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Mechanism of Action of Cyclic β-1,2-Glucan Synthetase from *Agrobacterium tumefaciens*: Competition between Cyclization and Elongation Reactions

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We have examined some aspects of the mechanism of cyclic β-1,2-glucan synthetase from *Agrobacterium tumefaciens* (235-kDa protein, gene product of the *chvB* region). The enzyme produces cyclic β-1,2-glucans containing 17 to 23 glucose residues from UDP-glucose. In the presence of added cyclic β-1,2-glucans (>0.5 mg/ml) (containing 17 to 23 glucose residues), the enzyme instead synthesizes larger cyclic β-1,2-glucans containing 24 to 30 glucose residues. This is achieved by de novo synthesis and not by disproportion reactions with the added product. This is interpreted as inhibition of the specific cyclization reaction for the synthesis of cyclic β-1,2-glucans containing 17 to 23 glucose residues but with no concomitant effect on the elongation (polymerization) reaction. Temperature and detergents both affect the distribution of sizes of cyclic β-1,2-glucans, but glucans containing 24 to 30 glucose residues are not produced. We suggest that the size distribution of cyclic β-1,2-glucan products depends on competing elongation and cyclization reactions.

Cyclic β-1,2-glucans are produced by species of *Agrobacterium* and *Rhizobium* and may be involved in nitrogen fixation (16), attachment of bacterial cells to plant hosts (1), and osmotic regulation (18). They also have potential industrial use as novel encapsulating agents (11).

The biosynthesis of cyclic β-1,2-glucans requires a membrane-bound enzyme (235-kDa protein) which becomes transiently radiolabelled with [U-^{14}C]glucose upon the addition of UDP-[U-^{14}C]glucose (12, 20, 21). Large quantities of cyclic β-1,2-glucans are synthesized by this protein under certain growth conditions and secreted outside the cell (5). A 65- to 75-kDa export protein contributes to this process (4, 8). After purification of a fraction containing the cyclic β-1,2-glucan products, high-performance liquid chromatography (HPLC) revealed that these glucans consisted of rings of between 17 and 23 glucose residues (cG_{17} to cG_{24}) (10). Even larger ring sizes have been detected (3). By using UDP-[U-^{14}C]glucose, Amemura (1) demonstrated that crude membrane preparations from *Agrobacterium tumefaciens* and *Rhizobium phaseoli* were able to synthesize a mixture of cyclic β-1,2-glucans. The distribution and relative amount of each glucan showed four general patterns depending on the species (1, 10). However, the mechanism of action of the synthetase has not been addressed. In this article, we suggest that the enzymatic mechanism involves competition between cyclization and elongation after the polymer reaches a critical size. Cyclic polymers of certain sizes can inhibit the cyclization reaction such that larger cyclic products result.

MATERIALS AND METHODS

Organism and culture media. *A. tumefaciens* C58 (wild type) was obtained from M. Rhodes, Agricultural and Food Research Council Institute of Food Research, Norwich, United Kingdom. The bacteria were grown in 0.5% (wt/wt) tryptone and 0.3% (wt/wt) yeast extract medium at 28°C with shaking (250 rpm) for 24 h. From a 0.1% (vol/vol) inoculation, the final A_{660} was 1.0 to 1.2. Bacteria (3 to 3.5 g [wet weight] per liter) were harvested by centrifugation at 23,000 × *g* for 2 h at 4°C, followed by a second centrifugation at 40,000 × *g* for 2 h at 4°C with an MSE high-speed 18 centrifuge. Bacteria were stored at −20°C.

Preparation of membrane fractions. Membrane fractions were prepared on the basis of a method described previously (21) but with substantial modification primarily to remove nucleic acids as follows. After thawing of the bacteria, 1 ml of 0.01 M Tris-Cl (pH 7.4)–0.15 M KCl was added per 0.3 g of bacteria, and the suspension was sonicated with an MSE Soniprep 150 apparatus (amplitude, ~22 μm) for a total of 15 min at 4°C. The sonicated suspension was centrifuged twice for 30 min at 40,000 × *g* (4°C), and the pellets were discarded. DNase and RNase (both from bovine pancreas) were added to the supernatant to a final concentration of 18 and 0.84 Kunitz units per ml, respectively. After 16 h at 4°C, streptomycin sulfate was added (2% [wt/vol]) and incubated for 30 min at 4°C and the mixture was centrifuged for 30 min at 18,000 × *g* at 4°C. The supernatant was then centrifuged for 2 h at 217,000 × *g* at 4°C (Beckman L8-M Ultracentrifuge with a 70.1 Ti rotor). The resulting pellet was carefully resuspended in 1 ml of 0.01 M Tris-Cl (pH 7.4)–0.15 M KCl and recentrifuged at 217,000 × *g* for 2 h at 4°C. The pellet was resuspended in 1 ml of the above buffer and designated membrane fraction I.

Assays. The reaction mixture contained 0.79 mM UDP-[U-^{14}C]glucose (1.39×10^{10} Bq/mol), 32 mM $MgCl_2$, 6.5 mM Tris-Cl (pH 7.4), 0.1 M KCl, and membrane fraction I in a total volume of 0.31 ml. For assays containing less UDP-[U-^{14}C]glucose, the radioactivity was 1.39×10^{11} Bq/mol. After 2 h at 30°C (unless otherwise stated), the reaction was stopped by immersion in a boiling water bath for 2 min. After centrifugation at 10,000 × *g* for 2 min, the supernatant was incubated with washed Amberlite resin (mixture of Amberlite IRA-400 anion-exchange resin [hydroxide form] and

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Amberlite IR-120 cation-exchange resin [hydrogen form]). After 10 min at 30°C with gentle mixing, the sample was centrifuged for 2 min at 10,000 × *g*. NaOH was added to the supernatant to 0.15 M and incubated for 10 min at 30°C. Any cyclic β-1,2-glucans produced were separated by high-performance anion-exchange chromatography–pulsed amperometric detection (HPAEC-PAD) (apparatus by Dionex) by a modification of the method of Koizumi et al. (9), as follows. A carbopac PA1 column (Dionex) was equilibrated in 0.15 M NaOH, and the cyclic β-1,2-glucans were eluted with 0.15 M NaOH–1.0 M sodium acetate. The PAD apparatus was equipped with a gold electrode (pulse sequence E1, +0.03 V; E2, +0.60 V; E3, −0.62 V). Fractions of either 0.2 or 1.0 ml (depending on the required resolution) were collected and counted for radioactivity. Alternatively, the amount of radiolabel was determined by using a radiochemical detector (Reeve) with 3 parts scintillant (Monoflow 4; National Diagnostics) to 1 part column eluent after neutralization with a membrane suppressor (Dionex) containing 0.4 M H_2SO_4. The radiolabel was used to quantify production of U-^{14}C-labelled cyclic β-1,2-glucans, and the results were confirmed by PAD. The protein concentration was estimated by using Coomassie blue G-250 protein assay reagent (Pierce), relative to bovine serum albumin. Scintillation counting was performed with samples (up to 0.5 ml) after samples were dried onto XtalScint Ready Caps (Beckman Instruments Ltd.).

Chromatography standards. The HPAEC-PAD was calibrated with a mixture of cyclic glucans, which were a kind gift from P. L. Sturla, Shell Research Ltd., Kent, United Kingdom. They were purified from this mixture to obtain individual cyclic β-1,2-glucans (cG_{17} to cG_{22}) as described previously (6). The standard mixture of cyclic β-1,2-glucans consisted of the following (percentage of total): cG_{17}, 4.6; cG_{18}, 10.9; cG_{19}, 30.4; cG_{20}, 22.2; cG_{21}, 14.9; cG_{22}, 9.9; cG_{23}, 1.9; cG_{24}, 1.6; cG_{25}, 0.4; cG_{26}, 0.5; cG_{27}, 0.6; cG_{28}, 0.7; cG_{29}, 0.5; cG_{30}, 0.4 (Fig. 1).

Kinetic analysis. K_m and V_{max} were calculated by the method of Wilkinson (17).

Preparation of [U-^{14}C]cG_{20}. U-^{14}C-labelled cyclic β-1,2-glucans were synthesized as described above. After HPAEC-PAD, the fractions corresponding to [U-^{14}C]cG_{20} were pooled and lyophilized. The concentration was estimated by scintillation counting.

RESULTS

The standard mixture of cyclic β-1,2-glucans was used to calibrate the HPLC (Fig. 1). Retention time increased with molecular mass, except for that for cG_{21}, which eluted before cG_{20}, in agreement with previous work (9).

Figure 2 shows the results of a typical assay of cyclic β-1,2-glucan synthetase. This system provided the basis for all of the following determinations and enabled both the specific activity (total cyclic β-1,2-glucans produced) and the amount of each cyclic β-1,2-glucan to be calculated. With this assay, the production of total cyclic β-1,2-glucans by membrane fraction I was linear up to a reaction time of 2 h. The specific activity for the protein used in this work was 0.34 ± 0.19 nmol/min · mg (14 separate cultures of bacteria and preparations of membrane fraction I). Quantitation of the peak areas for individual cyclic β-1,2-glucans produced from the standard assay described above gave the following distribution of products (percentage of total cyclic β-1,2-glucans with standard deviations from 12 determinations): cG_{17}, 2.7 ± 1.0; cG_{18}, 11.2 ± 3.6; cG_{19}, 17.6 ± 3.9; cG_{20}, 23.5 ± 2.4; cG_{21}, 20.6 ± 4.9; cG_{22}, 24.7 ± 4.2; cG_{23} and above, less than 1.

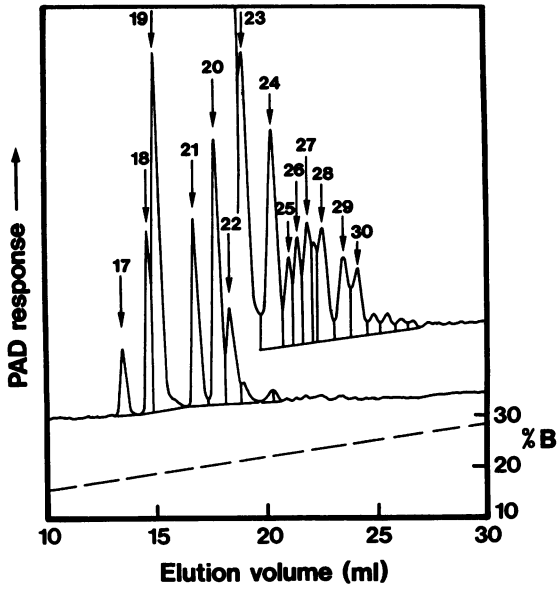

FIG. 1. Chromatography of cyclic β-1,2-glucans (standard mixture) on HPAEC with PAD. The arrows marked 17 to 22 show the retention times of standards consisting of one cyclic β-1,2-glucan. Other retention times, marked 23 to 30, were deduced as described in reference 14. Cyclic β-1,2-glucans (total, 10 μg) were applied to a Carbopac PA1 column in 0.15 M NaOH and eluted with a gradient (dashed line) of 0.15 M NaOH–1.0 M sodium acetate (solvent B). The inset shows the elution obtained by loading 200 μg of total cyclic β-1,2-glucans.

Reversal of the reaction. [U-^{14}C]cG_{20} was added to membrane fraction I–unlabelled UDP-glucose (0.79 mM)–$MgCl_2$ (32 mM) and incubated for 2 h. When analyzed by HPAEC, the only U-^{14}C-labelled compound that could be detected by radiocounting was [U-^{14}C]cG_{20}. Other cyclic β-1,2-glucans, including cG_{20}, were present as detected by PAD but were not U-^{14}C labelled. This experiment demonstrates that added cyclic β-1,2-glucans do not exchange glucose residues with growing cyclic β-1,2-glucans. Additionally, the synthetase does not catalyze exchange reactions between cyclic β-1,2-glucans that are already synthesized, i.e., external transglycosylation of soluble glucans does not occur. This clearly distinguishes the cyclic β-1,2-glucan synthetase from cyclodextrin glucanotransferase (EC 2.4.1.19). The latter enzyme acts by transglycosylation of maltooligosaccharides and/or already formed cyclodextrins in solution (14). Experimentally, therefore, the cyclic β-1,2-glucan synthetase reaction is not reversible, and there is no postsynthesis "shuffling" of cyclic β-1,2-glucans.

Inhibition of the reaction by individual cyclic β-1,2-glucans. The ability of individual cyclic β-1,2-glucans to act as inhibitors of cyclic β-1,2-glucan synthetase was examined. Figure 3 shows the effect of adding cG_{21} to the synthetase assay. There is a reduction in the overall amount of U-^{14}C-labelled cyclic β-1,2-glucans synthesized which arises from decreased amounts of all individual cyclic β-1,2-glucans. The greatest inhibition is, however, seen for the synthesis of [U-^{14}C]cG_{21}. The inhibition appears to reach saturation at >2 mg of cG_{21} per ml, so the synthesis of [U-^{14}C]cG_{21} at high concentrations of added cG_{21} is not completely abolished. The lowest rate of synthesis of [U-^{14}C]cG_{21} in the presence

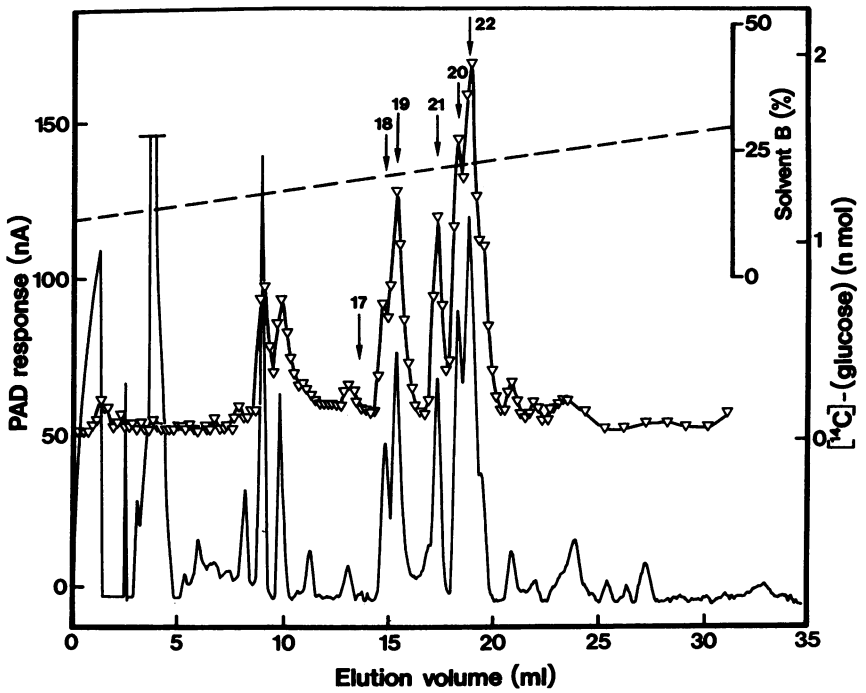

FIG. 2. Detection of U-^{14}C-labelled cyclic β-1,2-glucans by PAD and by radiochemical scintillation counting produced by membrane fraction I from UDP-[U-^{14}C]glucose. For membrane fraction I, UDP-[U-^{14}C]glucose and $MgCl_2$ were incubated and processed as described in Materials and Methods. U-^{14}C-labelled cyclic β-1,2-glucans were eluted with a gradient of 0.1 M NaOH–1.0 M sodium acetate (solvent B) (dashed line). The elution positions of standards (shown by arrows) were correlated to the response of the pulsed amperometric detector (solid line) and to the amount of radiolabel (▽). The measured radioactivity was used to internally calibrate the PAD.

of added cG_{21} is 35% of the control. At lower concentrations of UDP-[U-^{14}C]glucose, however, the rate of synthesis of cG_{21} is further decreased below 35% (see below).

The effect of adding cG_{18} or cG_{22} to the reaction mixture during synthesis of U-^{14}C-labelled cyclic β-1,2-glucans was examined. Added cG_{18} alone (1 mg/ml) had very little effect on the synthesis of [U-^{14}C]cG_{18}, but the synthesis of [U-^{14}C]cG_{20} was slightly increased and that of [U-^{14}C]cG_{22} was slightly reduced (Fig. 4). The total rate of synthesis of cyclic β-1,2-glucans was hardly affected by cG_{18}. In contrast, cG_{22} (1 mg/ml) decreased the total rate of synthesis by 30%, and the amount of [U-^{14}C]cG_{21} and [U-^{14}C]cG_{22} synthesized was decreased. This decrease was much more pronounced for [U-^{14}C]cG_{22} compared with the effect of adding cG_{21} (see above) (Fig. 4).

From the above results, a putative model for the synthesis of cyclic β-1,2-glucans was devised (Fig. 5). In this model, a linear β-1,2-glucan chain is built up, covalently attached to the protein, until 16 glucose residues are attached. After the addition of the 17th residue, either the glucan is then sterically able to adopt a configuration which the protein is able to cyclize, or, in a competing reaction, an additional glucose residue is added. This continues for all subsequent cyclic β-1,2-glucans. Thus, the cyclization reaction (denoted by rate constants k'_n) competes with the elongation reaction (rate constants k_n). The experiments described above clearly show that once cyclized, cyclic β-1,2-glucans do not reenter the reaction so the cyclization reaction is effectively irreversible. Further, to simplify the model, we assume that the rate constant, k_n, for addition of β-1,2-linked glucose to the covalently attached linear chain is the same whether the growing β-1,2-glucan is 16 or more glucose residues long.

If this model is valid, it would be expected that in the presence of sufficiently high concentrations of cyclic β-1,2-glucans containing 17 to 22 glucose residues, the major products of the reaction would be [U-^{14}C]cG_{23} and larger U-^{14}C-labelled cyclic β-1,2-glucans. Additionally, the total amount of U-^{14}C-labelled products, whatever the distribution, would be the same in both the presence and absence of inhibitor. The following experiments were designed to test this.

Effect of a mixture of cyclic β-1,2-glucans containing 17 to 22 glucose residues on the synthesis of U-^{14}C-labelled cyclic β-1,2-glucans. In the presence of a sufficiently high concentration of cyclic β-1,2-glucans containing 17 to 22 glucose residues, a completely different elution profile was observed after HPAEC-PAD of the assay mixture compared with that of a control sample (Fig. 6). The production of cG_{24} is increased, and cG_{25} to cG_{30} are produced. The latter were not present in significant amounts in the control.

The amounts of U-^{14}C-labelled cyclic β-1,2-glucans containing 24 to 30 glucose residues produced in the presence of cyclic β-1,2-glucans containing 17 to 22 glucose residues were calculated over a range of concentrations of UDP-[U-^{14}C]glucose (Table 1). The amounts of total synthesis and total synthesis in the control sample are very similar (Table 1). Only the higher substrate concentrations show some disagreement. These results demonstrate that if the synthesis of the normal range of glucans is inhibited by a sufficiently high concentration of products, the elongation reaction is unaffected and the linear β-1,2-glucans are channelled into equal amounts of larger polymers. This clearly demonstrates that added cyclic β-1,2-glucans have no effect on k but exert an inhibitory effect via k'_n. Since the specificity of inhibition by cyclic β-1,2-glucans of a single ring size is not absolute, the inhibition of synthesis of an individual cyclic

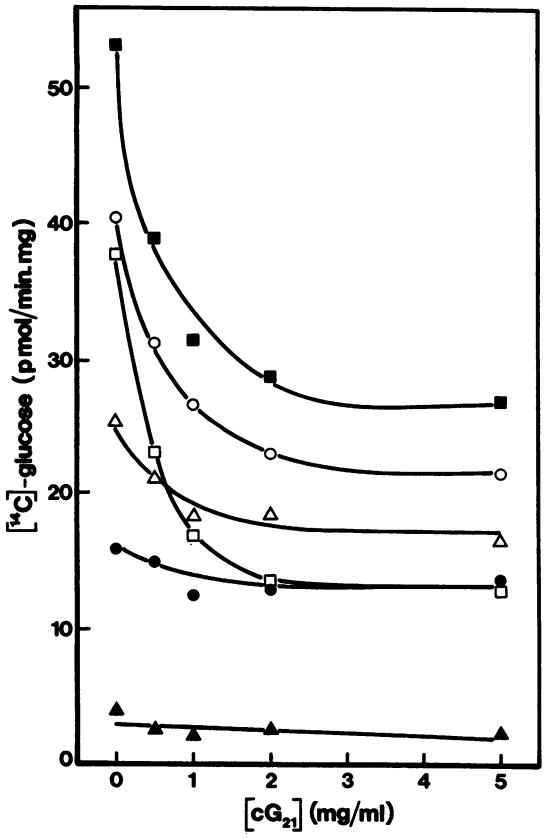

FIG. 3. Inhibition of cyclic β-1,2-synthetase by cG_{21}. Assays were performed in the presence of added cG_{21} (0 to 5 mg/ml), and the amounts of cG_{17} (▲), cG_{18} (●), cG_{19} (△), cG_{20} (○), cG_{21} (□), and cG_{22} (■) (all labelled with U-^{14}C) produced were estimated by radiodetection.

β-1,2-glucan is also affected by its closest neighbors. For example, cG_{21} decreases k'_{22} and k'_{20} almost to the same extents as k'_{21} (Fig. 4). During the reaction, synthesized U-^{14}C-labelled products are unlikely to be inhibitory since the concentration of added product required is more than 2 orders of magnitude larger than that synthesized in the standard assay.

Cyclization reaction. What factors, therefore, affect the relative values of k'? Examining the distribution of products from cyclic β-1,2-glucan synthetases from other organisms reveals that the proportion of each glucan varies with species (Table 2). It is therefore reasonable to assume that the properties of the protein determine the values of k'_n and their magnitudes relative to k. Also the rate of the elongation reaction varies from species to species, so each has an intrinsic value of k. We therefore postulate that changing the environment of the protein would yield changes in the proportions of each U-^{14}C-labelled cyclic β-1,2-glucan synthesized. The following experiments were set up to test this hypothesis.

Effect of temperature on the synthesis of U-^{14}C-labelled cyclic β-1,2-glucans. Assays were performed at between 10 and 45°C, and the amount of each cyclic β-1,2-glucan was estimated (Fig. 7). [U-^{14}C]cG_{17} production increases with temperature up to 35°C and then shows no further change. [U-^{14}C]cG_{18} increases up to 35°C and then declines slowly. [U-^{14}C]cG_{19} production is similar to that of [U-^{14}C]cG_{18}, but with a maximum at 30°C. [U-^{14}C]cG_{20}, [U-^{14}C]cG_{21}, and [U-^{14}C]cG_{22} exhibit a temperature optimum of ~20°C. It is clear, therefore, that changing the temperature of synthesis has a complex effect on the distribution of the product. Therefore, the rate of elongation, k, and each of the rates of cyclization, k'_n, vary with temperature, presumably because of changes in the protein conformation with temperature, and with some additional effects possibly contributed from variations in membrane fluidity.

Effects of detergents. Dilute concentrations of detergent can affect protein conformation without full denaturation. Furthermore, detergents will disrupt the membrane structure in which the cyclic β-1,2-glucan synthetase is located. The synthesis of cyclic β-1,2-glucans was therefore examined in the presence of a range of detergents (0.25% [wt/vol]). The relative activities obtained (no detergent, 100%) were as follows: 3-[(3-cholamidopropyl)-dimethyl-ammonia]-1-propanesulfonate (CHAPS), 83%; *n*-octyl-β-glucoside, 78%; Tween 20, 77%; cholic acid, 76%; Lubrol PX, 43%; Triton X-100, 40%. Since CHAPS had the least effect on the total activity, further experiments were conducted with this detergent.

The percentage of each cyclic β-1,2-glucan to the total amount was calculated as a function of CHAPS both above and below its critical micelle concentration (0.5% [wt/vol]) (Fig. 8). cG_{17} and cG_{18} show a clear increase with increasing concentrations of CHAPS. cG_{19} shows a small increase,

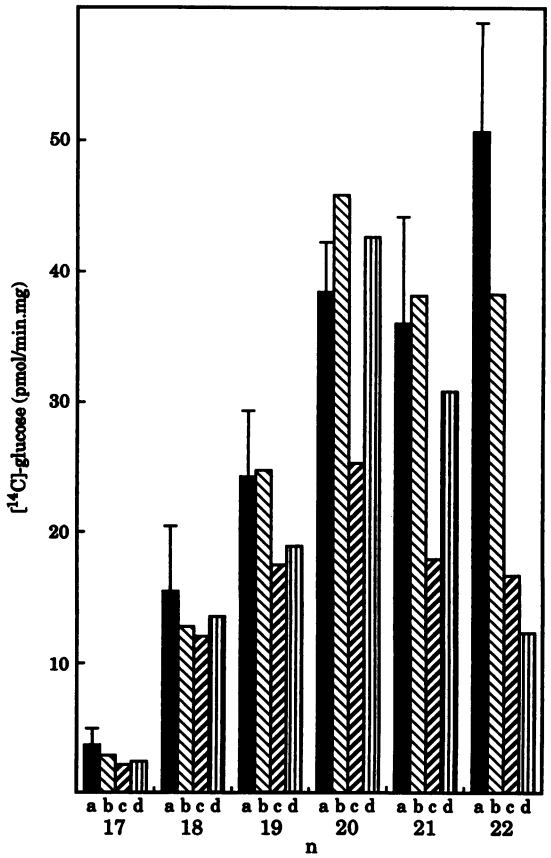

FIG. 4. Effects of the additions of cG_{18} (b), cG_{21} (c), and cG_{22} (d) (all 1 mg/ml) on the production of U-^{14}C-labelled cyclic β-1,2-glucans. The error bars on the control with no added cyclic β-1,2-glucans (a) represent standard deviations (12 measurements).

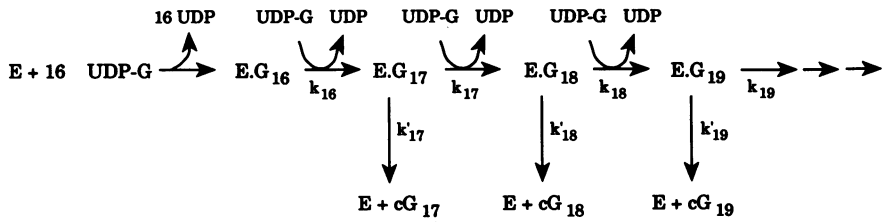

FIG. 5. Putative relationship between rate constants for cyclization (k'_n) and for chain elongation (k_n). E, cyclic β-1,2-glucan synthetase; UDP-G, UDP-glucose; G_n, linear β-1,2-glucan with n glucose residues.

whereas cG_{20}, cG_{21}, and cG_{22} all decrease as a percentage of the total cyclic β-1,2-glucan synthesized. It is therefore clear that the environment surrounding the protein strongly influences product distribution.

DISCUSSION

Two distinct mechanisms for the synthesis of exocellular polysaccharides (15) have been identified: a monomeric mechanism, in which nucleotide sugars are added sequentially to a growing chain, and a blockwise mechanism, in which nucleotide sugars are first polymerized to repeating units which are then joined onto a growing chain consisting of linked repeat units. The latter applies to complex polymers of many different sugars and linkages covalently attached to a membrane-bound lipid, as in xanthan biosynthesis (7). Because of the requirement for cyclization, the synthesis of cyclic polysaccharides is, however, unusual, although it is most likely to follow a monomeric mechanism. Compounds which are superficially similar, the cyclodextrins, do not require UDP-glucose but are hydrolytic products from the action of cyclodextrin glucanotransferase on starch. Thus, they are not metabolic products (2). Additionally, *Escherichia coli* produces linear β-1,2-glucans, but since these are not cyclic the mechanism of synthesis is obviously somewhat different. A large proportion of cyclic β-1,2-glucans secreted into the growth medium during growing are substituted (8). This substitution occurs after synthesis, since all cell extracts produce unmodified cyclic β-1,2-glucans (8). We have therefore not considered the substitution reactions in this mechanism since they clearly occur after the release of cyclic β-1,2-glucans from the 235-kDa protein.

The ChvA transport protein, however, may play a role in determining the product size distribution in vivo by maintaining the concentration of cyclic β-1,2-glucan in the periplasmic space at a (low) steady-state level so that it does not reach sufficiently high levels to be inhibitory. This protein does not affect the production of cyclic β-1,2-glucans in membrane fractions from broken cells (8).

We suggest that the mechanism of biosynthesis of cyclic β-1,2-glucans is as follows, on the basis of evidence presented here and from past work and rules suggested in reference 15. There is no requirement for an intermediate membrane-bound lipid (20). Rather, the initial reaction is that the 235-kDa protein reacts in a self-catalytic reaction (19) with UDP-glucose to form a 235-kDa protein with a glucose residue attached (E-G) to an unknown amino acid (1). Subsequent UDP-glucose molecules diffuse into the active site, and by transglycosylation reactions the linear

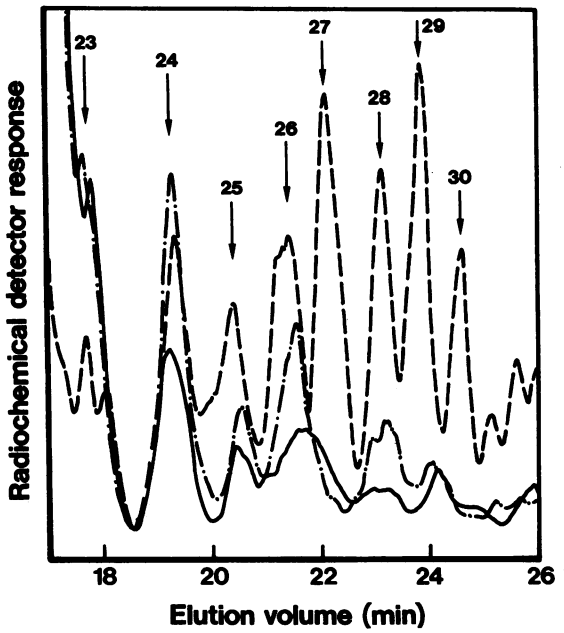

FIG. 6. Effect of the addition of a mixture containing cG_{17} (0.23 mg/ml), cG_{18} (0.54 mg/ml), cG_{19} (1.52 mg/ml), cG_{20} (1.11 mg/ml), cG_{21} (0.75 mg/ml), cG_{22} (0.49 mg/ml), cG_{23}, and β-1,2-glucans containing more glucose residues (all less than 0.1 mg/ml) on the production of U-^{14}C-labelled cyclic β-1,2-glucans. Assays were performed with no added cyclic β-1,2-glucans (solid line), with 10% of the above-listed concentrations (dashed line with dots), and with the above-listed concentrations (dashed line). Below 17 ml, the baseline of the latter drifts upward, without any clear peaks. The arrows indicate the elution positions of standards of cyclic β-1,2-glucans; numbers above the arrows indicate the numbers of glucose residues.

TABLE 1. Production of U-^{14}C-labelled cyclic β-1,2-glucans in the presence and absence of cyclic β-1,2-glucans

UDP-[U-^{14}C]glucose (μM)	Amt of [a]:			
	cG_{17} + cG_{18} + cG_{19} + cG_{20} + cG_{21} + cG_{22} (pmol/min · mg)		cG_{24} + cG_{25} + cG_{26} + cG_{27} + cG_{28} + cG_{29} + cG_{30} (pmol/min · mg)	
	Control	+cG mix	Control	+cG mix
20	19.0	ND	<1	20.8
25	26.6	ND	<1	29.4
30	36.4	ND	<1	29.6
50	50.2	ND	<2	48.6
100	119	ND	<2	71.8

[a] All β-1,2-glucans were labelled with U-^{14}C. cG mix concentrations were as in the legend to Fig. 6. The total cyclic β-1,2-glucan concentration was 5 mg/ml. ND, not detected.

TABLE 2. Proportions of cyclic β-1,2-glucans produced by three different species

Cyclic β-1,2 glucan with the following no. of glucose residues:	% β-1,2-glucan produced by:		
	A. radiobacter[a]	*R. phaseoli*[a]	*A. tumefaciens*
17	9	51	3
18	30	12	11
19	26	12	17
20	17	10	23
21	9	5	20
22	5	5	24
23	3	3	<1
24	1	2	<1

[a] Calculated from reference 12.

β-1,2-glucan increases in size. The reaction is unlikely to involve a blockwise synthesis, since the repeat unit is only one glucose residue (15). The reaction, on the basis of information from the synthesis of other polysaccharides such as hyaluronic acid from *Streptococcus* group A organisms, probably proceeds by a monomeric mechanism as follows:

$$\text{UDP-G} + \text{E} \rightleftharpoons \text{UDP} + \text{E-G}$$
$$\text{E-G} + \text{UDP-G} \rightleftharpoons \text{UDP} + \text{E-G-G}$$
$$\text{E-G-G} + \text{UDP-G} \rightleftharpoons \text{UDP} + \text{E-G-G-G}$$

and so the polymeric chain probably grows from the nonreducing end. No cyclic β-1,2-glucans smaller than those containing 17 glucose residues have been detected. Similarly, no linear β-1,2-glucan precursors have been detected,

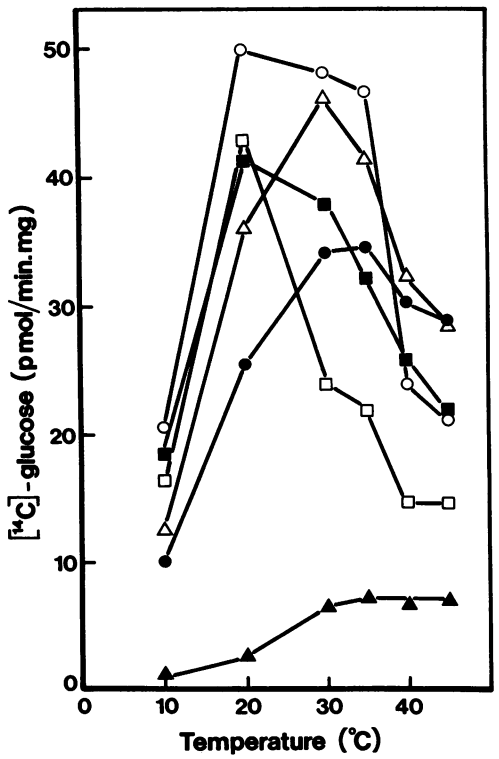

FIG. 7. Effect of temperature on the production of cG_{17} (▲), cG_{18} (●), cG_{19} (△), cG_{20} (○), cG_{21} (□), and cG_{22} (■), all labelled with U-^{14}C.

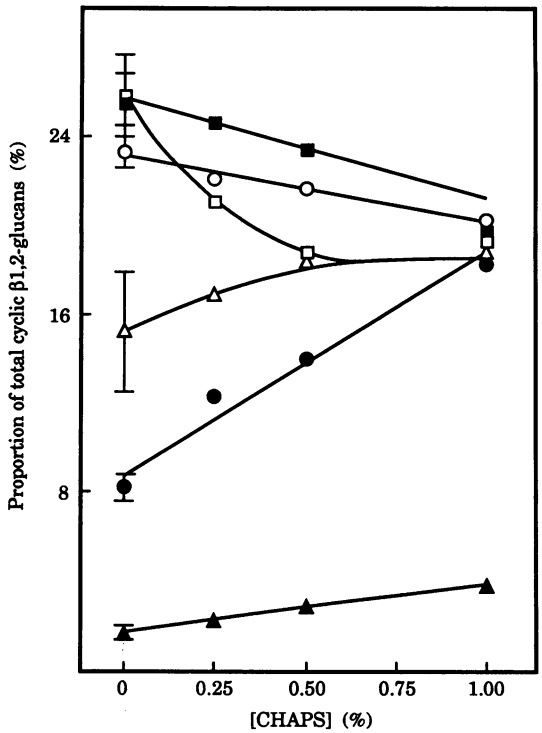

FIG. 8. Effect of CHAPS on the cyclic β-1,2-glucan synthetase reaction. For membrane fraction I, UDP-[U-^{14}C]glucose and $MgCl_2$ were incubated as described in Materials and Methods, together with various concentrations of CHAPS. The proportions of cG_{17} (▲), cG_{18} (●), cG_{19} (△), cG_{20} (○), cG_{21} (□), and cG_{22} (■) were estimated as percentages of the total U-^{14}C-labelled cyclic β-1,2-glucan production. Error bars represent standard deviations (four measurements).

showing that the growing chain remained attached to the protein (1). When the number of covalently attached glucose residues reaches 15 to 17, modelling calculations with β-1,2-glucan rings have shown that cyclization is energetically possible (13). Thus, the "tail" glucose (the first residue) is sterically able to fold back to meet the glucose residue which is attached to the protein. The folding back is probably aided by the structure of the active site. Once cyclization is sterically possible, the production of cyclic β-1,2-glucans then depends on the relative rates of cyclization and elongation, which in turn will depend on the precise arrangement of amino acid residues within the active site. This arrangement can be altered by the protein environment, leading to conformational changes and subtle alterations in the distribution of sizes of cyclic β-1,2-glucans. Both the elongation and cyclization reactions may be transglycosylation reactions. The donors would be UDP-glucose and the terminal glucose of the growing β-1,2-glucan chain, respectively. Thus, it is possible that the two reactions are similar but have different donors. A transglycosylation and disproportionation mechanism is observed for cyclodextrins, in which transglycosylation reactions can involve either the nonreducing end of the enzyme-bound maltooligosaccharide as the donor (cyclization) or the nonreducing end of a second maltooligosaccharide (in a ternary complex) as the donor (disproportionation) (2). In relatively high concentrations of product, individual cyclic β-1,2-glucans block the synthesis of glucans of the same size and to a lesser extent that of glucans of similar molecular weight. What is perhaps sur-

prising is that cG_{21} inhibits the reaction more than cG_{18}. This could be due to a higher binding affinity for cG_{21}, but this requires further experimentation. In the presence of the entire range of normal products (cG_{17} to cG_{22}), the cyclization reaction for production of cG_{17} to cG_{22} is inhibited but the elongation reaction is unaffected; this results in the synthesis of equivalent amounts (i.e., per glucose residue polymerized) of larger cyclic β-1,2-glucans (cG_{24} to cG_{30}).

It is still possible that there may be individual proteins which synthesize individual cyclic β-1,2-glucans. This would imply the existence of 14 or more modified proteins (either 14 gene products [unlikely] or posttranslationally modified proteins). In the absence of any positive evidence for this, the proposed mechanism should provide a framework for studies to further elucidate the action of this unusual bacterial system.

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