c-di-GMP-binding protein, a new factor regulating cellulose synthesis in *Acetobacter xylinum*

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Abstract A protein which specifically binds cyclic diguanylic acid (c-di-GMP), the reversible allosteric activator of the membrane-bound cellulose synthase system of *Acetobacter xylinum*, has been identified in membrane preparations of this organism. c-di-GMP binding is of high affinity (K_D 20 nM), saturable and reversible. The equilibrium of the reaction is markedly and specifically shifted towards the binding direction by K^+. The c-di-GMP binding protein, structurally associated with the cellulose synthase, appears to play a major role in modulating the intracellular concentration of free c-di-GMP and thus may constitute an essential factor in regulating cellulose synthesis in vivo.

Key words: Cellulose biogenesis regulation; Cyclic diguanylic acid; Binding protein; K^+; *Acetobacter xylinum*

1. Introduction

In *Acetobacter xylinum*, the membrane-bound UDP-glucose:1,4-β-D-glucan-4-β-glucosyl transferase (cellulose synthase, CS) performs the committed step in cellulose biosynthesis, and is subject to a multicomponent regulatory system [1–4]. Regulation is based on the novel nucleotide bis-(2′,5′)-cyclic diguanylic acid (c-di-GMP), a reversible allosteric activator, and the regulatory enzymes maintaining its intracellular turnover: diguanylate cyclase which catalyzes its formation from two molecules of GTP, and Ca^{2+}-sensitive c-di-GMP phosphodiesterase (PDE-A). The intracellular concentration of c-di-GMP determined in ^{32}P-labeled *A. xylinum* cells is one order of magnitude higher than both the K_a for the CS and the K_m for PDE-A [5]. These findings prompted us to investigate the possibility that c-di-GMP resides within the cell in a 'bound' form. In this paper we describe the isolation and partial characterization of a membranous c-di-GMP binding protein (CDGBP) whose binding action is specific, of high affinity, saturable and reversible. The equilibrium of the reaction is markedly and specifically displaced towards the binding direction by K^+. The binding protein structurally associated with the CS may functionally regulate its activity in vivo by modulating the intracellular level of free c-di-GMP.

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Abbreviations: CS, cellulose synthase; PDE, phosphodiesterase; c-di-GMP, cyclic diguanylic acid; CDGBP, c-di-GMP binding protein; WM, washed membranes; PEG-4000, polyethylene glycol-4000

2. Materials and methods

2.1. Cell growth and preparation of enzyme fractions

A. xylinum 1306-21 was grown in R-20 medium in the presence of 0.1% cellulase for 24 h [6]. Membranes prepared in the presence of 20% PEG-4000 were resuspended in 50 mM Tris-HCl (pH 7.5) containing 10 mM $MgCl_2$ and 1 mM EDTA (TME buffer), and recentrifuged at 18 000×*g* for 20 min. The resultant pellet, containing CS, PDE-A and CDGBP activities, resuspended in TME buffer comprises the 'washed membranes' (WM). To obtain PDE-A-free WM, a frozen and thawed regular preparation was homogenized, centrifuged and resuspended in TME buffer. CDGBP-free WM were derived from PDE-A-free WM by homogenization of the latter in 50 mM Tris-HCl pH 7.5 containing 3 mM EDTA, followed by centrifugation and resuspension of the pellet in TME buffer. The obtained supernatant, referred to as the 'EDTA eluate', is a partially purified preparation of CDGBP, containing 80% of the c-di-GMP binding activity and 5% of the protein relative to the PDE-A-free WM, and is devoid of detectable PDE-A and CS activities. Preparations free of either PDE-A or CDGBP retain their original CS activity. To obtain membranous protein-bound c-di-GMP, PDE-A-free WM (12.5 mg protein) was suspended in 50 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 2.5 mM $CaCl_2$, 1 mM dithiothreitol ('binding buffer') plus 1 mM KCl, incubated with 2 μM c-di-GMP for 15 min at 20°C and then centrifuged. The pellet was washed three times with 3 volumes of binding buffer containing KCl, and finally resuspended to the original volume in binding buffer lacking KCl. The resultant membrane preparation is devoid of CS activity which is lost during the incubation period [7]. For solubilization, native or CDGBP-free WM were resuspended with a Teflon homogenizer at 25 mg protein/ml in TME buffer containing 0.6% Triton X-100 and 20% glycerol. The suspension was gently shaken at 4°C for 1 h and then centrifuged at 100 000×*g* for 1 h. The supernatant represents the solubilized membranes.

2.2. Enzyme assays

CS was assayed [3] in the presence of 20 μM UDP-glucose and 1 μM c-di-GMP. PDE-A activity was determined as described [4]. CDGBP was assayed by determining the extent of [^{32}P]c-di-GMP binding to protein. Standard reaction mixtures (0.1 ml) contained 50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 5 mM $CaCl_2$, 5 mM KCl, 50 nM [^{32}P]c-di-GMP and PDE-A-free WM (10–35 μg protein) or EDTA eluate (1–6 μg protein). Following incubation at 4°C for 10 min, 1 ml of ice cold binding buffer containing 5 mM KCl was added and bound [^{32}P]c-di-GMP was separated from free substrate by vacuum filtration through nitrocellulose filters (Schleicher and Schuell BA-85). Filters were washed twice (within 30 s) with 5 ml buffer containing KCl and the bound radioactivity was determined. Binding to pre-boiled CDGBP preparations did not exceed 5% that of native samples and results were corrected accordingly.

2.3. Cation replacement

Replacement of cellular K^+ with Na^+ and determination of intracellular cation content were carried out as in [8] for *Escherichia coli*, except that treatment with diethanolamine was performed at pH 8.6 and the buffer used for the final wash and resuspension of cells was 25 mM demethylglutarate containing 110 mM NaCl and 5 mM NaH_2PO_4 (pH 6.0).

2.4. c-di-GMP determination

c-di-GMP (10–60 pmol) was assayed enzymatically by determining activation of the CS of *A. xylinum* as in [9], modified to 0.1 mg

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protein of CDGBP-free WM. c-di-GMP is heat stable, and samples containing protein-bound c-di-GMP were deproteinized by boiling for 3 min prior to assay. To determine intracellular c-di-GMP, cells were extracted with 0.6 M $HClO_4$, denatured protein was removed by centrifugation and the supernatant neutralized to pH 6.0 with 5 M K_2CO_3 and then recentrifuged to remove precipitated $KClO_4$. The resultant supernatant was assayed for c-di-GMP. The internal concentration of c-di-GMP (or cations where indicated) was calculated according to the assumption that 1 mg dry weight is associated within the cell with 4 μl water [10]. c-di-GMP and [^{32}P]c-di-GMP were prepared and purified by HPLC as described [4].

2.5. Protein assay and gel filtration

Protein content was determined according to Peterson [11] using bovine serum albumin as standard. Gel filtration analyses of the EDTA eluate and of solubilized membranes were carried out on calibrated columns (75×1.5 cm) of Sephacryl S-300 and Sepharose CL-6B, respectively. Columns were equilibrated and eluted with 50 mM Tris-HCl pH 7.5 containing 5 mM KCl.

3. Results and discussion

Perchloric extracts of *A. xylinum* were found to contain c-di-GMP at a level corresponding to a cellular concentration of 5–10 μM. The dinucleotide within the cell is mostly membrane-associated, since up to 80% of cellular c-di-GMP is detected in PDE-A-free WM prepared in the presence of 2.5 mM $CaCl_2$ (to inhibit residual PDE-A attack) and 5 mM KCl (to diminish dissociation of bound c-di-GMP, see below).

Under standard binding assay conditions PDE-A-free WM bind 25–35 pmol [^{32}P]c-di-GMP per mg protein. The labeled material released from the membranes by boiling is >98% c-di-GMP as judged by TLC analysis in various solvent systems [3]. The binding is highly specific to c-di-GMP, and shows the same nucleotide specificity as shown previously for CS activation and PDE-A activity. Thus cyclic dinucleotides such as c-di-AMP and c-di-CMP, which neither activate the CS nor inhibit its activation by c-di-GMP and have no effect on PDE-A activity, similarly do not interfere with [^{32}P]c-di-GMP binding. On the other hand, unlabeled c-di-GMP or c-di-IMP, which is also a potent CS activator and is degraded by PDE-A [5], block the radiolabeling of WM by 90% and 70% respectively when present in 10-fold molar excess.

The kinetics and binding characteristics of the CDGBP were examined, employing a partially purified preparation devoid of PDE-A and CS activities (EDTA eluate). At 4°C [^{32}P]c-di-GMP binding reaches a maximum within 5 min and remains constant for at least 40 min (Fig. 1). Reversibility of the binding is demonstrated following addition of 1000-fold molar excess of non-radioactive c-di-GMP to a binding assay at the point of maximal binding, as shown by the closed circles in Fig. 1. The resulting dissociation of [^{32}P]c-di-GMP

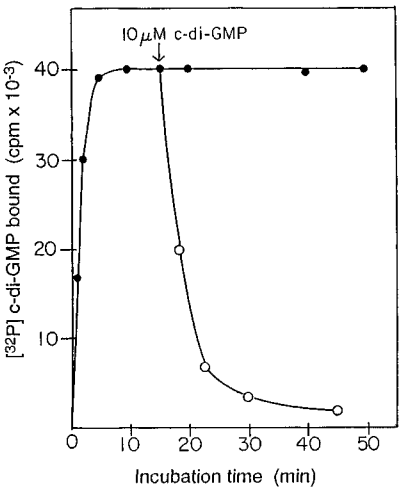

Fig. 1. Association and dissociation of [^{32}P]c-di-GMP binding. EDTA eluates of PDE-A-free washed membranes (6 μg protein) were incubated at 4°C with 10 nM [^{32}P]c-di-GMP for the designated times in the presence of 5 mM KCl and the binding was measured as described in Section 2. After equilibrium was reached at 15 min (arrow), 10 μM c-di-GMP was added to those incubation mixtures indicated by open circles.

displays a $t_{1/2}$ of 2–3 min and is 95% complete within 30 min. Reversibility of the reaction is also demonstrated by the rapid exchange of unlabeled protein-bound c-di-GMP with free [^{32}P]c-di-GMP. At 20°C binding reaches a maximum in less than 1 min. Binding of c-di-GMP increases linearly with protein concentration in the range 10–60 μg/ml, and is destroyed when the EDTA eluate is heated at 100°C for 1 min or exposed to proteases such as pronase (0.2 mg/ml), dispase (0.5 mg/ml) or proteinase K (0.2 mg/ml) for 20 min at 20°C. Binding is not affected by similar treatment with RNase A (100 μg/ml) or DNase I (270 U/ml). Gel filtration of the EDTA eluate yields one distinct peak of c-di-GMP binding activity corresponding to an apparent molecular mass of 200 kDa.

c-di-GMP binding is optimal at pH 7.5, has an absolute requirement for Mg^{2+} and is not affected by Mn^{2+} or Ca^{2+}. The rate of c-di-GMP binding is increased 2–3-fold by K^+ and half maximal stimulation occurs at 0.5 mM KCl. Stimulation is highly specific to K^+; it is not observed with Na^+, Li^+, Cs^+ or Rb^+ and is independent of the anion moiety (Cl^-, CH_3COO^- or I^-). K^+ does not affect the amount of c-di-GMP bound. When the binding of [^{32}P]c-di-GMP to the EDTA membrane eluate was determined at various concentrations of c-di-GMP, there was a progressive increase in binding, reaching saturation at about 60 nM. Scatchard analysis [12] of c-di-GMP binding in the presence or absence of

Table 1
Availability of protein-bound c-di-GMP for cellulose synthase activation and the effect of K^+

System	Cellulose synthase activity (cpm)
(1) CDGBP-free membranes	115
(2) Membranous protein-bound c-di-GMP	100
(3) System 1+system 2	7320
(4) System 1+system 2+KCl	650
(5) System 1+c-di-GMP	16500
(6) System 1+c-di-GMP+KCl	16000

Reaction mixtures (0.1 ml) contained 100 mM Tris HCl (pH 8.6), 10 mM $MgCl_2$, 2.5 mM $CaCl_2$, 40 μM UDP-D-[^{14}C]glucose, CDGBP-free washed membranes (0.08 μg protein, system 1) and cellulose synthase-inactivated membranous protein-bound c-di-GMP (1.0 μg protein, system 2). KCl and c-di-GMP were added to final concentrations of 5 mM and 0.25 μM, respectively. Incubation time was 5 min at 30°C. Reactions were terminated and incorporation of radioactivity into alkali-insoluble (1→4)-β-D-[^{14}C]glucan was determined as described in Section 2.

Table 2
Equilibrium of the c-di-GMP binding reaction

Equilibrium system	[^{32}P]c-di-GMP (cpm)			Free [^{32}P]c-di-GMP (% of total)
	Total	Free	Bound	
(1) CDGBP-[^{32}P]c-di-GMP−KCl	64 800	28 500	36 000	44
(2) CDGBP-[^{32}P]c-di-GMP+KCl (5 mM)	63 300	5 050	58 000	8

PDE-A-free WM (25 mg protein) were incubated for 15 min at 4°C with 2 μM [^{32}P]c-di-GMP (290 cpm/pmol) in 4 ml binding buffer containing 5 mM KCl and then centrifuged. The pellet was washed 3 times with 1 ml binding buffer containing 1 mM KCl and then eluted with 1 ml of 2 mM EDTA in 50 mM Tris-HCl pH 7.5 containing 1 mM KCl. 0.3 ml of the EDTA eluate (containing protein-bound [^{32}P]c-di-GMP and less than 0.2% free c-di-GMP) was diluted to 3.0 ml with binding buffer and allowed to equilibrate at 20°C for 30 and 60 min in the absence and presence of KCl. Aliquots of the mixtures were then filtered onto BA-85 membrane filters as described in Section 2 and the filters (containing bound [^{32}P]c-di-GMP) and the filtrates (containing free [^{32}P]c-di-GMP) were monitored for radioactivity. Total labeled c-di-GMP was determined in an unfiltered sample. TLC and autoradiography analysis of boiled unfiltered samples revealed c-di-GMP as the only labeled compound present. Similar results were obtained for the 30 and 60 min equilibrium periods.

KCl indicated a single high affinity binding site with a K_D of 25 nM and a B_{max} of 500 pmol/mg of protein. Hill plots of the data indicate a single class of binding sites with no cooperativity.

Membrane associated protein-bound c-di-GMP activates membranous cellulose synthase (Table 1). The availability of bound c-di-GMP for synthase activation is markedly reduced in the presence of K^+. The effect is specific to K^+; it is not observed with Na^+, Li^+, Cs^+ Rb^+ or NH_4^+ and is independent of the anion moiety. On the other hand, synthase activation by exogenously added free c-di-GMP is not affected by K^+. The bound cyclic dinucleotide is degraded under similar conditions by membranous PDE-A. The degradation of bound c-di-GMP, but not that of free c-di-GMP is similarly markedly reduced by K^+ (data not shown).

Considering the reversibility of the c-di-GMP binding reaction demonstrated above, the availability of bound c-di-GMP for synthase activation and PDE-A degradation is most probably due to the release of free c-di-GMP. The effect of K^+ on c-di-GMP availability raises the possibility that the cation shifts the equilibrium state of the reaction towards the binding direction. To assess the effect of K^+ on the release of free c-di-GMP from its protein-bound form, CDGBP-bound c-di-GMP preparations were allowed to equilibrate in the absence and presence of K^+ and then assayed for free c-di-GMP (Table 2). The results indicate that although the reaction is readily reversible, its equilibrium is indeed strikingly shifted towards the binding direction in the presence of K^+. Thus when equilibrated in the absence of K^+, 45% of initially bound c-di-GMP is liberated, whereas in the presence of K^+ only 8% is released. Such an equilibrium shift readily explains the effect of K^+ in reducing the availability of bound c-di-GMP for synthase activation and PDE-A degradation described above. Considering the high cellular K^+ level in *A. xylinum* (see below), the equilibrium shifting effect of this cation could account for the finding that most of the cellular c-di-GMP (5–10 μM) is in a bound form. Furthermore, the release of 8% of the cyclic dinucleotide from its protein-bound form, as expected for a steady state equilibrium in the presence of K^+ brings the cellular level of free c-di-GMP well within the concentration range predicted from the affinity of its known binding sites in vitro, namely those for the cellulose synthase and PDE-A [5].

To study the effects of K^+ on cellular c-di-GMP and in vivo cellulose synthesis at the physiological level, intracellular K^+ was replaced by extracellular Na^+ through K^+/H^+ and Na^+/H^+ antiport systems. K^+-depleted cells were then assayed for c-di-GMP content and, in a resting cell system, for [^{14}C]glucose conversion into cellulose and CO_2 [13]. Since *A. xylinum* cells are incapable of glycolysis, CO_2 evolution, being obligatorily coupled to ATP synthesis, represents a convenient indicator of energy metabolism [14]. Manipulation of internal cation content was achieved by modification to the simple method originally developed and demonstrated in *Vibrio alginolyticus* and *E. coli* [8]. The method, which does not induce cell plasmolysis, involves treatment of cells with diethanolamine and subsequent dilution in NaCl which results in replacement of intracellular K^+ by extracellular Na^+. The data in Table 3 show that *A. xylinum* cells washed with K^+ or Na^+ normally contain about 110 mM K^+, which correlates well

Table 3
Effect of replacement of cellular K^+ with Na^+ on cellulose synthesis, energy metabolism and intracellular c-di-GMP

Cell treatment and cation replacement	Intracellular concentration			Glucose conversion into	
	K^+ (mM)	Na^+ (mM)	c-di-GMP (μM)	cellulose (nmol/min/mg cells)	CO_2 (nmol/min/mg cells)
1) Diethanolamine $K^+ \rightarrow Na^+$	<1	114	0.7	0.4	3.2
2) Diethanolamine $K^+ \rightarrow K^+$	117	<1	4.3	3.8	3.4
3) Control Washing with KCl or NaCl	110	1–4	4.8	6.8	3.3

Cells harvested at the exponential phase of growth were employed. Replacement of cellular cations with diethanolamine was carried out as described in Section 2, employing either 140 mM NaCl or KCl as indicated. For total washing control experiments, harvested cells were washed 4 times with, and resuspended in 110 mM NaCl or KCl containing 25 mM dimethylglutarate and correspondingly 5 mM NaH_2PO_4 or KH_2PO_4 at pH 6.0. Intracellular cations and c-di-GMP concentrations were determined as described in Section 2. Glucose conversion to cellulose and CO_2 was determined as in [13] with reaction mixtures (1 ml) containing 1 mg cells (dry weight), 40 mM [U-^{14}C]glucose in 25 mM dimethylglutarate containing 110 mM NaCl or KCl and correspondingly 5 mM Na_2HPO_4 or KH_2PO_4 at pH 6.0.

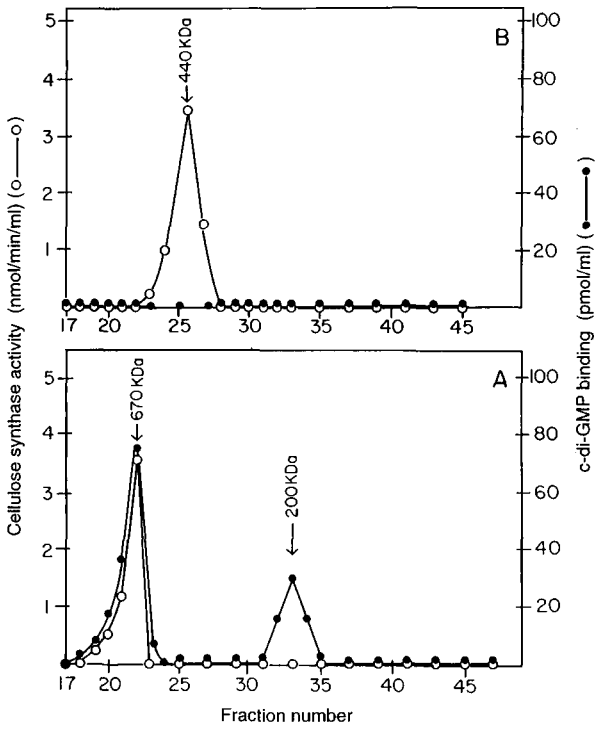

Fig. 2. Gel filtration of native and CDGBP-free solubilized membranes. Native washed membranes (A) and CDGBP-free washed membranes (B) solubilized in Triton X-100 were subjected to gel filtration on a calibrated Sepharose CL-6B column as described in Section 2. Fractions (1.5 ml) were assayed for cellulose synthase activity and [^{32}P]c-di-GMP binding.

with the osmolarity of the growth medium [15]. When intracellular K^+ was completely removed and replaced with Na^+, energy metabolism, as indicated by CO_2 evolution, was not impaired, whereas cellular c-di-GMP was depleted by 80% and cellulose synthesis was inhibited by 90% relative to K^+-loaded cells. The cellulose synthesizing capacity of the latter, although substantial, is not equal to control washed cells, most probably due to their repeated exposure to ethanolamine at pH 8.6. These findings are compatible with the reasoning that cellular K^+ plays a role in the release of c-di-GMP from its protein-bound form, in pace with the need of this nucleotide for the activation of the cellulose synthase which, as outlined below is probably structurally associated with CDGBP.

We have long been aware that the CS purified by the product entrapment procedure, which involves incubation of digitonin-solubilized membranes with excess UDPG and c-di-GMP [7], contains significant amounts of bound c-di-GMP (Weinhouse, H. and Benziman, M., unpublished data). The discovery of CDGBP raises the possibility that the c-di-GMP within the entrapped CS is in fact bound to its binding protein which in turn is strongly associated with the synthase. To verify this possibility, purified CS preparations derived from either native or CDGBP-free membranes were assayed for bound c-di-GMP (released by boiling) and for c-di-GMP binding. As expected, the former preparation contained bound c-di-GMP and exhibited a significant level of binding capacity (assayed by exchange with [^{32}P]c-di-GMP) whereas the latter was devoid of both (data not shown). The apparent linkage between the CS and CDGBP, manifested in the purified synthase, most probably reflects their original association within the membrane where both proteins natively reside. This is supported by the data presented in Fig. 2. Triton X-100-solubilized preparations of either native or CDGBP-free membranes were subjected to gel filtration and eluted fractions were analyzed for CS activity and c-di-GMP binding. In the case of native membranes 100% of the CS activity and 70% of the CDGBP activity comigrated as a single distinct peak corresponding to an apparent molecular mass of 600 kDa, while the residual CDGBP activity appeared in a second peak at 200 kDa, identical to that obtained for a partially purified CDGBP preparation (see above). On the other hand, with CDGBP-free membranes, CS activity eluted as a single peak of 400 kDa, similar to that previously reported for partially purified CS [7]. The 600 kDa peak most probably represents CS-associated CDGBP and supports the notion of structural association between the two proteins. Such association and the implied physical proximity of the two components within the membrane should be of considerable regulatory significance in targeting released c-di-GMP for synthase activation rather than its diversion to PDE-A degradation. In this connection it is noteworthy that although PDE-A is a membranous enzyme its activity was never detected in purified CS preparations.

We previously reported [5] that while in crude membrane preparations at effector concentrations below 0.5 μM the kinetics of CS activation by c-di-GMP display partially cooperative effects, they obey a Michaelis-Menten relationship in the case of purified enzyme. This apparent discrepancy is now readily explained with the discovery of the membranous high affinity CDGBP and as expected, when re-examined with CDGBP-free membranes the activation kinetics did not display a sigmoidal pattern even at extremely low effector concentrations (data not shown). At the physiological level however, the activation kinetics originally observed in native membranes is putatively of regulatory significance, since it probably reflects the situation within the cell in which the concentration of free c-di-GMP may well be within the range in which sigmoidal activation kinetics are observed.

The interrelationship between the newly discovered CDGBP and the various components of the CS of *A. xylinum*, as described here, introduces an additional level for regulation of cellulose biogenesis in vivo. The c-di-GMP generated by diguanylate cyclase binds primarily to the CDGBP, thus preserving it from degradation by PDE-A. The pace of the polymerization process is set by the cellular level of free c-di-GMP released from the CDGBP which it turn is kept at the appropriate level by cellular potassium. Structural association of CDGBP with the CS promotes the targeting of released c-di-GMP toward synthase activation and thus toward enhanced cellulose synthesis.

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