The Cyclic Diguanylic Acid Regulatory System of Cellulose Synthesis in Acetobacter xylinum

CHEMICAL SYNTHESIS AND BIOLOGICAL ACTIVITY OF CYCLIC NUCLEOTIDE DIMER, TRIMER, AND PHOSPHOTHIOATE DERIVATIVES

An unusual compound, cyclic-bis(3' → 5') diguanylic acid (c-di-GMP or cGpGp), is involved in the regulation of cellulose synthesis in the bacterium Acetobacter xylinum. This cyclic dinucleotide acts as an allosteric, positive effector of cellulose synthase activity in vitro (Kd = 0.31 μM) and is inactivated via degradation by a Ca2+-sensitive phosphodiesterase, PDE-A (Km = 0.25 μM). A series of 13 analogs cyclic dimer and trimer nucleotides were synthesized, employing a phosphotriester approach, and tested for the ability to mimick inhibitors of PDE-A activity, but at least one cyclic dinucleotide and trimer nucleotides were synthesized, employing a phosphotriester approach, and tested for the ability to mimick inhibitors of PDE-A activity, but at least one cyclic dinucleotide, cXpXp, which does not bind to cellulose synthase, is also a substrate for the degradation reaction, demonstrating that although the two enzymes share a similar, high degree of specificity for c-di-GMP, their cyclic dinucleotide binding sites are not identical. Phosphodiester bonds of activators in which an exocyclic oxygen is replaced with an atom of sulfur (cGp(S)pGp isomers) resist the action of PDE-A, and such derivatives may be prototypes for synthetic non-hydrolyzable c-di-GMP analogs.

Cellulose biogenesis is quintessential to the plant kingdom, where the deposition of fibrils within the cell wall is an integral process to growth and development (1). Cultures of Acetobacter xylinum produce a tough pellicle of high purity cellulose, and this Gram-negative bacterium constitutes a convenient model for morphological and biochemical investigations into the nature of cellulose formation. While in plant cell walls, cellulose fibrils are an intrinsic structural element, the mat-like pellicle of cellulose fibrils produced by A. xylinum is extracellular, apparently conferring a selective advantage to this obligative aerobe under static growth conditions (2).

In A. xylinum, cellulose fibrils are extruded into the external milieu from an array of discrete sites arranged along the cell surface, giving rise to a single ribbon structure composed of intertwined crystalline fibrils (3). The process of glucose polymerization into polyglucan chains and the assembly of chains into rigid fibrils have been postulated to be tightly coupled processes (4). Although the underlying organizing principles have not been defined, many aspects of the polymerizing mechanism are coming to light. Cellulose synthase, the enzyme responsible for the polymerization of glucose from UDP-glucose into the β-1,4-linked chain, has been isolated from this organism as a highly active membrane-bound catalytic unit (5). Comparison of various enzymatic levels in vitro (6), and of precursor concentrations in vivo (7), indicates that in the pathway from glucose, via glucose-6-phosphate, glucose-1-phosphate, and UDP-glucose, into polyglucan chain, the unique biosynthetic step catalyzed by cellulose synthase may be rate-limiting.

The cellulose synthase from A. xylinum is subject to a complex form of regulatory control which has not been encountered previously in a living system (5, 6, 8, 9). The basis of this regulation appears to lie in the intracellular concentration of an unusual cyclic guanylic nucleotide dimer (originally isolated as an incubation product of GTP with cell extracts (8, 9)) which functions as an allosteric effector of enzyme activity. In vitro, cellulose synthase activity displays nearly absolute dependence on the presence of nanomolar concentrations of this compound, which was conclusively identified in a recent report (10) as cyclic bis(3' → 5')-diguanylic acid (c-di-GMP or cGpGp). It is currently not known if c-di-GMP affects other cellular processes in addition to cellulose synthesis.

The pathways of synthesis and degradation of c-di-GMP are catalyzed by enzymes which occur in both soluble and extracellular forms (11). The cost of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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1 The abbreviations used are: c-di-GMP (or cGpGp), cyclic bis(3' → 5')-diguanylic acid; RP-HPLC, reverse phase high performance liquid chromatography; PEL, polyethyleneimine; BAP, bacterial alkalinephosphatase; THP, tetrahydropyranyl.
membrane-associated form in cell-free extracts. The formation of the nucleotide activator is attributed to diguanylate cyclase, a predominantly soluble enzyme, which condenses two molecules of GTP in a two-step reaction, via the intermediate linear triphosphate pppGpG. A membrane-bound phosphodiesterase, termed PDE-A, degrades the activator, cleaving a single phosphodiester bond in the cyclic structure to form the linear 5'-phosphoryl dimer pGpG (10, 11). This initial degradation product, which is devoid of stimulatory effect, is then further hydrolyzed to yield two molecules of 5'-GMP in a reaction attributed to a second phosphodiesterase, termed PDE-B. This latter phosphodiesterase has been deemed distinct from PDE-A on the basis that the ratio of PDE A to PDE B activity in membrane preparations is ap proximately 10:1, whereas in soluble extracts this ratio is reversed (11). Furthermore, in contrast to the PDE-B reaction, PDE-A activity is inhibited at low concentrations of Ca2+ ions, suggesting that this phosphodiesterase serves as an additional locus of regulatory control, maintained by this cation.

This novel system is dually intriguing as it is the only known form of regulation of a cellulose-synthesizing enzyme currently accessible to biochemical analysis, and since cyclic dinucleotides, such as c-di-GMP, have not been recognized previously to be of natural occurrence. The chemical synthesis of c-di-GMP (cGpG) by a modified hydroxybenzotriazolide phosphotriester approach is described here in full for the first time. Furthermore, several analogs of c-di-GMP, bearing modifications in either the ribose, phosphate, or guanine moieties, have been prepared with the objective of gaining more insight into the nature of the c-di-GMP regulatory system. Thus, various cyclic homodinucleotides, such as cBpBp (B = adenosine, cytidine, inosine, uridine, xanthosine, or deoxyguanosine), cyclic heterodinucleotides cBpGp, a tri nucleotide cGpGpGp, and a pair of c-di-GMP derivatives containing a chiral phosphorothioate linkage, cGp(S)Gp, were synthesized and tested for biological activity as effectors and substrates, respectively, of cellulose synthase and PDE-A.

MATERIALS AND METHODS

Chemicals—[U-14C]UDP-glucose and [α-32P]GTP were purchased from The Radiochemical Centre, Amersham, United Kingdom. 32P-Labeled c-di-GMP was prepared and purified as described (9). Bacterial alkaline phosphatase Type III and all chemical reagents and nucleotides, unless otherwise specified, were purchased from Sigma. Thin-layer plates of polyethyleneimine-cellulose were from Machery Nagel (polygram Cel 300 PEI). RP-18 HPLC columns were from Merck. A detailed description of synthetic procedures for novel cyclic nucleotides appears in the accompanying Miniprint Supplement. All nucleotides were >95% pure, as judged by HPLC.

Membrane Preparations—Membrane preparations from A. xylinum cells were prepared and stored as described previously (5).

Enzyme Assays—Cellulose synthase activity was assayed as described previously (5). The standard assay mixture contained, in a final volume of 0.1 ml, 50 mM Tris-HCl, pH 8.5, 20 mM MgCl2, 2.5 mM CaCl2, 0.1 mM EDTA, 20 μM [14C]GTP (50 cpm/nmol), membrane preparation (0.2-0.4 mg of protein), and additional components as indicated. Incubation was for 5 min at 30 °C. Reactions were terminated, and the 14C product formed was determined as described (5).

Standard PDE-A reaction mixtures contained, in a final volume of 0.1 ml, membrane preparations (1-5 μg of protein), 50 mM Tris-HCl, pH 8.5, 20 mM MgCl2, and either 32P-labeled c-di-GMP (250,000 cpm/reaction) or unlabeled nucleotides as indicated. Incubations were for 5 min at 30 °C. When employing radiolabeled substrate, reactions were terminated by application of a 20-μl aliquot to PEI-cellulose TLC plates. Following chromatography in 1.6 M KH2PO4, the residual radiolabeled substrate was detected by autoradiography, cut out, and counted (9, 11). Enzyme activity was calculated from the percentage of substrate consumed in the course of the incubation. Unlabeled reaction mixtures were assayed by reverse phase HPLC chromatography in KH2PO4, buffer as described below.

Reverse Phase HPLC—HPLC was performed on a Merck-Hitachi liquid chromatograph equipped with a 5-μm flow-through cell UV detector (259 nm), Ha-querling unit, and a Spectra-Physics 4100 computing integrator. Analytical runs, employing a 10-μl injection loop, were carried out at 1 ml/min in 0.1 M Na2HPO4, pH 4.8, on a RP-18 250 × 4-mm Lichrosphere column and a 50 × 4-mm RP-18 pre-column submerged in a 40 °C water bath. Unless otherwise specified, the following gradient of 20% acetonitrile in the above buffer was used: 0 min (0%); 5 min (0-5%); 10 min (5-15%); 15 min (5-12%); 37 min (12-75%); 48 min (75-100%). Preparative purifications were carried out using a RP-18 250 × 10-mm column run in 50 mM triethylammonium bicarbonate, pH 7.1, at 6 ml/min at room temperature, employing linear gradients (0-10%) of methanol.

RESULTS

Activation of Cellulose Synthase by c-di-GMP—In crude membrane preparations from A. xylinum, the rate of incorporation of glucose from UDP[14C]glucose into alkali-insoluble 1,4-β-D-[14C]glucan displays a low activity level when compared with the synthetic capacity of the intact cell (7). In the presence of c-di-GMP, stimulation of synthase activity ranges from 50 to up to 200-fold (8-10). This overall fold activation effect varies among individual enzyme preparations and thus has not been calibrated as a standard value. Under the assay conditions employed here, the rate of c-di-GMP-stimulated activity in membrane preparations, expressed per mg of cells, approximates 40% of that of the intact organism.

The kinetics of activation, as a function of c-di-GMP concentration, are relatively complex (Fig. 1). In the range of 0.3-4.0 μM, the dependence of cellulose synthase activity on effecter concentration manifests a typical Michaelis-Menten relationship, from which a Km value for c-di-GMP of 0.31 μM can be derived from the linear portion of the plot. Below this range, synthase activity falls off rapidly with the concentration of c-di-GMP, and overall kinetics of activation are suggestive of positively cooperative interactions. In order to examine this further, the stability of the activator in the assay system was examined, particularly since crude membranes contain phosphodiesterase activity which degrades c-di-GMP. In incubations carried out under parallel conditions in the presence of 32P-labeled c-di-GMP, partial degradation of the exogenously added activator was observed to be most prominent at the low initial concentrations of c-di-GMP, ranging from 2.5% (4 μM) to 50% (0.1 μM) (data not shown). This degradation can be attributed to the endogenous PDE-A activity of membrane preparations which, even when inhibited >99% by 2.0 mM CaCl2, apparently retains sufficient activity to effectively reduce the concentration of c-di-GMP in synthase assay mixtures. The data in Fig. 1 have been corrected for this reduction in activator concentration occurring in the course of the assay period by employing an average value obtained from the initial and final c-di-GMP concentrations. Thus, the deviation from typical Michaelis-Menten kinetics at low (<0.3 μM) concentrations of the activator cannot be accounted for on the basis of steady degradation. A more detailed kinetic analysis of c-di-GMP degradation under these particular assay conditions has not yet been performed. Notably, however, the kinetics of c-di-GMP activation of highly purified preparations of cellulose synthase (12), which is essentially devoid of PDE-A activity, do not

2 Portions of this paper (including part of "Materials and Methods" and Tables S1 and S-2) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are included in the microfilm edition of the Journal that is available from Waverly Press.
FIG. 1. Kinetics of c-di-GMP activation of cellulose synthase. Cellulose synthase activity in membrane preparations (200 μg of protein) was assayed in standard reaction mixtures in the absence and presence of various concentrations (0.1-4.0 μM) of c-di-GMP as described under “Materials and Methods.” The level of enzyme activity measured above basal level (8 pmol/min/mg protein) was used in the Eadie-Hofstee plot displayed. The data have been corrected for partial breakdown of c-di-GMP which occurs in the course of the assay. Residual c-di-GMP was determined in parallel incubation mixtures, containing [32P]-labeled c-di-GMP and unlabeled UDP-glucose, following termination of the reaction by addition of 10% trichloroacetic acid, centrifugation, and then thin-layer chromatographic analysis of the supernatant as described under “Materials and Methods” for standard PDE-A reaction assays. The values for c-di-GMP used to plot the curve were taken from the average of the initial and residual concentrations.

Analysis of the rate of production of alkali insoluble product as a function of UDP-glucose concentration (Fig. 2) in either the absence or presence of c-di-GMP yields a plot that is typical of Michaelis-Menten enzyme kinetics with respect to substrate. The apparent $K_m$ value (125 μM) calculated for UDP-glucose is independent of effector concentration, as reported previously for the activation produced by GTP and (diguanylate cyclase-containing) crude cell extract (5, 13). Rather, c-di-GMP stimulation of the enzyme is a result of an increase in the $V_{max}$ parameter of synthase activity.

Phosphodiesterase A Activity in Membrane Fraction—The hydrolysis of c-di-GMP to pGpG is defined as PDE-A activity. Employing an assay based on measuring the rate of conversion of [32P]-labeled c-di-GMP to its degradation products, resolved by thin-layer ion-exchange chromatography (9, 11), it has been possible to analyze the initial rate kinetics of the PDE-A reaction in membrane preparations (Fig. 3). The PDE-A activity conforms to typical Michaelis-Menten saturation kinetics with respect to the concentration of c-di-GMP (apparent $K_m$ = 0.25 μM). Under the conditions employed here (20 mM MgCl2), the $V_{max}$ parameter of the reaction is reduced by 53% in the presence of 100 μM CaCl2, whereas the apparent affinity for substrate appears to increase slightly. Much higher concentrations (>2 mM) of this cation are required to effectively inhibit the reaction by up to 99% (data not shown).

Synthesis of Cyclic Deoxyribonucleotide Analogs—The chemical synthesis of cyclic deoxyribonucleotides 8 (Scheme 2, Fig. 4) was achieved in two distinct stages by a modified hydroxybenzotriazole phosphotriester approach (14). In the first stage, as outlined in Scheme 1 (Fig. 4), a properly protected linear building block, enclosing 2 or 3 deoxyribo-
nucleotide units (i.e. 5 or 6) was synthesized employing the
bifunctional phosphorylating reagent 2a in a two-step phosphor-
ylation process (15). For the preparation of the phosphoro-
thioate-containing dimer 5 (X = S; B = GDP*), phosphorothio-
vating reagent 2b, applied previously for the synthesis of 3',5'-
cyclic ribonucleotide phosphorothioates (16) and the in-
roduction of a phosphorothioate linkage in 2,5-A derivatives
(17), was used for coupling 1 with 4. In the second stage
(see Scheme 2, Fig. 4), dimer 5 or trimer 6 was converted into
a building block suitable for cyclization (i.e. 7). For the
temporary protection of the 3'-terminal phosphodiester, the
allyl (18) group was introduced by coupling 5 or 6 with allyl
alcohol. After removal of the 4',4'-dimethoxytrityl group (19)
and the allyl function (18), an intramolecular condensation
was effected with 2,4,6-triisopropylbenzenesulfonyl-3-nitro-
1,2,4-triazole (TPSNT) (20), leading to the fully protected
cyclic di- or trimer 8.

Thus, nucleoside 1 was phosphorylated with 2a in dioxane
in the presence of pyridine. After 5 min, TLC analysis in-
dicated the complete formation of 3 (X = O). A slight excess of
4, having its 5'- and 3'-hydroxyl function unprotected, was
added. Work-up of the reaction mixture after 45 min, followed
by short column chromatography (21), afforded dimer 5 (X =
O) in an average yield of 75%.

A similar procedure was followed for the synthesis of dimer
5 (X = S) with the exception that phosphorylating agent 2b
was used for the condensation and N-methylimidazole was
added in the second phosphorylation step. After work-up,
the diastereomers were separated by short column chromato-
graphy and isolated in a total yield of 69%.

Trimer 6 (X = O; B = GDP*; R' = O-THP) was isolated in
81% yield after coupling 5 (X = O; B = GDP*; R' = O-THP), followed by work-up and
purification by short column chromatography.

Next, compounds 5 or 6 were co-evaporated with dioxane
and phosphorylated with 2a in the presence of a slight excess
of pyridine. After 5 min, when TLC analysis indicated com-
plete conversion of starting material into a compound with zero
mobility, allyl alcohol was added. After 30 min, work-up and
purification afforded pure 7 in an average yield of 79%.

The fully protected compounds 8 were deblocked by treat-
ment with oximate ions (22), followed by ammonolysis at
50 °C and in case R' = O-THP by acid hydrolysis (23). Crude
deprotected cyclic nucleotides 8 thus obtained were purified by
anion-exchange chromatography and converted into their
sodium salts. The homogeneity and identity of the depro-
ected cyclic deoxyribonucleotides 8 was assured by fast protein
liquid chromatography analysis, 'H and 31P NMR spectros-
copy, mass spectroscopy, and UV absorption (Table I). Minor
contaminations from some synthetic preparations (cXpXp,
cIpGp, cGpGpGp, and cGp(S)Gp:R- and Sp-diastereomers)
were removed by preparative HPLC. All of the compounds

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**Scheme 1**

R<sup>1</sup>-2-Chlorophenyl
R<sup>2</sup>-H or O-Tetrahydropropyl
B<sup>3</sup>-C-3,4-Cladophenyl

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**Scheme 2**

R<sup>1</sup>-2-Chlorophenyl
R<sup>2</sup>-H or O-Tetrahydropropyl
B<sup>3</sup>-C-3,4-Cladophenyl

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**Fig. 4. Scheme for the synthesis of cyclic deoxyribonucleotides.** See text for details.
Cyclic Dinucleotides in the Regulation of Cellulose Synthesis

Table I

Relevant data of deprotected cyclic deoxyribonucleotides

<table>
<thead>
<tr>
<th>Compound</th>
<th>$^{31}$P NMR at 85% HPO$_4$</th>
<th>$^{31}$H NMR in D$_2$O</th>
<th>$K_m$</th>
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</thead>
<tbody>
<tr>
<td>cGpGp</td>
<td>-0.62 7.84 (s; H-8)</td>
<td>252</td>
<td></td>
</tr>
<tr>
<td>cdGpGp</td>
<td>-0.11 8.09 (s; H-8)</td>
<td>245</td>
<td></td>
</tr>
<tr>
<td>cdGpdGp</td>
<td>-0.20 8.02 (s; H-8)</td>
<td>261</td>
<td></td>
</tr>
<tr>
<td>cdGp(S)Gp</td>
<td>-0.24 7.94 (s; H-8)</td>
<td>236</td>
<td></td>
</tr>
<tr>
<td>cGp(SP)Gp</td>
<td>-0.12 8.01 (s; H-8)</td>
<td>253</td>
<td></td>
</tr>
<tr>
<td>cIpGp</td>
<td>-1.03 7.95 (s; H-8)</td>
<td>251</td>
<td></td>
</tr>
<tr>
<td>cIpIp</td>
<td>-1.15 7.97 (s; H-8)</td>
<td>250</td>
<td></td>
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<tr>
<td>cIpIp</td>
<td>-1.55 7.73 (s; H-8)</td>
<td>253</td>
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<td>cXpGp</td>
<td>-0.74 8.04 (s; H-8)</td>
<td>250</td>
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<tr>
<td>cXpGp</td>
<td>-0.83 8.08 (s; H-8)</td>
<td>250</td>
<td></td>
</tr>
<tr>
<td>cCpGp</td>
<td>-0.92 7.84 (s; H-8)</td>
<td>261</td>
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<tr>
<td>cCpGp</td>
<td>-1.08 7.75 (s; 7.5 Hz; H-6)</td>
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<tr>
<td>cCpGp</td>
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<td>250</td>
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</tr>
<tr>
<td>cXpGp</td>
<td>-0.71 7.88 (s; H-8)</td>
<td>250</td>
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<tr>
<td>cCpCp</td>
<td>-1.21 8.02 (s; 7.6 Hz; H-6)</td>
<td>271</td>
<td></td>
</tr>
<tr>
<td>cCpCp</td>
<td>-1.21 7.96 (s; 7.6 Hz; H-5)</td>
<td>259</td>
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<tr>
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<td>cUpUp</td>
<td>-0.85 7.97 (s; 8.1 Hz; H-6)</td>
<td>262</td>
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</table>

Notes:
- $^{31}$P NMR in D$_2$O. Chemical shifts are in ppm/million relative to 85% HPO$_4$.
- $^{31}$H NMR in D$_2$O. Tetramethylammonium chloride was used as internal reference. Chemical shifts are in ppm/million relative to tetramethylsilane.

When present in standard assay mixtures at concentrations ranging from 1 to 100 μM, with the exception of the cyclic trimer, cGpGpGp, which produces a marginal (3–4-fold), but consistently observed, activation at concentrations above 50 μM. A number of noncyclic nucleotides related to the c-di-GMP structure, pGpG, GpG and 5'-GMP, also proved to be without effect when tested in this concentration range. The impotency of all of these compounds is due to a low affinity for the enzyme, as evidenced by the results of competition experiments wherein the nucleotides were tested for the ability to interfere with c-di-GMP stimulation of synthase activity. None of the compounds, when included at a concentration of 100 μM in standard assay mixtures containing 1 μM c-di-GMP affected the level of enzyme activity, when compared with mixtures containing 1 μM c-di-GMP alone (data not shown). Based on these results, the affinity of cellulose synthase for any of these structures is at least two orders of magnitude lower than that for the c-di-GMP activator. As with c-di-GMP, all of the activators appear to be relatively more effective at higher concentrations, i.e. display sigmoidal saturation curves.

Stimulation of cellulose synthase activity by c-di-GMP in crude membrane preparations is greatly enhanced in the presence of CaCl$_2$, which is a potent inhibitor of PDE-A activity (10, 11), and this salt (2.5 mM) is included in standard synthase reaction mixtures. When CaCl$_2$ was excluded from the reaction mixtures, the stimulation of activity produced by either the natural or synthetic activators was dramatically reduced (data not shown). To judge this effect more closely, aliquots from activator-containing standard reaction mixtures were tested for stimulatory activity following a preincubation period under conditions parallel to that of the synthase assay (Table II). In the absence of CaCl$_2$, each of the activators was destroyed in the course of the standard incubation period (not shown). However, although in general CaCl$_2$ preserves the activators, the analogs cIpIp and cGp(S)Gp are less protected, suggesting that their relative affinities might actually be 2–3-fold higher than those estimated from the saturation curves (Fig. 5). The results of more direct experiments (see below) confirm that the Ca$^{2+}$ requirement is attributable to inhibition of cyclic dinucleotide degrading phosphodiesterase (PDE-A) activity occurring in the membrane preparation.

Stability of Cyclic Dinucleotides and Related Compounds in the Presence of Membrane Preparations—The stability of the synthetic cyclic nucleotides, as well as of several other related phosphodiester-containing guanyl nucleotides, toward exposure to the phosphodiesterase activity of membranes was assessed employing reverse phase HPLC analysis of standard PDE-A reaction mixtures containing 10 μM (40 times the $K_m$ for c-di-GMP) nucleotide test substrate (Table III). Of all of the compounds tested, eight (cdGpGp, cdGpdGp, cIpGp, cIpIp, cXpGp, cXpGp, GpG and 5'-GMP, and cGp(S)Gp) are degraded at a pace comparable with that of c-di-GMP, judging by the rate of consumption of substrate or appearance of product(s). Both the linear dimer pGpG and the cyclic trimer cGpGpGp are also degraded, although at slower rates. Presumably, as is true for c-di-GMP hydrolysis which yields predominantly the linear dimer pGpG under these conditions, the cyclic nucleotide degradation products observed here result from the initial hydrolysis of a single phosphodiester bond in the molecule. However, aside from the case of the monophosphothioate derivatives (see below), the structures of the cyclic nucleotide degradation products have not been characterized, although their relatively high retention time values indicate that the major products detected in each case were not the intact nucleotide.
enzyme might occur. Orientations in which binding of the cyclic structure to the receptor presumably reflect preferences related to the rotational freedom of the entire molecule. Thus homodinucleotides (cGpGp, cXpXp, cdGpdGp, cIpIp) yield a single product, whereas heterodinucleotides (cdGpGp, cXpGp, cIpGp) give rise to two products, in yields consistent with a mechanism in which either of the two phospho-ester bonds in the cyclic dinucleotide is susceptible to cleavage. The degradation reaction, the number of products formed is consistent with a mechanism in which either of the two phospho-ester bonds in the cyclic dinucleotide is susceptible to cleavage, yielding the corresponding 5'-phosphorylated open dimer. Thus homodinucleotides (cGpGp, cXpXp, cdGpdGp, cIpIp) yield a single product, whereas heterodinucleotides (cdGpGp, cXpGp, cIpGp) give rise to two products, in yields which presumably reflect preferences related to the rotational orientations in which binding of the cyclic structure to the enzyme might occur.

None of the other cyclic dinucleotides tested, cApAp, cCpGp, cCpCp, and cUpUp, nor the cyclic monomer cGMP and linear dimer GpG, were judged to be labile to the PDE-A reaction conditions, even when the amount of membrane preparation employed in the degradation assay was increased to an amount sufficient to completely hydrolyze the equivalent of twice the quantity of c-di-GMP. In the case of the four cyclic dinucleotides, no marker compounds of possible hydrolytic products were available, and the possibility was considered that the apparent stability of these compounds might actually be due to a poor chromatographic resolution of products. However, attempts to improve the separation by modifying the conditions (i.e. methanol gradient) of elution in the HPLC analysis did not reveal UV-absorbing peaks in the reaction mixtures in addition to that of the original material. The observed stability of most of these compounds to PDE-A activity, namely, cApAp, cCpGp, cCpCp, and cUpUp, is compatible with their low affinity for the enzyme, as determined in inhibition assays (see below).

All of the cyclic dinucleotide degradations, as tested in Table II, are completely inhibited in the presence of 2.5 mM CaCl₂, implying that a common Ca²⁺-sensitive enzyme (i.e. PDE-A) executes each of these reactions. However, the results obtained from the preincubation assays mentioned above (Table III), in which the substrates were subjected to a 40-fold greater amount of membranes, indicated that the extent of this inhibition is disproportionate with regard to substrate. Indeed, when assayed at sub-saturating (100 μM) concentrations of CaCl₂, the hydrolysis of some analogs (cIpIp, cGp(S)Gp:R₃, cXpXp) proceeds relatively unimpeded (15–25% inhibition) relative to the 50–60% retarding effect observed on the hydrolysis rate of c-di-GMP and the other substrates (Table III). Under these conditions, the degradation rate of cGpGp was too slow to accurately estimate the inhibitory effect, although the hydrolysis of this compound is also probably impeded by Ca²⁺, since its stimulatory effect on synthase activity in membrane preparations was judged to be Ca²⁺-dependent. The same applies to the pGpG-degrading reaction in membrane preparations; in this case, employing radiolabeled substrate (10, 11) and the PEI-TLC assay, a 20% inhibition was observed at 2.5 mM CaCl₂ (not shown).

### Table II

<table>
<thead>
<tr>
<th>Activator</th>
<th>Supernatant volume added</th>
<th>Stimulation of cellulose synthase activity</th>
<th>Control</th>
<th>Preincubated</th>
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<tr>
<td>cGpGp</td>
<td>5</td>
<td>18,000</td>
<td>17,100</td>
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<tr>
<td></td>
<td>10</td>
<td>25,200</td>
<td>23,900</td>
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<td>cdGpGp</td>
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<td></td>
<td>10</td>
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<td></td>
<td>20</td>
<td>14,400</td>
<td>14,000</td>
<td></td>
</tr>
<tr>
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<td></td>
<td>40</td>
<td>11,200</td>
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<tr>
<td>cIpGp</td>
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<tr>
<td></td>
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<td>19,600</td>
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</tr>
<tr>
<td>cXpGp</td>
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<td>6,300</td>
<td>7,000</td>
<td></td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>12,000</td>
<td>11,200</td>
<td></td>
</tr>
<tr>
<td>cGp(S)Gp:</td>
<td>5</td>
<td>18,000</td>
<td>16,200</td>
<td></td>
</tr>
<tr>
<td>S₉</td>
<td>10</td>
<td>25,200</td>
<td>22,690</td>
<td></td>
</tr>
<tr>
<td>R₉</td>
<td>20</td>
<td>2,070</td>
<td>1,150</td>
<td></td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>4,530</td>
<td>2,550</td>
<td></td>
</tr>
</tbody>
</table>

are probably the corresponding linear dimers or trimer. Assuming that the same reaction pathway applies in every orientation, the number of products formed is consistent with a mechanism in which either of the two phospho-ester bonds in the cyclic dinucleotide is susceptible to cleavage, yielding the corresponding 5'-phosphorylated open dimer. Thus homodinucleotides (cGpGp, cXpXp, cdGpdGp, cIpIp) yield a single product, whereas heterodinucleotides (cdGpGp, cXpGp, cIpGp) give rise to two products, in yields which presumably reflect preferences related to the rotational orientations in which binding of the cyclic structure to the enzyme might occur.
in order to ascertain the relative stability of the modified phosphodiester bond towards PDE-A activity. Assuming that PDE-A action forms the corresponding 5'-phosphorylated linear dimer products from each of these, two products would be expected from each diastereomer if the hydrolysis of either phosphodiester bond in the molecule proceeds at equal rates. However, in the case of either diastereomer, only a single product was detected upon HPLC analysis of PDE-A reaction mixtures allowed to proceed until complete consumption of the substrate (Fig. 6, a, b, f, and g). The formation of a unique hydrolysis product (as indicated by co-chromatography of the reaction mixtures which yielded two distinct peaks) from each diastereomer suggests that each yields the corresponding 5'-phosphorylated 3'-terminal ribose structure in which the BAP-group and a modified internal structure, since neither BAP-Rp-diastereomers possess a mono-esterified phosphate group is contained in internal phosphodiester linkage, e.g. pG(S)pG. Further characterization supports this structural assignment. Incubation of the reaction mixtures with BAP yielded clearly distinguishable products (Fig. 6, c and h), and the reaction terminated by heating at 100 °C for 3 min, followed by centrifugation. The supernatants were analyzed by RP-HPLC as described under "Materials and Methods." Gradient solvent C: 0 min (0%); 5 min (0-5%); 10 min (5-12%); 15 min (12-75%); 37 min (75-0%). Reaction rates were estimated from the percent of total absorbance (i.e. substrate and product) appearing as product. The relative rate of degradation is the ratio of the observed rate to that of c-di-GMP in parallel incubations. Under these reaction conditions, typically 25% of the c-di-GMP substrate was converted to pGpG and <2% to 5'-GMP (280 s). Retention times recorded for other guanyl nucleotides were guanosine, 1015 ± 3' GMP, 467 ± 2' GMP, 896 ± 0.2% of the total absorbance. The effect of CaCl₂ on the degradation rate was tested in parallel incubation mixtures containing the addition of 100 μM CaCl₂.

### Nucleotide Inhibition of the PDE-A Reaction

To complement the degradation studies described above, and to assess the overall specificity of the PDE-A reaction, the initial degradation rate of 32P-labeled c-di-GMP (1 μM) was compared with reaction rates in the presence of equimolar and excess concentrations of various nucleotides (Table IV). With the exceptions of GDP, GTP, and ATP, all of the compounds tested were observed to inhibit the degradation of c-di-GMP, although to widely varying extents. In general, the most potent inhibitors are those cyclic dinucleotides (CdGpGp, CdGpdGp, CdGpGp, ClpIp, ClpIp, ClpIp, ClpIp, XpGp, XpGp, ClpIp, ClpIp, and ClpIp) and phosphothioate diastereomers) rated as good substrates of the degradation reaction. Interestingly, a gross correlation exists between the potency of these compounds as cellulose synthase activators and as PDE-A inhibitors; in general, the more effective activators are also more effective inhibitors. Albeit of slightly lower potency, several other guanyl nucleotides, cGpGp, pGpG, cGpGp, pppGpG, GpG, and GTP, are also inhibitors of the phosphodiesterase reaction. Although the former two compounds might possibly be substrates for the enzyme, the latter were judged to be >95% stable under the standard reaction conditions, by either HPLC analysis (cCpGp and GpG) or by PEI-TLC (pppGpG and GTP), employing radio-labeled material as substrate (10) (not shown). The other cyclic nucleotides tested, including cGMP, cGpGp, cGpGp, and cApAp, all of which are also >95% stable under the assay condition, produce detectable effects on the reaction rate only when present at 25-fold molar excess to substrate.

### DISCUSSION

Although the effect of c-di-GMP on cellulose synthase activity is clearly dramatic, the raison d'être of this unique control mechanism is still open to speculation. As an allosteric effector, c-di-GMP is unusual in that its stimulatory effect on the cellulose synthase reaction is manifested as an increase in the Vmax parameter of enzyme activity, rather than the
result of enhanced affinity for the UDP-glucose substrate. This type of regulation is usually associated with the physiological situation in which the intracellular concentration of substrate is well above the saturating level, although in _A. xylinum_ cells that of UDP-glucose probably does not exceed more than twice the synthase apparent _K_m (125 μM). Although this observation does not exclude the hypothesis that the rate of c-di-GMP turnover maintains synthase activity in step with the metabolic state of the cell, the cyclic nucleotide might be involved, as well, in an organizational role with regard to the mechanism of the synthase reaction. However, since the current assay method does not distinguish between the various stages of polymerization, such as polyglucan chain initiation, elongation, or termination, it has not been ascertained if the effect of c-di-GMP on the synthase reaction is to accelerate a specific step in this process. The recent finding that cellulose synthase in both highly (500-fold) purified (12) and immobilized (24) form retains its activity in step with the metabolic state of the cell, the cyclic nucleotide is integral to the structure of the enzyme. The partially sensitive to c-di-GMP suggests that the activator binding site is integral to the structure of the enzyme. The partially

### Table IV

<table>
<thead>
<tr>
<th>Nucleotide inhibition of the PDE-A reaction</th>
<th>Relative ( K_i )</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 μM</td>
</tr>
<tr>
<td>-----------------------------------</td>
<td>------</td>
</tr>
<tr>
<td>cGpGp</td>
<td>45</td>
</tr>
<tr>
<td>cGpCp</td>
<td>65</td>
</tr>
<tr>
<td>cdGpGp</td>
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<tr>
<td>cdGpdGp</td>
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<tr>
<td>cApGp</td>
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<tr>
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<tr>
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</tr>
<tr>
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</tr>
<tr>
<td>cApAp</td>
<td>&lt;5</td>
</tr>
<tr>
<td>cUpGp</td>
<td>&lt;5</td>
</tr>
<tr>
<td>cGp(S)Gp</td>
<td>&lt;5</td>
</tr>
</tbody>
</table>

The controls shown were carried out by addition of **H_2O** instead of **NaIO_4**. In additional controls performed, neither the phosphatase nor the **NaIO_4** treatment were observed to affect the intact analogs (prior to exposure to PDE-A). HPLC analysis was performed as described in Table III. Chromatograms a-e refer to S_3,-diastereomer and f-j to R_3,-diastereomer: a and f, zero times; b and g, PDE-A reaction mixtures; c and h, BAP treatment; d and i, isolated (NaIO_4, control) PDE-A products; and e and j, NaIO_4/Lysine treatment.
Cyclic Dinucleotides in the Regulation of Cellulose Synthesis

Cyclic dinucleotides observed under the conditions employed here warrant further examination. That cooperative interactions are not an inseparable regulatory property of the synthase has been demonstrated for c-di-GMP activation in highly purified enzyme preparations devoid of PDE-A activity.\(^3\) For a more definitive analysis of affinities and mechanisms, the sensitivity of the crude and purified forms of the synthase should be compared with regard to c-di-GMP and its analogs.

Considering the unusual structure of c-di-GMP, the high degree of specificity displayed by its molecular targets is not unexpected. In general, modifications in the c-di-GMP structure reduce the affinity of the molecule for both cellulose synthase and PDE-A, as judged by the ability to interact as activators, substrates, or inhibitors. In this respect, the significance of the cyclic structure is evident in the lower affinity of pGpG relative to that of c-di-GMP, by a factor of 10-fold for PDE-A and by at least 3 orders of magnitude for the synthase. The intact guanine rings, with the exception of the C-2 amino group, also appear to be particularly relevant to the biologically active cyclic dinucleotide structure, which is essentially devoid of activity in compounds containing alternative bases such as cApAp, cCpCp, cUpUp, and cCpGp. At the C-2 position, which has been studied in the most detail, the modifications have both partial and absolute effects on activity; substitution of the amine with carbonyl either reduces (cXpGp) or destroys (cXpXp) affinity for the synthase, whereas PDE-A also recognizes both molecules less effectively as substrates; replacement of the amine with hydrogen (cCpGp, cCpGp) results in compounds which are functional as both activators and substrates, but of reduced affinity. In addition, the importance of the C-6 carbonyl is demonstrated to the C-6 amine-bearing cApAp, which is neither a substrate nor effective inhibitor of this enzyme. The phosphoribosyl ring “backbone” also bears on the interaction of the molecule with its targets, as exemplified by the altered affinities afforded by substitution at the sugar hydroxyl (cdGpdGp) and at the exocyclic oxygen (cGp(S)pGp) of the phosphodiester bond. The inactivity of the dimeric structure is evident in the negligible affinity of both the lower homologue, cGMP, and the marginal affinity of the higher homologue, cGpGpGp, for either enzyme.

Given the 2-fold rotational symmetry of c-di-GMP, its protein binding sites might reflect a similar symmetrical disposition, as has been suggested from studies of cyclic dinucleotide inhibition of bacterial RNA polymerase (25). Comparison of the relative affinities of cdGpGp versus cdGdpGp, cGpGp versus cGdpGp, and cGpGp versus cGpGp, reveals that a given substitution is always more deleterious to the active structure when carried out in both GMP residues, suggesting that both participate in the recognition process. This might be taken as evidence that the cyclic dinucleotide binding occurs in either one of the two 180° rotational orientations and that both binding modes contribute to the observed potency of each analog. Such a situation would explain the apparent selectivity of PDE-A, which produces two products in varying yield, when presented with a heterodinucleotide (cdGpGp, cXpGp, cXpGp). The occurrence of alternate binding modes might explain the partial activity of the phosphothioate analog cGp(S)pGp in cellulose synthase; maximal activation of the enzyme may be prohibited even at apparent saturating concentrations of the activator due to competition between an “active” and an “inactive” binding orientation. Although this argument may also account for the potent effect on cellulose synthase activity of cXpGp relative to cXpXp, it might only be valid when the substitution is a purine, since cCpGp is of negligible affinity.

The foregoing analysis does not take into account the effect that substitutions can have on the overall conformation of the molecule. Cyclic dinucleotides can adopt a variety of asymmetrical conformations in solution (26) and crystalline state (27). The expectation that protein-nucleotide interaction might involve specific conformational properties which are not evident in the structural formula of the analog limits the conclusions regarding the relationships of structure to function and of alternate binding orientations.

The use of membrane preparations as a source of both synthase and PDE-A activities has facilitated the comparison of their c-di-GMP-binding sites, which would appear to be closely related, since under these conditions their respective apparent affinities (K\(_a\) = 0.31 \(\mu\)M; apparent K\(_m\) = 0.25 \(\mu\)M) are similar in value. However, although each of these enzymes are highly specific toward the c-di-GMP structure, PDE-A appears to be of wider specificity than cellulose synthase.

Their overlapping specificity is evident when considering that all of the cyclic dinucleotide activators of cellulose synthase serve as substrates in the PDE-A reaction, and a number of other cyclic dinucleotides (i.e. cdGpAp, cdCpAp, and cdIpIp) show low or no affinity for either enzyme. However, at least one compound, cXpXp, which lacks affinity for the synthase, is readily hydrolyzed by PDE-A activity. As noted, highly purified cellulose synthase (13) is essentially devoid of PDE-A activity,\(^4\) confirming that each is a distinct enzyme.

The PDE-A reaction is subject to inhibition by a variety of cyclic and linear guanyl nucleotides (cCpGp, pGpG, GpG), which are demonstrably lacking in affinity for the synthase. Thus, either the c-di-GMP-binding site of the phosphodiesterase accommodates structures which are not recognized at all by the regulatory site of the synthase, as is apparent the case for cXpXp, or the observed inhibition is not of a competitive nature. In this connection, it is interesting to consider the inhibitory effects on PDE-A activity of GTP and pppGpp, the phosphorylated structures of which sufficiently digress from that of the substrates and other inhibitors to suggest the involvement of an additional, possibly regulatory, site on the enzyme which responds to these compounds, particularly when considering that other, more closely related, guanosine derivatives such as cGMP, GDP, and GMP show little affinity. The physiological basis for this inhibition may lie in the fact that both GTP and pppGpp are substrates in the activator-forming diguanylate cyclase reaction. A more detailed analysis of the nature of guanyl nucleotide inhibition would facilitate the comparison of the overall degree of relatedness between the c-di-GMP-binding sites.

Throughout this discussion it has been assumed that the degradation of cyclic dinucleotides in the presence of membrane preparations is due to the action of a single highly specific enzyme, that is, PDE-A. This assumption is supported by the findings that all of the cyclic dinucleotide degradation reactions occur at similar rates and, without exception, all of the apparent substrates are potent inhibitors of PDE-A activity, as assayed employing c-di-GMP as substrate. Furthermore, all of the cyclic dinucleotide degradation reactions are inhibited at low CaCl\(_2\) concentrations.

The inhibitory effect of Ca\(^{2+}\) on the rate of hydrolysis of c-di-GMP appears to result from a reduction in the turnover number (V\(_{max}\)) of PDE-A. If the mechanism of inhibition were allosteric in nature, as via a specific Ca\(^{2+}\)-binding site which inactivates the enzyme when occupied, then the extent of the inhibition would be expected to be independent of substrate. However, the inhibitory effect of Ca\(^{2+}\) varies among the cyclic
dinucleotide substrates by up to a factor of 4-fold, suggesting that this divalent cation impedes catalysis through direct interaction with the substrate, possibly by competition with Mg$^{2+}$ ions, which are required for PDE-A activity (9, 11).

The structure-function analysis described here should be useful in the synthesis of additional analogs and new molecular tools with which to study the various components of the c-di-GMP regulatory system. For example, the finding that the phosphothioate linkage is relatively resistant toward PDE-A activity raises the prospect of obtaining a c-di-GMP analog which is a highly potent activator of cellulose synthase, but which is stable to enzymatic degradation. One structure which promises to fulfill these requirements is a diphosphothioate analog of c-di-GMP in which both phosphodiester bonds contain a substituted exocyclic oxygen in the $S$-configuration. This study has also led to the identification of functional groups which are relatively amenable to chemical modifications with regard to the preservation of biological activity, such as the exocyclic phosphodiester oxygen, the C-2 amino, and the ribosyl hydroxyl; this information should facilitate the design of affinity labels and columns, based on the c-di-GMP structure.

Six of the biologically active cyclic dinucleotides contain naturally occurring moieties, namely deoxyribose, inosine, and xanthine. When supplied with the corresponding nucleotide triphosphates, i.e. dGTP and ITP, crude diguanylate cyclase-containing cell extracts did not produce cellulose synthase activators in detectable yield (28). In preliminary experiments, severe chemical modifications, e.g. dGTP and ITP, crude diguanylate cyclase-containing cell extracts did not produce cellulose synthase activators in detectable yield (28).

REFERENCES


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Materials and Methods

Furidyl, tetrahydrofuran and diure were dried by refluxing with CaH₂ (3.6 g) and distilled. Benzyl alcohol was obtained from Merck (4.5 ml) and distilled. All solvents and chemicals were purchased from Sigma-Aldrich and used without further purification.

The synthesis of cyclic dinucleotides was performed by the solid-phase synthesis method. The resin was packed in the column and eluted with a gradient of acetonitrile-water (5:95) to (100:0) in buffer A (50 mM sodium tetraborate, pH 9.0). The eluted compounds were analyzed by HPLC and mass spectrometry.

Thecyclin synthase was obtained from Thermus thermophilus 758. The enzyme was purified by affinity chromatography on a nickel column. The enzyme was dialyzed against buffer B (50 mM sodium tetraborate, pH 9.0) before use.

The reaction mixture contained the cyclin synthase, the cyclic dinucleotide, and the substrate (100 µM). The reaction was initiated by the addition of the substrate and the reaction was monitored by HPLC and mass spectrometry.

The cyclin synthase was incubated with the cyclic dinucleotide and the substrate for 2 hours at 30°C. The reaction was stopped by the addition of dansyl chloride (100 µM). The reaction mixture was then analyzed by HPLC and mass spectrometry.

The data obtained were analyzed by SPPS software. The significance of the differences between the means was determined by ANOVA followed by the Bonferroni multiple comparison test.

The results are presented as the mean ± standard deviation. The data were analyzed by a paired t-test.

The cyclin synthase was purified by chromatography on a C18 column. The enzyme was dialyzed against buffer B (50 mM sodium tetraborate, pH 9.0) before use.

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P Ross, R Mayer, H Weinhouse, D Amikam, Y Huggirat, M Benziman, E de Vroom, A Fidder, P de Paus and L A Sliedregt


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