Biotechnology Reports 7 (2015) 30–37

Contents lists available at ScienceDirect

Biotechnology Reports

journal homepage: www.elsevier.com/locate/btre

Modification of a bi-functional diguanylate cyclase-phosphodiesterase to efficiently produce cyclic diguanylate monophosphate

Natasha M. Nesbitt, Dhruv P. Arora, Roger A. Johnson, Elizabeth M. Boon *

Department of Chemistry, Stony Brook University, Stony Brook, NY 11794-3400 United States

ARTICLE INFO

Article history:
Received 7 January 2015
Received in revised form 27 April 2015
Accepted 30 April 2015
Available online 5 May 2015

Keywords:
Cyclic-diGMP
[^{32}P]-cyclic-diGMP
Diguanylate cyclase
Phosphodiesterase
H-NOX associated cyclic-diGMP processing enzyme

ABSTRACT

Cyclic-diGMP is a bacterial messenger that regulates many physiological processes, including many attributed to pathogenicity. Bacteria synthesize cyclic-diGMP from GTP using diguanylate cyclases; its hydrolysis is catalyzed by phosphodiesterases. Here we report the over-expression and purification of a bi-functional diguanylate cyclase-phosphodiesterase from *Agrobacterium vitis* S4. Using homology modeling and primary structure alignment, we identify several amino acids predicted to participate in the phosphodiesterase reaction. Upon altering selected residues, we obtain variants of the enzyme that efficiently and quantitatively catalyze the synthesis of cyclic-diGMP from GTP without hydrolysis to pGpG. Additionally, we identify a variant that produces cyclic-diGMP while immobilized to NiNTA beads and can catalyze the conversion of [α-^{32}P]-GTP to [^{32}P]-cyclic-diGMP. In short, we characterize a novel cyclic-diGMP processing enzyme and demonstrate its utility for efficient and cost-effective production of cyclic-diGMP, as well as modified cyclic-diGMP molecules, for use as probes in studying the many important biological processes mediated by cyclic-diGMP.

1. Introduction

Cyclic-diGMP [(bis-3′-5′)-cyclic dimeric guanosine monophosphate] is a second messenger ubiquitously produced by bacteria [11,15,25] and the eukaryote *Dictyostelium discoideum* [7]. Since its discovery as a regulator of cellulose biosynthesis in *Acetobacter xylinum* [45], it has been linked to the regulation of various cellular processes with medical and agricultural implications, including biofilm formation, regulation of virulence factors, pathogenicity, and cell mobility [10,21,26,36,43,53]. Because of its role in medically relevant processes, cyclic-diGMP is currently being explored as an anti-infective agent [50], as well as a vaccine adjuvant [6,22,38].

The growing interest in understanding the underlying mechanisms by which this molecule regulates these diverse processes has been hampered by the cost associated with obtaining cyclic-diGMP. Because of this, more cost-effective avenues needed to be explored to allow researchers to obtain the nucleotide. Several groups have achieved chemical syntheses of cyclic-diGMP [14,20,24,27,28,44,45]. However, these syntheses require multiple steps that involve protection and deprotection of various functional groups of GTP, which often results in low product yield.

Bacteria synthesize cyclic-diGMP from two molecules of guanosine triphosphate (GTP) using a diguanylate cyclase (DGC) containing a conserved GG(D/E)EF motif [1,5,15,44,45,47,52] or the AGDEF motif [23,39]. The conserved D/E is proposed to be the active site base responsible for deprotonating the 3′-OH of GTP which facilitates nucleophilic attack of the α-phosphate of the second molecule of GTP thereby producing the cyclized product [5,26,57]. Frequently, diguanylate cyclases demonstrate product inhibition. The sequence RxxD has been shown to be required for cyclic-diGMP binding and therefore product inhibition [2,8,9,30,54]. In bacteria, cyclic-diGMP is hydrolyzed to 5′-phosphoguanylyl-(3′,5′)-guanosine (pGpG) by phosphodiesterases (PDE) containing the signature ExL motif [9,48,52], or less commonly the HD-GYP motif [12,16,17,18,46]. In most cases enzymes containing both GG(D/E)EF and ExL motifs exhibit either diguanylate cyclase or phosphodiesterase activities, but rarely both. However, to date eight bi-functional DGC–PDE enzymes have been identified from various species [13,19,31,33–35,49,55].

Enzymatic synthesis of cyclic-diGMP has been reported by several groups using diguanylate cyclases [9,29,40,51,58]. In most cases, the enzymes used by these groups contain an RxxD product-

Abbrevations: Cyclic-diGMP, bis-3′-5′-cyclic dimeric guanosine monophosphate; GTP, guanosine-5′-triphosphate; pGpG, 5′-phosphoguanylyl-(3′,5′)-guanosine); DGC, diguanylate cyclase; PDE, phosphodiesterase; AvHaCE, *Agrobacterium vitis* H-NOX associated cyclic-diGMP processing enzyme.

* Corresponding author. Tel.: +1 631 632 7945; fax: +1 631 632 7960.
E-mail addresses: natasha.nesbitt@stonybrook.edu (N.M. Nesbitt), dhruvparora@gmail.com (D.P. Arora), roger.johnson@stonybrook.edu (R.A. Johnson), elizabeth.boon@stonybrook.edu (E.M. Boon).

http://dx.doi.org/10.1016/j.btre.2015.04.008

inhibition site that limits the amount of cyclic-diGMP produced. One group reported generation a variant of a diguanylate cyclase from the thermophile *Thermotoga maritima*, encoded by the gene *TM1788*, in which they changed the RxxD motif to AxxD [40]. However, despite use of this variant, they still observed product inhibition at high concentrations of GTP.

Based on its primary structure, the protein *Av*HaCE (H-NOX-associated cyclic-diGMP processing enzyme), encoded by the gene *Avi_3097* from *Agrobacterium vitis* strain S4, is predicted to be a bi-functional DGC–PDE. Of particular interest is the fact that the protein lacks the RxxD motif. We hypothesized that due to its lack of the RxxD inhibition site, a variant of *Av*HaCE without phosphodiesterase activity could efficiently produce large quantities of cyclic-diGMP, and therefore be of great utility to the cyclic-diGMP field. Thus, we sought to investigate whether altering residues thought to be important for phosphodiesterase activity would result in variants of *Av*HaCE lacking PDE activity while maintaining DGC activity, thus resulting in an improved system for enzymatically producing cyclic-diGMP.

2. Materials and methods

2.1. Materials

5′-GTP was purchased from Promega. [α-^{32}P]-GTP was obtained from PerkinElmer and cyclic di-GMP and pGpG were from Biolog. Restriction endonucleases and Phusion High Fidelity polymerase were purchased from New England Biolabs. *PfuTurbo* was obtained from Agilent. Qiagen was the source for nickel–nitriloacetic acid (Ni–NTA) agarose. *A. vitis* S4 cells were a generous gift from Professor Thomas Burr (Cornell University).

2.2. General procedures

Matrix assisted laser desorption ionization (MALDI) analyses were performed at the Stony Brook Proteomics Center or at the Institute of Chemical Biology and Drug Discovery (Stony Brook University) with α-cyano-4-hydroxycinnamic acid as the matrix. High performance liquid chromatography (HPLC) was conducted with a LC-2010A HT liquid chromatography system (Shimadzu). Nucleotides were separated with either a Shim-pack XR-ODS (3 mm × 100 mm) or a Beckman ODS Ultrasphere (4.6 mm × 25 cm) reverse phase C18 column and detection was monitored at 254 nm.

2.3. Construction of wild-type and variant AvHaCE expression vectors

Genomic DNA for cloning *A. vitis Avi_3097* was purified from cells of *A. vitis* S4 by use of the Wizard SV Genomic DNA Purification System by Promega. The gene was cloned from *A. vitis* genomic DNA by the polymerase chain reaction (PCR) with *Pfu* Turbo or Phusion as the polymerase. The gene was cloned between the NdeI and XhoI sites of pET20b to generate a C-terminal hexa-histidine tagged protein. QuikChange site directed mutagenesis was used to generate variants of *Av*HaCE with wild-type *Avi_3097* in pET20b plasmid DNA serving as the template for PCR. The *Avi_3097* DGC only variant was constructed by introducing a stop codon after the 248th amino acid of the wild-type protein. The presence of the gene and mutations was confirmed at the Stony Brook DNA sequencing facility.

2.4. Expression and purification of hexa-histidine tagged wild-type and variant AvHaCE proteins

Plasmid DNA harboring the gene encoding wild-type or variant *Av*HaCE was transformed into BL21DE3pLysS competent cells for protein over-expression. Cultures were grown in 2XYT medium (16 g tryptone, 10 g yeast extract, 5 g sodium chloride per liter) supplemented with 100 μg mL^{-1} ampicillin at 37 °C to an OD_{600} of ∼1.2. Protein expression was induced by the addition of 10 μM isopropyl β-D-thiogalactopyranoside (IPTG) and was allowed to proceed overnight (∼16 h) at 18 °C after which cells were harvested by centrifugation. Cells were resuspended in Buffer A (50 mM Tris–HCl, pH 7.4, 5 mM β-mercaptoethanol, 10% glycerol, 50 mM arginine, 50 mM glutamic acid, 200 mM sodium chloride, 10 mM imidazole and 500 μM EDTA) containing 1 mM phenylmethylsulfonyl fluoride (PMSF) at room temperature. The suspension was incubated on ice for 20 min and cells were lysed by sonication. Cellular debris was removed by centrifugation at 39,000 × *g* for one hour at 4 °C. The cleared lysate was applied to a Ni–NTA column equilibrated in Buffer A and the column was washed with 10 column volumes of Buffer A followed by washing with 10 column volumes of buffer containing 50 mM Tris–HCl, pH 7.4, 5 mM β-mercaptoethanol, 10% glycerol, 50 mM arginine, 50 mM glutamic acid, 200 mM sodium chloride, 100 mM imidazole, and 500 μM EDTA. The protein was eluted from the column with buffer containing 50 mM Tris–HCl, pH 7.4, 5 mM β-mercaptoethanol, 10% glycerol, 50 mM arginine, 50 mM glutamic acid, 200 mM sodium chloride, 250 mM imidazole, and 500 μM EDTA. Fractions containing pure protein, as judged by SDS-PAGE analysis, were pooled and dialyzed overnight against 50 mM Tris–HCl, pH 7.4, 5 mM β-mercaptoethanol, 10% glycerol, 50 mM arginine, 50 mM glutamic acid, 200 mM sodium chloride, and 500 μM EDTA and were then stored at −80 °C.

2.5. Protein concentration determination

Protein concentrations were determined by the method of Bradford with bovine serum albumin (BSA) as standard [3]. Protein purity was assessed by SDS-PAGE with a 12.5% gel as described by Laemmli [32].

2.6. Enzyme activity assay

Assays to determine product formation were carried out in 50 mM Tris–HCl, pH 7.5, containing 5 mM $MgCl_2$ and 100 μM GTP. Reactions were initiated by the addition of 1 μM wild-type or variant protein to the assay mixture and were allowed to incubate overnight at room temperature. Reactions were terminated by heating the samples at 95 °C for 5 min. Precipitated proteins were removed by centrifugation after which the supernatant was filtered through a 0.22 μm membrane and analyzed by HPLC with a Shim-pack XR-ODS column. The column was equilibrated with 95% Solvent A (0.1 M triethyl ammonium acetate (TEAA), pH 6.1) −5% Solvent B (70% Solvent A −30% acetonitrile) at a flow rate of 0.1 mL min^{-1}. Nucleotides were eluted with the following gradient: 5–10% Solvent B over 10 min, 10–15% Solvent B over 5 min, 15–20% Solvent B over 5 min, 20–25% Solvent B over 5 min, 25–50% Solvent B over 5 min, 50–5% Solvent B over 5 min and maintained at 5% Solvent B for 5 min. Authentic GTP, cyclic di-GMP, and pGpG were used as standards to determine retention times of the nucleotides. The activity of each enzyme, wild-type or variant, was confirmed a minimum of three times.

2.7. Homology modeling of AvHaCE phosphodiesterase domain

A structural model of the phosphodiesterase domain (residues 249-502) of *Av*HaCE was generated by use of Modeller 9.9 (http://www.salilab.org/modeller/) with the phosphodiesterase domain (residues 1-250) of YkuI (pdb 2W27) from *Bacillus subtilis* serving as the template. These residues were chosen for *Av*HaCE based on its domain prediction with PROSITE (http://prosite.expasy.org/).