UNPRIMED GLUCAN BIOSYNTHESIS BY A PARTICULATE ADP-GLUCOSE-GLUCAN GLUCOSYL TRANSFERASE FROM AN ESCHERICHIA COLI MUTANT AND ITS STIMULATION BY A PROTEIN FACTOR

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SUMMARY

A particulate fraction of an E. coli mutant catalyses the transfert of glucose from ADP-glucose to glycogen but also to a methanol-insoluble product in the absence of primer. The last reaction requires the presence of albumin and either high concentrations of salts or a protein factor. This factor is present in the 158,000 x g supernatant of DF 2000 mutant and in the extracts of mutants lacking glycogen synthase.

INTRODUCTION

A double mutant of Escherichia coli isolated by Fraenkel (1), which lacks activities of both phosphoglucose isomerase and glucose-6-phosphate dehydrogenase, fails to grow on glucose but grows normally on gluconate. It has been shown by Fraenkel (2) that, when presented with glucose, this mutant accumulates internally high concentrations of glucose-6-phosphate and a polysaccharide containing D-glucose. This material has been isolated by Chambost et al. (3) and characterized as glycogen. The cells grown on D-gluconate, in the absence of glucose, contain all the enzymes for glycogen biosynthesis but are practically devoid of glucoside primer (3), this strain being severely restricted in glucose metabolism. However, when supplemented with D-glucose, these cells synthesize glycogen rapidly and without lag (3). Most of the enzyme preparations of glycogen synthases (4, 5, 6) have shown an absolute requirement for a glycogen-type glucan to accept glucose from ADP-glucose or UDP-glucose. However polysaccharide synthases have been found in Aerobacter aerogenes (7), in spinach leaves (8), potato tubers (9) and in a rat liver enzyme preparation (10) to be able to catalyze the de novo α-1,4 glucan synthesis.

In the present paper, we report the presence of an ADP-glucose-glucan glucosyltransferase in the particulate fraction of E. coli mutant, DF 2000. This preparation catalyzes the formation of a polyglucoside in the absence of added primer but requires the presence of bovine serum albumin and either high concentrations of some salts or a protein factor.

MATERIALS AND METHODS

- E. coli DF 2000 is a double mutant, derived from E. coli K10 (Hfr C), lacking both phosphoglucose-isomerase and glucose-6-phosphate dehydrogenase (1). This organism was a gift of Dr. Fraenkel (Harvard Medical School, Boston, Massachusetts). This strain was grown aerobically at 32° in a minimal salts medium (11) always supplemented with 3 µg of thiamine-HCl per ml and D-gluconate at 4 mg per ml. The cells were harvested in the logarithmic growth phase and washed with 0.05 M glycine-NaOH (pH 8.5) containing 0.015 M $MgCl_2$.

- The extracts were made in five volumes of 0.05 M glycine NaOH buffer (pH 8.5) containing 0.015 M $MgCl_2$ and 0.005 M dithiothreitol (DTT) by ultrasonic oscillation in a Branson Sonifier at maximal intensity for four periods of fifteen seconds. Cell-free extracts of E. coli DF 2000 were obtained by centrifugation at 1,000 x g for 30 minutes. The supernatant were then centrifuged at 158,000 x g for 90 min. The 158,000 x g pellet was suspended in the buffer used for extraction.

RESULTS

Particulate nature of glycogen synthase. When extracts of E. coli DF 2000, grown on gluconate without glucose, were prepared by sonic disruption in a medium of high magnesium concentration (15 mM) and subjected to a centrifugation at 158,000 x g, 87 to 94 % of the glycogen synthase activity (primed activity) were recovered in the pellet. Differential centrifugations showed that the enzyme is not associated with ribosomes but instead with membranes (Table I). Thus, when centrifugation was carried out at 40,000 x g in a medium of low magnesium concentration (1 mM) as much as 63.6 % of the activity was sedimented. In these conditions, membrane fragments are sedimented but not most of the ribosomes (14). On the other hand, when the log cells are converted to spheroplasts by treatment with

TABLE I - Sedimentation properties of glycogen synthase

	Percentage of synthase activity in supernatants	
	$MgCl_2$ (1 mM)	$MgCl_2$ (10 mM)
1,000 x g	100	100
40,000 x g (1 h)	36.4	27.2
100,000 x g (2 h 30)	12.0	13.0

The assay for activity was done at 37° C in presence of primer by the method of Preiss and Greenberg (13).

TABLE II - Requirements for unprimed synthesis

In presence of 0.1 M EDTA		In presence of 158,000 x g supernatant	
Reaction mixture	Glucose incorp. (nmole/30 min.)	Reaction mixture	Glucose incorp. (nmole/30 min.)
Complete	15.5	Complete	23.6
- EDTA	0.5	- supernatant	0.5
- Albumin	$\sim$ 0	- Albumin	0.4
- Glutathion	10.7	- Glutathion	10.1
		+ boiled supernatant	0.5

The complete system contained, in 200 µl : glycine-NaOH (pH 8.5), 5 µmoles ; [^{14}C] ADP-glucose, 40 nmoles (80,000 cpm) ; KCl, 5 µmoles ; glutathion, 2 µmoles ; bovine plasma albumin, 100 µg ; DF 2000 particulate preparation, 50 µl (20.4 µg of protein) and either 20 µl of 1 M EDTA or 20 µl of 158,000 x g supernatant. After 30 minutes at 32° C, the reaction was stopped by heating for 1 min. at 100° C and 0.5 mg of carried glycogen was added. The [^{14}C]-glucose incorporated into methanol-insoluble product was determined (13).

EDTA and lysozyme and then subjected to osmotic shock (15), only unsignificant amounts of the enzyme were liberated into the medium whereas 85 % of the activity is recovered in the pellet of lysed spheroplasts. Furthermore, the pattern of sedimentation of glycogen synthase, obtained by differential centrifugation (fig. 1) is similar to that of cytochrome b_1 which is known to be membrane linked (16).

TABLE III - Unprimed activity of the DF 2000 particulate fraction at different concentrations of ADP-glucose (in presence of 0.1 M EDTA).

[ADP-glucose] mM	[^{14}C]-glucose transferred (nmoles) 10'	20'	30'
0.2	2.6	6.1	10.8
0.8	2.6	7.9	16.7
2.0	2.86	7.45	14.45

The reaction mixture is described in Table II.

TABLE IV - Stimulation of unprimed synthesis by EDTA catalyzed by the particulate enzyme and by the soluble enzyme

	[^{14}C]-glucose transferred (nmoles/30 min.) 158,000 x g pellet	158,000 x g supernatant
- EDTA	0.4	0.2
+ EDTA	10.1	22.2

The reaction mixture is described in Table II. It contained 50 µl of extract (20 µg of pellet protein and 50 µg of supernatant protein).

Activation of de novo synthesis by EDTA and salts. The particulate fraction (158,000 x g pellet) catalyses in the presence of primer the synthesis of glycogen from ADP-glucose but is also able to catalyse the synthesis of a methanol-insoluble product in the absence of added glycogen if EDTA 0.1 M is added in the reaction mixture (Table II). Bovine plasma albumin is required for this reaction and glutathion gives a further stimulation (Table II). The rate of synthesis of unprimed product is not linear with time even in presence of high concentrations of ADP-glucose (Table III). If the extracts are prepared in the absence of magnesium, 50 to 60 % of the activity is solubilised and the soluble enzyme is also able to catalyze polyglucose synthesis

TABLE V - Effect of various salts on unprimed reaction

Salts	Conc. M	[^{14}C]-glucose transferred (nmoles)	
		15 min.	30 min.
EDTA	0.1	7.4	13.9
K acetate	1.0	3.2	6.8
Na citrate	0.5	3.5	5.9
$(NH_4)_2$ sulfate	1.0	2.7	4.8
K fluorure	1.0	4.9	9.0
1 molar KSCN, $NaClO_4$, NaBr and KCl are inactive			

Assays were performed as in Table II.

TABLE VI - Activation of unprimed synthesis by the extracts of mutants lacking glycogen synthase activity

Strains	MD_2	MD_3	6156	193	6157	B_{19}	B_{11}	1	7
Glucose transferred nmoles/30 min.									
at 32° C	0.5	0.6	20.2	21.0	19.0	18.0	11.0	20.0	19.0
at 37° C	-	-	-	-	3.6	-	2.4	0.95	

All these strains except 1 and 7 are derived from E. coli Hfr G6 ; MD2 and MD3 are strains carrying deletions of the maltose region and have practically no ADP-glucose pyrophosphorylase and glycogen synthase activity (20). The other strains are mutants lacking glycogen synthase activity ; strains 1 and 7 are derived from E. coli PA 601 (20). The cells were grown on synthetic medium supplemented with amino-acids as required (20) and glucose as carbon source. They were harvested in the logarithmic growth phase and washed with 0.05 M Tris-HCl buffer (pH 7.5). Cell-free extracts were obtained by centrifugation at 12,000 x g for 30 min. 20 µl of these extracts were added to the reaction mixture described in Table II.

in the absence of primer providing that EDTA is added to the reaction mixture (Table IV). Table V shows that high concentrations of some other salts, all belonging to the chastropic series (17), can stimulate the unprimed reaction in presence of albumine. These same salts have been shown to stimulate the enzyme of spinach leaves (18).

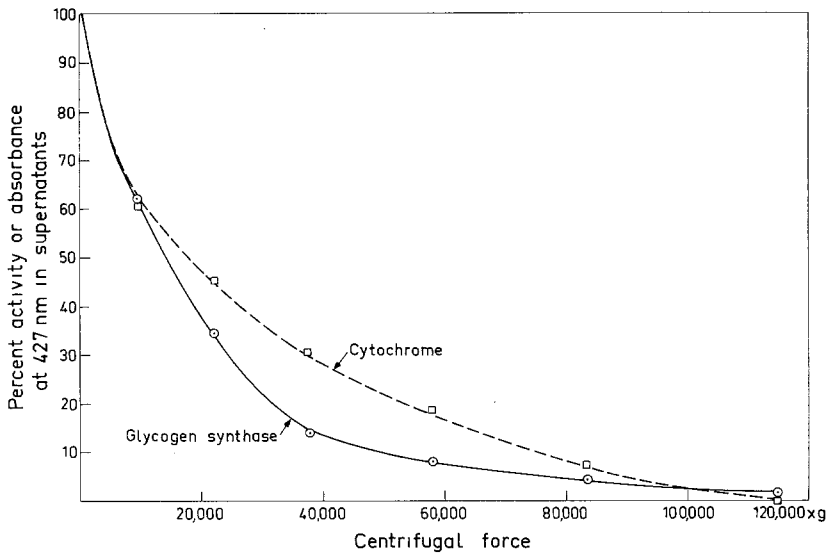

Fig. 1 - Sedimentation of glycogen synthase and cytochrome b_1 during differential centrifugation. Centrifugations were performed at 0-2° C in a Beckman L4 preparative ultracentrifuge equipped with a Ti50 rotor. Centrifugal forces are given at R_{max} and centrifugations are run during 90 min. The content of cytochrome b_1 is expressed as optical density unit at 427 nm (after the addition of hydrosulfite to the preparation).

Activation by a protein factor. The enzyme preparation recovered in the 158,000 x g pellet can also catalyse the unprimed synthesis from ADP-glucose in the presence of a protein factor which is contained in the 158,000 x g supernatant (Table II). This activator is heat labile and is precipitated between 0 and 30 % of saturation with ammonium sulfate. The very fact that, when the supernatant is boiled, it cannot be used, is evidence that no primer is present in the preparation. The presence of albumine is also required for this reaction (Table II).

In previous works (19, 20) we have isolated and studied several mutants of E. coli K12 altered in glycogen metabolism. The three genes of glycogen synthesis (glg A, glg B and glg C) corresponding respectively to glycogen synthase, branching enzyme and ADP-glucose pyrophosphorylase, have been localized and found to be closely linked, near the maltose region. Two strains carrying deletions of the maltose region lack also the three glg genes. Extracts of these strains are unable to activate the unprimed synthesis of glycogen (Table VI). This result indicates that the gene for the protein

factor may be in the glg region. Several mutants lacking glycogen synthase have been studied also for their ability to activate the unprimed synthesis. These mutants cannot synthesize glycogen since they lack glycogen synthase activity. However, the extracts of all the tested mutants are able to activate the unprimed synthesis catalysed by the particulate enzyme of the mutant DF 2000 (Table VI). They do not catalyse the de novo synthesis in presence of EDTA. The activation by this protein activator has been measured at 32° C because it is very sensitive to an increase of temperature. At 37° C, the activation is very low (Table VI).

DISCUSSION

In the enterobacteriaceae, 70-95 % of the α-1,4 glucan synthase is particulate (21) and this property can be attributed to enzyme binding to glycogen. The major part of this polysaccharide sediments in the same conditions. Fox et al. (22) have recently purified a glycogen synthase from an E. coli B mutant dereppressed in the levels of ADP-glucose pyrophosphorylase and glycogen synthase. The enzyme has been found in the particulate fraction with glycogen and could be solubilized only with treatment by α-amylase. This result seems to corroborate the hypothesis that the enzyme is bound to the glycogen. However, in extracts of DF 2000 strain which cells are devoid of glycogen, the major part of the glycogen synthase sediments with the particulate fraction. The enzyme does not appear to be associated with ribosomes but rather to membrane fragments. It may be possible that, in the cells which are devoid of glycogen, the enzyme is actually bound to the membrane but as the glycogen synthesis proceeds, the enzyme separates from the membrane owing to its greater affinity for glycogen.

The particulate fraction of DF 2000 mutant catalyses the transfert of glucose from ADP-glucose to glycogen but can also catalyse the synthesis of a methanol-insoluble product in the absence of primer. This last reaction requires in vitro the presence of albumine and EDTA. High concentrations of some other salts produce the same activation as EDTA. A similar effect of EDTA and salts has been observed by C. R. Krisman (10) with a partially purified enzyme from rat liver which catalyses the transfert of glucose to a trichloracetic acid-insoluble fraction. Ozbun et al. (8) have also demonstrated the stimulation of a spinach leave transferase by salts and EDTA. The mecha-

nism of stimulation by salts and albumin is unknown but the fact that EDTA also stimulates the de novo synthesis by the soluble enzyme shows that it has no effect on the binding of the enzyme with membrane. It could be explained by a change of conformation of the enzyme (18).

The de novo synthesis is also stimulated by a protein factor found in the soluble fraction of DF 2000 extracts. This factor, probably identical with the activator protein found in A. aerogenes (7), might be the factor required for the initiation of the glycogen biosynthesis in vivo. It is present in the extracts of several mutants lacking glycogen synthase. This activator may be either a specific protein inducing the appropriate conformation of the enzyme or a protein which serves as an primary acceptor for glucosyl units. However, as it is not required for the de novo synthesis in presence of EDTA or salts, the first hypothesis is more likely to be correct.

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