Differential Role of HAMP-like Linkers in Regulating the Functionality of the Group III Histidine Kinase DhNik1p*

Harsimran Kaur‡, Shikha Singh‡, Yogendra S. Rathore‡, Anupam Sharma‡, Kentaro Furukawa‡, Stefan Hohmann§, Ashish‡1,† and Alok K. Mondal‡2

From the °CSIR-Institute of Microbial Technology, Chandigarh 160036, India and the ‡Department of Chemistry and Molecular Biology, University of Gothenburg, Box 462, S-40530 Gothenburg, Sweden

Background: Detailed structural role of HAMP and HAMP-like linker domains in the functionality of Nik1 orthologs remains elusive.

Results: Shape-function readout of different mutants revealed functional distinction among HAMP-like linkers in DhNik1p.

Conclusion: H4b linker is critical to functioning of the protein.

Significance: First study providing structural insight in the functioning of the poly-HAMP module in Nik1 orthologs.

Nik1 orthologs are sensor kinases that function upstream of the high osmolarity glycerol/p38 MAPK pathway in fungi. They contain a poly-HAMP module at their N terminus, which plays a pivotal role in osmosensing as well as fungal death upon exposure to fludioxonil. DhNik1p is a typical member of this class that contains five HAMP domains and four HAMP-like linkers. We investigated the contribution of each of the HAMP-like linker regions to the functionality of DhNik1p and found that the HAMP4b linker was essential as its deletion resulted in the complete loss of activity. Replacement of this linker with flexible peptide sequences did not restore DhNik1p activity. Thus, the HAMP-like sequence and possibly structural features of this linker region are indispensable for the kinase activity of DhNik1p. To gain insight into the global shape of the poly-HAMP module in DhNik1p (HAMP1–5), multi-angle laser light and small angle x-ray scattering studies were carried out. Those data demonstrate that the maltose-binding protein-tagged HAMP1–5 protein exist as a dimer in solution with an elongated shape of maximum linear dimension ~365 Å. Placement of a sequence similarity based model of the HAMP1–5 protein inside experimental data-based models showed how two chains of HAMP1–5 are entwined on each other and the overall structure retained a periodicity. Normal mode analysis of the structural model is consistent with the H4b linker being a key to native-like collective motion in the protein. Overall, our shape-function studies reveal how different elements in the HAMP1–5 structure mediate its function.

Fungi contain a number of hybrid histidine kinases (HHK) that function as molecular sensors to regulate downstream signaling pathways (1, 2). Absence of sensor histidine kinase in higher animals make them an attractive therapeutic target for antifungal agents. Sln1p is the sole HHK in the yeast Saccharomyces cerevisiae and incidentally the first one discovered in fungi (3, 4). Sln1p is localized in the cell membrane and functions as an osmosensor to regulate the high osmolarity glycerol (HOG/p38) pathway (5). Besides Sln1p, group III HHK (Nik1 orthologs) have been shown to function as osmosensors in different species of fungi. Except for the organisms belonging to the “whole genome duplication” clade, group III HHK appears to be ubiquitous in fungi. In addition to their role as osmosensors in the stress-activated HOG/p38 pathway, they regulate morphogenesis, sporulation, and virulence factor expression in plant- and animal-pathogenic fungi (1, 2). Recent studies showed that the antifungal action of fludioxonil and iprodione is mediated through the group III HHK (6–8). The deletion of group III HHK renders fungal cells resistant to fludioxonil. Conversely, the heterologous expression of Nik1 orthologs in S. cerevisiae, which is naturally devoid of Nik1 orthologs, confers sensitivity to fludioxonil.

Structurally, Nik1 orthologs are quite distinct from Sln1p. They lack the membrane anchoring domain and mostly localize in the cytoplasm (9). They also have a unique N-terminal region consisting of multiple HAMP domain repeats. HAMP domains are widely distributed among proteins associated with signal transduction in prokaryotic and lower eukaryotic organisms, such histidine kinases, gadenyl cyclases, methyl accepting chemotaxis proteins and phosphatases (10). These domains are composed of ~50 amino acids with two amphipathic helices (11). HAMP domains have been established as signal transduction modules in prokaryotic sensor kinases, where single HAMP domains connect a membrane bound sensing (input) domain to the cytoplasmic output domain. Genetic and structural studies on bacterial transmembrane sensor kinases suggested stimulus induced conformational changes in the HAMP domain structure as the mode of signal transmission (12, 13). Nik1 orthologs have been identified from...
Functional Role of HAMP-like Linkers

Both pathogenic and nonpathogenic fungi. The number of HAMP domain repeats varies among different types of fungi. As per the SMART database, Nik1 orthologs from yeast contain five HAMP domains, whereas those from filamentous ascomycetes and basidiomycetes contain six and seven HAMP domains, respectively (14). Point mutations in the HAMP domains have been shown to affect fludioxonil sensitivity as well as osmosensing functions of Nik1 orthologs (8, 15, 16). The poly-HAMP module plays a very important role in the functionality of the group III HHK and appears to function both as sensor and regulator of its activity. However, the structural details of these phenotypes are not very clear.

We have previously characterized DhNik1p, a group III HHK from the halotolerant yeast Debaromyces hansenii. DhNik1p complements the snl1 deletion in S. cerevisiae and functions as a bona fide osmosensor (9). Like a typical group III HHK from yeast, DhNik1p contains five HAMP domains. Deletion analysis suggested that the HAMP domains are functionally distinct. Although the fourth domain is dispensable for histidine kinase activity of DhNik1p, it regulates the osmosensing function as well as the fludioxonil sensitivity mediated by the molecule (9, 17). In DhNik1p, the HAMP domains are interspersed with linker sequences. Recent studies suggested that these linker sequences also have HAMP-like features that are different from the canonical HAMP domains and therefore classified as divergent (18, 19). To understand the role of the linker regions in DhNik1p, we carried out functional analysis of different linker mutants. The results were correlated with a structural model of the dimeric form of the protein as it exists in solution.

MATERIALS AND METHODS

Yeast Strains and Plasmids—S. cerevisiae strains AMY1000 (MATa his3-1 leu2-0 met15-0 pSLN1::kanR::tetO7-TATA URA3::CMV-tTA ste11::hph) and NM1 (MATa snl1::LEU2 ste11::hph his3-200 leu2A1 ura3-3 52 trp1A63 with pRS-PTP2 URA3) (9) were used in this study. AMY1000 was constructed from S. cerevisiae strain R1158 (MATa his3-1 leu2-0 met15-0 URA3::CMV-tTA) (20) by inserting tetO7-TATA in the promoter region of the SLN1 gene and deleting the STE11 gene with a hygromycin-resistant gene cassette. S. cerevisiae strain YSH2472 (MATa leu2-3,112 trp1-1 his3-11,15 ade2-1 can1–100 snl1::LEU2 ssk1::kanMX ura3-1::YipSSRE-lacZ) was used as host for β-galactosidase reporter assay (17).

Construction of Plasmids Carrying Different Mutants of DhNik1—All mutant constructs were made by overlap extension PCR (21) using different primers, the sequences will be made available upon request. The mutant constructs E-AΔ1b, E-AΔ2b, E-AΔ3b, and E-AΔ4b carrying different single HAMP-like domain deletions was based on the boundaries predicted by SMART analysis. For E-AΔ1b, a construct designed to produce a DhNik1p mutant lacking the first HAMP-like domain (109–146 amino acid residues), two PCR were carried out utilizing primer pairs Dnik-NcoIF/E-A1b R and E-A1b F/Dnik1EcoRI_R. The PCR products were combined in third PCR using Dnik-NcoIF and Dnik1EcoRI_R primers. The resultant ~1.830-kb fragment was cloned in pDH Nik1 replacing the ~1.9-kb wild type sequence flanked by NcoI and EcoRI restriction sites. Similarly, E-AΔ2b (Δ200–238), E-AΔ3b (Δ292–330), and E-AΔ4b (Δ384–422), devoid of second, third, and fourth HAMP domains, respectively, were made. In another set of mutants, the deletions spanned the entire divergent HAMP domains to obtain F-LΔ1b (Δ106–149), F-LΔ2b (Δ197–241), F-LΔ3b (Δ289–333), and F-LΔ4b (Δ381–425).

To modify the length and residue composition of individual HAMP-like domains H1b, H2b, H3b, or H4b, they were replaced with an unrelated Gly-rich pentapeptide sequence -GGGGS to obtain L5Δ1b, L5Δ2b, L5Δ3b, and L5Δ4b mutants, respectively. Similarly, DhNik1p mutants wherein H1b, H2b, H3b, or H4b HAMP-like domain was replaced with three pentapeptide sequences in tandem-(GGGGS), were designated as L15Δ1b, L15Δ2b, L15Δ3b, and L15Δ4b, respectively. The mutants E-A41b, E-A42b, and E-A43b were made by replacing the H4b linker with H1b, H2b, and H3b, respectively. In all the mutant sets, each HAMP-like domain boundary marked by E-A residues was replaced.

To probe the role of the stutter elements in the activity of DhNik1p, mutants Δ1a, Δ2a, Δ3a, and Δ4a were constructed. In Δ1a, four amino acid residues (149LTNQ152) were deleted to remove stutter between H1b and H2 by overlap extension PCR strategy. For constructing Δ1a, two separate PCR were performed by utilizing the primer pairs, forward Dnik-NcoIF with the corresponding reverse mutagenic primer and the corresponding forward mutagenic primer with reverse Dnik1EcoRI_R to obtain the overlapping mutated products. These products were combined in third PCR using Dnik-NcoIF and Dnik1EcoRI_R primers. The amplified fragment was digested with NcoI and EcoRI and cloned into NcoI- and EcoRI-digested pDH Nik1. Similarly for Δ2a, Δ3a, and Δ4a, amino acid residues 241LTTQ244, 333LTNQ336, and 425LTSQ428, were deleted, respectively, using different primers.

Fludioxonil Sensitivity Assay—For fludioxonil sensitivity assay, freshly grown cultures (12 h at 28°C) were normalized to A600 1.0. Serial dilutions were made in sterile water, and 5 μl of a 10-fold dilution series were spotted onto agar plates supplemented with or without fludioxonil as indicated. Growth was monitored for 2–3 days at 30°C.

Western Blotting—Levels of dually phosphorylated Hog1p in S. cerevisiae strains AMY1000 expressing DhNik1p or its mutants were detected by Western blotting. Cells were grown in synthetic medium supplemented with appropriate amino acids (and doxycycline whenever required) until an A600 of 0.7–0.8 and either harvested as such or subjected to fludioxonil for 15 min before centrifugation. The resulting cell pellets were immediately frozen in liquid nitrogen. The total cell extract (20 μg of protein) from each sample was blotted onto nitrocellulose membrane and dually phosphorylated Hog1p was detected using an anti-dual phosphorylated p38 antibody (Cell Signaling Inc.) as described earlier (22). The level of Hog1p was detected in the same blot after re-probing with anti-Hog1p antibody (Y-215; Santa Cruz Biotechnology). Proteins were detected with the ECL kit (Amersham Biosciences).

β-Galactosidase Assay—S. cerevisiae strain YSH2472 expressing DhNik1p or its mutants were grown up to logarithmic phase in synthetic medium supplemented with appropriate amino acids at 28°C with or without 5 μg/ml of fludioxonil. For the β-galactosidase assay, 10 ml of each culture was recovered by centrifugation. Cells were washed once and resuspended in Z buffer (60 mM...
Na<sub>3</sub>HPO<sub>4</sub>, 40 mm Na<sub>2</sub>PO<sub>4</sub>·2H<sub>2</sub>O, 10 mm KCl, and 1 mm MgSO<sub>4</sub>·7H<sub>2</sub>O, pH 7.0). To permeabilize the cells, 1.0 ml of each culture (1.0 A<sub>600</sub>) was kept in liquid nitrogen for 1 min to then be heated at 37 °C for 3 min. This cycle was repeated three times. To start the reaction, 500 μl of Z buffer containing 37.4 mm β-mercaptoethanol and 200 μl of o-nitrophenyl-β-galactoside (4 mg/ml) were added to 500 μl of permeabilized cells. Samples were incubated at 30 °C until the appearance of a yellow color and the reaction was stopped by adding 500 μl of 1 m Na<sub>2</sub>CO<sub>3</sub>. The samples were centrifuged and absorbance (at 420 nm) was measured in the supernatants. β-Galactosidase activity was expressed as nanomoles of o-nitrophenyl-β-galactoside utilized per minute by 1 ml of culture, which was normalized to 1.0 A<sub>600</sub>. Values represent the mean ± S.D. of two independent pools of six transformants each.

Expression and Purification of Recombinant Proteins—The N-terminal region of DhNIK1p consisting of HAMP domains 1–3 (1–291 aa), HAMP domains 1–4 (1–383 aa), or HAMP domains 1–5 (1–480 aa) was expressed with an in-frame N-terminal maltose-binding protein (MBP) tag for the ease of protein purification. The different constructs (HAMP1–3 MBP, HAMP1–4 MBP, and HAMP1–5 MBP) or the vector pMAL-c2 was transformed into Escherichia coli strain ER2508 for overexpression and subsequent purification of MBP-tagged fusion proteins. Overnight cultures of these clones (at 37 °C in LB broth + 0.2% glucose supplemented with 100 μg/ml of ampicillin) were re-inoculated and grown to an A<sub>600</sub> of 0.5. Cells were then induced with 0.3 mm isopropyl 1-thio-β-d-galactopyranoside and further grown at 37 °C for 2 h. Cells were harvested and resuspended in lysis buffer (20 mm Tris, pH 7.4, 200 mm NaCl, 10% glycerol, 1 mm EDTA and protease inhibitor mixture, Roche Applied Science). After addition of lysozyme (0.1 mg/ml), the cells were disrupted by sonication. Recombinant MBP fusion protein from the supernatant fraction was then purified under native conditions, using amylose resin technology (Waters HPLC protein purification system, Waters Technology) coupled to a Waters HPLC protein purification system with a Shodex High Performance size exclusion column. Different HAMP constructs, namely HAMP1–3 MBP, HAMP1–4 MBP, and HAMP1–5 MBP at concentrations of 1, 0.786, and 0.697 mg/ml, respectively, were injected in a volume of 100 μl to the column equilibrated with buffer containing 20 mm Tris, pH 7.4, 0.2 mm NaCl, 10% glycerol, 1 mm EDTA, and 0.1 mm DTT at a flow rate of 0.5 ml/min. A BSA sample (5 mg/ml) was used as a reference to calibrate the system. The absolute molecular weights of individual peaks in the size exclusion chromatogram were determined using Astra software package version 6.0.3. A standard value of refractive index (dn/dc = 0.185 ml/g) was used for proteins.

Small Angle X-ray Scattering (SAXS) Data Collection—The SAXS data were collected at the X9 beam line (National Synchrotron Light Source, Brookhaven National Laboratory, Brookhaven, NY). 120 μl of each of the three different concentrations of MBP alone and HAMP1–5 MBP were used to collect SAXS data. Each sample and the match buffer were exposed to x-rays for 120 s in a quartz flow cell at 15 °C at a flow rate of 50 μl/min. To calibrate the beam intensity at zero angles, SAXS data sets were collected under identical conditions with five samples of hen egg lysozyme in 40 molarity NaOAc buffer, pH 3.8, containing 150 mm NaCl at varying predetermined concentrations using absorption at 280 nm (A<sub>280</sub>) = 2.5. Using the Python script-based programs written by Dr. Lin Yang (X9 Beam Line, National Synchrotron Light Source), the images recorded on the CCD were scaled and circularly averaged, and the buffer contribution was subtracted to obtain the scattering intensity (I) as a function of momentum transfer vector, Q (Q = (4πsinθ)/(λ)); where θ is the scattering angle and λ is the wavelength of the incident beam. All of the SAXS experiments described in this study were carried out in duplicate and the datasets were averaged prior to buffer subtraction and data analysis. Right after exposure to x-rays, the samples were collected in SDS-PAGE loading buffer and analyzed later for any degradation. Identical migration pattern between samples that stayed in the laboratory and the ones used for SAXS experiments supported no radiation-based damage.

SAXS Data Analyses—Kratky analysis (I(Q) × Q<sup>2</sup> versus Q plot) of the protein samples was carried out to assess the extent of inherent disorder in the scattering particles. Presuming globular nature of scattering species in solution, the Guinier approximation for globular particles was employed to estimate the radius of gyration (R<sub>G</sub>) of the scattering particles. According to this approximation, for a monodisperse sample of globular protein, a plot of ln(I(Q)) versus Q<sup>2</sup>, where Q × R<sub>G</sub> ≤ 1.3, should be linear and fit Equation 1.

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

Here I<sub>0</sub> is the intensity of scattering at zero angles and is directly proportional to the product of the molar concentration and the molecular mass of the scattering sample. It cannot be measured directly, but using Guinier approximation, it can be estimated by extrapolating the SAXS data to Q→0. Similarly the Guinier analysis for rod-like particles was employed to estimate the mean cross-sectional radius of the molecule, R<sub>C</sub>, given by Equation 2.

\[
\text{ln}(I(Q)) = \text{ln}(I_0) - (R_C^2/2) \times Q^2 
\]  
(Eq. 2)

Using estimated R<sub>G</sub> and R<sub>C</sub> values, the length of an ellipsoidal structure, L, can be estimated using the following relationship in Equation 3.

\[
L = \left[12(R_G^2 - R_C^2)^2\right]^{1/3} 
\]  
(Eq. 3)

In this study, the Guinier analyses were performed using the PRIMUS software package (23). Using the GNOM45 program, indirect Fourier transformation of the scattering data over the measured Q range computed a pairwise distribution function of inter-atomic vectors, P(r) (24), shown in Equation 4.

\[
P(r) = \left(\frac{1}{2\pi}\right) \int I(Q)Q \times r \sin(Q \times r) dQ 
\]  
(Eq. 4)

P(r) is essentially a histogram of the frequency of vector lengths connecting small volume elements within the entire volume.
Functional Role of HAMP-like Linkers

of the scattering particle. During indirect Fourier transformation, the $P(r)$ was considered as zero for vector lengths equal to 0 and the maximum linear dimension ($D_{\text{max}}$). The analyses also provided $R_p$ and $I_0$ values from the second moment and the start of $P(r)$, respectively. Programs available in the ATSAS package were used for SAXS data analysis in this study (25).

Ab initio Shape Reconstruction and Tertiary Structure Construction—By employing dummy residues and utilizing constraints provided within the experimentally measured SAXS $I(Q)$ profile, the three-dimensional shapes of the proteins were restored using the DAMMIF53 program (24). Ten models were generated without any predefined shape or symmetry bias and averaged using the DAMAVER suite of programs (26) to obtain a shape model that best represents the predominant solution shape of the protein.

Alongside, the sequence similarity based model of the full-length structure for HAMP1–5 was generated using the crystal structure of poly-HAMP domains from Pseudomonas aeruginosa soluble receptor Aer2 (PDB code 3LNR) (27). Modeller 9.10 program was used for the homology modeling and ModLoop was used for loop correction (28, 29). Gross dimensions of the models were compared with the SAXS data based information. The best fitting model was considered for further refinement. Of the 480 residues in HAMP1–5, only 415 residues could be modeled leaving 55 residues at the N-terminal and 10 residues at the C-terminal. Based on information from MALSS and SAXS, the dimeric HAMP1–5 model was generated, and the coordinates were energy minimized using the Newton program from the Tinker suite of programs to a root mean square deviation gradient cut-off of 0.1 kcal/mol/Å. To understand low frequency motions possible in the structural model of HAMP1–5, normal mode analysis was performed using the ELNemo server (30). Because normal mode provides directionality to the conformational change, we used a range of perturbation amplitudes to calculate the conformational changes. After finalizing the perturbation to 200 in symmetric manner, similar calculations were done for the mutants, and the same mode was considered for comparison of mutation rendered change in the collective motions.

Figure Generation and Graphical Representations—Open source software programs, including PyMOL and SPDB viewer were used for figure generation. Origin5.0 was used for data analysis and graphical representation.

RESULTS

Functional Analysis of DhNik1p Linker Deletion Mutants in S. cerevisiae—DhNik1p contains five canonical HAMP domains (H1, H2, H3, H4, and H5) that are interspersed with four HAMP-like linkers in DhNik1p (H1b, H2b, H3b, and H4b) (Fig. 1A). These linker regions are also highly conserved among Group III HHK. To determine their role in the functionality of DhNik1p, we constructed deletion mutation in each of them separately. Two sets of deletion mutants were made. The first set was comprised of the mutants E-AΔ1b (Δ109−146), E-AΔ2b (Δ200−238), E-AΔ3b (Δ292−330), and E-AΔ4b (Δ384−422) where the individual linker domain was deleted based on the boundaries predicted by SMART analysis. In the second set, the deletions spanned the entire divergent HAMP domains to obtain F-LΔ1b (Δ106−149), F-LΔ2b (Δ197−241), F-LΔ3b (Δ289−333), and F-LΔ4b (Δ381−425). To determine the effect of each deletion mutation for the functionality of DhNik1p, we tested the ability of these mutants to complement the shn1 mutation in S. cerevisiae. For this, we transformed both sets of deletion mutants into S. cerevisiae strain AMY1000 and checked the growth of the transformants on plates containing doxycycline. In strain AMY1000, the SLN1 gene is under control of the doxycycline repressible tetO7-promoter and therefore the addition of doxycycline blocks expression of SLN1. This kills yeast cells due to constitutive activation of the HOG pathway. Previously, we have shown that DhNik1p can complement the lethality caused by the SLN1 deletion in S. cerevisiae (9). Accordingly, the S. cerevisiae strain AMY1000 expressing DhNik1p grows in the presence of doxycycline. Mutants E-AΔ1b, E-AΔ2b, and E-AΔ3b could also support growth of the AMY1000 strain in the presence of doxycycline indicating that they were functional. However, cells expressing the mutant E-AΔ4b failed to grow on doxycycline plates showing that this construct was not functional (Fig. 1B). These mutants showed comparable levels of in vivo expression as checked by immunoblotting with HA-tagged constructs (data not shown). In the other set, mutants F-LΔ2b or F-LΔ3b enabled growth on doxycycline plates, whereas mutant F-LΔ4b did not mediate growth (Fig. 1B). Therefore, the deletions of HAMP-like linkers H2b, H3b, and H4b have similar functional consequences irrespective of the domain boundaries used for creating them. Interestingly, the mutant F-LΔ1b failed to grow on the doxycycline plate. Immunoblotting with the HA-tagged construct confirmed its in vivo expression (data not shown). Therefore unlike E-AΔ1b, F-LΔ1b is non-functional.

Substitution of HAMP-like Linkers H1b, H2b, and H3b with Flexible Linkers Does Not Abrogate DhNik1p Function—A recent study with a soluble receptor protein Aer2 of prokaryotic origin, which contains successive and interwoven HAMP domains also shows a parallel four-helix bundle architecture for divergent HAMP domains (27). Amino acid sequences of the HAMP-like linkers present in DhNik1p showed features of the divergent HAMP domain, which could be important for signal propagation (18). Therefore, we investigated whether conservation of the structural feature of the divergent HAMP domain was important for the activity of DhNik1p. For this, we replaced the HAMP-like linkers with unrelated Gly-rich peptide sequences that were known to be more flexible and having low helix-forming propensity. Mutants L5Δ1b, L5Δ2b, L5Δ3b, and L5Δ4b were constructed by replacing H1b, H2b, H3b, and H4b linkers individually with the pentapeptide sequence -GGGGS. In the case of L5Δ1b, L5Δ2b, L5Δ3b, and L5Δ4b, the linkers were replaced with three pentapeptide sequences in tandem -GGGGS. Boundaries of the linker regions in these mutants were similar to those in E-AΔ1b, E-AΔ2b, E-AΔ3b, and E-AΔ4b. The plasmids carrying these mutants were transformed into S. cerevisiae strain AMY1000. The growth patterns of the transformants were assessed on SD plates containing doxycycline (Fig. 1C). The strains carrying mutants L5Δ1b, L5Δ2b, and L5Δ3b grew well on doxycycline plates. A similar growth pattern was also observed for strains carrying mutants L15Δ1b, L15Δ2b, and L15Δ3b. The replacement of H1b,
H2b, or H3b with either GGGGS or (GGGGS)₃ in DhNik1p did not affect its ability to suppress the growth defect of sln1 mutation. However, exchanging the H4b linker with either -GGGGS or -(GGGGS)₃ sequences in DhNik1p led to non-functional kinase mutants as they failed to grow on doxycycline plates. These results indicate the importance of the HAMP-like sequence and structural feature for the linker H4b.

FIGURE 1. Functional analysis of HAMP-like linker mutants of DhNik1p. A, schematic representation of the amino-terminal domain structure of DhNik1. Each HAMP domain comprises of AS1 and AS2 helix joined by a flexible connector. Each HAMP-like domain comprise an HAMP-like AS1 and HAMP-like AS2 helix joined by a HAMP-like connector. Sequences covering the HAMP-like linker deletions spanning residues E-A are highlighted in red, whereas F-L deletions include additional residues that are underlined and highlighted in purple. B, growth pattern of S. cerevisiae strain AMY1000 transformed with DhNik1 or the indicated deletion mutants on minimal SD-agar plates without histidine and with or without 10 μg/ml of doxycycline (Dox) at 28 °C. Experiments were repeated three times with similar results. C, growth pattern of S. cerevisiae strain AMY1000 transformed with DhNik1 or mutants where the HAMP-like linker was replaced with amino acid sequence GGGGS (L5₁b, L5₂b, L5₃b, L5₄b) or (GGGGS)₃ (L15₁b, L15₂b, L15₃b, and L15₄b) on minimal SD-agar plates without histidine and with or without 10 μg/ml of doxycycline at 28 °C. Experiments were repeated three times with similar results.
Functional Role of HAMP-like Linkers

The HAMP-like Linker H4b Is Pivotal for DhNik1p Kinase Function—In S. cerevisiae, Sln1p regulates the HOG pathway negatively. Therefore, shutting off the expression of SLN1 in strain AMY1000 with doxycycline resulted in the activation of Hog1p (Fig. 2A). The detection of phosphorylated Hog1p in these cultures at the indicated time points was determined by immunoblotting. B, level of phosphorylated Hog1p in AMY1000 expressing DhNik1 incubated with doxycycline (Dox) for different times. C, S. cerevisiae strain AMY1000 carrying DhNik1 or different linker mutants. E-A-D1b, E-A-D2b, E-A-D3b, and E-A-D4b were grown on SD minimal media without histidine and supplemented with 10 μg/ml of doxycycline at 28 °C until A600nm = 0.8. Total protein extract from these cells was analyzed by Western blotting using anti-phospho-p38 antibody for phospho-Hog1 (P-Hog1) and anti-Hog1 antibody for total Hog1p. D, immunoblot with mutants L5-D1b, L5-D2b, L5-D3b, and L5-D4b. E, immunoblot with mutants L15-D1b, L15-D2b, L15-D3b, and L15-D4b.

We moved on to determine the extent of Hog1p phosphorylation in AMY1000 cells carrying mutants where the HAMP-like linkers were replaced with flexible glycine-rich linkers by Western blotting. As shown in Fig. 2, D and E, the cells expressing L5-D4b and L15-D4b mutants showed intense Hog1p phosphorylation indicating that these mutants were non-functional and therefore incapable of regulating the HOG pathway. However, activation of Hog1p was not observed in cells expressing the other mutants suggesting that they all retained kinase activity. Collectively, the deletion and substitution analysis unequivocally demonstrate the essential role of the HAMP-like linker H4b in DhNik1p activity.

HAMP-like Linkers Influence Fludioxonil Sensitivity of DhNik1p—Fludioxonil is an important fungicide extensively used in post-harvest treatment of fruits, seeds, and other agricultural practices to control plant-associated spoilage fungi. Fungicidal action of fludioxonil involves inhibition of group III HKK resulting in constitutive activation of the HOG pathway. Therefore, we studied whether the HAMP-like linkers had any role in modulating the fludioxonil sensitivity conferred by DhNik1p. For this, the growth of S. cerevisiae strain AMY1000 expressing DhNik1p or different mutants of DhNik1p carrying deletion in linker domains were checked by dilution spotting on SD plates containing 25 μg/ml of fludioxonil (Fig. 3A). The mutant E-A-D1b did not confer growth on fludioxonil plates and sensitivity toward the drug was quite similar to that conferred by DhNik1p. In contrast, mutants E-A-D2b or E-A-D3b conferred fludioxonil resistance. Similarly, we also tested fludioxonil sensitivity conferred by the mutants with the flexible linker. Cells expressing the mutant L5-D1b remained sensitive to fludioxonil, whereas those expressing mutants L5-D2b and L5-D3b were completely resistant to the drug (Fig. 3B). Thus, these results indicated that the HAMP-like linker H1b was dispensable for fludioxonil sensitivity of DhNik1p, whereas linkers H2b and H3b played an important role in this process.

Earlier studies showed that the toxicity of fludioxonil against S. cerevisiae cells expressing group III HKK including DhNik1p is based on activation of the Hog1p MAPK (17). Therefore, we determined the level of phosphorylated Hog1p in S. cerevisiae strain AMY1000 expressing linker deletion mutants by immunoblotting after exposing the cells to fludioxonil. In E-A-D1b expressing cells exposed to fludioxonil the levels of phosphorylated Hog1p was higher than in the untreated control (Fig. 3C). Similarly, the cells expressing wild type DhNik1p also showed activated Hog1p upon fludioxonil treatment. In comparison, the cells expressing mutants E-A-D2b or E-A-D3b showed an elevated level of phosphorylated Hog1p in untreated samples and the level did not change even after fludioxonil treatment. Comparable results were obtained with DhNik1p mutants where HAMP-like linkers were replaced with a flexible linker (Fig. 3D).

We next monitored the in vivo effect conferred by fludioxonil on the histidine kinase activity of linker mutants using an SSRE-lacZ reporter system (17). In this system, the SSRE-lacZ reporter activity depends on phosho-relay from DhNik1p to
FIGURE 3. Fludioxonil sensitivity of the different DhNik1p mutants. A, S. cerevisiae AMY1000 cells transformed with pRS423, DhNik1, or HAMP-like linker deletion mutants E-A_Δ1b, E-A_Δ2b, E-A_Δ3b, or E-A_Δ4b or B, flexible linker mutants LS_Δ1b, LS_Δ2b, or LS_Δ3b, or LS_Δ4b were grown on SD-His plates and SD-His plates supplemented with doxycycline (10 μg/ml) and fludioxonil (Flu) (25 μg/ml) at 28 °C for 3 days before taking photographs. Experiments were repeated three times with similar results. C, immunoblotting analysis. Total cell extract from the respective culture not exposed to fludioxonil (∼) was loaded as control. Blots were analyzed using anti-phospho-p38 antibody for phospho-Hog1 (P-Hog1) and anti-Hog1 antibody for total Hog1 (Hog1). E, histidine kinase activity of HAMP-like linker mutants: β-galactosidase activity of complementing the sln1 mutation in S. cerevisiae strain AMY1000 expressing DhNik1p and HAMP-like linker deletion mutants E-A_Δ1b, E-A_Δ2b, or E-A_Δ3b or D, flexible linker mutants LS_Δ1b, LS_Δ2b, or LS_Δ3b. Cells were grown in SD minimal media without histidine at 28 °C until an A_600nm ∼0.8 and treated with fludioxonil (5 μg/ml) for 15 min. Total cell extract from these samples was used for Western blotting analysis. Total cell extract from the respective culture not exposed to fludioxonil (∼) was loaded as control. Blots were analyzed using anti-phospho-p38 antibody for phospho-Hog1 (P-Hog1) and anti-Hog1 antibody for total Hog1 (Hog1). F, histidine kinase activity of HAMP-like linker mutants: β-galactosidase activity of complementing the sln1 mutation in S. cerevisiae strain AMY1000 expressing DhNik1p and HAMP-like linker deletion mutants E-A_Δ1b, E-A_Δ2b, or E-A_Δ3b or D, flexible linker mutants LS_Δ1b, LS_Δ2b, or LS_Δ3b. Cells were grown in SD minimal media without histidine at 28 °C until an A_600nm ∼0.8 and treated with fludioxonil (5 μg/ml) for 15 min. Total cell extract from these samples was used for Western blotting analysis. Total cell extract from the respective culture not exposed to fludioxonil (∼) was loaded as control. Blots were analyzed using anti-phospho-p38 antibody for phospho-Hog1 (P-Hog1) and anti-Hog1 antibody for total Hog1 (Hog1). G, histidine kinase activity of HAMP-like linker mutants: β-galactosidase activity of complementing the sln1 mutation in S. cerevisiae strain AMY1000 expressing DhNik1p and HAMP-like linker deletion mutants E-A_Δ1b, E-A_Δ2b, or E-A_Δ3b or D, flexible linker mutants LS_Δ1b, LS_Δ2b, or LS_Δ3b. Cells were grown in SD minimal media without histidine at 28 °C until an A_600nm ∼0.8 and treated with fludioxonil (5 μg/ml) for 15 min. Total cell extract from these samples was used for Western blotting analysis. Total cell extract from the respective culture not exposed to fludioxonil (∼) was loaded as control. Blots were analyzed using anti-phospho-p38 antibody for phospho-Hog1 (P-Hog1) and anti-Hog1 antibody for total Hog1 (Hog1). H, histidine kinase activity of HAMP-like linker mutants: β-galactosidase activity of complementing the sln1 mutation in S. cerevisiae strain AMY1000 expressing DhNik1p and HAMP-like linker deletion mutants E-A_Δ1b, E-A_Δ2b, or E-A_Δ3b or D, flexible linker mutants LS_Δ1b, LS_Δ2b, or LS_Δ3b. Cells were grown in SD minimal media without histidine at 28 °C until an A_600nm ∼0.8 and treated with fludioxonil (5 μg/ml) for 15 min. Total cell extract from these samples was used for Western blotting analysis. Total cell extract from the respective culture not exposed to fludioxonil (∼) was loaded as control. Blots were analyzed using anti-phospho-p38 antibody for phospho-Hog1 (P-Hog1) and anti-Hog1 antibody for total Hog1 (Hog1). I, histidine kinase activity of HAMP-like linker mutants: β-galactosidase activity of complementing the sln1 mutation in S. cerevisiae strain AMY1000 expressing DhNik1p and HAMP-like linker deletion mutants E-A_Δ1b, E-A_Δ2b, or E-A_Δ3b or D, flexible linker mutants LS_Δ1b, LS_Δ2b, or LS_Δ3b. Cells were grown in SD minimal media without histidine at 28 °C until an A_600nm ∼0.8 and treated with fludioxonil (5 μg/ml) for 15 min. Total cell extract from these samples was used for Western blotting analysis. Total cell extract from the respective culture not exposed to fludioxonil (∼) was loaded as control. Blots were analyzed using anti-phospho-p38 antibody for phospho-Hog1 (P-Hog1) and anti-Hog1 antibody for total Hog1 (Hog1). J, histidine kinase activity of HAMP-like linker mutants: β-galactosidase activity of complementing the sln1 mutation in S. cerevisiae strain AMY1000 expressing DhNik1p and HAMP-like linker deletion mutants E-A_Δ1b, E-A_Δ2b, or E-A_Δ3b or D, flexible linker mutants LS_Δ1b, LS_Δ2b, or LS_Δ3b. Cells were grown in SD minimal media without histidine at 28 °C until an A_600nm ∼0.8 and treated with fludioxonil (5 μg/ml) for 15 min. Total cell extract from these samples was used for Western blotting analysis. Total cell extract from the respective culture not exposed to fludioxonil (∼) was loaded as control. Blots were analyzed using anti-phospho-p38 antibody for phospho-Hog1 (P-Hog1) and anti-Hog1 antibody for total Hog1 (Hog1). K, histidine kinase activity of HAMP-like linker mutants: β-galactosidase activity of complementing the sln1 mutation in S. cerevisiae strain AMY1000 expressing DhNik1p and HAMP-like linker deletion mutants E-A_Δ1b, E-A_Δ2b, or E-A_Δ3b or D, flexible linker mutants LS_Δ1b, LS_Δ2b, or LS_Δ3b. Cells were grown in SD minimal media without histidine at 28 °C until an A_600nm ∼0.8 and treated with fludioxonil (5 μg/ml) for 15 min. Total cell extract from these samples was used for Western blotting analysis. Total cell extract from the respective culture not exposed to fludioxonil (∼) was loaded as control. Blots were analyzed using anti-phospho-p38 antibody for phospho-Hog1 (P-Hog1) and anti-Hog1 antibody for total Hog1 (Hog1). L, histidine kinase activity of HAMP-like linker mutants: β-galactosidase activity of complementing the sln1 mutation in S. cerevisiae strain AMY1000 expressing DhNik1p and HAMP-like linker deletion mutants E-A_Δ1b, E-A_Δ2b, or E-A_Δ3b or D, flexible linker mutants LS_Δ1b, LS_Δ2b, or LS_Δ3b. Cells were grown in SD minimal media without histidine at 28 °C until an A_600nm ∼0.8 and treated with fludioxonil (5 μg/ml) for 15 min. Total cell extract from these samples was used for Western blotting analysis. Total cell extract from the respective culture not exposed to fludioxonil (∼) was loaded as control. Blots were analyzed using anti-phospho-p38 antibody for phospho-Hog1 (P-Hog1) and anti-Hog1 antibody for total Hog1 (Hog1). M, histidine kinase activity of HAMP-like linker mutants: β-galactosidase activity of complementing the sln1 mutation in S. cerevisiae strain AMY1000 expressing DhNik1p and HAMP-like linker deletion mutants E-A_Δ1b, E-A_Δ2b, or E-A_Δ3b or D, flexible linker mutants LS_Δ1b, LS_Δ2b, or LS_Δ3b. Cells were grown in SD minimal media without histidine at 28 °C until an A_600nm ∼0.8 and treated with fludioxonil (5 μg/ml) for 15 min. Total cell extract from these samples was used for Western blotting analysis. Total cell extract from the respective culture not exposed to fludioxonil (∼) was loaded as control. Blots were analyzed using anti-phospho-p38 antibody for phospho-Hog1 (P-Hog1) and anti-Hog1 antibody for total Hog1 (Hog1). N, histidine kinase activity of HAMP-like linker mutants: β-galactosidase activity of complementing the sln1 mutation in S. cerevisiae strain AMY1000 expressing DhNik1p and HAMP-like linker deletion mutants E-A_Δ1b, E-A_Δ2b, or E-A_Δ3b or D, flexible linker mutants LS_Δ1b, LS_Δ2b, or LS_Δ3b. Cells were grown in SD minimal media without histidine at 28 °C until an A_600nm ∼0.8 and treated with fludioxonil (5 μg/ml) for 15 min. Total cell extract from these samples was used for Western blotting analysis. Total cell extract from the respective culture not exposed to fludioxonil (∼) was loaded as control. Blots were analyzed using anti-phospho-p38 antibody for phospho-Hog1 (P-Hog1) and anti-Hog1 antibody for total Hog1 (Hog1). O, histidine kinase activity of HAMP-like linker mutants: β-galactosidase activity of complementing the sln1 mutation in S. cerevisiae strain AMY1000 expressing DhNik1p and HAMP-like linker deletion mutants E-A_Δ1b, E-A_Δ2b, or E-A_Δ3b or D, flexible linker mutants LS_Δ1b, LS_Δ2b, or LS_Δ3b. Cells were grown in SD minimal media without histidine at 28 °C until an A_600nm ∼0.8 and treated with fludioxonil (5 μg/ml) for 15 min. Total cell extract from these samples was used for Western blotting analysis. Total cell extract from the respective culture not exposed to fludioxonil (∼) was loaded as control. Blots were analyzed using anti-phospho-p38 antibody for phospho-Hog1 (P-Hog1) and anti-Hog1 antibody for total Hog1 (Hog1).
Functional Role of HAMP-like Linkers

FIGURE 4. Functional analysis of the stutter mutants. A, growth pattern of S. cerevisiae strain AMY1000 expressing different stutter mutants on minimal SD-agar plates without histidine in the absence and presence of 10 μg/ml of doxycycline (Dox) after 3 days at 28 °C. The experiment was repeated three times with similar results. B, fludioxonil sensitivity of the AMY1000 strain transformed with DhNik1 or stutter mutants on SD-His plates and SD-His plates supplemented with doxycycline (10 μg/ml) and fludioxonil (25 μg/ml) after 3 days at 28 °C. The experiment was repeated three times with similar results. C, immunoblot showing fludioxonil-induced Hog1p phosphorylation in S. cerevisiae strain AMY1000 expressing DhNik1p or different stutter mutants Δ1a, Δ2a, or Δ3a. Cells were grown in SD-minimal media without histidine at 28 °C until an A600,0 = 0.8 and treated with fludioxonil (5 μg/ml) for 15 min. Total cell extract from these samples was used for blotting. Total cell extract from the respective culture not exposed to fludioxonil (−) was loaded as control. Blots were analyzed using anti-phospho-p38 antibody for phospho-Hog1p (P-Hog1) and anti-Hog1 antibody for total Hog1 (Hog1). D, β-galactosidase assay to determine the histidine kinase activity of stutter mutants. β-Galactosidase activity in yeast strain YSH2472 harboring pDhNik1 or stutter mutants Δ1a, Δ2a, Δ3a, or Δ4a was determined after growth in minimal liquid media in the absence (dark) or presence of 5 μg/ml of fludioxonil (light gray). Experiments were repeated with two independent pools of six transformants each. β-Galactosidase activity is expressed as nanomole of o-nitrophenyl-β-galactoside utilized per minute by 1 ml of culture, which was normalized to 1.0 A600.

ferred fludioxonil resistance and fludioxonil induced Hog1p phosphorylation was not observed in these mutants (Fig. 4, B and C). In comparison, S. cerevisiae cells expressing Δ1a did not grow in the presence of fludioxonil and these cells exhibited robust phosphorylation of Hog1p upon exposure to fludioxonil. We also investigated the in vivo effect of stutter deletions on the kinase activity of DhNik1p using the SSRE-lacZ reporter system. In the absence of fludioxonil, cells expressing the Δ1a mutant showed 2-fold higher β-galactosidase activity than cells expressing DhNik1p and the addition of fludioxonil to the growth medium caused a substantial reduction of the β-galactosidase activity. These results indicated that mutation in the 1a stutter element did not affect histidine kinase activity, which remained as sensitive to fludioxonil as that of wild type DhNik1p (Fig. 4D). In comparison, the cells expressing Δ2a and Δ3a mutants showed reduced β-galactosidase activity (30–40% activity to that of DhNik1p), which was not inhibited by fludioxonil (Fig. 4D). β-Galactosidase activity in cells expressing the Δ4a mutant was as low as vector control, consistent with a non-functional mutant. Thus, it appeared that the stutter element 4a, which followed the linker 4b was crucial for kinase activity of DhNik1p, whereas 1a was dispensable. The deletion of stutter elements 2a and 3b did not abolish the kinase activity of DhNik1p and therefore were not essential.

Phenotype analysis of linker deletion mutants as well as the stutter mutants alluded that the 4b linker was functionally distinct from others linkers although it shared common HAMP-like sequence features. We therefore set out to determine whether other linkers could functionally replace the 4b linker. For this, mutant constructs E-A41b, E-A42b, and E-A43b were transformed into S. cerevisiae strain AMY1000 and the growth pattern of the transformants were checked on SD plates containing doxycycline. The strains carrying mutants E-A41b and E-A43b did not grow on the doxycycline plate (Fig. 5A) and exposure to doxycycline activated Hog1p in these mutants (Fig. 5B). These results indicated that they were non-functional mutants. The mutant E-A42b grew well in the presence of doxycycline (Fig. 5A), and as well as could suppress Hog1p activation in the absence of Sln1p (Fig. 5B). However, it was resistant to fludioxonil (Fig. 5C) indicating that it was a constitutively active kinase mutant (14, 17). To have a quantitative measure of the activity of E-A42b, we performed an in vivo β-galactosidase assay. E-A42b showed a high level of kinase activity. In the presence of fludioxonil activity of the mutant remained quite high although at a reduced level (~3-fold) (Fig. 5D) and therefore was not sensitive to fludioxonil. The phenotypes of all the mutants were also confirmed using yeast strain NM1 having a different sln1 mutation (data not shown).

Size Exclusion Chromatography-MALLS Experiments, HAMP1–5 MBP Exists as Dimer in Solution—Structurally, HAMP domains have been shown to be entwined on each other forming dimeric states. To examine whether HAMP1–5 MBP existed as a homodimer, we carried out size exclusion chromatography coupled with MALLS. HAMP1–5 MBP showed a single peak profile for the protein (Fig. 6). In comparison to BSA, which was used as standard, the retention volume and profile suggested a molecular mass of 161 kDa for the eluting protein. This result clearly established its dimeric status in solution. Similar experiments established the dimeric status also for the other HAMP constructs, e.g. HAMP1–3MBP (145 kDa) and HAMP1–4 MBP (149 kDa) (data not shown).

Small Angle X-ray Scattering of HAMP1–5 MBP—To obtain structural insights into the poly-HAMP domains in DhNik1p, we collected SAXS data of HAMP1–5 MBP at three different protein concentrations varying from ~0.3 to 0.8 mg/ml (Fig. 7). An identical slope of the Guinier region of the low Q data in the
Functional Role of HAMP-like Linkers

given concentration range confirmed the absence of any concentration-dependent aggregation or inter-particle effect in the protein samples (Fig. 7A). The Guinier analysis presuming the protein to have globular and rod-like shape profiles showed \( R_g \) of HAMP1–5 MBP to be 107.3 ± 0.3 Å and \( R_c \) of 17.4 ± 0.5 Å (Table 1). Insets in Fig. 7A showed the linear region of Guinier approximation presuming globular and rod-like shapes, above and lower, respectively. The downward rolling over of the data at low \( Q \) in the lower inset confirmed that HAMP1–5 MBP adopts a globular shape. This was surprising, because the significantly greater values of \( R_g \) in comparison to \( R_c \) supported an elongated shape of the HAMP1–5 MBP, and Equation 3 suggested the scattering shape to be of about 366 ± 1.1 Å in length. Kratky analysis of the SAXS datasets showed a peak-like profile that upheld a globular shape in the HAMP1–5 MBP molecules lacking inherent disorder (Fig. 7B, inset). Presuming the mono-disperse scattering nature of HAMP1–5 MBP molecules in solution, indirect Fourier transformation of the data sets over a much wider angular range (\( Q \) range of 0.004 to 0.2 Å\(^{-1}\)) provided a good solution at \( D_{max} \sim 360 \pm 010 \) Å, which also computed an \( R_g \) of 107.5 ± 0.5 Å (Fig. 7B and Table 1). The ripples in the calculated \( P(r) \) profiles indicated an extended shape of the molecules with multiple domains. Analysis of the inter-atomic vectors perpendicular to the long axis of the molecules showed a peak-shoulder profile with a peak at ~17 Å and a shoulder at 42 Å (Fig. 7C). This suggested that along the girth of the HAMP1–5 MBP protein, there were two sets of thicknesses possible, some domains with a cross-sectional dimension about 17 Å and others with almost double the size, in different possible combinations (Fig. 7D). Mass estimation from Porod approximation of the HAMP1–5 MBP suggested it to be around 188 kDa. This along with earlier MALS results clearly indicated that the HAMP1–5 protein was a dimer and the global shape of the protein in solution possibly had some latent motion that increased its apparent size in solution.

The scattering shape of HAMP1–5 MBP was generated by averaging 10 individual solution shapes, which were calculated using SAXS data acquired from samples with the highest concentration (i.e. 0.8 mg/ml) (Fig. 8). The structure showed a rod-like shape of the molecule with a slight bulge on one side. To compare the overall shape profile we placed the homology model for HAMP1–5 inside the volume of the SAXS-based envelope for HAMP1–5 MBP (35). Additionally, we carried out data analysis of the SAXS \( I(Q) \) profiles from solutions of MBP, the tag used with HAMP1–5 (data not shown). Considering a much wider \( Q \) range (0.003 to 0.3 Å\(^{-1}\)) and the monodisperse
globular nature of MBP, indirect Fourier transformation provided a good solution at $D_{\text{max}}$ of 65 Å with an $R_g$ close to 22.5 Å (Table 1). The predominant solution scattering shape of MBP was restored by averaging 10 individual dummy residue models derived from the SAXS data of MBP at a protein concentration of 0.7 mg/ml (data not shown). Interestingly, manual placement of the crystal structure of MBP (PDB code 3HPI, chain A) showed a good fit with the SAXS-derived envelope volume (32). This confirmed that MBP predominantly exists as monomer in solution and the dimeric state of HAMP1–5 MBP was driven intrinsically. Inertial axes alignment of the SAXS data-based and sequence similarity based models suggested that the actual global shape of the HAMP1–5 MBP possessed

---

**TABLE 1**

Structural parameters computed for HAMP1–5 MBP and MBP alone, at different protein concentrations

| Protein Sample | Concentration [mg/ml] | Guinier analysis | Indirect Fourier transformation |
|----------------|-----------------------|------------------|--------------------------------|
|                |                       | $R_g$ [Å] | $R_c$ [Å] | $L$ [Å] | $R_g$ [Å] | $R_c$ [Å] | $D_{\text{max}}$ [Å] |
| HAMP1–5 MBP    | 0.8                   | 107.7     | 17.9    | 367.9      | 107.5     | 17.4    | 366       |
|                | 0.5                   | 107.3     | 17.4    | 366.8      | 108.0     | 17.7    | 368       |
|                | 0.3                   | 107.0     | 17.1    | 365.9      | 107.0     | 17.6    | 365       |
| MBP            | 0.7                   | 22.5      | 22.4    | 65.0        |           |         |           |
|                | 0.5                   | 22.7      | 22.3    | 65.2        |           |         |           |
|                | 0.3                   | 22.2      | 22.5    | 65.5        |           |         |           |
some degrees of freedom mainly perpendicular to the long dimension of the protein, a factor that we could not comprehend from the static three-dimensional sequence similarity based model of HAMP1–5.

**Normal Mode Analysis for Homology Modeled HAMP1–5**—Unlike other prokaryotic poly-HAMP containing sensor kinases, DhNik1p is unique in having HAMP-like linkers interspersed with the HAMP domains such that each HAMP and HAMP-like domain pair form a 90-residue repeat unit with the adjoining 90-residue repeat units marked by a stutter (Fig. 9A). The presence of extended periodicity in HAMP1–5 in the form of HAMP-like domains called for the need to have stutters before the beginning of HAMP domains 2–5 in DhNik1p, which could help in the regulatory function (phasing) of the protein. We selected the most collective motion representing the low frequency normal mode of the model of HAMP1–5, showing the highest collectivity (which corresponded to global motion of HAMP 1–5) (Fig. 9). We found that along with the extreme ends of HAMP1–5 (i.e. residues both from the N- and C-terminal, which were synchronous in terms of magnitude), the residues from HAMP domains 1 and 5, 2 and 4, and HAMP-like linkers 1 and 4 showed concerted (in-phase) motion as seen by their $R^2$ values. Comparison of the $R^2$ profiles of the stutter mutants with those of the native protein showed no observable differences between the two (Fig. 9, C–E). Unlike stutter mutants, F-L deletion mutants showed high mobility and out of phase displacements for the extreme N- and C-terminal residues for L5Δab and L15Δab mutants, both of which showed non-functionality in our biochemical experiments (data not shown). Interestingly, the E-AΔ1b mutant showed increased displacement as compared with the native protein in the N-terminal region, however, unlike the F-LΔ1b mutant, the displacement for this mutant was in-phase with that of the native type and was also found to be functional in our biochemical experiments (Fig. 9C). The flexible peptide linker mutants L5Δab and L15Δab have out of phase displacements for most
Functional Role of HAMP-like Linkers

parts of the HAMP1–5, which indicated that any mutation in the fourth HAMP-like linker deviates the functioning of the HAMP1–5 from its native type (Fig. 9D and data not shown). It is noteworthy to mention that any mutation in the second and third HAMP-like linker does not render them non-functional, which is also supported by our normal mode analysis, where we see no out of phase displacements throughout the HAMP1–5 upon deletion of residues in these two regions.

DISCUSSION

Nik1 orthologs are an important class of sensor kinases that functions upstream of the HOG/p38 MAPK pathway in fungi (2). In their N terminus, they contain a poly-HAMP module, which is essential for osmosensing as well as cell death upon exposure to the antifungal agent fludioxonil (2, 6, 9, 33–36). DhNik1p is a typical molecule of this class and contains five HAMP domains and four HAMP-like linkers (Fig. 1A). In this study, we investigated the contribution of each of the HAMP-like linker regions on the functionality of DhNik1p. Our analysis showed that the HAMP4b linker is essential as its deletion resulted in the complete loss of activity. Replacement of this linker with flexible peptide sequences did not restore activity. Thus, the HAMP-like sequence and possibly structural features of this linker region are indispensable for the kinase activity of DhNik1p. In contrast, the deletion mutant E-A42b as well as mutants carrying the flexible peptide linkers (L5Δ1b and L15Δ1b) could complement sln1 and suppress activation of Hog1p in the absence of Sln1p. They also exhibited in vivo kinase activity quite comparable with wild type DhNik1p as measured by indirect β-galactosidase assay (Fig. 3E). Like that of DhNik1p, the in vivo activity of these mutants was highly sensitive to fludioxonil and the expression of these mutants in S. cerevisiae strain AMY1000 conferred fludioxonil sensitivity to the host. These results clearly indicated that the H1b linker was dispensable for the activity of DhNik1. The mutants with deletion or replacement of H2b or H3b linkers were functional as they showed phenotypic complementation and could suppress Hog1p phosphorylation. However, their activity was 3–4-fold lower than that of DhNik1p. These mutants conferred fludioxonil resistance and behaved like a constitutively active kinase. Thus, although H2b and H3b linkers were not essential for kinase activity they were crucial for both fludioxonil sensitivity and osmosensing. The H4b linker was functionally distinct from others although they share common features. To determine whether the position or the sequence of the H4b linker was important, it was replaced by H1b, H2b, or H3b. Only the H2b linker could partially substitute the functionality of the H4b linker. It could suppress the lethality of Sln1p deletion but failed to confer fludioxonil sensitivity (Fig. 5, A and C). The in vivo assay showed that the mutant E-A42b had higher kinase activity (Fig. 5D). Interestingly, although fludioxonil reduced the activity of the mutant, it retained sufficient kinase activity that was quite comparable with the wild type protein observed earlier. The activity of E-A42b appeared to be biased toward the kinase-on state. The H2b linker was most closely related to H4b in terms of similarity (82%) and identity (67%) and this could be the reason for the observed phenotypes. Thus, the sequence of the H4b linker is important for its differential role.

In prokaryotic trans-membrane receptors, the HAMP domain acts as signal relay modules (37, 38). Structurally, HAMP domains are arranged as symmetric homodimeric parallel coiled coils with each protomer having two α-helices, AS1 and AS2, connected by a flexible linker segment (12, 13, 27, 37). The four-helix bundle is held together by a hydrophobic core. In response to the upstream input signal, the four-helix bundle undergoes reversible conformational changes that regulate the activity of the downstream kinase domain (31, 39). The presence of the phase stutter at the end of the AS2 helix had been proposed to play an important role in this regard (19, 39). DhNik1p contains four stutter elements following the AS2 helix of each HAMP-like linker. An essential role of the phase stutters in signal relay has been proposed for poly-HAMP containing bacterial sensor HK (27). To determine the role of these phase stutters in mediating signal propagation through the HAMP repeats in DhNik1p, we constructed deletions mutants Δ1a (149–152 aa; LTNQ), Δ2a (241–244 aa; LTTQ), Δ3a (333–336 aa; LTNQ), and Δ4a (425–428 aa; LTSQ). Deletion of stutter element 1a in DhNik1p did not affect its ability to complement the Sln1p function in S. cerevisiae and confer fludioxonil sensitivity. In the in vivo β-galactosidase assay, the Δ1a mutant conferred a 2-fold higher activity than DhNik1p, which could be inhibited by fludioxonil. Thus, the deletion of 1a did not affect the functionality of DhNik1p. In contrast, complete loss of function was observed with the Δ4a mutant. The mutants, Δ2a and Δ3a, retained their ability to complement the sln1 function, exhibited only ~25% activity in vivo. They also showed resistance to fludioxonil indicating the constitutive nature of these mutants.

Previous studies indicated that HAMP domains in Nik1 orthologs function as sensor and regulate activity of the downstream histidine kinase module. However, structural insight into the functioning of these poly-HAMP modules is lacking. To obtain mass distribution and low-resolution molecular envelopes in the poly-HAMP module, we performed SAXS with HAMP1–5 MBP, which contained all the HAMP and HAMP-like linker domains of DhNik1p. SAXS data indicated an extended shape of the poly-HAMP module. We also generated a sequence similarity based model for the homodimeric poly-HAMP module of DhNik1p using PDB 3LNR as template in which the consecutive HAMP and HAMP-like linkers in DhNik1p formed a di-HAMP concatenated structure. Importantly, the homology model fully satisfied the SAXS-based molecular envelope and the calculated collective motion of the native protein and its mutants correlated with the functional readouts. Our shape-function studies promote that regulation of the histidine kinase activity by the upstream poly-HAMP module could be through an in-line mechanism than the side-on interaction as suggested earlier (9). Several recent studies indicated variations in the HAMP domain structure arising out of different helix rotation, helix translation, and helix-helix crossing angle (12, 13, 27). Similarly, a different mechanism of signal propagation through HAMP domains had been proposed. Two distinct conformational states, inter-convertible through helix rotation or translation, represent the on-off state of the HAMP domains (12, 40, 41). In comparison, a dynamic structure for the HAMP domain has also been proposed (31).
Such a structure with varying bundle stability could impose a biphasic control output (39). The *P. aeruginosa* chemoreceptor, Aer-2, is the only other system where functioning of the poly-HAMP module has been studied. In Aer-2, the PAS-sensing domain appeared to regulate the alternative HAMP structure of the successive C-terminal HAMP domains through an in-line mechanism (40). In the case of DhNik1p, the absence of any input sensor domain made the system more interesting and intriguing. Our normal mode analysis revealed that some of the HAMP and HAMP-like linker domains showed larger movements and therefore were more dynamic than others. The signal generated from the dynamic state of the H1 HAMP domain is propagated through the concerted motions of the HAMP and HAMP-like linkers to the H5 domain. The H4b linker domain plays an essential role for the “on-off” transition of poly-HAMP conformation, whereas the H3b and H2b linkers appeared to have a facultative role in signal propagation. The HOG pathway maintains a basal level of signaling even under ambient conditions and which allows more rapid response and fine tuning of signaling thresholds upon stress treatments (42, 43). The poly-HAMP structure can provide such a dynamic switch through the concerted motion that could be disrupted in the changing cytoplasmic environment under high osmolarity stress. Thus, the distinct roles played by the HAMP-linkers in DhNik1p could represent another interesting facet of the HAMP signaling.

Acknowledgments—We acknowledge the use of National Synchrotron Light Source, Brookhaven National Laboratory, which was supported by the United States Department of Energy, Office of Science, Office of Basic Energy Sciences, under Contract number DEAC02-98CH10886. We acknowledge the technical help provided by R. Sharma and D. Bhatt.

**REFERENCES**

1. Catlett, N. L., Yoder, O. C., and Turgeon, B. G. (2003) Whole-genome analysis of two-component signal transduction genes in fungal pathogens. *Eukaryot. Cell* 2, 1151–1161
2. Bahn, Y. S. (2008) Master and commander in fungal pathogens: the two-component system and the HOG signaling pathway. *Eukaryot. Cell* 7, 2017–2036
3. Ota, I. M., and Varshavsky, A. (1993) A yeast protein similar to bacterial two-component regulators. *Science* 262, 566–569
4. Maeda, T., Wurgler-Murphy, S. M., and Saito, H. (1994) A two-component system that regulates an osmosensing MAP kinase cascade in yeast. *Nature* 369, 242–245
5. Posas, F., Wurgler-Murphy, S. M., Maeda, T., Witten, E. A., Thai, T. C., and Saito, H. (1996) Yeast Hog1 MAP kinase cascade is regulated by a multistep phosphorylase mechanism in the SLN1 YPD1 SK1 two-component osmosensor. *Cell* 86, 865–875
6. Motoyama, T., Kadowaki, K., Ohira, T., Ichishi, A., Fujimura, M., Yamauchi, I., and Kudo, T. (2005) A two-component histidine kinase of the rice blast fungus is involved in osmotic stress response and fungicidal action. *Fungal Genet. Biol.* 42, 200–212
7. Ochiai, N., Fujimura, M., Motoyama, T., Ichishi, A., Usami, R., Horikoshi, K., and Yamaguchi, I. (2001) Characterization of mutations in the two-component histidine kinase gene that confer fluoroquinolone resistance and osmotic sensitivity in the os-1 mutants of *Neurospora crassa*. *Pest Manag. Sci.* 57, 437–442
8. Yoshimi, A., Tsuda, M., and Tanaka, C. (2004) Cloning and characterization of the histidine kinase gene Dic1 from *Cochliobolus heterostrophus* that confers dicarboximide resistance and osmotic adaptation. *Mol. Genet. Genomics* 271, 228–236
9. Meena, N., Kaur, H., and Mondal, A. K. (2010) Interactions among HAMP domain repeats act as an osmosensing molecular switch in group III histidine kinases from fungi. *J. Biol. Chem.* 285, 12121–12132
10. Aravind, L., and Ponting, C. P. (1999) The cytoplasmic helical linker domain of receptor histidine kinase and methyl-accepting proteins is common to many prokaryotic signalling proteins. *FEMS Microbiol. Lett.* 176, 111–116
11. Applemann, J. A., and Stewart, V. (2003) Mutational analysis of a conserved signal-transducing element: the HAMP linker of the *Escherichia coli* nitrate sensor NarX. *J. Bacteriol.* 185, 89–97
12. Hulko, M., Berndt, F., Gruber, M., Linder, J. U., Truffault, V., Schultz, A., Martin, J., Schultz, J. E., Lupas, A. N., and Coles, M. (2006) The HAMP domain structure implies helix rotation in transmembrane signaling. *Cell* 126, 929–940
13. Swain, K. E., and Falke, J. J. (2007) Structure of the conserved HAMP domain in an intact, membrane-bound chemoreceptor: a disulfide mapping study. *Biochemistry* 46, 13659–13667
14. Randhawa, A., and Mondal, A. K. (2013) The sixth HAMP domain negatively regulates the activity of the group III HK containing seven HAMP domains. *Biochem. Biophys. Res. Commun.* 438, 140–144
15. Cui, W., Beever, R. E., Parkes, S. L., Weeds, P. L., and Templeton, M. D. (2002) An osmosensing histidine kinase mediates dicarboximide fungicide resistance in *Botryotinia fuckeliana* (*Botrytis cinerea*). *Fungal Genet. Biol.* 36, 187–198
16. Miller, T. K., Renault, S., and Selitrennikoff, C. P. (2002) Molecular dissection of alleles of the osmotic-1 locus of *Neurospora crassa*. *Fungal Genet. Biol.* 35, 147–155
17. Furukawa, K., Randhawa, A., Kaur, H., Mondal, A. K., and Hohmann, S. (2012) Fungal fluoroquinolone sensitivity is diminished by a constitutively active form of the group III histidine kinase. *FEMS Lett.* 586, 2417–2422
18. Dunin-Horkawicz, S., and Lupas, A. N. (2010) Comprehensive analysis of HAMP domains: implications for transmembrane signal transduction. *J. Mol. Biol.* 397, 1156–1174
19. Stewart, V., and Chen, L.-L. (2010) The S helix mediates signal transmission as a HAMP domain coiled-coil extension in the NarX nitrate sensor from *Escherichia coli* K-12. *J. Bacteriol.* 192, 734–745
20. Maan, S. N., Davierwala, A. P., Haynes, J., Moffat, J., Peng, W.-T., Zhang, W., Yang, X., Pootoolal, J., Chua, G., Lopez, A., Trochesset, M., Morse, D., Krogan, N. J., Hiley, S. L., Li, Z., Morris, Q., Grigull, J., Mitsakakis, N., Roberts, C. J., Greenblatt, J. F., Boone, C., Kaiser, C. A., Andrews, B. I., and Hughes, T. R. (2004) Exploration of essential gene functions via titratable promoter alleles. *Cell* 118, 31–44
21. Ho, S. N., Hunt, H. D., Horton, R. M., Pullen, J. K., and Pease, L. R. (1989) Site-directed mutagenesis by overlap extension using the polymerase chain reaction. *Gene* 77, 51–59
22. Sharma, P., Meena, N., Aggarwal, M., and Mondal, A. (2005) *Debaryomyces hansenii*, a highly osmo-tolerant and halo-tolerant yeast, maintains activated DhoG1p in the cytoplasm during its growth under severe osmotic stress. *Curr. Genet.* 48, 162–170
23. Konarev, P. V., Volkov, V. V., Sokolova, A. V., Koch, M. H. J., and Svergun, D. I. (2003) PRIMUS: a Windows PC-based system for small-angle scattering data analysis. *J. Appl. Crystallogr.* 36, 1277–1282
24. Svergun, D. I. (1999) Restoring low resolution structure of biological macromolecules from solution scattering using simulated annealing. *Biophys. J.* 76, 2879–2886
25. Petoukhov, M. V., Konarev, P. V., Kikhney, A. G., and Svergun, D. I. (2007) *J. Appl. Crystallogr.* 40, s223–s228
26. Volkov, V. V., and Svergun, D. I. (2003) Uniqueness of ab initio shape determination in small-angle scattering. *J. Appl. Crystallogr.* 36, 860–864
27. Airola, M. M., Watts, K. J., Bilwes, A. M., and Crane, B. R. (2010) Structure of concatenated HAMP domains provides a mechanism for signal transduction. *Structure* 18, 436–448
28. Eswar, N., Webb, B., Marti-Renom, M. A., Madhusudhan, M. S., Eramian, D., Shen, M. Y., Pieper, U., and Sali, A. (2007) Comparative protein structure modeling using MODELLER. *Curr. Protoc. Protein Sci.* Chapter 2, Unit 2 9
29. Sánchez, R., and Sali, A. (2000) Comparative protein structure modeling: introduction and practical examples with modeller. *Methods Mol. Biol.* **143**, 97–129
30. Suhre, K., and Sanejouand, Y.-H. (2004) ElNe´mo: a normal mode web server for protein movement analysis and the generation of templates for molecular replacement. *Nucleic Acids Res.* **32**, W610–W614
31. Zhou, Q., Ames, P., and Parkinson, J. S. (2009) Mutational analyses of HAMP helices suggest a dynamic bundle model of input-output signalling in chemoreceptors. *Mol. Microbiol.* **73**, 801–814
32. Gould, A. D., and Shilton, B. H. (2010) Studies of the maltose transport system reveal a mechanism for coupling ATP hydrolysis to substrate translocation without direct recognition of substrate. *J. Biol. Chem.* **285**, 11290–11296
33. Yoshimi, A., Kojima, K., Takano, Y., and Tanaka, C. (2005) Group III histidine kinase is a positive regulator of Hog1-type mitogen-activated protein kinase in filamentous fungi. *Eukaryot. Cell* **4**, 1820–1828
34. Ochiai, N., Fujimura, M., Oshima, M., Motoyama, T., Ichishi, A., Yamada-Okabe, H., and Yamaguchi, I. (2002) Effects of iprodione and fludioxonil on glycerol synthesis and hyphal development in *Candida albicans*. *Biosci. Biotechnol. Biochem.* **66**, 2209–2215
35. Bahn, Y.-S., Kojima, K., Cox, G. M., and Heitman, J. (2006) A unique fungal two-component system regulates stress responses, drug sensitivity, sexual development, and virulence of *Cryptococcus neoformans*. *Mol. Biol. Cell* **17**, 3122–3135
36. Hagiwara, D., Matsubayashi, Y., Marui, J., Furukawa, K., Yamashino, T., Kanamaru, K., Kato, M., Abe, K., Kobayashi, T., and Mizuno, T. (2007) Characterization of the NikA histidine kinase implicated in the phosphorylation signal transduction of *Aspergillus nidulans*, with special reference to fungicide responses. *Biosci. Biotechnol. Biochem.* **71**, 844–847
37. Falke, J. J., and Hazelbauer, G. L. (2001) Transmembrane signaling in bacterial chemoreceptors. *Trends Biochem. Sci.* **26**, 257–265
38. Watts, K. J., Johnson, M. S., and Taylor, B. L. (2008) Structure-function relationships in the HAMP and proximal signaling domains of the aerotaxis receptor *Aer*. *J. Bacteriol.* **190**, 2118–2127
39. Zhou, Q., Ames, P., and Parkinson, J. S. (2011) Biphasic control logic of HAMP domain signalling in the *Escherichia coli* serine chemoreceptor. *Mol. Microbiol.* **80**, 596–611
40. Airola, M. V., Huh, D., Sukomon, N., Widom, J., Sircar, R., Borbat, P. P., Freed, J. H., Watts, K. J., and Crane, B. R. (2013) Architecture of the soluble receptor Aer2 indicates an in-line mechanism for PAS and HAMP domain signaling. *J. Mol. Biol.* **425**, 886–901
41. Ferris, H. U., Dunin-Horkawicz, S., Mondejar, L. G., Hulkó, M., Hantke, K., Martin, J., Schultz, J. E., Zeth, K., Lupas, A. N., and Coles, M. (2011) The mechanisms of HAMP-mediated signaling in transmembrane receptors. *Structure* **19**, 378–385
42. Hohmann, S. (2009) Control of high osmolarity signalling in the yeast *Saccharomyces cerevisiae*. *FEBS Lett.* **583**, 4025–4029
43. Macia, J., Regot, S., Peeters, T., Conde, N., Solé, R., and Posas, F. (2009) Dynamic signaling in the Hog1 MAPK pathway relies on high basal signal transduction. *Sci. Signal.* **2**, ra13