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UDP-Glucose: (1→3)-β-Glucan Synthases from Mung Bean and Cotton

DIFFERENTIAL EFFECTS OF Ca^{2+} AND Mg^{2+} ON ENZYME PROPERTIES AND ON MACROMOLECULAR STRUCTURE OF THE GLUCAN PRODUCT

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ABSTRACT

A re-examination of the kinetic properties of UDP-glucose: (1→3)-β-glucan (callose) synthases from mung bean seedlings (*Vigna radiata*) and cotton fibers (*Gossypium hirsutum*) shows that these enzymes have a complex interaction with UDP-glucose and various effectors. Stimulation of activity by micromolar concentrations of Ca^{2+} and millimolar concentrations of β-glucosides or other polyols is highest at low (<100 micromolar) UDP-glucose concentrations. These effectors act both by raising the V_{max} of the enzyme, and by lowering the apparent K_m for UDP-glucose from >1 millimolar to 0.2 to 0.3 millimolar. Mg^{2+} markedly enhances the affinity of the mung bean enzyme for Ca^{2+} but not for β-glucoside; with saturating Ca^{2+}, Mg^{2+} only slightly stimulates further production of glucan. However, the presence of Mg^{2+} during synthesis, or $NaBH_4$ treatment after synthesis, changes the nature of the product from dispersed, alkali-soluble fibrils to highly aggregated, alkali-insoluble fibrils. Callose synthesized *in vitro* by the Ca^{2+}, β-glucoside-activated cotton fiber enzyme, with or without Mg^{2+}, is very similar in size to callose isolated from cotton fibers, but is a linear (1→3)-β-glucan lacking the small amount of branches at C-0-6 found *in vivo*. We conclude that the high degree of aggregation of the fibrils synthesized with Mg^{2+} *in vitro* is caused either by an alteration of the glucan at the reducing end or, indirectly, by an effect of Mg^{2+} on the conformation of the enzyme. Rate-zonal centrifugation of the solubilized mung bean callose synthase confirms that divalent cations can affect the size or conformation of this enzyme.

Essentially all higher plants contain a UDP-glucose: (1→3)-β-glucan (callose) synthase. This enzyme is largely found on the plasma membrane, and in most cases is latent and only becomes activated by perturbed conditions which lead to some loss of membrane permeability (5, 16). Some years ago, Ray (26) named this enzyme Glucan Synthetase II, and assayed it at high concentrations of UDP-Glc in the absence of divalent cations, although others have reported that activity can be enhanced by Mg^{2+} (6), and variable stimulation by β-glucosides has frequently been observed (12, 24). More recently, the enzyme has been shown to be activated by Ca^{2+} (7, 8, 16, 17, 24), a finding which could provide an explanation for the activation of callose synthesis *in vivo* under perturbed conditions where influx of this cation might be expected to occur (16).

It is clear that confusion exists over the exact effector requirements for this enzyme and the conditions which should be used to obtain optimal activity *in vitro*. Since the enzyme is often used as a plasma membrane marker (25, 26), and since it may play an important role in defense mechanisms in plants (1) and has even been suggested to be an altered form of cellulose synthase (4, 14), we felt it was important to clarify the kinetic properties of callose synthase from several plant sources. In addition, we compare in detail the structure of the product synthesized *in vitro* in the presence of different combinations of effectors to that of the callose produced *in vivo* during cotton fiber development, one of the relatively rare cases where callose is synthesized *in vivo* in a nonperturbed state (13, 21).

MATERIALS AND METHODS

Materials. Mung beans (*Vigna radiata*) were purchased locally, soaked overnight in aerated water, and grown for 3 to 5 d at 28°C in complete darkness. Cotton (*Gossypium hirsutum*, Acala SJ-2) was grown in growth chambers with cycles of 12 h light at 28°C and 12 h dark at 21°C. Exo-(1→3)-β-glucanase (from *Basidiomycetes* sp QM 806) was kindly provided by Dr. K. Matsuda (Tohoku University, Japan), endo-(1→3)-β-glucanase (from *Rhizopus*) was from E. T. Reese (U.S. Army Labs, Natick, MA), and crude elicitor fractions from fungal and sycamore cell walls were from P. Albersheim (University of Georgia). Sirofluor was from Australia Biosupplies, Sepharose CL-6B from Pharmacia, Aquamix from WestChem, and Protease K, lichenase (a contaminant in an α-amylase preparation from *Bacillus subtilis*), and unlabeled UDP-Glc were purchased from Sigma. UDP-[U-^{14}C]Glc was purchased from ICN, digitonin was from Serva, and laminaribiose was from CalBiochem.

Enzyme Isolation and Assay. Cotton fibers were harvested 18 to 22 d postanthesis and a 5,000*g* membrane pellet was prepared as described previously (7). A 100,000*g* membrane pellet and digitonin-solubilized enzyme from hypocotyls and roots of etiolated mung beans were prepared as described previously (29) using a homogenization buffer of 50 mM Tris/HCl (pH 7.3) containing 5 mM EDTA, 5 mM EGTA, 0.5 mM phenylmethylsulfonyl fluoride and 0.05 mM leupeptin. Enzyme was solubilized from membranes resuspended to 3 to 5 mg protein/ml in 50 mM

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Tris/HCl (pH 7.3), 20% (v/v) glycerol, and 1% (w/v) digitonin. After incubating for 30 min at 4°C and centrifuging for 1 h at 100,000*g*, 30 to 60% of the total enzyme activity was solubilized. In our experience, good activity can be obtained by homogenization with a variety of buffers in the pH range 7 to 8, with or without protease inhibitors, although Tris/HCl is somewhat inhibitory at higher concentrations. Enzyme preparations were stored at −80°C in buffer containing 20% (v/v) glycerol and were stable for many months.

Except where indicated, assays were carried out using the membrane-bound form of the enzymes. The composition of a typical complete reaction mix was 20 mM Hepes/KOH (pH 7.3), 5 mM $CaCl_2$, 10 mM cellobiose, 0.01% digitonin, 100 μM UDP-[^{14}C]Glc (200,000 cpm/assay), and 10 to 50 μg of protein, in a final volume of 200 μl. However, in the cases where cotton fiber glucan produced *in vitro* was compared to the *in vivo* glucan, the reaction mixtures contained laminaribiose in place of cellobiose and 10 mM Bistrispropane/Hepes (pH 7.5) replaced Tris/HCl as buffer. Except for a few cases noted in the text, reactions were linear with respect to time at 25°C for at least 15 min. Reactions were routinely terminated by addition of 3 ml of 66% ethanol containing 0.5 mM EDTA, carrier cellulose was added, and the mixes chilled at −20°C for at least 30 min. They were then filtered onto Whatman GF/C glass fiber filters, washed three times in 66% ethanol, once in chloroform:methanol (1:1), and the filters counted in Aquamix. Protein was determined by a modification of the Lowry assay for membrane proteins (22). At concentrations below 20 μM, free Ca^{2+} concentrations were established by the use of a Ca^{2+}-EGTA buffer, with the free Ca^{2+} concentration calculated using a computer program designed by J. Walton, ARCO Plant Cell Research Institute, Dublin, CA; these calculations are based upon the dissociation constant for Ca^{2+}-EGTA as a function of pH and Mg^{2+} concentration. They do not take into account any interactions of Ca^{2+} with membranes and/or UDP-Glc; it may be for this reason that we find a 2- to 3-fold variation in the apparent affinity of the enzymes for Ca^{2+} in different experiments.

Product Entrapment Procedure. Digitonin-solubilized mung bean enzyme was partially purified (estimated 50–200-fold) by a modification of the product entrapment procedure of Kang *et al.* (15). Enzyme was incubated for 30 min at 25°C and then 90 min at 4°C in 5 mM cellobiose, 5 mM unlabeled UDP-Glc, 2.5 mM $CaCl_2$, and 2.5 mM $MgCl_2$. A visible white precipitate of glucan formed which was centrifuged (53,000*g*, 30 min) through a layer of glycerol to form a pellet which also contained trapped glucan synthase. This pellet was homogenized in 50 mM Tris/HCl (pH 7.5), centrifuged to remove glucan, and the supernatant was reincubated with substrate and effectors and recentrifuged as before. The second pellet was homogenized, centrifuged, and the supernatant used for EM studies.

Polysaccharide Structure Determinations. (1→3)-β-Glucan was isolated from cotton fibers harvested 19 d postanthesis. Fresh locules were frozen in liquid N_2, and fibers, freed from seeds, were grouped to a powder in liquid N_2, and then homogenized with ice-chilled 1 M $HClO_4$. The mixture was centrifuged at 0°C, the supernatant discarded, and the pellet was resuspended in water, neutralized with NaOH, and dialyzed against distilled H_2O for 3 d at 4°C. This insoluble material was extracted sequentially 3 times each for 30 min in water (85°C), 4% KOH/0.1% $NaBH_4$, and 24% KOH/0.1% $NaBH_4$ (11). About 65% of the total (1→3)-β-glucan was found in the 4% KOH/0.1% $NaBH_4$ extract and this glucan was further purified by precipitation at pH 4.0 and subsequent solubilization with 4% KOH, then neutralized, and treated with Protease K at 40°C for 6 h, followed by dialysis against distilled H_2O and freeze-drying (yield 210 mg from 100 locules).

The mol wt of glucans were estimated by gel filtration on a column (1 × 100 cm) of Sepharose CL-6B equilibrated with 0.1 M NaOH. Samples of [^{14}C]glucan synthesized *in vitro* were solubilized by overnight extraction at 40°C in 24% KOH/0.1% $NaBH_4$ prior to gel filtration; glucan synthesized *in vivo* and purified as above was solubilized and applied to the column in 0.1 M NaOH. Total carbohydrate was determined by the phenol-sulfuric acid method (9).

Optical rotation was determined with a standard polarimeter in the laboratory of Dr. C. Ballou, UC Berkeley. Neutral sugars were determined as their alditol acetates (2) by GLC on a DB-225 capillary column (0.25 mm × 15 m; J & W Scientific) at 200°C. Methylation analysis was done by the method of Harris *et al.* (10). Partially methylated alditol acetates were separated on the same capillary column with a temperature program of 2°/min from 130 to 195°C. Radioactive derivatives were quantitated by Radio-GLC (Packard Instruments) using a glass column (0.5 × 200 cm) of OV-225 at 160°C. ^{13}C-NMR spectra were recorded at 50°C at 200 MHz on a Varian XL300 NMR spectrometer; samples were dissolved in Me_2 SO-*d*6.

Enzymic hydrolyses of glucan were carried out in 1 ml reaction mixtures containing 5 mg of cotton fiber glucan, 0.2 mg of enzyme, 10 mM sodium acetate (pH 5.0), and a few drops of toluene; incubation was for 12 h at 40°C, and was terminated by heating in a boiling water bath for 5 min. Rates of hydrolysis were calculated from the relative amounts of reducing sugar (Somogyi method) (11) to total sugar (phenol-sulfuric acid method; 9). The hydrolysates were deionized with Dowex 50W (H^+ form), dried under N_2, dissolved in 0.5 ml water and chromatographed on Whatman 3MM paper in 1-propanol:ethyl acetate:water (3:2:1). Carbohydrate from different areas of the chromatogram was quantitated (9) following elution in water.

Electron Microscopy. Digitonin-solubilized mung bean enzyme, as a crude preparation or partially purified by product entrapment, was incubated for 30 min at 21°C with 10 mM UDP-Glc, 10 mM cellobiose, 5 mM $CaCl_2$ with or without 5 mM $MgCl_2$. Reaction mixtures were then mounted on carbon-coated Formvar grids and negatively stained with 1% uranyl acetate containing 1 mg/ml bacitracin. Samples were observed with a Philips 420 electron microscope at 80 kV.

RESULTS

Effector Requirements for Callose Synthases. Table I shows an example of the effects of various compounds on the production of ethanol- and alkali-insoluble glucan using membranes or 1%-digitonin solubilized membrane proteins from mung beans. We have presented similar results for cotton fiber and soybean preparations in conference proceedings (7, 8). Digitonin at low concentrations (0.01–0.05%) stimulates activity in membrane preparations, presumably due to unmasking of activity outside-out vesicles. Consistient with this, digitonin increases the V_{max} but has no effect on the apparent K_m for UDP-Glc (not shown). Omission of cellobiose results in some reduction in glucan production; however, if the glycerol present in our enzyme preparations is also removed, very little glucan is produced. Alternatively, if divalent cations are removed by addition of EDTA, the enzyme is virtually inactive: in terms of total ethanol-insoluble glucan produced, only Ca^{2+}, and not Mg^{2+}, is required in conjunction with cellobiose and/or glycerol for maximal activity. Thus, at 100 μM UDP-Glc, the enzyme shows a nearly absolute dependence for activity on the presence of both Ca^{2+} and a sugar or other polyol. Mg^{2+}, however, appears to play a more complex role in modulating the nature of the glucan produced, as removal of Mg^{2+} from the reaction mix results in a marked change in the solubility of the glucan produced. Whereas the presence of Mg^{2+} usually does not result in much stimulation of total glucan synthesis in the presence of saturating Ca^{2+}, Mg^{2+} is absolutely required for production of alkali-insoluble glucan.

Table I. *Effector Requirements for Mung Bean Callose Synthase Activity*

Reactions contained 100 μM UDP-[^{14}C]Glc (9700 cpm/nmol), 5 mM Tris/HCl (pH 7.3), 0.01% digitonin, 0.22 M glycerol, 13 μg membrane protein or 9 μg solubilized protein and other constituents as indicated. Incubation was for 10 min at 25°C. Alkali-insoluble glucan was determined by diluting and incubating reactions for 2 h at 21°C in 2 ml 24% (w/v) KOH prior to collection and water washing of products on filters.

Additions (5 mM each) to Assay	Glucan Produced			
	Membrane-bound enzyme		Digitonin-solubilized enzyme	
	Ethanol-insoluble	Alkali-insoluble	Ethanol-insoluble	Alkali-insoluble
	nmol Glc incorporated/min·mg protein			
$CaCl_2$, $MgCl_2$, CB[a]	24.9	30.2	34.8	40.3
As above, minus digitonin	11.3	12.7	ND[b]	ND[b]
$CaCl_2$, $MgCl_2$	16.9	20.4	20.8	21.3
As above, minus glycerol[c]	<1	<1	ND[b]	ND[b]
EDTA, CB	<1	<1	<1	<1
EDTA, $MgCl_2$, CB	1.0	1.1	2.7	2.9
$CaCl_2$, CB	23.4	<1	24.6	<1

[a] Cellobiose. [b] Not determined. [c] Membranes washed to remove glycerol.

Table II. *Concentrations of β-Glucoside or Polyol Required for Half-Maximal Callose Synthase Activity*

All reactions contained 100 μM UDP-[^{14}C]Glc, 5 mM $CaCl_2$, and 5 mM $MgCl_2$, and were incubated for 10 min at 25°C. Those for the mung bean enzyme contained 5 mM Tris/HCl (pH 7.3) and 10 μg protein; for the cotton fiber enzyme, 5 mM Hepes/KOH (pH 7.0) and 22 μg protein. V_{max} values (not shown) were essentially the same for all compounds tested.

	Apparent K_a	
	Mung bean	Cotton fiber
	mM	
Laminaribiose	0.2	0.09
Cellobiose	0.4	0.13
Gentiobiose	1.2	>1
Glucose	2.6	1.0
Maltose	7	1.5
Sucrose	15	ND[a]
Glycerol	200	ND[a]

[a] Not determined.

Table II shows the estimated concentration of a variety of sugars or polyols required for half-maximal activity in the presence of saturating Ca^{2+} and Mg^{2+}. Although β-linked di-glucosides are the most effective compounds that promote activity, at higher concentrations a variety of sugars or polyols are also activators and yield V_{max} values very close to those obtained with saturating cellobiose. Digitonin, which contains β-glucosidic linkages, cannot replace cellobiose. With the cotton fiber enzyme, we also tested the effect of addition, at up to milligram amounts per assay, of carboxymethylcellulose, cotton fiber (1→3)-β-glucan or xyloglucan, or crude elicitor fractions from fungal or sycamore cell walls, but none could substitute for cellobiose. Some limited stimulation was obtained with cellotetraose, laminaritetraose and laminaripentaose, but on a molar basis these were far less effective than the corresponding disaccharides. Using the mung bean enzyme, we determined that cellobiose affects both the V_{max} and the apparent K_m for UDP-Glc when assayed in the presence of saturating Ca^{2+} and Mg^{2+} (Fig. 1). Note that the V *versus* S plots shift from hyperbolic to sigmoidal in the absence of cellobiose.

Interactions between Ca^{2+} and Mg^{2+}. The mung bean enzyme, like those from soybean (17) and sugar beet (24), has a high affinity for Ca^{2+} (Fig. 2). Good activation in the absence of Mg^{2+} occurs with 5 to 10 μM Ca^{2+}, and the affinity is enhanced by the presence of Mg^{2+}, with activation then occurring at 0.1 to 2 μM Ca^{2+}. In contrast, Mg^{2+} has no effect whatsoever on the affinity for cellobiose (not shown). Ca^{2+} affects both the V_{max} and the apparent K_m for UDP-Glc (Fig. 3) as does cellobiose (Fig. 1), and V *versus* S plots shift from hyperbolic to sigmoidal at very low concentrations of either effector. Mg^{2+} stimulates activity in the presence of limiting Ca^{2+}, consistent with the interpretation that Mg^{2+} enhances the affinity of the enzyme for Ca^{2+} (Fig. 2). Note that the V versus S plot is sigmoidal and the apparent K_m considerably higher in the absence of any divalent cations, an assay condition used frequently in earlier studies with this enzyme. We also noted that glucan production with time often shows a lag under these conditions (not shown; also found for the maize enzyme, N. Carpita, personal communication).

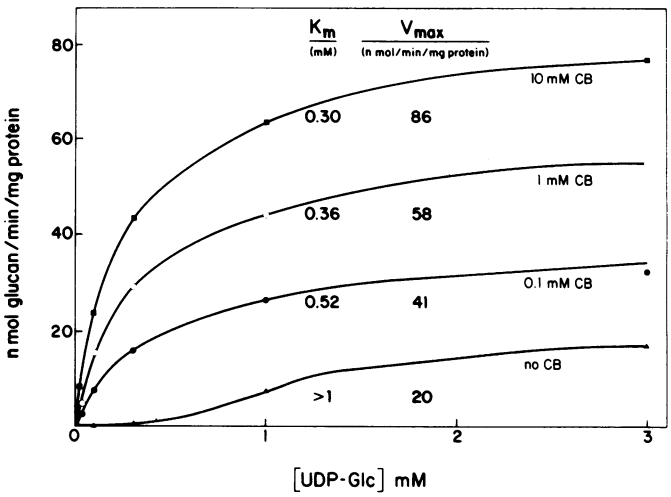

FIG. 1. Effect of variation in concentration of UDP-Glc and cellobiose in the presence of saturating $CaCl_2$ and $MgCl_2$ on mung bean callose synthase activity. Reactions, containing 5 mM each of $CaCl_2$ and $MgCl_2$, 50 mM Hepes/KOH (pH 7.8), 0.01% digitonin, 14 μg of membrane protein, and other constituents as indicated, were incubated for 10 min at 25°C. K_m and V_{max} values were calculated from Lineweaver-Burk plots or the direct linear (1/V *versus* S/V) technique or (in the case of very sigmoidal curves) were estimated by eye from the plots shown.

The effect of increasing Mg^{2+} concentration on stimulating the production of alkali-insoluble glucan by both the membrane-bound and digitonin-solubilized forms of the mung bean enzyme is shown in Figure 4. These plots are highly sigmoidal, yielding *n* values in Hill plots of 2.6 and 2.9 for the membrane-bound

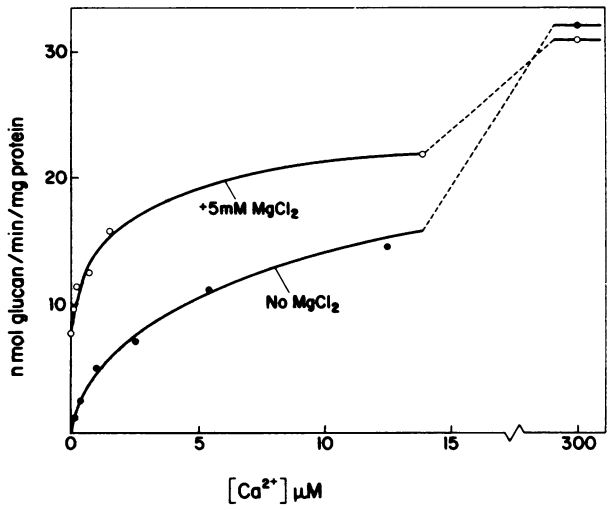

FIG. 2. Activity of mung bean callose synthase as a function of concentration of free Ca^{2+}. Reactions, containing 250 μM UDP-[^{14}C]Glc, 5 mM cellobiose (CB), 0.01% digitonin, 20 mM Hepes/NaOH (pH 7.3), 10 μg of membrane protein, 1 mM EGTA, and variable amounts of $CaCl_2$, with (O) or without (●) 5 mM $MgCl_2$, were incubated for 15 min at 25°C. Free Ca^{2+} was estimated as described in "Materials and Methods."

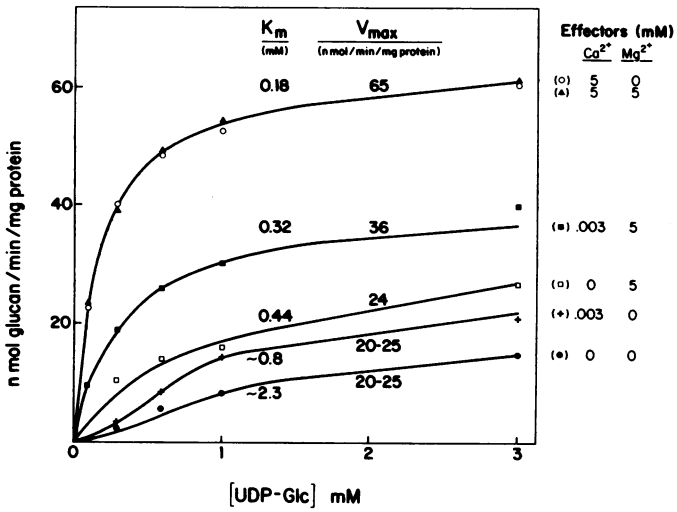

FIG. 3. Effect of variation in concentration of UDP-Glc, $CaCl_2$, and/or $MgCl_2$ in the presence of saturating levels of cellobiose on mung bean callose synthase activity. Reactions, containing 10 mM cellobiose, 0.01% digitonin, 20 mM Hepes/NaOH (pH 7.3), 24 μg of membrane protein, and other constituents as indicated, were incubated for 5 and 10 min at 25°C. 'Zero' Mg^{2+} and Ca^{2+} was obtained by inclusion of 5 mM EDTA in the reaction, 'zero' Ca^{2+} by inclusion of 5 mM EGTA, and 0.003 mM Ca^{2+} by use of a Ca^{2+}-EGTA buffer.

and solubilized forms respectively (n value = slope of line for $V/[V_{max}-V]$ *versus* $\log_{10}[Mg^{2+}]$). Addition of Mg^{2+} at the end of the reaction did not alter the solubility of the glucan produced, indicating that Mg^{2+} is probably not causing direct aggregation of the glucan. These results are consistent with a cooperative conformational change in the enzyme induced by Mg^{2+}, which in turn affects the macromolecular structure of the product synthesized.

The results shown in Table III show that the presence of $NaBH_4$ during alkaline extraction can prevent solubilization of the glucan product synthesized in the absence of Mg^{2+}. This would seem to indicate that this glucan may simply be more

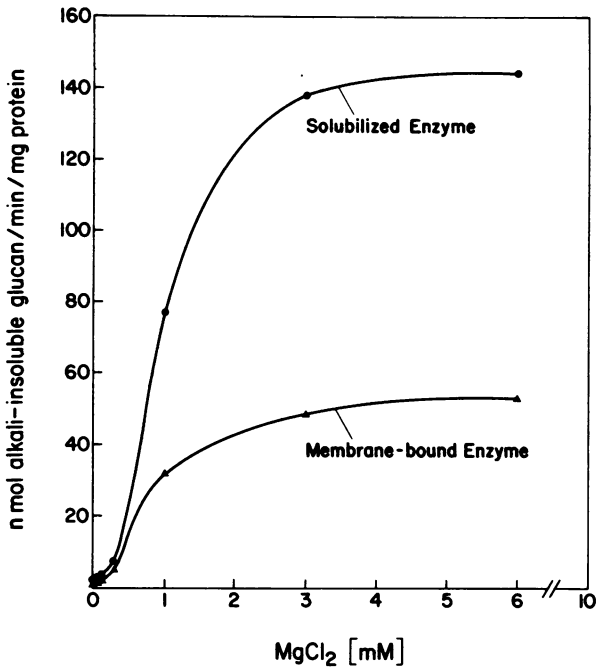

FIG. 4. Effect of variation in $MgCl_2$ concentration in the presence of saturating $CaCl_2$ and cellobiose on the production of alkali-insoluble glucan by mung bean callose synthase. Reactions, containing 100 μM UDP-[^{14}C]Glc, 5 mM Tris/HCl (pH 7.3), 0.02% digitonin, 5 mM $CaCl_2$, 5 mM cellobiose, 0.22 M glycerol, 13 μg membrane protein or 9 μg solubilized protein, and varying $MgCl_2$, were incubated 10 min at 25°C. Alkali-insoluble glucan was analyzed as described in Table I.

susceptible to alkaline degradation ('base-peeling'), a phenomenon that can be prevented if the reducing end of the glucan is protected by borohydride reduction. However, other results, also shown in Table III, do not support this conclusion. First, the product synthesized in the absence of Mg^{2+} is solubilized within 1 min in alkali at 21°C; furthermore, it can then be precipitated by ethanol from the KOH solution, indicating no loss of labeled monomer from the reducing end. Even after 3 h in alkali lacking $NaBH_4$, more than half of the product can be precipitated with ethanol.

To clarify further this difference in product structure, we examined with transmission EM the glucan produced by solubilized mung bean enzyme in the presence of Ca^{2+} and cellobiose with or without Mg^{2+}. The difference in chemical solubility of the glucan is clearly reflected in the state of the fibrils, which are dispersed if synthesized in the absence of Mg^{2+} (Fig. 5A), but highly aggregated in its presence (Fig. 5B). No fibrils were observed in any incubations lacking UDP-Glc.

We also noted the frequent occurrence of 20 to 30 nm diameter doughnut-shaped structures in the aggregated glucan and wondered if these could represent synthase complexes (arrows, Fig. 5B). Solubilized enzyme was purified 50–200-fold by a 'product entrapment' procedure ("Materials and Methods" and Ref. 15), incubated with UDP-Glc and effectors and examined by EM (Fig. 5C). Numerous doughnut-shaped structures were seen, and we occasionally observed glucan fibrils protruding from the center of such a structure (inset, Fig. 5C). If this represents a callose synthase complex, it is a very large enzyme (20–30 nm diameter after negative staining).

Figure 6 shows the behavior of the digitonin-solubilized mung bean enzyme during velocity sedimentation in glycerol gradients. In the presence of 5 mM EDTA (or 5 mM EGTA or no chelator at all, not shown), the enzyme sediments as a relatively sharp peak ahead of the bulk of the solubilized protein. This indicates

Table III. *Effects of Mg^{2+}, Alkali, and Borohydride on Solubility of Glucan Product*

For mung bean enzyme, reactions contained 20 μg membrane protein, 7.5 mM Hepes/KOH (pH 7.3), 0.01% digitonin, 0.25 mM UDP-[^{14}C]Glc (4000 cpm/nmol), 5 mM cellobiose, 5 mM $CaCl_2$, ±5 mM $MgCl_2$; incubation was for 10 min at 25°C prior to treatment and filtration. For cotton fiber enzyme, reactions contained 6 μg membrane protein, 1.25 mM Bistrispropane/Hepes (pH 7.5), 10 mM cellobiose, 5 mM $CaCl_2$, 0.01% digitonin, 0.1 mM UDP-[^{14}C]Glc (10,000 cpm/nmol), ±5 mM $MgCl_2$; incubation was for 15 min at 25°C.

Treatment after Reaction Prior to Filtering	Glucan Recovered on Filters			
	Mung bean enzyme		Cotton fiber enzyme	
	No $MgCl_2$	+$MgCl_2$	No $MgCl_2$	+$MgCl_2$
	nmol Glc/min·mg protein			
66% ethanol, −20°C, 30 min	35.6	46.5	26.7	33.1
H_2O, 21°C, 30 min	35.8	48.5	21.7	26.7
24% KOH, 21°C, 2 h	0.3	43.2	ND[a]	ND[a]
24% KOH/0.5 M $NaBH_4$, 21°C, 2 h	42.5	36.2	ND[a]	ND[a]
24% KOH, 100°C, 20 min	0.7	25.1	1.0	17.7
24% KOH/0.5 M $NaBH_4$, 100°C, 2 h	41.3	59.5	19.2	25.7

Minutes in 24% KOH ±$NaBH_4$ at 21°C after Incubation Minus $MgCl_2$	Mung Bean Enzyme			
	Filtered directly from KOH		Precipitated in 66% ethanol from KOH	
	No BH_4	+BH_4	No BH_4	+BH_4
0			41.1	
1	1.3	22.1	43.0	49.1
10	0.8	31.7	43.4	49.0
30	0.6	36.4	41.7	50.4
60	0.5	37.9	36.1	44.6
180	1.0	42.9	35.2	48.3

[a] Not determined.

again that the callose synthase is of high mol wt: Jack bean urease (18.6 S; 480,000 mol wt) sediments about half as fast as does the callose synthase under these conditions.

Moreover, in the presence of 5 mM $MgCl_2$ (or 5 mM $CaCl_2$, not shown) the enzyme sediments even faster and appears more heterogeneous in size and/or shape (Fig. 6). The amount of the shift and the width of the peak in the presence of Mg^{2+} or Ca^{2+} is somewhat variable from run to run. The level of divalent cation required to increase the sedimentation rate of the enzyme in this way was found to be between 1 and 5 mM, which is in the physiological range for the concentration of Mg^{2+}, but not of Ca^{2+}, found *in vivo*, suggesting a role for Mg^{2+} in modulating enzyme size *in vivo*.

Comparison of the Structure of Cotton Fiber (1→3)-β-Glucan Synthesized *in Vitro* and *in Vivo*. Developing cotton fibers are unusual in that they transiently synthesize (1→3)-β-glucan at the onset of secondary wall cellulose synthesis (13, 21). The cotton fiber is, therefore, a useful system in which to compare the nature of the (1→3)-β-glucan produced *in vitro* with that produced in an unperturbed state *in vivo*.

Staining of cotton fibers with Sirofluor, a compound that fluoresces on specific interaction with (1→3)-β-glucans (28), showed a relatively uniform distribution of the glucan throughout the length of the fiber wall (not shown). This is similar to the previous finding of Waterkeyn (30) who used the less specific dye, aniline blue.

The glucan produced *in vivo* has been reported to be largely a linear (1→3)-β-glucan (13, 21). Fine-structure analysis of this glucan is reported here for the first time. This glucan is distributed in all the fractions extracted from fibers (13, 21), and under our conditions, 4% KOH extracted a major portion (about 65% as judged by methylation analysis). When purified further from the 4% KOH extract by acid precipitation and protease digestion, the polysaccharide was homogeneous by chromatography on Sepharose CL-6B with an average mol wt in the range of 50,000 to 200,000 (Fig. 7A). This mol wt is quite similar to the size of the glucan synthesized *in vitro* in the presence of Ca^{2+} (Fig. 7B) or Ca^{2+} plus Mg^{2+} (Fig. 7C); however, only about half of the glucan synthesized with Ca^{2+} and Mg^{2+} was solubilized by the overnight extraction at 40°C in 24% KOH/0.1% $NaBH_4$ prior to gel filtration. Thus, within the limits available for analysis, it would seem that the Mg^{2+}-dependent change in solubility of the glucan (Table III), is not due to any major change in size of the individual glucan chains.

The $[\alpha]_D$ value of the purified glucan extracted from fibers was +3° in 0.1 M NaOH, and it contained glucose as the sole sugar. GLC profiles of alditol acetates from fully methylated glucan showed three peaks corresponding to 2,3,4,6-tetra-*O*-methylglucose, 2,4,6-tri-*O*-methylglucose, and 2,4-di-*O*-methylglucose in the proportion of 1:23:1 based on peak areas, and the structures were confirmed by GLC-MS (not shown). No 2,3,6-tri-*O*-methylglucose derivative, indicative of (1→4)-linked glucose, was found. Therefore, the native glucan backbone is (1→3)-linked and has a small proportion of branches at C-O-6.

This structure for the cotton fiber glucan was further confirmed by enzymic degradation. It was 48% digested by an *endo*-(1→3)-β-glucanase and completely digested by an *exo*-(1→3)-β-glucanase. Hydrolysis products with the *endo*-enzyme were glucose, laminaribiose, and a small amount of laminaritriose. Exoglucanase digestion yielded glucose and gentiobiose in a molar ratio of about 20:1. Because this enzyme degrades from the nonreducing end of chains, one cannot determine the chain length of the branches, and can only conclude that the branches commence with (1→6)-linked glucose residues which may or may not be extended further by additional (1→3)-linked glucosyl residues. The size of the glucan, as judged by gel filtration, was not changed following incubation with *B. subtilis* lichenase (an *endo*-[1→3, 1→4]-β-glucanase that cleaves adjacent to [1→4]-bonds), further

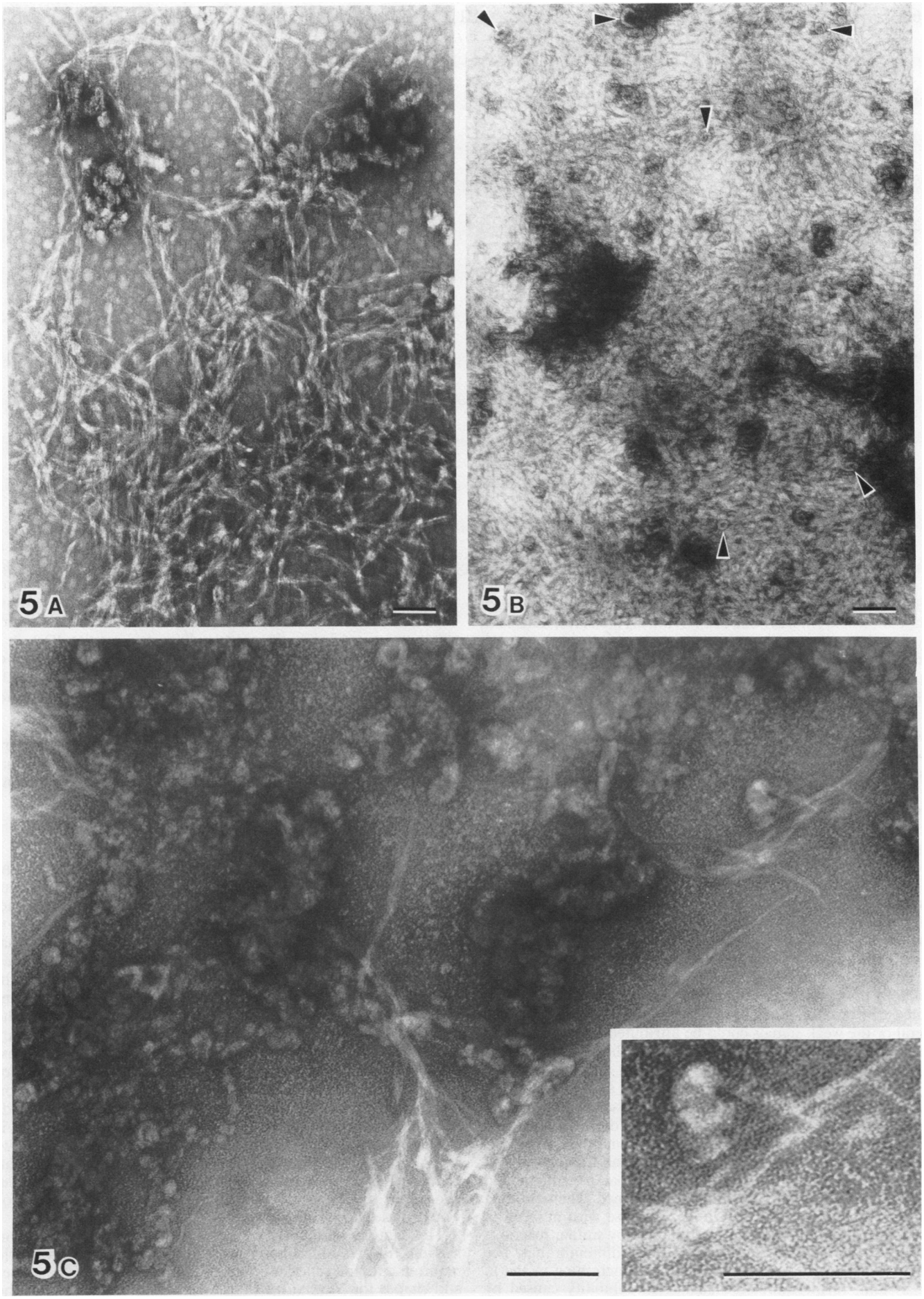

FIG. 5. Electron micrographs of fibrils synthesized by digitonin-solubilized mung bean callose synthase. A, Dispersed fibrils. Reaction contained 10 mM unlabeled UDP-glucose, 10 mM cellobiose, 5 mM $CaCl_2$, and crude digitonin-solubilized enzyme. Incubation time prior to fixation was 30 min at 21°C. B, Aggregated fibrils. Reaction contents as A, but with the inclusion of 5 mM $MgCl_2$. C, As for A, but enzyme was partially purified 50–200-fold by product entrapment (see "Materials and Methods"). Bar = 0.1 μm.

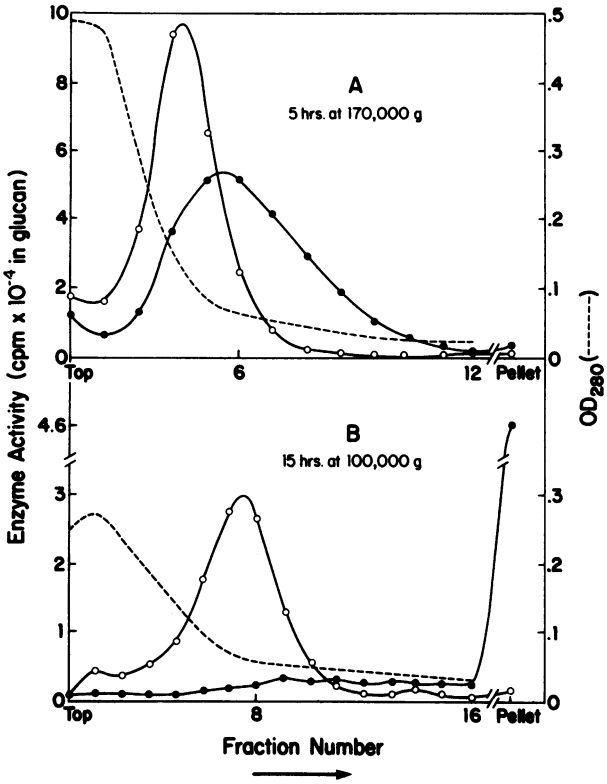

FIG. 6. Glycerol gradient centrifugation of digitonin-solubilized mung bean callose synthase. One-half ml (1–2 mg protein) of solubilized enzyme preparation in 1% digitonin and 50 mM Tris/Cl (pH 7.5) containing either 5 mM EDTA (O) or 5 mM $MgCl_2$ (●) was layered onto 15 to 30% (v/v) linear glycerol gradients containing 12.5 mM Hepes/KOH (pH 7.3) and 5 mM EDTA (O) or 5 mM $MgCl_2$ (●). Sedimentation was in a Beckman SW 40 rotor at 4°C with the g forces and times indicated. (----), OD_{280} for gradient plus EDTA. OD_{280} values for gradient with $MgCl_2$ (not shown) were similar to those for the EDTA gradients. Although digitonin was not included in these gradients, we have found it to enhance recovery of activity without substantially altering sedimentation patterns.

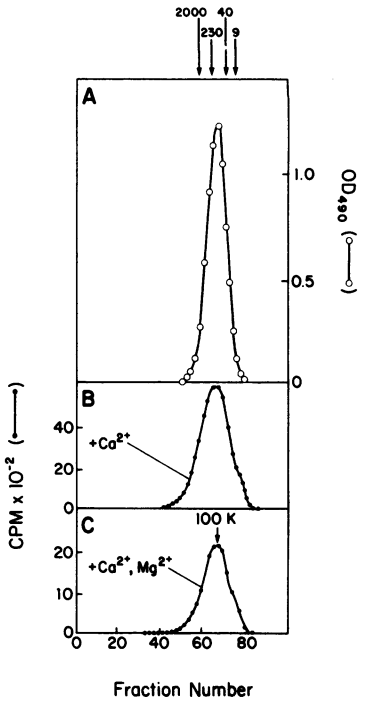

FIG. 7. Gel-filtration of cotton fiber (1→3)-β-glucan synthesized *in vivo* or *in vitro*. Samples were chromatographed on a Sepharose CL-6B (1 × 100 cm) column equilibrated in 0.1 M NaOH. Mol wt standards were Blue Dextran (2000 kD), pea xyloglucan (230 kD), and dextrans (40 and 9 kD). A, Purified (1→3)-β-glucan isolated from cotton fibers; glucan was quantified by the phenol/sulfuric acid reaction (OD_{490}); B and C, [^{14}C]glucan synthesized *in vitro* (15 min, 25°C) in the presence of 5 mM laminaribiose, 5 mM $CaCl_2$, without (B) or with (C) 5 mM $MgCl_2$. [^{14}C]Glucan was solubilized by overnight incubation of reaction mixtures at 40°C in 24% KOH/0.1% $NaBH_4$.

supporting the conclusion that the polymer does not contain any (1→4)-β-linkages interspersed between (1→3)-β-linkages.

^{13}C-NMR further confirmed this structure showing 6 intense signals and 7 weak signals. Of the intense signals, a peak at 103.9 ppm could be assigned to the β-anomeric carbon at C-1; a split signal at 86.1 to 86.2 ppm to the C-3 of the (1→3)-linkage, typically shifted downfield (as in laminarin) from the characteristic position of an unsubstituted C-3; signals at 77.0 ppm, 74.6 ppm, 69.6 ppm, and 62.2 ppm could be attributed to the unsubstituted carbon atoms at C-5, C-2, C-4, and C-6, respectively. Several of the weak signals could be attributed to carbon atoms in the branched glucosyl residues. No peak indicative of a linkage at C-4 was detected.

The [^{14}C]glucan synthesized *in vitro* was a linear (1→3)-β-glucan as judged by results of similar analyses. There was no indication of branching. The only radioactive derivative detected during methylation analysis was 2,4,6-tri-*O*-methylglucose; the product was resistant to lichenase digestion, and digestion with the *exo*-(1→3)-β-glucanase yielded only radioactive glucose and no gentiobiose. Identical results were obtained when product from reactions containing Ca^{2+} and cellobiose with or without Mg^{2+} was analyzed, or with glucan obtained by similar reactions using detached fibers (6) as opposed to isolated membranes. The C-O-6 branching enzyme is therefore not functioning in the *in vitro* system; furthermore, the change in solubility caused by Mg^{2+} is not due either to a change in chain length or in pattern of branching. Absence of any detectable radioactivity in terminal glucose residues indicates that the enzyme is polymerizing long β-glucan chains *in vitro*.

DISCUSSION

The mung bean and cotton fiber callose synthases clearly show a complex interaction with UDP-Glc, Ca^{2+}, Mg^{2+}, and β-glucosides or polyols. Although the traditional assay for this enzyme, performed in the absence of added divalent cations and >50 μM UDP-Glc, affords fair activity, it clearly is not the optimal condition. Ray (26) suggested these conditions because they allowed one to distinguish this plasma membrane activity from the much lower levels of Mg^{2+}-dependent-(1→4)-β-glucan synthase found in the Golgi. Having now a clearer picture of the kinetic properties of callose synthases, we recommend that they be assayed in the presence of 0.01 to 0.03% digitonin, 5 mM cellobiose, saturating Ca^{2+} (concentrations up to 5 mM are not inhibitory in our experience), and a UDP-Glc concentration close to the apparent K_m of 0.2 to 0.3 mM. Since Mg^{2+} affords little additional stimulation of activity under these conditions, it can be omitted to avoid possible interference with (usually low) levels of other Mg^{2+}-dependent synthases; however, the glucan produced in the absence of Mg^{2+} will be largely alkali-soluble, and assays should therefore measure production of ethanol- or water-insoluble glucan.

The data presented here clearly explain why the literature on this enzyme contains many contradictory reports of variable requirements for cellobiose and Mg^{2+}, and variable K_m values and shapes for V *versus* S plots. We found originally that the

cotton fiber enzyme displayed sigmoidal kinetics and low affinity for UDP-Glc (6), but we later observed hyperbolic kinetics and a much lower K_m (7); these apparently contradictory results are now reconciled, since the former study was done with EDTA-extracted fibers assayed in the absence of Ca^{2+}, and the latter was done with enzyme assayed in the presence of Ca^{2+}. Similarly, if isolation and/or assay buffers contain sucrose or glycerol in high concentration, then these may partially substitute for cellobiose. Another potential source of confusion about this enzyme arises from the reports of Kauss *et al.* (17) that the soybean enzyme can be converted to a Ca^{2+}-independent form by proteolysis. Although their data are convincing for soybean, we have been unable to observe this effect with the cotton or mung bean enzymes. It remains to be seen how widespread this phenomenon is, but it should be kept in mind in any studies with callose synthases. These workers also reported that organic polycations can enhance the activity of the soybean enzyme at limiting concentrations of Ca^{2+} (18); this effect of polycations resembles one of the effects which we observe for Mg^{2+}, and it may be that all of these cations bind at the same site, a site which is separate from, but which influences, the Ca^{2+}-binding site on the enzyme.

The very low apparent K_a for Ca^{2+} for these enzymes in the presence of physiological levels of Mg^{2+} supports the notion that small variations in levels of free intracellular Ca^{2+} probably form a key regulatory mechanism for callose synthesis *in vivo* (16). It is not at all clear, however, what compound(s) *in vivo* fulfills the requirement for β-glucoside or polyol. We have recently observed that 100,000*g* supernatants from crude cotton fiber extracts do contain a factor which can replace cellobiose, but in order to see this effect is it necessary to heat the supernatant at 100°C for 5 min to remove a heat-labile inhibitor of the enzyme. Radioactive cellobiose or laminaribiotol (T Hayashi, DP Delmer, unpublished data) or cellodextrins (20) were not incorporated into product, suggesting that these activators do not serve as primers; rather, the kinetic studies described here suggest that they serve as allosteric effectors.

Mg^{2+} shows unusual effects on the enzyme; in addition to enhancing the affinity for Ca^{2+}, the presence of Mg^{2+} seems to cause some cooperative, conformational change and/or aggregation of the enzyme and also results in synthesis of a more insoluble, aggregated form of glucan. The Mg^{2+}-induced change in the macromolecular structure of the glucan product; however, is not due to any detectable change in the primary structure or size of the individual glucan chains synthesized. Thus, Mg^{2+} may instead be causing some realignment of the multiple active sites in the enzyme complex so as to favor more extensive association of the nascent glucan chains during polymerization. An effect of Mg^{2+} on the structure at the reducing end of the glucan is also possible, as indicated by the protective effect of $NaBH_4$ against solubilization of the glucan in alkali. The results of Table III in sum do not support the conclusion that solubilization is due to extensive alkaline degradation, but they do suggest that the structure of the polymer at the reducing end is critical and might differ between product synthesized in the presence or absence of Mg^{2+}. Although the size of the glucan synthesized *in vitro* by the cotton fiber enzyme is remarkably similar to that produced *in vivo*, it does lack the low level of branching observed *in vivo*. A similar loss or marked reduction in branching activity *in vitro* has also been observed for (1→3)-β-glucan synthesis in yeast and other fungi (19).

These studies also indicate that the mung bean callose synthase, as solubilized in digitonin, is of high mol wt (>500,000). Other recent studies using gel filtration of the soybean enzyme (M Thelen, DP Delmer, unpublished data), native PAGE of enzyme from several sources (29), and radiation inactivation of the red beet enzyme (27), also indicate that callose synthases are large enzymes. The intriguing notion that these enzymes may be altered forms of cellulose synthase complexes (4, 14) can never be proven until they are characterized in a pure state, but it is worth noting that the doughnut-shaped structures seen by us (Fig. 5) are quite similar in size to the rosettes and terminal globules seen in freeze-fracture studies and proposed to be cellulose synthase complexes (3). Similarly, we note that Mg^{2+} has been indicated as playing a role in maintaining the structural integrity of the terminal complexes in *Oocystis* (23), a result which may be relevant to the effects of Mg^{2+} observed in this study on the conformation of mung bean callose synthase.

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