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2014

Gupta, S., & Bhattacharjya, S. (2014). Characterization of the near native conformational states of the SAM domain of Ste11 protein by NMR spectroscopy. *Proteins: Structure, Function, and Bioinformatics*, 82(11), 2957-2969.

<https://hdl.handle.net/10356/80215>

<https://doi.org/10.1002/prot.24652>

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Structure, Function, and Bioinformatics

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Journal:	<i>PROTEINS: Structure, Function, and Bioinformatics</i>
Manuscript ID:	Prot-00188-2014.R1
Wiley - Manuscript type:	Research Article
Date Submitted by the Author:	n/a
Complete List of Authors:	Gupta, Sebanti; Nanyang Technological University, School of Biological Sciences Bhattacharjya, Surajit; Nanyang Technological University, School of Biological Sciences
Key Words:	nmr, SAM domain , signaling , unfolding , near native

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Review

Characterization of the Near Native Conformational States of the SAM domain of Ste11 protein by NMR Spectroscopy

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Short title: Urea induced near native conformational change in Ste11 SAM.

Key word: Nuclear magnetic resonance, low-energy alternate states, near-native conformations, Ste11 SAM domain.

Funding Sources: We thank Ministry of Education (MOE, ARC18-13), Singapore for financial support.

Abbreviations

SAM, Sterile alpha motif; SAM domain, Sterile alpha motif domain; NMR, nuclear magnetic resonance; HSQC, heteronuclear single quantum coherence.

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Abstract

The sterile alpha motif or SAM domain is one of the most frequently present protein interaction modules with diverse functional attributions. SAM domain of the Ste11 protein of budding yeast plays important roles in mitogen-activated protein kinase (MAPK) cascades. In the current study, urea-induced, at sub-denaturing concentrations, structural and dynamical changes of the Ste11 SAM domain have been investigated by NMR spectroscopy. Our study revealed that a number of residues from helix 1 and helix 5 of the Ste11 SAM domain display plausible alternate conformational states and largest chemical shift perturbations at low urea concentrations. Amide proton (H/D) exchange experiments indicated that helix 1, loop and helix 5 become more susceptible to solvent exchange with increased concentrations of urea. Notably, helix1 and helix5 are directly involved in binding interactions of the Ste11 SAM domain. Our data further demonstrate that the existence of alternate conformational states around the regions involved in dimeric interactions in native or near native conditions.

Introduction

Proteins undergo conformational excursion under native solution conditions whereby the natively folded conformation of a protein, or the lowest energy state, is highly populated (>99%) with other conformational states of higher energy are sparsely populated. Correlations of protein structure, folding and function of proteins require characterization of not only the folded states but also these lowly populated conformational states¹⁻³. The structural fluctuations of protein molecules can range from local conformational changes to a large scale or global opening of the folded structure. Folding models of protein molecules describe conformational variations in a funnel shaped free-energy landscapes¹⁻³. The population and energy level of the excited states of proteins can be modulated by temperature and denaturants⁴⁻⁸. NMR spectroscopy has been serving as a key technique to elucidate the high energy states of proteins⁸⁻¹¹. NMR experiments carried out at sub-denaturing conditions (in presence of low concentrations of chaotropes) including amide proton temperature dependent, amide proton exchange with solvent D₂O or H/D exchange, relaxations measurements have provided valuable insights into the excited states of proteins at the near native conditions^{4-7, 12-14}. Notably, the temperature dependent amide proton chemical shift change of proteins can identify structural changes or alternate conformations close to the native state, whereas partially folded states or folding intermediates of proteins are recognized by H/D exchange^{4, 6, 7, 14-17}.

The sterile alpha motif or SAM domain is a multifunctional protein module involved in diverse function ranging from cell signaling, transcription repression, synaptic scaffold to translational control¹⁸⁻²⁰. SAM domain was originally identified with single copy of a 65-70 residues during

the multiple sequence alignment studies in Ste11 and Ste50 proteins from MAP-kinase signaling cascade of budding yeast along with few other proteins involved in the development of fruit flies^{21, 22}. To date, SAM domains have been identified in more than 1300 proteins in all genomes and only comparable to the number of SH2 (Src homology 2) domain detected among different organisms^{20, 23}. Unlike SH2 and SH3 (Src homology 3) domains, SAM domains reflect functional diversity and even a close homologue can perform various functions¹⁸. SAM domains have been known to be involved in homo and hetero - oligomerizations²⁴⁻²⁷ and can also interact with other non-SAM containing proteins²⁸⁻³⁰. Extensive self-association of SAM domain is evident in case of TEL and Yan, necessary to regulate transcription of DNA^{31, 32}. Further, SAM domain of the Shank protein has been found to bind with metal ions Zn^{+2} that helps in stabilization of a multi-protein assembly at the neural synapses³³. Additionally, binding interaction of SAM with RNA has been described in case of Smg protein from *Drosophila melanogaster* and Vts 1 protein from yeast^{34, 35}. Furthermore, SAM domains showed detectable interactions with the lipid membrane^{36, 37}. Very recently, SAM domain of Sla1p has been found to be involved in clathrin mediated endocytosis process³⁸. NMR spectroscopy and x-ray crystallography based structure determination of SAM domains from several proteins demonstrated a conserved fold consisting of five helices of different length^{25, 32, 33, 39-41}.

Despite of vast importance of the conserved SAM fold in diverse mode of functions, studies on structural stability, folding properties and dynamics of these domains are sparse^{36, 42, 43}. The 3-D structure of the Ste11 SAM domain determined by NMR showed that the SAM domain of Ste11 exists as a homo-dimer in solution⁴⁴. The interfacial interactions of the dimeric structure of Ste11 SAM, involving residues of helix 4 and helix 5, are found to be critical for the stability and folded structure^{42, 44}. The smaller size and the dimeric assembly of the SAM domain of

Ste11 protein have prompted us to utilize Ste11 SAM as a model for the folding and dynamics study. Ste11 is one of the major components of MAP kinase pathway of budding yeast⁴⁴⁻⁴⁶. The interaction between Ste11 and Ste50 is mediated by their SAM domains, important for activation of Ste11 kinase^{46,47}. In this work, we have examined conformational and dynamical characteristics of the Ste11 SAM domain under sub-denaturing concentrations of urea. We have employed temperature dependent amide proton chemical shift changes, ¹⁵N relaxation, H/D exchange experiments to probe alternate conformations of the Ste11 SAM domain.

MATERIALS AND METHODES

Protein expression and purification: Ste11 SAM (D37-R104)⁴⁴ domain from *Saccharomyces cerevisiae* was sub cloned into the pET14b vector with a thrombin cleavable N-terminal His₆ tag and over expressed into *Escherichia coli* BL21 (DE3) cells, grown at 37 °C either in LB or M9 medium containing [¹⁵N] ammonium chloride (Cambridge Isotope Laboratories). The cells were induced with 1mM IPTG at an OD₆₀₀ of 0.6-0.7. After incubating for 6-12 hours at 16 °C for protein production, cells were centrifuged and resuspended in 20 mM Tris-hydrochloride buffer of pH 8.0. The cell solution was lysed by sonication and the supernatant was applied to Nickel-NTA column for His tag-based purification (Qiagen). Strongly bound target protein was eluted with buffer containing 500 mM imidazole. The purified protein was extensively dialyzed against 10mM sodium phosphate buffer, containing 300 mM NaCl and 20 mM β-ME at pH 5.8. Sample of Ste11 SAM was loaded onto a Hiload Superdex 75 10/300 GL analytical column with the AKTA FPLC UPC-900 system (GE Healthcare UK Ltd., England). The column was equilibrated with 10 mM sodium phosphate buffer containing 300 mM sodium chloride and 20 mM β-ME,

pH 5.8. The sample was eluted at a flow rate of 0.5 ml/min and detected at 276nm. The amino acid sequence of Ste11 SAM was numbered as D1- R68.

NMR spectroscopy: All the NMR experiments were performed on a Bruker DRX 600 MHz spectrometer equipped with an actively shielded cryoprobe. NMR data were processed using the NMRPipe and NMRDraw^{48, 49} suite and analyzed by Sparky (T.D. Goddard and D.G. Kneller, University of California, San Francisco). The chemical shifts were directly or indirectly referenced to DSS.

Urea titration experiment: Urea titration experiments were recorded at 298 K for Ste11 SAM domain by adding aliquots from a 10 M urea stock solution, prepared in 10 mM sodium phosphate buffer, 300 mM NaCl and 20 mM β-ME, pH 5.8 into the NMR tube containing the ¹⁵N labeled native Ste11 SAM at 0.6 mM concentration. The samples were allowed to equilibrate for 20 min before the recording of two-dimensional ¹H-¹⁵N HSQC spectra. The reductions in the intensity of HSQC cross peaks as a function of the denaturant concentration was fitted to the equation⁵⁰,

$$Y_{\text{obs}} = (\alpha_N + \beta_N[D]) \div 1 + \exp \{- [m [D] - C_m/RT]\}$$

Here Y_{obs} represents the intensity of a HSQC peak, α_N and β_N are intercept and slope of the pre and post unfolding regimes, m is the slope at the midpoint of the unfolding transition, $[D]$ is the concentration of the denaturant and C_m is the denaturant concentration at the midpoint of the transition, RT is $0.57 \text{ kcal mol}^{-1}$. The urea induced chemical shift changes in ¹⁵N and HN resonances was determined using the following equation which is designated as chemical shift perturbation (CSP), $\Delta(H,N) = [\{(\Delta_H)(W_H)\}^2 + \{(\Delta_N)(W_N)\}^2]^{1/2}$

where W_H and W_N are weighting factors for 1H and ^{15}N chemical shifts, respectively ($W_H=1$, $W_N=0.154$)⁵¹ and Δ_H and Δ_N are the chemical shift difference for amide proton and ^{15}N respectively.

Temperature dependence of amide proton chemical shift: Temperature dependent amide proton chemical shift for Ste11 SAM were performed by recording series of HSQC spectra at seven different temperatures, 283 K, 286 K, 289 K, 292 K, 295 K, 298 K and 301 K. These experiments were done for native and four different concentrations of denaturant (0.5 M, 0.75 M, 1.0 M and 1.5 M urea) in 10 mM sodium phosphate buffer, 300 mM NaCl, 20 mM β -ME, pH 5.8. For each amide proton, a straight line was fitted to the chemical shift temperature dependence and subtracted from the experimental points to get a residual plot, as described in previous studies^{4, 7}.

Hydrogen-Deuterium exchange experiment: To study amide proton exchange, lyophilized Ste11 SAM was dissolved in D_2O buffer of 10 mM sodium phosphate, 300 mM NaCl and 20 mM β -ME at pH 5.8. A series of two dimensional 1H - ^{15}N HSQC spectra were recorded within an interval of 30 min. Hydrogen exchange rates (k_{ex}) were fitted to a single exponential decay equation, $I(t) = I(0) * \exp(-K_{ex} * t)$. Where, $I(t)$ and $I(0)$ are the intensities at the given time t and $t = 0$ respectively and K_{ex} is the rate constant of the hydrogen-deuterium exchange reaction. The protection factors were determined from a ratio of calculated intrinsic exchange rates of the amide protons of Ste11 SAM domain and experimentally measured exchange rates⁵². The H-D exchange experiments were recorded for native as well as four different urea concentrations, 0.25 M, 0.5 M, 0.75 M and 1.5 M.

Relaxation measurement: The ^{15}N longitudinal ($R_1 = 1/T_1$) and transverse ($R_2 = 1/T_2$) relaxation rate constants of ^{15}N labeled Ste11 SAM domain samples, in 10 mM sodium phosphate buffer,

300 mM NaCl, 20 mM β -ME, pH 5.8, were determined by collecting a series of ^1H - ^{15}N HSQC spectra at different time interval with sensitivity enhancement in a Bruker DRX 600MHz at 298K temperature⁵³. The relaxation delays for T_1 measurement were 5, 40, 80, 130, 210, 330, 470, 630, 800, 1000, 3000 ms with repeating the experiments at the time points of 80 ms and 470 ms for error estimation. For T_2 measurement the data were acquired with delays of 14.4, 28.8, 43.2, 57.6, 72.0, 86.4, 100.8, 115.2, 129.6, 144.0, 158.4 ms with the duplicate points at 72.0 ms for error estimation. The relaxation rate constants were determined by fitting the cross peak intensities to a mono-exponential function^{44,54}. Steady state ^{15}N - $\{^1\text{H}\}$ heteronuclear NOE experiments were performed with and without the saturation of the ^1H resonances. ^1H saturation was accomplished by 120° pulses spaced at 5 ms interval for a period of 3 s with an inter-scan relaxation delay of 2 s. All these experiments were repeated for two different denaturant (urea) concentrations; 0.5 M and 2 M. The T_1 and T_2 relaxation experiments were repeated twice and a total of 2048 complex data points with 96 or 128 complex increment were collected for these relaxation experiments. For ^{15}N - $\{^1\text{H}\}$ hetero-nuclear NOE experiments, spectra were recorded with 2048 complex data points with 256 complex t_1 points. Further analysis of the backbone dynamic parameters was carried out according to the Lipari-Szabo model-free method by using the program Tensor2⁵⁵. The model free parameters were extracted using the experimentally determined ^{15}N relaxation rate parameters and the heteronuclear NOE values. An isotropic diffusion model was used to obtain order parameters (S^2 , S_f^2 , S_s^2) and internal mobility (τ_e and K_{ex}) following model selection procedure as described by Mandel et. al⁵⁶.

RESULTS

Urea induced unfolding of dimeric Ste11 SAM domain: In a previous study, we investigated urea induced unfolding of the native and interfacial mutants of Ste11 SAM at 150 mM NaCl⁴².

The 3-D structure of the homo-dimeric Ste11 SAM domain was determined at 300 mM NaCl⁴⁴. Here, we have examined urea mediated global unfolding transitions of the Ste11 SAM at 300 mM NaCl. To monitor the unfolding of Ste11 SAM, a series of HSQC spectra were collected with increasing urea concentrations, ranging from 0.5 M to 6 M. We have observed reduction in intensity of HSQC cross peaks of the native dimer as urea concentrations were increased beyond 2 M. ¹⁵N-¹H HSQC spectra indicated that SAM domain assumes largely unfolded conformations at 6 M urea (Supplementary Fig. S1). The equilibrium unfolding of the Ste11 SAM domain was further fitted into two state unfolding transitions (see Materials and Methods). Analyses of these transition curves and midpoint of transition (C_m) indicated that the native like global fold of the domain are sustained upto 3.2 M urea (Supplementary Fig. S2). Consequently, we have restricted our structural and dynamics analysis at ≤ 2 M urea concentrations ensuring perturbations within the near native states of the domain.

Urea induced chemical shift changes of Ste11 SAM: Amide proton and ¹⁵N chemical shifts are sensitive probes for the environment of a given residue in a protein structure⁵⁷. We have calculated the chemical shift perturbations (CSP) of the Ste11 SAM domain to determine plausible denaturant binding regions. Figure 1 (panel A) shows the bar diagram plotting of combined chemical shift changes of ¹⁵N and ¹HN at 2 M urea concentration. Effect of urea on the chemical shifts at 0.5 M concentration was less pronounced and an average CSP has been estimated to be to 0.01 ppm that eventually increased to 0.018 ppm in the presence of 1 M urea (Supplementary Fig. S3). Residues F9, V10, L12, E15, T20, Q21, Y22, Q28, L31, V32, T33, E34, I44, L45, S63, F66 and R68 have experienced above average CSP in 1 M urea (Supplementary Fig. S3). As noted, in 2 M urea average CSP increases to 0.024 ppm whereby more residues e.g. F9, V10, Q11, L12, L14, E15, G18, Q21, Y22, I27, L31, V32, T33, E34, I44,

L45, A47, N51, L57, S63, S65, F66, Q67 and R68 exhibited higher (≥ 0.024 ppm) chemical shift changes (Fig. 1A). The changes in chemical shift perturbations at 2 M urea are mapped onto the structure of SAM domain (Fig.1B).

Temperature dependence of the amide proton chemical shift at sub-denaturing concentrations

of urea: The amide proton chemical shift generally follows a linear dependence with variation of temperatures⁵⁸. However, amide protons of proteins may delineate non-linear temperature dependence⁴. Such observation has been correlated with the plausible existence of alternate conformational states of higher free energies⁴. We have examined residues of Ste11 SAM domain accessing alternate conformations at native and near native conditions. ¹⁵N-¹H HSQC spectra of the SAM domain were recorded as a function of temperature within the range of 283K to 301K at 0 M, 0.5 M, 0.75 M, 1 M and 1.5 M urea concentrations (see Materials and Methods). Note that the ¹⁵N-¹H HSQC spectra recorded in the entire temperature range are qualitatively similar and well below the melting temperature. Most of the residues of SAM domain showed linear temperature dependence as expected, however, 2, 4, 2, 3 and 5 residues out of 68 depicted curved temperature profile in presence of 0 M, 0.5 M, 0.75 M, 1.0 M and 1.5 M urea concentrations, respectively. Figure 2 shows representative examples of non-linear temperature dependence of selected residues. As can be seen, more residues exhibited curved profile with the increased concentration of urea. We have also noticed various trends in temperature profiles between the native and different urea solutions. Residue L12, Q21, K38, G49, I59, K62, S63 and Q67 define curved temperature dependency either at native condition or only at a single urea concentration (Fig. 3A). Nonetheless, residues e.g. F13, C19, D43 and I46 demonstrated non-linear profile in two of the five different buffer conditions. Interestingly, temperature dependence curves of residues F13, C19 and D43 appeared to be changing from convex to concave or vice

versa with increasing urea concentrations. Residues delineating curved temperature dependence are highlighted onto the structure of Ste11 SAM domain structure (Fig. 3B).

Dynamical Changes of the Ste11 SAM domain at dub-denaturing concentration of urea: The backbone dynamics and the motional characteristics of Ste11 SAM domain were investigated by measuring the ^{15}N relaxation parameters, R_1 ($1/T_1$) and R_2 ($1/T_2$) at native, 0.5 M and 2 M urea concentrations. ^{15}N transverse relaxation rate (R_2) serves as a useful marker for local conformational transitions occurring at milli-micro second time scale while longitudinal relaxation rate (R_1) is only sensitive to nanosecond to picosecond time scale motion⁵⁹. Additionally, the ^{15}N R_2 is also a parameter that may be modulated for global conformational transitions⁵. The difference in R_1 (ΔR_1) and R_2 (ΔR_2) for individual residue at 0.5 and 2.0 M urea with native condition, 0 M urea, are shown (Fig. 4). At 0.5 M urea, there was no significant change in R_1 rate except for residue T33. However, increased R_1 values ($\geq 2.5\text{s}^{-1}$) were evident for a few residues including Q21, V50 and G54 in 2 M urea (Fig. 4A). Similarly, in case of R_2 relaxation, the motional changes were very modest at 0.5 M urea concentration whereas more deviations from native were observed at 2 M urea. A number of residues including F9, L12, Y22, E34, Y39, L45, D55, L57 and L60 were found to be experiencing escalated change in R_2 with a ΔR_2 value $\geq 5.5\text{s}^{-1}$ at 2 M urea (Fig. 4B). The negative ΔR_2 for these residues may indicate a probable conformational exchange at this condition. An analysis of average R_2 values for the five helices of Ste11 SAM in native, 0.5 M and 2 M urea revealed significant dynamical changes for helix 1 and helix 5, in 2 M urea, compared to the native solution condition (Fig. 4C).

Internal mobility of the Ste11 SAM domain was further analyzed, for the native protein and in 0.5 M urea, using Model-free approach (see Methods) (Figs. 5 and 6). The generalized order parameter (S^2) describes the spatial extent of motion of the N-H bond vector at ps-ns time scale

and provides information of atomic scale residue flexibility at this time regime. A high structural rigidity is specified by the order parameter value ~ 1 whereas the unrestricted bond vectors should display the S^2 value of zero. The average S^2 values of Ste11 SAM domain were observed to be 0.85 ± 0.06 and 0.82 ± 0.04 at native and 0.5 M urea concentration, respectively, indicating a global rigidity of the SAM domain under both conditions. However, a comparison of motional characteristics of the residues in secondary structural elements revealed that average S^2 value of the helix 5 decreased from 0.86 in native to 0.68 at 0.5 M urea while S^2 values appeared to be comparable for other helices under these conditions (Fig. 5C). These observations indicated that the residues in helix 5 of the Ste11 SAM may be undergoing a somewhat more conformational fluctuation in 0.5 M urea. The K_{ex} parameter describes motions occurring at μ s-ms time scale. The K_{ex} values ranged from 1.20 to 10.48 sec^{-1} at native and 1.48 to 11.57 sec^{-1} at 0.5 M urea, respectively. Eighteen residues of the native Ste11 SAM domain demarcated contribution to conformational exchange on micro-millisecond time scale whilst more residues, increased to twenty one, showed K_{ex} contribution in 0.5 M urea (Fig. 6). Among residues revealing K_{ex} dependency, T20, Q21, V31, L40, V50, N51 were from the loop region of the dimeric structure of the Ste11 SAM domain. A Model-free analysis of the Ste11 SAM domain at 2 M urea was precluded due to unfavorable ^{15}N - ^1H heteronuclear NOE spectra.

H-D exchange at sub-denaturing urea concentrations: H-D exchange NMR of proteins at sub-denaturing concentrations of chaotropes is a useful method for the elucidation of folding-unfolding transitions at native-like solution conditions¹⁵⁻¹⁷. H-D exchange experiments, for Ste11 SAM domain, were carried out at native and four different urea concentrations, 0.25 M, 0.5 M, 0.75 M and 1.5 M urea. Figure 7 shows ^{15}N - ^1H HSQC spectra of Ste11 SAM domain in buffer containing D_2O recorded after 360 min. of exchange at various concentrations of urea. There

were diminution in HSQC peak intensity and disappearance of cross-peaks with increasing concentrations of urea. The native state H-D exchange studies of Ste11 SAM domain revealed a faster exchange of residues F9, V10, E16, T20, Q21, D24, S25, N30, E34, E35, Y39, K42, I44, N51-R56, S63-F66 apart from the amide and carboxy terminal residues. Moreover, residues G18 and K62 were protected only at native condition. For better appreciation toward the changes in exchange rate of different amide protons with increasing denaturant concentration, we have analyzed H-D exchange data in terms of protection factor ratio (urea/native) at 0.25 M, 0.75 M and 1.5 M urea. Note, protection factor of amide protons were determined from a ratio of calculated intrinsic exchange rates of amide protons of the Ste11 SAM domain and experimentally measured exchange rates⁵². As can be seen, most of the residues demonstrated a decrease in protection factor ratio with increased urea concentrations. However, residues from helix 1, loop and helix 5 were found to be more susceptible (protection factor ratio ≤ 0.1) for exchange at higher urea concentrations (Fig. 8). Additionally, we have noticed interesting trends in amide proton exchange with increasing denaturant (0 M to 1.5 M). Residues L12, F26 and L48 were highly protected (> 0.5 PF ratio) only up to 0.25 M urea concentration while I17, L40 and L45 were less accessible for solvent exchange even at 1.5 M urea (Fig. 8). Notably, some residues e.g. E15, I37, D43, L48 and R61 delineated almost no change in protection factor ratio with increment of denaturant and probably represent the slowest exchanging amides (Fig. 8). Residues demarcate distinct features in H-D exchange experiments are highlighted onto the structure of Ste11 SAM domain (Fig. 9).

DISCUSSION

Apart from the structure, the dynamical characteristics and conformational transitions of a protein are similarly important not only for its functional aspects but also to understand the free

energy landscape. The resemblances between the folding and molecular recognition during interactions (existence of the thermodynamically stable native structure, the size and diversity of the conformational space) are already well accepted⁶⁰. During folding and also in signaling events, the energy landscape remains populated with numerous conformational states with various energy differences. The rate of inter-conversion between these different states is important for the protein functions which can vary from the nanosecond to micro-milli seconds time scale⁶¹. Stability and flexibility of the structure of protein molecules appear to be determining the extent of ruggedness of the underlying energy landscape during the molecular events like folding and binding. The knowledge about the near-native conformations is important as these minor populated states control the structural adaptability, inter-conversion rate between the native and alternative states and perturbation of the energy landscape by external effectors of a protein⁶²⁻⁶⁴.

In the current work, we have examined conformational and dynamical changes of the Ste11 SAM domain at sub-denaturing concentrations of urea. The native structure of the Ste11 SAM domain, at 300 mM NaCl, remains folded at 2.5 M as judged by the wide chemical shift dispersions and lack of new cross-peaks corresponding to the unfolded states. The global unfolding was evident only at 6 M urea concentration (Supplementary Fig. S1). The retention of global native fold can be approximated from the residue specific C_m values of Ste11 SAM (Supplementary Fig. S2) (the lowest value is 3.32). Hence, the structural changes of dimeric Ste11 SAM have been investigated within 2 M urea concentration which is well below the unfolding transition zone. Note, an urea induced unfolding study, by NMR, also demonstrated that the SAM domain of DLC-1 assumes unfolded state ~ 6 M urea with midpoint of transition ~ 3 M urea⁴³. Furthermore, chaotropes mediated global unfolding of SAM domains have been

investigated in previous studies^{36,42,43}, the conformational and dynamical changes of SAM domain under sub-denaturing concentration of denaturant has not been reported earlier.

Residues of the Ste11 SAM domain undergoing alternative conformational states: The existence of the curved profile of the amide proton chemical shift along with the temperature is an indication of accessible alternative conformational states^{6,7}. The alternative forms can be considered as the local unfolded state; composed of approximately five residues which are centered on the residue that shows curved temperature dependence^{6,7,65}. Under sub-denaturing concentrations of urea, other alternative states can be seen as the denaturant may decrease the energy difference between the native and the excited states^{4,6}. Ste11 SAM domain shows that two (F13 and G49) out of sixty eight of its residues were responding in non-linear fashion toward temperature changes (Fig. 2). However, with the increased urea concentrations, more residues, L12, C19, Q21, K38, D43, I46, I59, K62, S63 and Q67, with alternative conformations were detected (Fig. 2). These residues are mapped onto helix 1, helix 4, helix 5 and in loop regions of the SAM domain structure (Fig. 3). In other words, these structural elements of the Ste11 SAM domain are likely to be excursing higher energy conformational states under sub-denaturing conditions. Notably, helix 4 and helix 5 are found to be directly involve in homo (ste11 SAM) or hetero (Ste 50 SAM) oligomerization of Ste11 SAM domain^{42,44}. Residues from helix 1 and loop between helix1 and helix 2 also have been reported to be involved in Ste50 SAM domain interactions^{39,44}. The striking change of the temperature profile from convex to concave or vice versa (F13, C19 and D43) may be an indication of alteration of local structural forms even at low urea concentration. We have looked further for plausible structural origin of non-linear temperature dependence. Interestingly, the side chains of K62, S63 and F13 are in close contact in the 3-D structure of the SAM domain. Also, in this region side chains of I59 and C19 are

found to have complementary packing interactions (Supplementary Fig. S4). It may be likely that the aromatic ring flipping and/or inter-side chain hydrogen bonds could be the responsible for local structural changes leading to curved temperature profiles. Furthermore, several of these residues in helix 1, helix 4 and helix 5 also delineated above average CSP values in the presence of urea, indicting their plausible involvement in binding with the denaturant (Fig. 1, panels A and B).

Dynamical changes of the Ste11 SAM domain under sub-denaturing conditions: The ^{15}N relaxation and H-D exchange experiments were performed to examine the dynamics of SAM domain at sub-denaturing concentrations of urea. There were limited changes in ^{15}N longitudinal relaxation or R_1 values at the sub-denaturing concentrations, 0.5 and 2 M, of urea as this parameter is less sensitive toward conformational transitions induced at low denaturant concentration (Fig 4A), whereas, such conformational transitions or alternative conformations can contribute more to transverse relaxation or R_2 . A conspicuous sequence wise variation was observed in R_2 as the urea concentration was increased from 0 to 2 M (Fig. 4, panels B and C). Enhancement in the R_2 rate was depicted by the residues F9, L12, Y22, E34, Y39, L45, D55, L57 and L60 at 2 M urea located largely at helix 1, helix 5 and loop regions (Fig. 4, panels B and C). The augmented R_2 rate may reflect the possible occupancy of the exchanged conformations by these segments of the Ste11 SAM domain. Model free analyses of the Ste11 SAM domain in native and 0.5 M urea also indicated conformational fluctuations of the helix 5 (Figs. 5C and 6). We have also attempted to examine dynamical characteristics of Ste11 SAM domain, at sub denaturing urea concentrations using relaxation dispersion NMR, however, none of the residues of the Ste11 SAM showed any response to these experiments, possibly due to the absence of conformational time scale required for relaxation dispersion studies. Equilibrium H/D exchange

experiments at sub-denaturing conditions can probe conformational stability or structural regions affected by denaturant. At the native state of Ste11 SAM domain, amide protons of most of the secondary structural regions (helix 1 – helix 5) were found to be highly protected. We find that additions of sub-denaturing concentrations of urea have caused a progressive enhancement of the exchange of a number of amide protons of the Ste11 SAM domain as judged by the dramatic reduction in protection factor ratios (Figs. 8 and 9). We find that residues from helix 1, loop and helix 5 were even more prone to exchange with solvent in the presence of low sub-denaturing denaturant concentration of urea (Figs. 8 and 9).

Implications in folding and interactions: Our results, urea induced chemical shift change, temperature profile of amide protons, ^{15}N relaxation and H-D exchange experiments, obtained for the Ste11 SAM domain at sub-denaturing solution conditions containing urea might have potential implications in folding energy landscape and binding interactions of the SAM domain. Collectively, we find that among the five helices, residues in helix 1, helix 5 are primarily responsible for urea induced chemical shift perturbations and demonstrate alternate conformational states. These helical segments are also dynamically affected in comparison to other helices in the structure of Ste11 SAM domain. It may be noted that the helix 5 of the Ste11 SAM domain, and also for other canonical SAM domain, is longer in length. It may be postulated that the long helices of proteins may display higher stability due to more number of hydrogen bonds. However, our results show that the long helix 5 of the Ste11 SAM delineates most of the conformational and dynamical variations at the near native conditions. Such characteristics of the helix 5 may be linked with plausible functional requirement of the Ste11 SAM domain. It is noteworthy that helix 5 and helix 1, are involved binding interactions with Ste50 SAM domain^{39, 42, 44}. A number of residues of the helix 5 are also found to be engaged in

interactions at the dimeric interface of the Ste11 SAM domain⁴⁴. It is therefore appealing to speculate that these high energy conformational states might have implications in binding with other SAM domains. Remarkably, alternate conformational states for the substrate binding sites are observed for other proteins including SUMO-1, ubiquitin and NEDD8^{4, 66}. In these proteins, high energy conformational states are involved in substrate interactions. Considering the overlap between the near native folding and molecular recognition in the energy landscape, we hypothesize that the regions accessing alternate conformational states of the Ste11 SAM might be potentially involved in determining the ruggedness of the free energy landscape.

ACKNOWLEDGMENT

We would like to acknowledge Dr. Anirban Bhunia for helping with NMR experiments.

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Figure Legends

Figure 1: Urea induced chemical shift changes of the Ste11 SAM domain: (Panel A) A bar diagram showing combined chemical shift difference of ^{15}N and HN resonances of each residue of the SAM domain between native and 2M urea. The residues experiencing above average chemical shift change are marked. The dotted line is indicating the average chemical shift difference. The secondary structural elements are shown at the bottom. (Panel B) A ribbon representation of the three dimensional structure of the Ste11 SAM domain (pdb: 1X9X) highlighting residues showing above average combined chemical shift changes in 2 M urea (the blue and orange spheres indicate residues with $\text{CSP} \geq 0.04$ ppm and $0.024 \leq \text{CSP} < 0.04$ ppm, respectively). Figure was prepared using PyMOL program.

Figure 2: Non-linear temperature dependence of the amide chemical shifts of the Ste11 SAM domain in native and in sub-denaturing conditions: Representative examples of residues of the Ste11 SAM domain showing non-linear or curved temperature profile as a function of temperatures of the amide proton chemical shifts at 0 M, 0.5 M, 0.75 M, 1 M and 1.5 M urea. The right panel shows a few examples of residues delineating linear temperature dependence. Error bars indicate chemical shift measurement error, which was estimated to be ± 0.003 ppm.

Figure 3: Residues of the Ste11 SAM domain with curved temperature profiles: (panel A) Summary of the residues showing curved temperature dependence of the Ste11 SAM domain in native and in urea: 0.5 M, 0.75 M, 1 M and 1.5 M concentrations. The filled circle represents residues displaying non-linear behavior. The secondary structures of the native Ste11 SAM

domain are shown above amino acid sequence. (Panel B) A ribbon representations of the three-dimensional structure of the Ste11 SAM domain (pdb: 1X9X) highlighting (in sphere) residues showing curved temperature profiles in native, 0.5 M, 0.75 M, 1 M and 1.5 M urea concentrations. Residues showing non-linear behavior are excusing alternate conformational states.

Figure 4: Comparison of the dynamical characteristics of the Ste11 SAM Domain at native and near-native condition: Bar diagrams showing differences between R_1 (panel A) and R_2 (panel B) of residues of the Ste11 SAM domain at native and two different urea concentrations (0.5 M and 2 M). The residues exhibit significantly higher ΔR_2 values are marked (panel B). These residues may experience the enhancement of conformational exchange with the increment of urea concentration. The bar diagram (panel C) compares average R_2 values of the residues belonging to the five helices of the of Ste11 SAM domain at 0 M, 0.5 M and 2 M urea concentration.

Figure 5: Comparison of the order parameter of the Ste11 SAM Domain at native and near-native condition: The order parameter (S^2) of Ste11 SAM domain as function of amino acid sequence at native (panel A) and in 0.5 M urea (panel B). The dotted line indicates the average order parameter value. (panel C) The average S^2 value of the residues comprising five helices of the Ste11 SAM domain. A reduced average S^2 value of helix 5 in 0.5 M urea indicates its comparative flexibility at pico-nano second time scale.

Figure 6: Comparison of the exchange rates of the Ste11 SAM Domain at native and near-native condition: Bar diagram showing the chemical exchange contribution (K_{ex}) estimated

from Model free analyses of the Ste11 SAM domain as a function of amino acid sequence at 0 M urea (black bar) and 0.5 M urea (gray bar).

Figure 7: Urea induced change in the H-D exchange profile of Ste11 SAM domain: ^{15}N - ^1H HSQC spectra of Ste11 SAM domain after 360 min exchange against solvent D_2O at native condition and in presence of 0.25 M, 0.5 M, 0.75 M and 1.5 M urea. Samples were prepared by dissolving lyophilized powder in a D_2O buffer of 10 mM sodium phosphate, pH 5.8, containing 300 mM NaCl and 20 mM β -ME with/without adequate amount of urea. The protein concentrations for H/D exchange studies were fixed at 0.5 mM.

Figure 8: Urea induced change in protection factor of the amide protons of Ste11 SAM domain: The plot shows the residue specific change in protection factor with the increment of urea concentration from 0 M to 1.5 M. The solid filled box, circle and triangles represent protection factor ratios (urea/native) at 0.25 M, 0.75 M and 1.5 M urea concentrations. The solid line indicates the ratio of 0.5 while the dashed line demarcates the ratio of 0.1. The residues with protection factor ratio >0.5 can be considered as highly protected while those below 0.1 are more solvent accessible.

Figure 9: Changes in solvent accessibility of different regions of Ste11 SAM with increasing urea concentration: A ribbon representation of the three-dimensional structure of the Ste11 SAM domain (pdb: 1X9X) highlighting residues either with less protection factor (protection factor ratio ≤ 0.27) or with high protection factor (protection factor ratio ≥ 0.5) in pink and blue spheres, respectively. The urea concentrations are mentioned above the structure.

Figure1
69x30mm (300 x 300 DPI)

Figure2
154x133mm (300 x 300 DPI)

A

B

Figure3
216x264mm (300 x 300 DPI)

Figure4
47x12mm (300 x 300 DPI)

Figure5
279x633mm (300 x 300 DPI)

Figure6
102x78mm (300 x 300 DPI)

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Figure7
98x54mm (300 x 300 DPI)

Figure8
153x131mm (300 x 300 DPI)

Figure9
37x7mm (300 x 300 DPI)

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