minute* (Duerksen and Halvorson, 1958) provided a basis for some of the studies included in the present paper.

MATERIALS AND METHODS

Organisms. β -Glucosidase is produced constitutively by the yeast strains *S. fragilis* Y610 and *S. dobzhanskii* Y1974. Higher constitutive amounts of the enzyme are synthesized by a hybrid of the above species, *S. fragilis* Y610 $\times$ *S. dobzhanskii* Y1974, and, therefore, the majority of the present experiments were performed with this hybrid. All of these yeasts were obtained from L. J. Wickerham.

Mutant yeasts with low constitutive levels of β -glucosidase were obtained by ultraviolet irradiation of cultures suspended in distilled water. Colonies negative for growth on cellobiose were selected, transferred into succinate synthetic medium, and the differential rate of constitutive β -glucosidase synthesis was determined.

Conditions of growth and enzyme determination. Yeast cultures were grown overnight in succinate (0.2%) synthetic medium (pH 5.4), and, periodically, samples were removed and assayed for β -glucosidase; the differential rate of enzyme synthesis ($\Delta E/\Delta OD$) was determined as previously described (Duerksen and Halvorson, 1959). A unit of enzyme is defined as that amount of enzyme necessary to bring about an optical density change of 0.001 per min at 400 m μ per 3 ml of reaction mixture at 30 C, employing 10^{-3} M *p*-nitrophenyl- β -glucoside as substrate. During the course of this study the control level of β -glucosidase synthesis has slowly increased, pre-

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sumably owing to selection of high enzyme-producing strains in succinate synthetic medium. The qualitative and quantitative response to metabolic control, however, has remained the same.

α -Glucosidase and β -galactosidase were assayed by the procedures of Halvorson and Elias (1958) and Lederberg (1950), employing the chromogenic substrates, *p*-nitro-phenyl- α -D-glucoside (α -pNPG), and *o*-nitro-phenyl- β -D-galactoside (ONPG), respectively.

Reagents. Ethyl- β -thio-D-glucoside (TEG) was synthesized by R. Young by mercaptolysis of glucose (Lemieux, 1951). The removal of glucose and other contaminants was accomplished by chromatography in 80% acetone on Whatman powdered cellulose. After chromatography the compound was twice crystallized from ethanol-ethyl acetate, and the crystals were dried in vacuo (65 C, mp, 99.5 C). The *p*-nitro-phenyl- β -D-glucoside (β -pNPG) and the ONPG were purchased from the California Corp. for Biochemical Research. The α -pNPG was synthesized by J. W. Gorman by the method of Montgomery, Richtmyer, and Hudson (1942). Sources of other compounds used have been described previously (Duerksen and Halvorson, 1958).

RESULTS

Effects of glucosides. The differential rate ($\Delta E/\Delta OD$) of constitutive β -glucosidase synthesis by the hybrid yeast *S. fragilis* Y610 $\times$ *S. dobzhanskii* Y1974 growing on succinate synthetic medium is approximately one-half that of the fully induced *Rhodotorula minute* (Duerksen and Halvorson, 1959). Among a number of glucosides and thioglucosides tested for induction of the inducible yeast, methyl- β -D-glucoside (β -MG), methyl- β -thio-D-glucoside (TMG), and TEG were found to be good inducers. The addition of these compounds to cultures of the hybrid yeast in succinate synthetic medium elicited a two- to threefold increase in the rate of enzyme synthesis. This stimulation of β -glucosidase synthesis in the hybrid yeast by inducers raised the level of enzyme to approximately the same level as in the induced *Rhodotorula*. In Fig. 1 the $\Delta E/\Delta OD$ is shown to be increased from a basal level of 172 to induced levels of 500 (β -MG) and 393 (TEG). Maximal induction was obtained with 10^{-2} M β -MG or TEG. The synthesis of β -glucosidase by the hybrid yeast is, therefore, semi-constitutive.

FIG. 1. Constitutive and inducible synthesis of β -glucosidase in the hybrid yeast *Saccharomyces fragilis* $\times$ *S. dobzhanskii*. Inducers, 10^{-2} M methyl- β -D-glucoside (β -MG) and 5×10^{-3} M ethyl- β -thio-D-glucoside (TEG), were added as indicated.

Duerksen and Halvorson (1959) reported that phenyl- β -thio-D-glucoside (TPG) would competitively inhibit induction of β -glucosidase by TEG in the *Rhodotorula*. In the hybrid, on the other hand, TPG did not inhibit constitutive synthesis or synthesis induced by glucose, β -MG, or TEG.

Some delay in the onset of induced synthesis in the hybrid yeast was apparent from the curves showing induction in Fig. 1. Examples of a lag in the induction of β -galactosidase in *E. coli* have been attributed either to the induction of a galactoside permease (Cohn, 1957) or to the mobilization of a messenger system (Pardee and Prestidge, 1961). The possibility that a β -glucoside permeability system in the hybrid yeast is responsible for the delay in induction has not as yet been examined. Although one must assume from the constitutive synthesis of enzyme that the flow of messenger is already proceeding, the production of messenger may be further influenced by the presence of inducer. The gratuitous induction with TEG eliminates the possibility that the induction lag could arise from hydrolysis of the glucoside followed by induction by glucose.

The physical-chemical properties of the purified enzyme produced constitutively by the hybrid yeast were compared with those of the purified, induced enzyme from *R. minute* (Hu et al., 1960). Marked similarity of the enzymes was observed with the exception of their behavior

towards natural β -glucosides. The affinity constants of the natural β -glucosides for the constitutive enzyme were ten times higher than for the inducible enzyme. It was of interest, therefore, to determine whether the constitutive and inducible yeasts differed with respect to inducibility by natural β -glucosides. These compounds showed little or no induction of enzyme in the inducible *R. minute* (Duerksen and Halvorson, 1959), but were effective inducers of the semiconstitutive system (Table 1). The range of inducing compounds is, therefore, greater for the hybrid yeast than for the *Rhodotorula*. Both parent yeast strains, *S. fragilis* Y610 and *S. dobzhanskii* Y1974, are semiconstitutive for β -glucosidase production (Table 2). Accordingly, the hybrid nature of the yeast *per se* is not responsible for the semiconstitutive state.

TABLE 1. Induction of β -glucosidase in the yeast hybrid *Saccharomyces fragilis* $\times$ *S. dobzhanskii* by natural β -glucosides*

Substance	Inducing activity $\Delta E/\Delta OD$ (% control)
Control (no addition).....	100
Arbutin.....	328
Salicin.....	550
Esculin.....	635
Amygdalin.....	625
Cellobiose.....	138

* Cells were grown in succinate synthetic medium containing 10^{-2} M β -glucosides. Differential rates of enzyme synthesis were determined as outlined in Materials and Methods.

Repression and the lack of inducer reversal. Cohn and Horibata (1959) found that the inducible synthesis of β -galactosidase in *Escherichia coli* was subject to repression by a number of carbon sources. These workers observed that inducer would partially reverse the repression by glucose, suggesting that catabolic repression led to the production of an intermediate which can compete with the inducer for some site involved in controlling the rate of enzyme synthesis.

The semiconstitutive synthesis of β -glucosidase in the hybrid yeast is also sensitive to repression by glucose (MacQuillan et al., 1960). One interesting feature of this repression was the inability of inducers to reverse the inhibition even at minimal, repressing concentrations of glucose (10^{-3} M). The examination of compounds leading to repression of constitutive β -glucosidase synthesis was extended to include a number of substances (Table 3). As in the case of glucose inhibition, repression by those compounds tested was not reversed by inducer. Figure 2 shows that lactate repressed constitutive β -glucosidase synthesis, whether the inducer was added previously or not. The lag in induction is due to contaminating glucose in β -MG and serves to reiterate that inducer will not offset glucose effects. In lactate repression, as with glucose repression, preinduction (Fig. 2) did not confer resistance to catabolic repression of enzyme synthesis.

Induction by glucose. Although concentrations of glucose of 10^{-3} M or above led to repression of constitutive β -glucosidase synthesis, lower concentrations showed a stimulatory effect

TABLE 2. β -Glucosidase synthesis in various yeast strains and mutants*

Strain	$\Delta E/\Delta OD$		Strain	$\Delta E/\Delta OD$	
	Constitutive	Induced		Constitutive	Induced
<i>S. fragilis</i> $\times$ <i>S. dobzhanskii</i>	180	504	<i>S. dobzhanskii</i>	84	104
M42-19.....	152	—	M19-1	42	95
M42-17.....	120	—	M19-5	19	—
M42-24.....	80	—	M19-6	9	—
M42-8.....	50	—			
M42-12.....	27	27			
M42-7.....	22	112			
M42-4.....	10	29	<i>S. fragilis</i>	30	120
M42-9.....	6.6	9.0			

* For determination of the differential rates of synthesis of β -glucosidase, the various yeasts were grown aerobically in succinate synthetic medium at 30 C. Where indicated, induced (β -MG) was added at 10^{-2} M.

TABLE 3. *Repressed rates of constitutive β -glucosidase synthesis in yeast in response to various carbon sources**

Repressing compound	$\Delta E/\Delta OD$
Succinate (control).....	320
Methanol.....	210
Pyruvate.....	155
tert-Amyl alcohol.....	84
Lactate.....	83
Isopropanol.....	70
Ethanol.....	46
n-Butanol.....	44
Acetate.....	40
Glucose.....	21
Acetaldehyde.....	0
Isoamyl alcohol.....	0

* Inhibitors were added at a final concentration of 0.5% to cultures growing in succinate synthetic medium.

FIG. 2. Lactate inhibition of β -glucosidase synthesis and the lack of reversal by inducer. An overnight culture of the hybrid yeast in succinate synthetic medium was divided into two 100-ml amounts in 250-ml flasks. β -MG was added to one flask to give a concentration of 10^{-2} M. Flasks were shaken in a 30°C water bath. After about one generation time (3 hr) each culture was divided and transferred to two 125-ml flasks. Lactate at a final concentration of 10^{-2} M was then added to one flask containing inducer and to one flask without inducer. Flasks were then reincubated on the shaker.

(Fig. 3). The high rates of enzyme synthesis induced by low glucose concentrations exhibited an optimum around 3×10^{-4} M and even exceeded the differential rate of enzyme synthesis induced by TEG or β -MG. Examination of the effects on constitutive β -glucosidase synthesis of varying amounts of the other inhibitors listed in Table 3 failed to detect other inducing agents. The results with other metabolic repression agents are typified by the results with lactate (Fig. 4), which was inhibitory at high concentrations and has no effect at low concentrations. It thus appears that a low concentration of glucose itself or a derivative of glucose is acting as an inducing agent. The alternative conclusion, that low glucose inhibits the synthesis of an internal repressor, appears less likely unless the synthesis of an internal repressor is subject to a narrower range of inhibitors than is β -glucosidase synthesis.

The inductive effect of TEG could not be superimposed upon the inductive effect of glucose. Although this finding suggests a common induction pathway, glucose and TEG may act at different sites. For example, low glucose concentrations might inhibit aporepressor synthesis, whereas TEG might compete with repressor function.

The specificity of glucose induction was examined by studying the effects of four hexoses on the formation of α - and β -glucosidase and β -galactosidase in the hybrid yeast (Table 4). Glucose in low concentrations induced both glucosidases, whereas high concentrations repressed enzyme synthesis. Fructose repressed in high or low concentrations, and mannose appeared to induce α -glucosidase, but repress β -glucosidase. Galactose repressed β -glucosidase, had no effect on α -glucosidase synthesis, and induced the formation of β -galactosidase. Thus, induction of these carbohydrases by hexoses appears to exhibit some specificity.

Derepression. When the kinetics of induction of β -glucosidase synthesis were examined at varying glucose concentrations, several features of the process were observed (Fig. 5). (i) At low concentrations (10^{-4} M) glucose had a direct inductive effect. The induction was almost immediate and no prior phase of repression occurred. (ii) When concentrations of glucose were used which initially led to repression of β -glucosidase synthesis, the subsequent rate of synthesis which occurred during the inductive phase (derepression) was

FIG. 3. Effect of glucose on constitutive β -glucosidase synthesis. A culture of the hybrid yeast, grown overnight in succinate synthetic medium was diluted with fresh medium, then subdivided into 125-ml amounts in 250-ml flasks containing various concentrations of glucose.

FIG. 4. Inhibition of constitutive β -glucosidase synthesis by lactate. A culture of the hybrid yeast grown overnight in succinate synthetic medium was diluted with fresh medium and then subdivided into 125-ml amounts in 250-ml flasks containing various concentrations of lactic acid.

TABLE 4. Specificity of hexose induction effects on three glycosidases of the hybrid yeast *Saccharomyces fragilis* $\times$ *S. dobzhanskii**

Enzyme	Constitutive	$\Delta E/\Delta OD$							
		Glucose		Fructose		Mannose		Galactose	
		10^{-2} M	10^{-4} M	10^{-2} M	10^{-4} M	10^{-2} M	10^{-4} M	10^{-2} M	10^{-4} M
β -Glucosidase	324	<1.2	636	34	272	88	132	19	252
α -Glucosidase	14.4	2.0	68.5	3.5	10.3	33.6	8.7	12.4	4.7
β -Galactosidase	<1.2	<1.2	<1.2	<1.2	<1.2	<1.2	<1.2	2.7	18.6

* See Fig. 3 for details.

greater than that found for direct induction. In Fig. 5 the differential rates of synthesis are 790 for 10^{-4} M glucose induction and 1,420 for derepression following inhibition by 5×10^{-4} M glucose.

These results suggest differences in the regulatory controls involved in induction or derepression. An optimal period of repression appears to exist for maximal rates of derepressed synthesis. Longer phases of repression with concentrations of glucose higher than ca. 5×10^{-4} M lead to rates of synthesis which are about four-fold that of the control constitutive level. The results plotted in Fig. 3, therefore, are probably composed of a derepression phenomenon superimposed upon direct induction by glucose. The conclusion that glucose induction and derepression are not identical is supported by the finding shown in Fig. 6. Cells which were first exposed to lactate to repress β -glucosidase synthesis, upon washing and removal to succinate synthetic medium show a derepression effect. In this instance, repression must first occur before stimulated rates of synthesis can be demonstrated. No direct induction of β -glucosidase synthesis by lactate at any concentration was observed.

The possibility remained that during glucose

FIG. 5. Kinetics of glucose induction of β -glucosidase synthesis in yeast. Glucose was added as indicated by the arrow.

FIG. 6. Derepression of β -glucosidase synthesis after lactate inhibition. The hybrid yeast was grown in succinate synthetic medium (200 ml in a 500-ml flask). At the time indicated, the culture was divided into two 95-ml amounts in 250-ml flasks, one of which received lactate (1%), incubated for 2 hr, and again was divided into two equal parts; one-half of each culture was reincubated immediately in 125-ml flasks. The other halves of the two cultures were centrifuged, washed in sterile succinate synthetic medium at 30 C, resuspended in fresh succinate medium to the original volume, and reincubated.

repression an inhibitor of β -glucosidase activity was synthesized. Glucose itself, for example, is an inhibitor of the constitutive enzyme (Hu et al., 1960). Repeated washing of lyophilized, glucose-grown cell pellets in phosphate buffer did not alter the level of enzyme, nor could an inhibitor of β -glucosidase be demonstrated in extracts of glucose- or lactate-grown cells. When strains containing low levels of enzyme were isolated by ultraviolet irradiation, a gradation of stable constitutive enzyme levels extending to mutants with very low rates of synthesis was observed. Table 2 lists a selected series of these mutants and shows, at least for some of the mutants tested, that inducibility is variable. No fully inducible or absolute negative mutants for β -glucosidase were found.

DISCUSSION

The semiconstitutive state of β -glucosidase synthesis in the present study itself presents an interesting facet to the problem of control. In the example studied here where induction is only two- or threefold over control constitutive levels, the internal mechanism of control appears to be somewhat ineffectual. In *S. lactis*

(Herman and Halvorson, *unpublished data*) noninducibility of β -glucosidase synthesis is dominant, suggesting the presence of a repressor mechanism for β -glucosidase synthesis. In the present studies on the specificity of hexose induction in the hybrid yeast, glucose or a near derivative of glucose appears to be the inducer.

In the hybrid yeast, glucose may act as an inducer or a repressor of β -glucosidase synthesis. Such observations might be expected for a two-component repressor (Szilard, 1960). For example, glucose at high concentrations might be the precursor of large amounts of both aporepressor and corepressor (Jacob and Monod, 1961), although low concentrations of glucose could provide small amounts of one (e.g., aporepressor), but higher amounts of the other. If glucose and β -glucosides induce at a common site, β -glucosides should reverse repression by glucose. The evidence thus far demonstrates that inducers of β -glucosidase synthesis do not reverse glucose repression (MacQuillan et al., 1960).

Since repressors are generally assumed to act at the level of the operator gene, the inhibition of constitutive β -glucosidase synthesis by glucose poses a dilemma. If constitutive synthesis were the result of internal inducer action, then conceivably the glucose repressor could act at the internal inducer site. Another site of action of exogenous inducers would then have to be assumed. On the other hand, constitutive synthesis could be the result of o^c or i^- mutations (Jacob and Monod, 1961); o^c mutations are resistant to inhibition at the repressor level, and i^- mutants would exhibit repression if an alternate route of repressor synthesis existed. In the latter case, one could imagine that semi-constitutive synthesis in the hybrid yeast occurs because of the low rate of synthesis of an internal repressor (leaky i mutant) whose action can be reversed by inducers. However, the existence of an inducer-resistant, glucose repression suggests that two types of repressors control β -glucosidase synthesis. Hauge et al. (1961) have recently reported that the level of ribosomal-bound β -glucosidase in the hybrid yeast is higher from glucose-repressed cells than from cells grown on succinate, suggesting that glucose inhibits the release of enzyme from the ribosome.

The inhibitory effects of high concentrations

of glucose may also function at more than one site for β -glucosidase synthesis. This is suggested by the behavior of cells exposed to 5×10^{-4} M glucose (Fig. 5). A transient inhibition of β -glucosidase synthesis is followed by a rate of enzyme formation three- to fourfold higher than that of constitutive synthesis. This accelerated rate of enzyme synthesis could be understood if glucose inhibited (i) a stereospecific enzyme which couples aporepressor and corepressor, or (ii) release of β -glucosidase from the template. The latter effect would be overcome rapidly, but the former would require that the cytoplasmic levels of this enzyme be replenished. During the time required to accumulate the coupling enzyme a period of complete derepression would occur, perhaps sufficient to account for the derepressed rate of β -glucosidase synthesis observed.

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