Open Interface and Large Quaternary Structure Movements in 3D Domain Swapped Proteins: Insights from Molecular Dynamics Simulations of the C-Terminal Swapped Dimer of Ribonuclease A

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ABSTRACT Bovine pancreatic ribonuclease (RNase A) forms two three-dimensional (3D) domain swapped dimers. Crystallographic investigations have revealed that these dimers display completely different quaternary structures: one dimer (N-dimer), which presents the swapping of the N-terminal helix, is characterized by a compact structure, whereas the other (C-dimer), which is stabilized by the exchange of the C-terminal end, shows a rather loose assembly of the two subunits. The dynamic properties of monomeric RNase A and of the N-dimer have been extensively characterized. Here, we report a molecular dynamics investigation carried out on the C-dimer. This computational experiment indicates that the quaternary structure of the C-dimer undergoes large fluctuations. These motions do not perturb the proper folding of the two subunits, which retain the dynamic properties of RNase A and the N-dimer. Indeed, the individual subunits of the C-dimer display the breathing motion of the β-sheet structure, which is important for the enzymatic activity of pancreatic-like ribonucleases. In contrast to what has been observed for the N-dimer, the breathing motion of the two subunits of the C-dimer is not coupled. This finding suggests that the intersubunit communications in a 3D domain swapped dimer strongly rely on the extent of the interchain interface. Furthermore, the observation that the C-dimer is endowed with a high intrinsic flexibility holds interesting implications for the specific properties of 3D domain swapped dimers. Indeed, a survey of the quaternary structures of the other 3D domain swapped dimers shows that large variations are often observed when the structural determinations are conducted in different experimental conditions. The 3D domain swapping phenomenon coupled with the high flexibility of the quaternary structure may be relevant for protein-protein recognition, and in particular for the pathological aggregations.

INTRODUCTION

Many proteins perform their biological function in oligomeric states (Goodsell and Olson, 2000). Oligomeric proteins present several advantages when compared to the monomeric counterparts. Multimeric proteins often show increased thermal and/or chemical stability, and modulated activity through cooperative effects. Furthermore, oligomerization also leads to the formation of large protein assembly with minimal amounts of DNA. This aspect minimizes the number of errors that may occur in transcription.

The aggregation of monomers to form an oligomeric protein is a complex molecular process whose details are still obscure. Generally, the transition from monomeric to oligomeric states relies on the generation of novel chain-chain interfaces, which are progressively stabilized by evolution. Recently, a great attention has been devoted to a different mechanism denoted as three-dimensional (3D) domain swapping (Bennett et al., 1995; Schlunegger et al., 1997). The aggregation via 3D domain swapping is based on the mutual exchange of a domain of one chain with the same domain of a second chain (Bennett et al., 1994, 1995; Schlunegger et al., 1997; Newcomer, 2001, 2002; Liu and Eisenberg, 2002). Accordingly, the structure of the oligomeric protein is stabilized by contacts that preexist in the momomeric form (Closed-interface or C-interface) as well as by novel chain-chain interfaces (Open-interface or O-interface) (Bennett et al., 1995; Schlunegger et al., 1997).

Among 3D domain swapped proteins, pancreatic-like ribonucleases have played an important role, as they were the subjects of several pioneering studies (see, for example, Crestfield et al., 1962). Structural evidences on the occurrence of 3D domain swapping in ribonucleases were derived more than 20 years ago for bovine seminal ribonuclease (BS-RNase) (Capasso et al., 1983). This enzyme also exhibits the intriguing characteristic of forming two equilibrium isomers: the swapped (M $\times$ M) (Mazzarella et al., 1993) and the nonswapped (M = M) (Piccoli et al., 1992; Berisio et al., 2003) dimer. More recently, structural investigations carried out on the two dimers formed by bovine pancreatic ribonuclease (RNase A) in a variety of experimental conditions (Crestfield et al., 1962; Park and Raines, 2000; Gotte et al., 2003) have demonstrated that they

Submitted June 29, 2004, and accepted for publication December 2, 2004.

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Abbreviations used: 3D, three-dimensional; MD, molecular dynamics; RMSD, root mean square deviations; RMSF, root mean square fluctuations; RMSIP, root mean square inner product; RNase A, bovine pancreatic ribonuclease; C-dimer, C-terminal swapped dimer of ribonuclease A; N-dimer, N-terminal swapped dimer of ribonuclease A.

0006-3495/05/03/2003/10 $2.00

doi: 10.1529/biophysj.104.048611

adopt two different types of 3D domain swapping (Liu et al., 1998, 2001). Indeed, in one dimer (hereafter denoted as C-Dimer) the swapped fragment is the C-terminal β-strand (Liu et al., 2001), whereas in the other one (N-dimer) the exchanged region is the N-terminal α-helix (Liu et al., 2001). As a consequence, the N- and the C-dimer display completely different shapes as the N-dimer presents a very compact structure (Liu et al., 1998), whereas C-dimer only shows a very loose O-interface (Liu et al., 2001) (Fig. 1).

Despite the increasing interest for 3D domain swapping, the available data on the dynamic properties of 3D domain swapped dimers are rather limited. The first extensive characterizations of the dynamic behavior of a 3D domain swapped dimer compared to its monomeric counterpart have been reported only very recently (Sekijima et al., 2003; Japelj et al., 2004; Merlino et al., 2004b). In previous investigations, we demonstrated that the functional collective motions of the β-sheet structure (Merlino et al., 2002; Vitagliano et al., 1998, 2002), characteristic of pancreatic-like ribonucleases (Merlino et al., 2003), are coupled in the two subunits of the N-dimer, thus suggesting a possible mechanism for the regulation of its catalytic activity (Merlino et al., 2004b).

Here, we report the results of molecular dynamics studies of the C-dimer in water. The simulations reveal that the C-Dimer exhibits extensive movements of the two subunits. These findings, together with an examination of the quaternary structure variations of other 3D domain swapped dimers indicate that 3D domain swapping may be an effective mechanism to generate oligomers endowed with an unusual flexibility. Furthermore, the coupling of the breathing motions of the two subunits observed in the N-Dimer, is not detected in the C-dimer. These results underline the role of the compactness of the O-interface in the intersubunit communications.

METHODS

System and definitions

Following the definition of Eisenberg and co-workers (Bennett et al., 1995; Schlunegger et al., 1997), in a 3D domain swapped dimer, the interface that preexists in the monomeric form is termed Closed interface (C-interface) (Fig. 1). On the other hand, the interface that forms upon dimerization is denoted as Open interface (O-interface). The loop connecting the body with its swapped fragment of each subunit, termed hinge peptide, undergoes a dramatic structural transition in the 3D domain swapping process.

The C-dimer is characterized by an elongated shape and by a rather loose O-interface (Fig. 1) (Liu et al., 2001). The swapped fragment is represented by the C-terminal β-strand (residues 116–124). Each subunit is composed by three α-helices and a V-shaped β-sheet constituted by two arms that will be hereafter denoted as V_1 and V_2. V_1 consists of residues 61–63, 71–75, and 105–111 of one subunit and of residues 116–124 of the other, whereas V_2 embodies residues 42–46, 82–87, and 96–101. The three helices encompass residues 3–13 (helix I), 25–35 (helix II), and 50–60 (helix III). The C-interface consists of two strands (105–111 and 116–124) of the V_1 arm. The hinge peptide and the main body of the protein are composed by residues 112–115 and 1–111, respectively. The O-interface is limited to the pairing of the two hinge peptides (Fig. 1). The local structure of this region corresponds to a short β-sheet interrupted by Pro-114. Because some catalytic residues belong to the C-terminal β-strand region, in the C-dimer, two different structural units containing all the functional residues, each composed by the body of one subunit and the C-terminal strand of the other, may be identified.

Simulation procedure

The molecular dynamics simulations were performed with the program GROMACS (van der Spoel et al., 1994). The starting model for the simulation was the molecule composed of the A/B chains taken from the Protein Data Bank (PDB) entry 1F0V (Liu et al., 2001). The dimer was immersed in a rectangular box containing simple-point-charge water molecules (Berendsen et al., 1981). The ionization state of charged residues was set to mimic a neutral pH environment (Merlino et al., 2002). To neutralize the system, eight water molecules were replaced by chloride counterions. For comparative purposes, the molecular dynamics studies were carried out by using the same protocol applied for the simulation of RNase A (Merlino et al., 2002) and the N-dimer (Merlino et al., 2004b).

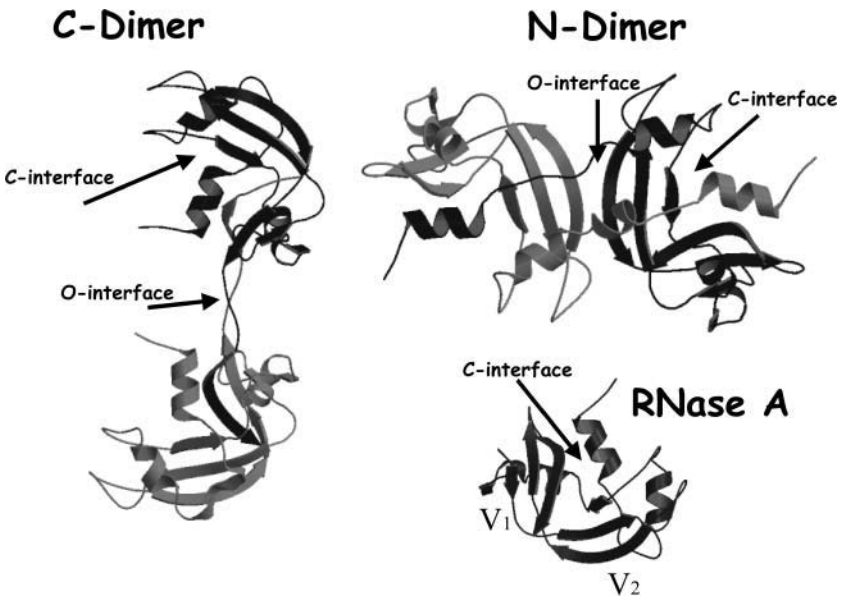

FIGURE 1 Ribbon diagram of RNase dimers.

Briefly, to allow relaxation of solvent molecules, the energy of the system was preliminarily minimized by keeping fixed the protein atoms. The resulting system, containing 58,264 atoms, was submitted to 20 ps of molecular dynamics at 300 K. The energy of the system was then minimized without restraints, before starting constant temperature molecular dynamics at 300 K. The overall timescale of the simulation was 3 ns. All bond lengths were constrained using the SHAKE algorithm (Ryckaert et al., 1977). Nonbonded cutoffs of 10 Å for Lennard-Jones and 13 Å for Coulomb potentials were used. A dielectric constant of $\varepsilon = 1$ and an integration time step of 0.002 ps were used. The coordinates were saved at every 0.2 ps.

To verify the reliability of the findings of this MD study, a second independent 3-ns simulation has been carried out at 300 K using a different set of initial velocities. The results of the two simulations are in good agreement. This represents a good indication of the completeness of the conformational sampling of each simulation. Therefore, only the results obtained from one simulation will be described.

Analysis of the trajectories

To assess the quality of the simulations, the trajectories were checked using GROMACS routines and the Metaphor software suite (M. A. Ceruso, unpublished data). To determine coupling between intra- and interstructural unit motions in the C-dimer, atomic correlation matrices were calculated and normalized as

$$C_{ij} = \langle \Delta R_i \cdot \Delta R_j \rangle / (\langle \Delta R_i \cdot \Delta R_i \rangle \langle \Delta R_j \cdot \Delta R_j \rangle)^{1/2},$$

where ΔR_i and ΔR_j are the displacements from the mean positions of the i^{th} atom and the j^{th} atom, and $\langle\ \rangle$ represent the time average over the equilibrated portion of the trajectory. C_{ij} values vary in the interval $-1 < C_{ij} < 1$. To eliminate the contribution of relative subunit motions, interstructural unit correlation matrices were generated after an independent fit of each structural unit trajectory on the same reference. To compare the motions of the two structural units, a difference atomic correlation matrix was built with elements defined as

$$\Delta C_{ij} = C_{ij}(A) - C_{ij}(B),$$

where $C_{ij}(A)$ and $C_{ij}(B)$ are the values of C_{ij} calculated for the two structural units. ΔC_{ij} values vary in the range $-2 < \Delta C_{ij} < 2$.

Essential degrees of freedom were extracted from the trajectories according to the essential dynamics method (Amadei et al., 1993) that is based on the construction of the covariance matrix of the coordinate fluctuations. The covariance matrix is diagonalized to obtain the eigenvectors and eigenvalues that provide information about correlated motion throughout the protein. The eigenvectors represent the directions of motion and the eigenvalues the amount of motion along each eigenvector. The eigenvectors are then sorted according to their eigenvalues in descending order. Usually, the first 10–20 eigenvectors suffice to describe almost all conformational substates accessible to the protein (Ceruso et al., 1999).

The internal fluctuations of the C-dimer and N-dimer structural units and of the RNase A were compared by evaluating their principal subspaces (first 10 eigenvectors). As already done to compare the concerted motions of several protein systems (Ceruso et al., 1999, 2003; Grottesi et al., 2002; Merlino et al., 2002, 2003), the overlap of the principal subspaces of each pair of proteins was evaluated by estimating root mean square inner product (RMSIP).

The RMSIP is defined as:

$$\sqrt{\frac{1}{10}\sum_{i=1}^{10}\sum_{j=1}^{10}(\eta_i^a \eta_j^b)^2},$$

where $\eta_i^a$and η_j^b are the i^{th} and j^{th} eigenvectors from the set *a* and set *b*, respectively.

The RMSIP was also used to ascertain the convergence in the essential space between two halves of the equilibrated trajectories and to evaluate the similarity of the two trajectories.

The analyses of the internal fluctuations were performed omitting the peptide regions 15–22 and 112–115, whose local structure changes in the dimerization process that leads to the formation of the N- and the C-dimer, respectively.

To ensure that the structural and dynamic properties of N-dimer derived from the simulations are free from initial nonequilibrium effects, all of the analyses were performed in the portion of trajectories from 1200 to 3000 ps. In this region the essential subspace converged as revealed by calculating the RMSIP value between the two halves of the trajectory (Table 1).

Selection and analysis of domain swapped dimers endowed with high mobility

A survey of the PDB database (Berman et al., 2002) was carried out to identify 3D domain swapped dimers that exhibit significantly different quaternary structures as a consequence of mutations, ligand binding, and/or differences in crystal packing. The search was performed on the January, 2004 release of the PDB, which contains ~22,000 protein structures.

To evaluate the rearrangement of the structural units occurring in different structures of a given domain swapped dimer, a stepwise superimposing procedure was carried out using the programs LSQKAB of the CCP4 suite (Bailey, 1994) and DynDom (Hayward and Lee, 2002). In particular, as already described elsewhere (Vitagliano et al., 1998; Merlino et al., 2004c), we evaluated the rotation required to overlay the second structural unit, after the optimal superimposition of the first.

RESULTS

C-Dimer shows extensive movements of its quaternary structure

To evaluate the stability of the MD simulation, several time-dependent properties of the C-dimer were analyzed. As shown in Fig. 2 *A* (*black line*), the C-dimer shows high fluctuations of its quaternary structure during the simulation. Indeed, the overall C^{α} root mean square deviation (RMSD) of the structure during the simulation versus the starting x-ray model fluctuates between 2 and 6 Å.

It should be noted, however, that the individual structural units (see Methods for the definition) of the protein are very stable, with RMSD close to 2 Å (Fig. 2 *A*, *gray lines*). The stability of each unit is confirmed by the analysis of the time evolution of the secondary structure elements and of the total number of hydrogen bonds. The overall stability of the system is also corroborated by the trend of the potential interaction energy, which is virtually constant during the whole trajectory.

These data indicate that the C-dimer undergoes large variations of its quaternary structure that do not perturb the proper folding of each subunit. These variations are also evidenced by the time evolution of the distance between the centers of mass of the two structural units of the dimer (Fig. 2 *B*). Interestingly, although the system significantly alters its quaternary structure during the simulation the total solvent accessible surface area display a limited fluctuation with a mean value of $725.7 \pm 3.5\ nm^2$, which is in close agreement

TABLE 1 Root mean square inner products for projection of the first 10 C^{α} eigenvectors of a set onto the first 10 of another

	C-Dimer Structural unit 1	C-Dimer Structural unit 2	N-Dimer Structural unit 1	N-Dimer Structural unit 2	RNase A
C-Dimer structural unit 1	0.78*	0.69	0.68	0.63	0.65
C-Dimer structural unit 2		0.70*	0.73	0.75	0.71
N-Dimer structural unit 1			0.75*†	0.70†	0.68†
N-Dimer structural unit 2				0.77*†	0.74†
RNase A					0.78*†

*The RMSIP between the first 10 eigenvectors of the two halves of each trajectory was calculated as reference values.
†From Merlino et al., 2004b.

with that (727.2 nm^2) derived from the x-ray structure of C-Dimer (PDB code 1F0V). Similarly, limited fluctuations of hydrophobic (mean value 278.1 ± 1.7 nm^2) and hydrophilic (mean value 446.2 ± 2.2 nm^2) solvent exposed regions are observed. Finally, it is worth mentioning that the C-dimer preserves the overall twofold symmetry during the simulation. For most of the simulation, the two structural units are related by a rotation of 168–180°. Only occasionally (between 2700 and 2800 ps) this rotation is close to 150°. These findings, along with a visual inspection of a number of structures extracted from the trajectory, suggest that the fluctuations of the quaternary structure essentially correspond to a hinge-bending motion of the two structural units (Fig. 2 *C*). A significant twist of the C-dimer structure is only observed in the region 2700–2800 ps (Fig. 2 *C*).

The motions of two structural units of the C-dimer are similar

Fig. 3 *A* shows the C^{α} root mean square fluctuations (RMSF) of the two structural units of the C-dimer. The two structural units show comparable fluctuations. The atomic correlation matrices are also extremely similar, as shown by the difference atomic correlation matrix, which does not exhibit any significant ($\Delta C_{ij} > 0.7$) feature (Fig. 3 *B*). The concerted motions were also investigated by using the root mean square inner product (RMSIP; see Methods for the definition) between the first 10 eigenvectors derived from the diagonalization of the covariance matrix of the C-dimer structural unit trajectories (Table 1). The high value of the RMSIP (0.69) represents a further indication that the two structural units of the dimer undergo the same motions.

The motions of the C-dimer structural units are similar to those of the N-dimer and to RNase A

The fluctuations of the two structural units of the C-dimer are very similar to those observed for the N-dimer (Fig. 3 *C*) (Merlino et al., 2004b) and for RNase A (Merlino et al., 2002), although in the last case the region 50–60 displays a slightly higher mobility (Fig. 3 *A*). In addition, as observed for the N-dimer (Merlino et al., 2004b) and RNase A (Merlino et al., 2002), the fluctuations of the C^{α} atom of the C-dimer structural units derived from the simulations are in good agreement with the experimentally determined B-factors (Fig. 3 *D*).

The principal subspace derived for the C-dimer structural units largely overlaps that of RNase A and of the N-dimer units, as shown by the RMSIP values reported in Table 1. As expected on the basis of this similarity, the analysis of the

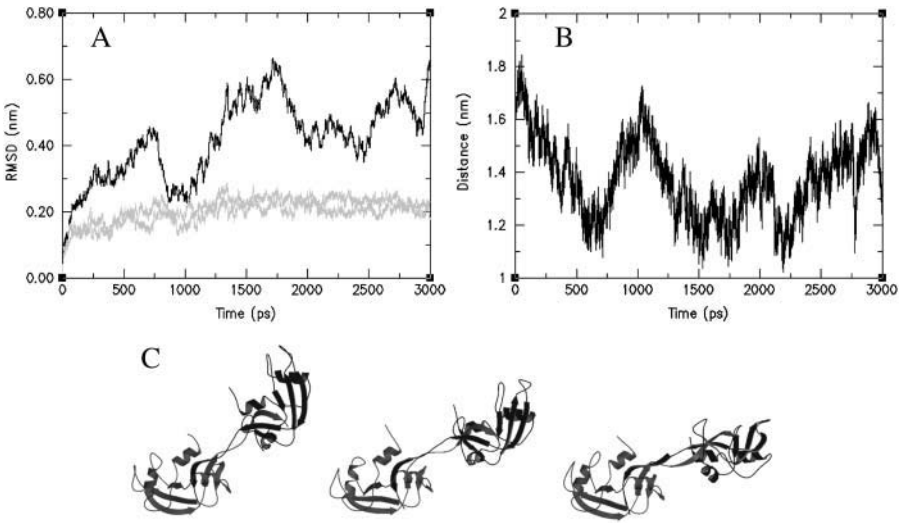

FIGURE 2 (*A*) Root mean square positional deviations from the x-ray starting structure computed on C^{α} atoms of the dimer (*black line*) and of the bodies of the two subunits (*gray lines*) as function of time, (*B*) distances between the centers of mass of the two structural units as function of time, and (*C*) snapshots from the simulation.

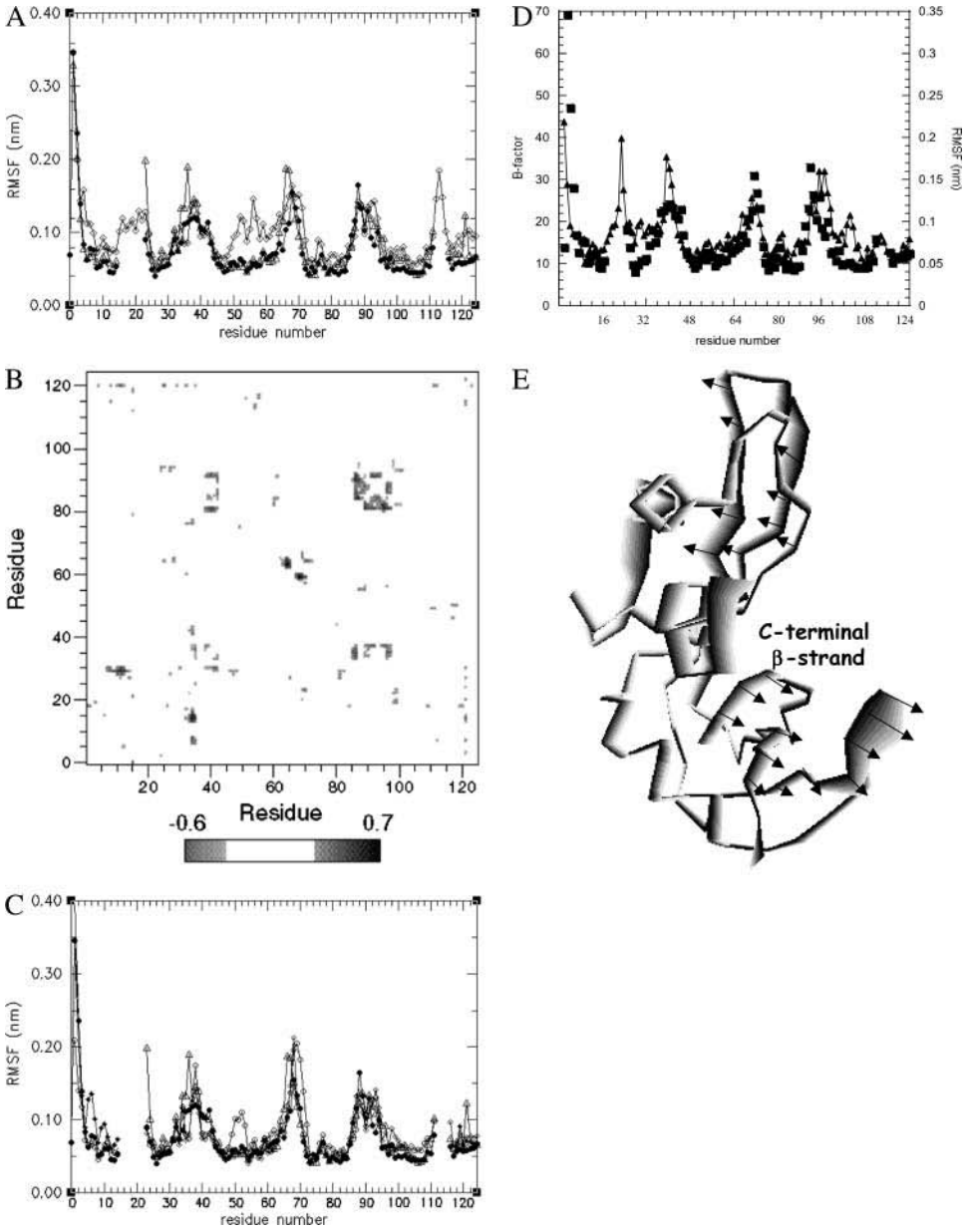

FIGURE 3 (*A*) Root mean square fluctuations of the C-dimer amino acids calculated from the simulation in comparison with those of RNase A (◇). The symbols ● and △ refer to the A and B subunits, respectively. (*B*) Difference intrastructural unit correlation matrix; (*C*) root mean square fluctuations of the C-dimer amino acids in comparison with those of the N-dimer (♦ and ○, for the A and B subunits, respectively). (*D*) Comparison between the A subunit B-factor derived from the x-ray structure (▲) and the root mean square fluctuations of the A subunit C^{α} atoms of the C-dimer (■). (*E*) Motions of one of the two structural units along the first principal eigenvector. A gray-scale is used to represent the motions in a film-like fashion. Vectors are used as qualitative indicators of the direction of the C^{α} atom motions.

first eigenvectors indicates that the β-sheet regions of the C-Dimer units exhibit the breathing motions (Fig. 3 *E*), which has been well characterized in experimental and theoretical studies carried out on RNase A (Berisio et al., 2002; Merlino et al., 2002; Vitagliano et al., 2002), N-dimer (Merlino et al., 2004b), as well as on other members of the pancreatic-like superfamily (Vitagliano et al., 1998; Merlino et al., 2003, 2004b).

This is in line with the general finding that the dynamic properties strongly depend on the architecture of the proteins (Ceruso et al., 1999, 2003, 2004; Merlino et al., 2003, 2004a). However, it should be pointed out that, in the case of the C-dimer, the β-sheet scaffold of each unit of the protein has a composite nature, because one of the β-strands comes from the partner chain. Thus, the presence of a swapped strand in the β-sheet region of the protein does not prevent the breathing motion characteristic of pancreatic-like ribonucleases.

Breathing motions of two C-dimer structural units are not coupled

The analysis of the interstructural unit correlated motions of the C-dimer was conducted by evaluating atomic correlation matrices. In Fig. 4, *A* and *B*, positive and negative correlations are reported. As can be noted from the figure, the correlation matrices for C-dimer do not show any significant peaks (C_{ij} is always <0.3), indicating that the

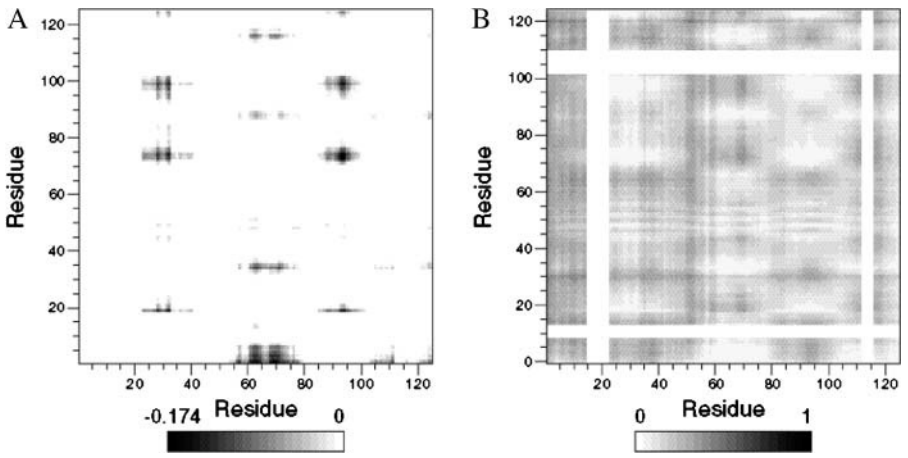

FIGURE 4 Positive (*A*) and negative (*B*) interstructural unit correlation matrices. As described in the Methods section, in this analysis the residues 16–22 and 112–115 have been omitted.

breathing motions of the two structural units (see above) are not correlated. It is worth mentioning that a completely different picture was derived for the N-dimer (Merlino et al., 2004b), where a strong correlation of these motions, likely transmitted through the extended β-sheet structure of the O-interface, was observed. Therefore, the N- and the C-dimer display different trends for the coupling of the structural unit breathing motions, although the local and global flexibility of individual units is very similar.

Motions at the C- and O-interface

The C-interface, which corresponds to the interactions between the two β-strands 105–111 and 116–124, is well preserved during the simulation. Indeed, the hydrogen bonds established among the main-chain atoms of the two strands are thoroughly conserved, as observed for RNase A (Merlino et al., 2002). As a representative example of this general trend, the hydrogen bond formed by the O atom of the Val-116 of the first subunit and N atom of Glu-111 of the other is shown in Fig. 5 *A*. The dynamic behavior of the active site region of the C-dimer is also similar (data not shown) to that reported for RNase A (Vitagliano et al., 2000; Merlino et al., 2002).

The analysis of the dynamic properties of the O-interface provides interesting information. This region consists of a short antiparallel β-sheet formed by residues 111–113 of one subunit and 111–113 of the other (Liu et al., 2001). Notably, the main-chain hydrogen bonds, which characterize the antiparallel β-sheet, are rather well preserved during the simulation, despite the large overall motions of the dimer: as an example, the hydrogen bonds between the N and O atoms of the Asn-113 of the two subunits are shown in Fig. 5, *B* and *C*. In the analysis of the dynamic properties of the O-interface, a particular attention was devoted to the hydrogen bond between the side chains of Asn-113 of the two subunits. Eisenberg and co-workers (Liu et al., 2001) suggested that this Asn-Asn H-bond could be considered as an example of the polar zipper interactions occurring in amyloid fiber formation. As shown in Fig. 5 *D*, this hydrogen bond is present in several phases of the trajectory. However, the specific geometry of the two Asn side chains changes during the trajectory, with the formation of the hydrogen bond that involves both the $O^{\delta 1}$ and the $N^{\delta 2}$ atoms of the two residues.

Extensive movements in 3D domain swapped dimers

The large interstructural unit motions of the C-dimer prompted us to carry out a survey on other 3D domain swapped dimers to verify whether extensive movements of the quaternary structures can be considered a common feature of 3D domain swapping proteins. This hypothesis was checked by analyzing the structures of 3D domain swapped dimers, which were either reported in different crystal forms or solved by NMR. In particular, we evaluated the rotation required to overlay the second structural units, after the optimal superimposition of the first (Table 2).

As shown in the table, many 3D domain swapped dimers, whose structure has been determined in different contexts, present large quaternary structure variations. Extensive variations occur for wild-type cyanovirin between the crystal and the solution state (Yang et al., 1999; Bewley and Clore, 2000; Barrientos et al., 2002). Large quaternary structure variations are also compatible with the NMR data on human cystatin (Staniforth et al., 2001). A remarkable reorganization of the quaternary structure are also observed comparing the structures of the transcription factor NusG from *Aquifex aeolicus* derived from two crystal forms (Knowlton et al., 2003), and two dimers of the Grb2-SH2-Ac-pYYNV complex present in the same asymmetric unit (Schiering et al., 2000). Smaller but significant reorganization of the quaternary structure are observed for BS-RNase (Vitagliano et al., 1998; Merlino et al., 2004c), whose quaternary structure is tightly constrained by two intersubunit disulfide bridges (Mazzarella et al., 1993). It is also worth noting that

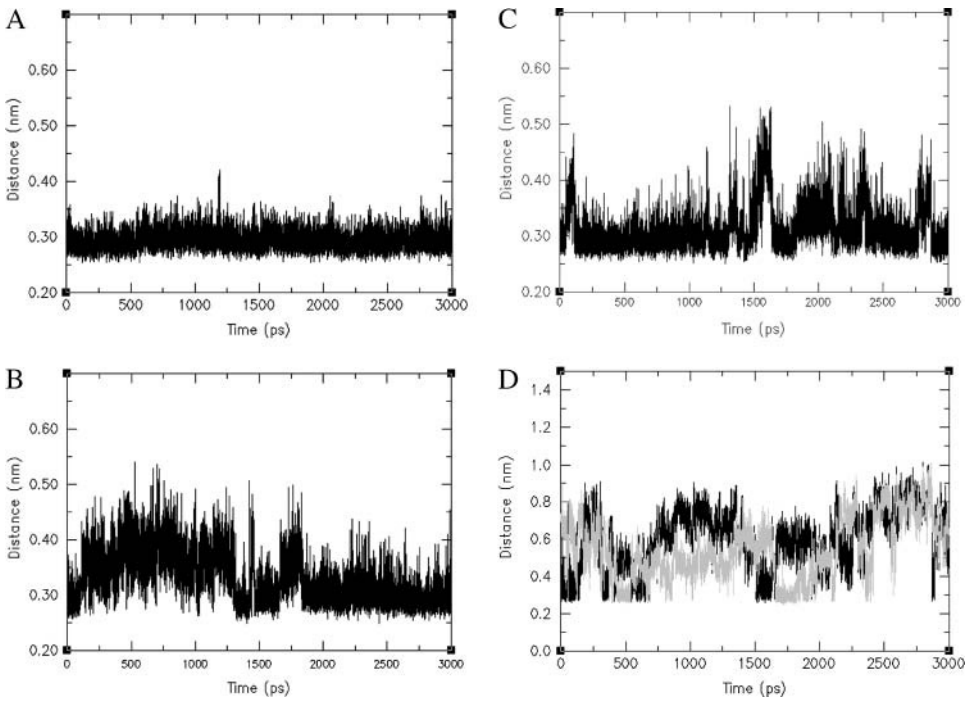

FIGURE 5 Interactions in the C- and O-interface regions are: (*A*) distance between O atom of Val-116 of the first subunit and N atom of Gln-111 of the other as function of time; (*B*) distance between O atom of Asn-113 of the first subunit and N atom of Asn-113 of the other as function of time; (*C*) distance between N atom of Asn-113 of the first subunit and O atom of Asn-113 of the other as function of time; and (*D*) distance between Nd2 atom of Asn-113 of the first subunit and Od1 atom of Asn-113 of the other as function of time and vice versa.

single point mutations significantly alter the structure of protein L (O'Neill et al., 2001) and cyanovirin (Botos et al., 2002), and to a minor extent of the T4 endonuclease VII (Raaijmakers et al., 1999, 2001). On the other hand, little variations are observed in two structures of the nerve factor binding domain of the TrkA receptor (Ultsch et al., 1999; Robertson et al., 2001) and minor alterations are produced by single point mutations in rat Cd2 (Murray et al., 1998) and by ligand binding in the Fha domain of cell cycle checkpoint protein Chfr (Stavridi et al., 2002). Finally, huge variations are observed comparing homologous 3D domain swapped dimers. Indeed, a rotation of 130° is observed when the structures of the N-dimer (Liu et al., 1998) and of the human pancreatic ribonuclease dimer (Canals et al., 2001) are compared. Similarly, large reorganizations are detected by superimposing the ligand binding domains of TrkA, TrkB, and TrkC receptor structures (Ultsch et al., 1999), 41–44% sequence identity, and comparing the structures of human (Janowski et al., 2001) and chicken cystatin (Staniforth et al., 2001).

DISCUSSION

The structural characterization of RNase A dimers carried out by Eisenberg and co-workers (Liu et al., 1998, 2001) has highlighted several fascinating features: i), a protein can swap both N- and C-terminal ends; ii), the overall quaternary structure of the two dimers is completely different; and iii), the 3D domain swapping may be important for protein misfolding and aggregation. With the aim of gaining dynamic information on these intriguing systems, we have undertaken a thorough investigation of these dimers by molecular dynamics simulations. In previous articles, we studied the dynamic properties of the RNase A monomer (Merlino et al., 2002) and of the N-dimer (Merlino et al., 2004b). The results of these studies have shown that the monomer and the individual structural units of the N-dimer are endowed with a similar breathing motion of the β-sheet

TABLE 2 Quaternary structure variation of 3D domain swapping dimers

Protein	PDB codes	Rotation angle (°)*
BS-Rnase	1BSR/1D9X	7
Bovine/human Rnase	1A2W/1H8X	130
Protein L	1K50/1K53	57
Cyanovirin	1J4V/1L5B	48
	1J4V/1L5E	148
	1J4V/1LOM	82
TrkA/TrkB	1WWA/1WWB	38
TrkA/TrkC	1WWA/1WWC	38
TrkB/TrkC	1WWB/1WWC	157
RatCD2	1A64/1A6P	1
	1A64/1A7B	6
T4 Endonuclease VII	1EN7/1E7D	22
	1EN7/1E7L	18
Grb2-SH2	1FYR	21
Transcription factor NusG	1NPP/1NPR	13
Fha domain of Chfr	1LGP/1LGQ	17
Human/chicken cystatin	1N9J/1G96	106
TrkA	1WWA/1HE7	5
C-Dimer	MD conformers in Fig. 2 *C*/1F0V	52,32,58

*The rotation angle is the angle required to superimpose the structural unit 2, after the optimal fitting of the structural unit 1.

structure. Interestingly, the motions of the structural units of the N-dimer are highly coupled (Merlino et al., 2004b). Here we report a molecular dynamic study on the C-terminal swapped dimer of RNase A. As far as local and global motions of the individual structural units are concerned, the findings of these investigations are in line with the previously reported results (Merlino et al., 2002, 2004b). Indeed, the active site and the overall breathing motion of the units strictly follow the trends that emerged from the studies on RNase A and the N-dimer. As for the case of the N-terminal end exchange (Merlino et al., 2004b), the swapping of the C-terminus does not affect the local dynamic behavior of the enzyme, despite the fact that the swapped strand is part of the β-sheet structure.

However, the MD investigation on the C-dimer shows distinctive features. In contrast to the data reported for the N-Dimer (Merlino et al., 2004b), the β-sheet motions of the two structural units are not coupled in the C-dimer. In other words, from the dynamic point of view the two structural units of the C-dimer are independent. This finding may be ascribed to the different assembly of the two dimers. In fact, the N- and the C-dimer are characterized by different O-interface areas, being that the N-dimer is more compact. Therefore, the larger interface of the N-dimer is able to transmit interunit communications, whereas the loose connection of the two structural units in the C-dimer does not. This observation suggests that large intersubunit surfaces are essential for signal transmission and could be essential for allosteric modulation.

The results of the MD simulation also show that the loose O-interface allows large fluctuations of the quaternary structure of the C-dimer. Specifically, the RMSD of the whole dimer from the crystallographic structure fluctuates in a wide range (2.0–6.0 Å), despite the fact that main bodies of the two subunits present almost constant RMSD values (~2.0 Å). A different trend was observed for the N-dimer, which presents a rather constant RMSD (~3.5 Å) from the starting x-ray structure in the equilibrated region of the trajectory (Merlino et al., 2004b). The large fluctuations of the C-dimer suggest that the x-ray structure is likely conditioned by crystal packing. It should be recalled that the C-dimer crystals were grown in the presence of the substrate analog dCpdG, which links different molecules of the dimer in the crystal. This interaction, important for packing, favors the crystallization process (Liu et al., 2001).

An analysis of the structures deposited in PDB shows that large quaternary structure movements are frequent among 3D domain swapped dimers. Particularly evident is the conformational variability of cyanovirin, which displays three distinct quaternary structures (Yang et al., 1999; Bewley and Clore, 2000; Barrientos et al., 2002). Indeed, large variations are not only observed when crystal and NMR models are compared, but also between structures derived from different crystal forms (Yang et al., 1999; Bewley and Clore, 2000; Barrientos et al., 2002). Similarly, the lowest-energy NMR conformers of cystatin display very diverse subunit orientations (Staniforth et al., 2001). Very recently, Janowski et al. (2004) have reported that the different domain swapping dimers contained in the asymmetric unit of a modified form of cystatin show a significant degree of variation, as a consequence of the high flexibility of the open interface.

In 3D domain swapped dimers, large structural variations may also be induced by single point mutations, mainly occurring in the hinge peptide region. An uncommon quaternary structure variability is also observed among homologous proteins that generate 3D domain swapped dimers.

The unusual motions of the 3D domain swapped dimers reported here are due to the peculiar way in which they are assembled. An intrinsic difference can be identified between nonswapped and swapped dimers. In the former, dimerization requires the formation of a novel interface surface, which should be large enough to prevent dissociation. On the contrary, the stability of a swapped dimer may be ensured by the C-interface, which preexists in the monomer, and does not necessarily depend on the contacts between the main bodies of the two subunits. In other words, large interfaces between the main bodies of the two subunits, which are important for providing stability in nonswapped dimer, are optional in the swapping ones. Notably, the two swapped dimers of RNase A represent two emblematic cases: indeed, the N-dimer presents a rather rigid O-interface with limited structural flexibility, on the other hand the C-dimer, which is characterized by a loose O-interface, undergoes large structural fluctuations.

Therefore, it can be suggested that the swapped dimers endowed with loose O-interfaces may undergo large fluctuations, if the hinge peptide is sufficiently flexible. It should be noted that the inspection of the interacting surfaces observed in 3D domain swapped dimers showed that the surface area of the O-interface is often quite limited (Schlunegger et al., 1997) with few hydrogen bonds and salt bridges (Sinha et al., 2001). Consequently, the absence of strongly stabilizing interactions between the two units does not constrain their motion, by producing a lower barrier than would need to be overcome in such extensive motions.

The high flexibility may also be an important factor that contributes to the formation of ''loose'' 3D swapping dimers and higher oligomers. Indeed, it can be speculated that the entropy costs associated with the oligomerization processes are somewhat limited. In other words, in a dimer, the loose O-interface allows a large conformational freedom, which reduces the unfavorable entropic contribution to dimer formation.

Several biological roles have been proposed for the 3D domain swapping. Among these, it has been suggested that it can be important for the modulation of catalytic actions (Vitagliano et al., 1999), for immunosuppressive and antitumoral activities (Vescia and Tramontano, 1981; Cafaro et al., 1995) and for the formation of the amyolid fibrils (Liu

et al., 2001). Here we show that high flexibility is a distinctive property of several 3D domain swapped dimers. This finding suggests the intriguing idea that the coupling of 3D domain swapping with the high flexibility could be important for a number of biological processes that rely on protein-protein recognition step, including the pathological aggregations.

This work was supported by Italian Ministero dell'Istruzione, Università e Ricerca (PRIN 2002) and Regione Campania 'Legge 5'.

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