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Efficient Enzymatic Production of the Bacterial Second Messenger c-di-GMP by the Diguanylate Cyclase YdeH from *E. coli*

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Abstract Cyclic di-GMP (c-di-GMP) is an almost universal bacterial second messenger involved in the regulation of cell surface-associated traits and the persistence of infections. GGDEF and EAL domain-containing proteins catalyse c-di-GMP synthesis and degradation, respectively. We report the enzymatic large-scale synthesis of c-di-GMP, making use of the GGDEF domain-containing protein YdeH from *Escherichia coli*. Overexpression and purification of YdeH have been established, and the conditions for c-di-GMP synthesis were optimised. In contrast to the chemical synthesis of c-di-GMP, enzymatic c-di-GMP production is a one-step reaction that can easily be performed with the equipment of a standard biochemical lab. The protocol allows the production of milligram amounts of c-di-GMP within 1 day and paves the way for extensive biochemical and biophysical studies on c-di-GMP-mediated processes.

Keywords c-di-GMP · Diguanylate cyclase · GGDEF domain · Enzymatic synthesis · *E. coli*

Introduction

Bis-(3′-5′)-cyclic di-GMP (c-di-GMP) is an important bacterial second messenger involved in the regulation of a number of complex physiological processes. C-di-GMP was first identified 20 years ago as an activating factor of cellulose synthase in *Acetobacter xylinum* [1], but in the mean time, it has been shown to play a central role, among others, in the transition between a motile, single-cell state to a sessile, surface-attached state found in biofilms [2–5]. Its relevance for the virulence of pathogenic bacteria is well established [2–6].

C-di-GMP is synthesised by the condensation of two GTP molecules. This reaction is catalysed by the GGDEF domain of diguanylate cyclases (DGCs) [7–10], whereas EAL [7, 11–13] and HD-GYP [14] domains hydrolyse the compound to yield the linear pGpG

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dinucleotide. All these catalytic domains are typically found in combinations with other, mostly sensory or regulatory, domains and control the cellular level of c-di-GMP.

Much about the mechanisms of c-di-GMP signalling in bacteria has still to be discovered, in particular since only a few targets of c-di-GMP have been identified. Characterised c-di-GMP receptors comprise PilZ domains [15] and degenerated GGDEF and EAL domains [16, 17]. Furthermore, the GEMM riboswitches have recently been discovered to regulate gene expression via c-di-GMP binding [18–20].

Recent studies have demonstrated that exogenous c-di-GMP treatment inhibits adhesive *Staphylococcus aureus* cell-to-cell interactions and biofilm formation [21] and that it is effective also in a mouse model [22]. These findings make c-di-GMP an interesting candidate as a potential antimicrobial agent. It was demonstrated that c-di-GMP stimulates the immune system to prevent bacterial infections and is therefore evaluated as a potential vaccine adjuvant candidate [23–25]. It was also reported that c-di-GMP inhibits cancer cell proliferation in vitro and could therefore be used as a therapeutic agent [26].

In order to carry out extensive studies on c-di-GMP-mediated processes, sufficient supply of this compound is crucial. The reported chemical syntheses of c-di-GMP are multistep reactions, which make them time-consuming, expensive and inefficient [27–33]. Most of them make use of phosphotriester, phosphoamidite or H-phosphonate chemistry based on air- or water-sensitive reagents and involve several chromatographic purification steps.

In contrast, DGC-catalysed synthesis of c-di-GMP from GTP appears straightforward, as has been reported for PleD, VCA0956 and WspR [12, 13, 34–37]. However, all these DGCs show potent allosteric product inhibition with K_i values in the low micromolar range. Furthermore, the purified proteins are rather unstable in solution. Recently, a DGC from a thermophilic organism (*Thermotoga maritima*) has been employed for the enzymatic synthesis of c-di-GMP [38]. A fragment comprising only its DGC domain with the allosteric inhibition site (I-site) mutated to impede product inhibition has been exploited. Large amounts (about 20 mg) of c-di-GMP were obtained employing 1 mg of this modified version of the enzyme and by replenishing repeatedly the reaction mixture with the substrate GTP.

In this paper, we report a large-scale production procedure of c-di-GMP employing, as an alternative enzyme, YdeH from *Escherichia coli*. This enzyme consists of an N-terminal domain of unknown fold and a C-terminal GGDEF domain. We have shown previously [39] that YdeH, in contrast to most of the other DGCs, is a constitutively active dimer and exhibits a turnover of 1.6 min^{-1} and a K_m of 17 μM. The YdeH catalysed reaction shows non-competitive product inhibition only at comparatively large c-di-GMP concentration (K_i=44 μM), in contrast to the aforementioned DGCs. Furthermore, even at very high c-di-GMP concentration (>1 mM), YdeH shows residual activity (15%) [39]. We show that with this enzyme, complete conversion of GTP to c-di-GMP can be achieved within hours, yielding milligram amount of product.

Material and Methods

Expression and Purification of YdeH

C-terminally His6-tagged YdeH was expressed in the pET28b plasmid in the *E. coli* Rosetta strain. The transformed cells were cultivated at 37 °C in Luria–Bertani medium supplemented with ampicillin (100 μg/mL) and chloramphenicol (34 μg/mL) until the OD_{600} had reached 0.7. Gene expression was induced by adding 1 mM isopropyl-β-D-thiogalactopyranoside

(IPTG). After 4 h of incubation, the cells were harvested by centrifugation (6,800×*g*, 10 min, 4 °C) and the pellets were frozen at −20 °C.

Prior to lysis, the frozen cell pellets were thawed and resuspended in Ni-A buffer (50 mM NaH_2PO_4, pH 7.5, 200 mM NaCl, 10 mM imidazole, 50 mM L-glutamic acid, 50 mM L-arginine). After the addition of DNAse (2.5 μg/mL; Sigma) and EDTA-free protease-inhibitor cocktail (Roche, 1 tablet/50 mL buffer), the cells were disrupted with a French press (Thermo Spectronic) at 15,000 psi. The lysate was cleared by centrifugation (28,000×*g*, 45 min, 4 °C), and the supernatant was filtered (0.22 μm) and loaded onto a 5-mL HisTrap column (GE Healthcare). After washing the column with Ni-A buffer (10 column volumes (CV)), the protein was eluted with a linear gradient of imidazole from 10 to 500 mM in 10 CV. The pooled fractions were concentrated to 1 mL and further purified by size-exclusion chromatography using a Superdex 75 16/60 column (GE Healthcare) and SEC buffer (20 mM Tris–HCl, pH 7.6, 150 mM NaCl, 50 mM L-glutamic acid, 50 mM L-arginine). The chromatographic runs were carried out with an Äkta Purifier FPLC unit and monitored with Unicorn software. The purification process was monitored by SDS-PAGE followed by Coomassie blue R-250 staining. Protein concentration was determined by measuring the absorbance at 280 nm (ε_{280}=39,880 $M^{-1}cm^{-1}$).

Enzyme Assay

The initial rate of c-di-GMP synthesis was measured by following the substrate consumption using ion exchange chromatography. The reaction mixture (100 μL) contained 2 μM purified YdeH, 100 μM GTP (Sigma), and 5 mM $MgCl_2$. The effect of pH on the activity of YdeH was tested in 50 mM MES, pH 6.5–7.5, 50 mM Tris–HCl, pH 7.5–8.5, and 50 mM CHES, pH 9.0–10.0. Also, the effect of different salts (LiCl, NaCl and KCl) and NaCl concentrations (25 mM–1.5 M) was investigated. The reaction was stopped by heating of the sample for 2 min at 99 °C. Subsequently, 100 μL of the reaction mixture was diluted in 900 μL 5 mM NH_4HCO_3, pH 8.0, filtered (0.22 μm) and loaded on an ion-exchange column (ResourceQ 1 mL, GE Healthcare). The nucleotides were separated with a gradient from 0.005 to 1 M NH_4HCO_3, pH 8.0, in 14 CV. The amount of substrate and reaction product was determined by integration of the UV absorption (253 nm) peaks. The procedure was calibrated with GTP (Sigma) and c-di-GMP (Biolog, Bremen) standards of known concentrations.

Enzymatic Production of c-di-GMP

For the synthesis of c-di-GMP, 2 μM YdeH was incubated with 500 μM GTP in 50 mM Tris–HCl, pH 7.5, 50 mM NaCl and 5 mM $MgCl_2$ for 5 h. The progress of the reaction was followed by withdrawing samples (100 μL) that were inactivated by heat denaturation. Subsequently, the enzyme was removed by filtering and the sample content analysed by ion-exchange chromatography. After completion of the reaction, the mixture was heated for 5 min at 99 °C, centrifuged (4,500×*g*, 10 min, room temperature) and filtered (0.22 μm). For purification of the reaction product, Et_3NHCO_3 to a final concentration of 5 mM was added to the reaction mixture and loaded on a reversed-phase chromatography column (1.7 mL per 1 mL resin Resource RPC, GE Healthcare). After washing the column with 5 mM Et_3NHCO_3 (10 CV), c-di-GMP was eluted with a linear gradient of ethanol from 0% to 50% in 10 CV. The reversed-phase chromatography was carried out with an Äkta Purifier FPLC unit and monitored with Unicorn software. The c-di-GMP-containing fractions were pooled and lyophilised. The powder was dissolved in water and the concentration was

determined by measuring the optical density at 253 nm of an aliquot diluted to 10 μM (assuming an ε_{253} of 23,700 $M^{-1}cm^{-1}$ as reported in [29]).

Analytical Methods

Purified c-di-GMP was analysed by high-performance liquid chromatography coupled to mass spectrometry. The samples were diluted 50-fold with 10 mM NH_4OAc, pH 6.4, and injected into a Supercosil™ LC-18-T column (Supelco). The run was performed at a flow rate of 0.7 mL/min using a linear gradient (0–50%) of acetonitrile. The electron spray ionisation mass spectrometry with a time-of-flight analyser was performed using a microTOF Focus system (Bruker Daltronics). The capillary voltage was 4500 V, and the end-plate offset was 500 V (negative mode); the dry temperature was 200 °C, the dry gas flow was 9 L/min, and the nebulizer pressure was 2 bar.

To test the purity of the obtained c-di-GMP, a sample was analysed by ^{1}H-nuclear magnetic resonance (NMR) spectroscopy (Bruker Advance 600) in 10 mM Tris–HCl (D11), pH 7.4 (Cambridge Isotope Laboratories, Inc.), 150 mM NaCl, 50 μM EDTA at 298 K. For comparison, spectra of synthetic c-di-GMP (Biolog) and GTP (Sigma) were acquired.

Results and Discussion

Expression and Purification of YdeH

YdeH expression was tested in different *E. coli* strains and at different temperatures. In all conditions, most of the expressed YdeH was found in inclusion bodies. The highest amount of soluble protein was achieved in the Rosetta strain at 37 °C, 3–4 h after IPTG induction. YdeH was purified to homogeneity using a two-step purification procedure, consisting of Ni-affinity and size-exclusion chromatography. To achieve a protein concentration above 0.8 mg/mL, the addition of arginine and glutamic acid to the buffers was essential to avoid protein aggregation [40]. The obtained protein was pure and no degradation fragments were present, as demonstrated by the occurrence of a single band on SDS-PAGE (Fig. 1). The final yield was about 5 mgL^{-1} culture.

Optimisation and Characterisation of the Enzymatic Reaction

Substrate and product amounts were analysed by ion-exchange chromatography. A peak at a somewhat smaller elution volume than that of the final product was observed in the

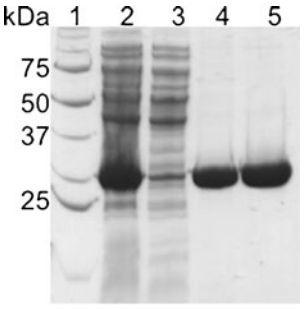

Fig. 1 SDS-PAGE of the purification of YdeH. A 12% (*w*/*v*) gel loaded with: *lane 1* molecular weight marker, *lane 2* cell extract, *lane 3* soluble fraction of the cell extract, *lane 4* purified YdeH after Ni column, *lane 5* purified YdeH after size-exclusion chromatography

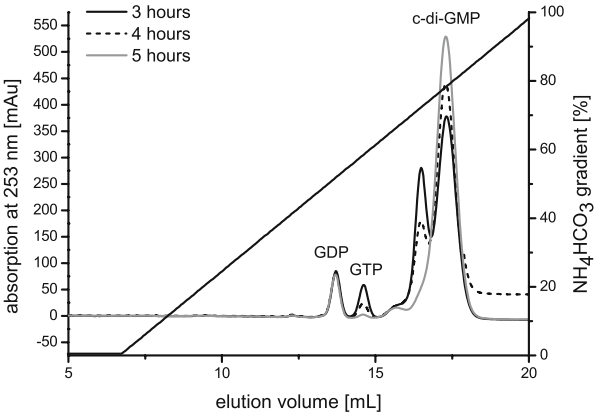

Fig. 2 Time course of the synthesis of c-di-GMP. The samples were analysed using ion-exchange chromatography. After 5 h, the reaction is virtually complete. The leftmost small and constant peak corresponds to a GDP contamination in the commercial GTP batch

chromatograms taken at early to intermediate time points, but was absent after completion of the reaction (Fig. 2). As demonstrated by MS, this transient peak did not correspond to a reaction intermediate, but had the mass of c-di-GMP. Probably, the two peaks correspond to different oligomeric states of c-di-GMP.

The optimum pH for the synthesis of c-di-GMP was found to be at pH 7.5 in Tris buffer (Fig. 3). Variation of the salt (LiCl, NaCl, KCl) had only a marginal effect. The enzymatic activity was virtually the same in 50 mM NaCl and 50 mM KCl, but was found to be reduced by about 20% in 50 mM LiCl. Similarly, variation of the NaCl concentration (25 mM–1.5 M) had no significant effect on activity. Therefore, we chose a comparatively low NaCl concentration (50 mM) to be compatible with the requirements for the subsequent c-di-GMP purification procedure.

Enzymatic c-di-GMP Production and Purification

For the large-scale synthesis of c-di-GMP, the molar ratio of enzyme to substrate has been optimised in order to obtain complete conversion within a convenient period. Using 2 μM YdeH and 500 μM GTP, virtually complete substrate conversion was achieved within 5 h,

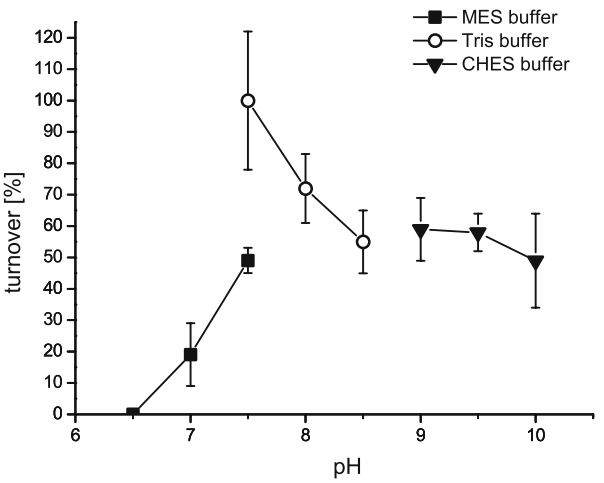

Fig. 3 pH dependence of the enzymatic activity of YdeH. The optimum reaction velocity for the synthesis of c-di-GMP was found in 50 mM Tris–HCl, pH 7.5

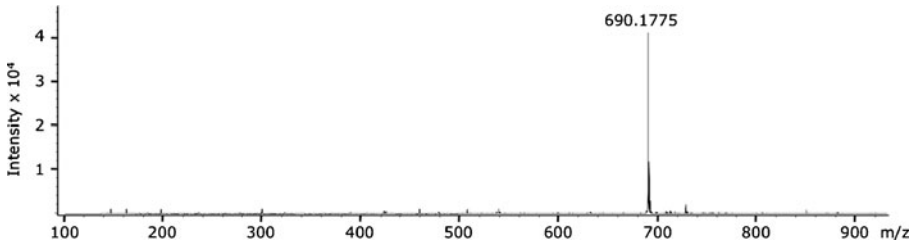

Fig. 4 Convoluted mass spectrum of enzymatically produced c-di-GMP. The measured mass of 690.1775 g/mol is in agreement with the calculated mass of 690.0870 g/mol

as shown in Fig. 2. Initially, the reaction volume was 0.1 mL, but was eventually upscaled to 0.5 L without any detrimental effect. C-di-GMP was purified on a reversed-phase column with ethanol as eluent. The Resource RPC resin was chosen because of its stability at basic pH values. The mobile phase was adjusted so as to prevent binding of the mono-nucleotides GDP and GTP. Ethanol has the advantage of being non-toxic compared to other typically used eluents such as acetonitrile or methanol. The procedure yielded 75 mg of pure c-di-GMP employing 30 mg of YdeH. Considering the ease of YdeH purification, further optimisation of the procedure by recycling of the enzyme as in [38] was not considered.

Product Analysis

LC/MS clearly identified the reaction product as c-di-GMP (Fig. 4). No impurities were detected in the chromatogram. The observed mass of c-di-GMP was in perfect agreement with the calculated mass. In addition, the ^{1}H-NMR spectra of purified c-di-GMP completely coincided with the spectrum of chemically synthesised c-di-GMP and showed no traces of the substrate GTP (Fig. 5).

Conclusions

Here, we have described an alternative way for the enzymatic synthesis of the bacterial second messenger c-di-GMP. We use the DGC YdeH from *E. coli*, which is an ideal

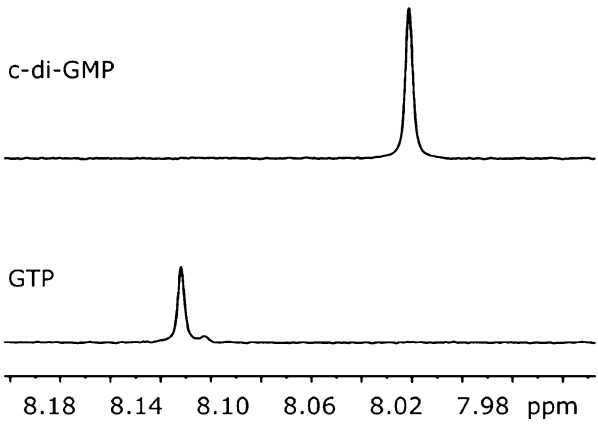

Fig. 5 Part of the ^{1}H-NMR spectrum (600 MHz) showing the chemical shift of H8 of enzymatically produced c-di-GMP (*top*, δ=8.04) and of GTP (*bottom*, δ=8.13) as reference. Comparison demonstrates that the c-di-GMP sample was free of GTP. Chemical shifts are relative to H_2O (δ=4.77, 298 K)

candidate because it is constitutively active in vitro and exhibits only weak product inhibition; thus, c-di-GMP concentrations of up to 0.25 mM can be achieved. Our system allows producing 2.5 mg of pure c-di-GMP per milligram of YdeH with standard biochemical lab equipment.

Enzymatic c-di-GMP production outperforms chemical synthesis with respect to time, costs and number of chromatographic purification steps [27–33]. Moreover, most of the chemical syntheses are based on air- and water-sensitive reagents. The enzymatic procedures described in the literature [12, 13, 34–37], however, employ rather unstable DGCs that, in addition, show strong product inhibition and therefore do not yield complete substrate conversion. The recently described procedure using a DGC from a thermophilic organism (*T. maritima*) [38] solved the solubility issue and provides the best specific yield so far (20 mg/mg enzyme), but had to be mutated in the allosteric inhibition site (I-site) to abolish product inhibition.

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