

Two Forms of Yeast Glycogen Synthetase and Their Role in Glycogen Accumulation

Lucia B. Rothman-Denes and Enrico Cabib

NATIONAL INSTITUTE OF ARTHRITIS AND METABOLIC DISEASES, NATIONAL INSTITUTES OF HEALTH,
BETHESDA, MARYLAND

Communicated by E. R. Stadtman, April 28, 1970

Abstract. The glycogen content of yeast rises dramatically just before the onset of the stationary phase of growth. Concomitantly, a rapid increase was found in the glucose 6-phosphate-independent (I) activity of glycogen synthetase, as well as in the total amount of enzyme. A mutant (GS 1-36) was obtained, which did not accumulate glycogen during growth. The synthetase from this strain was in the glucose 6-phosphate-dependent (D) form at all times. The total enzymatic activity of the mutant also increased sharply at the end of the logarithmic phase, although its maximal value was only one third that of the parent strain. Incubation with glucose of wild type resting cells from the logarithmic phase resulted in a slow accumulation of glycogen, which was accelerated after 20 min. At the same time a transformation from the D to I form of the enzyme was detected. The same slow initial rate of glycogen deposition was found with stationary cells of mutant GS 1-36, but the rate gradually declined to zero, rather than accelerating.

The interconversion of the I and D forms was obtained with extracts from cells harvested during different phases of growth. Examination of the properties of the I and D forms showed that the latter was much more strongly inhibited by ATP at low glucose 6-phosphate concentration. These findings clearly establish the fundamental role of the I form in glycogen accumulation. When taken together with previous results, they also show that the physiological significance of the I-D interconversion depends on the concentration of glucose 6-phosphate. Under certain conditions glucose 6-phosphate appears to regulate directly the activity of the predominant form of the enzyme.

Introduction. Previous work from this laboratory has shown that ATP and ADP, as well as other anions, are allosteric inhibitors of yeast glycogen synthetase (UDP-glucose:glycogen- α -4-glucosyltransferase, E.C.2.4.1.11), and that glucose 6-phosphate (Glu-6-P) counteracts the inhibition.^{1,2} Similar results were obtained with the enzymes from muscle,³ liver,^{4,5} and *Blastocladiella*.⁶ It was proposed^{2,3} that this interaction between metabolites might be the principal mechanism for the regulation of glycogen synthesis in yeast, whereas in higher organisms it would be superimposed upon the interconversion between Glu-6-P-dependent (D) and Glu-6-P-independent (I) forms.⁷ However, when glycogen accumulation was studied as a function of growth, it could not be correlated

with variations in the level of metabolites.⁸ On the other hand, changes in the sensitivity of glycogen synthetase to Glu-6-P were detected. These changes, the interconversion of two forms of glycogen synthetase *in vitro*, and some properties of the two forms constitute the substance of the present report.

Materials and Methods. Yeast growth: *Saccharomyces cerevisiae* S288C, a haploid strain of mating type α , was grown in a yeast extract-peptone-glucose medium⁹ on a reciprocating shaker at 30°C. Resting cells were incubated with glucose and salts as previously described,¹⁰ and maintained at a constant concentration of glucose.

Mutagenesis: Strain α S288C was mutagenized with ethylmethane sulfonate according to Fink.⁹ After enrichment in minimal medium,⁹ samples were spread on minimal agar and incubated at 30°C. The resulting colonies were stained with iodine, according to Chester,¹¹ and those differing in color from the wild type were immediately transferred to plates of minimal agar. Mutant GS 1-36 was yellowish-white after staining, in contrast to the parent, which gave a brown color.

Extract for measuring enzyme activity and interconversion of I and D forms: Extracts from lyophilized cells yielded erratic results when used to determine changes in the ratios of independence during growth, especially in the late logarithmic phase. Preparations from spheroplast lysates showed excellent reproducibility. Therefore the latter procedure was adopted, despite the danger of changes in the ratio of independence (see below), which might occur during the incubations with EDTA- β -mercaptoethanol and with snail extract. Spheroplasts were prepared and washed as already described,¹² except for the substitution of 0.55 M mannitol for 0.6 M KCl as osmotic stabilizer. The following manipulations were made at 0–5°C. For each gram (wet weight) of yeast used, 0.05 M imidazole-acetate, pH 7, containing 50 mM EDTA and 20 mM KF, was added to the spheroplast pellet to a final volume of 1 ml. The suspension was sonicated for two periods of 15 sec each, using the small probe of the Branson Sonifier at low power (setting no. 2), and centrifuged for 15 min at 20,000 $\times g$. The pellet was resuspended in the imidazole-EDTA-KF mixture up to the original volume. The supernatant fluid and pellet fractions were used without further treatment, to assess the "total" amount of glycogen synthetase (see below). For determinations of the ratio of independence (see *Enzyme assay*), samples of the supernatant fluid were filtered through small Sephadex G-25 columns, previously equilibrated with the same imidazole-EDTA-KF mixture used above. The preparations for the interconversion experiments (see Fig. 4) were obtained and filtered through Sephadex as above, but 0.05 M imidazole-acetate, pH 7, containing 1 mM EDTA was substituted for the imidazole-EDTA-KF mixture throughout.

Preparation of I and D forms for kinetic measurements: The D and I enzymes were obtained from lyophilized cells from the logarithmic and stationary phase, respectively, by centrifugation of extracts in the presence of glycogen, as described elsewhere.¹³

Enzyme assay: Two incubation mixtures were used, which corresponded to assays A and B. For assay A, the mixtures contained 5 mM UDP-¹⁴C-glucose, 50 mM glycylglycine, pH 7.5, 10 mM EDTA, 30 mg/ml of glycogen, and enzyme, in a total volume of 50 μ l. Assay B had the same composition except for UDPG, which was 0.4 mM, and the buffer, which was 50 mM succinate-cacodylate, pH 6.5. Both assays were performed in the absence and in the presence of 10 mM Glu-6-P. Incubation was for 10 min at 30°C. The following steps were as previously described.³ For counting, the washed glycogen pellet was transferred to a vial with two 0.5-ml portions of water. 10 ml of toluene-Triton X-100 (2:1) scintillation mixture¹⁴ were added, and the radioactivity was measured in a Packard liquid scintillation spectrometer. The ratio of independence¹⁵ is defined as the activity without Glu-6-P divided by the activity with 10 mM Glu-6-P, the result being multiplied by 100. The "total" activity is that measured in the presence of 10 mM Glu-6-P.

Glycogen: Glycogen was determined as reported previously.¹⁰

Results. In the present work a haploid strain of yeast, *S. cerevisiae* α S288C, was used, so that mutants could be obtained. Figure 1A shows the course of

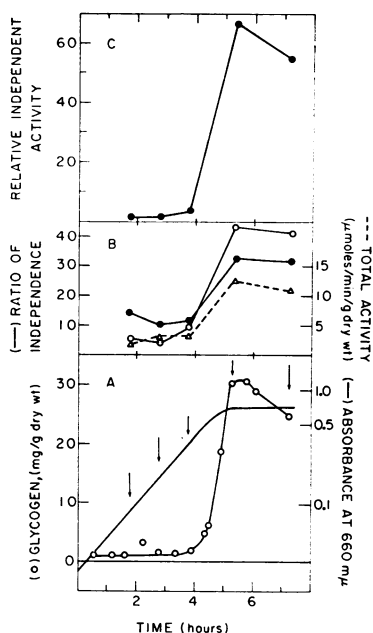


FIG. 1

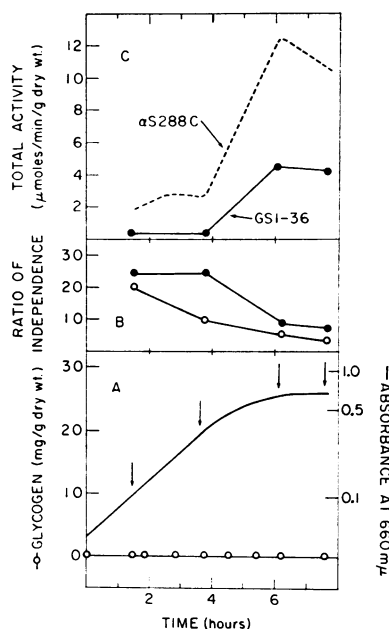


FIG. 2

Fig. 1.—Changes in glycogen content and in glycogen synthetase activity during yeast growth. In A, samples were taken for enzyme measurement (shown in B and C) at the times indicated with arrows. In B, the total activity (broken line) is the sum of "total" activities of the supernatant fluid and of the pellet from the $20,000 \times g$ centrifugation, as determined with assay A (see *Methods*). The ratio of independence was measured in a Sephadex-filtered sample of the supernatant fluid with assay A (—●—) and B (—○—). The values in C were obtained by multiplying in each case the "total" activity by the ratio of independence, as measured with assay B. The value for the first point was set as 1 and the others were normalized accordingly.

Fig. 2.—Changes in glycogen content and in glycogen synthetase activity during growth of mutant GS 1-36. For A and B see legends of Fig. 1. In C, the "total" activity is compared with that of Fig. 1B, from which the data for the broken line were taken.

accumulation of glycogen in the wild-type strain, as a function of growth. The polysaccharide content was extremely low during the logarithmic phase, but increased steeply just before and during the beginning of the stationary phase. Figure 1B indicates that glycogen synthetase extracted from cells in logarithmic growth had little activity in the absence of Glu-6-P. Coincident with the rise in glycogen level, both the total activity of the enzyme and the ratio of independence increased sharply. The rise in I activity appeared even larger when the assay was conducted under conditions approaching more closely those believed to prevail *in vivo*, i.e., 0.4 mM UDPG and pH 6.5,^{2,10} (assay B) as shown in Figure 1B. When both the increase in "total" activity and in the ratio of independence were taken into account, the over-all change in I activity was more than 60-fold (see Fig. 1C), in striking parallelism with the increase in glycogen.

In order to assess independently the importance of the rise in "total" activity and in the ratio of independence, use was made of a "glycogenless" mutant, GS 1-36, derived from strain αS288C (see *Methods*). Figure 2 presents the behavior of this mutant in an experiment analogous to that of Figure 1. As can be seen

from Figure 2A, there was no accumulation of glycogen throughout the entire growth cycle, including the stationary phase. During that period, the ratio of independence of glycogen synthetase was declining,¹⁶ rather than increasing. The "total" activity was very low during the logarithmic phase, but later increased 15-fold, as compared to a 4-fold increase for the parent strain. However, the maximal value was intermediate between those obtained with the logarithmic and stationary cells of α S288C. This experiment suggested that the D activity was not functional *in vivo*. In view of the relatively low level of total enzymatic activity in the mutant, other evidence was sought to corroborate these results.

It has been reported¹⁰ that resting yeast cells accumulate glycogen when incubated with glucose and salts. As shown in Figure 3, such an accumulation does occur with cells of α S288C, which were harvested at the stationary phase. In logarithmic phase cells, the increase in glycogen content was very slow in the beginning, but it became faster after about 20 min of incubation. In analogy with the results obtained with growing cells (Fig. 1), a concomitant increase in the ratio of independence of glycogen synthetase occurred, as illustrated in

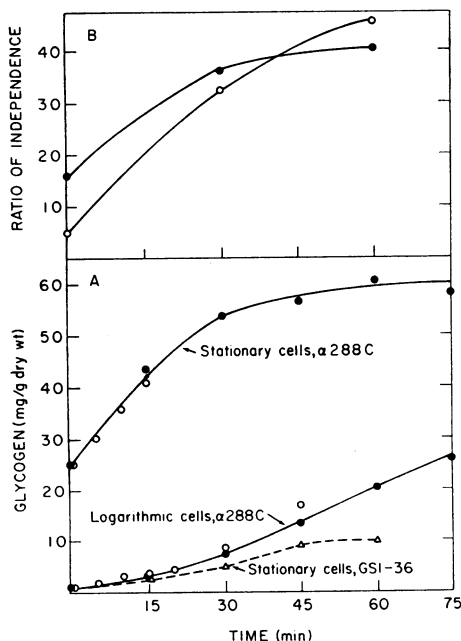


FIG. 3.—Accumulation of glycogen during incubation of resting cells in glucose. For the conditions see *Methods*. Logarithmic phase cells were collected at an absorbance of 0.1 at 660 m μ and stationary phase cells 1 hr after growth had stopped. In A, empty and filled circles refer to different experiments. In B, ratios of independence were determined for the enzymes from logarithmic cells under the two conditions of Fig. 1B, and the same symbols are used here.

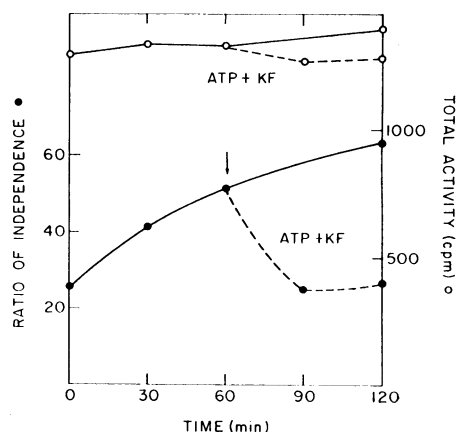


FIG. 4.—Interconversion between I and D forms of glycogen synthetase. A Sephadex-filtered extract, prepared from spheroplasts as described under *Methods*, was incubated at 30°C in the presence of 4 mM Mg²⁺. At 60 min (arrow), ATP and KF were added to a portion of the incubation mixture, to obtain concentrations of 4 and 20 mM, respectively. Samples were taken at different times and the ratio of independence and "total" activity were determined with assay A.

Figure 3B. The amount of "total" enzyme per gram of yeast had increased only 10% after 30 min and 70% after 60 min. With stationary phase cells of mutant GS 1-36 the initial rate of glycogen accumulation was similar to that of the logarithmic cells of the parent, but the rate decreased and was finally reduced to zero, in marked contrast with the acceleration found with the parent (Fig. 3A). When the stationary phase cells were incubated with glucose, formation of spheroplasts was accompanied by very extensive lysis, concomitant with large losses in enzyme activity. Thus, no data on the ratio of independence could be obtained for the incubated stationary cells of either parent or mutant strain.

It is of interest that the proportion of enzyme found in the $20,000 \times g$ sediment appeared to be related to the glycogen content of the cells. Thus, in the five samples of Figure 1B, the recovery of total enzyme in the pellet was 10, 16, 8, 40, and 46%, respectively, while the corresponding values for Figure 2B were 12, 6, 6, and 6%. Those for the logarithmic phase cells in the experiment of Figure 3 were 8% at time 0, 36% after 30 min of incubation, and 44% after 60 min.

Interconversion of glycogen synthetase forms *in vitro*: The observed changes in the ratio of independence might be ascribed, at least in some cases, to the preferential synthesis of one form of the enzyme, rather than to an interconversion between the two forms, of the type found in animal tissues. Therefore, it was important to demonstrate the interconversion *in vitro*. Figure 4 shows an experiment in which a Sephadex-treated extract from the late logarithmic phase was incubated in the presence of Mg^{2+} . The ratio of independence of glycogen synthetase increased steadily. When ATP and fluoride¹⁷ were added at 60 min, there was a sudden drop in the independence ratio, which returned to the zero time value; the "total" activity changed little throughout the experiment. In other experiments,¹³ Mg^{2+} was found to be a requirement for the D to I conversion, and ATP plus Mg^{2+} for the opposite reaction, as would be expected in analogy to the muscle enzymes.⁷ Both interconversions could be demonstrated in extracts from either logarithmic or stationary phase cells of $\alpha S288C$, as well as of GS 1-36.¹³ In the latter case, the interconversions appeared to be more sluggish, but it could not be decided whether the defect was in the glycogen synthetase or in the converting enzymes, since complete separation between them has not been achieved.

Kinetic properties of the D and I form: The response of the D and I forms to Glu-6-P was examined in the absence and in the presence of ATP. It may be observed in Figure 5 that the behavior of the yeast enzymes was strikingly similar to that of their muscle counterparts.³ At low levels of Glu-6-P, the D enzyme from either the logarithmic cells of the parent or the stationary cells of the mutant was more strongly inhibited by ATP than the I form from the stationary phase of the parent. The twofold activation of the I enzyme by Glu-6-P in the absence of ATP is not due to contamination with the D form, because the latter has a much higher Michaelis constant for the sugar phosphate.¹³

Discussion. The experimental evidence indicates that a conversion of D to I form of glycogen synthetase takes place in the yeast cell, in response to certain

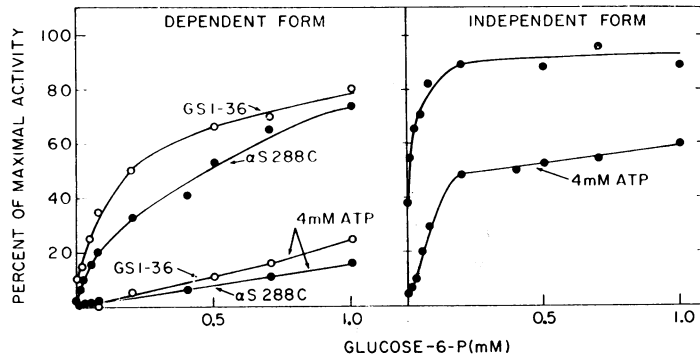


FIG. 5.—Effect of Glu-6-P on the activity of D and I form, in the presence of ATP. For the preparation of the enzymes see *Methods*. The enzyme from GS 1-36 was obtained from stationary phase cells. Determinations were made using assay B, with additions of Glu-6-P and ATP, as indicated. The activity is expressed in percentage of that obtained with 10 mM Glu-6-P, in the absence of ATP.

stimuli, the nature of which is not yet clear at the molecular level (Figs. 1 and 3). Interconversions between the two forms have also been demonstrated *in vitro*. During growth, the D to I transformation is accompanied by an increase in total activity, which may be caused by synthesis of new enzyme. Mutant GS 1-36 also shows this increase, but appears to be unable to effect the conversion of D to I form *in vivo*, as shown by the results of Figure 2 and by the limited accumulation of glycogen in Figure 3. Extracts from the mutant do carry out both D-I interconversions, albeit at a reduced rate, and the locus of the defect in this strain remains to be established.

The central problem of this study is: How does the interconversion between D and I forms of glycogen synthetase affect the formation of glycogen *in vivo* under different conditions? The scheme presented in Figure 6 attempts to answer this question in terms of the concentration of Glu-6-P in the cell, taking into account the presence of inhibitors, such as ATP and ADP. The effect of the conversion is shown at three arbitrary levels of Glu-6-P, low, intermediate,

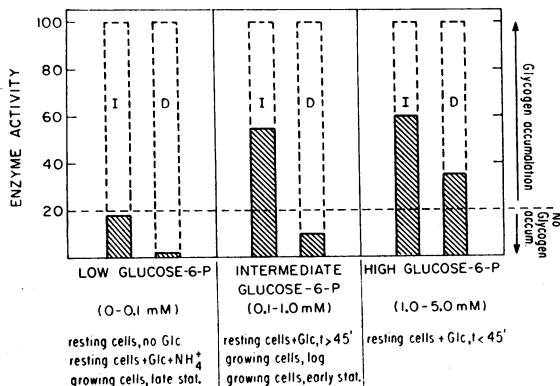


FIG. 6.—Effect of the Glu-6-P level on the relative activity of I and D forms. The height of the broken line bars denotes the "total" activity as measured with 10 mM Glu-6-P and in the absence of inhibitors. The hatched area represents the actual activity in the presence of 4 mM ATP (or a mixture of ATP and ADP), at the indicated levels of Glu-6-P. Abbreviations: stat., stationary; 45', 45 min; Glc, glucose.

and high. The activities are based on the conditions of Figure 5, that is, at 0.4 mM UDPG and 4 mM ATP, which would be close to the concentrations *in vivo*,¹⁰ but the values are to be considered only as a framework for discussion. It is assumed that a certain turnover of glycogen will always occur, and that a minimal rate of synthesis, shown as the horizontal broken line at 20% of maximal enzymatic activity, is necessary to offset degradation of the polysaccharide. Variations in the rate of degradation are provisionally disregarded.¹⁸ According to this scheme, when Glu-6-P is very low, even complete conversion into the I form will not cause glycogen accumulation. This situation will prevail in resting cells in the absence of glucose¹⁰ or in the presence of NH_4^+ ,¹⁰ and in cells which have entered the stationary phase, where the glucose of the medium has been completely utilized and a drop in polysaccharide content actually takes place (see Fig. 1). In each of these cases the ratio of independence was found to be high. On the other hand, at intermediate levels of Glu-6-P, conversion of D into I form will be decisive for the deposition of glycogen. This situation would occur during the logarithmic and early stationary phases of growth. The increase in total amount of enzymatic activity, not considered in Figure 6, will multiply the effect of the conversion and explain the precipitous increase in glycogen seen in Figure 1. Mutant GS 1-36, being unable to carry out the D to I conversion *in vivo*, cannot take advantage of the increase in total activity.

At very high concentrations of Glu-6-P, even the D form of the enzyme may be stimulated to the point where accumulation of glycogen becomes possible. This condition appears to prevail for some time during the incubation of resting cells with glucose¹⁰ and would explain the initial slow accumulation found with both logarithmic cells of the wild type and stationary cells of mutant GS 1-36. At later stages, as Glu-6-P falls into the "intermediate" level, the mutant will stop making glycogen, while the parent, being able to bring about the D to I transformation, will maintain and actually increase the rate of synthesis of the polysaccharide.

According to this formulation, the physiological significance of the D-I interconversion would depend, both in yeast and muscle,^{3,19} on the concentration of Glu-6-P. In addition, changing levels of the sugar phosphate may directly influence the activity when a single form of the enzyme predominates. This would be the case, for instance, when ammonium ions are added to stationary phase resting cells,¹⁰ or during incubation of mutant GS 1-36 in glucose, as indicated above.

We are greatly indebted to Dr. Gerald Fink for providing strain α S288C and for making his manuscript on the procedure for mutagenesis available prior to publication. We also wish to thank Dr. F. A. Keller for help with some of the experiments and for discussions, and Drs. G. Ashwell, W. B. Jakoby, and M. F. Singer for useful criticism.

Abbreviations: Glu-6-P, glucose-6-phosphate; I and D, glucose-6-phosphate-independent and glucose-6-phosphate-dependent activity of glycogen synthetase, respectively.

* Requests for reprints may be addressed to Dr. E. Cabib, Laboratory of Biochemistry and Metabolism, Building 10, Room 9N-119, National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, Bethesda, Md. 20014.

¹ Rothman, L. B., and E. Cabib, *Biochemistry*, 6, 2098 (1967).

² *Op. cit.*, 6, 2107 (1967).

- ³ Piras, R., L. B. Rothman, and E. Cabib, *Biochemistry*, **7**, 56 (1968).
- ⁴ De Wulf, H., W. Stalmans, and H. G. Hers, *Eur. J. Biochem.*, **6**, 545 (1968).
- ⁵ Gold, A. H., *Biochemistry*, **9**, 946 (1970).
- ⁶ Plessman Camargo, E., R. Meuser, and D. Sonneborn, *J. Biol. Chem.*, **244**, 5910 (1969).
- ⁷ Larner, J., *Trans. N. Y. Acad. Sci.*, **29**, 192 (1966).
- ⁸ Rothman, L. B., and E. Cabib, unpublished results.
- ⁹ Fink, G., in *Methods in Enzymology*, ed. H. Tabor and C. W. Tabor (New York: Academic Press, in press), vol. 17A.
- ¹⁰ Rothman, L. B., and E. Cabib, *Biochemistry*, **8**, 3332 (1969).
- ¹¹ Chester, V. E., *Nature*, **214**, 1237 (1967).
- ¹² Behrens, N. H., and E. Cabib, *J. Biol. Chem.*, **243**, 502 (1968).
- ¹³ Rothman, L. B., and E. Cabib, manuscript in preparation.
- ¹⁴ Patterson, M. S., and R. C. Greene, *Anal. Chem.*, **37**, 854 (1965).
- ¹⁵ Belocopitow, E., M. M. Appelman, and H. N. Torres, *J. Biol. Chem.*, **240**, 3473 (1965).
- ¹⁶ The results obtained with logarithmic phase cells are of doubtful value because of the extremely low activity of the enzyme in those samples.
- ¹⁷ Fluoride inhibits the D to I conversion.¹³
- ¹⁸ Previous work has shown that glycogen turnover is small in resting cells incubated with glucose.¹⁰
- ¹⁹ Piras, R., and R. Staneloni, *Biochemistry*, **8**, 2153 (1969).