Characterization of Cyclophilin from Thaumarchaeota

*Nitrosopumilus maritimus*: Implications on the Diversity of Chaperone-like Activity in the Archaeal Domain

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**ABSTRACT:** The Archaea constitute separate domain of life and show resemblance with bacteria in their metabolic pathways while showing similarity with eukaryotes at the level of molecular processes such as cell division, DNA replication, protein synthesis, and proteostasis. However, the molecular machinery of archaea can be considered a simpler version of that found in eukaryotes because of the absence of multiple paralogs for any given molecular factor. Therefore, archael systems can possibly be used as a model system for understanding the eukaryotic protein folding machinery and thereby may help to address the molecular mechanism of various protein (mis)foldings and diseases. In the process of protein folding, the cis–trans isomerization of the peptide–prolyl bond is a rate-limiting step for the correct folding of proteins. Different types of peptidyl–prolyl cis–trans isomerases can accelerate this reaction, e.g., cyclophilin, FKBP, and parvulin. Among the five phyla of the archaeal domain, homologs of the cyclophilin protein are found only in two. Here we have characterized a cyclophilin from an archaeal organism, *Nitrosopumilus maritimus* (NmCyp), belonging to the phylum Thaumarchaeota. Like other known cyclophilins, NmCyp also possesses PPIase activity that can be inhibited by cyclosporine A. Generally, archael proteins are expected to possess differential thermal stability due to their adaptation to extreme environmental niche conditions. However, NmCyp exhibits low thermal stability and starts to aggregate beyond 40 °C. The properties of NmCyp are compared to those reported for the cyclophilin from another archael organism, *Methanobrevibacter ruminantium*. The current study sheds light on the differential behavior of cyclophilin proteins from two different phyla of archaee.
limited information is available for cyclophilins from archael organisms.2,19−21

Archaea are considered to be a connecting link between eukaryotes and prokaryotes.22,23 Protein folding in the archaea is fascinating because archael species can grow under extreme environmental conditions like high temperature, extreme pH, and high salt, which are normally thought to be disruptive for protein structure and function.24 Several studies have suggested that chaperones play a major role in overcoming various kinds of stresses and help the organism maintain a viable proteome. PPIase proteins like cyclophilins may be important for the maintenance of the cellular viability in archaea.25 However, cyclophilins are present only in two phyla of archaea: Eurarchaeota and Thaumarchaeota. The biophysical and biochemical characterization of a cyclophilin from an archael organism, Methanobrevibacter ruminantium of phylum Eurarchaeota, was reported recently.26 No cyclophilin homolog from any member of Thaumarchaeota has been characterized yet. The present work is an effort to establish the similarities and differences in the cyclophilins from the two archael groups. In this work, we have selected the Cyp protein from Nitrosopumilus maritimus, an aquamarine organism that belongs to the order Nitrosofilales of the phylum Thaumarchaeota, for further characterization.27 Thaumarchaeota represents a distinctive phylum of the domain Archaea that encompasses ammonia-oxidizing organisms from the soil, hot springs, and marine waters. All the Thaumarchaeota discovered so far live under autotrophic conditions and fixed CO₂, but some are dependent on bacteria for organic materials.27 For further analysis, the NmCyp protein (cyclophilin from Nitrosopumilus maritimus) was overexpressed in E. coli and the purified protein was used for various functional assays including biophysical characterization.

2. Results

The cyclophilin homologs were searched in the predicted proteomes of all the 196 archael organisms in the NCBI database for which the whole genome has been sequenced, using P. torridus cyclophilin as the seed sequence.21 A total of 122 cyclophilin homologs were identified. The length of the protein homologs identified from archael organisms ranged from 145 to 980 amino acids. It was observed that some cyclophilin-like proteins were present as multidomain proteins, where a pro-isomerase superfamily domain was found at the N-terminus of the cyclophilin domain resulting in a much longer protein length (approximately 900 amino acids). All of such proteins (16 protein homologs) were found in 15 organisms of phylum Thaumarchaeota. These organisms also had a smaller protein length (approximately 900 amino acids). All of such proteins (16 protein homologs) were found in 15 organisms of phylum Thaumarchaeota. These organisms also had a smaller average of hydropathy (GRAVY) values of cyclophilins from Thaumarchaeota is still unknown. The grand average of hydropathy (GRAVY) values of cyclophilins from all the organisms of Thaumarchaeota lie between −0.2 and −0.4, which predict the hydrophilic nature of cyclophilins from Thaumarchaeota (Figure S2A and Table S1).

The physicochemical properties of NmCyp and MrCyp recombinant proteins are compared (Table S2). The NmCyp protein consists of a total of 158 residues. Of them, 22 and 17 residues are positively and negatively charged, respectively. The grand average of hydropathy (GRAVY) is negative (−0.42), which indicates that the protein is hydrophilic and therefore might be soluble in water. The cyclophilin from phylum Eurarchaeota (MrCyp) has a higher GRAVY score (−0.251) as compared to NmCyp, which implies that NmCyp is less hydrophobic than MrCyp (Figure S2B). The aliphatic index of a protein is defined as the relative volume of the protein occupied by the aliphatic side chains. A high aliphatic index is often taken as an index of a higher thermostability of a protein.30 The aliphatic index was calculated to be 66.01 and 72.04 for NmCyp and MrCyp, respectively, suggesting the higher thermostability of MrCyp over NmCyp.

2.2. Functional Annotation and Modeled 3D Structure of the NmCyp Protein. To explore the conserved residues in the archael cyclophilin protein NmCyp, the protein sequence was aligned with other cyclophilins, human CypA, Geobacillus kaustophilus, E. coli, and Methanobrevibacter ruminantium, which have been studied before. This sequence comparison with known sequences suggests 16 amino acids (R45, F50, M51, Q53, T69, Y74, N75, A93, Q96, S100, S103, Q104, F106, F114, L115, and Y119) to be part of the active site in NmCyp. The 158 amino acid long NmCyp has only one domain, i.e., PpiB (3−143 amino acids). The sequence comparison of NmCyp with the previously known MrCyp suggests that several binding site residues like R45, F50, M51, Q53, A93, Q96, S100, S103, Q104 are common among both the organisms (Figure 1). Few other amino acid sites appear to be conserved among archael cyclophilins (apart from the binding site residues), and their relevance requires further study.

Structures of many bacterial and eukaryotic cyclophilins are known, but no structural information is available for any archael cyclophilin. We therefore modeled the structure of NmCyp using the Geobacillus kaustophilus structure (PDB ID: 2MVZ) as a template via the homology modeling approach using the Modeller 9.17 software.31 Figure 2 shows the modeled structure of NmCyp. A total of two alpha helices and
eight antiparallel β-strands are present and make a signature cyclophilin fold. The electrostatic surface was generated with the help of PyMOL. The accuracy of the modeled structure was further validated after a refinement process using Ramachandran map calculations computed with the RAM-PAGE software. In the Ramachandran map, total numbers of residues that lie in favored, allowed, and outlier regions are 94.2, 5.8, and 0%, respectively. It is evident that the conserved binding site residues lie in the biggest cavity of the protein. The extra stretch of eight amino acids observed in Figure S1 lies near the active site of NmCyp. The cavity burrow of NmCyp appears to be neutral in charge but is surrounded by positively and negatively charged residues.

2.3. Functional Characterization of NmCyp.

To functionally characterize the overexpressed NmCyp protein, several assays like the PPIase and chaperone-like activity assay were performed with the purified protein. PPIase activity was performed against the target peptide, N-succinyl-alala-pro-phe-p-nitroaniline. The aggregation prevention assay for chaperone-like activity was performed against bovine carbonic anhydrase II (BCAII) and citrate synthase (CS) as substrate proteins.

2.3.1. Peptidyl-prolyl Cis–Trans Isomerase Assay. The PPIase activity of NmCyp was estimated using the standard PPIase assay. The peptide N-Suc-alala-pro-phe-p-nitroaniline was used as a substrate, and the reaction was measured at 15 °C for 20 min. The PPIase activity assay of NmCyp shows that it accelerates the rate of cis–trans isomerization of the substrate as compared to the control reaction (Figure 3). The PPIase activity of NmCyp was also confirmed by incubating the protein with its inhibitor cyclosporine A (CsA). CsA is the known inhibitor of cyclophilin and so is expected to inhibit the PPIase activity of NmCyp. In the presence of CsA (40 μM), the PPIase activity of NmCyp diminished significantly. Different concentrations of CsA below and above 40 μM were also tried; however, the reaction never reached saturation, which makes the calculation of the reaction rate unreliable. Therefore, the data accumulated at 40 μM CsA concentration are used. The speeding up of peptidyl-prolyl cis to trans isomerization upon adding PPIase in the reaction mixture as well as its inhibition upon adding the inhibitor CsA proves that NmCyp acts as a PPIase. The initial rate of cis–trans isomerization for the substrate only is 0.36 ± 0.02 min⁻¹, which was enhanced 1.4-fold (0.52 ± 0.02 min⁻¹) upon the addition of the NmCyp protein. The rate of reaction is significantly reduced (approximately 4-fold) (0.13 ± 0.02 min⁻¹) upon the addition of the inhibitor.

2.3.2. Chaperone-like Activity of NmCyp. Chaperones are known to prevent the aggregation of proteins by binding to their exposed hydrophobic sites. Such chaperone activity has been reported in a variety of cyclophilins earlier, so it was
imperative to study if NmCyp does the same. To assay the chaperone-like function, bovine carbonic anhydrase II (BCAII) and citrate synthase (CS) are frequently used as substrates, and therefore, these were selected for assaying the chaperone activity of NmCyp. In the first experiment, different molar ratios of NmCyp (0.75 and 1.50 μM) were incubated with BCAII (0.75 μM) at 65 °C (Figure 4A; only the 1:1 result is shown). It was observed that with the increase in incubation time (10 min) at 65 °C, NmCyp starts to aggregate, resulting in increased intensity from 10 to 200 A.U. This study highlights that NmCyp is unstable at high temperatures, so it could possibly not prevent the aggregation of other substrate proteins like BCAII.

To overcome the limitation of the temperature sensitivity of NmCyp when assaying chaperone-like activity with the BCAII substrate, citrate synthase (CS) was tried as an alternate substrate because this substrate is known to become inactivated and aggregate at a lower temperature, i.e., 45 °C.35 Thus, different molar ratios of NmCyp were incubated with citrate synthase, and the aggregation kinetics of the reaction were recorded. It was observed that NmCyp alone starts aggregating at 45 °C (Figure 4B). It shows no protective effect on the aggregation of CS. Collectively, from these studies, we concluded that NmCyp is unable to prevent the thermal aggregation of the aggregation-prone proteins probably because of its own instability at higher temperatures; thus, the chaperone-like function of NmCyp could not be assayed at high temperatures. To overcome the limitation of high temperatures, we had performed the assay with a chemical denaturant, but due to less stability of NmCyp with the denaturant, it was difficult to measure the chaperone-like activity.

**Figure 4.** Thermal aggregation prevention of BCAII and citrate synthase by NmCyp. (A) The aggregation prevention of BCAII with the NmCyp protein using 1:1 molar concentration. (B) The aggregation prevention of citrate synthase with varying concentrations of the NmCyp protein. The zoom-in view shows the spectra of citrate synthase at 45 °C. In the figure, A.U. stands for “arbitrary units”.

**Figure 5.** CD and fluorescence spectra of native NmCyp. (A) Far-UV CD spectrum of native NmCyp from 200 to 250 nm. (B) Near-UV CD spectrum of native NmCyp from 260 to 350 nm. (C) Fluorescence emission spectrum of NmCyp on excitation at 280 nm. In the figure, A.U. stands for “arbitrary units”.

2.4. Structural Characterization of NmCyp. CD and fluorescence spectroscopies are efficient tools for the measurement of the secondary and tertiary structure as well as folding—unfolding properties of proteins.36,37 The far-UV CD spectrum showed the predominance of \( \beta \)-sheet content followed by \( \alpha \)-helices, a typical signature secondary structure of cyclophilin proteins. The positive ellipticity in the near-UV CD spectrum at 290 nm signifies the presence of tryptophan residue in the native NmCyp protein (Figure 5 A,B). The fluorescence emission spectrum of the native NmCyp structure, and the value of \( \lambda_{\text{max}} \) indicates that this tryptophan is buried in the native structure. This \( \lambda_{\text{max}} \) was further used for monitoring the changes in the NmCyp tertiary structure when the protein was exposed to extreme conditions of temperature, pH, and chemical denaturants.

2.5. Thermostability of the NmCyp Protein. Molecular chaperones are generally expected to exhibit high thermal stability. The effect of temperature (25—90 \(^\circ\)C) on the structural stability of the NmCyp protein was studied by far-UV CD, near-UV CD, and fluorescence spectroscopies. CD spectra recorded at different temperatures reveal that the secondary and tertiary structure of NmCyp protein remains stable only up to 40 \(^\circ\)C (Figure 6A). Beyond 40 \(^\circ\)C, significant changes in the ellipticity values (mdeg) of both far-UV and near-UV scans signify the low stability of NmCyp (Figure 6A,C). The refolding studies, the protein was unable to regain its secondary and tertiary structure (spectra of native and refolded protein do not overlap) due to irreversible aggregation. This indicates that the thermal unfolding of the NmCyp protein is irreversible (Figure 6B,D).

The fluorescence emission of tryptophan is highly sensitive to the environment polarity and is a useful tool to measure the conformational changes in proteins. The \( \lambda_{\text{max}} \) (314 nm) of the NmCyp protein does not show any shift up to 40 \(^\circ\)C, which indicates the stability of NmCyp up to 40 \(^\circ\)C (Figure 6E). Beyond 45 \(^\circ\)C, a significant red shift in \( \lambda_{\text{max}} \) was observed (314 to 354 nm). The increase in emission intensity and red shift in \( \lambda_{\text{max}} \) indicates that the tryptophan residue of the NmCyp protein gets exposed to a polar environment, which might be
due to the unfolding of the protein’s tertiary structure after 45 °C (Figure 6E). It is well known that a completely exposed tryptophan in an aqueous buffer is expected to have a $\lambda_{\text{max}}$ of over 350 nm.31,38,39

To check the reversibility in the unfolding process, the NmCyp protein was cooled again from 90 to 25 °C in a stepwise manner. The $\lambda_{\text{max}}$ of the unfolded protein (354 nm) does not shift back to the native $\lambda_{\text{max}}$ (314 nm) and remains the same as the unfolded protein (354 nm) (Figure 6F). This suggests that the unfolding of NmCyp is irreversible. In contrast, its comparison with the reported cyclophilin from another archaeal organism (MrCyp) suggests that MyCyp can maintain its structure up to 65 °C and tends to regain the folded structure on cooling from 90 to 25 °C.20

2.6. Reversible Unfolding of NmCyp under the Influence of Urea. The effect of the chemical denaturant on the secondary and tertiary structure of NmCyp was monitored using far-UV, near-UV, and intrinsic fluorescence spectroscopy. In the presence of urea from 0 to 2.5 M, no significant change in the secondary structure of NmCyp was

Figure 7. Equilibrium unfolding and refolding of NmCyp with different concentrations of urea. (A) Far-UV CD spectra of NmCyp at different urea concentrations. (B) Near-UV CD spectra of the native NmCyp at different urea concentrations. (C) Unfolding data (222 nm) of NmCyp at different concentrations of urea fitted into a two-state model. (D) Refolding of NmCyp after diluting the urea.

Figure 8. Equilibrium unfolding and refolding of NmCyp with different concentrations of urea using fluorescence spectroscopy. (A) Emission spectra of NmCyp at different concentrations of urea. (B) Emission spectra of the refolded protein on diluting the denaturant concentration till 0.2 M. (C) Comparison of $\lambda_{\text{max}}$ of the unfolded and refolded NmCyp protein in the presence of urea. (D) The pattern of the unfolding of NmCyp monitored by the change in fluorescence emission intensity. The data were fitted to a three-state equation, and thermodynamic parameters were calculated31−44 (Table S4). In the figure, A.U. stands for “arbitrary units”.

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observed. After 2.5 M urea concentration, a significant fraction of the secondary structure was lost as inferred from the change in ellipticity signal at 222 nm (Figure 7A). The change in ellipticity at 290 nm (in the near-UV range) reflects a loss of the tertiary structure of the protein at 2.5 M urea concentration. At 290 nm, a positive ellipticity was mainly caused by tryptophan residue, and no change in intensity was observed up to 2.5 M urea; however, beyond 2.5 M, there was a drastic decrease in the intensity with an increase in urea concentration (Figure 7B). This observation suggests the loss of the secondary and tertiary structure of NmCyp beyond 2.5 M urea concentration. The unfolding experiment data obtained from CD were further fitted into a two-state equation (Figure 7C). The obtained m (cooperativity) and Cm (mid-point of unfolding) from fitted data were used for the calculation of $\Delta G_{\text{UH}_2\text{O}}$, and the obtained value was 2.7 ± 0.97 kcal mol$^{-1}$ (Table S3). During refolding studies, the highly concentrated unfolded NmCyp protein was diluted with a refolding buffer up to the minimum possible denaturant concentration in the solution. The far-UV CD spectra show that after diluting the denaturant concentration, NmCyp tends to refold back to its native structure (Figure 7D). The spectra of the unfolded and refolded protein overlap with each other, signifying the reversibility of the NmCyp denaturant mediated unfolding.

Urea unfolding probed by intrinsic fluorescence was helpful in giving a detailed picture of structural changes. The protein structure is intact up to 1.5 M urea concentration as there is neither any shift in $\lambda_{\text{max}}$ nor any change in fluorescence emission intensity. At 2.5 M urea concentration, a sharp shift in $\lambda_{\text{max}}$ from 314 to 364 nm was observed. However, the fluorescence emission intensity does not change significantly. A large red shift of 50 nm in the emission maxima is considered a signature of change in the environment of aromatic residues. On further increase in concentration of the denaturant to 4 M, a significant increase in emission intensity was observed, while the $\lambda_{\text{max}}$ is 364 nm (Figure 8A). In the last phase, where the urea concentration varies from 4 to 8 M, the fluorescence intensity and $\lambda_{\text{max}}$ remain constant, suggesting that the protein is completely unfolded at 4 M urea. The comparison of unfolding profiles obtained from the CD and fluorescence data helped us to conclude that the tertiary structure compactness of the proteins loosened at a lower urea concentration of ~1.10

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**Figure 9.** ANS-based extrinsic fluorescence studies of NmCyp at different urea concentrations. (A) ANS-based emission spectra of the protein in the presence of an increasing concentration of urea. (B) A change in emission maxima ($\lambda_{\text{max}}$) on increasing concentration of urea.

**Figure 10.** Equilibrium unfolding and refolding of NmCyp in the presence of GdnHCl. (A) Far-UV CD spectra from 200 to 250 nm at different concentrations of GdnHCl (0−7 M). (B) Near-UV CD spectra from 260 to 350 nm at different concentrations of GdnHCl (0−6 M). (C) Far-UV CD spectra of refolded proteins on diluting the denaturant. (D) GdnHCl dependent unfolding of NmCyp monitored by the change in mdeg values by CD spectroscopy. The data were fitted to a two-state equation, and thermodynamic parameters were calculated. (E) Normalized data of the NmCyp unfolded and refolded protein at different concentrations of GdnHCl.
M followed by the complete unfolding of the secondary and tertiary structure at higher urea concentrations.

During the refolding studies, the $\lambda_{\text{max}}$ of unfolded protein (361 nm) reverts to 314 nm (native protein), which shows that the unfolded protein refolds back to its native structure (Figure 8B,C). The unfolding experiment data obtained by the fluorescence spectrophotometer was further fitted into a three-state equation, and thermodynamic parameters were calculated for NmCyp (Figure 8D and Table 2). A comparison of NmCyp unfolding in the presence of urea with MrCyp under similar conditions\(^{20}\) (Figure 8A) suggests that NmCyp is significantly less stable than MrCyp. This pattern also coincides with the lesser thermal stability of NmCyp in comparison to MrCyp.

The ANS dye was used to estimate the exposure of hydrophobic patches upon the unfolding of NmCyp under the influence of urea. The $\lambda_{\text{max}}$ of the native protein in the presence of ANS was $\sim$508 nm. As the concentration of urea was increased from 0.1 to 0.5 M, a slight blue shift in the $\lambda_{\text{max}}$ along with an increase in fluorescence intensity was observed. The blue shift in the $\lambda_{\text{max}}$ and increased emission fluorescence intensity signify the exposure of the hydrophobic structure in the presence of urea. When the urea concentration was further increased from 1 to 4 M, a red shift from $\sim$508 to 520 nm was observed, indicating the unfolding of the tertiary structure of the NmCyp protein. After the 4 M urea concentration, there was no significant red shift or increase in intensity, which indicates the complete unfolding of the NmCyp protein (Figure 9A,B).

2.7. Equilibrium Unfolding and Refolding of NmCyp in the Presence of GdnHCl Using CD and Fluorescence Spectroscopy. To observe the effect of GdnHCl on the structural stability of NmCyp, GdnHCl-induced denaturation was measured with different probes. The NmCyp protein maintains its native structure only up to 1.0 M GdnHCl concentration. A decrease in CD signals (change in the ellipticity values) was observed when the concentration of GdnHCl increased beyond 1.0 M (Figure 10). The loss of ellipticity (mdeg values) at $[\theta]_{322}$ signifies the loss in the secondary structure of a protein. Similarly, the tertiary structure of the NmCyp protein was also lost beyond 1 M GdnHCl concentration (Figure 10). The change in ellipticity beyond 1.0 M remains constant, which signifies the complete unfolding of the NmCyp protein at this denaturant concentration. The data were further fitted into a two-state equation to estimate the thermodynamic parameters of NmCyp (Figure 10 and Table S3).\(^{20,40-42}\) The mid-point of unfolding ($C_{\text{MNU}}$) and cooperativity ($m$) for NmCyp is 0.75 $\pm$ 0.02 M and 4.45 $\pm$ 0.48 kcal mol$^{-1}$ M$^{-1}$, respectively.

The unfolding transition of the NmCyp protein was also monitored by plotting the change in $\lambda_{\text{max}}$ (emission wavelength) and fluorescence intensity at different GdnHCl concentrations. Like the urea unfolding profile, we found that the native structure of the protein is intact up to 0.3 M GdnHCl. The increase in GdnHCl concentration beyond 0.3 M results in a significant red shift in the $\lambda_{\text{max}}$ with a decrease in fluorescence emission intensity. At 3 M GdnHCl concentration, we found a significant increase in fluorescence emission intensity in addition to a shift in $\lambda_{\text{max}}$ from 314 to 350 nm (Figure 11A). The red shift in fluorescence emission indicates that the Trp (W67) residue is getting more exposed to the polar environment because of the unfolding of the protein in the presence of GdnHCl. On addition of GdnHCl, the intensity and $\lambda_{\text{max}}$ follow a sigmoidal-shaped curve, which is an indication of protein denaturation. It is clear from the plot that NmCyp loses its structure at a very low concentration of GdnHCl. The unfolding data fit best into the three-state equation to obtain the thermodynamic parameters (Figure 11B and Table S4).\(^{41-44}\)

During refolding studies, the NmCyp protein (unfolded in the presence of a highly concentrated GdnHCl) was diluted with a refolding buffer to the minimum possible denaturant concentration, where NmCyp regained its native secondary and tertiary structure after incubation for 1 h. This shows that NmCyp has the capacity to refold back to its native structure after denaturation with GdnHCl (Figure 11C,D). Gibbs free energy change as calculated for MrCyp (10.37 $\pm$ 1.5 kcal/mol) and NmCyp (4.8 kcal/mol) in the presence of GdnHCl suggested that the latter is significantly less stable than the former.

2.8. ANS Binding Reveals Surface Hydrophobicity in Unfolded NmCyp. The emission maxima of ANS bound to the NmCyp protein were monitored as a function of the increasing concentration of GdnHCl (Figure 12A). When the concentration of GdnHCl increased from 0 to 1 M, there were a slight blue shift in the emission maxima and an increase in intensity signifying the conformational changes in the structure even at lower GdnHCl concentrations. When the GdnHCl concentration was increased from 2.0 to 3.0 M, there was a slight red shift (508 to 516 nm) in $\lambda_{\text{max}}$ that suggests that NmCyp unfolds on increasing GdnHCl concentration. Beyond 3 M GdnHCl concentration, no significant shift in the $\lambda_{\text{max}}$ was observed, which indicates that the unfolding of the protein structure was complete at this concentration and all the hydrophobic patches were exposed to the polar environment (Figure 12B).

2.9. Effect of pH on NmCyp Structural Stability. No significant conformational changes in the secondary and tertiary structure of NmCyp were observed at alkaline pH.
This suggests that NmCyp is more stable at a pH range (6–11) as compared to an acidic pH range (<5).

The accessibility of tryptophan residue often varies from a completely buried to partially solvent-exposed state depending upon the folding and unfolding states of any protein. NmCyp at neutral pH has an emission maximum at 314 nm, which indicates that the single tryptophan residue (W67) is buried inside the protein core. Fluorescence emission spectra at acidic pH show a drastic red shift in $\lambda_{\text{max}}$ (314 to 345 nm) suggesting that the NmCyp protein unfolds under these conditions (Figure 14). The fluorescence intensity and $\lambda_{\text{max}}$ remain unchanged in the pH range (5–11), which indicate the stability of NmCyp at the pH range (5–11). The ANS fluorescence intensity was maximum at the acidic pH range (1–5), which suggests that hydrophobic patches are more exposed at acidic pH as compared to pH 7.0. The spectra of NmCyp at alkaline pH are like native spectra (pH 7), and overlapping spectra signify that the tertiary structure of protein is not affected by alkaline pH (Figure 14). NmCyp is more stable at the pH range (6–11) as compared to the acidic pH (pH < 5).

**Figure 12.** ANS-based extrinsic fluorescence studies of NmCyp at different GdnHCl concentrations. (A) Emission spectra of the protein in the presence of increasing concentrations of GdnHCl. The concentration of ANS used was 50 $\mu$M. (B) Change in emission maxima ($\lambda_{\text{max}}$) on increasing concentration of GdnHCl. In the figure, A.U. stands for “arbitrary units”.

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**Figure 13.** Effect of pH on the secondary and tertiary structure of NmCyp. (A) Far-UV CD spectra of NmCyp at different pHs (1–11). (B) A zoom-in view of the regions clustered close together in the pH range 5–11 for better visualization. (C) Near-UV CD spectra of NmCyp at different pHs (1–11). (D) A zoom-in view of the regions clustered close together in the pH range 5–11 for better visualization.

### 3. DISCUSSION

This is the first elaborate study of biochemical and biophysical characterization of any cyclophilin from Thaumarchaeota. Sixteen genomes of organisms from Thaumarchaeota have been sequenced up to now, and 15 of these contain the cyclophilin gene (Figure S1). From the alignment of protein sequences, it has been observed that a unique stretch of 7–10 amino acids is present in the cyclophilin homologs from Thaumarchaeota organisms, which is absent in cyclophilin orthologs coming from the organism of different phyla. This stretch lies close to the active site and may influence the function of this protein. The functional activity analysis of NmCyp established that the characteristic PPIase activity and its inhibition by CsA are conserved in this protein (similar to those seen earlier in cyclophilins from *Methanobrevibacter ruminantium* (MrCyp) of phylum Euryarchaeota) (Figure 3). Additionally, both NmCyp and MrCyp are similar in their response to pH changes as both exhibit structural stability in the pH range of 6–11 (Figures 13 and 14). In contrast, the structural loss and aggregation of NmCyp beyond 40 °C made...
it inept for the aggregation prevention assay of different aggregation-prone substrates, e.g., citrate synthase and BCAII. In our previous study, it was observed that the overexpression of MrCyp in *E. coli* cells made them more resistant to heat shock (at 45 °C), suggesting the chaperone-like activity of this protein in enhancing cell viability *in vivo*.20 Yet, a similar assay was not feasible for NmCyp since our current studies establish that it is a thermolabile protein that starts aggregating at 40 °C (Figure 6). Therefore, the chaperone-like activity, especially under conditions of heat stress, could not be generalized for all the archaeal cyclophilins.

Our current study suggests that NmCyp is more tolerant of chemical denaturation but is quite sensitive to thermal stress since it folds back in a reversible manner after the removal of denaturing agents but does not recover from the heat stress (Figures 7 and 10). A comparison between the MrCyp and NmCyp suggests that MrCyp is more resistant to urea denaturation, tolerating up to 6 M denaturant concentration, while NmCyp loses its structure in a lower urea concentration of 4 M urea. A similar pattern was also observed for their stability in GdnHCl. NmCyp unfolding—refolding in the presence of chemical denaturing conditions fits best to the two-state model (as observed by CD experiments), while a better fit to the three-state model (suggesting an additional intermediate state) was observed with fluorescence studies.

The quantitative estimation of Gibbs free energy changes shows that NmCyp is significantly destabilized over the highly stable PPIase MrCyp from phylum Euryarchaeota (Tables S3 and S4). This again suggests the higher stability of MrCyp over NmCyp. In conclusion, the collective comparison of chemical and thermal stabilities and PPIase activity between two PPIases from two different phyla suggests that although the PPIase activity may be the basic conserved function of the cyclophilin protein, the thermal stability and chaperone-like activity

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**Figure 14.** (A) The intrinsic fluorescence of NmCyp at different pHs from 1 to 11. Panel 1 shows a zoom-in view of the spectra recorded at pH 5–11 (for better visualization), and panel 2 shows the change in $\lambda_{max}$ and emission intensity at different pHs. (B) The ANS-mediated extrinsic fluorescence of NmCyp at different pHs ranging from 1 to 11. Panel 3 shows a zoom-in view of the spectra recorded at pH 6–11, and panel 4 shows the change in $\lambda_{max}$ and intensity of NmCyp in the pH range 1–11 after incubating with 50 μM of ANS. In the figure, A.U. stands for “arbitrary units”.

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(especially under heat stress) may be an add-on feature not necessarily found in all cyclophilins.\textsuperscript{35,46} The study may therefore pave the path for more studies that could deduce the evolutionary history of the different functions in cyclophilin proteins.

4. MATERIALS AND METHODS

4.1. Sequence Selection, Multiple Sequence Alignment, and Phylogenetic Tree Analysis of Archaeal Cyclophilin Proteins. A total of 122 protein sequences for cyclophilins from different archaeal species were retrieved from NCBI and downloaded in FASTA format. To remove the redundancy of sequences, CD-HIT\textsuperscript{47} was used with an identity threshold of 100%. The cyclophilins from a total of 106 archaeal organisms were selected for further analysis and were aligned by using different multiple alignment tools, i.e., Clustal Omega,\textsuperscript{49} Guidance,\textsuperscript{49} and T-COFFEE,\textsuperscript{50} to find the best alignment pattern and sequence conservation among the cyclophilin sequences. ProtParam was used to calculate the physicochemical properties, e.g., molecular weight, pI value, amino acid composition grand average of hydropathicity (GRAVY), and aliphatic index for the protein sequence of cyclophilin from \textit{Nitrosopumilus maritimus} (NmCyp). In the ExPasy server,\textsuperscript{51} the ProtScale tool was used for amino acid scale representation (Kyte and Doolittle hydrophobicity scale).\textsuperscript{52}

4.2. Functional Annotations of Selected Cyclophilin Sequences. Conserved domains and motifs were analyzed by sequence similarity with other members of the cyclophilin family and various protein databases like CDD-BLAST\textsuperscript{53} and InterProscan.\textsuperscript{54} InterProscan provides functional analysis of proteins by classifying them into families and predicting domains and functionally important sites.

4.3. Secondary and Tertiary Structure Prediction. The PDBSum\textsuperscript{55} and CDD-BLAST were used for predicting the secondary structure features. There is currently no structure available for any archaeal cyclophilin, so we modeled the structure of NmCyp using Modeller 9.17.\textsuperscript{32} Several parameters were taken into consideration when selecting the template for modeling, such as sequence identity (%), query coverage, and conserved domains. The electrostatic potential surface diagram and cartoon structure were visualized by PyMOL.\textsuperscript{56} The accuracy of the modeled structures was further validated using Ramachandran map calculations computed with the RAMPAGE software.\textsuperscript{33}

4.4. Cloning of the NmCyp Gene and Heterologous Overexpression of the NmCyp Protein. The gene encoding cyclophilin from \textit{Nitrosopumilus maritimus} was artificially synthesized and further subcloned into a pET28a (+) vector. Once the gene sequence was confirmed, the pET28a (+) NmCyp recombinant plasmid was transformed in \textit{E. coli} BL21-DE3. The expression of NmCyp was checked in auto induction media (AIM) and grown at 37 °C and 180 rpm. The pelleted cells were then resuspended in a lysis buffer (25 mM Tris, 100 mM NaCl, 10% glycerol, and 1 mM PMSF) and lysed through sonication. The overexpression of the recombinant protein was verified by western blot. An overexpressed NmCyp protein having a His-tag at the N-terminus was purified by affinity chromatography using a Ni\textsuperscript{2+}-NTA resin (Qiagen). The mixture was incubated at 4 °C for 3 h and eluted with an increasing concentration of imidazole (50–500 mM). The fractions obtained after elution were dialyzed against 1× PBS buffer at 4 °C to remove imidazole. After dialysis, the sample was subjected to size exclusion chromatography column to obtain the pure homogeneous protein. All the collected fractions were analyzed by separation on 15% SDS-PAGE at each step. The final protein yield was estimated to be ~36 mg/L. A molar extinction coefficient of 9970 M\textsuperscript{−1} cm\textsuperscript{−1} at 280 nm was used to determine the protein concentration.

4.5. Functional Activity Assays of the NmCyp Protein. 4.5.1. PPIase Activity Assay. The PPIase activity of the purified protein was estimated by a chymotrypsin-coupled reaction that exploits the conformational selectivity of chymotrypsin toward the chromogenic substrate of N-succinyl-Ala-Ala-Pro-Phe-4-nitroanilide.\textsuperscript{57} The assay is based on the difference in absorbance determined for the cis and trans isomers of Suc-Ala-Ala-Pro-Phe-4-nitroanilide. The peptide substrate N-succinyl-Ala-Ala-Pro-Phe-4-nitroanilide (Sigma) was resuspended in 0.47 M LiCl/trifluoroethanol (TFE) to produce a cis-proline isomer, and a stock solution of 5 mM was constituted. Chymotrypsin used in the study was dissolved in 0.1 mM HCl. Chymotrypsin dissolved in the assay buffer (35 mM HEPES-KOH, pH 7.8) served as the blank. The reaction was initiated by the addition of substrate (35 μM) to chymotrypsin, and then NmCyp (1.4 mM) in the same assay buffer was added to this mixture to study its effect on the PPIase activity. The PPIase activity was monitored by recording the absorbance at 390 nm every 0.1 s for 20 min. Inhibition of the reaction in the presence of the specific inhibitor cyclosporine A (CsA) was also checked by adding the inhibitor to the assay mix 30 min before the start of the reaction and incubating at 4 °C. The change in absorbance at 390 nm was measured, and experimental data fitting was performed using Origin 9.0. The catalytic efficiency of NmCyp was calculated using relation \( K_{cat}/k_m = (K_p - K_n)/E \), where \( K_p \) and \( K_n \) are the first-order rate constant of reaction in the presence and absence of the enzyme, respectively, \( E \) is concentration of the PPIase enzyme.

4.5.2. Chaperone-like Activity Assay. The chaperone activity of NmCyp was checked by estimating its ability to prevent the aggregation of bovine carbonic anhydrase (BCAII) and citrate synthase (CS).\textsuperscript{35,58} The substrate protein BCAII (0.75 μM) was placed at 65 °C in the thermostat cuvette holder of the Cary Eclipse Fluorescence Spectrophotometer to check the independent aggregation pattern of BCAII. The excitation and emission wavelength for BCAII was set at 400 nm and a slit width of 5 nm. The extent of light scattering of BCAII was monitored for 15 min. BSA was used as a negative control in both the experiments. The different molar ratios of NmCyp (1:1 and 1:2) and BCAII (0.75 μM) were also tried to see the concentration-dependent aggregation inhibition. Another substrate, citrate synthase (CS 0.15 μM), was also used for estimating the aggregation prevention efficiency at 45 °C. The same parameters mentioned above were used for measuring the aggregation kinetics. Concentration-dependent aggregation inhibition was tried at different molar ratios of NmCyp and CS.

4.6. Secondary and Tertiary Structure Estimation of the NmCyp Protein. Far-UV and near-UV CD measurements were recorded using a JASCO J-815 CD spectrometer. Near-UV CD measurements in the range of 250–300 nm were performed with 0.2 mg/mL protein using a cuvette of path length 1 mm. Far-UV CD measurements were recorded in the range of 260–350 nm using a higher protein concentration of 5 mg/mL in a cuvette of path length 1 mm. The data were
recorded for both the ranges using a scan speed of 100 nm/min and a spectral bandwidth of 1.0 nm. Three spectra were collected for both the ranges in the continuous mode and averaged to minimize the noise. The buffer was subtracted from the spectra to avoid buffer contribution.

Changes in the tertiary structures of the protein were analyzed by intrinsic fluorescence spectroscopy. The spectra were recorded on a spectro-fluorimeter (Cary Eclipse) using a quartz cuvette of 10 mm path length. NmCyp was excited at a wavelength of 280 nm with a slit width of 5 nm, and the fluorescence emission spectra were collected from 300 to 400 nm. A final concentration of 0.2 mg/mL of NmCyp in the PBS buffer was used for all unfolding studies.

4.7. Thermal Unfolding and Refolding Studies of NmCyp Using CD and Fluorescence Spectroscopy. For CD spectroscopy based thermal denaturation studies, NmCyp was diluted in the PBS buffer and placed in a 1 nm quartz cuvette. The samples were heated from 25 to 90 °C, incubating the sample at each temperature step for 5 min. Far-UV and near-UV spectra at all were collected at an interval of 5 °C. The millidegree (mdeg) values obtained were plotted against the respective temperatures. For the fluorescence-based thermal denaturation studies, 0.2 mg/mL NmCyp protein in a 10 nm quartz cuvette was placed in the thermostat holder of the spectrofluorimeter. At each selected temperature ranging between 25 and 90 °C, the protein was incubated for 5 min. The sample was excited at a wavelength of 280 nm, and spectra were recorded in the 300–400 nm range. The fluorescence intensity was plotted against the respective temperatures.

For far-UV and near-UV CD spectroscopy based thermal refolding studies, 90 °C heat denatured NmCyp was gradually cooled to 25 °C with 5 min incubation at each temperature (5 °C intervals). A continuous far-UV and near-UV scan was taken in the range of 250–190 and 260–350 nm, respectively, at each temperature step from 90 to 25 °C. The obtained mdeg data were plotted against the respective temperature.

For the fluorescence-based thermal refolding studies, 90 °C heat denatured 0.2 mg/mL NmCyp was gradually cooled to 25 °C after incubating for 5 min at each temperature step. The denatured protein was excited at a wavelength of 280 nm, and a continuous scan was recorded over the wavelength range of 300–400 nm from 90 to 25 °C. The fluorescence intensity values obtained were plotted against the respective temperatures.

4.8. Chemical Denaturant Mediated Unfolding and Refolding of NmCyp Monitored by CD Spectroscopy. For equilibrium unfolding studies, the protein samples were prepared by incubating 0.2 mg/mL of proteins with different concentrations of GdnHCl (0–8 M) and urea (0–8 M) for 30 min at 25 °C in the PBS buffer containing the respective amount of GdnHCl or urea. All the measurements were performed in both far-UV region (250–200 nm) and near-UV (260–350 nm) ranges. Each spectrum was corrected for contribution from a urea (0–8 M) or GdnHCl (0–8 M) containing a buffer solution. The obtained millidegree values were plotted against the respective urea and GdnHCl concentrations. For the equilibrium unfolding transition curve, data obtained at 222 nm were plotted with the respective concentration of the denaturant.

The highly concentrated NmCyp protein was incubated with 6 M urea and GdnHCl. The resulting unfolded protein was diluted, resulting in a final concentration of protein of 0.2 mg/mL and denaturant concentration of 0.2 M. The far-UV scan was recorded for the NmCyp protein in the range of 250–200 nm. For the spontaneous refolding, the far-UV CD signal for each refolded sample at 222 nm was recorded. Each signal was corrected for the contribution of the buffer solution containing urea and GdnHCl each. To see the percentage reversibility, the mdeg values obtained from the refolding data were plotted with unfolding data obtained from the equilibrium unfolding experiment.

4.9. Chemical Denaturant Mediated Unfolding Studies of NmCyp Monitored by Intrinsic Fluorescence Spectroscopy. For studying the effect of the chemical denaturant on the tertiary structure of MrCyp and NmCyp, urea and GdnHCl were used as denaturants. Samples were prepared by incubating 0.2 mg/mL protein with different concentrations of urea (0–8 M) and GdnHCl (0–8 M) at 25 °C for 30 min in the PBS buffer. The equilibrated samples were excited at 280 nm, and the emission spectra were recorded between 300 and 400 nm with excitation and emission slit width of 5 nm each. To see the equilibrium transition, the relative fluorescence intensity observed at 314 nm for NmCyp was plotted against the respective denaturant concentrations.

The highly concentrated protein sample was unfolded with 6 M urea and GdnHCl at 25 °C. The unfolded protein was diluted in a way resulting in a final concentration of 0.2 mg/mL and the denaturant concentration being diluted to 6 to 0.2 M in the PBS buffer. The refolding mixture was incubated for 1 h at 25 °C. The refolded protein was excited at 280 nm, and emission fluorescence spectra were recorded at 300–400 nm. To see the extent of reversibility of the unfolding transition, the relative fluorescence intensity at 314 nm (NmCyp) of each refolded sample was plotted with the unfolding transition curve obtained from the equilibrium unfolding experiment. The unfolding transition curves (urea and GdnHCl) were fitted to a two-state and three-state model for the calculation of thermodynamic stability parameters of the protein. 20,40,41,43

4.10. Data Fitting. The urea and GdnHCl induced unfolding data obtained from CD and fluorescence spectroscopy were further fitted into two- and three-state models. The two-state denaturation equilibrium indicates only native (N) and unfolded (U) states, while the three-state denaturation equilibrium indicates native (N), intermediate (I), and unfolded (U) states of a protein that are significantly present in the transition process. The equilibrium for the two-state and three-state models can be represented by Keq as follows: 41,43,44

For the two-state model:

\[ N \rightleftharpoons U \]  
\[ K_{NU} = \text{equilibrium constant for reaction } N \rightleftharpoons U. \]

The observed signal of the protein from both CD and fluorescence intensity observed at 314 nm for NmCyp (c) at any concentration of the denaturant is given by the sum contribution of all the two states as

\[ S_N(c) = S_{NU}(c) + S_U(c) \]

\[ f_N(c) \text{ and } f_U(c) \text{ are the fractions of two states at different urea and GdnHCl concentrations of } c, \text{ and } S_N \text{ and } S_U \text{ are the signal for pure N and U states, respectively.} \]

The fractions \( f_N \text{ and } f_U \) are also related to the equilibrium constant \( K_{NU} \) of unfolding transition from \( N \rightleftharpoons U \) and hence related to corresponding free energy changes \( \Delta G_{\text{NU}} \), as follows:

For the two-state equilibrium equation:
\[ f_N = 1/1 + K_{NU} = 1/[1 + \exp(\Delta G_{\text{NU}}/RT)] \]  
(3)

\[ f_U = \exp(\Delta G_{\text{NU}}/RT)/1 + \exp(-\Delta G_{\text{NU}}/RT) \]  
(4)

where \( R \) is the gas constant and \( T \) is the absolute temperature. The free energy changes of unfolding are known to vary linearly with denaturant concentration such that

\[ \Delta G_{\text{NU}} = \Delta G_{\text{NU}}^{0} + m_{\text{NU}} \cdot c \]  
(5)

So from combining eqs 1–5, we can get the equation shown below for the two-state model:

\[ S_{\text{obs}} = S_N + S_U \exp[-(\Delta G_{\text{NU}}/RT)] \]  
+ \[ \exp[-(\Delta G_{\text{NU}}/RT)] \]  
(6)

Here, we assume that \( S_N \) and \( S_U \) are linearly dependent on the denaturant concentration, so \( S_N = a_1 + b_1 \cdot c \) and \( S_U = c_1 + p_1 \cdot c \), where \( a_1 \), \( b_1 \), \( c_1 \), and \( p_1 \) are constants obtained from the intercept and slope of the native, intermediate, and unfolded state baseline, respectively. By using all the above-discussed equations, the denaturant mediated unfolding data were analyzed and thermodynamic parameters were calculated.

For the three-state model:

\[ N \rightleftharpoons I \rightleftharpoons U \]  
(7)

\[ K_{NI} = \text{equilibrium constant for reaction N \rightleftharpoons I} \]  
\[ K_{IU} = \text{equilibrium constant for reaction I \rightleftharpoons U} \]  
\[ K_{NU} = \text{equilibrium constant for reaction N \rightleftharpoons U} \]  

The observed signal of protein from both CD and fluorescence intensity of the protein \([S_{\text{obs}}(c)]\) at any concentration of the denaturant is given by the sum contribution of the entire three-state model as

\[ S_{\text{obs}}(c) = S_N f_N(c) + S_I f_I(c) + S_U f_U(c) \]  
(8)

\( f_N(c), f_I(c), \) and \( f_U(c) \) are the fractions of three states at different urea and GdnHCl concentrations of \( c \) and \( S_N, S_I, \) and \( S_U \) are the signal for pure N, I, and U states, respectively.

The fractions \( f_N, f_I, \) and \( f_U \) are also related to equilibrium constants \( K_{NI} \) and \( K_{NU} \) of the unfolding transition from \( N \rightleftharpoons I \) and \( N \rightleftharpoons U \), respectively, and hence are related to corresponding free energy changes \( \Delta G_{NI} \) and \( \Delta G_{NU} \) as follows:

\[ f_N = 1/(1 + K_{NI} + K_{NU}) \]  
= \[ 1/[1 + \exp(\Delta G_{NI}/RT)] \]  
\exp(\Delta G_{NU}/RT) \]  
(9)

\[ f_I = K_{NI}/(1 + K_{NI} + K_{NU}) \]  
= \[ \exp(\Delta G_{NI}/RT) + \exp(\Delta G_{NU}/RT) \]  
(10)

\[ f_U = K_{NU}/(1 + K_{NI} + K_{NU}) \]  
= \[ \exp(\Delta G_{NI}/RT) + \exp(\Delta G_{NU}/RT) \]  
(11)

where \( R \) is the gas constant and \( T \) is the absolute temperature. The free energy changes of unfolding are known to vary linearly with denaturant concentration such that

\[ \Delta G_{NI} = \Delta G_{\text{NI}}^{0} + m_{\text{NI}} \cdot c \]  
\[ \Delta G_{NU} = \Delta G_{\text{NU}}^{0} + m_{\text{NU}} \cdot c \]  

\( \Delta G_{\text{NI}}^{0} \) and \( \Delta G_{\text{NU}}^{0} \) are \( \Delta G_{NI} \) and \( \Delta G_{NU} \) at the 0 M denaturant concentration. \( m_{\text{NI}} \) represents the dependence of respective free energy change on the denaturant concentration \( c \) and the co-operativity of transition.

So, from combining eqs 1–4, we can get the equation shown below for three-state model:

\[ S_{\text{obs}}(c) = S_N + S_I \exp[-(\Delta G_{\text{NI}}^{0} + m_{\text{NI}} \cdot c)/RT] + S_U \exp[-(\Delta G_{\text{NU}}^{0} + m_{\text{NU}} \cdot c)/RT]/1 + \exp[(\Delta G_{\text{NI}}^{0} + m_{\text{NI}} \cdot c)/RT]/1 \]  
+ \[ \exp[-(\Delta G_{\text{NU}}^{0} + m_{\text{NU}} \cdot c)/RT]/1 \]  
(11)

Here, we assume that \( S_N, S_I, \) and \( S_U \) are linearly dependent on the denaturant concentration \( (c) \), so \( S_N = a_1 + b_1 \cdot c, S_I = c_1 + p_1 \cdot c \), and \( S_U = c_1 + g_1 \cdot c \), where \( a_1, b_1, c_1, p_1, c_1, \) and \( g_1 \) are constants obtained from the intercept and slope of the native, intermediate, and unfolded state baseline, respectively. By using all the above-discussed equations, the denaturant mediated unfolding data were analyzed and thermodynamic parameters were calculated.

4.11. Spectrofluorimetric Estimation of the Surface Hydrophobicity of NmCyp Proteins. ANS serves as a sensitive fluorogenic substrate for detecting exposed hydrophobic sites of a protein. The native and unfolded proteins (mediated by urea and GdnHCl) were incubated with ANS (50 \( \mu \)M) for 1 min at 25 \( ^\circ \)C before recording the spectra. The samples were excited at 390 nm, and emission spectra were recorded in the range of 400–600 nm with excitation and emission slit width of 5 and 2.5 nm, respectively. The protein in the PBS buffer with a concentration of 0.2 mg/mL was used for all the experiments. The buffer containing ANS and denaturant (Urea/GdnHCl) served as the blank.

4.12. pH-Dependent Secondary and Tertiary Structural Changes in NmCyp as Monitored by CD and Fluorescence Spectroscopy. The samples of the NmCyp protein were prepared in buffers of different pHs ranging from 1 to 11. The spectrum for all the protein samples was recorded in both the far-UV (250–190 nm) and near-UV (350–260 nm) range. Each spectrum was corrected for the buffer solution of the particular pH. The effect of pH on the tertiary structure was also monitored using fluorescence spectroscopy. The samples were excited at 280 nm, and the emission spectra were collected from 300 to 400 nm at 25 \( ^\circ \)C. To see the effect of pH on the surface hydrophobicity of NmCyp, the protein was incubated with 50 \( \mu \)M ANS and excited at 390 nm; emission spectra at different pHs were recorded in the range of 400–600 nm.
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Author Contributions
V.K. and M.G. planned the whole study. All the experiments except the revised enzymatic assay (PPIase assay) were done by Anchal and Vineeta Kaushik, respectively. V.K. and M.G. analyzed the data and wrote the manuscript. All authors have given approval to the final version of the manuscript.

Notes
The authors declare no competing financial interest.
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ABBREVIATIONS
Cyp-cyclophilin; Nm-Nitrosopumilus maritimus; PPIase-peptidyl prolyl cis-trans isomerases; CsA-cyclosporine A; BCAII-bovine carbonic anhydrase; CS-citrate synthase

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