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BIOSYNTHESIS OF GLYCOGEN IN *NEUROSPORA CRASSA*

PURIFICATION AND PROPERTIES OF THE UDPGLUCOSE:GLYCOGEN 4-α-GLUCOSYLTRANSFERASE

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Summary

The *Neurospora crassa* glycogen synthase (UDPglucose:glycogen 4-α-glucosyltransferase, EC 2.4.1.11) was purified to electrophoretic homogeneity by a procedure involving ultracentrifugation, DEAE-cellulose column chromatography, $(NH_4)_2SO_4$ fractionation and 3-aminopropyl-Sepharose column chromatography. The final purified enzyme preparation was almost entirely dependent on glucose-6-*P* and had a specific activity of 6.9 units per mg of protein. The subunit molecular weight of the glycogen synthase was determined by electrophoresis in sodium dodecyl sulfate-polyacrylamide gel to be 88 000—90 000. The native enzyme was shown to have a molecular weight of 270 000 as determined by sucrose density gradient centrifugation. Thus, the glucose-6-*P*-dependent form of the *N. crassa* glycogen synthase can exist as trimer of the subunit. Limited proteolysis with trypsin or chymotrypsin converted the glucose-6-*P*-dependent form of the enzyme into an apparent glucose-6-*P*-independent form. The enzyme was shown to catalyze transfer of glucose from UDPglucose to glycogen as well as to its phosphorylase limit dextrin, but not to its β-amylase limit dextrin. Moreover, glucose, maltose and maltotriose were not active as acceptors.

Introduction

Glycogen synthase (UDPglucose:glycogen 4-α-glucosyltransferase, EC 2.4.1.11), the rate-limiting enzyme for glycogen synthesis, is present in mam-

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

mals [1,2] and yeast [3,4] in two forms which are interconvertible by phosphorylation and dephosphorylation. The phosphorylated enzyme, glycogen synthase D, requires glucose-6-*P* for its activity, whereas the dephosphorylated enzyme, glycogen synthase I, is active irrespective of the presence or absence of glucose-6-*P*. The two forms of the enzyme have been also observed with *Neurospora crassa* [5,6]. Recently, homogeneous preparations of glycogen synthase have been obtained from mammals [7—17], yeast [18] and bacteria [19,20]. Although some information on subunit structure and kinetic mechanism is available on the microorganism glycogen synthases, i.e. those from *Saccharomyces cerevisiae* [18] and *Escherichia coli* [20], purification of these enzymes have been hampered by their high affinity for glycogen.

In this paper, we describe a purification method of glycogen synthase from *N. crassa*. Advantage has been taken of the fact that the enzyme is readily separated from endogenous glycogen by DEAE-cellulose column chromatography and adsorbed on 3-aminopropyl-Sepharose. The molecular weight of the native enzyme and that of its subunit have been determined. We also report the properties of the glycogen synthase, especially the primer dependence of the reaction catalyzed by the enzyme.

Materials and Methods

Materials. UDP[U-^{14}C]glucose was purchased from New England Nuclear Corp. Rabbit liver glycogen, bovine serum albumin, glucose-6-*P*, NADP, beef liver catalase and rabbit muscle glycogen phosphorylase *a* were purchased from Sigma Chem. Co.; yeast glucose-6-*P* dehydrogenase from Oriental Yeast Ind. Co.; β-amylase from Worthington Biochem. Co.; trypsin and chymotrypsin from Seikagaku Kogyo Co.; Nonion NS-120 from Nippon Yushi Co. Pullulan and pullulanase were generous gifts from Hayashibara Co. Maltotriose was prepared from pullulan by digestion with pullulanase. *N. crassa* glycogen was isolated and purified as reported previously [21]. β-Amylase and phosphorylase limit dextrins of the *N. crassa* glycogen were prepared by exhaustive treatment of the glycogen with β-amylase or phosphorylase. 3-Aminopropyl-Sepharose was prepared as described by Shaltiel and Er-El [22].

Buffer solutions. Buffer A comprised 50 mM glycine, 5 mM mercaptoethanol, 0.05 mM phenylmethylsulfonyl fluoride and 1 mM EDTA. It was adjusted to pH 8.0 with NaOH. Buffer B had basically the same composition as that of buffer A but comprised 25% (v/v) glycerol instead of EDTA.

Assay of glycogen synthase. The standard incubation mixture contained 6 μmol of glycyl-glycine/NaOH buffer (pH 8.6), 0.2 μmol UDP[^{14}C]glucose (specific activity, $1.5 \cdot 10^5$ cpm/μmol), 0.5 mg glycogen and 0.25 μmol glucose-6-*P*. For the assay of glucose-6-*P*-independent activity of glycogen synthase, glucose-6-*P* was omitted from the incubation mixture. Reaction was started by adding 15 μl of the enzyme to 35 μl of the substrate solution. After incubation for 15 min at 30°C, a 50 μl portion of the incubation mixture was applied to a column (0.5 × 5 cm) of anion-exchange resin (Dowex 1 x8, Cl^- form, 50—100 mesh), and the column was immediately eluted with 1.0 ml of water. The radioactivity in the eluate was determined with a scintillation spectrometer using 10 ml of NT scintillator [23]. One unit of the synthase activity

was defined as 1 μmol of glucose transferred in 1 min under the above conditions.

Protein determination. Protein was determined by the method of Lowry et al. [24] after being precipitated with trichloroacetic acid. Bovine serum albumin was used as a standard.

Electrophoresis. Polyacrylamide gel electrophoresis under non-denaturing conditions was carried out according to a modification of the method of Williams and Reisfeld [25]. Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate was carried out according to the procedure described by Weber and Osborn [26] using 7% polyacrylamide gel. The reference polypeptides for determination of subunit molecular weight were γ-globulin, bovine serum albumin, ovalbumin and chymotrypsinogen.

Sucrose density gradient centrifugation. Sucrose density gradient centrifugation was carried out as described by Martin and Ames [27]. Using a linear gradient of 5—20% (w/v) sucrose in buffer A, a 50-μl portion of the enzyme solution was loaded on the gradient (final volume, 4.4 ml). Centrifugation was carried out at 30°C and 35 000 rev./min for 6 h. Fractions of 7 drops each (about 0.13 ml) were collected. Estimate of the molecular weight was obtained using catalase (250 000 [28]) and glucose-6-*P* dehydrogenase (101 600 [29]) as marker proteins.

Digestion of glycogen synthase with protease. The glycogen synthase was incubated at 30°C with trypsin or chymotrypsin (corresponding to 2% of the substrate protein) in buffer B, from which phenylmethylsulfonyl fluoride was omitted. At appropriate intervals, aliquots were withdrawn for measurement of enzyme activity in the absence and in the presence of glucose-6-*P*. As a control, a parallel assay was run without addition of protease.

Results

Enzyme purification

All purification steps were carried out at 0—4°C.

(1) Extraction of the enzyme. A wild type strain of *N. crassa* (6068, IFO) was grown as reported previously [21]. The mycelia used for preparation of the enzyme were harvested in the late logarithmic phase (2.5—3 days after inoculation). The mycelia were collected with coarse filter paper on a Buchner funnel and rinsed sufficiently with distilled water. The mycelia pads (45 g, wet weight) were homogenized with a suitable amount of quartz sand in 100 ml of buffer A. After cell debris were removed by centrifugation at 1500 × *g* for 15 min, the supernatant was further centrifuged at 20 000 × *g* for 30 min. The supernatant fluid at 20 000 × *g* was diluted to 100 ml with buffer A.

(2) Isolation of 100 000 × g particulate fraction. The supernatant fluid at 20 000 × *g* was centrifuged at 100 000 × *g* for 120 min. The 100 000 × *g* particulate was homogenized in 100 ml of buffer B by glass homogenizer. Almost all of the total glycogen synthase activity applied to the ultracentrifugation was recovered (Table I). The ratio of the glucose-6-*P*-independent activity to the total activity was, however, decreased from 0.83 to 0.46 at this step (Table II).

(3) DEAE-cellulose chromatography. The 100 000 × *g* particulate fraction was applied to a DEAE-cellulose column (4.5 × 14 cm) which was equilibrated

TABLE I

PURIFICATION OF *N. CRASSA* GLYCOGEN SYNTHASE

Purification step	Volume (ml)	Activity (units)	Specific activity (units/mg)	Yield (%)
1. 20 000 × *g* supernatant	100	38 511	0.023	100
2. 100 000 × *g* pellet	100	39 415	0.051	102 *
3. DEAE-cellulose	165	26 285	0.208	68
4. 45% $(NH_4)_2SO_4$) precipitate	10	25 341	1.633	66
5. 3-Aminopropyl-Sepharose	20	16 384	6.884	43

* Overestimated value should be due to the experimental error.

with buffer B. The column was first washed with 800 ml of buffer B, and then eluted with a linear gradient of 0—0.5 M NaCl in buffer B. The endogenous glycogen was washed out through the column and fractions containing glycogen synthase emerged at about 0.3 M NaCl. There was effective separation of the glycogen synthase from endogenous glycogen particle. The glucose-6-*P*-independent activity of the enzyme emerged at the same fractions as those of the total activity (data not shown). Glycogen synthase fractions were pooled and used for further purification.

(4) Ammonium sulfate fractionation. Finely powdered $(NH_4)_2SO_4$ was added to the pooled fractions to make 45% saturation and the mixture was adjusted to pH 8.0 by addition of NH_4OH. After stirring for 60 min, the precipitate was collected by centrifugation at 25 000 × *g* for 60 min and dissolved in a minimal amount of buffer B. Then the enzyme solution was dialyzed for 16 h against 1 l of buffer B.

(5) 3-Aminopropyl-Sepharose chromatography. The ammonium sulfate precipitate fraction, 10 ml, was adsorbed onto a 3-aminopropyl-Sepharose column (1.4 × 9 cm) equilibrated with buffer B. The column was washed with 30 ml of buffer B, and then eluted with a linear gradient of 0—0.4 M NaCl in buffer B. As shown in Fig. 1, the glycogen synthase was eluted as a single peak at approx. 0.2 M NaCl. About 65% of the total activity applied to the column was recovered. Fig. 1 shows that glucose-6-*P*-independent activity of the enzyme was eluted in the same fractions as those of the total activity. But the

TABLE II

CHANGES OF RATIO OF THE GLUCOSE-6-*P*-INDEPENDENT ACTIVITY TO THE TOTAL ACTIVITY OF GLYCOGEN SYNTHASE DURING PURIFICATION STEPS

Purification step	Activity (units)		Ratio —Glc-6-*P*/+Glc-6-*P*
	—Glc-6-*P*	+Glc-6-*P*	
1. 20 000 × *g* supernatant	31 967	38 511	0.83
2. 100 000 × *g* pellet	18 175	39 415	0.46
3. DEAE-cellulose	12 785	26 285	0.49
4. 45% $(NH_4)_2SO_4$ precipitate	9 720	25 341	0.38
5. 3-Aminopropyl-Sepharose	3 673	16 384	0.22

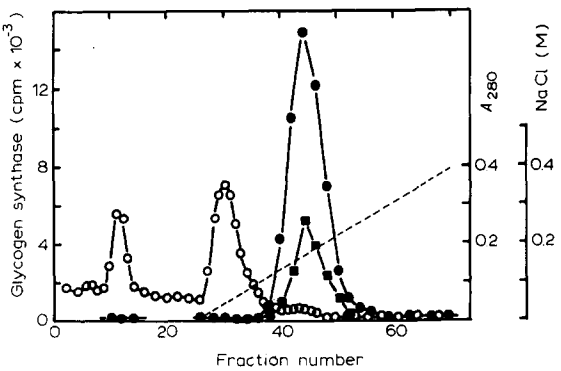

Fig. 1. Elution pattern of the glycogen synthase from a 3-aminopropyl-Sepharose column. —●—, total glycogen synthase activity; —■—, glucose-6-*P*-independent activity; —○—, protein. Fractions of 2 ml were collected and assayed for enzyme activity under the optimum conditions. Details are given in the text.

ratio of the glucose-6-*P*-independent activity to the total activity was further decreased from 0.38 (ammonium sulfate fractionation step) to 0.22 (Table II). The active fractions (40—50) were pooled, dialyzed against 1 l of buffer B, and then quick-frozen in a solid CO_2/acetone bath and stored at —85°C. The purified enzyme was stable for several months when stored at —85°C in the presence of buffer B.

The results of purification of the enzyme are given in Table I. The isolated enzyme had a specific activity of 6.9 units per mg of protein. The enzyme was purified approx. 300-fold from the 20 000 × *g* supernatant fraction with 43% recovery of the activity. In the initial crude extracts, the glycogen synthase was considerably more active in the absence of glucose-6-*P*, but the independent activity was decreased during the purification step and the purified glycogen synthase was mostly glucose-6-*P*-dependent form (Table II).

Purity of the enzyme preparation

The purified preparation of glycogen synthase showed only one protein staining band after polyacrylamide gel electrophoresis under non-denaturing conditions and the enzyme activity was in good correspondence to the protein band (Fig. 2). Electrophoresis on sodium dodecyl sulfate gels also showed a single protein band (Fig. 2). The molecular weight of the synthase subunit was calculated to be 88 000—90 000 by comparison of its relative mobility with that of the standard proteins.

Sucrose density gradient centrifugation

The enzyme sedimented as a single peak in sucrose density gradient centrifugation (data not shown). The glycogen synthase migrated slightly faster than beef liver catalase and very much faster than yeast glucose-6-*P* dehydrogenase. The apparent molecular weight for the synthase was determined to be approx. 270 000 using catalase and glucose-6-*P* dehydrogenase as references for molecular weight. This result shows that the glucose-6-*P*-dependent form of *N. crassa* glycogen synthase appears to behave as a trimer during sucrose density gradient centrifugation.

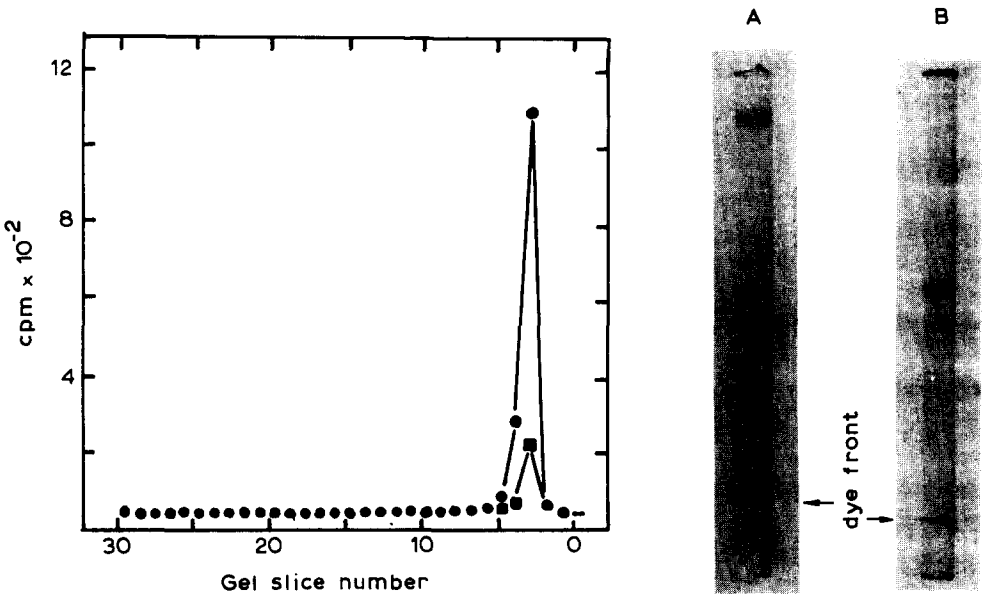

Fig. 2. Polyacrylamide gel electrophoresis of purified *N. crassa* glycogen synthase. Gel A: Electrophoresis of the native enzyme. The enzyme (20 μg) was subjected to gel electrophoresis at 4°C for 60 min, applying 3 mA per column. Duplicate columns were run, one column being stained with Coomassie Brilliant Blue to visualize the protein bands, the other cut into 2-mm segments which were assayed for activity. Each segment was soaked in 100 μl of buffer B for 12 h at 4°C and the elute was used for enzyme activity. The activity profile obtained is shown in the diagram. —●—, total synthase activity; —■—, glucose-6-*P*-independent activity. Gel B: Electrophoresis in the presence of sodium dodecyl sulfate. The enzyme (10 μg) was subjected to gel electrophoresis (7% gel) at room temperature for 4 h, applying 7 mA per column. Protein bands were stained with Coomassie Brilliant Blue.

Properties of the glycogen synthase

Optimum pH range of the synthase was between pH 8.2 and 9.0 in 0.12 M glycyl-glycine/NaOH buffer and a maximal activity was obtained at 30°C. The glycogen synthase was stable at 4°C for 24 h in the pH range of 7.4—8.2 (data not shown).

Effect of metal ions and various reagents

The effect of several metal ions on the enzyme activity was examined (Table III). Divalent metal cations, Mg^{2+}, Mn^{2+}, Ca^{2+} and Fe^{2+}, as well as Fe^{3+} gave no significant effect on the synthase activity. The synthase was somewhat inhibited by Co^{2+} and was strongly inhibited by 1 mM Cu^{2+}, Zn^{2+} and Hg^{2+}. As shown in Table III, the synthase was also strongly inhibited by 1 mM sodium dodecyl sulfate and phenylmercuric acetate. However, EDTA, iodoacetic acid and sodium thioglycolate gave no significant effect on the synthase.

Digestion of glycogen synthase with protease

Purified preparation of glucose-6-*P*-dependent form of the glycogen synthase was digested with trypsin or chymotrypsin, and the change in the enzyme activity was followed (Fig. 3). The total activity of the synthase did not change during the first 15 min period of the incubation with trypsin but then

TABLE III

EFFECT OF VARIOUS METAL IONS AND REAGENTS ON THE ACTIVITY OF GLYCOGEN SYNTHASE

Final concentration of metal ions was 1.0 mM, and that of chemical reagents was 1.0 mM, except for *p*-chloromercuribenzoate (0.1 mM).

Compound	Remaining activity (%)
None	100
$MgCl_2 \cdot 6H_2O$	101
$MnCl_2 \cdot 4H_2O$	106
$CaCl_2 \cdot 2H_2O$	100
$CoCl_2 \cdot 6H_2O$	77
$FeCl_2$	95
$FeCl_3$	99
$CuCl_2 \cdot 2H_2O$	11
$ZnCl_2$	22
$HgCl_2$	5
EDTA	82
Iodoacetic acid	90
Phenylmercuric acetate	52
p-Chloromercuribenzoate	81
Sodium thioglycolate	86
Sodium dodecyl sulfate	7

decreased to 60% of the initial activity after 60 min. On the other hand, the activity assayed in the absence of glucose-6-*P* increased rapidly from the beginning and declined slowly after 30 min. Thus, the ratio of the glucose-6-*P*-independent activity to the -dependent activity showed an increase from 0.12 to 0.43 during the first 15 min period of incubation, without any appreciable loss in total activity. With chymotrypsin, total activity of the synthase remained constant throughout the incubation (60 min). Simultaneously, gradual conversion of the glucose-6-*P*-dependent form of the synthase to glucose-6-*P*-inde-

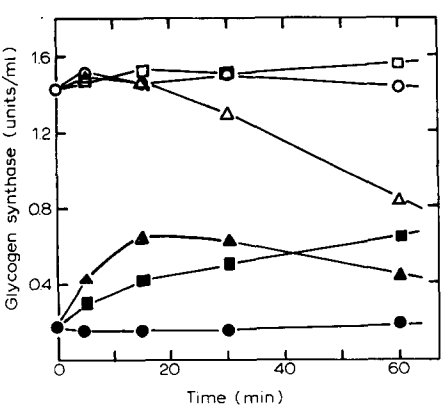

Fig. 3. Effect of proteases on the activity of glycogen synthase, glucose-6-*P*-dependent form. For conditions see in the text. Aliquots were withdrawn at different times and the activity was measured either in the absence or in the presence of glucose-6-*P*. Open symbols and solid symbols represent the activity in the presence and in the absence of glucose-6-*P*, respectively. ○, ●, control; △, ▲, trypsin; □, ■, chymotrypsin.

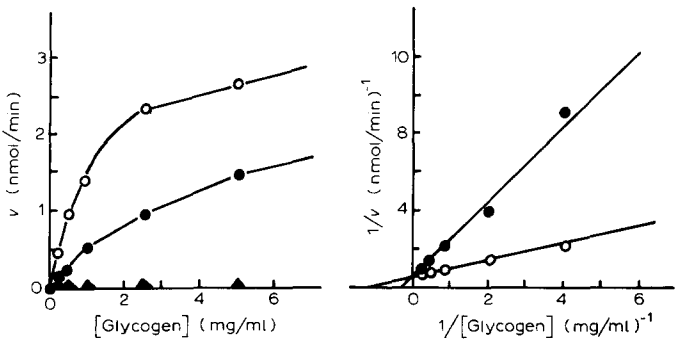

Fig. 4. Various primer saturation kinetics of the purified *N. crassa* glycogen synthase. Glycogen synthase was assayed under the conditions (in the presence of glucose-6-*P*) described in Materials and Methods at the final concentrations of primer indicated. Double-reciprocal concentration of the same data are shown in the diagram on the right. —○—, *N. crassa* glycogen; —●—, phosphorylase limit dextrin of *N. crassa* glycogen; —▲—, β-amylase limit dextrin of *N. crassa* glycogen. Details are in the text.

pendent form occurred until after 60 min and the value of the ratio of the two synthase activities was raised to 0.41.

Activity with primer

Effect of several primers in various concentrations on the enzyme activity was examined as shown in Fig. 4. In the absence of added primer, no enzyme activity was observed. These results show that the purified enzyme preparation contains no endogenous glycogen. Unlike the undegraded glycogen and its phosphorylase limit dextrin, its β-amylase limit dextrin seemed to be inactive as a primer (Fig. 4, left). Different slopes were observed in the reciprocal plots of the reaction rate versus primer concentration with two different primers, the undegraded glycogen and phosphorylase limit dextrin of the glycogen (Fig. 4, right). Although the V values of the both primers were almost equal, the apparent K_m value of the phosphorylase limit dextrin (6.7 mg/ml) was higher than that of the undegraded glycogen (1.5 mg/ml) under the standard conditions. Glucose and maltooligosaccharides (maltose and maltotriose) were examined for their ability to act as acceptors (Table IV). Glucose was found to

TABLE IV

ACTIVITIES OF OLIGOSACCHARIDE ACCEPTORS OF GLYCOGEN SYNTHASE

Acceptor	Concentration	Glucose transferred	
		μmol/min per mg protein	%
Glucose	100 mM	0	0
	200 mM	0	0
Maltose	100 mM	0.128	1.9
	200 mM	0.304	4.4
Maltotriose	100 mM	0.047	0.7
	200 mM	0.100	1.5
N. crassa glycogen	10 mg/ml	6.854	100

be inactive as an acceptor, whereas very low activity was seen for maltose and maltotriose.

Discussion

The *N. crassa* glycogen synthase was extracted from the mycelia harvested in the late logarithmic phase and was purified to satisfactory homogeneity. Purification of glycogen synthase has often encountered difficulty in separating the enzyme from endogenous glycogen, for the enzyme is in most cases firmly associated with the glycogen in vivo. The liberation of the synthase from the glycogen has been conventionally performed by digestion of the glycogen with α-amylase [8] or endogenous phosphorylase [16]. Uncertainty remains, however, as to whether the purified enzyme preparations are completely free of glycogen. Fortunately, application of DEAE-cellulose chromatography has enabled us to separate the *N. crassa* glycogen synthase successfully from endogenous glycogen. For the final purification of glycogen synthase, chromatography on aminoalkyl-Sepharose has often been employed. Thus, the rabbit muscle [22,30] and the *E. coli* [20] glycogen synthases were purified with the aid of aminoalkyl-Sepharose. With the *N. crassa* enzyme, 3-aminopropyl-Sepharose was found to be useful to purify the enzyme to electrophoretic homogeneity. As can be seen from Table II, the ratio of the glucose-6-*P*-independent activity to the total activity of the enzyme was decreased to almost one-fourth of the initial value at the end of the final purification step. If regulation of glycogen synthesis in *N. crassa* is conducted by competitive action of the allosteric effectors, as is considered to be the case in yeast [31,32] and other fungi [33], decrease of the glucose-6-*P*-independent activity may be attributed to removal of the effectors during the purification process. However, other possibilities cannot be neglected, for the enzyme may be converted into the glucose-6-*P*-dependent form by phosphorylation.

The value of specific activity, 6.9 units per mg of protein, of the purified enzyme is at a level similar to, or somewhat lower than, those reported for the mammalian enzymes (5—35 units per mg of protein) [7—17], but much lower than those of the yeast [18] and the *E. coli* [20] enzymes (93 and 505 units per mg of protein, respectively). The subunit molecular weight of the *N. crassa* enzyme (88 000—90 000), as determined by sodium dodecyl sulfate electrophoresis, seems to be similar to those of the mammalian enzymes (85 000—90 000) [9,13,16], but significantly different from that of the yeast (77 000) [18] or bacterial (49 000) [20] enzyme. The molecular weight of the native enzyme was determined to be 270 000 by sucrose density gradient centrifugation. Hence, the native form of this enzyme appears to be composed of three identical subunits. As has been reported, some mammalian glycogen synthases, such as the rabbit muscle synthase D [8] and the rat liver synthase D [11], are present in trimers. However, other enzymes have been found to occur in different degrees of aggregation. For instance, the rabbit muscle synthase I [7], the swine kidney synthase I [12] and the yeast synthase D [18] are reported to be tetramers, whereas the rabbit liver enzymes, both I and D forms, exist as dimers [15]. The active forms of the *E. coli* enzyme are reported to occur as dimers, trimers and tetramers [20]. In spite of these findings, however, no satisfactory

explanation has been given for the relation between activity and degree of aggregation of glycogen synthase.

Recently, Huang and Robinson [17] have reported that the purified human placental glycogen synthase D activity, is stimulated by Mg^{2+}, Mn^{2+} or Ca^{2+}. A similar activation effect of Mg^{2+} has been reported with the enzyme preparations from different sources [34,35]. However, activity of the purified *N. crassa* glycogen synthase was not stimulated by divalent metal cations (Table III). Several glycogen synthases have been reported to be readily inactivated by sulfhydryl group reagents, *p*-chloromercuribenzoate, *p*-hydroxymercuribenzoate and 5,5'-dithiobis(2-nitrobenzoic acid) [18]. The *N. crassa* enzyme was not significantly inactivated by sodium thioglycolate and *p*-chloromercuribenzoate, but lost almost half of its activity by treatment with phenylmercuric acetate (Table III). A strong inactivation effect was also seen for sodium dodecyl sulfate. Other reagents, such as EDTA and iodoacetic acid, did not affect the enzyme to a great extent.

Although the interconversion of glycogen synthase is mainly conducted by phosphorylation and dephosphorylation, irreversible conversion of one form of the enzyme to the other form by limited proteolysis has also been reported. With muscle glycogen synthase, limited proteolysis with trypsin converted the glucose-6-*P*-independent form into the glucose-6-*P*-dependent form, but not vice versa [36,37]. On the contrary, treatment with trypsin or subtilisin converted the dependent form of the yeast enzyme into an apparent independent form [38]. With the *N. crassa* enzyme, treatment of the glucose-6-*P*-dependent form with trypsin or chymotrypsin brought about conversion into an apparent glucose-6-*P*-independent form (Fig. 3). Thus, the *N. crassa* glycogen synthase was shown to be more similar to the yeast enzyme. The different effect of proteolysis may reflect the difference of fine structure of the allosteric sites of the two types of the enzyme.

It is now generally accepted that most glycogen synthases are active only in the presence of added primer. In general, high-molecular-weight polymers are effective as acceptor molecules. However, low-molecular-weight oligosaccharides also function as primers though at much lower rates. The efficiency of the oligosaccharides as primers is different according to the variety of the enzyme. Thus, the rabbit muscle glycogen synthase has been reported to be able to utilize both glucose and maltose as glucosyl acceptors, in place of glycogen [30]. Maltose and maltotriose were effective as primers for the transfer of glucose from ADPglucose in the *E. coli* glycogen synthase reaction, although the former was a rather poor acceptor [20]. However, no activity was seen for glucose in the bacterial glycogen synthase reactions [20]. In common with other enzymes, the purified *N. crassa* glycogen synthase was shown to require a primer for glycogen synthesis. As shown in Table IV, glucose was inactive as a primer in the *N. crassa* glycogen synthesis and either maltose or maltotriose showed little priming activity. Moreover, a distinct difference was observed in the priming activity between β-amylase limit dextrin and phosphorylase limit dextrin of the glycogen. Whereas the former was virtually inactive as a primer, as shown in Fig. 4, the latter showed considerable activity. The apparent K_m value of the phosphorylase limit dextrin was 4.4 times as large as that of the glycogen itself (Fig. 4), although V values of both primers were almost equal.

It appears that the affinity of the primers for glycogen synthase may be affected by the change of their outer chain length. These results suggest the existence of another enzyme system responsible for the de novo synthesis of the primer in vivo. A suggestion proposed by Drucker [39] that the conidia of *N. crassa* lack an appreciable amount of reserve polysaccharides, may support this view.

Our previous paper [40] has demonstrated that soluble enzyme preparation of *N. crassa* catalyzes the transfer of glucose from UDPglucose not only into the endogenous glycogen, but also into the trichloroacetic acid-insoluble fraction. The latter product was suggested to serve as a primer for the subsequent glycogen synthesis. However, clear evidence could not be obtained as to whether these two transfer reactions were catalyzed by a single enzyme or by two different enzymes. Our present results that the purified *N. crassa* glycogen synthase cannot utilize short-chain primers, suggest the existence of another enzyme system responsible for the de novo synthesis of the primer in vivo.

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