Molten Globules

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The RING Domain of the Scaffold Protein Ste5 Adopts a Molten Globular Character with High Thermal and Chemical Stability**

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Abstract: *Ste5 is a scaffold protein that controls the pheromone response of the MAP-kinase cascade in yeast cells. Upon pheromone stimulation, Ste5 (through its RING-H2 domain) interacts with the β and γ subunits of an activated heterodimeric G protein and promotes activation of the MAP-kinase cascade. With structural and biophysical studies, we show that the Ste5 RING-H2 domain exists as a molten globule under native buffer conditions, in yeast extracts, and even in denaturing conditions containing urea (7 M). Furthermore, it exhibits high thermal stability in native conditions. Binding of the Ste5 RING-H2 domain to the physiological Gβ/γ (Ste4/Ste18) ligand is accompanied by a conformational transition into a better folded, more globular structure. This study reveals novel insights into the folding mechanism and recruitment of binding partners by the Ste5 RING-H2 domain. We speculate that many RING domains may share a similar mechanism of substrate recognition and molten-globule-like character.*

Ste5 is a scaffold protein that controls the pheromone response MAP-kinase cascade in yeast.[1] Upon stimulation by pheromone, Ste5 interacts with the β and γ subunits of activated heterodimeric G protein through its RING-H2 domain and facilitates activation of the MAP-kinase cascade. The structural basis for RING-H2 domain function remains to be clarified. Our structural and biophysical experiments using differential scanning calorimetry (DSC) and nuclear magnetic resonance spectroscopy (NMR) indicate that the *S. cerevisiae* Ste5 RING-H2 domain (scSte5 RH2) exhibits a molten globule fold under standard buffer conditions, in yeast extracts, and even in the presence of urea (7 M). Upon binding of the physiological Gβ/γ (Ste4/Ste18) ligand, scSte5 RH2 adopts a better folded globular structure. This study presents novel insights into the mechanism of folding and recruiting binding partners by scSte5 RH2, which may well be features of other RING domains. Small globular proteins are generally thought to fold cooperatively to a well-defined state, a process that can often be described by a two-state model.[2] Deviation from this mechanism, particularly non-cooperative folding, is typical for molten globules (MG) and dry molten globules (DMG).[3] Features of molten globules, such as loose structural heterogeneity and assembly of dynamic conformers, have been reported to play important roles in molecular recognition and enzymatic catalysis.[4]

With DSC, we determined the heat capacity profile of the domain as a function of temperature in standard buffer conditions (phosphate (50 mM), NaCl (150 mM), β-mercaptoethanol (2 mM), pH 7.0; Figure 1). The observed molar heat capacity (c_p) exhibits an unusual, almost linear increase at 10–110 °C. As indicated by the sharp drop in the molar heat capacity, scSte5 RH2 aggregates at about 110 °C. Native globular proteins that unfold according to a two-state model usually exhibit a large cooperative heat capacity peak of unfolding and a defined pretransition phase. Thus, the diffuse non-cooperative nature of the scSte5 RH2 melting transition indicates a heterogeneous ensemble of conformations that are

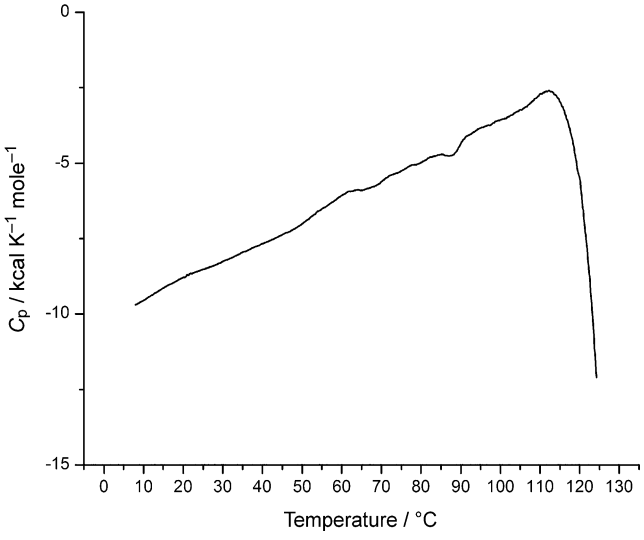

Figure 1. Molar heat capacity (c_p) of the Ste5 RING-H2 domain (scSte5 RH2) vs. temperature. Differential scanning calorimetry (DSC) measurements were conducted in an aqueous solution of scSte5 RH2 (150 μM) in standard buffer solution: phosphate (50 mM), NaCl (150 mM), β-mercaptoethanol (2 mM), pH 7.0. The diffuse non-cooperative melting transition is characteristic of a molten globule state; aggregation occurs at about 110 °C.

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characteristic for a molten globule state, which has been previously observed in other domains.[5] The species scSte5 RH2 exhibits very high thermal stability, which was also confirmed by NMR spectra measured at 298 K, 328 K, and 358 K (Supporting Information, Figure S9). The slope of the molar heat capacity as a function of temperature is $27.3 \times 10^{-3}\,J\,K^{-2}\,g^{-1}$, which is approximately four times larger than typical values for folded proteins ($[6–8] \times 10^{-3}\,J\,K^{-2}\,g^{-1}$).[6] This demonstrates that the domain shows significant conformational dynamics, as is typical in several known molten globules.

Further evidence that scSte5 RH2 adopts a molten globule state was obtained from NMR measurements. First, we measured a two-dimensional (2D) ^{1}H-^{15}N heteronuclear correlation (HSQC) spectrum of scSte5 RH2 in the same standard conditions as in the DSC experiments above. The broad signals, indicating exchange between several conformations and the small chemical shift dispersion (Figure 2a), are typical for a molten globule. The monomeric state of scSte5 RH2 and the integrity of the zinc coordination were confirmed by several experiments (for details see: Figures S7, S8, S10, and S11, and Table S3).

After adding urea (7 M) the HSQC spectrum (Figure 2b), and, consequently, the general fold, remained remarkably similar. Changes that are characteristic of protein unfolding, such as resonances moving towards random coil positions, narrowing of line-widths, or loss of the coordinated zinc ion, were not observed. Based on these observations, we analyzed whether scSte5 RH2 also exists as a molten globule in a buffer that mimics in vivo conditions. A [^{1}H,^{15}N]-HSQC spectrum of the domain in yeast extracts (Figure 2c) is very similar to the one observed in standard buffer conditions, thus suggesting that scSte5 RH2 may exist as a molten globule in vivo.

For a better understanding of the dynamic behavior of scSte5 RH2 we measured [^{1}H,^{15}N]-HSQC spectra at 5, 25, and 45 °C (Figure 3). Usually, resonance linewidths of proteins decrease with increasing temperature, owing to shorter rotational correlation times (τ_c). Most resonances follow this rule (Figure 3). However, some peaks observed at low temperatures are not observed at higher temperatures, which is indicative of a conformational exchange that is typical for molten globules. Other resonances showed the opposite behavior (green circles in Figure 3; see also Figure S1), which could mean that a resonance represents a single conformation at 4 °C, resulting in a narrower linewidth than at 45 °C, where different conformers interconvert on an intermediate timescale. We also observed some peaks doubling at all temperatures investigated, but for different resonances (Figure S1). The observation of two resonances for a particular nucleus indicates two slowly exchanging conformers. Together, our NMR and DSC data strongly supports the conclusion that scSte5 RH2 exists as a heterogeneous structural ensemble in solution, with the ability to adopt multiple conformations in widely different experimental conditions.

For a more detailed analysis of the NMR spectra, we performed chemical shift assignments for the backbone resonances (Table S1) in denaturing conditions (Figure 4; see also Table S4). Because of severe overlap of resonances in the central region of the [^{1}H,^{15}N] correlation spectra, the backbone resonance assignment could only be completed to 87%. Well-dispersed resonances represent residues that are positioned in the vicinity of cysteine and histidine residues that coordinate a zinc cation: L55, S56, Q58, E59, C60, and L61 (Figure 4). TALOS+ analysis (Table S2) and 3D ^{15}N-resolved [^{1}H,^{1}H]-NOESY data (Figure S3; for the peak list see Table S5) revealed typical H^{α}-H^{N} and H^{N}-H^{N} NOE patterns for α-helical and β-strand secondary structures for parts of the polypeptide.[8] Figure 4b summarizes the secondary structure elements identified in scSte5 RH2. The first β-

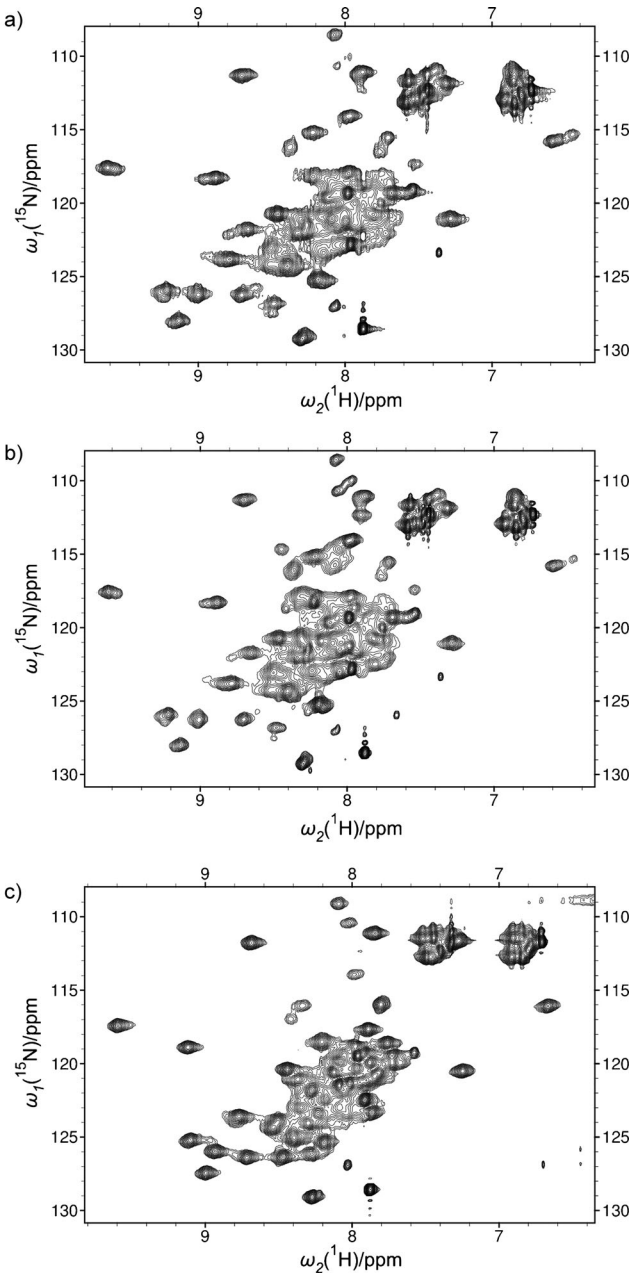

Figure 2. 500 MHz two-dimensional (2D) ^{1}H-^{15}N correlation NMR spectra of the ^{15}N-labeled Ste5 RING-H2 domain in different buffers measured at 25 °C; concentration (200 μM) determined by PULCON.[7] a) Standard buffer (see Figure 1). b) Standard buffer plus urea (7 M). c) Yeast extract.

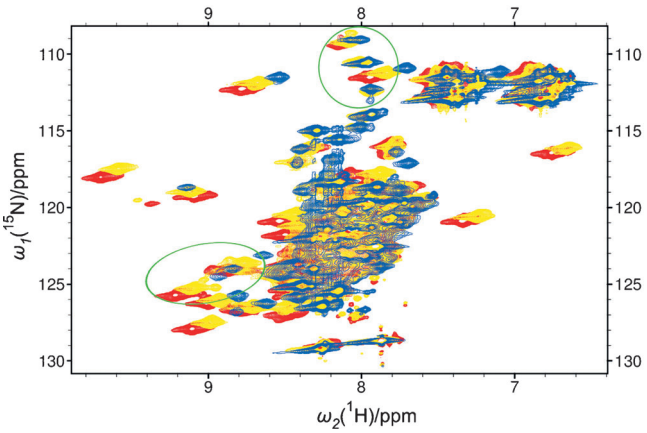

Figure 3. Overlay of three NMR spectra of scSte5 RH2 (shown in Figure 2a) measured at 5 °C (blue), 25 °C (yellow), and 45 °C (red) in standard buffer conditions. Green circles show resonances that demonstrate unusual line broadening with increasing temperatures or peak doubling at different temperatures (see text); detailed expansions and mapping of the amino acid sequence are presented in the Supporting Information (Figures S1 and S12).

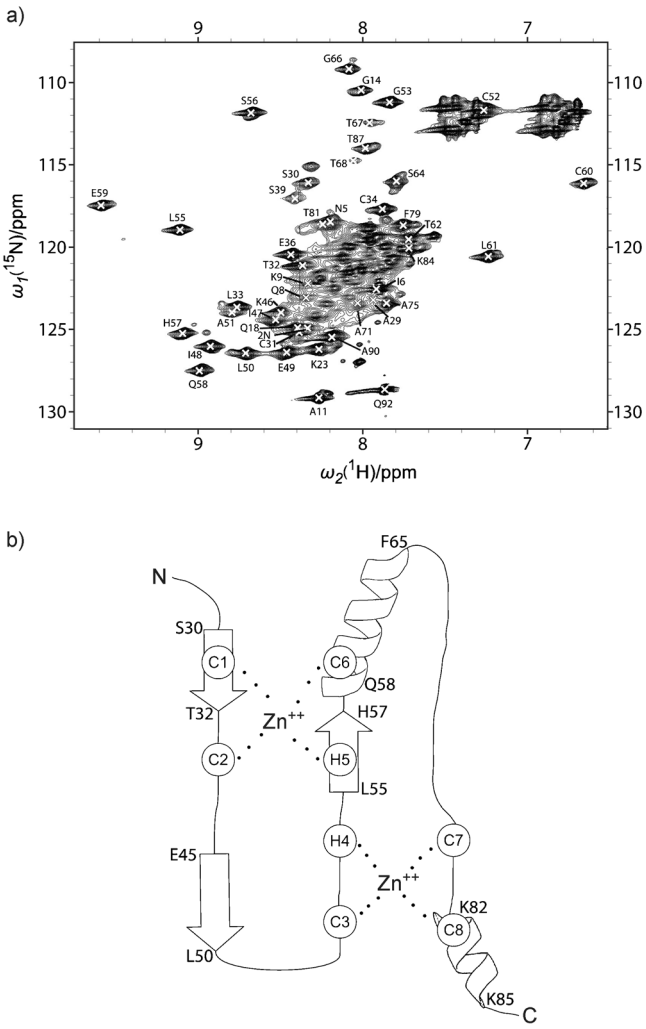

Figure 4. a) 2D ^{1}H-^{15}N correlation spectrum of scSte5 RH2 (in denaturing buffer; see Figure 2b) measured at 900 MHz and 25 °C. Backbone resonance assignments (Table S4) are indicated by white crosses labeled with the amino acid code and sequence number. Assignments of the central part are given in Figure S2. b) Zn^{2+} coordination and secondary structure elements in scSte5 H2 in standard buffer conditions (see Figure 1).

strand (β_1; residues S30–T32) includes the cysteine residue C31 involved in zinc coordination. A second β-strand (β_2) is formed from E45–L50. The third short β-strand (β_3, L55–H57) comprises the zinc-coordinating H57. In addition, NOE patterns demonstrate the presence of two short α-helices Q58–F65 (α_1) and K82–K85 (α_2). Overall, our structural analysis shows that the molten globule of scSte5 RH2 comprises disordered parts and regions with predominantly regular secondary structure elements.

Based on our data, scSte5 RH2 adopts similar conformational states in standard buffer conditions, in the presence of urea, and in yeast extracts. The varying solvent conditions do, however, influence the dynamic behavior. Therefore, we recorded heteronuclear NOE data (Figure S4) to roughly characterize the dynamics of the amide groups in the polypeptide backbone. Average heteronuclear NOE values of scSte5 RH2 in yeast extract and in normal buffer conditions was high (over 0.7), which reflects the rigidity of the backbone,[9] and confirms that scSte5 RH2 is not in a random-coil state. In urea (7 M) the protein backbone was more dynamic, producing smaller heteronuclear NOE values, especially between residues 31 and 58. Possibly urea loosens the hydrogen bond network, which could reduce the propensity for secondary structure elements. Few residues situated in the vicinity of the zinc coordination site exhibit similar heteronuclear NOE values in all three conditions (Figure S4).

The peculiar molten globular state of scSte5 RH2 raises the question of whether this conformational state binds the natural ligands and whether the binding brings about a major conformational change. The physiological binding partners are the β (Ste4) and γ (Ste18) subunits of the G protein.[10] Binding was monitored by [^{1}H,^{15}N]-HSQC NMR spectroscopy, both with and without the addition of G protein subunits β/γ. Upon interaction with the binding partners, scSte5 RH2 underwent a dramatic conformational rearrangement (Figure S5). The resonances became more dispersed and narrower in linewidth. These changes provide evidence that, upon interaction with the Ste4/Ste18 heterodimer, the active form of scSte5 RH2 escapes the molten globule state at least partially and adopts a better-defined and less-dynamic structure.

We have shown that the free domain exists as a molten globule, both in vitro and in conditions closely mimicking the in vivo situation. This type of molten globule consists of a heterogeneous ensemble of sub-states undergoing dynamic exchange. The domain is partially folded owing to its zinc clusters, and includes some secondary structure elements. Even in urea (7 M) the zinc clusters are preserved, whereas other structural elements become less pronounced. Other zinc-finger domains show a collapse of their global fold upon urea treatment (Figure S6), which may indicate a loss of zinc coordination. The large conformational change of scSte5 RH2 in the presence of the natural ligands Ste4/Ste18 could

represent a structural plasticity, which facilitates the recognition of multiple ligands and which allows it to play an important role in other processes, such as signaling in the case of Ste5. Our unpublished results of the Far1 RING domain show signatures of a molten globular state similar to scSte5 RH2. Thus, it is tempting to speculate that this folding state could be a common feature of many RING domains. Alternatively folded RING domains could constitute another state of structural organization that differs from folded and unfolded ones, as previously proposed.[4b,11]

From over 300 mammalian RING domains, the largest subspecies of ubiquitin ligating enzymes (E3 ligases) in the ubiquitin proteasome system (UPS), only a few atomic structures have been determined, and few details of their enzymatic activities are known.[12] In the case of alternatively folded proteins like molten globules, different conformers could represent low-populated enzymatically active states responsible for different molecular functions.[13]

As a further aspect of our studies on scSte5 RH2, we found exceptional stability against thermal and chemical treatment. The high stability was remarkable, and to our knowledge it is the first example of the preservation of a molten globule state in denaturing conditions, although studies of proteins in denaturing conditions have shown partial, residual ordering.[14] These findings contrast with the general view that molten globules have poor thermal and chemical stability.[15] Often molten globules are related to misfolding, or are even regarded as irrelevant. Possibly, the stability of scSte5 RH2 emphasizes its importance to fulfill essential molecular functions, such as the activation of a signaling cascade under stressful conditions.[10] Stable MGs able to promiscuously bind multiple ligands could be of great evolutionary advantage in simple organisms like bacteria or yeasts. The simultaneous coexistence of several conformations of the Ste5 RING domain would favor a conformational selection of binding partners like the ubiquitin conjugating enzymes (E2) without adopting a defined fold, as in the cases of *Methanococcus jannaschii* chorismate mutase (MjCM) and glutathione transferase A1-1 (GSTA1-1).[4b,11] On the other hand, Ste5 clearly forms a more globular structure upon binding G proteins. Proteins able to interact with several molecular partners in different binding modes may be evolutionarily old because of their adaptation, and switch between induced-fit and conformational selection, which could be a convenient way to share different functions. Furthermore, some molten globule proteins, such as bacterial type III secretion chaperones, have been reported to bear evolutionarily conserved autoinhibitory properties, which is abolished by a structural change upon binding with its partner.[16] Therefore, we speculate that the alternatively folded RING domains may play more sophisticated and significant biological roles than have been thus far imagined.

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