Structural Basis of Functional Diversification of the HD-GYP Domain Revealed by the *Pseudomonas aeruginosa* PA4781 Protein, Which Displays an Unselective Bimetallic Binding Site

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ABSTRACT

The intracellular level of the bacterial secondary messenger cyclic di-3',5'-GMP (c-di-GMP) is determined by a balance between its biosynthesis and degradation, the latter achieved via dedicated phosphodiesterases (PDEs) bearing a characteristic EAL or HD-GYP domain. We here report the crystal structure of PA4781, one of the three *Pseudomonas aeruginosa* HD-GYP proteins, which we have previously characterized *in vitro*. The structure shows a bimetallic active site whose metal binding mode is different from those of both HD-GYP PDEs characterized so far. Purified PA4781 does not contain iron in the active site as for other HD-GYPs, and we show that it binds to a wide range of transition metals with similar affinities. Moreover, the structural features of PA4781 indicate that this is preferentially a pGpG binding protein, as we previously suggested. Our results point out that the structural features of HD-GYPs are more complex than predicted so far and identify the HD-GYP domain as a conserved scaffold which has evolved to preferentially interact with a partner GGDEF but which harbors different functions obtained through diversification of the active site.

IMPORTANCE

In bacteria, the capability to form biofilms, responsible for increased pathogenicity and antibiotic resistance, is almost universally stimulated by the second messenger cyclic di-GMP (c-di-GMP). To design successful strategies for targeting biofilm formation, a detailed characterization of the enzymes involved in c-di-GMP metabolism is crucial. We solved the structure of the HD-GYP domain of PA4781 from *Pseudomonas aeruginosa*, involved in c-di-GMP degradation. This is the third structure of this class of phosphodiesterases to be solved, and with respect to its homologues, it shows significant differences both in the nature and in the binding mode of the coordinated metals, indicating that HD-GYP proteins are able to fine-tune their function, thereby increasing the chances of the microorganism to adapt to different environmental needs.

The dinucleotide cyclic di-GMP (c-di-GMP) serves as a core signal in the coordinated lifestyle switch of most bacteria in response to different environmental stimuli. In particular, high levels of c-di-GMP generally flag hostile conditions and result in reduced motility, attachment, and biofilm formation, while low levels of c-di-GMP indicate that it is time to go back to virulence and cell division (1–5). The intracellular levels of c-di-GMP are fine-tuned by the opposite action of dedicated enzymes: diguanylate cyclases (DGCs) and phosphodiesterases (PDEs) (6). The first class, characterized by a GGDEF domain, synthesize c-di-GMP from two GTP molecules, while the second one hydrolyzes it. PDEs are further divided into two unrelated superfamilies of hydrolases: the more abundant EAL proteins, which degrade c-di-GMP to the linear dinucleotide pGpG, and the HD-GYP proteins, which are able to hydrolyze c-di-GMP into two molecules of GMP. Bacteria display a wide variety of genes encoding these three enzymatic domains, which are often fused with additional regulatory modules. Furthermore, GGDEF and EAL domains can also be found within the same protein (the so-called hybrid proteins), indicating the high level of complexity of c-di-GMP metabolism. While GGDEF and EAL proteins have received considerable attention in the last decade, HD-GYP proteins, which were identified later (7, 8), have fallen behind for several years, and many issues regarding their role and function are still discussed. For example, it is not known whether HD-GYPs are functional dupli-

cates of EAL enzymes emerging from convergent evolution or whether they have a different role within more complex regulatory pathways. If this is the case, since HD-GYPs are not ubiquitous in bacteria, the presence of genes encoding these domains may well be considered a hallmark of bacterial species with more sophisticated biofilm-mediated survival strategies, such as *Pseudomonas aeruginosa* (9, 10) or *Vibrio cholerae* (11). Indeed, HD-GYPs have been shown to interact with GGDEF domains within multi-

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zyme complexes, while EAL domains seem to interact with GGDEF domains only in hybrid proteins (i.e., when they are fused within the same polypeptide chain) (12, 13). From a structural point of view, HD-GYPs are metal binding proteins named after their conserved motifs, but while the HD residues clearly serve as metal ligands, the role of the GYP triad is not so clear. Indeed, it was shown that mutation of the GYP signature renders the protein RpfG unable to interact with its specific GGDEF partners (12), while leaving the catalytic activity unaffected (14). Moreover, it should be recalled that it is not excluded that degenerated HD-GYPs (i.e., mutated in the HD-GYP signature), though catalytically inactive, may still function as c-di-GMP or even pGpG receptors (15, 16). Recently, these puzzling issues benefited from the first two solved structures for this class of proteins. The first, Bd1817 (16), is a degenerated protein from *Bdellovibrio bacteriovorus*, while the second, PmGH, is an active enzyme from *Persephonella marina* (14). Both proteins were purified and crystallized as iron binding proteins; however, the two metal binding sites differ markedly, with Bd1817 showing a bi-iron center, while PmGH binds three iron ions with different oxidation states. Therefore, the authors of the latter structure suggested that the HD-GYP superfamily should be further divided in two subgroups, based on the presence of a bimetallic or a trimetallic center, and highlighted the need for further structural data to fully elucidate the features of this intriguing class of enzyme (14). We have recently presented a detailed biochemical characterization of two of the three HD-GYP proteins encoded by the genome of the human pathogen *Pseudomonas aeruginosa* (15), namely, PA4781 and PA4108, which were shown to affect c-di-GMP levels *in vivo* (the third, PA2572, displays a degenerated YN-GYP signature) (17). Here we move a step forward and report the crystal structure of PA4781, coupling a structural study to its previous *in vitro* characterization (15). Strikingly, the structure shows a bimetallic active site whose metal binding mode is different from those of both Bd1817 and PmGH. Purified PA4781 did not contain iron in the active site as for previously reported HD-GYPs (14, 16, 18), and we show that it binds to a wide range of transition metals with similar affinities. Moreover, the structural features of PA4781 are in agreement with our previous data, indicating that this is preferentially a pGpG binding protein (15).

MATERIALS AND METHODS

Protein expression and purification. PA4781 full-length protein and two N-terminal deletion mutants (namely, PA4781-G112start and PA4781-D174start, lacking the REC domain and the REC domain plus the S-helix, respectively) were expressed with an N-terminal histidine tag and purified as described in reference 15. After the affinity chromatography step (performed on an Ni²⁺-loaded high-performance [HP] chelating column [GE Healthcare]), the N-terminal histidine tag was removed by proteolysis with thrombin (Sigma) (3 to 5 U/mg of fusion protein). The digestion mix was then loaded on an HP chelating column (GE Healthcare); thrombin was eluted in the flowthrough fraction, while the proteins were eluted at 100 mM imidazole. An additional step of size exclusion chromatography (SEC) was introduced to purify the dimeric fraction of the proteins to homogeneity. Both full-length PA4781 and PA4781-G112start were eluted as dimers, while PA4781-D174start was monomeric in solution. The protein was concentrated and used for crystallization trials. However, both the truncated mutants showed a high propensity for aggregation and precipitation at protein concentrations higher than 1 mg/ml. This drawback could not be avoided by changing ionic strength, pH, detergents, or additives of the protein buffer, and therefore, for these mutants only low concentrations could be explored during crystallization trials.

TABLE 1 Data collection and refinement statistics for PA4781_{HD-GYP}

Parameter	Value for PA4781 _{HD-GYP} ^a
Data collection statistics	
Beamline	BESSY ID-14.1
Detector	CCD MarMosaic 225
Space group	P4 ₁
Cell dimensions	
<i>a</i> = <i>b</i> , <i>c</i> (Å)	93.98, 78.47
Resolution range (Å)	46.9–2.20 (2.27–2.20)
CC(1/2) (%) ^b	99.6 (68.5)
<i>I</i> /σ(<i>I</i>)	9.9 (2.4)
Completeness (%)	100 (100)
Reflections	
Total no. observed	531,410 (46,360)
Multiplicity	15.3 (15.3)
Wilson plot <i>B</i> value	27.0
Refinement statistics	
Resolution range (Å)	46.99–2.20
No. of unique reflections used	31,313
<i>R</i> _{work} / <i>R</i> _{free} ^c	18.3/21.7 (29.9/29.6)
Mean <i>B</i> -factor (Å ²) (no. of atoms)	
Protein	
Chain A	34.7 (1,695)
Chain B	37.2 (1,690)
Ni ions	21.7 (4)
Water molecules	35.1 (102)
RMSD	
Bond length (Å)	0.019
Bond angle (°)	1.816
Ramachandran (<i>n</i>, %)	
Favored	424, 98.4
Allowed	7, 1.6
MolProbity ^d (score, percentile)	1.45, 99

^a Values in parentheses refer to the highest-resolution shell.

^b Percentage of correlation between intensities from random half-data sets.

^c *R* values, defined as (Σ|*F*_o − |*F*_c||)/Σ|*F*_o|, describe the difference between the experimental observations and the calculated values.

^d The MolProbity score combines the clashscore, rotamer, and Ramachandran evaluations into a single score, normalized to be on the same scale as X-ray resolution.

Crystallization and data collection. PA4781 full-length protein was extensively tested for crystallization. The best hit, square base bipyramidal single crystals, was obtained from the PGA screen (Molecular Dimensions) using the Phoenix crystallization robot (Art Robbins) and could be reproduced only once. Crystals appeared after 3 months in a droplet formed by mixing equal volumes of an 80 μM fresh (i.e., never frozen) protein solution (in 20 mM Tris [pH 7.6], 20 mM NaCl, 5 mM MgCl₂) with the reservoir solution purchased from Molecular Dimensions (0.1 M cacodylate [pH 6.5], 0.2 M KSCN, 0.2 M KBr, 3% polyethylene glycol 20,000 [PEG 20K], 3% poly-γ-glutamic acid polymer [PGA]). Crystals were cryoprotected using 20% glycerol. The best single crystal diffracted up to a 2.2-Å resolution. Diffraction data have been collected on BL14.1 operated by the Helmholtz-Zentrum Berlin (HZB) at the BESSY II electron storage ring (Berlin-Adlershof, Germany) (19). Data were processed using XDS (20, 21) and Pointless and Aimless (22). Crystals are tetragonal with space group P4₁ and the following unit cell dimensions: *a* = *b* = 93.98 Å; *c* = 78.47 Å. Final statistics are reported in Table 1.

Structure solution. Data were phased by molecular replacement using the monomeric PmGH (PDB code 4MCW [14]) as a search model in MOLREP (23). Cycles of model building and refinement were carried out using Coot (24) and Refmac5 (25). The final model consists of two molecules (one dimer) per asymmetric unit and contains only the HD-GYP

domain of PA4781. No electron density was observed for the first 150 N-terminal residues. However, crystal packing of these P4₁ crystals leaves no space to fit the lacking REC domain. Final statistics are reported in Table 1.

XRF. X-ray fluorescence (XRF) spectra were collected at Elettra Synchrotron (Trieste, Italy) on an XRD1 beamline. Several nondiffracting crystals were washed in their mother liquor and fished in the same loop before flash freezing in liquid nitrogen. The crystals were then exposed to an 11.3-keV (1.1-Å) X-ray beam. Fluorescence emission was recorded from 3.0 to 12.0 keV. As a control, the same spectra were recorded also for the pin alone and for the pin plus mother liquor.

ICP-MS. Inductively coupled plasma mass spectrometry (ICP-MS) analyses were performed on a Bruker (Bremen, Germany) 820-MS ICP-MS equipped with a MicroMist (0.4 ml/min) glass nebulizer. The operating conditions were as follows: plasma flow, 18.0 liters/min; auxiliary flow, 1.80 liters/min; sheath gas, 0.18 liter/min; nebulizer flow, 1.00 liter/min; radio frequency (RF) power, 1.40 kW; pump rate, 4 rpm; and stabilization delay, 30 s. Samples for ICP-MS were prepared by denaturation of an 82 μ M protein solution (in 150 mM NaCl, 20 mM Tris-HCl [pH 7.8]) with HCl and trichloroacetic acid (TCA), followed by addition of EDTA. The samples were then heated at 80°C for 30 min, and the precipitated protein was removed by centrifugation. As a control, double-distilled water and the protein buffer were subjected to the same protocol. See the supplemental material for details on chemicals and method of analysis.

Preparation of apo proteins. Semiapo (in which one site is metal free) and fully apo (in which both sites are metal free) proteins were prepared by incubation in a solution composed of 50 mM EDTA, 150 mM NaCl, and 20 mM Tris-HCl (pH 6) for 30 min at 37°C or 24 h at 25°C, respectively. After this step, the sample was centrifuged at 13,000 rpm for 15 min, and EDTA was removed by gel filtration on a fast-performance liquid chromatography (FPLC) column (Superdex 75 10/300; GE Healthcare) in 150 mM NaCl and 20 mM Tris-HCl (pH 7.2). The extinction coefficient of both semiapo and fully apo, as calculated by means of the bicinchoninic acid (BCA; Sigma-Aldrich) assay, was the same as for the holoprotein reported in reference 15, i.e., 0.59 (mg/ml)⁻¹ cm⁻¹.

ITC. Isothermal titration calorimetry (ITC) experiments were carried out using an iTC200 microcalorimeter (MicroCal). Aliquots (1.5 μ l) of metal solution (0.1 mM) were injected into a 5 μ M PA4781 (either semiapo or fully apo) solution at 25°C. Data were fitted using the “one-binding-site model” or “sequential binding mode” of the MicroCal version of ORIGIN. The heat of binding (ΔH), the stoichiometry (n), and the dissociation constant (K_D) were then calculated from plots of the heat evolved per mole of ligand injected versus the molar ratio of ligand to protein using the software provided by the vendor. Eventual metal-induced unfolding effects were ruled out by circular dichroism (CD) spectrum analysis (see Fig. S1 in the supplemental material).

Multiple-sequence alignment and phylogenetic analysis. PSI-BLAST was employed to find sequences closely related to PA4781 from *Pseudomonas aeruginosa*, which was used as a probe (26). Fifty-eight nonredundant (identity threshold < 95%) sequences were retrieved from the NCBI (27). A multiple-sequence alignment was obtained by means of the ClustalW tool (28). The alignment was then used for the secondary-structure prediction (PhD server [29]) and for detection of evolutionarily conserved residues (30). Evolutionary analysis was conducted with MEGA5 (31). To this end, the maximum likelihood method based on the model of Whelan and Goldman was used (32). The initial tree for the heuristic search was obtained by applying the neighbor-joining method to a matrix of pairwise distances estimated using a JTT model.

Molecular docking. Molecular docking was carried out by means of Molegro Virtual Docker (MVD) software (CLCbio). Ligands were built and energy was minimized by using the PRODRG server (33). Flexible torsions were automatically detected by MVD and manually checked for consistency. The three-dimensional structure of PA4781 was prepared by automatically assigning bond orders and hybridization, and by adding

explicit hydrogens, charges, and Tripos atom types. Crystallographic water molecules were ignored and excluded from docking computations. A search space with a 15.0-Å radius, centered on the metal ligand binding site, was used for docking. A grid-based MolDock score with a grid resolution of 0.30 Å was used as scoring function for docking. MolDock SE was used as docking algorithm. For each ligand, 10 runs were defined. Similar poses (root mean square deviation [RMSD] < 1.2 Å) were clustered, and the best-scoring one was taken as representative. Other docking parameters were fixed at their default values. After docking, energy optimization of hydrogen bonds was performed.

Protein structure accession number. The coordinates and structure factors determined in this study have been deposited in the Protein Data Bank with accession number 4R8Z.

RESULTS AND DISCUSSION

PA4781 is an HD-GYP protein, which is activated by phosphorylation of its N-terminal regulatory domain (REC domain). A dimerization helix (sensing helix or S-helix) connects the REC domain to the C-terminal HD-GYP catalytic domain (Fig. 1A). The protein displays a partially degenerated signature, with the tyrosine of the GYP motif replaced by a phenylalanine (HD-GFP). We set up extensive crystallization trials of three different constructs of PA4781 (i.e., REC-helix-HD-GYP, helix-HD-GYP, and HD-GYP alone); crystals were detected only in a single plate of the full-length protein after 3 months. The structure was solved by molecular replacement using the PmGH monomer (PDB code 4MCW [14]) as search model. Inspection of the electron density maps and crystal packing revealed the presence of only the catalytic domain, and the model could be built unambiguously only starting from Arg-151 as the N-terminal residue. The protein underwent autolysis during the crystallization process, yielding crystals of the HD-GYP domain alone.

Crystal structure of PA4781 HD-GYP domain. The protein crystallized as a dimer, with each monomer displaying a bimetallic binding site (Fig. 1). The two HD-GYP domains displayed the expected all- α fold already observed for PmGH (14) and Bd1817 (16), with the first 5 helices forming a calix around the bimetallic binding site. The active-site cavity is flanked by the very long loop between α 4 and α 5 (loop 4/5), which comprises the GYP signature (GYP loop; GFP in the case of PA4781), while the HD signature is located in the α 2-helix (His-220 and Asp-221). Access to the active site is restricted by the presence of two loops, loop 2/3 (lid) and loop 5/6, making the whole domain look similar to a crab claw.

Bimetallic binding site and structural comparison with Bd1817 and PmGH. The coordination of the two metal ions is octahedral, with the axial directions almost parallel. The distance between the two metals is 5.4 Å. A total of six side chains are involved in coordination, belonging to five histidine residues and to the HD signature aspartate, with the latter coordinating both ions (Fig. 1C); the remaining five positions (two for M1 and three for M2) are occupied by water molecules. Three more aspartate residues and three water molecules contribute to an H-bond network that further stabilizes the metal center (Fig. 1C).

As shown in Fig. 2, the active site of PA4781 is different from those of both Bd1817 and PmGH. Although displaying two metal ions in the active site, the positions of the metals and the binding mode of PA4781 are very different from the bimetallic center observed in Bd1817 (Fig. 2A). The distance between the two metals in PA4781 is 2 Å longer than in Bd1817 (5.4 versus 3.4 Å): this increased distance does not allow a bridging water molecule to

FIG 1 Crystal structure of the PA4781 HD-GYP domain. (A) Domain organization of full-length PA4781 and of the cleaved fragment present in the crystals. (B) Cartoon representation of the dimer (side and top views). The S-helices are highlighted in different colors. (C) The bimetallic center. Shown is an electron density map ($2F_o - F_c$, contoured at 1.5 σ) of the crucial residues coordinating the two metal ions (in green). Water molecules belonging to the octahedral coordination (dark gray) are highlighted in red, while those contributing to the key H-bond network (light gray) have a lighter coloring. (D) Monomeric structure reporting the helix numbering and highlighting important regions such as the GYP loop and the lid. On the right side, a surface representation shows the active-site cavity.

coordinate both ions, as observed in Bd1817 (16). Moreover, in PA4781, the M2 has only three protein ligands, while in Bd1817, four residues and two water molecules coordinate each iron. The different coordination and position of M2 arises mainly from the position of helix 1, carrying the coordinating residues His-150, in the *Bdellovibrio* protein; this helix is shifted toward the active site, consequently reducing the distance between the metal ions and allowing Asn-265 to participate in M2 coordination as the fourth ligand. However, the shift of helix 1, and the resulting binding mode, is apparently peculiar to the degenerated fold of Bd1817 that displays a reduced volume of the metal binding cavity, with the lid region (loop 2/3) occluding the access to the active site. In contrast, given the high sequence similarity and the similar domain organization (including the dimerization S-helix), PA4781 and PmGH superpose very well (Fig. 2B). Compared to PmGH,

the active-site entrance of PA4781 is reduced by the more closed conformation of the two clamps (the lid loop and loop 5/6) (Fig. 2B), while the structural overlap of the very long GYP loops of the two proteins is striking and is discussed in detail below. The main difference between PA4781 and PmGH is at the level of the exposed loop connecting the S-helix with $\alpha 1$ (loop S/1) (Fig. 2B); the very different conformation of this loop in the two proteins also accounts for the active-site diversity. After the *Persephonella* structure was solved, the authors classified the HD-GYP family into two evolutionarily distinct groups based on the sequence conservation of loop S/1 (14). The first subfamily, characterized by the signature motif E(D)TG in this region, is supposed to display a trinuclear metal center, since a crucial carboxylate ligand (Glu-185), bridging two metal sites of the trimetallic center in PmGH, is conserved or replaced with an aspartate. In the second

FIG 2 Superposition of the PA4781 HD-GYP domain with known structures. (A) Superposition of PA4781 (sand) with Bd1817 (PDB code 3TM8 [16]) (blue). The bottom portion shows the metal binding sites of the two proteins. Protein ligand side chains are shown as sticks; the two metals of PA4781 are represented as yellow spheres, while the two iron ions of Bd1817 are colored in dark blue. All the nonprotein ligands were omitted for clarity. (B) Superposition of PA4781 (same coloring as in panel A) with PmGH (PDB code 4MCW [14]) (purple). The three iron ions of PmGH are colored in purple.

subgroup this carboxylate is replaced by an aromatic residue (Tyr or Phe), and therefore, the HD-GYPs clustering in this subfamily are expected to harbor a bimetallic center with seven ligands as in Bd1817 (14). Indeed, due to the sequence similarity to PmGH, PA4781 clustered in the first group of trimetallic proteins (14), although it displayed a nonconsensus motif in loop S/1 [PRS instead of E(D)TG (see Fig. S2 in the supplemental material)]. Our data indicate that in PA4781 the conformation of this loop is completely different from that in PmGH and does not contribute any important residue to the active site (Fig. 2B), which shows six ligands instead of eight and a bimetallic center, thus confirming that the conformation of loop S/1 is important to shape the metal binding network, as suggested in reference 14. A multiple-sequence alignment and a new phylogenetic analysis of sequences closely related to PA4781 highlighted other structural features, which may be used to further cluster these proteins (Fig. 3; see also Fig. S3 in the supplemental material). However, since it is very difficult to predict the conformation of loop regions, and a single loop (loop S/1) appears to affect dramatically the structure and the binding mode of the metal center, these data confirm that more crystallographic data will be required for a full structural characterization of the HD-GYP family. Additionally, and surprisingly, PA4781 was shown to be an unselective transition metal binding protein.

On the nature of the metals. Recombinant PA4781 was expressed in *Escherichia coli* and purified by immobilized metal ion affinity chromatography (IMAC) as Bd1817 and PmGH, which

were both purified with iron bound in the active site (although if expressed in the presence of divalent metal ions, PmGH incorporated these metals at the peripheral binding sites, while the middle position was always occupied by iron). However, purified PA4781 did not bind iron, as determined by tripyridyl-S-triazine (TPTZ) colorimetric assay (34) (data not shown). Therefore, the nature of the bound metal was determined by X-ray fluorescence (XRF) spectra on nondiffracting crystals and by inductively coupled plasma mass spectrometry (ICP-MS) on the purified protein, and the metal was shown to be nickel (see Fig. S4 in the supplemental material). Since PA4781 was purified by IMAC on a nickel-loaded resin (chelating Sepharose HP), this result was highly suspicious and seemed biased by the purification protocol. Therefore, we performed a new purification using zinc instead of nickel in the IMAC step. Interestingly, ICP-MS analysis of this purified protein showed that PA4781 incorporated zinc during purification (data not shown). Prompted by the evidence that PA4781 is apparently able to exchange the active-site metals, we investigated in more detail the interaction of PA4781 with biologically active transition metals *in vitro*, in order to assess whether there was a specific metal showing higher binding affinity. To analyze the specificity and the thermodynamics of the interaction, binding of metals listed in Table 2 to PA4781 was directly measured using isothermal titration calorimetry (ITC). First, we removed the metal content of “as purified” PA4781 (i.e., two Ni^{2+} ions) by incubation of the protein with EDTA. Interestingly, it was possible to modulate the metals’ occupancy in the active site by means of different incuba-

FIG 3 Phylogenetic tree of 58 HD-GYPs closely related to PA4781. Evolutionary analysis for 58 closely related sequences indicates that PA4781 clusters with 25 HD-GYP proteins, which display two insertions, corresponding to loop 1/2 and loop 3/4 (proteins colored in gray). Within this group, PA4781 belongs to a small subgroup of proteins displaying a nonconsensus motif in loop S/1 (proteins in magenta). PA4781 and PmGH are boxed for clarity. For multiple-sequence alignment, see Fig. S3 in the supplemental material.

tion times with EDTA, 0.5 h or 20 h, which populated two different species of PA4781: a semiapo form (in which one site is metal free) and a fully apo form (in which both sites are metal free). These results indicate that the two binding sites have different affinities for the metal ions; therefore, here we refer to the metal binding sites of PA4781 as high- and low-affinity sites (H-site and L-site).

ITC experiments were then run on both species in order to discriminate the reactivity of each site. The titration profile of both semiapo and fully apo is depicted in Fig. 4. The dissociation constant (K_D) and all the additional thermodynamic parameters, obtained by fitting the enthalpy curve, are reported in Table 2 and in Fig. S5 in the supplemental material, respectively.

As expected for specific binding, integration of the titration

TABLE 2 Metal affinity (K_D) and stoichiometry of binding to semiapo or fully apo PA4781 obtained by ITC^a

Metal	Fully apo		Semiapo	
	n	K_D (μ M)	n	K_D (μ M)
Ni ²⁺	2	0.04–0.43	1.02	0.39
Co ²⁺	2	0.01–0.70	1.01	0.11
Zn ²⁺	2	0.10–1.00	2	0.70–3.00 ^b
Mn ²⁺	2	0.01–0.64	1.20	0.50
Fe ²⁺	ND	ND	0.89	0.64
Cu ²⁺	ND	ND	0.82	0.75
Mg ²⁺	ND	ND	ND	ND
Fe ³⁺	ND	ND	ND	ND

^a In cases where the stoichiometry of binding (n) was 2, data were fitted with the sequential binding mode equation, imposing the binding stoichiometry; errors are within $\pm 20\%$ for titrations to fully apo and to semiapo for zinc and within 5% for the other titrations. ND, not detected.

^b These values were apparent, given that a displacement equilibrium of the metal bound to H-site was also taking place.

peaks on semiapo produced a sigmoidal enthalpy curve for each metal (Fig. 4, black symbols) and a binding stoichiometry of 1, with the exception of Mg²⁺ and Fe³⁺, which did not bind (data not shown), and of Zn²⁺, which diverged from this trend (see below). Under these experimental conditions, the L-site appears to be unselective, since all the metals tested showed comparable binding affinities ($\Delta G \approx 9$ kcal/mol); the positive entropic factor ($-\Delta S$) in the thermodynamic profile indicates that metal coordination to this site involves a conformational change (see Fig. S5).

The peculiar properties of binding of Zn²⁺ to PA4781 allowed us to use this metal as a probe of the reactivity of the two metal sites. Binding of Zn²⁺ to the semiapo protein showed two events (Fig. 4), contrary to expectations given that the semiapo PA4781 has, in principle, the sole L-site available. It is likely that Zn²⁺ binds to both the L-site and the H-site, implying Ni²⁺ displacement (Fig. 5A) from the latter site. Interestingly, in the holoprotein, in which both metal sites are occupied by nickel, zinc is able to displace only one metal site and not both, as could be expected on the basis of the results on the semiapo protein (Fig. 5A; see also Fig. S6 in the supplemental material). Altogether, our data suggest that accessibility of the second site to Zn²⁺ seems to be dependent on the occupancy of the other site. We can speculate that metal binding to the L-site locks the H-site geometry, possibly via the conformational change occurring upon binding to the L-site, in agreement with the thermodynamic data discussed above (Fig. 5A). This behavior suggests a cross talk between the two sites, also unveiled by analysis of metal binding to the fully apo protein (Fig. 4, white symbols), which could only be fitted with a sequential binding mode equation for two sites, indicating that metal binding to the H-site triggers the binding to the L-site. Among the metals assayed, only Ni²⁺, Zn²⁺, Co²⁺, and Mn²⁺ bind to fully apo PA4781; Cu²⁺ and Fe²⁺ do not. However, the cross talk between the two binding sites hampers the unequivocal determination of the thermodynamic parameters of metal binding to the sole H-site, whose metal affinity, under these experimental conditions is at least 1 order of magnitude higher than binding to the L-site (Table 2). Based on the experimental data presented above, we can propose a scheme of the cross talk between the two metal binding sites of PA4781, depicted in Fig. 5B.

Overall, these *in vitro* results indicate that the H-site is more

selective than the L-site, but not enough to display a preferential affinity for a specific metal. On the other hand, binding of metals to the H-site positively affects the L-site, which, in turn, is competent to bind a wide range of metal ions with submicromolar affinity. Nevertheless, a chaperone-mediated binding of a specific metal to PA4781 *in vivo* cannot be excluded (35). In our previous work, the enzymatic activity of recombinant PA4781 (“as purified”) was measured only in the presence of Mn²⁺ and Mg²⁺ and was shown to be very low (15). Therefore, we measured the catalytic activity of PA4781 after reconstitution of the fully apo protein with Ni²⁺, Zn²⁺, Co²⁺, or the sole Mn²⁺ in order to assess whether binding of other metals could significantly enhance the catalysis. We used both c-di-GMP and pGpG as substrates, but none of these metals was found to affect catalysis (see Fig. S7 in the supplemental material). Coupled with the binding data, these results suggest that in PA4781, the bimetallic center may play a structural more than a catalytic role. This hypothesis is supported by thermal denaturation experiments showing that binding of divalent metals ions to the fully apo protein shifts the apparent melting temperature from a minimum of 5°C (Co²⁺) to a maximum of 12°C (Ni²⁺, Zn²⁺, and Mn²⁺) (see Fig. S8). Since PA4781 expression was recently shown to be induced when cells are grown in Mg²⁺-limited medium (36), another intriguing hypothesis is that PA4781 may have evolved to be a metal sensor within the complex c-di-GMP-mediated biofilm regulation of *Pseudomonas aeruginosa*.

Substrate binding and allosteric regulation. We have previously suggested that the active site of PA4781 may be designed to specifically bind pGpG (the linear product of c-di-GMP hydrolysis), due to the presence of the nonconsensus Glu-314 instead of a conserved alanine, which would generate a steric clash with the distal phosphodiester group of c-di-GMP (15). The structure of PA4781 confirms that the bulky side chain of Glu-314 points into the active-site cleft, and superposition of c-di-GMP, as observed in complex with PmGH (PDB code 4MDZ [14]), shows that Glu-314 would actually clash with the ligand in such a *cis* conformation (Fig. 6A). Accordingly, *in silico* docking of c-di-GMP into the active site yielded the cyclic dinucleotide in a linear *trans* conformation, compatible with Glu-314 steric hindrance (Fig. 6A). However, in this *trans* conformation, both of the phosphate groups of c-di-GMP are too far from the two metal ions for the reaction to take place. Indeed, the PA4781-E314A mutant was shown to bind c-di-GMP with a 17-fold-higher affinity (15). In contrast, docking of the more flexible pGpG resulted in two possible binding modes, since pGpG is not a symmetric molecule and therefore can enter the active site in two possible orientations, as illustrated in Fig. 6B and C. In both cases one phosphate group is near enough to the bimetallic center to replace one water ligand of both metals, and in the illustration in Fig. 6B, Lys-224 is also at H-bonding distance from the phosphate bridging oxygen (i.e., the hypothetical leaving group). Therefore, the active-site cleft of PA4781 seems to be designed to preferentially bind pGpG rather than c-di-GMP.

Moreover, since we previously shown that phosphorylation of the N-terminal REC domain triggers a conformational change of the HD-GYP domain, which renders the active site accessible (15), we could also speculate on the activation mechanism of HD-GYP proteins displaying a similar domain organization. Our working hypothesis is that phosphorylation of the N-terminal REC domain triggers a conformational change of the lid region, through the

FIG 4 Binding of selected metals to fully apo (□) and semiapo (■) PA4781. Analysis by ITC measurements was performed by titrating 5 μ M PA4781 with 100 μ M metal solution in 150 mM NaCl and 20 mM Tris-HCl (pH 7.2). Graphs show the integrated energy values normalized for injected protein. Binding isotherms were fitted using a single binding site model for the semiapo experiments (with the exception of the Zn^{2+} titration) and a sequential binding model for two sites for the other titrations. All measurements were done at least in duplicate.

torsion of the S-helices, ultimately resulting in an opening of the active site (see Fig. S9 in the supplemental material). The gating function of the lid loop may be a common feature of HD-GYPs sharing the same domain organization as PA4781 and PmGH (i.e., regulatory domain/S-helix/HD-GYP). This loop connects helices $\alpha 2$ and $\alpha 3$, and both of these helices are part of the dimeric interface interacting directly with the S-helices, whose role in response regulators is to allosterically transmit a conformational change from a regulatory (sensing) domain to a catalytic or DNA binding domain (37–40) (see Fig. S9). Together with a similar activation mechanism, PA4781 and PmGH also share an identical structure for the GYP loop.

The GYP loop. The GYP motif (GFP in PA4781) is located in

the very long loop connecting helices $\alpha 4$ and $\alpha 5$ and displaying three turns; in PA4781 it consists of 20 residues (from His-281 to Pro-300). Surprisingly, within the HD-GYP family, this is the region with the highest degree of conservation, indicating that this loop is likely involved in HD-GYP function(s). Dow and colleagues initially suggested extension of the GYP signature to HH EXXDGXGYP (41), including other conserved residues (e.g., the two initial M1-coordinating histidine residues), and after the structure of PmGH was solved, the signature was further refined to include an isoleucine (HHEXXDGGXGYPXXXXXXXXXI) (14). The GYP loop of PA4781 and of PmGH show an astonishing degree of structural conservation that was not observed before between PmGH and Bd1817 (Fig. 7). This likely correlates with

FIG 5 Proposed scheme of the cross talk between metal binding sites. (A) Titration of the semiapo protein (H-site occupied by nickel) with zinc results in occupancy of both the H- and L-sites, while when the holo protein is titrated, zinc is able to displace nickel from only one site (likely the L-site). This suggests that occupancy of the L-site locks the metal bound to the H-site. (B) Scheme for the sequential metal (M) binding proposed for the PA4781 protein. Occupancy of the H-site induces a conformational change that favors binding of the metal to the L-site (possibly by reorienting the common bidentate-ligand Asp-221), which, in turn, locks the H-site.

Bd1817 having a deletion in the GYP signature (G_P), while in PA4781 all key residues of this region are conserved. Such a structural invariance is unquestionably related to HD-GYP function(s). Ryan and colleagues have shown that for RpfG from *Xanthomonas campestris*, the GYP signature is primarily involved in protein-protein interactions (PPI) with specific GGDEF domains (12), and this hypothesis is supported by the mutational analysis performed on PmGH indicating that mutation of the GYP signature residues does not significantly affect catalysis (14). The structure of PA4781 sheds light on the conformation of the GYP loop, when conserved, allowing each signature residue to be precisely assigned to a specific role, as illustrated in Fig. 7. In particular, only those residues that determine the loop conformation are conserved; these include residues contributing H-bonds or hydrophobic contacts, together with those allowing peculiar peptide conformations like glycine or proline. The whole loop is constrained in its rigid body conformation by this conserved network of interactions, but it is anchored to the helical core of the HD-GYP domain by only two hydrophobic interactions: (i) on one side between the conserved Trp-285 and the backbone of loop 6/7 (accordingly, we suggest extension of the GYP loop signature to HHEXWDGXGYPXXXXXXXXXI, where the tryptophan residue is underlined) and (ii) on the other side by the tyrosine (Phe-290 in the case of PA4781) of the GYP motif, whose aromatic ring is inserted between the C β of His-282 and Gln-247 side chains like a piton, keeping the whole loop in position (Fig. 7). This interaction is also responsible for the kink of helix 3, which is forced to bend at the level of Gln-247 by the steric hindrance of Phe-290 side chain, ultimately affecting also the position of the lid region. In contrast, in Bd1817, where the tyrosine of the GYP motif is missing (G_P), helix 3 is not bent and the lid is closed, occluding the active-site entrance.

Conclusions. This is the third structure of an HD-GYP protein

FIG 6 Docking of c-di-GMP and pGpG in the active site of PA4781. (A) Superposition of c-di-GMP, as observed in complex with PmGH (in white; PDB code 4MDZ [14]) and docked (in blue) into the active site of PA4781. The side chain of Glu-314 (yellow) hampers optimal binding of the cyclic dinucleotide to assume a linear conformation characterized by docking of a guanine in a very different position with respect to the *cis*-conformer observed in PmGH. (B and C) Docking of pGpG (purple) into the active site of PA4781. The linear dinucleotide docked in two possible orientations with the two bases swapping their positions. Residues interacting with the ligand are shown in light green. In both cases, (i) the bridging phosphate interacts with the bimetallic center; (ii) one guanine makes a favorable π -cation interaction with Arg-311, while the other contacts residues belonging to the Lid region; and (iii) the conserved Lys-224, which is supposed to protonate the leaving group, is in close proximity with the bridging phosphate.

FIG 7 Structural features of the GYP loop. (Left) Structure superposition between the GYP loop regions of PA4781 (blue) and PmGH (purple), showing a complete conservation of the backbone conformation. (Center) Conserved H-bonds (green dashed lines) and hydrophobic network (cyan dashed lines) of the GYP loop region. Notice that the conserved residues of the motif (HHEXWDGXGYPXXXXXXI) are implicated in this network, suggesting that structural conservation is related with function. (Right) Detail of the hydrophobic stacking interaction of F-290 responsible for the kink of helix 3.

to be solved, and comparison with two homologues, especially with PmGH, while highlighting important structural determinants of the HD-GYP proteins, confirms that this fascinating class of PDEs still needs to be fully understood. PA4781 shows a bimetallic center that is different from those of both Bd1817 and PmGH. Superposition of the metal binding sites shows that the coordination and position of the first metal are conserved in the three proteins (four protein ligands in the same position), while diversification is obtained through the different ways by which the additional metal ion(s) is bound. These differences arise from the conformation of loop S/1, which may or may not provide additional metal-coordinating ligands and therefore constitutes a crucial structural determinant of the HD-GYP domain. However, the conformation of this loop depends on both the protein sequence and the nature of the N-terminal domain(s) of each protein. Furthermore, the unselective binuclear metal binding site of PA4781 indicates that the nature of the bound metal(s) is a further variable that has to be considered. In our opinion, this variability does not allow a straightforward structure/function prediction, based on sequence alignment or homology modeling techniques, for the members of the HD-GYP family. Contrary to the structural features of the GGDEF and EAL domains, which appear to be invariant, the three structures solved so far appear to have very different structural features correlating with diverse functions: Bd1817 is supposed to be a receptor, PmGH is an active enzyme, and for PA4781, the lack of appreciable catalytic activity in the presence of the various metals assayed strongly indicates that the primary role of PA4781 is not to hydrolyze nucleotides, as already predicted in our previous work (15). Therefore, the results presented here further underline that the structural portrait of this class of phosphodiesterases is far from complete. On the other hand, the astonishing sequence and structural conservation of the exposed GYP loop between PA4781 and PmGH strongly suggests that the HD-GYP domain is a conserved scaffold whose main role is to allow protein-protein interactions with partner GGDEF domains while achieving a different function(s) through diversification of the active-site cavity and the N-terminal regulatory domains.

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