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YEAST GLYCOGEN SYNTHETASE IN THE GLUCOSE 6-PHOSPHATE-INDEPENDENT FORM: A CASE OF COLD LABILITY WITHOUT MAJOR CHANGES IN MOLECULAR SIZE

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SUMMARY

The form of yeast glycogen synthetase independent of glucose 6-phosphate is reversibly inactivated by cold in the absence of substrates. Addition of glycogen or of a high concentration of glycerol completely prevents the inactivation, whereas glucose 6-phosphate, uridine diphosphate glucose and $(NH_4)_2SO_4$ afford partial protection. Sedimentation coefficients of 11.2 S at 30 °C and 10.1 S at 0 °C were calculated from centrifugation in sucrose density gradients. This small difference appears to be due to a change in conformation rather than to dissociation of the enzyme into subunits in the cold. The glucose 6-phosphate-dependent form of glycogen synthetase does not display cold sensitivity.

INTRODUCTION

This laboratory is presently engaged in the study and purification of yeast glycogen synthetase (UDPglucose:glycogen α-4-glucosyltransferase (EC 2.4.1.11)[1]. As a part of this study, a method was developed[2] for the separation, in the absence of glycogen, of the glucose-6-*P*-dependent (D) and -independent (I) forms of the enzyme. Under these conditions the activity of the I form was found to be decreased by previous incubation at 0 °C, an effect which was studied in some detail because of its obvious relevance to further purification of the enzyme. One of the findings was that the enzyme showed only a small change in sedimentation coefficient with temperature. This result contrasts with recent reports[3,4] according to which the I form from rabbit muscle and rat liver shows aggregation at low temperature, and also with the properties of most cold-labile enzymes whose hydrodynamic properties have been studied. Because of this unusual behavior it seemed of interest to communicate our results at the present time.

Abbreviations: I, glucose 6-phosphate-independent form of glycogen synthetase; D, glucose 6-phosphate-dependent form of glycogen synthetase.

MATERIALS

Uridine diphosphate [$^{14}C_6$]glucose (specific activity 227 Ci/mole) and Aquasol were obtained from New England Nuclear Corp. Nonradioactive UDPglucose and shellfish glycogen were purchased from Sigma Chemical Company. Chromatographic sheet (ITLC-SG type, 20 cm × 20 cm) was the product of Gelman Instrument Company. Yeast glucose 6-phosphate dehydrogenase was obtained from Boehringer–Mannheim Corporation and bovine liver catalase from Worthington.

METHODS

Preparation of D and I forms of glycogen synthetase

D and I forms of yeast glycogen synthetase were prepared by DEAE-cellulose chromatography at 4 °C according to the methods described earlier[2]. Both enzyme preparations were concentrated by ultrafiltration through an Amicon UM-10 membrane, dialyzed against 0.045 M Tris–HCl buffer, pH 7.5, containing 1 mM mercaptoethanol and 10% glycerol and stored at −20 °C until used. The enzyme preparations were stable for several months under these conditions.

Assay of glycogen synthetase activity

Glycogen synthetase was assayed by a method based on the same principle as that described by Thomas *et al.*[5]. The assay mixture contained 1 μmole of glycylglycine buffer, pH 7.5, 0.2 μmole of EDTA, 0.6 mg of shellfish glycogen, 0.1 μmole of [^{14}C]UDPglucose (specific activity $4.7 \cdot 10^5$ cpm/μmole), 0.2 μmole of glucose-6-*P* and enzyme in a total volume of 25 μl. Glucose-6-*P* was omitted when the independent activity was measured. Incubation was at 30 °C for 2 min and the reaction was stopped by adding 5 μl of glacial acetic acid. Aliquots of the reaction mixture (25 μl) were applied to 1.5 cm × 9.8 cm strips of Gelman chromatographic sheet, 1.5 cm above the bottom edge. The strips were dried with warm air and developed ascendingly in a solvent system of 66% ethanol containing 0.1 M ammonium acetate. After developing for 12 min the strips were taken out and dried again with warm air. In this solvent system the reaction product ([^{14}C]glycogen) remained at the origin, whereas [^{14}C]UDPglucose moved with the solvent front. A 2.3-cm segment of each strip, from 1.3 cm above to 1.0 cm below the starting line, was cut out and inserted into a scintillation vial. After adding 10 ml of Aquasol the radioactivity was measured in a scintillation counter.

One unit of activity is defined as that amount of enzyme which catalyzes the incorporation of 1 μmole of glucose per min into glycogen.

Assay of the temperature sensitivity of glycogen synthetase

The assay was performed by incubating glycogen synthetase at various temperatures in the presence of 10 mM glycylglycine, pH 7.5, and 5 mM magnesium acetate. At timed intervals, aliquots (usually 3 μl) were withdrawn for the determination of glycogen synthetase under standard assay conditions.

Other methods

Sucrose density gradient centrifugation was carried out by the procedure of

Martin and Ames[6] with an SW-65 rotor in a Beckman L2-65B ultracentrifuge. Catalase was assayed according to Beers and Sizer[7] and glucose 6-phosphate dehydrogenase as described by Warburg and Christian[8]. Proteins were determined by the method of Lowry *et al.*[9].

RESULTS

Recovery of D and I-form glycogen synthetase after DEAE-cellulose column chromatography

During purification of glycogen synthetase by DEAE-cellulose column chromatography at 4 °C, it was found that the recovery of the I form was always lower than that of the D form. As shown in Table I, when an undialyzed crude extract, containing enzyme mainly in the D form[2], was chromatographed on a DEAE-cellulose column, 87% of the total activity applied was recovered as D-form enzyme. When

TABLE I

RECOVERY OF YEAST GLYCOGEN SYNTHETASE AFTER DEAE-CELLULOSE COLUMN CHROMATOGRAPHY

The separation of D and I forms of glycogen synthetase was carried out at 4 °C according to the methods described earlier[2]. Concentration of the pooled fractions was performed in an Amicon ultrafiltration cell with a UM-10 membrane at 4 °C. The pooled enzyme fractions were usually concentrated about 10 times, then dialyzed against 0.045 M Tris–HCl buffer, pH 7.5, containing 1 mM mercaptoethanol and 10% glycerol. The dialyzed enzymes were then distributed in several tubes and kept at −20 °C. The activation step was carried out by incubating the concentrated enzyme at 30 °C for 20 min. in the presence of 10 mM glycylglycine buffer, pH 7.5, and 5 mM magnesium acetate. After 20 min incubation, aliquots were withdrawn for the measurement of glycogen synthetase activity.

	*Pooled fractions (%)**		*After concentration (%)**		*After activation (%)**	
	I	*D*	*I*	*D*	*I*	*D*
Undialyzed crude extract	—	87	—	86.5	—	86.5
Dialyzed crude extract	40	—	56.5	—	82	—
Undialyzed and dialyzed crude extract (equal portions)**	22.8	46	24.6	43.8	39.6	43.8

* Total activity applied to the column taken as 100%.
** Total activity (I + D) equal to that applied to the other two columns.

the same amount of dialyzed crude extract, containing enzyme in the I form[2], was separated under identical conditions, only 40% of the total activity applied was recovered as I form, and no significant activity was detected in the fractions which corresponded to the elution area of the D form. When equal portions of dialyzed and undialyzed crude extracts containing equal activities of I and D form respectively, were applied to a DEAE-cellulose column and chromatographed as described previously, 46% of the total activity applied was recovered as D-form enzyme, and only 22.8% as I-form enzyme. Concentration of the pooled I form by ultrafiltration increased the yield somewhat. Significant increase in the recovery of the I form was achieved when the concentrated pooled fractions were incubated in the presence of 10 mM glycylglycine, pH 7.5, and 5 mM magnesium acetate at 30 °C for 20 min

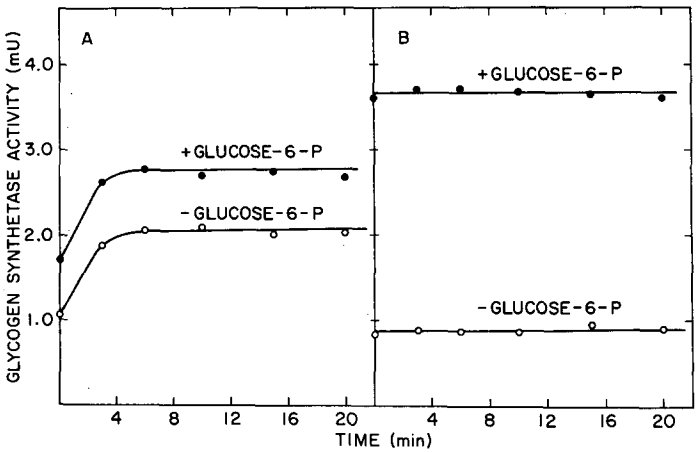


Fig. 1. Effect of incubation at 30 °C upon the D and I forms of glycogen synthetase. Incubation mixtures containing 1 μmole of glycylglycine buffer, pH 7.5, 0.5 μmole of magnesium acetate and either 0.41 mg, as protein, of I form (A) or 0.18 mg of D form (B) in a total volume of 0.1 ml, were kept in an ice bucket at 0 °C. At zero time, the incubation mixtures were transferred to a 30 °C water bath, and 3-μl aliquots were withdrawn at various time intervals for the measurement of glycogen synthetase activity, both in the presence and absence of glucose-6-*P* at 30 °C for 2 min.

(Table I). After this incubation, the recovery of the I form was comparable to that of the D form. Tris buffer is as effective as glycylglycine buffer, and the addition of 5 mM magnesium acetate only accelerates the rate of activation[10].

Effect of incubation at 30 °C on the I and D forms of glycogen synthetase

From the results of Table I, it is evident that the I and D forms obtained from DEAE-cellulose columns behave differently upon incubation at 30 °C. A more detailed comparison between the two forms showed (Fig. 1) that the I form undergoes a gradual activation when it is assayed either in the presence or absence of glucose-6-*P*. No such activation was observed for the D form. The degree of activation of I form upon incubation at 30 °C varied somewhat from one preparation to the next but ranged from 1.5 to 3-fold. The ratio of independence[1] of the I form also increased to some extent during incubation at 30 °C.

Time course of the reaction with I-form glycogen synthetase

The time course of the reaction catalyzed by the I form was determined before and after incubation of the enzyme at 30 °C. With previously activated enzyme a linear increase in incorporation was obtained, whereas untreated preparations gave rise to a curve of increasing slope (Fig. 2). This result suggests that some activation may occur during incubation under assay conditions. During the short incubation time used in the standard assay, *i.e.* 2 min, the conversion appears to be negligible.

Reversibility of temperature effects

After the I form was activated by incubation at 30 °C, its activity remained stable on further incubation (Fig. 3). Successive cycles of cooling and heating resulted in parallel decreases and increases of activity.

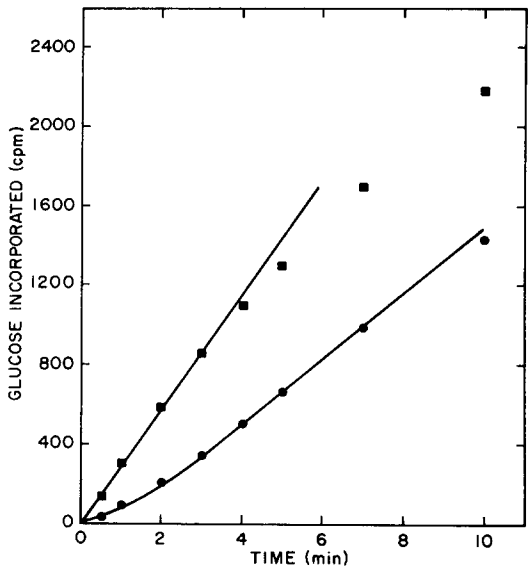


Fig. 2. Effect of previous incubation on the time course of the reaction with I form. Incubation of the enzyme at 30 °C before the assay, where indicated, was carried out for 20 min under the conditions of Fig. 1. The assay mixture contained 5 μmoles of glycylglycine buffer, pH 7.5, 1 μmole of EDTA, 3 mg of shellfish glycogen, 0.5 μmole of [^{14}C]UDPglucose (total activity 235 000 cpm), 1 μmole of glucose-6-*P* and 22 μg, as protein, of I form in a total volume of 0.125 ml. During the following incubation at 30 °C, 5-μl samples were withdrawn at various time intervals and pipetted onto a chromatographic strip (see Methods) at the place where 5 μl of glacial acetic acid had just been spotted. The activity is expressed in cpm incorporated into glycogen. I form without previous incubation, ●—●, I form after 20 min incubation, ■—■.

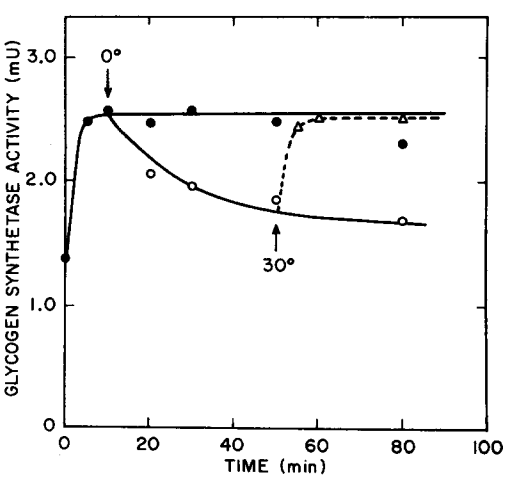
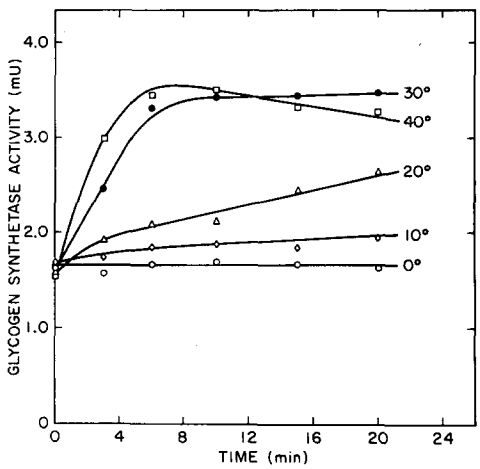


Fig. 3. Reversibility of cold inactivation. The content of the incubation mixture was as described under Fig. 1. Samples were transferred to the appropriate temperature where indicated by the arrows. Aliquots were withdrawn at different times from each incubation mixture and the activity measured under standard assay conditions. Enzyme incubated at 30 °C, ●—●; at 30 °C, then at 0 °C, ○--○; at 30 °C, then at 0 °C, finally again at 30 °C, △—△.



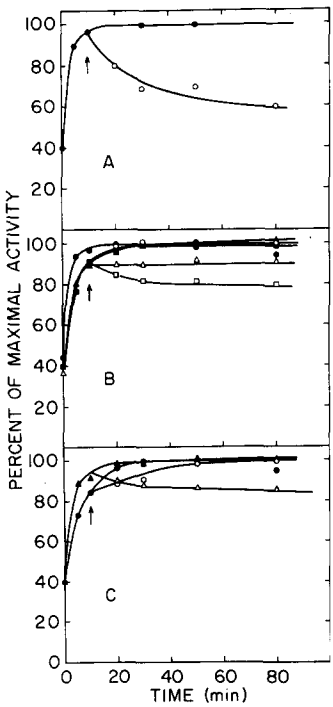


Fig. 4. Effect of incubation temperature on the activation of the I form. The experiments were carried out as described under Fig. 1. The amount of I form used in the prior incubation mixture was 1.3 mg of protein.

Fig. 5. Effect of different substances on cold inactivation. The conditions for incubation and assay were as described under Fig. 1. All effectors when added were present in the mixture from the beginning of the incubation at 30 °C. At 10 min (arrows) samples from each mixture were transferred to a bath at 0 °C (open symbols). (A) Control, ●—● and ○—○. (B) 30 mg/ml of shellfish glycogen added, ●—● and ○—○; 10 mM glucose-6-*P* added, ▲—▲ and △—△; 10 mM UDPglucose added, ■—■ and □—□. (C) 45% glycerol added, ●—● and ○—○; 10 mM $(NH_4)_2SO_4$ added, ▲—▲ and △—△.

Effect of incubation temperature on rate of activation

The I form of glycogen synthetase was incubated at various temperatures and then assayed at 30 °C with the results shown in Fig. 4. No activation was observed upon incubation at 0 °C but increasing temperature resulted in an increased activation rate. At 40 °C an optimal rate was reached, however the enzyme began to lose activity upon prolonged incubation.

Effect of various substances on the temperature sensitivity of the I form

The I form obtained from DEAE-cellulose columns did not contain glycogen. Since the enzyme binds strongly to glycogen[1] and therefore might well be attached to the polysaccharide *in vivo*, it was of interest to study the effect of glycogen on cold inactivation. It was indeed found (Fig. 5B) that glycogen, while not preventing activation at 30 °C, afforded complete protection against inactivation at low temperatures. Other substances were partially or totally effective in preventing cold inactivation. The co-substrate UDPglucose provided only moderate protection, whereas

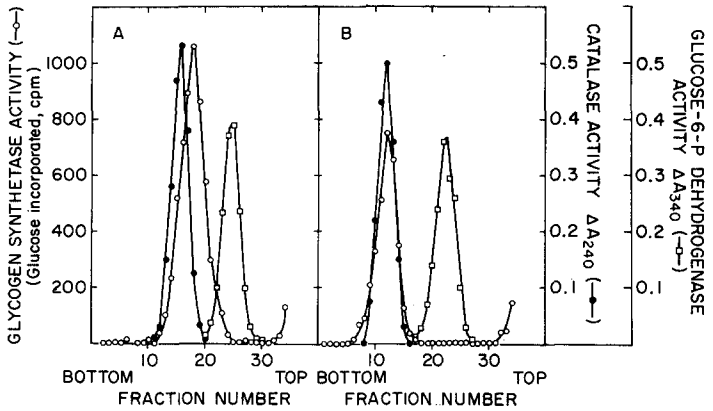


Fig. 6. Sucrose density gradient centrifugation of I-form enzyme at 0 °C (A) and 30 °C (B). Samples of 0.1 ml, containing glycogen synthetase (0.3 mg of protein) bovine liver catalase and yeast glucose 6-phosphate dehydrogenase were layered on 5 ml of a 5–20% sucrose density gradient in 10 mM glycylglycine buffer, pH 7.5, and 5 mM magnesium acetate. Samples for the 30 °C centrifugation were incubated for 20 min at this temperature prior to layering. Centrifugation was performed at 60 000 rev./min for 360 and 200 min, at 0 and 30 °C respectively, in an SW-65 rotor. Fractions of 0.14 ml were collected and assayed for glycogen synthetase, ○—○; catalase, ●—●; and glucose 6-phosphate dehydrogenase, □—□. The small amounts of glycogen synthetase which remained at the top of the gradient appear to be artifactual, since in other experiments some activity of the marker enzymes was also found at the same location.

the allosteric effector glucose-6-*P* was more efficient (Fig. 5B). Glycerol at 45% was an excellent protector, and 10 mM $(NH_4)_2SO_4$ was partially effective (Fig. 5C). If one defines protection as the percentage of total decrease in activity, as observed in the control at 80 min, which is prevented by any given substance, the results are as follows: 100% protection for glycogen and glycerol; 77% for glucose-6-*P*; 63% for $(NH_4)_2SO_4$ and 50% for UDPglucose. KCl at 10 mM was totally ineffective.

Sucrose density gradient centrifugation

Cold inactivation of enzymes usually has been correlated with dissociation or aggregation of the enzyme molecules. To ascertain if such was the case with yeast glycogen synthetase, the sedimentation behavior of the I form was determined at 0 and 30 °C with the use of sucrose density gradients[6]. A small decrease in the sedimentation coefficient was observed at the lower temperature (Fig. 6). Using bovine liver catalase and yeast glucose-6-*P* dehydrogenase as markers, sedimentation coefficients of 11.2 S and 10.1 S were calculated at 30 and 0 °C, respectively. This would correspond to apparent molecular weights of 243 000 and 202 000, respectively.

DISCUSSION

Exposure of glycogen-free preparations of yeast glycogen synthetase in the I form to 0 °C resulted in a loss of activity, which could be reversed by rewarming. Glycogen totally prevented the inactivation, whereas UDPglucose and glucose-6-*P* afforded partial protection. In contrast to the results of Smith and Larner[3] and McVerry and Kim[4], who found extensive aggregation of rabbit muscle or rat liver glycogen synthetase in the I form upon cooling, the yeast I form only showed a

small decrease in sedimentation coefficient at low temperature. This effect is so modest, that it appears to be due to a change in the shape of the molecule rather than to a dissociation into subunits. Therefore, the enzyme may exist in at least two interconvertible configurations. Both temperature and the presence of certain ligands affect the equilibrium between these forms. Since the D form of the synthetase appears to be completely resistant to cold inactivation, it may be concluded that the occurrence of phosphate groups at certain locations of the molecule results in the stabilization of one of the configurations. Temperature affects not only the equilibrium but also the rate of the transition, as indicated by the results of Fig. 4.

A perusal of the literature shows that about 20 enzymes have been found to exhibit cold lability[3,11–19]. In most cases, when the hydrodynamic behavior of the protein was determined the inactivation was found to be accompanied by a large decrease in sedimentation coefficient, which was interpreted as a dissociation of the enzyme into subunits. In one of the few occasions in which an increase in sedimentation rate occurred[11], the enhancement of this effect by an increased dilution of the enzyme suggested that dissociation preceded the aggregation[13]. Cold lability was therefore interpreted as a direct result of enzyme dissociation, which would be caused by the weakening of hydrophobic interactions at low temperatures[13]. In the case of yeast glycogen synthetase I form and of ribulose diphosphate carboxylase[15] no gross change in sedimentation behavior with temperature has been found. The loss in activity at low temperature appears to result from a change in configuration of the protein. These findings, added to the fact that a change in conformation prior to dissociation was detected with pyruvate carboxylase[14], suggest that dissociation is not a "*sine qua non*" condition for cold inactivation. Indeed, it is quite possible that in most cases the primary event is a conformational transition which may or may not be followed by a dissociation. The protection afforded by substrates and allosteric effectors supports this idea. The availability of cold-sensitive enzymes which do not exhibit gross variations in molecular size at low temperatures may help in elucidating this problem. The glycogen synthetase system may be especially appropriate for this purpose, because of the presence of a natural "control", the D form, which is not cold-labile.

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