

Mechanism of 3D domain swapping in bovine seminal ribonuclease

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3D domain swapping (3D-DS) is a complex protein aggregation process for which no unique mechanism exists. We report an analysis of 3D-DS in bovine seminal ribonuclease, a homodimeric protein whose subunits are linked by two disulfide bridges, based on NMR and biochemical studies. The presence of the covalent bonds between the subunits stabilizes the unswapped dimer, and allows distinct evaluation of the structural and dynamic effects of the swapping with respect to the dimerization process. In comparison with the monomeric subunit, which, in solution has a compact structure without any propensity for local unfolding, both swapped and unswapped dimers show increased flexibility. NMR analysis, together with urea denaturation and hydrogen–deuterium exchange data, indicates that the two dimers have increased conformational fluctuations. Furthermore, we found that the rate-limiting step of both the swapping and unswapping pathways is the detachment of the N-terminal helices from the monomers. These results suggest a new general mechanism in which a dimeric intermediate could facilitate 3D-DS in globular proteins.

Structured digital abstract

• [BS-RNase](#) and [BS-RNase bind](#) by [nuclear magnetic resonance](#) ([View interaction](#))

Introduction

Despite the renewed interest in 3D domain swapping (3D-DS), partly because of its association with the aggregation mechanism of amyloidogenic proteins [1,2], the details of the process are not known, and nor can the tendency of a protein to swap be predicted [3]. According to the Eisenberg scheme [4], 3D-DS is prompted by the partial opening of a monomer structure, which is followed by the association of the partially unfolded proteins: in the swapped dimer, a new contact region between the two subunits is established, defining the so-called open interface, and the two dislocated regions reconstitute the previous inter-

face (called closed). This mechanism appears to be plausible, and only the first step has been revised, because experimental data have suggested two alternative scenarios: local partial unfolding of monomers in several cases [5], but total monomer unfolding in other cases [6,7]. Following the partial unfolding scheme, the Gibbs energy balance associated with 3D-DS should consist of: (a) the energy loss necessary to detach the swapping domain and unfold the hinge region, where prolines are often present [8]; (b) a loss in translational entropy for dimerization; and (c) an energy gain resulting from the new interactions occur-

Abbreviations

3D-DS, 3D domain swapping; BS-RNase, seminal bovine ribonuclease; H–D, hydrogen–deuterium; HSQC, heteronuclear single quantum coherence; mBS, monomeric derivative of seminal bovine ribonuclease; PDB, Protein Data Bank; PF, protection factor.

ring at the open interface. On the other hand, in the total unfolding scheme, the energy loss for the first step is significantly larger, and the probability of such an event is considerably lower. However, by comparing the kinetic and thermodynamic parameters of several proteins swapping under different experimental conditions, Liu and Huang recently suggested the general occurrence of total unfolding [9]. They propose that this intermediate step should be necessary not only in the case of proteins requiring chemical or physical stress to swap (such as RNase A and cyanovirin-N), for which global unfolding has already been suggested [6,7], but also for the C-terminal domain of severe acute respiratory syndrome coronavirus main protease, for which other authors have found swapping without unfolding [5].

In all of the above-mentioned cases, 3D-DS is always associated with a monomer-to-swapped dimer conversion, which complicates the analysis, because of the need to consider the loss in translational entropy. Therefore, in this study we have investigated the swapping mechanism in an interesting protein representing a special case of intramolecular 3D-DS. Bovine seminal ribonuclease (BS-RNase) is, indeed, biosynthesized as a monomer, but it is converted into a homodimer through two disulfide bonds, between Cys31 and Cys32 of one subunit and Cys32 and Cys31, respectively, of the other subunit. The native protein is isolated as a mixture of unswapped ($M=M$) and swapped ($M\times M$) isoforms, in a molar equilibrium ratio $[M\times M]/[M=M]$ of 2 : 1 [10]. Interestingly, the swapping induces further entangling of the BS-RNase subunits, and elicits additional biological properties of the protein. BS-RNase shows selective cytotoxicity for tumor cells, which is strongly related to the enzymatic activity [11]. Moreover, besides the RNA hydrolytic function, other essential features that endow BS-RNase with these additional properties are the ability to strongly interact with the tumor cell membranes [12,13], which results in protein internalization via endocytosis [14], and the ability to evade the binding of RNase inhibitor [15], a protein found at high concentrations in the cytosol that strongly binds, and inhibits, most monomeric ribonucleases [16,17]. It has been proposed that this latter property depends on the swapped isoform, because it retains a dimeric structure even under the reducing cytosol conditions [18,19], which hamper the binding by RNase inhibitor.

From a structural point of view, the occurrence of the intersubunit disulfide bridges makes BS-RNase a unique case among swapping proteins, because the swapped [Protein Data Bank (PDB) entry: [1BSR](#)] and

unswapped (PDB entry: [1R3M](#)) isoforms show very minor structural differences, not only at the closed interface, but also at the open interface. Furthermore, the identical quaternary structure provides the unique opportunity to study the sole effects of the interchange of the swapping elements. *In vitro*, BS-RNase dislocates its N-terminal helices under very mild temperature and pH conditions, which necessarily involve only local unfolding. In the past, this feature has been attributed to the high flexibility of its 16–22 hinge region [20,21], where a proline is present, as in most cases of 3D-DS [8]. However, mutagenesis studies have suggested that the hinge sequence is not responsible for the swapping process [22]. Furthermore, the monomeric derivative of BS-RNase (mBS) in solution behaves like a compact, globular protein, without any detectable propensity for local unfolding [23].

Here, we report an analysis of the interchange of the N-terminal helices in BS-RNase based on a combination of NMR and kinetic data, and we show how the dimerization process can facilitate the 3D-DS process.

Results

NMR studies

A comparison of the structural properties of the two dimeric, purified BS-RNase isoforms was performed by means of NMR measurements. Swapped and unswapped conformers showed a superimposable set of resonances (Fig. 1A), indicative of a very similar structure, in line with X-ray diffraction data. Comparison of backbone chemical shifts with those of mBS revealed that only a few resonances were shifted (Fig. 1A), most of them belonging to residues located in close proximity to the open interface and the hinge loop. Thus, with simple $^1\text{H}/^{15}\text{N}$ -HSQC experiments, it was possible to monitor the swapping process by incubating the samples of the unswapped dimer at 37 °C and acquiring spectra at 25 °C (to suppress any further swapping) every 2 h over several days. Given the high degree of similarity between the NMR spectra, most signals of the two isoforms overlapped to a high degree. Nevertheless, we were able to monitor the process by following the intensity of a few peaks corresponding to residues located in different regions of the protein surface, i.e. at the open interface (Leu28), and in the β -sheet region (Asp83, Lys98, and Thr99). An overlay of the respective regions in the $^1\text{H}/^{15}\text{N}$ -HSQC spectra at different times during the swapping process is shown in Fig. 1B. The time dependence of the signal intensity, over the whole time interval explored, is

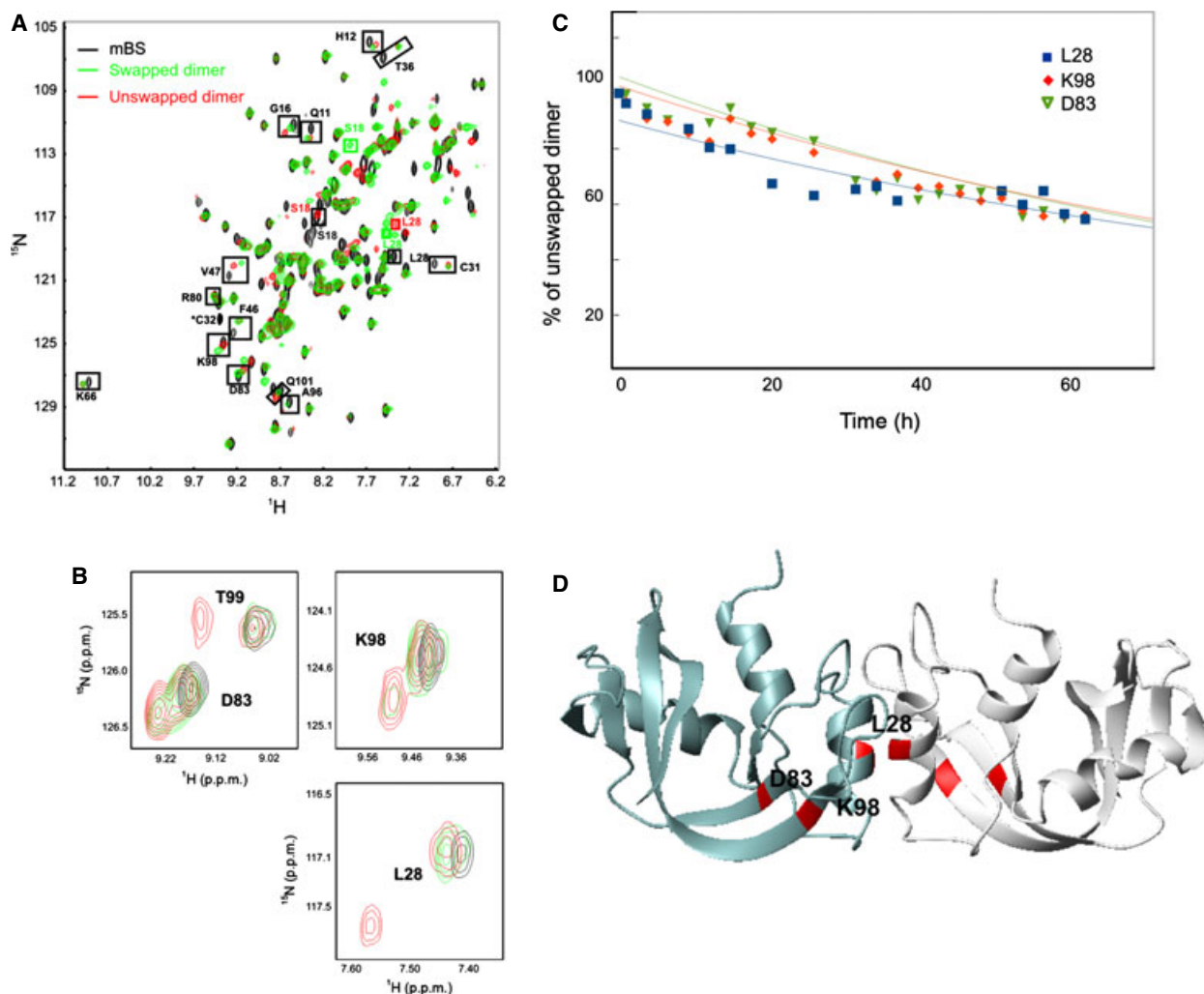


Fig. 1. BS-RNase isoforms in solution. (A) Comparison of $^1\text{H}/^{15}\text{N}$ -HSQC spectra of monomeric, unswapped and swapped isoforms. (B) Conversion of the unswapped isoform into the MxM/M=M mixture, through the overlay of $^1\text{H}/^{15}\text{N}$ -HSQC spectra of the Leu28, Lys98, Thr99, and Asp83, obtained at different experimental time points. Black: peaks of the residues at the beginning of the experiment, when only the unswapped dimer is present in solution. Green: peaks obtained halfway through the experiment. Red: the HSQC spectrum of the mixture of swapped and unswapped isoforms at the end of the experiment. (C) Time dependence of Leu28, Asp83 and Lys98 signal intensities followed by the use of $^1\text{H}/^{15}\text{N}$ -HSQC during the swapping process. (D) Structure of the unswapped dimer (PDB code: [1R3M](#)), with the positions of Leu28, Asp83 and Lys98 highlighted in red.

shown in Fig. 1C. The peak intensities decreased in a similar manner for all of the residues, even in regions far away from the dislocating helices, indicating that the swapping process affects the local environment of the protein residues in a concerted way, but we have not used these data to try to obtain rate constant values. Furthermore, in none of the spectra did we have evidence of populated equilibrium intermediates. In addition, the hinge peptide linking the N-terminal helix to the main protein body was characterized by similar conformational flexibility in both dimers, as indicated by the $^1\text{H}/^{15}\text{N}$ heteronuclear NOE signals (Fig. S1). On the other hand, the flexibility of this region in both

dimers is smaller than in the same region in mBS, as a consequence of the interactions occurring between the side chains of hinge residues and the corresponding partner subunit [22]. This result indicates that the conformational flexibility of the hinge peptide is not sufficient to account for the propensity to dislocate the N-terminal helices, as it has been previously shown that, in aqueous solution, mBS behaves like a stable globular protein, without any tendency to detach the N-terminal helix [23,24]. As the dimeric forms showed an increased propensity to detach the N-terminal helices, a further analysis of the protein conformational fluctuations in solution was performed by com-

paring the early stages of urea-induced denaturation and the hydrogen–deuterium (H–D) exchange rates of both BS-RNase isoforms with those of mBS by means of NMR measurements. Figure 2A shows the effects of the addition of 1 M urea on mBS, by showing the residues whose chemical shifts were displaced more than 0.1 p.p.m. upon addition of 1 M urea, and the residues that underwent a displacement between 0.05 and 0.1 p.p.m. The same data are reported in the form of a bar-graph in Fig. S2. Figure 2B,C shows the effect of the addition of 1 M urea on unswapped and

swapped BS-RNase. Comparison of the three panels of Fig. 2 indicates that urea accessibility was increased in both dimers with respect to mBS, as, in the presence of 1 M urea, both dimers were affected by urea addition more than mBS. The different behavior between monomer and dimers was more evident at the interface regions, at the so-called deamidation loop (region 65–72), and at the C-terminal region. In addition, the N-terminal helices proved to be more accessible in the unswapped dimer than in the swapped one, in agreement with the higher conformational stability of the latter isoform, which is more abundant in solution. Furthermore, significant differences in regions crucial for swapping between monomer and dimers emerged at higher urea concentration, as shown in Fig. S3. This shows differences between the two dimers, indicating that the N-terminal helices and the hinge peptides were more affected in the unswapper isoform than in the swapped isoform. Additional evidence for the increased conformational flexibility of the dimeric isoforms emerged from the H–D exchange experiments. On the basis of the protection factors, calculated as described in Experimental procedures, residues were divided into three classes: slow-exchanging, medium-exchanging, and fast-exchanging (Fig. 3). The comparison of Fig. 3A with Fig. B,C suggests a significant difference between the monomeric and dimeric isoforms. Most residues in the N-terminal helix passed from the slow-exchange regime in mBS (Fig. 3A) to the medium-exchange regime in the swapped isoform (Fig. 3B), and even to the fast-exchange regime in the unswapped isoform (Fig. 3C). Again, among the dimers, the unswapped isoform showed the highest

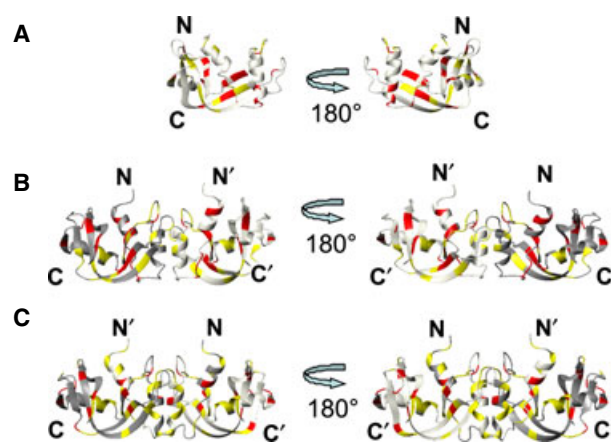


Fig. 2. Chemical denaturation of BS-RNase isoforms. Urea (1 M) denaturation of monomeric (A), unswapped (B) and swapped (C) BS-RNase. Residues whose amide protons shift more than 0.05 ppm but less than 0.1 ppm are shown in yellow. Residues whose amide protons shift more than 0.1 ppm are shown in red.

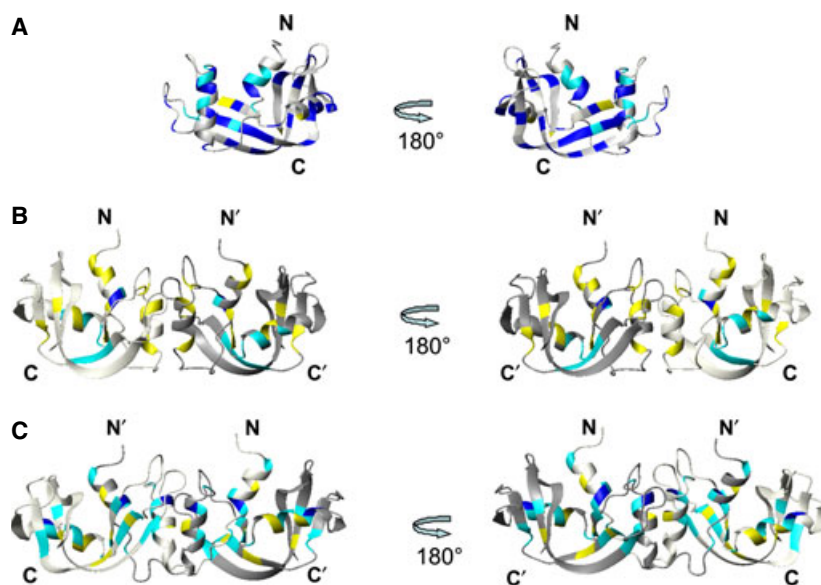


Fig. 3. Solvent accessibility of BS-RNase isoforms. Hydrogen exchange measurements in monomeric (A), unswapped (B) and swapped (C) BS-RNase. PFs are mapped onto the mBS structure with the following color code: residues with $PF > 1 \times 10^4$ are shown in dark blue, those with $10 \times 10^3 < PF < 1 \times 10^4$ are shown in cyan, and those with $PF < 10 \times 10^3$ are shown in yellow. Residues that could not be analyzed are shown in gray.

conformational fluctuations in solution. Overall, the experimental data reported in Figs 2 and 3 suggest that dimerization increases the accessibility and the conformational flexibility of the regions playing the fundamental role in the domain swapping of BS-RNase, and that, in particular, the unswapped dimer is the most plastic form in solution.

Interconversion kinetic analysis

To complete our investigation of swapping in BS-RNase, we investigated the temperature dependence of the rate of conversion of both isoforms by using a well-established biochemical protocol for the quantitative evaluation of isoform amounts, which has already been used to monitor the time course of both the swapping and unswapping processes in native and recombinant BS-RNase [10,22], as well as in some mutants [22,25]. It is worth noting that, as in all other known cases of 3D-DS, the isoform interconversion reactions can be studied solely over a very narrow temperature range, as indicated in Fig. S4A,B. A standard kinetic analysis allowed the evaluation of the rate constants for the two processes, on the assumption that both swapping and unswapping follow pseudo-first-order kinetics at the different temperatures. These rate constant values were used to construct two Arrhenius plots (Fig. S4C). Both swapping and unswapping yielded the same activation enthalpy value (99 ± 12 kcal mol⁻¹ and 97 ± 11 kcal mol⁻¹, respectively). This value corresponds, within the limits of experimental error and with a change of the sign, to twice the enthalpy change associated with the binding of the S-peptide to the S-protein: (a) $-(43 \pm 2)$ kcal mol⁻¹ at 25 °C and pH 5.0, via direct isothermal titration calorimetry measurements [26]; (b) $-(44 \pm 3)$ kcal mol⁻¹ at 39 °C and pH 7.0, via a specific analysis of differential scanning calorimetry data for the denaturation of S-peptide–S-protein complexes [27]; and (c) $-(48 \pm 3)$ kcal mol⁻¹ from the kinetics of dissociation of S-peptide from S-protein at 30 °C and pH 6.7 [28,29]. This finding indicates that: (a) the rate-limiting step of both the swapping and the unswapping pathways is the detachment of the two N-terminal α -helices from the main body of the monomers; and (b) swapping and unswapping occur via local partial unfolding. This does appear to be plausible, because, for both isoforms, the productive kinetic intermediate should necessarily have the two N-terminal helices detached, and so the magnitude of the activation enthalpy should be similar. However, the kinetics of the swapping process are markedly faster than those of the unswapping process. At 37 °C, $k(M=M \rightarrow M \times M) = 0.186$ h⁻¹,

whereas $k(M \times M \rightarrow M=M) = 0.041$ h⁻¹. This might be because the two closed interfaces in the swapped isoform are not independent of each other, because they are composed of residues belonging to both monomers, and so the probability of having both the N-terminal helices detached is lower than in the unswapped isoform. It is worth recalling here that the closed interface hosts the catalytic residues of BS-RNase (His12, Lys41, and His119), which, in the swapped isoform, are contributed by the two different monomers. The crosstalk between the two active sites of the swapped isoform is confirmed by the negative cooperativity characterizing its catalytic mechanism; the occupation of one active site hinders the occupation of the other active site by a substrate molecule. This should lead to a lower value of the pre-exponential factor (which is related to the activation entropy) in the Arrhenius equation for the unswapping process (Fig. S4), giving an increase in the lower kinetic constant values. It should be emphasized that, at equilibrium, the swapped form is favored, having a concentration approximately twice that of the unswapped form (at 37 °C), but the standard Gibbs energy difference between the two isoforms amounts to only 0.5 kcal mol⁻¹ at 25 °C (i.e. $\Delta G = -RT \ln K$). This fact cannot be explained only on a structural basis, because it implies a delicate balance of several factors, among which are the accessibility and dynamics of the solvation shell that, as suggested by the H–D exchange and urea susceptibility data described above, are quite different between the two isoforms.

Discussion

The singularity of BS-RNase, isolated as a mixture of swapped and unswapped homodimeric isoforms that interconvert under mild conditions, allows separate evaluation of the effects of dimerization and swapping processes. This represents an interesting opportunity with respect to all other cases reported in the literature, which are limited to monomer–swapped dimer conversion. On the other hand, the presence of multiple, interconverting, BS-RNase isoforms (monomer, unswapped dimer, swapped dimer, and reduced-swapped dimer), together with some possible intermediates [30], makes a comprehensive study of the features of the different dimeric isoforms in solution very complicated. Our comparative analysis among the structural and dynamic features of the swapped and unswapped dimers with respect to mBS indicates that dimerization promotes swapping, influencing the protein dynamics and conformational fluctuations. This is also in agreement with the increased susceptibility to

trypsin cleavage of the two isolated dimeric isoforms in comparison with mBS, reported in a careful biochemical study [31].

According to our data, as summarized in Fig. 4 (pathway A), we propose that the new interactions established at the open interface should facilitate swapping, not only by preparing the correct scaffold [20], but also by increasing conformational fluctuations, thus favoring the transient local unfolding of the N-terminal helices and hinge regions reported in Eisenberg's scheme (pathway B), which culminates in the dislocation of N-terminal helices.

If this mechanism were also correct for other proteins, the tendency to swap would depend not only on the presence of 'strained regions' in the monomeric structure [32], but also on the presence of a potentially good open interface. Studies on RNase A derivatives seem to confirm this hypothesis, as mutants engineered with the two interchain disulfide bridges and the BS-RNase hinge yielded unswapped dimers that dislocated N-terminal ends under mild, physiological conditions [33]. The observed increase in RNase A domain-swapped aggregates induced by crowding agents is also in line with this hypothesis [34].

In conclusion, it is not possible to determine whether the BS-RNase pathway of 3D-DS represents a unique case, or whether it can be considered to be a more general scheme, even though it is tempting to speculate that BS-RNase cannot be the only protein that has evolved in this direction, and dimerization

could precede and favor swapping in other proteins. This hypothesis emphasizes the role of the two interchain disulfide bridges, because the unswapped isoform may constitute a special 'covalent' case of an unswapped noncovalent dimer that could represent an obligatory intermediate species, even in typical (i.e. monomer-to-dimer) 3D-DS processes.

Experimental procedures

Protein samples

The expression and purification of labeled and unlabeled proteins have been described in detail elsewhere [35]. The amount of swapped protein as a function of temperature was evaluated as described by Ercole *et al.* [36].

Swapping–unswapping rates

The kinetics of the swapping–unswapping process was followed at four different temperatures: 30 °C, 33 °C, 35 °C, and 37 °C.

For the kinetic analysis, interconversion rate constants at the various temperatures were obtained by fitting the first data points on the assumption of first-order kinetics.

The rate constants were determined at the beginning of the two processes, and so they should not be representative of the situation at thermodynamic equilibrium. At 37 °C, the ratio $k(M=M \rightarrow M \times M) = 0.186 \text{ h}^{-1} / k(M \times M \rightarrow M=M) = 0.041 \text{ h}^{-1}$ does not correspond to $K = 2$.

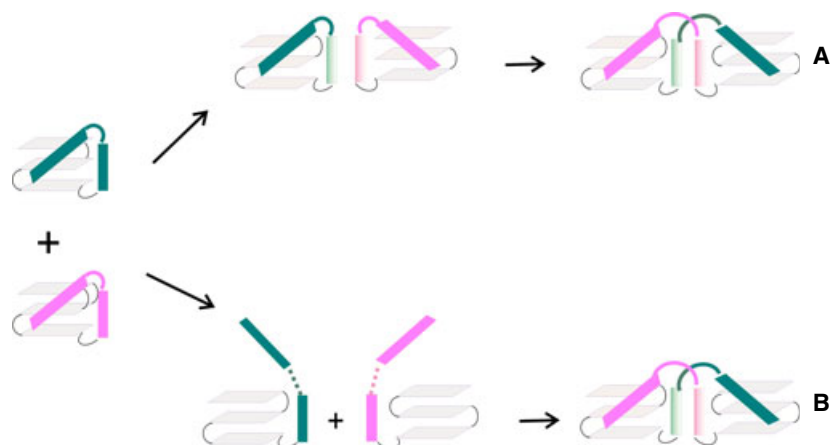


Fig. 4. Mechanisms of domain swapping. Each monomeric subunit has helical elements involved in the swapping process, respectively indicated by green and pink rectangles. The regions constituting the new ('open') interface in the dimer are also colored. According to our scheme (pathway A), the dimerization establishes intersubunit interactions (represented by faded rectangles) that destabilize the dimer structure and promote the dislocation of exchanging moieties under mild conditions. In this scheme, the 'open interface' is formed before the swapping. For comparison, the 3D-DS mechanism according to the Eisenberg scheme is reported in pathway B. It consists of the partial opening of the monomeric structure, followed by the association of the partially unfolded proteins to form the swapped dimer. In this scheme, both 'open' and 'closed' interfaces are formed in the final step of the process.

NMR measurements

NMR spectra for structure refinement were acquired at 298 K on Bruker spectrometers operating at 500 MHz, 600 MHz, 700 MHz, and 750 MHz, equipped with triple-resonance gradient probes. Data were processed with NMRPIPE [37], and visualization of spectra, peak-picking and analysis were performed in NMRVIEW [38]. Chemical shift assignments were obtained from standard 3D triple-resonance experiments performed on $^{15}\text{N}/^{13}\text{C}$ -labeled samples of unswapped and swapped dimers [39]. To follow the conversion of the swapped dimer in the mixture of unswapped and swapped dimers, a series of $^1\text{H}/^{15}\text{N}$ heteronuclear single quantum coherence (HSQC) spectra was recorded at 298 K. Each experiment at 298 K was followed by warming at 310 K for 2 h and subsequent cooling at 298 K to acquire the next HSQC spectrum.

The urea-induced unfolding of BS-RNase was performed by adding solid urea (urea_{d4}; Sigma Aldrich) directly to the NMR tube. The urea concentration was increased by 1 M at each titration step up to a total final concentration of 7 M. In order to keep the protein concentration and pH constant, we prepared a different protein sample for each point of the titration, and for each of them we adjusted the pH to 5.7. To check for the possible volume increase caused by the addition of solid denaturant, the length of the sample in the NMR tube was measured at each step, and found to be constant (within the limits of experimental error). The unfolding process was monitored with a series of 2D HSQC experiments performed at 298 K.

As described by Piccoli *et al.* [10], lyophilization affects the stability of the unswapped isoform, for this reason hydrogen exchange experiments were performed by dilution of the ^{15}N -labeled NMR dimeric protein samples 10-fold with 99.9% D₂O, and concentration in a stirred ultrafiltration cell (Amicon, Millipore). H–D exchange rates were determined by fitting a first-order exponential equation to the peak heights versus time data:

$$I(t) = I(0)\exp(-k_{\text{obs}}t)$$

where $I(t)$ represents the cross-peak height at time t , $I(0)$ the original cross-peak height, k_{obs} the observed rate of H–D exchange, and t the time in seconds. The intrinsic H–D exchange rates (k_{rc}) were calculated with the web-server program SPHERE (<http://www.fccc.edu/research/labs/roder/sphere>; [40]). The protection factor (PF) for each detectable NH group was calculated from the following equation:

$$\text{PF} = k_{\text{rc}}/k_{\text{obs}}$$

Accordingly, we divided the exchanging protons into slow-exchanging ($\text{PF} > 1 \times 10^5$), medium-exchanging ($1 \times 10^5 < \text{PF} < 1 \times 10^3$) and fast-exchanging ($\text{PF} < 1 \times 10^3$).

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Supporting information

Additional supporting information may be found in the online version of this article at the publisher's web site:

Fig. S1. $^1\text{H}/^{15}\text{N}$ heteronuclear NOEs of swapped (M×M) and unswapped (M=M) BS-RNase isoforms.

Fig. S2. Chemical shift perturbation upon addition of 1 M urea to monomeric, unswapped and swapped BS-RNase.

Fig. S3. Urea denaturation of monomeric, unswapped and swapped BS-RNase.

Fig. S4. Kinetics of swapping and unswapping processes in BS-RNase.