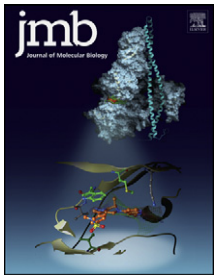

Structural and Enzymatic Characterization of a cAMP-Dependent Diguanylate Cyclase from Pathogenic *Leptospira* Species

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Abstract

Leptospira interrogans serovar Copenhageni is a human pathogen that causes leptospirosis, a worldwide zoonosis. The *L. interrogans* genome codes for a wide array of potential diguanylate cyclase (DGC) enzymes with characteristic GGDEF domains capable of synthesizing the cyclic dinucleotide c-di-GMP, known to regulate transitions between different cellular behavioral states in bacteria. Among such enzymes, LIC13137 (Lcd1), which has an N-terminal c*G*MP-specific phosphodiesterases, *a*denylyl cyclases, and *F*hlA (GAF) domain and a C-terminal GGDEF domain, is notable for having close orthologs present only in pathogenic *Leptospira* species. Although the function and structure of GGDEF and GAF domains have been studied extensively separately, little is known about enzymes with the GAF-GGDEF architecture. In this report, we address the question of how the GAF domain regulates the DGC activity of Lcd1. The full-length Lcd1 and its GAF domain form dimers in solution. The GAF domain binds specifically cAMP (K_D of 0.24 μM) and has an important role in the regulation of the DGC activity of the GGDEF domain. Lcd1 DGC activity is negligible in the absence of cAMP and is significantly enhanced in its presence (specific activity of 0.13 s^{-1}). The crystal structure of the Lcd1 GAF domain in complex with cAMP provides valuable insights toward explaining its specificity for cAMP and pointing to possible mechanisms by which this cyclic nucleotide regulates the assembly of an active DGC enzyme.

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Introduction

Different forms of purine nucleotides have emerged as second messengers, including cyclic mononucleotides [3′,5′-cyclic adenosine monophosphate (cAMP) and 3′,5′-cyclic guanosine monophosphate (cGMP)], cyclic dinucleotides (c-di-GMP, c-di-AMP, and cAMP-GMP) [1–4], and linear nucleotides (ppGpp and pppGpp) [5,6], all with important roles in regulating bacterial behavior in response to internal and external signals [7]. A universal eubacterial mechanism of signal transduction is mediated by the ubiquitous second messenger bis-(3′→5′) cyclic dimeric guanosine monophosphate (c-di-GMP) that controls various processes, such as biofilm production, adaptation to the external environment, production of virulence factors, and motility [8,9]. c-di-GMP is synthesized by GGDEF domain-containing diguanylate cyclase (DGC) enzymes, which convert two molecules of GTP into one molecule of c-di-GMP and two molecules of pyrophosphate. The c-di-GMP molecule can be subsequently hydrolyzed to pGpG and GMP by specific phosphodiesterases (PDEs) that contain EAL and HD-GYP domains, respectively [8,10–12].

Most DGCs do not consist of stand-alone GGDEF domains but rather often have one or more N-terminal

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domains that regulate DGC activity by sensing specific chemical signals and mediating dimerization required for catalysis of the bimolecular reaction [13]. Of the more than 26,000 proteins annotated to contain GGDEF domains in the Pfam database, 438 have an architecture in which the GGDEF domain is immediately preceded by a c*G*MP-specific PDEs, *a*denylyl cyclases, and *F*hlA (GAF) domain [14]. GAF domains, named based on the proteins in which they were first identified, have been shown to bind to different ligands, including the cyclic nucleotides cAMP and/or cGMP, heme, and biliverdin [15]. Despite forming one of the largest domain families found in all kingdoms of life [16], the GAF domain's structural and functional diversity is poorly characterized. For example, the GAF domains found in human cyclic nucleotide PDEs have been shown to have a variety of cNMP binding modes, making it difficult to discern their ligand binding specificity [17]. GAF domains usually form dimers, and ligand-induced conformational changes in the dimer often have an important role in the regulation of protein activity [18,19].

The bacterium *Leptospira interrogans* serovar Copenhageni causes leptospirosis disease, a worldwide zoonosis that affects various mammals including humans [20]. In 5–15% of leptospirosis cases, the clinical symptoms may progress to a severe disease condition, which can cause jaundice, renal failure, meningitis, hypotension, bleeding, multiple organ failure, pulmonary hemorrhages and, in some cases, death [21,22]. The ability of some *Leptospira* species to grow in different conditions, such as in soil, water, and in different mammalian organs and the bloodstream, suggests that the bacteria should possess different signaling pathways to sense the external environment in order to appropriately modify bacterial biochemistry, physiology, and behavior. Therefore, we can expect that c-di-GMP will have an important role in the *Leptospira* life cycle. However, to date, almost nothing is known about c-di-GMP signaling in *Leptospira.*

LIC13137 from *L. interrogans* serovar Copenhageni is a two-domain protein with an N-terminal GAF domain, predicted to be a cyclic mononucleotide (cNMP) receptor, and a C-terminal GGDEF domain predicted to be an active DGC (Fig. S1). Interestingly, close LIC13137 orthologs, with >23% identity, are exclusively found in pathogenic species of the *Leptospira* genus, such as *L. interrogans, L. santarosai*, *L. kirschneri, L. noguchii, L. weilii, L. kmetyi*, *L. alexanderi*, *L. mayottensis, L. alstonii*, and *L. borgpetersenii,* but are not found in opportunistic or non-pathogenic *Leptospira* species, such as *L. biflexa, L. yanagawae*, *L. terpstrae*, *L. meyeri, L. licerasiae, L. vanthielii, L. wolbachii, L. wolffii, L. fainei, L. broomii*, and *L. inadai.* LIC13137 has only one paralog, LIC12273 (23% identity), with GAF-GGDEF domain architecture in the *L. interrogans* genome. LIC12273 has orthologs in both pathogenic and non-pathogenic *Leptospira* species (Fig. S2a). The low sequence similarity of the LIC13137 and LIC12273 GAF domains suggests that they probably bind different ligands (Fig. S2b and c).

Here, we describe the crystal structure of the LIC13137 GAF domain in complex with its ligand, cAMP. Structural analysis offers important insights to explain its ability to discriminate cAMP from cGMP and allows us to propose a structural mechanism for cAMP-dependent control of DGC activity. We show that the enzyme's DGC activity, $k_{cat} = 0.13\ s^{-1}$, is strongly dependent on cAMP binding, with dissociation constant of 0.24 μM. These results suggest that LIC13137 is a potential node for the integration between cAMP and c-di-GMP signaling in *L. interrogans.* Based on our analysis of LIC13137's function, we named this enzyme Lcd1 for *L*eptospira c*A*MP-dependent *D*GC *1*. Our results demonstrate a direct involvement of cAMP in the production of c-di-GMP by Lcd1.

Results and Discussion

The GAF domain of Lcd1 binds cAMP

Lcd1, coded by the LIC13137 gene, has two domains, an N-terminal GAF domain (residues 28–165) and a C-terminal GGDEF domain (residues 178–326) with a predicted linker (residues 166–177) between them. It is difficult, based on amino acid

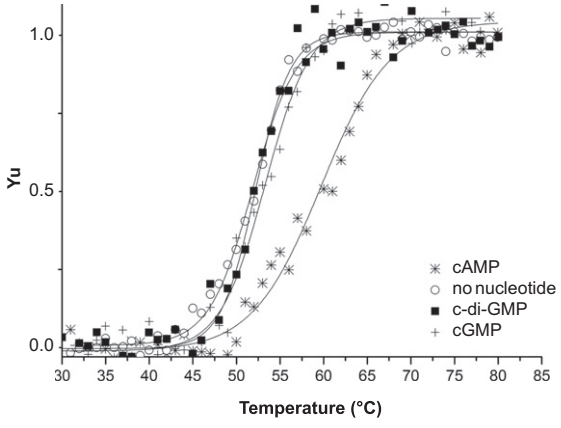

Fig. 1. Lcd1 binds cAMP. Conformational stability of $Lcd1_{GAF\_1–180}$ (15 μM) in the presence of different cyclic nucleotides (100 μM). The denaturation assays were accompanied by measuring $[\theta]_{220nm}$ between 30 °C and 80 °C, and the signal was converted to a fraction of unfolded protein (Yu) as described in Material and Methods. The denaturation temperatures (T_m) were calculated from the temperature at which Yu = 0.5. No nucleotide, T_m = 52 °C; cAMP, T_m = 59 °C; c-di-GMP, T_m = 52 °C; cGMP, T_m = 52.5 °C.

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