Evidence on How a Conserved Glycine in the Hinge Region of HapR Regulates Its DNA Binding Ability

LESSONS FROM A NATURAL VARIANT

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HapR has been recognized as a quorum-sensing master regulator in Vibrio cholerae. Because it controls a plethora of disparate cellular events, the absence of a functional HapR affects the physiology of V. cholerae to a great extent. In the current study, we pursued an understanding of an observation of a natural protease-deficient non-O1, non-O139 variant V. cholerae strain V2. Intriguingly, a nonfunctional HapR (henceforth designated as HapRV2) harboring a substitution of glycine to aspartate at position 39 of the N-terminal hinge region has been identified. An in vitro gel shift assay clearly suggested the inability of HapRV2 to interact with various cognate promoters. Reinstatement of glycine at position 39 restores DNA binding ability of HapRV2 (HapRV2G), thereby rescuing the protease-negative phenotype of this strain. The elution profile of HapRV2 and HapRV2G proteins in size-exclusion chromatography and their circular dichroism spectra did not reflect any significant differences to explain the functional discrepancies between the two proteins. To gain insight into the structure-function relationship of these two proteins, we acquired small/wide angle x-ray scattering data from samples of the native and G39D mutant. Although Guinier analysis and indirect Fourier transformation of scattering indicated only a slight difference in the shape parameters, structure reconstruction using dummy amino acids concluded that although HapR adopts a “Y” shape similar to its crystal structure, the G39D mutation in hinge drastically altered the DNA binding domains by bringing them in close proximity. This altered spatial orientation of the helix-turn-helix domains in this natural variant provides the first structural evidence on the functional role of the hinge region in quorum sensing-related DNA-binding regulatory proteins of Vibrio spp.

Studies on the quorum-sensing signal network of Vibrio cholerae have produced a rich harvest of data where the periodic appearance and performance of two regulatory proteins, namely LuxO and HapR, determine the fate of a plethora of disparate cellular events (1). Of these, HapR has been given the status of a master regulator because it controls a wide range of diverse physiological activities, thus exerting a profound influence on the survival and pathogenic potential of this bacterium. Collectively, it represses biofilm development and the production of primary virulence factors (2) while it stimulates the production of HA/protease (3), promotes chitin-induced competence (4), increases resistance to protozoan grazing (5), enhances the survival against oxidative stress (6), and controls the expression of the gene encoding Hcp (7). In a recent effort, Zhu and co-workers have elegantly characterized additional novel direct targets of HapR and illustrated two distinct binding motifs (motif 1 and motif 2) in all target promoters (8). Because it modulates a multitude of diverse cellular parameters, the absence of a functional HapR affects the physiology of V. cholerae to a great extent. Being a master regulatory protein of a quorum-sensing circuit, a great deal of work has therefore been dedicated to understanding the various structural and functional aspects of HapR. Although previous analysis has identified certain residues contributing to the DNA binding activity of HapR (9), the role of residues in the hinge region has not been evaluated in this context. While unraveling the necessary cause of a protease-negative phenotype of a non-O1, non-O139 strain of V. cholerae, we discovered a variant HapR harboring a glycine to aspartate substitution in the hinge region. Herein, our structure-function results underscore the significance of a hinge region glycine moiety at position 39 in mediating HapR interaction with its cognate promoters.

MATERIALS AND METHODS

Bacterial Strains and Methods—The bacterial strains and plasmids used in this study are listed in supplemental Table 1. V. cholerae strains were derived from a non-O1, non-O139 strain V2, serogroup O37. Strains were maintained at −70 °C in Luria-Bertani (LB) medium containing 20% glycerol. Escherichia coli BL21 (DE3) (Novagen) was used for the overexpression of proteins. All strains were propagated at 37 °C in liquid with agitation or on solid (1.5% agar) in Luria broth unless mentioned otherwise. For the protease assay, V. cholerae strains were grown with aeration at 37 °C in tryptic soya broth without agar.
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dextrose (TSB-D). When appropriate, the growth medium was supplemented with ampicillin (100 µg ml⁻¹) or chloramphenicol (17 µg ml⁻¹). All antibiotics were purchased from Sigma-Aldrich and GE Healthcare. Media ingredients were purchased from Himedia and Difco. To disrupt the chromosomal copy of luxO, conjugation was conducted between recipient V. cholerae strain V2 and donor E. coli SM10: λ pir transformed with pSVM (supplemental Table 1). luxO mutants of V2 were screened by streaking onto thiosulphate-citrate-bile salt-sucrose plates containing ampicillin (100 µg ml⁻¹). Disruption was further confirmed by Southern hybridization. The resulting strain was designated as V2-SVM (supplemental Table 1). To disrupt hapR in V2, a similar strategy was adopted where conjugation was carried out between recipient V2 and donor E. coli SM10: λ pir harboring pCD (supplemental Table 1). hapR mutants of V2 were screened by streaking onto TCBS plates containing chloramphenicol (17 µg ml⁻¹). Disruption was further confirmed by Southern hybridization. The recombinant strain was named V2S-RV2G.

Protease Assay—Protease activity was measured using an azocasein assay as described earlier (10). Briefly, wild-type and recombinant derivatives of V. cholerae strain V2 (supplemental Table 1) were grown in TSB-D containing chloramphenicol (17 µg ml⁻¹) and ampicillin (100 µg ml⁻¹) accordingly, with agitation to stationary phase at 37 °C. 100 µl of stationary phase culture supernatant was incubated with 100 µl of azocasein (5 mg ml⁻¹ in 100 mM Tris, pH 8.0) for 1 h at 37 °C. The reaction was stopped by the addition of 400 µl of 10% trichloroacetic acid. After centrifugation, supernatant was transferred to 700 µl of 525 mM NaOH, and the A was determined at 442 nm. One azocasein unit was defined as the amount of enzyme producing an increase of 0.01 A unit/h.

Site-specific Mutagenesis—A D39G mutation in the hapRV2 ORF was generated on plasmid pSV2 (supplemental Table 1) using a Gene Tailor mutagenesis kit from Invitrogen according to the manufacturer’s guidelines. The primers are listed in supplemental Table 2. Positive clones were checked by sequencing. One such clone designated as pSV2G was further transformed into V2S, and the recombinant strain was designated as V2S-RV2G.

Protein Purification and Electrophoretic Gel Mobility Shift Assay with Promoter Regions of aphA, hapA, and vc0900—HapRV2 (Asp190) and HapRV2G (D39G) proteins were purified by Ni²⁺-nitrilotriacetic acid chromatography. The wild-type gene and aspartate variants of HapR were cloned into the NdeI-BamHI site of the pET15b vector (Novagen) to generate an N-terminal His₆-HapR fusion protein. All clones were confirmed by sequencing and transformed into E. coli BL21 (DE3). After induction with 0.4 mM isopropyl 1-thio-β-D-galactopyranoside, HapR proteins were purified through Qiagen Ni²⁺-nitrilotriacetic acid columns. All proteins were dialyzed overnight in a solution of buffer A containing 10 mM Tris, pH 7.9, 100 mM KCl, 0.1 mM EDTA, 0.1 mM DTT, 5% glycerol. Gel mobility shift assay was done essentially as described earlier (9). Briefly, three fragments of 399, 665, and 467 bp corresponding to promoter regions of aphA, hapA, and vc0900, respectively, were amplified with primer pairs as listed in supplemental Table 2. The fragments were gel-purified and end-labeled with [γ-³²P]dATP using T4 polynucleotide kinase (New England Biolabs). The binding reaction was carried out with 4 ng of labeled fragment in 10 mM Tris-HCl, pH 7.9, 1 mM EDTA, 1 mM DTT, 60 mM KCl, 10% glycerol, 5 µg of BSA, and 1 µg of poly(dI:dC) in a 20-µl reaction volume for 20 min at 26 °C. The reaction mixture was applied to a 5% polyacrylamide gel and subjected to electrophoresis in 1× Tris-acetate-EDTA, pH 8.5, at 4 °C. The gel was dried and autoradiographed to examine the shift of the band.

Circular Dichroism Measurement—HapRV2 and HapRV2G were examined by circular dichroism using a Jasco J-810 spectropolarimeter. Measurements in the far ultraviolet region (250–190 nm) were performed on protein solutions (0.2 mg/ml) employing a cell with path length of 0.1 cm at 25 °C. The mean residue ellipticity, [θ], was calculated using a mean residue molecular mass of each protein. Each spectrum reported is an average of 10 scans.

Molecular Weight Determination—HapRV2 and HapRV2G were dialyzed overnight in buffer A. All proteins were subjected to molecular sieve chromatography using a Bio-Sil SEC 125 analytical column (300 × 7.8 mm) (Bio-Rad) and “Biologic Duo flow” chromatography system (Bio-Rad). Elution volumes were determined by monitoring the absorbance at both 230 and 280 nm. All mutant proteins were eluted at the same volume as determined for wild-type HapR.

Source of Protein Samples for SAXS Experiments—The two proteins were purified to homogeneity from the AKTA explorer FPLC system using an S200 column and concentrated using Millipore membrane concentrators with 10 kDa. UV-visible absorption results suggested that the concentrations of proteins HapRV2 and HapRV2G were about 2.8 and 2.5 mg/ml, respectively. To estimate the beam intensity at zero angles, hen egg white lysozyme purchased from ACROS Organics (Morris Plains, NJ) was dissolved, dialyzed, and purified by gel filtration in 40 mM sodium acetate buffer, pH 3.8, containing 150 mM NaCl.

Synchrotron SAXS/WAXS Data Acquisition and Processing—The SAXS data were collected at beam line X9 at the National Synchrotron Light Source (Brookhaven National Laboratory). Two charge-coupled detectors simultaneously collected data at small (SAXS) and wider (WAXS) angles. The wavelength of the beam was 0.873 Å, and the ratio of the detector distance from the two charge-coupled detectors to the diameter of charge-coupled detector was 20.8. 45 images were collected under identical conditions/set-up. The images recorded on two charge-coupled detectors from protein solutions were circularly averaged, buffer subtracted, and scaled to obtain relative scattering intensity (I) as a function of momentum transfer vector, Q (Q = 4πsinθ/λ), where λ is the beam wavelength and θ is the scattering angle. The SAXS and WAXS intensity profiles were scaled and merged using the Q data between 0.12 and 0.2 Å⁻¹. All SAXS experiments were carried out in duplicate. No protein appeared to have suffered degradation during exposure to x-rays as characterized by the migration pattern in SDS-PAGE.

5 The abbreviations used are: TSB-D, tryptic soya broth without dextrose; PDB, Protein Data Bank; SAXS, small angle x-ray scattering; WAXS, wide angle x-ray scattering.
**SAXS Data Analysis**—Guinier approximation was employed to estimate the $R_G$ of the scattering particle. According to this approximation, for a monodisperse sample of globular protein, a plot of $\ln(I(Q))$ versus $Q^2$, where $Q \times R_G \leq 1.3$, should be linear and fits into the following equation,

$$\ln[I(Q)] = \ln[I_0] - (R_G^2/3) \times Q^2$$  \hspace{1cm} (Eq. 1)

where $I_0$ is defined as the intensity of scattering at zero angles, is directly proportional to the product of molar concentration and molecular mass of the scattering sample, and can be approximated by extrapolating SAXS data to $Q \sim 0$ (12). $R_G$ is defined as the root mean square of all elemental volumes from the center of mass of the particle, weighted by their scattering densities and is characteristic of the overall shape of the molecule. For this study, Guinier analysis was performed using the Primus software package (13). Using GNOM45 software (14), indirect Fourier transformation of the scattering data over the measured $Q$ range computed a pairwise distribution function of interatomic vectors, $P(r)$ (Equation 2).

$$P(r) = \frac{1}{2\pi} \int I(Q)Q \times r \sin(Q \times r) dQ$$  \hspace{1cm} (Eq. 2)

$P(r)$ is a histogram of the frequency of vector lengths connecting small volume elements within the entire volume of the scattering particle. During indirect Fourier transformation, $P(r)$...
was considered to be zero for vector lengths equal to 0 and $D_{\text{max}}$. The analysis also provided $R_g$ and $I_0$ from the second moment and the start of $P(r)$, respectively. Kratky analysis of the HapRV2 and HapRV2G molecules was carried out by interpreting the shape of the $I(Q)Q^2$ versus $Q$ plot.

Ab Initio Structure Restoration—Employing dummy residues and constraints provided within the SAXS profile, the three-dimensional shape of the two proteins was restored using the DAMMINIQ program (15). Ten models were generated without any predefined shape or symmetry bias. An average model that best represented all of the individual solutions was generated using the DAMAVER suite of programs (16). For HapRV2G, SUPCOMB software was used to superimpose the inertial axes of our SAXS data-based model and its known crystal structure (Protein Data Bank ID code 2PBX) in an automated manner (17).

DNA-HapR Docking Calculations—For DNA-protein docking, we used the GRAMM program freely available from Prof. Vasker’s website. We used parameters for high resolution docking with an increase in repulsion factor to 50. 100 low energy models were written out for each run. For interaction analysis, the model of the complex with lowest energy interacting with the helix-turn-helix binding region was selected (and presented). For DNA duplex, we used promoter regions of VC0900 (motif 1), VCA0865 (motif 2), and VC2647 (motif 2).

Graphical Analysis and Representations—Open Source PyMOL 0.99rc6 was used for graphical analysis and figure generation.

RESULTS AND DISCUSSION

Identification of a Nonfunctional HapRV2 That Is Unable to Stimulate Protease Production in V. cholerae—The aim of the present study was to investigate the molecular mechanism leading to the protease-negative phenotype of V. cholerae strain V2 (Fig. 1A). In the light of current knowledge, this phenotypic behavior could be explained in the following manners: (i) existence of a nonfunctional HapR (18), (ii) a problem in protease secretory machinery, or (iii) repression of hapR due to constitutively active LuxO (11). To begin with, a chromosomal copy of luxO was disrupted, and resultant strain V2-SVM (V2/H9004 luxO) was found to remain protease-negative, thus ruling out any possibility of a constitutively active LuxO-mediated suppression of protease production (Fig. 1A). As the protease-negative phenotype could also be due to mutations occurring in HapR (6), the correctness of gene encoding hapR was examined in strain V2. Together, sequence analysis and ClustalW alignment of HapRV2 (GenBank accession number DQ379712) identified a single point mutation (GAT to GAT) that converts a glycine 39 to aspartate (G39D) (supplemental Fig. 1). This glycine along with other residues is also conserved in other HapR homologs (Fig. 1B and supplemental Fig. 1). There are a total of nine α helices where the first three helices of each HapR monomer form the putative DNA binding domain. The HTH motif lies between helices α2 and α3. The remaining six α-helices (α4 to α9) are located in the large C-terminal domain. Interestingly, the conserved glycine-rich hinge region (G34IGRGG39) that links the α1 and α2 helices has been highlighted in the crystal structure resolved for HapR dimer (PDB ID code 2PBX) (Fig. 1C). We carried out rigid-body docking calculations to gain insight into the putative manner in which DNA duplexes belonging to motif 1 and motif 2 may prefer to interact (Fig. 1D and supplemental Fig. 3). Interestingly, in the low energy models of all complexes, the Phe55 of HapR was within the interaction zone to the oligonucleotides. Earlier, the role of this residue of HapR in its DNA binding ability has been proven experimentally (9). The docking results indicate that both motif 1 and motif 2 duplexes may prefer to interact grossly in the same manner, but differences do remain in the
angle and rotated position while interacting with HapR. The nucleotides that play the contact points with the HapR dimer, as seen in the low energy structural models, are depicted in supplemental Fig. 4. Possibly, this might be the reason for differential binding efficacy of the HapR to different motifs which eventually leads to a varied response level in eliciting function.

To determine its functionality, recombinant construct of hapRV2 (pSV2) was transformed into V2S (hapR disrupted strain of V2) to generate V2S-RV2 (supplemental Table 1). In addition, V2S was also transformed with empty vector (V2S-C) and hapRPL91 (V2S-RPL91) where latter served as a positive control. Protease activity was measured in the cell-free culture supernatants of V2S-C, V2S-RPL91, and V2S-RV2. In contrast to V2S-RPL91, the remaining strains turned out to be protease-negative (Fig. 1A), thus raising the possibility of V2 being a hapR mutant. These data also suggested that there is no defect in the protease secretory pathway in this strain. Unlike HapRV2, glycine 39 is conserved in HapRPL91 and other functional HapR (supplemental Fig. 1). To further confirm, glycine was restored at position 39 by site-directed mutagenesis, and the recombinant construct hapRV2G was able to rescue the protease-negative phenotype of strain V2.

Evaluate DNA Binding Ability of Variant HapRV2—HapR justifies its role as a master regulator by interacting with a range of cognate promoter sequences. To assess the DNA binding ability of HapRV2, a gel shift assay was employed with hapA, aphA, and vc0900 promoter regions. It should be noted that the promoter region of vc0900 contains a motif 1 binding site whereas the promoter regions of hapA and aphA contain a motif 2 binding site. Unlike its functional counterpart HapRV2G, HapRV2 fails to bind to any of these promoter regions, thus indicating a compromise in binding ability of HapRV2 (Fig. 2).
Molecular Weight and Secondary Structure Determination of HapRV2 and HapRV2G
—Being a prominent member of TetR family transcriptional regulators, it has been suggested that HapR acts as a dimer (9, 10). There remains a possibility that substitution of glycine with aspartate at position 39 might affect the dimer stability and/or its overall structure which eventually might be perturbing the function of this regulatory protein. To delve further into the structural aspects, we estimated the molecular weight of these proteins by size-exclusion chromatography (supplemental Fig. 2) and carried out circular dichroism analysis of HapRV2 and HapRV2G (Fig. 3). Interestingly, no significant difference was observed in the gel filtration elution profiles of the purified His-tagged HapRV2 and HapRV2G proteins and their corresponding circular dichroism spectra. Even, K2D analysis of the CD data (in the range of 250–190 nm) suggested that only a minor loss of α helical content (<4%) occurred as a result of the G39D mutation. Overall, this low resolution information helped in ruling out a grossly misfolded shape of the HapRV2 protein, but lacked in explaining the loss of DNA binding function.

Structure Reconstruction from SWAXS Data—To aid us in visualizing the structure-function role, the crystal structure of HapR has been resolved in an unliganded form, which illustrates a dimeric, two-domain molecule having an N-terminal DNA binding domain and C-terminal dimerization domain (PDB ID code 2PBX) (3). In both chains of dimer, the conserved G34IGRGG39 forms an unstructured link between the helix 1 and helix 2. Although the molecular docking based model of the HapR-DNA complex illustrates the significance of Phe55 in the DNA binding domain in mediating the interaction (9), it does not elucidate any role of the glycine hinge region in enabling the DNA binding ability of HapR. Analysis of the crystal structure revealed that to affect the turn structure in the hinge region, two of the four glycines (Gly34 and Gly36) occupied the D-side of the Ramachandran map with ϕ, ψ values 99, 148, and 68, −12, respectively. On the other hand, the residues Gly38 and Gly39 adopted backbone torsion angles −162, 146, and −136, 175, respectively. We could possibly reason that to affect the turn structure with some flexibility, nature used and retained the only coding residues capable of adopting conformation on the right side of Ramachandran map, but still the answer to the question that how the naturally occurring mutation in HapRV2 resulted in a protease-negative strain remained elusive.

To gain an insight into the global architecture of HapR as a function of mutation, small/wide angle x-ray scattering (SWAXS) experiments were carried out. The measured SWAXS intensity data from the samples of HapRV2 and HapRV2G and their Guinier region are presented in Fig. 4A. Presuming the globular nature of the scattering species, the slope of the linear fit of Guinier analysis over the Q range of
0.004–0.057 Å⁻¹ and 0.004–0.054 Å⁻¹ for HapRV₂ and HapRV₂G, provided an R₀ of 23.4 ± 0.2 and 24.3 ± 0.1 Å, respectively. Importantly, the ability to fit a linear equation to the low angle region confirmed a complete lack of aggregation in the samples. The P(r) analysis over a wider Q range (0.004–1.0 Å) provided a more complete estimation of the structural parameters specific to the predominant shape preferred by these proteins in solution (Fig. 4B). The P(r) calculated for functional (HapRV₂G) and its inactive variant (HapRV₂) showed a single peak profile with a Dₘₚₐₓ of 78 and 82 Å, and an R₀ of 23.8 ± 0.3 and 24.7 ± 0.6 Å, respectively. Indirect Fourier transformation suggested that the I₀ values for the samples of HapRV₂ and HapRV₂G were ~410 and 350 units, respectively. Based on an estimated I₀/c value of 10 units for lysozyme and considering a mass of 46 kDa (as both these proteins form a dimer stabilized by an intermolecular disulfide bond), we estimated the concentration of the HapRV₂ and HapRV₂G proteins to be ~2.6 and 2.3 mg/ml, respectively. The only differences lie in the proteins at higher Q or smaller dimensions in real space. To gain visual insight into how Gly to Asp mutation is reorienting the DNA binding domains, we modeled the global structure of HapR and its inactive mutant. An average of 10 individual solutions of the model restored for HapR confirmed that it adopts a “Y”-shaped globular structure which agrees well with the crystal structure solved for the same protein (PDB ID code 2PBX) (Fig. 4B). Superimposition of inertial axes of our model and crystal structure concluded that the two binding domains of HapR are in an open geometry suitable for binding to DNA. Surprisingly, comparison of a similar model calculated for HapRV₂ with the model generated for HapR brought forth a dramatic loss of the open architecture of DNA binding domains (Fig. 4B). Overlay of the core models computed for HapRV₂G and HapRV₂ clearly demonstrated how there is a large scale redistribution of scattering shape only on one end of the dimeric molecule, very likely the DNA-binding portion (Fig. 4C). Taking together our molecular and structural biology results, we present here first structural evidence on the critical contribution of a hinge glycine residue in regulating the DNA binding ability of HapR (Fig. 4D). Similarity in secondary structural content but difference in global structure suggests that the mutation-driven shape change is localized in the hinge region which may occur in the protein without significant change in its global energy.

Accumulated evidences underpin the significance of hinge region as a multifunctional domain of various DNA-binding proteins (19–21). In a recent effort, Hopfner and co-workers have elegantly resolved the architecture of a hinge domain of eukaryotic SMC (structural maintenance of chromosomes) proteins and showed how the hinge domain fold is conserved from prokaryotes to eukaryotes. Their data also illustrate the evolution of the hinge domain within eukaryotic SMC proteins to serve specific functions. The glycine hinge region of HapR is also conserved in other TetR family quorum sensing regulatory proteins (supplemental Fig. 2). It would be interesting to examine how the conserved residues in the glycine hinge region of HapR and HapR homologs help them to discern their target genes. Additional studies are planned to address this issue.

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