Salt Dependence Conformational Stability of the Dimeric SAM Domain of MAPKKK Ste11 from Budding Yeast: A Native State H/D Exchange NMR Study

Anirban Bhunia[1, 3,ψ*], Humaira Ilyas[2ψ] and Surajit Bhattacharjya[1*]

[1]School of Biological Sciences, 60 Nanyang Drive, School of Biological Sciences, Singapore 637551, Singapore

[2]Department of Biophysics, Bose Institute, P-1/12 CIT Scheme VII (M), Kolkata 700 054, India

[3]Current address: Department of Biophysics, Bose Institute, P-1/12 CIT Scheme VII (M), Kolkata 700 054, India

[ψ]Both authors contributed equally

***Corresponding Authors**

Anirban Bhunia
Phone: (0091) (33) 2569 3336
Email: anirbanbhunia@gmail .com or bhunia@jcbose.ac.in

Surajit Bhattacharjya
Phone: (0065) 6316 7997
Email: Surajit@ntu.edu.sg

ABSTRACT: The sterile α motif, also called the SAM domain, is known to form homo or heterocomplexes that modulate diverse biological functions through regulation of specific protein-protein interactions. The MAPK pathway of budding yeast *Saccharomyces cerevisiae* comprises a three-tier kinase system akin to mammals. The MAPKKK Ste11 protein of yeast contains a homodimer SAM domain, which is critical for transmitting cues to the downstream kinases. The structural stability of the dimeric Ste11 SAM is maintained by hydrophobic and ionic interactions at the interfacial amino acids. Urea induced equilibrium unfolding process of the Ste11 SAM domain is cooperative without evidence of any intermediate states. The native state H/D exchange under sub-denaturing conditions is a useful method for the detection of intermediate states of proteins. In the present study, we investigated the effect of ionic strength on the conformational stability of the dimer using the H/D exchange study. The hydrogen exchange behavior of the Ste11 dimer under physiological salt concentration reveals two metastable partially folded intermediate states, which may be generated by a sequential and cooperative unfolding of the five helices of the fold. These intermediates appear to be the dominant species for the dynamic and reversible unfolding of the Ste11 SAM domain, underlining a significant pathway for its folding kinetics *via* hydrophobic collapse. In contrast, higher ionic concentration eliminates this cooperativity between the stabilizing pairs of helices.

INTRODUCTION

The sterile alpha motif (SAM) domain is recognized as a conserved protein-protein interaction module.[1-3] SAM domains are found to be expressed constitutively across several eukaryotic organisms[4] modulating diverse biological processes through the formation of homo or heterocomplexes.[5-7] Since their first identification SAM domains are known to adopt a globular structure characterized by five core helices,[8-11] which may or may not self-associate and bind to other SAM domains to bring about an array of regulatory functions, including cell signaling, enzyme localization, cell migration, and transcription and translation regulation, among others.[1, 11-13]

One such SAM domain is found in the N-terminal region (residues D37-R104) of Ste11, a protein kinase in the MAPK signaling pathway of *Saccharomyces cerevisiae*, where it plays a role in cell-type-specific transcription and signal transduction.[14, 15] Ste11 SAM domain interacts with the SAM domain of an adaptor protein, Ste50.[16, 17] Additionally, the Ste11 SAM domain has been shown to exist as a homodimer[14] in solution characterized by the canonical SAM-fold of five helices. Our previous work demonstrated that hydrophobic amino acid residues present at the dimer interface play a key role in maintaining the structural integrity of the Ste11 SAM domain, thus aiding the downstream interaction with Ste50 and modulating the MAPKKK functions.[18] In particular, the interfacial mutants, L57R, and L60R depicted reduced stability without any detectable interactions with Ste50 SAM. Also, interestingly, the structural stability of the dimeric Ste11 SAM was found to be determined by the salt concentrations in solution.[14, 15] At low salt concentration, the dimeric structure of the Ste11 SAM domain undergoes a time dependent structural transformation into helical oligomers of various sizes.[14,15] However, the molecular mechanism of the salt-dependent structural stability of the Ste11 SAM is not clearly understood. We hypothesize that partially unfolded intermediate states of Ste11 SAM may be responsible for the structural changes into helical aggregates under low salt concentration. However, the equilibrium

urea induced unfolding data of Ste11 SAM demonstrated a cooperative process without any evidence for populated folded monomers or partially folded intermediate states.[18] Therefore, we have utilized native state H/D exchange, at sub-denaturing concentrations of urea, for the characterization of lowly populated partially unfolded states of the Ste11 SAM domain.

As per the Boltzmann distribution, under native state conditions, any protein molecule has a small population of transient state intermediates occupying a higher than ground state energy level, called the partially unfolded forms (PUFs) and even under mildly denaturing condition protein molecules will cycle through these globally unfolded states.[19, 20] The prolonged hydrogen exchange (HX) behavior thus can be utilized to study the global protein unfolding pathway; the identification and study of these intermediates, therefore, pave the way for elucidating the mechanism of protein cooperativity as well protein folding and unfolding.[21-28] The hydrogen exchange study of native protein under mild denaturing conditions is realized through different procedures.[29-31] It reveals the potential metastable, partially unfolded intermediates, their interaction (exchange) behavior with the solvent, as well as the denaturant, that occur in the steps leading up to the completely unfolded state.[32-34] Such transitions are mostly cooperative and may involve a sequential unfolding of the structural units in tandem with their underlying counterparts.[35, 36]

In the present study, we elucidate the effect of monovalent salt in stabilizing the hydrophobic interfaces of the Ste11 SAM domain under urea-induced mild denaturing conditions. Our results indicate that the increase in salt concentration, up to 300 mM stabilizes the symmetrical dimer by increasing the free energy of unfolding (ΔG) with the midpoint of the complete denaturation process averaged at ~1.3M urea concentration. Further, native-state hydrogen/deuterium (H/D) exchange studies revealed that at a physiological concentration (150 mM) of salt, the Ste11 SAM domain undergoes cooperative unfolding in which the interfacial residues contributed by helix 4 and 5, and aided by helix 2 open first (Partially

Unfolded Form 1 or PUF1) thus exposing the buried residues from helix 1 and 3 (PUF2), allowing a stepwise unfolding of the protein. This sequential unfolding event, therefore, highlights the critical role of hydrophobic collapse in helix 1 and 3 to be the driving force for initiating folding of the Ste11 SAM dimer. In contrast, native state H/D exchange at 300 mM salt concentration, partially unfolded forms, could not be detected whereby the native dimeric state of the Ste11 SAM is in equilibrium with a globally unfolded state. We surmise that the partially unfolded states of Ste11 SAM populated at low salt concentration may be responsible for aggregation into helical polymers. Taken together, this study highlights an important aspect of SAM stabilization *via* the hydrophobic collapse that aids proper protein folding along with a subtle hint regarding the role of ionizable residues in stabilizing the native SAM domain at a lower concentration of salt.

MATERIALS AND METHODS

Protein Expression and Purification

Saccharomyces cerevisiae Ste11 SAM (D37-R104) (**UniProtKB – P23561**) domain[14] was purified as described previously.[18] Briefly, Ste11 SAM (D37-R104) was sub-cloned into pET14b vector with a thrombin cleavable His_6 tag and overexpressed in *E. coli* BL21 (DE3) by growing at 37°C either in LB or M9 medium containing [^{15}N] ammonium chloride (Cambridge Isotope Laboratories, USA). Upon reaching an OD_{600} of 0.6-0.7, the cells were induced with 0.3 mM IPTG for 6-12 hours at 15°C, to allow protein production. Next, cells were centrifuged and re-suspended in 20 mM Tris-hydrochloride buffer, pH 8.0. The cell suspension was lysed by sonication, and the supernatant was applied onto activated nickel-NTA columns for His-tag affinity purifications (Qiagen). The protein-loaded column was washed extensively with appropriate buffers to remove any unwanted and weakly bound proteins. The target protein was eluted with 500 mM imidazole and dialyzed against 10 mM sodium phosphate buffer containing 150 mM NaCl and 20 mM β-mercaptoethanol at pH 5.8. The amino acid sequence of Ste11 SAM was numbered as D1-R68.

The mutant protein L57R was generated using the QuickChange mutagenesis (Stratagene) kit, and the mutation was confirmed by DNA sequencing.

Circular Dichroism Measurements

A Chirascan circular dichroism (CD) spectrometer (Applied Photophysics Ltd., U.K.) was used to record the far-UV CD spectra of the dimeric Ste11 SAM with increasing concentration of urea at different salt concentrations, and study the changes in the folded population. The experimental sample was prepared using 10 μM of dimeric Ste11 SAM protein dissolved in 10 mM sodium phosphate, 20 mM β-mercaptoethanol, and varying concentration of NaCl (10, 50, and 150 mM) at pH 5.8. The sample was equilibrated for 15 minutes, with increasing concentrations of urea (0-6 M) before data recording. The spectra were collected from 190 to 230 nm at 20 °C using a 0.01 cm path length sandwich cuvette. Baseline correction was done, and an averaged scan of three spectral scans was reported. The data collection was performed with a spectral bandwidth of 1 nm and a step size of 0.4 nm for an improved signal-to-noise ratio. The fraction folded population of dimeric Ste11 SAM was estimated by normalizing the value of molar ellipticity (θ_M) obtained at 222 nm at different concentrations of urea relative to that observed in the absence of urea.

NMR Spectroscopy

All the NMR experiments were performed on a Bruker DRX 600 MHz spectrometer equipped with an actively shielded cryoprobe. The NMR data were processed using the NMRPipe[37] and NMRDraw[38] suite and analyzed by Sparky (T.D. Goddard and D.G. Kneller, University of California, San Francisco). The chemical shifts were indirectly referenced to DSS.

NaCl Titration Experiment

To study the effect of varying NaCl concentration, the amide proton exchange was recorded. Lyophilized powder of Ste11 SAM was dissolved in a D_2O buffer of 10 mM sodium phosphate, 20 mM β-mercaptoethanol, and varying concentration of NaCl (50, 150, and 300 mM) at pH 5.8. A series of two-dimensional ^{1}H-^{15}N HSQC spectra were recorded within 30 min interval, and hydrogen exchange rates (k_{ex}) were fitted to a single-exponential decay equation as described in *Data Fitting* to calculate the protection factor.

A similar experiment was also conducted with ^{15}N-labeled interfacial mutant L57R, prepared in a D_2O buffer of 10 mM sodium phosphate buffer, 150 mM NaCl, and 20 mM β-mercaptoethanol, pH 5.8, to deduce the role of interfacial residues.

Urea Denaturation Experiment

Titration experiments with urea were recorded at 15°C for the Ste11 SAM domain. 0.4 mM ^{15}N-labeled native Ste11 SAM was titrated with aliquots from a 9 M stock solution of urea, prepared in 10 mM sodium phosphate buffer, 20 mM β-mercaptoethanol, and varying concentration of NaCl (10, 50, and 150 mM) at pH 5.8. The samples were equilibrated for 20 minutes, and two-dimensional ^{1}H-^{15}N HSQC spectra were recorded. The HSQC cross-peaks intensity reduction as a function of the denaturant concentration was fitted to the following equation,[39]

$$Y_{obs} = \frac{(\alpha_N + \beta_N[D])}{1 + exp\left\{-\left[\frac{m([D] - Cm)}{RT}\right]\right\}} \qquad \text{Eq. 1}$$

where Y_{obs} represents the intensity of an HSQC peak, α_N and β_N are the intercept and slope of the pre-unfolding regimes, respectively, m is the slope at the midpoint of the unfolding transition, [D] is the concentration of the denaturant, C_m is the denaturant concentration at the midpoint of the transition, and RT is 0.57 kcal mol^{-1}.

Additionally, two-dimensional ^{1}H-^{15}N HSQC spectra were also recorded for 0.4 mM ^{15}N-labeled native Ste11 SAM treated with sub-denaturing concentration (~1.5 M) urea, prepared in 10 mM sodium phosphate buffer, 20 mM β-mercaptoethanol, and varying concentration of NaCl (150, and 300 mM) at pH 5.8.

Native State Hydrogen-Deuterium Experiment

Two-dimensional ^{1}H-^{15}N HSQC spectra were recorded for 0.4 mM ^{15}N-labeled native Ste11 SAM, titrated with sub-denaturing concentration (from 0 to 1.5 M) urea, prepared in a D_2O buffer of 10 mM sodium phosphate buffer, 20 mM β-mercaptoethanol, and varying concentration of NaCl (150, and 300 mM) at pH 5.8, within 30 min interval. The intrinsic residue-specific free energy of unfolding was

calculated and plotted against the concentration of urea to ascertain the unfolding patterns at different salt concentrations.

Data Fitting

The decreasing peak intensity obtained over time for each residue under the aforementioned conditions of two-dimensional ^{1}H-^{15}N H/D exchange NMR experiments were fitted to a single-exponential decay equation,

$$I(t) = I(0) \times exp(-k_{ex} \cdot t) \qquad \text{Eq. 2}$$

Where, I(0) and I(t) are the amide proton intensities at time 0, and t mins, respectively; and k_{ex} is the rate constant of the observed hydrogen-deuterium exchange reaction.

Next, the protection factor (PF) was calculated by taking a ratio of the calculated intrinsic exchange rate[40] (k_{int}) and the experimentally determined exchange rate of the unprotected amide proton (k_{ex}).

$$PF = k_{int}/k_{ex} \qquad \text{Eq. 3}$$

The free energy of exchange (ΔG_{HX}) was further deduced by using the equation (4) as below,

$$\Delta G_{HX} = -RTlnK_{op} \qquad \text{Eq. 4}$$

$$or, \Delta G_{HX} = -RTln\,(k_{ex}/k_{int}) \qquad \text{Eq. 5}$$

where K_{op} is the equilibrium rate constant for protein unfolding, R is the universal gas constant (0.008314 kJ mol^{-1} K^{-1}), and T is the temperature in Kelvin.

RESULTS

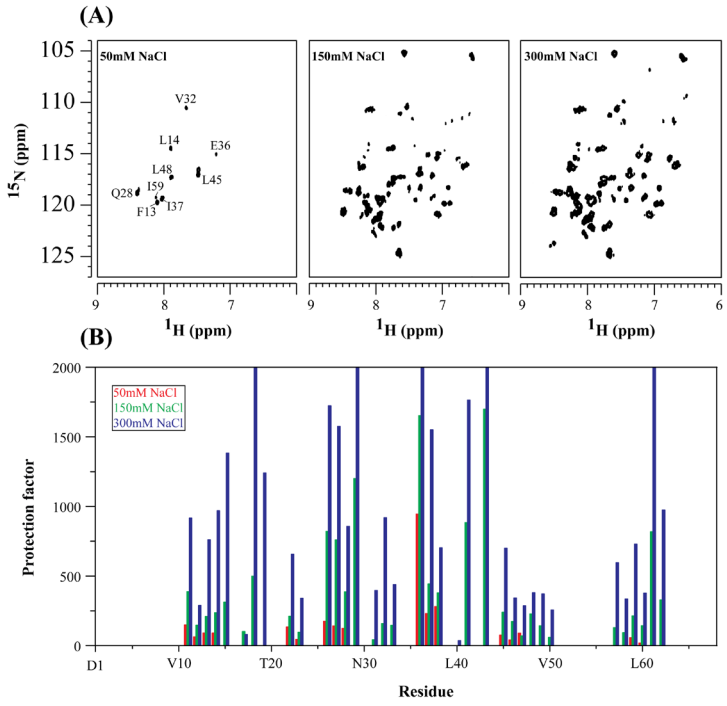
The Ste11 SAM domain is stabilized by salt.

The conformational stability of the Ste11 SAM was deduced by a series of native-state H/D exchange studies in the absence of any denaturant, recorded with increasing concentration of sodium chloride (NaCl) from 50 mM to 300 mM. As shown in Figure 1A, ^{1}H-^{15}N HSQC spectra of Ste11 SAM were characterized by an increasing number of HSQC cross-peaks of higher intensity with increasing

concentration of NaCl. At 50 mM NaCl concentration majority of the backbone amide of the native Ste11 SAM exchanged with the solvent D_2O within 60 minutes. A comparison of the protection factor (PF) of the amide protons, calculated from the HSQC cross-peaks intensity profile, for individual amino acid residues at different concentrations of NaCl depicted the same (Figure 1B). Due to the fast H/D exchange, we could calculate the PF for only sixteen residues at 50 mM concentration of NaCl. Significantly, the low value of the PF was observed for the majority of the residues at this concentration of salt. However, upon increasing salt concentration up to 300 mM, the value of PF increased by several folds, signifying overall conformation stability and integrity provided by NaCl. It is important to note that residues F13, L14, F26, I27, V32, I37, and I59, involved in hydrophobic packing that forms the signature five-fold helices remained protected. The same was also indicated by the value of free energy of unfolding (ΔG_{UN}) for the five helices, which increased with increasing salt concentration (Figure 1C). Such a linear increase (Supporting information Figure S1) in ΔG_{UN} upon an increase in salt concentration indicated native state stabilization *via* Hofmeister effects. Additionally, residues like Q11, E15, Q28, E36, D41, and R61 with the propensity to form salt bridge interaction remain stabilized. Furthermore, it should be noted that interfacial hydrophobic residues from helix 4 and helix 5 (*viz*. I44, I46, A47, I53, L57, and L60), implicated with stabilization of the dimeric interface, depicted a comparatively much less stabilization upon an increase in the salt concentration.

Urea Induced Unfolding of the Native Ste11 SAM Domain

Figure 2 summarizes the unfolding characteristics of the native Ste11 SAM dimer under the varying concentration of NaCl, from 10 to 150 mM, when titrated with increasing concentration of urea. Unfolding behavior was initially monitored using far-UV CD spectra. As shown in Figure 2A, the change in the folded population was deduced using the value of the molar ellipticity (θ_M) recorded at 222 nm and normalized relative to the same recorded in the absence of the denaturant urea. The global unfolding study of the Ste11 SAM dimer was highly cooperative and depicted a gradual increase in the mid-point of denaturation from ≈ 2.8 M of urea at 10 mM NaCl to ≈ 4.2 M at 150 mM NaCl. This highlights the stabilizing effect of salt on the conformational stability of the native Ste11 SAM dimer.



(C)

ΔG (*kcal/mol*)	50mM	150mM	300mM
Helix 1	4.54	5.09	6.14
Helix 2	4.63	5.29	6.25
Helix 3	5.36	5.65	6.59
Helix 4	4.33	5.25	6.29
Helix 5	3.14	5.02	6.13

Figure 1. (A) Representative ^{1}H-^{15}N HSQC spectra of the native Ste11 SAM in the presence of different salt concentrations after 60 minutes against solvent D_2O. (B) Bar diagram showing the distribution of the protection factor of the amide protons of the native Ste11 SAM in different salt conc. (C) Comparison of free energy change at the atomic level in the presence of varying salt concentrations.

Furthermore, a series of two-dimensional ^{1}H-^{15}N HSQC spectra were also recorded under similar conditions. Attenuation of the HSQC cross-peaks intensity of the native Ste11 SAM dimer was observed in all

the experimental set-ups as a function of urea concentration (data not shown). This equilibrium-unfolding event was further analyzed by the two-state unfolding transition, and the mid-point of unfolding transition (C_m) was calculated at different NaCl concentrations (Supporting information Figure S2). The C_m values from NMR experiments corroborated well with the CD data. A plot of the residue-specific difference (ΔC_m) in the value of C_m at 10 mM and 150 mM NaCl gave a clear indication that residues like F9, V10, L12, and F13 of helix 1 show maximum stability, indicated by high ΔC_m value. This further depicts the stabilization of the native Ste11 SAM dimer with increasing concentration of NaCl by stabilization of key hydrophobic and aromatic amino acid residues (Figure 2 B-C). Of note, the value of ΔC_m was averaged at ~1.25 M urea (Figure 2B), making it an ideal condition to study residue-specific cooperativity that allows the unfolding, as also indicated by the unperturbed amide proton resonance of the ^{1}H-^{15}N HSQC spectra, especially for residues F9, V10, L12, F13, F26, and F66 forming a hydrophobic hub that stabilizes the individual Ste11 SAM helix fold (Figure 3).

Taken together, these results highlight the native stability of Ste11 SAM dimer to urea induced denaturation, with complete unfolding at [urea] of 6 M, and the underlying cooperativity of the unfolding event.

Salt Concentration Influences the Mechanism of Unfolding.

Two-dimensional ^{1}H-^{15}N HSQC experiments were conducted to deduce the residue-specific H/D exchange of Ste11 SAM dimer upon treatment with 1.5 M urea under the varying concentration of NaCl. A plot of the reside-specific free energy of exchange as a function of urea concentration gave insights into the nature of unfolding events as well as helped to differentiate between the urea-dependent unfolding event and urea-independent local fluctuation events.

As shown in figure 4, the value of ΔG_{HX} remained independent of urea concentration at the initial points

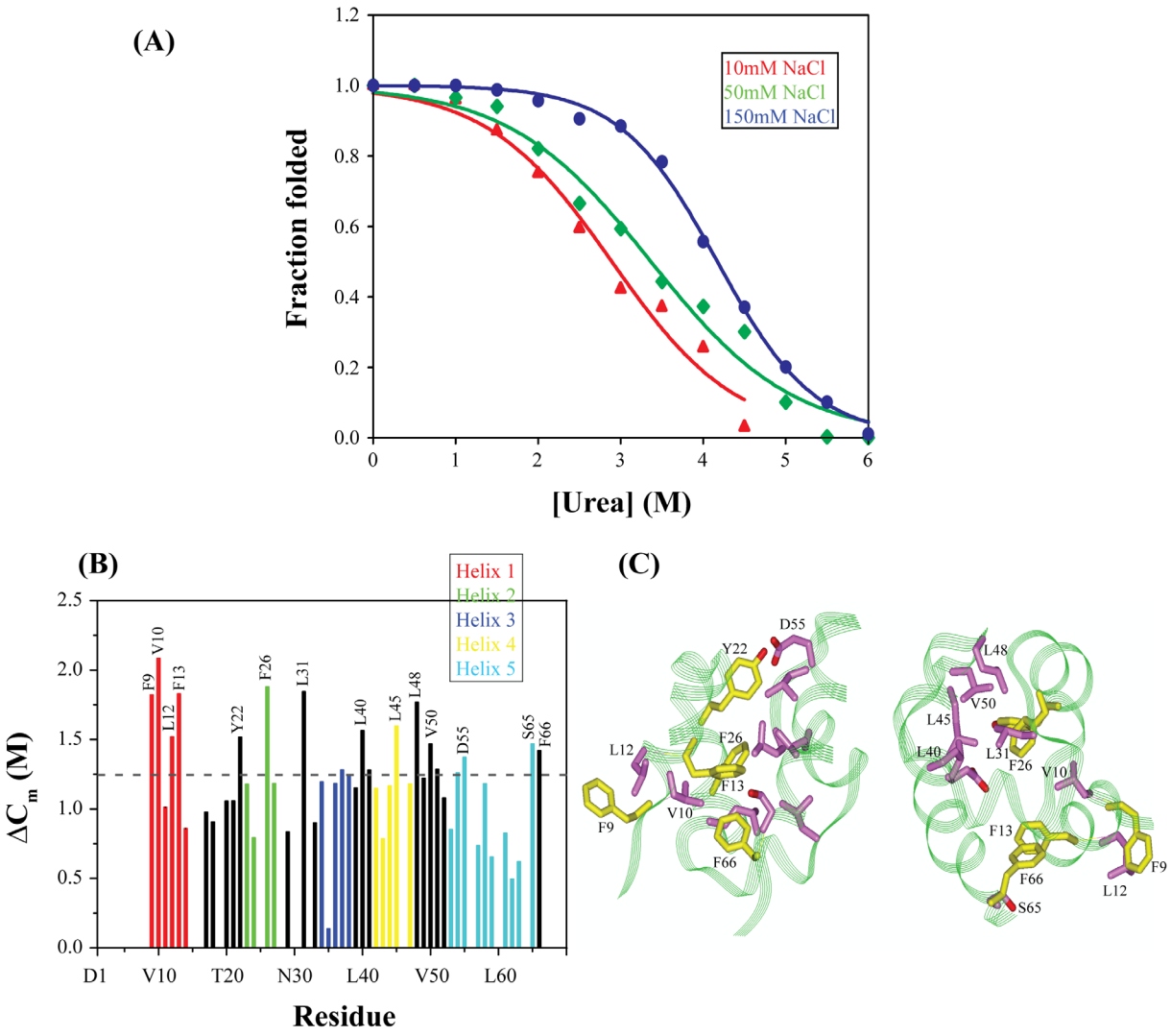


Figure 2. (A) Change in the native folded population of the dimeric Ste11 SAM with increasing concentrations of urea at different salt concentrations (B) Bar diagram depicting the change of concentration of urea at the midpoint (C_m) of unfolding transition as a function of the residue of the native Ste11 SAM domain. The dashed line indicates the average ΔC_m value of the Ste11 SAM domain to be ≈ 1.25 M. (C) Structural positions of the amino acids of the dimeric Ste11 SAM domain is illustrated.

of titration, indicating local structural fluctuations to be the dominant forces that allow transient

deprotection of the amide protons. This is especially true for residues L23, and I27 from helix 2, and I46, L57, I59, and L60, from helix 4 and 5, already established to be crucial for stabilization of the dimer interface, that merge to an almost similar free energy of unfolding (ΔG_{HX}), initiating the unfolding of the Ste11 SAM dimer, and forming the first cooperative unit of unfolding (PUF1). This triggers the opening of helix 1 and 3, the second cooperative unit

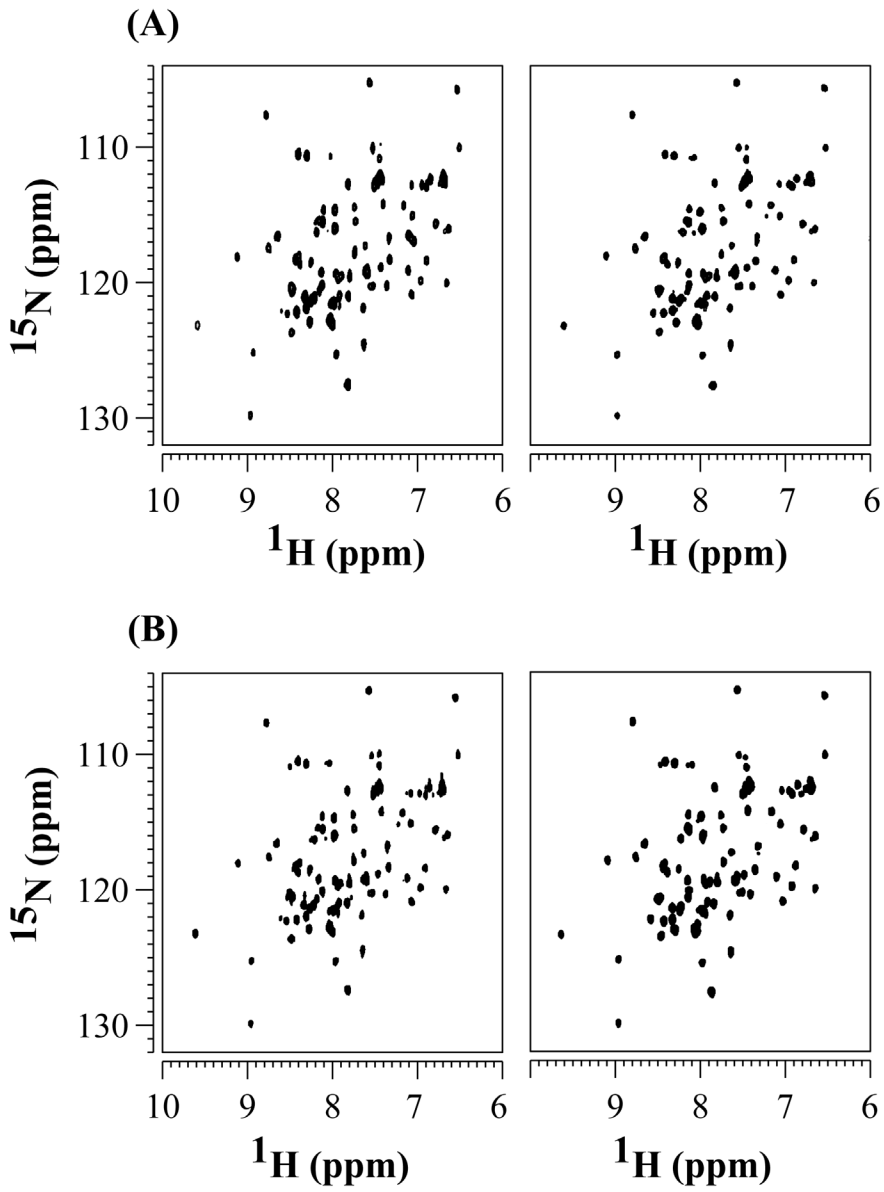


Figure 3. ^{1}H-^{15}N HSQC spectra of Ste11 SAM in the absence (left panel) and presence of 1.5 M urea (right panel) recorded at (A) 150 mM, and (B) 300 mM NaCl concentration. The denaturant does not change the conformation of the dimeric Ste11 SAM as the chemical shifts of the amide protons are not affected. The NMR experiment was performed with Bruker DRX 600 MHz, equipped with cryoprobe.

of unfolding (PUF2) (Figure 4 C and D). The residues from these helices are dominated by a similar

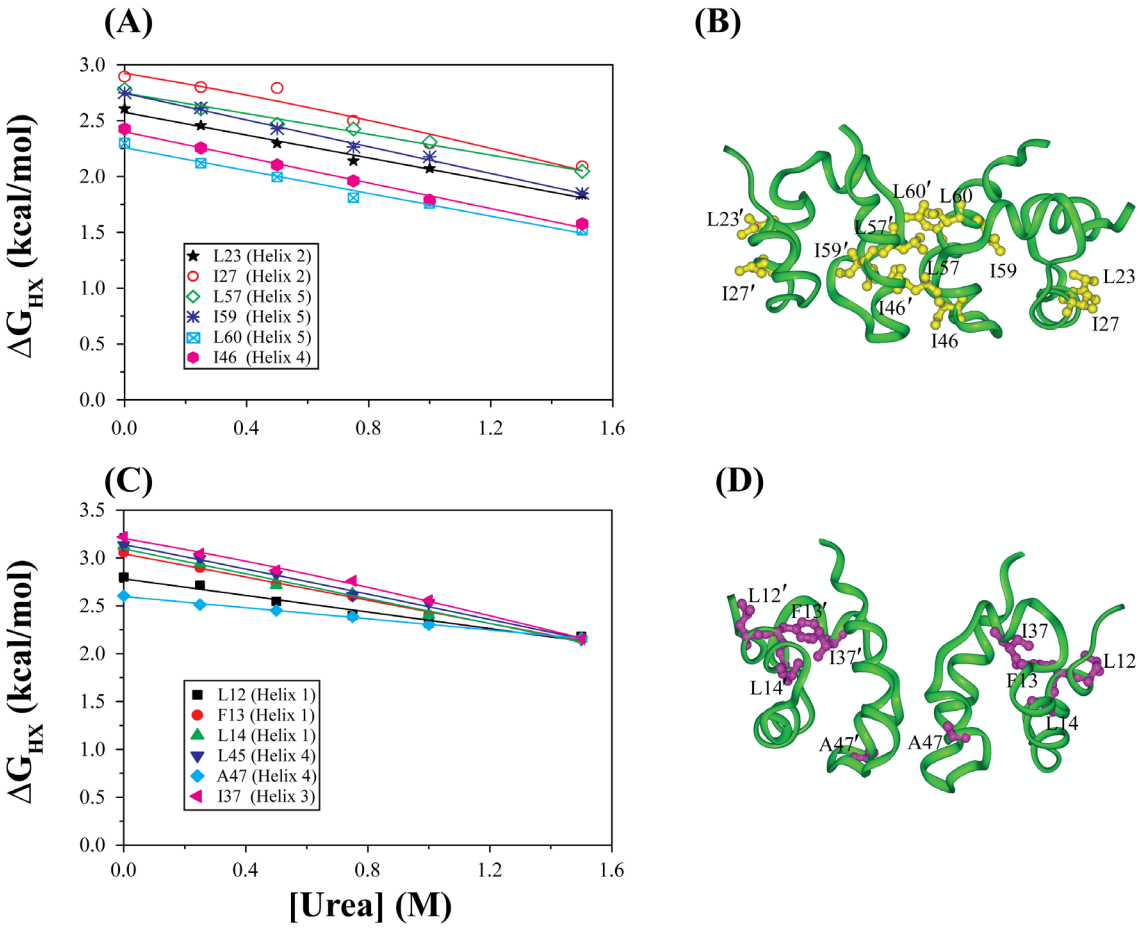


Figure 4. (A and C) Native state hydrogen-deuterium exchange results of Ste11 SAM. HSQC experiments were carried out at a protein concentration of 0.4 mM in 10 mM sodium phosphate buffer, 150 mM NaCl and 20 mM β-mercaptoethanol, pH 5.8. (B and D) Corresponding structural positions of amino acids of dimeric Ste11 SAM is illustrated. The NMR experiment was performed with Bruker DRX 600 MHz, equipped with cryoprobe.

opening event, allowing a stepwise unfolding initiation, caused by a sufficiently high concentration of urea (≈ 1.5 M), but still far below the global unfolding transition. Taken together, the overlay of the transition curve of residues from a discrete region of the dimer at 150 mM NaCl indicates cooperativity among those regions at the initial stage of dimer unfolding, hinting at the possible transiently unfolded intermediates.

In contrast, at 300 mM NaCl, the transition curve shows a close juxtaposition for residues corresponding to different regions of the protein dimer with no detectable transient intermediate populations (Figure 5

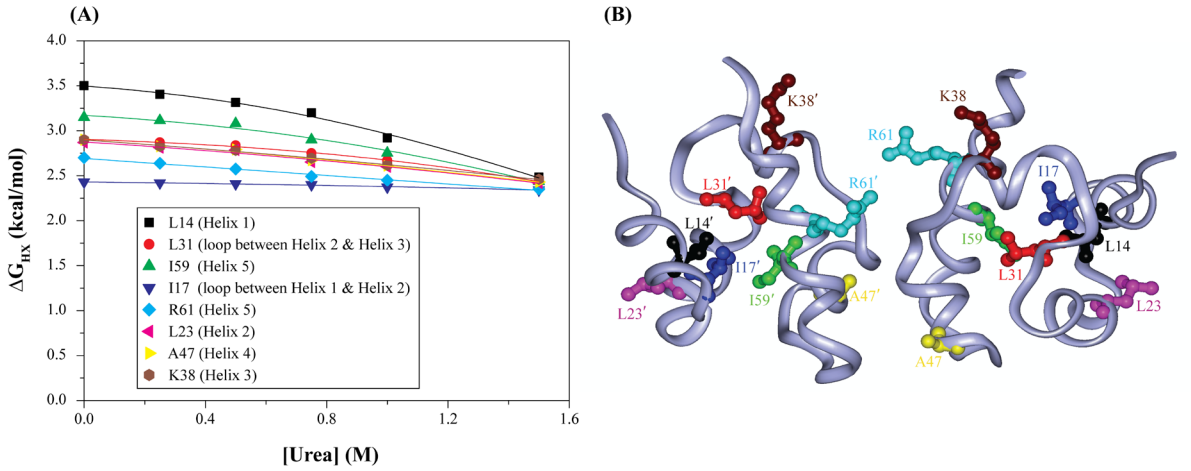


Figure 5. (A) Native state hydrogen-deuterium exchange results of Ste11 SAM. HSQC experiments were carried out at a protein concentration of 0.4 mM in 10 mM sodium phosphate buffer, 300 mM NaCl and 20 mM β-mercaptoethanol, pH 5.8. (B) Corresponding structural positions of amino acids of dimeric Ste11 SAM is illustrated. The NMR experiment was performed with Bruker DRX 600 MHz, equipped with cryoprobe.

A and B). Additionally, few positively charged residues like K38 and R61 also participate in the global unfolding kinetics. An important point of consideration here is the fact that residues from helix 1 (L12, F13, and L14) are comparatively more stabilized, highlighting their prime importance in the structural and functional attributes of the Ste11 SAM protein.

Conformational Stability of the Interfacial Mutant L57R

Unfolding studies with the Ste11 SAM dimer constituting the L57R mutant was conducted by recording the H/D exchange experiments at 150 mM NaCl. The mutant dimer depicted complete global unfolding as the backbone amide protons were almost entirely exchanged with the solvent deuterium. Thus the value of free energy change could be calculated only for 11 residues (Figure 6). It is worth mentioning that the average value of the free energy change was ≈ 2.1 kcal/mol, corroborating closely with the free energy change of PUF2. This could be attributed to the loss of the stabilizing effect of the L57 residue, indicating

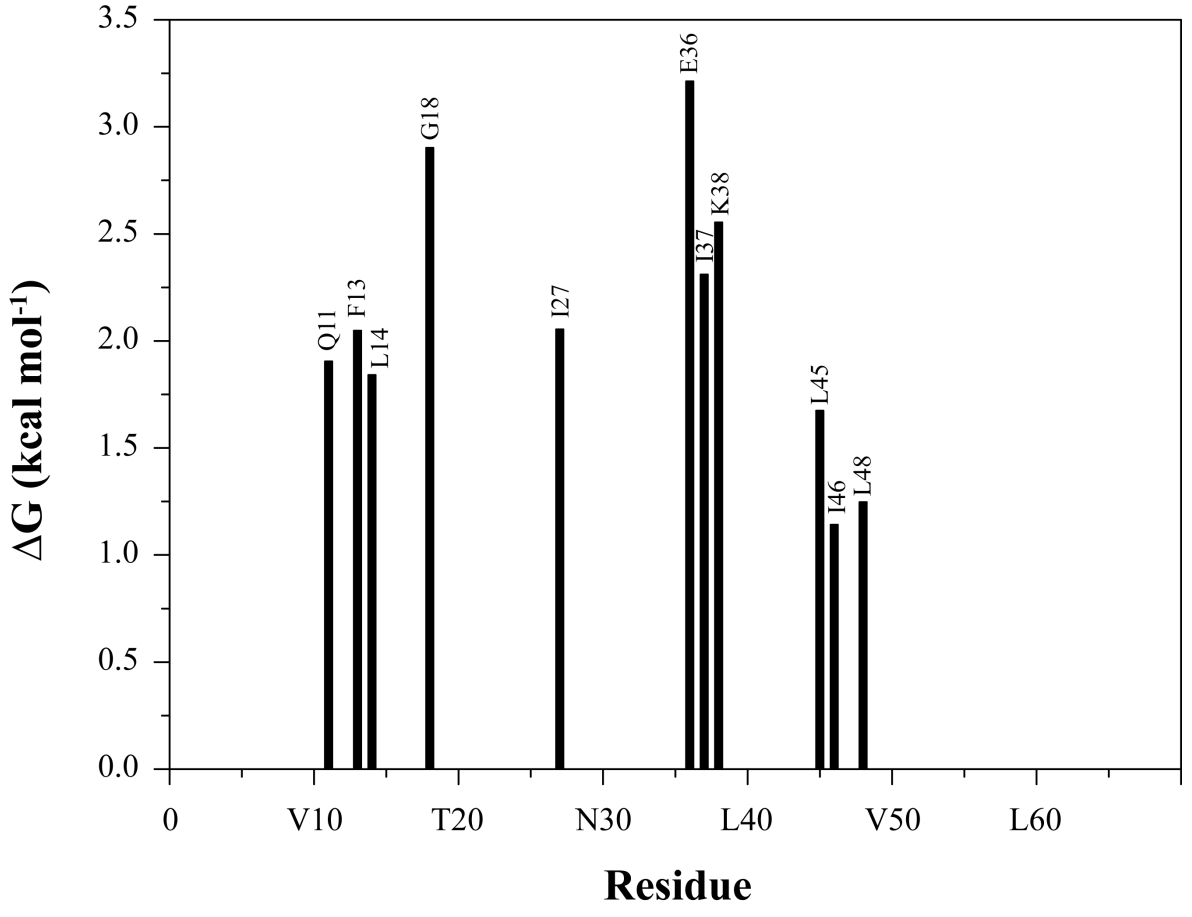


Figure 6. Bar diagram showing the free energy change of L57R Ste11 SAM mutant protein at the residue level estimated from the H/D experiment in the presence of 150 mM of NaCl. The experiment was performed with Bruker DRX 600 MHz, equipped with cryoprobe.

that salt-induced stabilization of the Ste11 SAM protein and the cooperative unfolding event is aided by dimer stabilization by the interfacial amino acid residues.

DISCUSSION

Protein folding is a complex multi-level procedure with vast biological implications.[28, 41-43] As such, a wide number of opposing hypotheses have emerged, trying to explain the different folding behaviors exhibited by the huge repertoire of proteins found in nature.[35, 44, 45] Of this, the classical pathway assumed that the proteins have a well-defined folding pathway, which is strewn with certain distinct intermediate forms. In contrast, the new-view pathway inferred that proteins fold into their native structures *via* multiple routes and intermediates, pictured in the form of a funnel-shaped reaction landscape. The sparsely

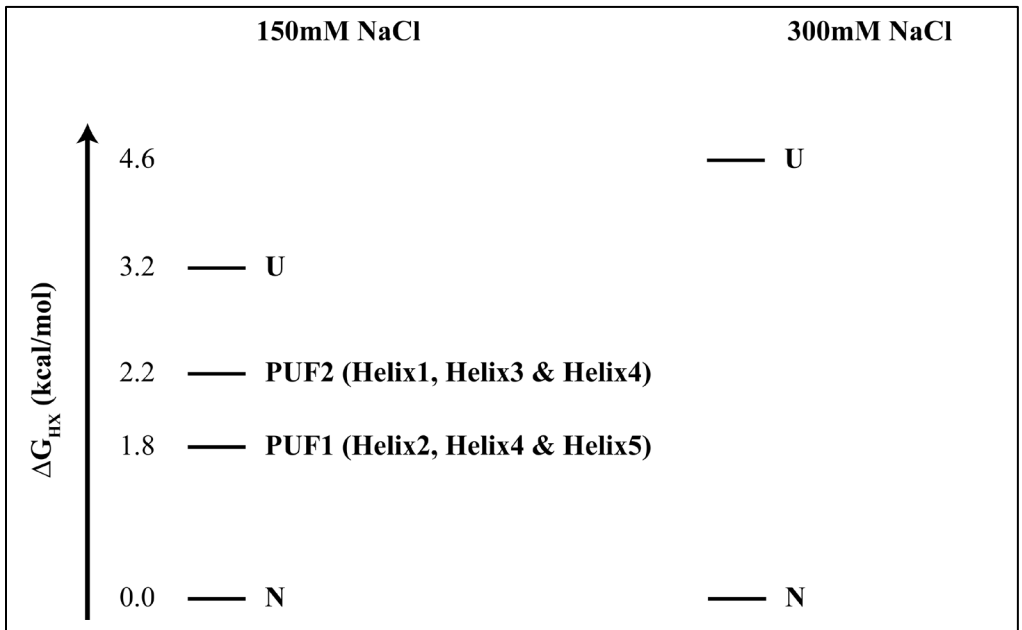


Figure 7. Energy diagram depicting the unfolding kinetics of the Ste11 SAM domain under differential NaCl condition, upon treatment with a sub-denaturing concentration of urea. At 150 mM NaCl concentration, mild treatment with urea generates sequential unfolding with two partially unfolded forms (PUF 1 and 2), which lead to a complete global unfolding. These intermediates, however, do not appear at 300 mM NaCl concentration.

populated structural intermediates with near-native conformation and varying energy levels help to regulate the protein structure and its adaptability, the equilibrium between the folded and unfolded states, as well as the effect of an external molecule like ligand, denaturant, etc. on the energy landscape.[46, 47] Hence, the disparity in assumptions and the heightened necessity to understand protein folding makes it very important to study the structural attributes of the intermediates occurring between the native and unfolded states. However, their short lifetime (<1 s) and low population make it difficult to probe their structural features using basic biophysical techniques.[48] Additionally, an understanding of the factors regulating protein stability and their correlation to the molecular structure is an important aspect for understanding the forces involved in folding and unfolding kinetics. Studying these dynamical attributes and structural configurations act as an important tool to clear our understanding of the free energy landscape as well as the functional characteristics of the protein.

A meaningful way to study the protein folding intermediates is by way of structural perturbations in the native protein folding process, caused by altering one of the folding parameters like pH, temperature, ionic strength, etc.[23] In the current work, we have explored the effect of monoprotic salt, NaCl, on the conformational stability of the Ste11 SAM dimer domain using NMR-based hydrogen-deuterium exchange (HX) studies, at sub-denaturing concentrations of urea. Earlier studies have reported upon the ability of high concentrations of salt to allow the accumulation and stabilization of folding/refolding intermediates *via* a salt-induced collapse state.[49, 50]

The native structure of the SAM domain undergoes global unfolding only at a concentration as high as 6 M urea, by reduction of the energy gap between the native and excited states.[18] Therefore the metastable unfolded structural intermediates were generated using a low concentration of urea, lying much below the unfolding transition region as indicated by the well-dispersed chemical shifts of the amide protons and lack of any new cross-peaks corresponding to the unfolded forms. It should be noted that native-state

hydrogen-deuterium exchange studies allow detection and probing of the infinitesimally populated folding intermediates under mildly destabilizing conditions, thus allowing to deduce the detailed mechanism of denaturant action and protein unfolding.[51] It should be noted that though chaotropic mediated unfolding of the SAM domains from different proteins has been extensively studied and reported,[18, 52] the effect of salt concentration on the conformation and dynamics remains unreported.

Mechanism of salt-induced stabilization of Ste11 SAM

The H/D exchange profile of Ste11 SAM recorded with increasing concentration of NaCl indicate salt-induced stabilization. This could occur through several different mechanisms. Firstly, the non-specific Coulombic effect allows the specific binding of salt ions to the surface charge of the Ste11 SAM, thus helping to reduce the repulsive charge-charge interactions.[53, 54] The stabilization of residues like Q11, E15, Q28, E36, D41, and R61, having the propensity to form salt bridge interactions, support this mechanism. It has been observed that salts promote stability *via* specific anion binding and interaction. Ste11 SAM has two clusters of negatively charged residues, and specific binding with Na^+ can be assumed to increase the overall stability. Such effect has earlier been reported for Barster, an inhibitor protein of the extracellular RNase, barnase, in the bacterium *Bacillus amyloliquefaciens*.[50]

However, such ion binding and stabilization are usually observed at a low concentration of salts (generally below 200 mM) and exhibit a dependence on the logarithmic salt concentration.[54] However, Ste11 SAM depicts a linear dependence on the concentration range of NaCl studied, indicating an additional mechanism of salt-induced stabilization. An alternative would be to assume that at higher concentration, NaCl promotes preferential hydration of the loci containing the charged residues imparting thermodynamic stability. This is the so-called Hofmeister effect or the ion-specific effect, observed at a salt concentration above 100 mM.[54] It helps to stabilize proteins by decreasing the solubility of the non-polar residues and thus strengthening hydrophobic interactions, as was visible by the high PF of residues

F13, L14, F26, I27, V32, I37, and I59, involved in hydrophobic packing that forms the signature five-fold helices. Also, the 'salting out' effect is a common phenomenon observed at a higher concentration of salt, facilitating protein-protein interaction *via* hydrophobic interaction.

Sub-denaturing urea concentration generate dynamical structural changes in Ste11 SAM

Amide protons of several residues distributed among all five helices of Ste11 SAM remain highly protected from solvent D_2O exchange. As the majority of the critical residues remain protected due to hydrogen bonding and/or burial in the native structure, the urea induced unfolding of Ste11 SAM is thus significantly influenced by the concentration of NaCl in the solution. Titration with increasing concentration of urea probed at different concentration of NaCl (ranging from 10 mM to 150 mM) by recording a series of far-UV CD spectra and two dimensional ^{1}H-^{15}N HSQC spectra and fitting into a two-state unfolding transition revealed an upward shift in the midpoint of transition (C_m), with no apparent unfolding till 2 M of urea concentration. The complete unfolding of the native Ste11 SAM domain occurred only at about 6 M concentration of urea, allowing us to study the structural and dynamics perturbations at <2 M urea concentrations. The addition of sub-denaturing concentration of urea enables perturbations to occur only at the near-native conformation of the protein *via* gradual unfolding and generation of partially unfolded forms. Such intermediates have low structural rigidity due to local fluctuations causing hydrogen bond breakage and thus exhibit a heightened rate of amide proton exchange, depicted by the reduction in the PF when studied using equilibrium hydrogen-deuterium exchange experiments. As such, it allows studying the structural motifs destabilized by the denaturant urea and the effect of NaCl concentration in imparting conformational stability. Additionally, these intermediates also give an insight into the secondary structural cooperative units, or foldons, which are present in the protein[35, 55]. A comparison of the studies performed at 150 mM and 300 mM NaCl

concentration delineate the underlying stabilizing effect of salt on the dimer structure and also reveal the structural units of unfolding.

NaCl concentration has the varying effect of Ste11 SAM stabilization

At 150 mM NaCl concentration, the amide protons in helix 2, 4, and 5 merge to an almost similar ΔG_{HX} forming a cooperative unit (PUF1, a foldon) *viz.* residues in these structural entities unfold simultaneously upon mild treatment with urea (Figure 7, left side). Additionally, this unfolding event is promoted by initial urea-independent local fluctuations that help in deprotecting the buried amide protons. The dominance of the Hofmeister effect over the Coulombic effect can justify the initiation of Ste11 SAM unfolding from Helix 5, having several exposed positively charged residues. This is followed by destabilization of helix 1, 3, and 4 (PUF2), which were relatively more stable due to the prevailing hydrophobic patch and thus highlighting the role of initial hydrophobic collapse in aiding correct and proper folding of Ste11 SAM. PUF2 is generated by an initial exchange through local fluctuations, only to be dominated by more extensive unfolding kinetics promoted by a higher concentration of urea perturbing the hydrogen bond interactions. The opening of this second cooperative unit brings out the final transition leading up to complete global unfolding. It can, therefore, be concluded that at 150 mM NaCl concentration, a small number of native-like intermediates occupy discrete free-energy wells that could help to outline a stepwise or sequential folding/unfolding pathway.

However, at 300 mM NaCl, the value of ΔG_{HX} decreases sharply for all measurable amide protons simultaneously, indicating the dominance of a sizeable unfolding reaction that generates single high energy unfolded state in a globally cooperative manner. However, earlier studies under sub-denaturing urea concentration had revealed that amongst the five helices fold of Ste11 SAM, residues present in Helix 1 and Helix 5 were chiefly responsible for urea induced chemical shift perturbations (CSPs) and depicted alternate conformational states.[31] Additionally, Helix 1 and Helix 5 are involved in binding

interactions with Ste50 SAM domain while intimate interactions among several hydrophobic residues and ionic interactions between Helix 4 and Helix 5, at the C-terminus of the molecule help in the stabilization of the Ste11 SAM dimer domain. This could indicate a fast two-step folding starting with an initial transient closed to open form transition, allowing the exchange of the exposed amide protons with the solvent, with no apparent visible intermediate (Figure 7, right side). This means that either the intermediates fail to exist or the initial barrier in the folding/unfolding pathway is rate limiting such that the intermediates cannot be visualized.

Studies with the L57R Ste11 SAM mutant further support our claim highlighting helix 4 and 5 as the epicenter of unfolding initiation. The role of the interfacial residues in dimer stability has already been probed.[18] Of them, L57 residue appears to be a key player involved in a mutual packing hydrophobic interaction that imparts global stability. Additionally, the almost similar values for the free energy change of the L57R mutant dimer and PUF2 highlights the structural instability and dynamicity promoted due to the L57R mutation. The loss of conformational stability shown by the L57R mutant could be supported to (a) the weaker packing interaction between the residues R57, I44′, and I46′, present at the interface, and (b) the probable ionic repulsion with the nearby positively charged residues in the neighboring subunits and salt ions. This loss of interfacial integrity promotes the faster exchange of residues in helix 4 and 5, thus catalyzing the global unfolding events and indicating the primary role of L57R in aiding salt-induced stabilization of the Ste11 SAM dimer, and making helix 4 the starting point for native Ste11 SAM unfolding to occur.

A polypeptide chain holds much more information than merely encoding a stable native structure, concealing enormous propensities of adopting alternatives states which may impart newer functionality.[56] We hypothesize that these partially unfolded metastable intermediates play an essential role in defining the functional attributes of Ste11 SAM by allowing interaction with appropriate signaling partners by way

of domain swapping.[57] Additionally, resolving a protein into its various cooperative units assures the elucidation of their functional and biological evolution.

CONCLUSION

The method of native-state hydrogen-deuterium exchange NMR utilized here to gain an insight into the folding behavior of the Ste11 SAM domain. The results obtained so far substantiate an interesting underlying conformational representation and corroborate well with the already established structure of the same. The protein can be assumed to constitute two independent but cooperative structural units, called foldons, which interact sequentially with one other, initiating the protein folding pathway, thus leading up to functionally sound folding kinetics. Additionally, various studies also ascertain that these substructures or foldons influence and guide the functional and evolutionary properties of the protein in which they occur.[28, 55]

ASSOCIATED CONTENT

Supporting Information.

Increase of ΔG_{UN} with increasing salt concentration (Figure S1); bar diagram depicting the mid-point of unfolding transition (C_m) as a function of residues (Figure S2).

AUTHOR INFORMATION

Corresponding Author

*Anirban Bhunia (anirbanbhunia@gmail.com or bhunia@jcbose.ac.in)

*Surajit Bhattacharjya (Surajit@ntu.edu.sg)

Present Addresses

† Department of Biophysics, Bose Institute, P-1/12 CIT Scheme VII(M), Kolkata 700 054, India.

Author Contributions

‡AB and HI contributed equally.

Funding Sources

This work was supported by grants from The Ministry of Education (MOE), Singapore.

ACKNOWLEDGMENT

HI would like to thank Bose Institute for Senior Research Fellowship.

ABBREVIATIONS

SAM, Sterile α motif; MAPK, Mitogen-Activated Protein Kinase; MAPK, Mitogen-Activated Protein Kinase Kinase; D_2O, Deuterium Oxide; CD, Circular Dichroism; H/D, Hydrogen/Deuterium Exchange; NMR, Nuclear Magnetic Resonance; PF, Protection Factor; PUF, Partial Unfolded Forms; HX, Hydrogen Exchange; HSQC, Heteronuclear Single Quantum Coherence.

REFERENCES

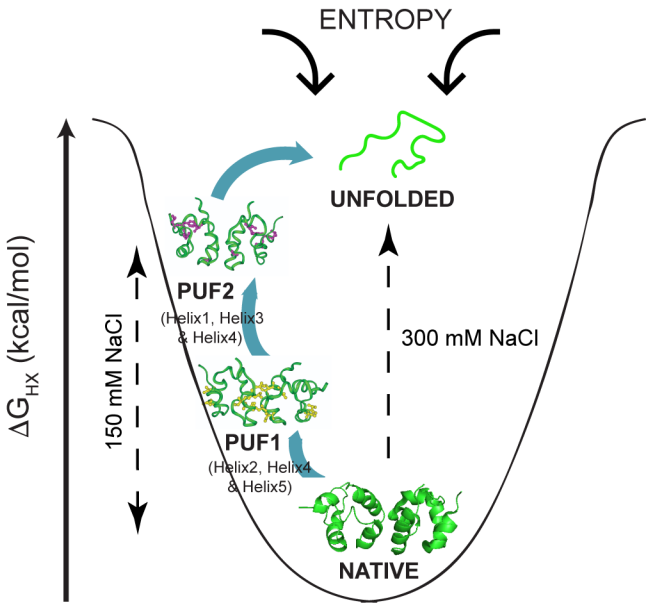
[1] Kim, C. A.; Bowie, J. U. (2003) SAM domains: uniform structure, diversity of function. Trends Biochem. Sci. 28, 625-628.
[2] Qiao, F.; Bowie, J. U. (2005) The many faces of SAM. Sci. STKE 2005, re7.
[3] Schultz, J.; Ponting, C. P.; Hofmann, K.; and Bork, P. (1997) SAM as a protein interaction domain involved in developmental regulation. Protein Sci. 6, 249-253.
[4] Stapleton, D.; Balan, I.; Pawson, T.; Sicheri, F. (1999) The crystal structure of an Eph receptor SAM domain reveals a mechanism for modular dimerization. Nat. Struct. Biol. 6, 44-49.
[5] Ramachander, R.; Kim, C. A.; Phillips, M. L.; Mackereth, C. D.; Thanos, C. D.; McIntosh, L. P.; Bowie, J. U. (2002) Oligomerization-dependent association of the SAM domains from Schizosaccharomyces pombe Byr2 and Ste4. J. Biol. Chem. 277, 39585-39593.
[6] Kim, C. A.; Sawaya, M. R.; Cascio, D.; Kim, W.; Bowie, J. U. (2005) Structural organization of a Sex-comb-on-midleg/polyhomeotic copolymer. J. Biol. Chem. 280, 27769-27775.

[7] Poirel, H.; Lopez, R. G.; Lacronique, V.; Della Valle, V.; Mauchauffé, M.; Berger, R.; Ghysdael, J.; Bernard, O. A. (2000) Characterization of a novel ETS gene, TELB, encoding a protein structurally and functionally related to TEL. Oncogene 19, 4802-4806.
[8] Peterson, A. J.; Kyba, M.; Bornemann, D.; Morgan, K.; Brock, H. W.; Simon, J. (1997) A domain shared by the Polycomb group proteins Scm and ph mediates heterotypic and homotypic interactions. Mol. Cell Biol. 17, 6683-6692.
[9] Thanos, C. D.; Goodwill, K. E.; Bowie, J. U. (1999) Oligomeric structure of the human EphB2 receptor SAM domain. Science 283, 833-836.
[10] Kim, C. A.; Gingery, M.; Pilpa, R. M.; Bowie, J. U. (2002) The SAM domain of polyhomeotic forms a helical polymer. Nat. Struct. Biol. 9, 453-457.
[11] Qiao, F.; Song, H.; Kim, C. A.; Sawaya, M. R.; Hunter, J. B.; Gingery, M.; Rebay, I.; Courey, A. J.; Bowie, J. U. (2004) Derepression by depolymerization; structural insights into the regulation of Yan by Mae. Cell 118, 163-173.
[12] Pawson, T.; Nash, P. (2003) Assembly of cell regulatory systems through protein interaction domains. Science 300, 445-452.
[13] Kim, C. A.; Phillips, M. L.; Kim, W.; Gingery, M.; Tran, H. H.; Robinson, M. A.; Faham, S. Bowie, J. U. (2001) Polymerization of the SAM domain of TEL in leukemogenesis and transcriptional repression. EMBO J. 20, 4173-4182.
[14] Bhattacharjya, S.; Xu, P.; Gingras, R.; Shaykhutdinov, R.; Wu, C.; Whiteway, M.; Ni, F. (2004) Solution structure of the dimeric SAM domain of MAPKKK Ste11 and its interactions with the adaptor protein Ste50 from the budding yeast: implications for Ste11 activation and signal transmission through the Ste50-Ste11 complex. J. Mol. Biol. 344, 1071-1087.
[15] Bhattacharjya, S.; Xu, P.; Chakrapani, M.; Johnston, L.; Ni, F. (2005) Polymerization of the SAM domain of MAPKKK Ste11 from the budding yeast: implications for efficient signaling through the MAPK cascades. Protein Sci. 14, 828-835.
[16] Wu, C.; Leberer, E.; Thomas, D. Y.; Whiteway, M. (1999) Functional characterization of the interaction of Ste50p with Ste11p MAPKKK in Saccharomyces cerevisiae. Mol. Biol. Cell 10, 2425-2440.
[17] Stevenson, B. J.; Rhodes, N.; Errede, B.; Sprague, G. F. (1992) Constitutive mutants of the protein kinase STE11 activate the yeast pheromone response pathway in the absence of the G protein. Genes Dev. 6, 1293-1304.
[18] Bhunia, A.; Domadia, P. N.; Xu, X.; Gingras, R.; Ni, F.; Bhattacharjya, S. (2008) Equilibrium unfolding of the dimeric SAM domain of MAPKKK Ste11 from the budding yeast: role of the interfacial residues in structural stability and binding. Biochemistry 47, 651-659.
[19] Englander, S. W. (2000) Protein folding intermediates and pathways studied by hydrogen exchange. Annu. Rev. Biophys. Biomol. Struct. 29, 213-238.
[20] Henzler-Wildman, K.; Kern, D. (2007) Dynamic personalities of proteins. Nature 450, 964-972.
[21] Krishna, M. M.; Hoang, L.; Lin, Y.; Englander, S. W. (2004) Hydrogen exchange methods to study protein folding. Methods 34, 51-64.
[22] Bhavesh, N. S.; Sinha, R.; Mohan, P. M.; Hosur, R. V. (2003) NMR elucidation of early folding hierarchy in HIV-1 protease. J. Biol. Chem. 278, 19980-19985.
[23] Bai, Y. (2006) Protein folding pathways studied by pulsed- and native-state hydrogen exchange. Chem. Rev. 106, 1757-1768.
[24] Bai, Y.; Sosnick, T. R.; Mayne, L.; Englander, S. W. (1995) Protein folding intermediates: native-state hydrogen exchange. Science 269, 192-197.

[25] Gupta, S.; Bhattacharjya, S. (2014) NMR characterization of the near native and unfolded states of the PTB domain of Dok1: alternate conformations and residual clusters. PLoS One 9, e90557.
[26] Jaravine, V. A.; Rathgeb-Szabo, K.; Alexandrescu, A. T. (2000) Microscopic stability of cold shock protein A examined by NMR native state hydrogen exchange as a function of urea and trimethylamine N-oxide. Protein Sci 9, 290-301.
[27] Connell, K. B.; Horner, G. A.; Marqusee, S. (2009) A single mutation at residue 25 populates the folding intermediate of E. coli RNase H and reveals a highly dynamic partially folded ensemble. J. Mol. Biol. 391, 461-470.
[28] Lim, S. A.; Bolin, E. R.; Marqusee, S. (2018) Tracing a protein's folding pathway over evolutionary time using ancestral sequence reconstruction and hydrogen exchange. eLife 7, e38369.
[29] Kumar, A.; Srivastava, S.; Mishra, R. K.; Mittal, R.; Hosur, R. V. (2006) Local structural preferences and dynamics restrictions in the urea-denatured state of SUMO-1: NMR characterization. Biophys. J. 90, 2498-2509.
[30] Kumar, A.; Srivastava, S.; Hosur, R. V. (2007) NMR characterization of the energy landscape of SUMO-1 in the native-state ensemble. J. Mol. Biol. 367, 1480-1493.
[31] Gupta, S.; Bhattacharjya, S. (2014) Characterization of the near native conformational states of the SAM domain of Ste11 protein by NMR spectroscopy. Proteins 82, 2957-2969.
[32] Dyson, H. J.; Wright, P. E. (2005) Elucidation of the protein folding landscape by NMR. Methods Enzymol. 394, 299-321.
[33] Dyson, H. J.; Wright, P. E. (2001) Nuclear magnetic resonance methods for elucidation of structure and dynamics in disordered states. Methods Enzymol. 339, 258-270.
[34] Zhou, Y.; Yang, D. (2017) Equilibrium folding dynamics of meACP in water, heavy water, and low concentration of urea. Sci. Rep. 7, 16156.
[35] Englander, S. W.; Mayne, L. (2014) The nature of protein folding pathways. Proc. Natl. Acad. Sci. USA 111, 15873-15880.
[36] Dyson, H. J.; Wright, P. E. (2004) Unfolded proteins and protein folding studied by NMR. Chem. Rev. 104, 3607-3622.
[37] Delaglio, F.; Grzesiek, S.; Vuister, G. W.; Zhu, G.; Pfeifer, J.; Bax, A. (1995) NMRPipe: a multidimensional spectral processing system based on UNIX pipes. J. Biomol. NMR 6, 277-293.
[38] Johnson, B. A. (2004) Using NMRView to visualize and analyze the NMR spectra of macromolecules. Methods Mol. Biol. 278, 313-352.
[39] van Mierlo, C. P.; van den Oever, J. M.; Steensma, E. (2000) Apoflavodoxin (un)folding followed at the residue level by NMR. Protein Sci. 9, 145-157.
[40] Bai, Y.; Milne, J. S.; Mayne, L.; Englander, S. W. (1993) Primary structure effects on peptide group hydrogen exchange. Proteins 17, 75-86.
[41] Dill, K. A.; Ozkan, S. B.; Shell, M. S.; Weikl, T. R. (2008) The protein folding problem. Annu. Rev. Biophys. 37, 289-316.
[42] Wang, M.; Kaufman, R. J. (2016) Protein misfolding in the endoplasmic reticulum as a conduit to human disease. Nature 529, 326-335.
[43] Dobson, C. M. (2002) Getting out of shape. Nature 418, 729-730.
[44] Anfinsen, C. B. (1972) The formation and stabilization of protein structure. Biochem. J. 128, 737-749.
[45] Dill, K. A.; Chan, H. S. (1997) From Levinthal to pathways to funnels. Nat. Struct. Biol. 4, 10-19.
[46] Verkhivker, G. M.; Bouzida, D.; Gehlhaar, D. K.; Rejto, P. A.; Freer, S. T.; Rose, P. W. (2002) Complexity and simplicity of ligand-macromolecule interactions: the energy landscape perspective. Curr. Opin. Struct. Biol. 12, 197-203.

[47] Whitten, S. T.; García-Moreno E, B.; Hilser, V. J. (2005) Local conformational fluctuations can modulate the coupling between proton binding and global structural transitions in proteins. Proc. Natl. Acad. Sci. USA 102, 4282-4287.
[48] Hansen, D. F.; Feng, H.; Zhou, Z.; Bai, Y.; and Kay, L. E. (2009) Selective characterization of microsecond motions in proteins by NMR relaxation. J. Am. Chem. Soc. 131, 16257-16265.
[49] Goto, Y.; Adachi, M.; Muta, H.; So, M. (2018) Salt-induced formations of partially folded intermediates and amyloid fibrils suggests a common underlying mechanism. Biophys. Rev. 10, 493-502.
[50] Pradeep, L.; Udgaonkar, J. B. (2002) Differential salt-induced stabilization of structure in the initial folding intermediate ensemble of barstar. J. Mol. Biol. 324, 331-347.
[51] Dyson, H. J.; Wright, P. E. (1998) Equilibrium NMR studies of unfolded and partially folded proteins. Nat. Struct. Biol. 5 Suppl., 499-503.
[52] Barrera, F. N.; Garzón, M. T.; Gómez, J.; Neira, J. L. (2002) Equilibrium unfolding of the C-terminal SAM domain of p73. Biochemistry 41, 5743-5753.
[53] Beauchamp, D. L.; Khajehpour, M. (2012) Studying salt effects on protein stability using ribonuclease t1 as a model system. Biophys. Chem. 161, 29-38.
[54] Tadeo, X.; López-Méndez, B.; Castaño, D.; Trigueros, T.; Millet, O. (2009) Protein stabilization and the Hofmeister effect: the role of hydrophobic solvation. Biophys. J. 97, 2595-2603.
[55] Englander, S. W.; Mayne, L. (2017) The case for defined protein folding pathways. Proc. Natl. Acad. Sci. U S A 114, 8253-8258.
[56] Wildes, D.; Anderson, L. M.; Sabogal, A.; Marqusee, S. (2006) Native state energetics of the Src SH2 domain: evidence for a partially structured state in the denatured ensemble. Protein Sci. 15, 1769-1779.
[57] Miller, K. H.; Marqusee, S. (2011) Propensity for C-terminal domain swapping correlates with increased regional flexibility in the C-terminus of RNase A. Protein. Sci. 20, 1735-1744.

For Table of Contents use only


ENTROPY
UNFOLDED
PUF2
(Helix1, Helix3
& Helix4)
PUF1
(Helix2, Helix4
& Helix5)
NATIVE
150 mM NaCl
300 mM NaCl
ΔG_HX (kcal/mol)