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An HD-GYP Cyclic Di-Guanosine Monophosphate Phosphodiesterase with a Non-Heme Diiron−Carboxylate Active Site

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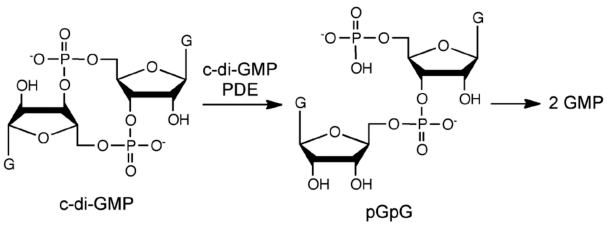
Supporting Information

ABSTRACT: The intracellular level of the ubiquitous bacterial secondary messenger, cyclic di-(3′,5′)-guanosine monophosphate (c-di-GMP), represents a balance between its biosynthesis and degradation, the latter via specific phosphodiesterases (PDEs). One class of c-di-GMP PDEs contains a characteristic HD-GYP domain. Here we report that an HD-GYP PDE from *Vibrio cholerae* contains a non-heme diiron−carboxylate active site, and that only the reduced form is active. An engineered D-to-A substitution in the HD dyad caused loss of c-di-GMP PDE activity and of two iron atoms. This report constitutes the first demonstration that a non-heme diiron−carboxylate active site can catalyze the c-di-GMP PDE reaction and that this activity can be redox regulated in the HD-GYP class.

Cyclic di-(3′,5′)-guanosine monophosphate (c-di-GMP) is a widespread bacterial second messenger, regulating processes such as biofilm formation and virulence.[1] C-di-GMP phosphodiesterases (c-di-GMP PDEs) catalyze hydrolysis of c-di-GMP to pGpG, which is subsequently hydrolyzed to GMP, as shown in Scheme 1.

Scheme 1. c-Di-GMP PDE Activity



Two distinct classes of c-di-GMP PDEs, EAL and HD-GYP, are named for domains containing the eponymous amino acid sequence motifs.[1] Both classes are annotated as metal-dependent phosphohydrolases and invariably contain additional protein domains involved in signaling or activity regulation. Although a large body of genetic evidence supports the c-di-GMP PDE function of HD-GYP domains, only one example of an isolated HD-GYP protein with c-di-GMP PDE activity has been reported.[2] HD-GYP domains are a subclass of the HD domain superfamily, characterized by a His,Asp divalent metal-binding sequence motif.[3] The EAL class requires Mn^{2+} and/or Mg^{2+}, but the active site metal ions in the HD-GYP class have not been established.[1] A gene (VCA0681) in the cholera-causing bacteria, *V. cholerae*$_{ET}$, encodes a protein with tandem HD and HD-GYP domains, hereafter referred to as HD-[HD-GYP] (see Figure S1 in the Supporting Information). Overexpression of HD-[HD-GYP] in *V. cholerae*$_{ET}$ resulted in decreased c-di-GMP levels.[4] An equivalent strain expressing a variant encoding a D-to-A substitution in the HD motif of the HD-GYP domain (HD-[HA-GYP]) showed a phenotype consistent with loss of c-di-GMP PDE activity. However, the HD-[HD-GYP] was not isolated or further characterized. Here we show that the isolated HD-[HD-GYP] exhibits c-di-GMP PDE activity with iron as the only detectable transition metal and that the iron is contained in non-heme His,carboxylate-ligated dimetal sites.

The HD-[HD-GYP] with tandem N-terminal 6×His-MBP affinity/solubility tags was overexpressed in *Escherichia coli*. The protein was purified using an MBP affinity column but not a His-tag affinity column. When a ferrous salt was added to the cultures at the time of induction of protein expression, the as-isolated tagged HD-[HD-GYP] reproducibly contained ∼4 irons/protein monomer and only trace levels of other transition metals. When a manganous salt instead of the ferrous salt was added to the cultures, the purified protein contained substoichiometric amounts of both metals (see the Supporting Information). Therefore, the results reported below are for proteins isolated from the ferrous salt-spiked cultures. All results were obtained at room temperature in 50 mM MOPS pH 7.3.

The UV−vis absorption spectrum (Figure 1) of the as-isolated (∼4 Fe/monomer) HD-[HD-GYP] shows the characteristic features of non-heme oxo/carboxylato-bridged diferric ($Fe^{3+}Fe^{3+}$) sites with terminal His and carboxylate ligands.[5] These features include the double absorption between 300 and 400 nm ($\varepsilon_{350\ nm}$ ∼ 7700 M^{-1} cm^{-1} per 2Fe) and shoulder at ∼490 nm. The anaerobic sodium dithionite-reduced protein showed the expected featureless absorption spectrum (apart from a very minor heme contaminant) of the corresponding diferrous ($Fe^{2+}Fe^{2+}$) sites. The reduced protein rapidly recovered the as-isolated protein's spectral features upon re-exposure to air. The as-isolated protein showed a weak $g_{av} < 2$ EPR signal (∼6% of diiron sites) characteristic of the mixed-valent $Fe^{II}Fe^{III}$ oxidation level,[5] which was enhanced by substoichiometric dithionite addition (Figure S2 in the

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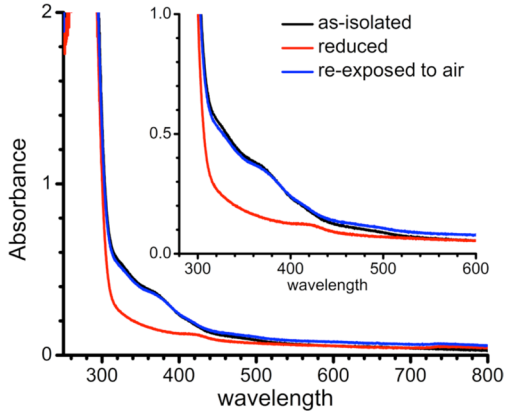

Figure 1. UV−vis absorption spectra of HD-[HD-GYP].

Supporting Information). Neither iron binding nor the spectral features were observed in the separately expressed and isolated 6×His-MBP.

Figure 2 shows that the reduced but not the as-isolated HD-[HD-GYP] catalyzes complete conversion of c-di-GMP into

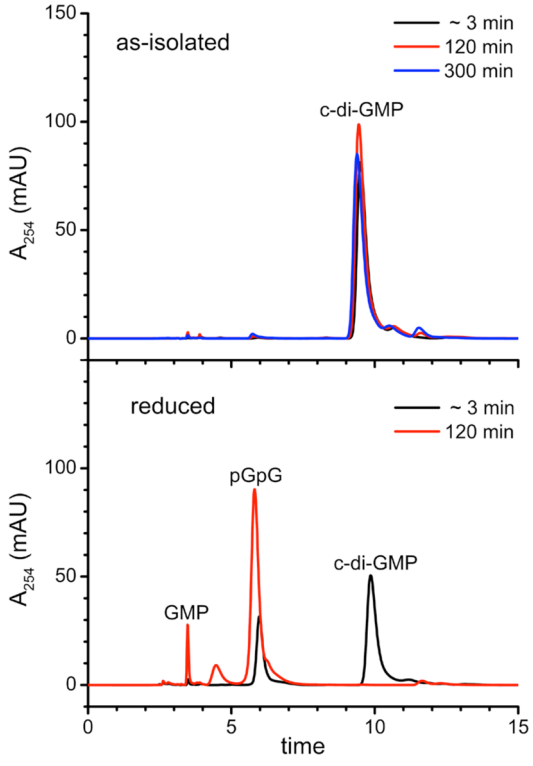

Figure 2. HPLC traces for c-di-GMP PDE activity of HD-[HD-GYP] in the as-isolated and reduced forms.

pGpG and a very minor portion of GMP. Mn^{2+} (10 mM) added to the assay mixture had no significant effect on activity of the reduced enzyme (Figure S3 in the Supporting Information). The reduced HD-[HD-GYP] showed very low pGpG PDE activity and no detectable c-GMP PDE activity (Figure S4 in the Supporting Information). The putative active site D-to-A variant, HD-[HA-GYP], was reproducibly isolated with ∼2 mol Fe/mol protein monomer and had no significant c-di-GMP PDE activity either as-isolated or reduced (Figure S5 in the Supporting Information). The variant containing the D-to-A substitution in the non-GYP HD motif, HA-[HD-GYP], contained ∼3 mol of Fe/mol of protein monomer, and this reduced protein showed c-di-GMP PDE activity indistinguishable from that of the wild type protein (Figure S5 in the Supporting Information).

The SWISS-MODEL web server (http://swissmodel.expasy.org/) was used to generate a structural model from the amino acid sequence of HD-[HD-GYP]. The server modeled the HD and HD-GYP domains separately. Figure 3 shows the modeled

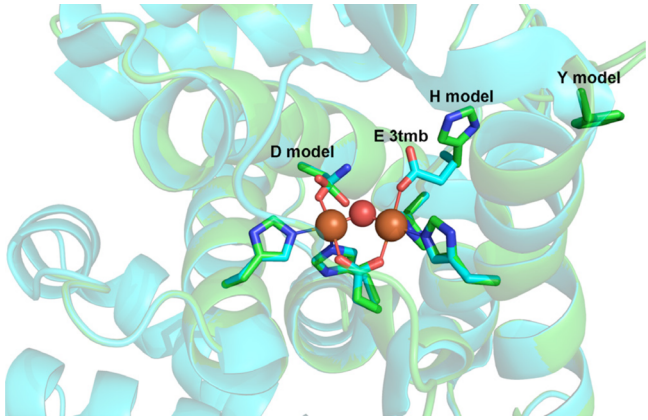

Figure 3. Model of the HD-GYP domain of HD-[HD-GYP] (green) superimposed onto the template structure, PDB entry 3tmb (cyan). Orange and red spheres represent irons and bridging solvent oxygen, respectively, in 3tmb. The modeled iron ligand residues are highlighted in the amino acid sequence in Figure S1 in the Supporting Information.

HD-GYP domain superimposed onto the server-chosen template structure, PDB entry 3tmb, which contains a solvent/Asp-bridged non-heme diiron site.[6] The model essentially superimposes residues homologous to the diiron-bridging Asp and the two terminal His ligands to each iron of the 3tmb template. The modeled HD-GYP domain diiron site structure is fully consistent with the UV−vis and EPR spectral features. The bridging aspartate ligand in the model corresponds to that substituted in the iron-deficient, inactive D-to-A variant, HD-[HA-GYP]. The 3tmb protein was referred to as an "unconventional HD-GYP protein", because it lacks the Y of the GYP motif and was reported to have no c-di-GMP PDE activity.[6] The Y of the GYP domain in the model ("Y model" in Figure 3) is not within metal-ligating distance. The HD-GYP domain model predicts an Asp ("D model" in Figure 3) in place of a terminal Asn ligand in 3tmb. A more notable difference is that a terminal Glu ligand in 3tmb ("E 3tmb" in Figure 3) is absent in the modeled HD-GYP domain. The model instead superimposes the main chain atoms of a His residue ("H model" in Figure 3) onto those of E 3tmb. This His residue is highly conserved in HD-GYP proteins.[1] We found that a simple rotation around the beta carbon can superimpose the NE2 of H model onto the ligating OE2 atom of E 3tmb. These diiron coordination differences could explain the lack of c-di-GMP PDE activity reported for the 3tmb protein. An alternative explanation is that the activity assay may not have been conducted on the reduced 3tmb protein.

A structural model of the N-terminal (non-GYP) HD domain of HD-[HD-GYP] was generated using as template PDB entry 2ogi, which is annotated as an HD-domain phosphohydrolase and contains a diiron site, but is otherwise uncharacterized. The modeled HD domain diiron site (Figure

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S6 in the Supporting Information) is structurally very similar to that of the HD-GYP domain model, and the modeled bridging Asp ligand corresponds to that substituted in the active HA-[HD-GYP] variant. This structural similarity explains why the protein is isolated with ∼4 iron atoms and why the spectra do not distinguish two different diiron sites. Despite this similarity the individual HD motif D-to-A variants show that the c-di-GMP PDE activity resides in the HD-GYP domain. This observation is consistent with the phenotypes associated with overexpression of VCA0681 and its D-to-A variant in *V. cholerae*$_{ET}$.[4] Our observation that the reduced but not oxidized HD-[HD-GYP] diiron sites are active provides potential mechanisms for both direct and allosteric redox regulation of c-di-GMP PDE activity in at least some members of the HD-GYP class. We have previously demonstrated allosteric redox regulation by the hemerythrin-like non-heme diiron domain in a *V. cholerae*$_{ET}$ diguanylate cyclase, which catalyzes the biosynthesis of c-di-GMP.[7] This redox regulation of both biosynthesis and degradation of c-di-GMP could constitute an adaptive response to the variable environments in the *V. cholerae*$_{ET}$ life cycle.[4]

■ ASSOCIATED CONTENT

Supporting Information

Experimental procedures, amino acid sequence, EPR spectra, HPLC traces, HD domain model, Figures S1−S6. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS USED

c-di-GMP, cyclic di-(3′,5′)-guanosine monophosphate; PDE, phosphodiesterase; pGpG, 5′-phosphoguanylyl-(3′→5′)- guanosine; *V. cholerae*$_{ET}$, *Vibrio cholerae* O1 biovar El Tor str. N16961; HD-[HD-GYP], *V. cholerae*$_{ET}$ VCA0681 protein containing tandem HD and HD-GYP domains; MBP, maltose binding protein; MOPS, *N*-morpholinopropanesulfonic acid; HPLC, high performance liquid chromatography; c-GMP, cyclic (3′,5′)-guanosine monophosphate

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