

Research Article

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Head or tail? A molecular dynamics approach to the complex structure of TNF-associated factor TRAF2

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Abstract: Tumor necrosis factor receptor-associated factor proteins (TRAFs) are trimeric proteins that play a fundamental role in signaling, acting as intermediaries between the tumor necrosis factor (TNF) receptors and the proteins that transmit the downstream signal. The monomeric subunits of all the TRAF family members share a common tridimensional structure: a C-terminal globular domain and a long coiled-coil tail characterizing the N-terminal section. In this study, the dependence of the TRAF2 dynamics on the length of its tail was analyzed *in silico*. In particular, we used the available crystallographic structure of a C-terminal fragment of TRAF2 (168 out of 501 a.a.), TRAF2-C, and that of a longer construct, addressed as TRAF2-plus, that we have re-constructed using the AlphaFold2 code. The results indicate that the longer N-terminal tail of TRAF2-plus has a strong influence on the dynamics of the globular regions in the protein C-terminal head. In fact, the quaternary interactions among the TRAF2-C subunits change asymmetrically in time, while the movements of TRAF2-plus monomers are rather limited and more ordered than those of the shorter construct. Such findings shed a new light on the dynamics of TRAF subunits and on the protein mechanism *in vivo*, since TRAF monomer–trimer equilibrium is crucial for several reasons (receptor recognition, membrane binding, hetero-oligomerization).

Keywords: molecular dynamics, quaternary interaction, TRAFs, protein contact networks

Introduction

Protein oligomerization, which is the association of monomeric subunits into a large and complex structure, is a widespread molecular process in living organisms, as demonstrated by the large number of oligomers found in cells as well as in the list of the structures present in the protein data bank (PDB) [1]. Oligomerization provides both structural (higher stability) and functional (finer regulation) advantages, thus increasing biological performances of proteins and enzymes [2]. The case of TNF (tumor necrosis factor) receptor-associated factor proteins (TRAFs) is paradigmatic. TRAFs are trimeric proteins that mediate the signal transduction from receptors (such as TNFRs) to the downstream signaling cascades [3]. For such a crucial role, the members of the TRAF family have become a very popular object of the study in the past two decades [4]. In particular, TRAF malfunctioning and polymorphisms have been identified as a main source of human diseases, ranging from cancer to inflammation [5,6].

All members of the TRAF family share a common tridimensional assembly (Figure 1). Each monomer is constituted by two distinct parts, namely, a N-terminus tail, TRAF-N – containing a few zinc-finger regions, and a RING domain (with the exception of TRAF1) – and a TRAF-C domain, characterized by a number of interactions among the three protein chains [4]. The TRAF-C section (which includes ~230 amino acids per chain) is in turn organized in two distinct sub-domains: a globular, β -sandwich C-terminal region and an N-terminal helical section, which participates in the formation of a long coiled-coil tail, together with the two homologous segments of the other two subunits.

The two domains play both a relevant biological role but perform different functions. While the C-terminal is

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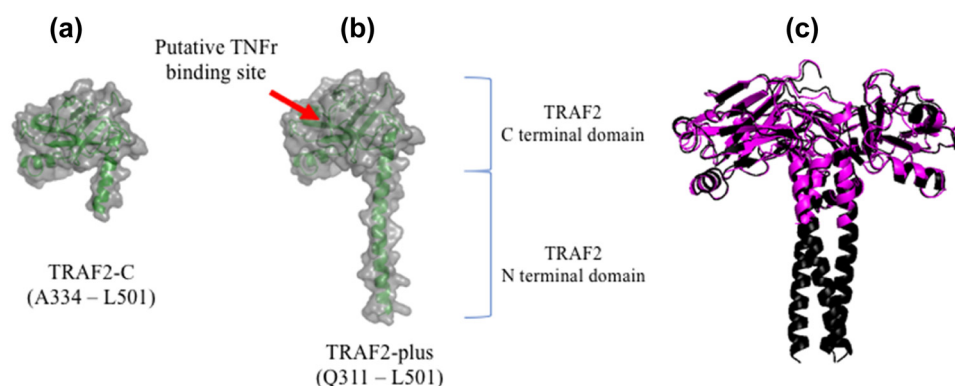


Figure 1: Molecular graphics rendering of monomeric TRAF2-C (panel a, 1ca4.pdb) and monomeric TRAF2-plus (panel b, AlphaFold2 prediction). The molecular surface is represented in gray, and the secondary structure in green. The figure shows the elongated structure of TRAF2-plus with respect to TRAF2-C. The molecular graphics rendering of trimeric TRAF2-plus (panel c, black) is also reported in the third panel. The crystallographic structure of TRAF2-C (pdb 1ca4) has been superimposed (panel c, mauve) for comparison.

involved in the receptor recognition mechanism, the N-terminal stalk contains different regions that bind downstream effector molecules [4]. The impact of TRAF oligomerization on their functions (and in particular on TRAF2) pertains from the direct [7,8] and indirect [9] binding to TNF receptors, to the interaction with downstream effectors, such as the cellular inhibitors of apoptosis [10–12], and to the RING–RING dimeric formation. Two crystallographic structures of TRAF2 are available so far. The first one (pdb code: 1CA4) includes the 168 amino acids [8] of the protein primary structure at the C-terminus (from position 334–501) and corresponds to the soluble construct used for *in vitro* experiments [13,14]. This C-terminal fragment, from now on addressed as TRAF2-C, includes the three large globular heads and only 14 residues of the trimer coiled-coil tail. Fluorescence correlation spectroscopy measurements [14] have revealed that such short version of TRAF2 dissociates into monomeric species at low concentration (<100 nM). The monomers display an enhanced capacity in membrane binding [15], also inducing an inward membrane vesiculation, in the presence of the ganglioside GM1 [16]. *In silico* studies have suggested that the three subunits form dynamic clusters, in which two of them, alternately, display highly correlated motions, while the third acts more independently [17,18].

The second available crystallized construct (pdb code: 1QSC) is 23 residues long (from position 311 to 501) with respect to TRAF2-C [7], but the refined 3D structure contains only residues 323–501 (i.e., 179 amino acids). For both crystals of TRAF2, the putative binding sites of the membrane receptors have been localized on the external surface of the C-terminal globular heads, at the level of a large groove, involving three protein beta-strands [7,8].

In this study, we have obtained a structural model of the longer protein, addressed as TRAF2-plus (191 residues, positions 311–501), and then analyzed the dynamics of the two molecular fragments (TRAF2-C and TRAF2-plus) through *in silico* simulations (as detailed in Methods). The results suggest that the longer tail of TRAF2-plus reduces the degrees of freedom of the globular heads, preserving the roughness of the trimer surface that characterizes the crystallographic structures. A new regulatory role for the coiled-coil section might be envisaged, that is, a control of the TRAF monomer–trimer equilibrium and a synchronization of the conformational changes occurring at the level of the protein head. Such findings might explain the peculiar behavior of the shorter construct (TRAF2-C) in the presence of biological membranes and suggest new insights on the mechanism of TRAF oligomerization.

Methods

The TRAF2-C protein structures (pdb-id: 1ca4, 1qsc) present in the PDB contain at most the residues from 311 to 501. We obtained the TRAF2-plus structure from residue 311 to 501 by using the AlphaFold-Multimer (AF2-multimer) algorithm [19,20] that can predict homomeric and heteromeric protein structures. In order to obtain the TRAF2-plus structure, we performed AF2-multimer calculations on the available online notebooks. We obtained a set of scored structures, and we computed the RMSD between the AF2-multimer structures and the TRAF2-C one, choosing the TRAF2-plus structure whose RMSD value was the smallest.

Molecular dynamics (MD)

Classical MD simulations of the TRAF2-plus trimer were performed starting with the structure obtained from AF2-multimer and using the GROMACS package [21,22] with GROMOS43A1 force field [39]. MD simulations were carried out in the NpT ensemble at 288 and 310 K by following exactly the same equilibration procedure as already reported [17,23] and here reported.

The temperature is held fixed at (288 or 310 K) using the V-rescale thermostat [24] with a coupling time of 0.1 ps. The pressure is kept constant at the reference pressure of 1 bar with a coupling time of 1 ps and an isothermal compressibility of $4.5 \times 10^{-5} \text{ bar}^{-1}$, exploiting the features of the Berendsen barostat [25]. The simple point charge model is used for water molecules. The simulation box is cubic (with a side of 10.3 nm); TRAF2 monomer is immersed in 33,366 water molecules, and an appropriate number of Na^+ or Cl^- counterions are added to have a wholly neutral system. MD simulations are performed at neutral pH. Periodic boundary conditions are used throughout the simulation, and the particle mesh Ewald algorithm is used to deal with the long-range Coulomb interactions [26]. A time step of 2 fs was used. A non-bond pair list cut-off of 1.0 nm was used, and the pair list was updated every ten steps. TRAF2-plus trimer is initially relaxed in vacuum via a steepest descent minimization. Then, the appropriate amount of counterions and water is added. At this point, the solvent is relaxed by a few steps (10 ps) of NVT MD at 200 K, leaving the solute untouched. Then, the whole system, solute and solvent, is equilibrated for 50 ps in the NVT ensemble at the appropriate temperature (288 or 310 K). This is the situation from which the final 500 ns long NpT MD simulation at 288 or 310 K is started.

Protein contact networks (PCNs)

The PDB files, reporting the structural information of the TRAF2-C and the TRAF2-plus proteins, have been modeled as networks, where nodes are the protein residues and the links between nodes represent the noncovalent significant intramolecular interactions.

The mathematical representation of such networks, from now on referred to PCNs, is the adjacency matrix, whose generic element is defined as:

$$A_{ij} = \begin{cases} 1 & \text{if between the } i\text{th and the } j\text{th residues a link exists} \\ 0 & \text{otherwise.} \end{cases}$$

A link exists between two nodes if their Euclidean distance lies within a given range, in our case if the distance lies in the range 4–8 Å, to account only for significant noncovalent interactions between residues.

The node degree k_i is the number of links that a node has with other nodes, and in the perspective of PCNs, it describes the local stability of protein structure.

Network clustering is based on the adjacency matrix and node degree: it helps in identifying densely interconnected regions of the protein structural network, which has been demonstrated to correspond to functional domains [27–29].

We applied the spectral clustering method, based on the computation of the Laplacian matrix L defined as:

$$L = D - A,$$

where D is the degree matrix (D), defined as a diagonal matrix where the nonnull elements on the diagonal d_{ii} are the corresponding node degree k_i , and A is the adjacency matrix (A). Cluster identification is based on the components of the eigenvector v_2 corresponding to the second minor eigenvalue of L [30,31].

Analysis of subunit–subunit interface

The analysis of the subunit interfaces has been performed using the COCOMAPS software [32] and the PISA service at the European Bioinformatics Institute [33]. In particular, a cutoff of 8 Å has been used to identify each couple of amino acids belonging to different interfaces, in contact with each other. The distances of such quaternary interactions have been then used to evaluate S_{index} as described in the text.

Results

Analysis of the overall TRAF2-plus dynamics vs TRAF2-C

The TRAF2-plus structure at time $t = 0$ has been obtained using the AlphaFold2 AI system (see Methods) and superimposed to the available crystallographic file of TRAF2-C (pdb code: 1CA4), as shown in Figure 1 (right panel). TRAF2-C retains most of its overall, native, structural features in the temperature range of 15–37°C [17]. For such reason, MD simulations have been carried out both at 288 and at 310 K, and the corresponding RMSD values are reported in Figure 2.

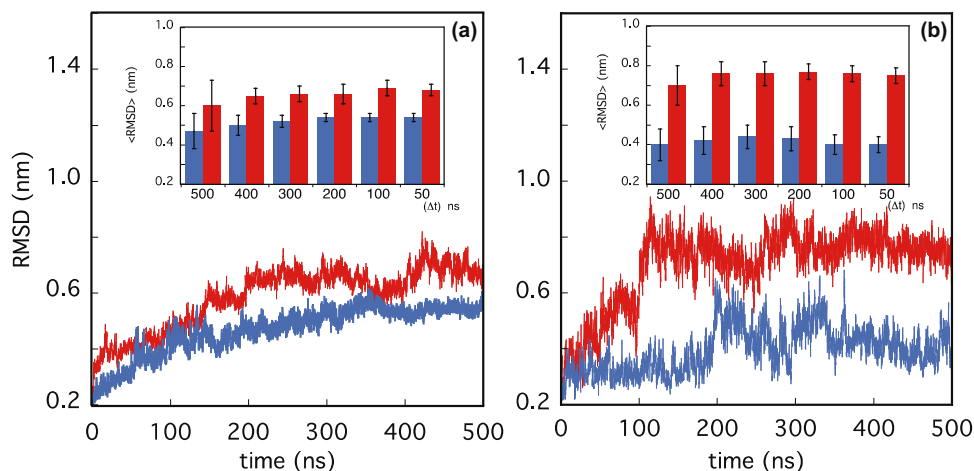


Figure 2: RMSD time course of the TRAF2-C backbone (panel a) at 310 K (red line) and 288 K (blue line). In panel b, the RMSD of TRAF2-plus backbone at 310 K (in red) and 288 K (in blue) is shown. Insets: average RMSD values and their corresponding standard deviations. The average RMSD has been evaluated for the whole simulation (500 ns) and in the last 400, 300, 200, 100, and 50 ns, at both temperatures.

The TRAF2-C dynamics at 288 K is characterized by an almost monotonic increase between $t = 0$ and $t = 300$ ns (Figure 2), followed by oscillations around an average RMSD value of about 0.54 ± 0.02 nm (Figure 2a, inset). A more rapid increase occurs at 310 K ($0 < t < 200$ ns), followed by larger fluctuations, which converge to a slightly higher average RMSD value, namely, 0.68 ± 0.03 nm. The dynamics of TRAF2-plus at 310 K displays an even faster behavior, with an RMSD increase in the first ~ 120 ns (Figure 2) and a final value of about $\sim 0.75 \pm 0.03$ nm

(Figure 2b, inset). At 288 K, a much slower dynamics takes instead place, with a final displacement of about 0.40 ± 0.05 nm. The structural changes occurring in the secondary and tertiary structure of the two proteins at 310 K are more explicitly shown in Figure 3, where the initial ($t = 0$) and final ($t = 500$ ns) respective cartoons have been superimposed. The graphic rendering and the analysis of the secondary structure content demonstrate that the major transformations that take place in TRAF2-plus mainly concern the coiled-coil section, which is twisted with respect to the initial

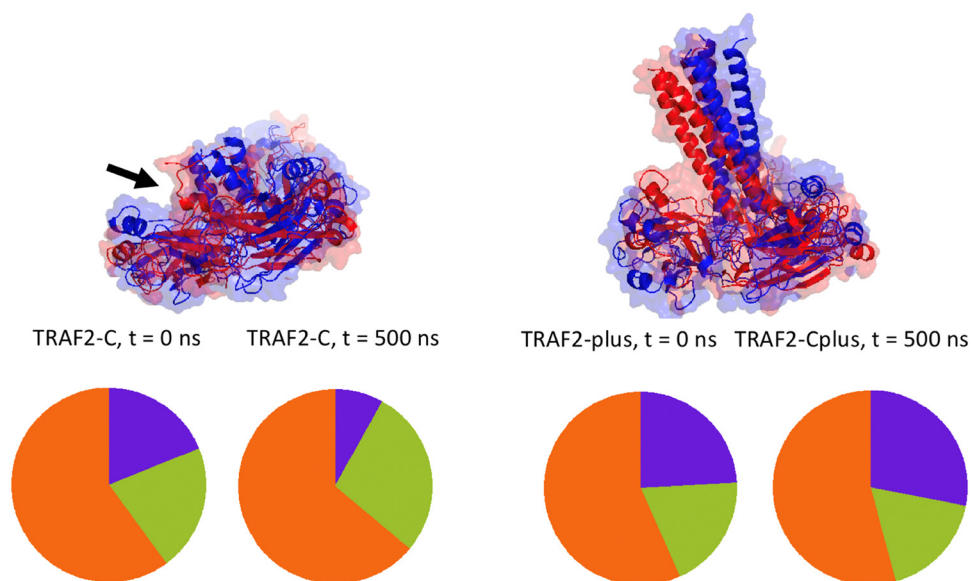


Figure 3: Structures at 310 K of the simulated TRAF2-C (left) and TRAF2-plus (right) at $t = 0$ ns (blue) and $t \approx 500$ ns (red). The secondary structure elements are shown in the cartoon modality (α -helices as cylinders and as flat strands) and their percentage contents quantified in the form of pie charts (helices = purple, β -structures = orange, coil = green). The little black arrow included in the picture indicates the partial unfolding of the coiled-coil tail in TRAF2-C.

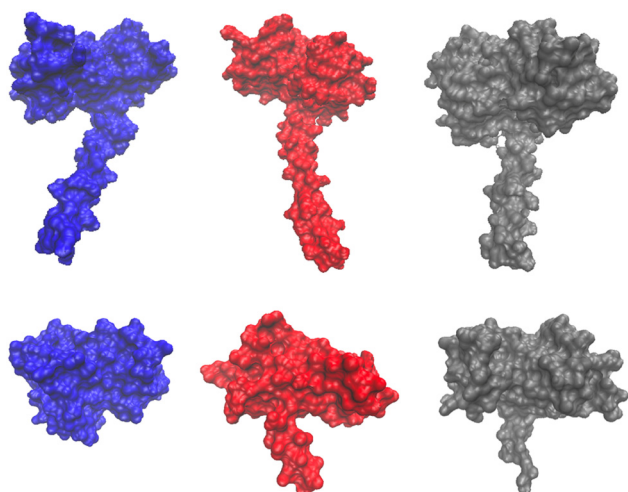


Figure 4: Simulated structures of the single chains of TRAF2-plus (upper illustrations) and TRAF2-C (lower drawings) at 200 ns.

position, with the three long helices still fully folded. In the case of TRAF2-C, instead, a partial loss of α -helix secondary structure is observed, mainly ascribable to the partial loosening of the N-terminal tail (Figure 3).

The analysis of the respective monomeric structures at 310 K is reported in Figure 4 at time $t = 200$ ns, that is, when almost ~95% of the RMSD change has already occurred, for both proteins (Figure 2). The structural details of the single chains have been obtained using a surface rendering, which better shows the shape of each subunit. Indeed, in this kind of representation, the N-terminal tail of the A-subunit cannot be distinguished in TRAF2-C, as it is

bent toward the protein head. The N-terminal helix of subunit C is also partially unstructured, while all the monomers of TRAF2-plus maintain the fully folded tail that characterizes the protein structure at time $t = 0$. In order to corroborate such findings, an analysis of the principal component (PCA) of motion has been carried out at 310 K. In Figure 5, the cartoons of TRAF2-C and TRAF-plus at 0, 250, and 500 ns are reported, while the movies of both constructs along the first eigenvector are available in the files included in the supplementary materials.

Such analysis put in evidence that the principal movement of TRAF2-plus involves the bending of the protein C-terminal section toward the coiled-coil helices, causing an increase in the value of the angle between the other side of the protein head and the long tail (Figure 5). In TRAF2-C, a distancing of the three short helices in the N-terminal section is evident already at 250 ns, together with the partial unfolding of the coiled-coil structure (Figure 5), diagnostic of a major structural instability of the shorter trimeric construct.

Effects on the subunit interface and on the C-terminal shape

The details on the protein quaternary structure dynamics have been obtained evaluating the average distance between each center of mass of three TRAF2 subunits (AB, BC, and CA subunits), at the two different temperatures (Figure 6). The

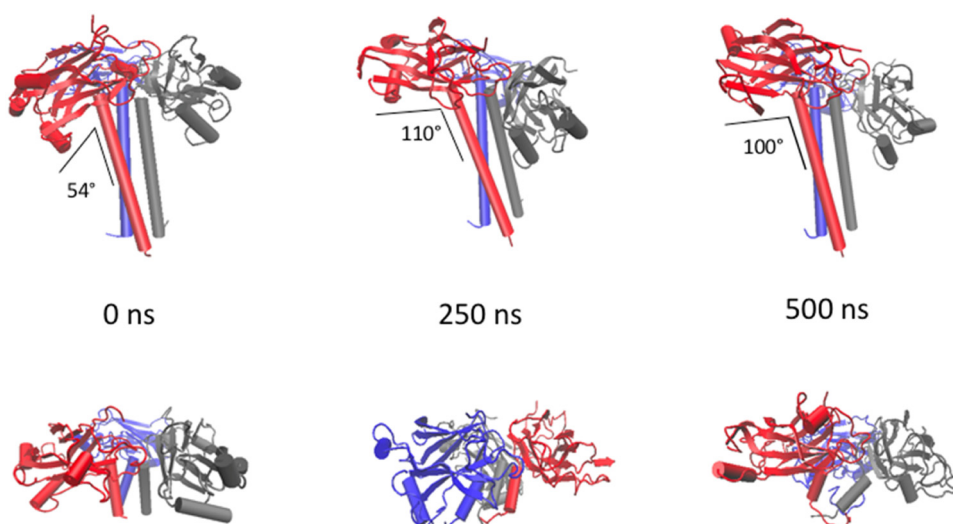


Figure 5: Simulated structures of TRAF2-plus (upper illustrations) and TRAF2-C (lower drawings) at different instants of the simulation. The three subunits, A, B, and C, are plotted in blue, red, and gray, respectively, and the secondary structure elements reported in the cartoon modality (α -helices as cylinders and β -structures as flat strands).

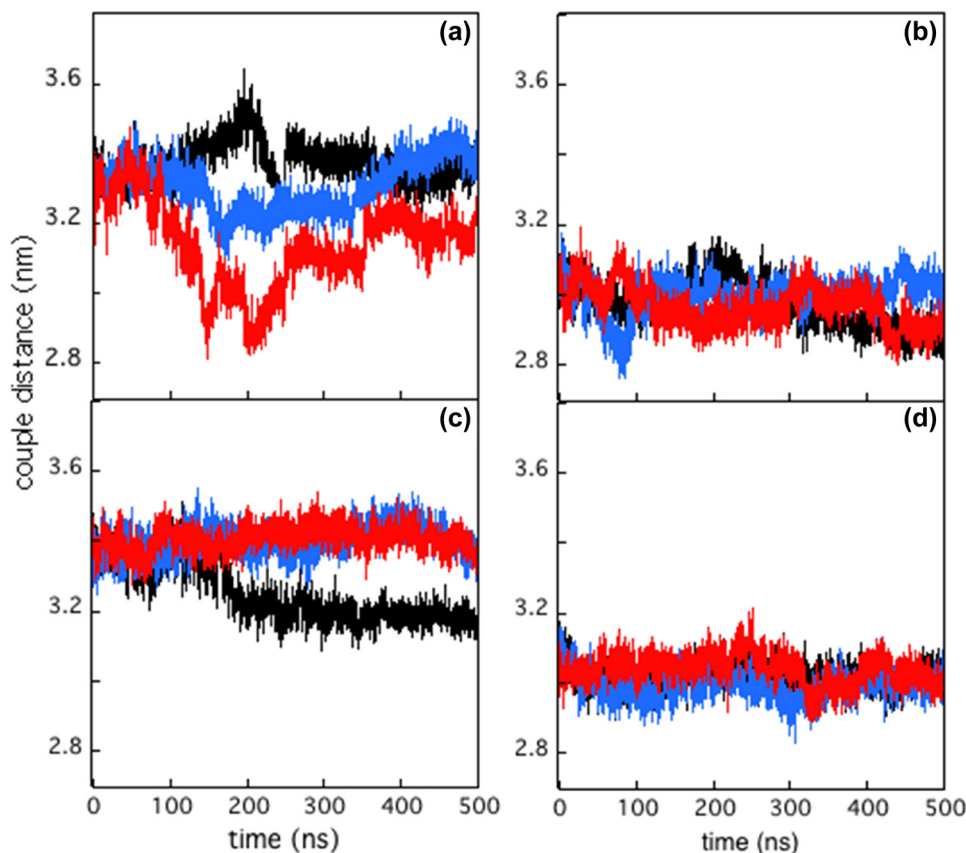


Figure 6: Couple distances of TRAF2-C (310 K, panel a and 288 K panel c) and TRAF2-plus (310 K panel b and 288 K, panel d) subunits as a function of time. The black, red, and blue lines correspond to the couple of AB, BC, and CA subunits, respectively.

distancing and re-approaching movements of the three subunits are particularly pronounced in TRAF2-C at 310 K, while such effects are comparatively small in the case of TRAF2-plus, suggesting that the longer coiled-coil tail reduces the protein degrees of freedom.

The enhanced mobility observed in the simulation of TRAF2-C has important consequences at the level of the protein quaternary structure. In Figure 7, top views of the TRAF2-C and TRAF2-plus simulated structures are reported at $t = 0, 250$, and 500 ns. The graphic rendering demonstrates that in TRAF2-plus, the balance of the interactions within each couple of subunits is only marginally affected by the limited movements of the protein subunits (Figure 7IV, V and VI). The larger conformational changes that characterize the TRAF2-C dynamics display, instead, an asymmetric configuration (Figure 7II and III) and the arise of unstructured segments (Figure 7III) at the subunit interfaces with respect to TRAF2-plus (Figure 7VI). Moreover, in TRAF2-C at 250 and 500 ns, a progressive re-shape of the molecular surface, affecting the local tertiary structure at the level of the putative TNFr binding site, occurs.

The extension of monomer–monomer interactions and their effects on the protein topology have been characterized by PCNs analysis, an approach that identifies the coordination of different domains and subunits in multimeric proteins [34–36]. In detail, the polypeptide structure is represented in terms of an amino acid network, where clusters are the sets of residues preferentially interconnected, and not necessarily coincident with the subunits of protein [37]. As shown in Figure 8, the whole head of TRAF2-plus (colored in green) acts like one, single unit, while the long tail appears to be organized in two clusters (yellow and red), distinguished by a different association degree. The segments characterized by a stronger interconnection are shown in the graphic rendering of Figure 8 and include the residues in the first portion of the coiled-coil tail, which roughly corresponds to the cluster in red reported in Figure 8a. The topology of TRAF2-C at 200 ns is completely different, as two chains form a large aggregate, while the third subunit is more independent (Figure 8c). In this case, main interactions are distributed along the interface that lies between the two clusters, involving both the helical tails and the protein heads, as shown in Figure 8d.

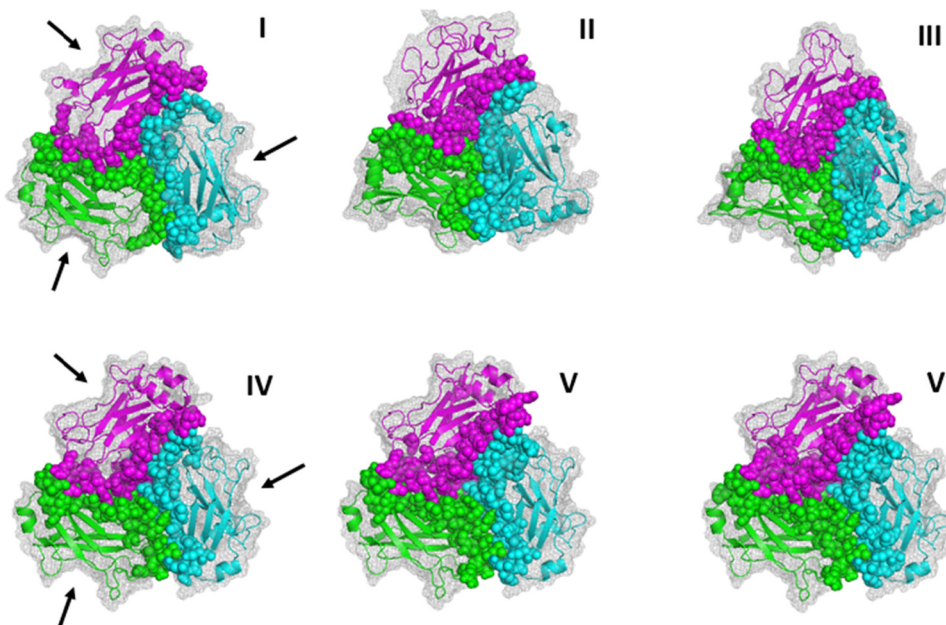


Figure 7: Top view of TRAF2-C at 0, 250, and 500 ns in panels I, II, and III, respectively. The three subunits are colored in green, cyan, and purple. The cartoon rendering represents the secondary structure, while the spheres correspond to the amino acids located at the subunit interfaces. The analogous TRAF2-plus structures at 0, 250, and 500 ns are reported in panels IV, V, and VI. The black arrows in the structures at $t = 0$ ns indicate the putative binding site to the TNF receptor.

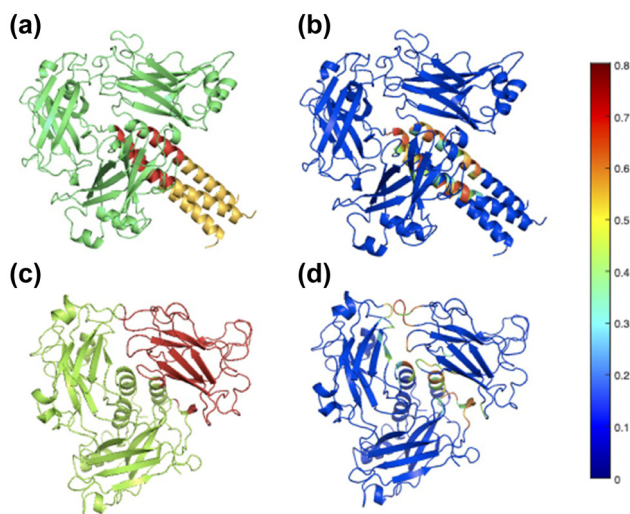


Figure 8: Clustering representation of TRAF2-plus (panel a) and TRAF2-C (panel c). In panels b and d, the corresponding distribution of the strongest interactions that characterize the two TRAF constructs is reported on a color scale (0–1) that ranges from blue to red.

The strained state produced at the intersection among the three monomers has also important structural consequences on the roughness of the trimer external surface. The frames obtained at intermediate states of the simulation (~200–250 ns) suggest that changes of different extent characterize the two constructs (Figures 4 and 7). In particular,

the concavity of the three bights present in the protein crystallographic structure (Figure 7, black arrows) is progressively reduced in the course of time in the case of TRAF2-C, while the minor effects occur in TRAF2-plus (Figure 7).

Evaluation of order/disorder at the level of the protein quaternary interactions

The order/disorder transition has been followed along the simulation introducing a surface index, S_{index} , defined as follows:

$$S_{\text{index}}^t = \frac{1}{n} \sum_i^n D_i(t),$$

which is the sum of all the distances at time t , $D_i(t)$, between residue pairs at the subunit interface, divided by the number of couples, n . The S_{index} values along the simulation are plotted in Figure 9, for both proteins.

The larger overall mobility of TRAF2-C (Figures 6 and 7) produces two important effects on the S_{index} values: (i) wider oscillations with respect to the case of TRAF2-plus and (ii) a smaller average value, $\langle S_{\text{index}} \rangle$, driven by the closing of the couples BC and CA (Figure 6, panel a). Such asymmetric behavior at the subunit interfaces of TRAF2-C has been quantified introducing an order

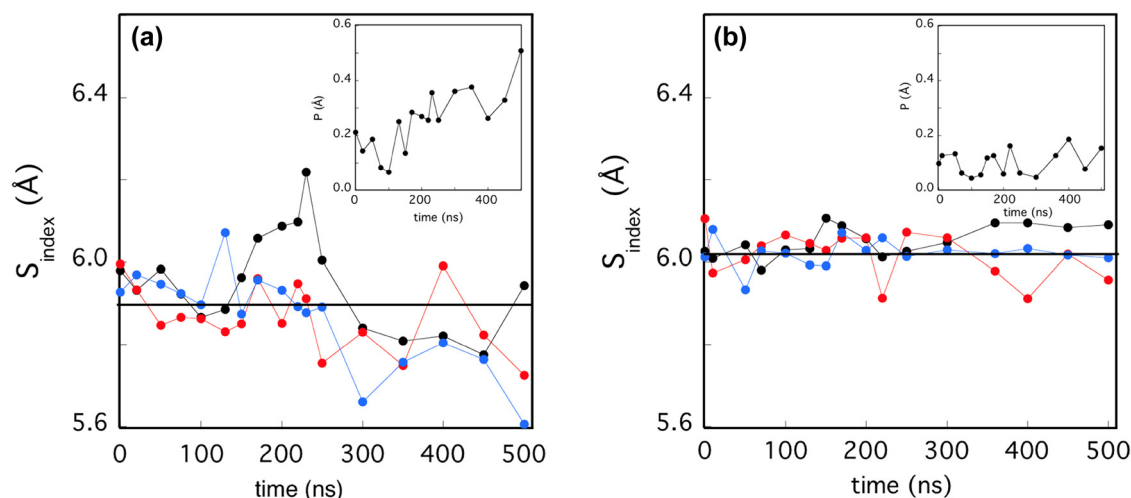


Figure 9: Time dependence of the S_{index} parameter for TRAF2-C (panel a) and TRAF2-plus (panel b). The black, blue, and red symbols correspond to the AB, BC, and CA interfaces, respectively. The horizontal black line corresponds to the average value, $\langle S_{\text{index}} \rangle$. In the two insets, the dependence of the order parameter, P , is reported as a function of time.

parameter, $P(t)$, defined as the sum of the three absolute deviation values of S_{index} with respect to the average $\langle S_{\text{index}} \rangle$, at each time t :

$$P_t = \sum_{i=1}^3 |S_{\text{index}}^i(t) - \langle S_{\text{index}} \rangle|.$$

As shown in the insets of Figure 9, $P(t)$ is a randomly oscillating function for TRAF2-plus, while it increases up to three times its initial values in the case of TRAF2-C, as expected from a more disordered distribution of quaternary interactions at the three monomer–monomer interfaces.

Discussion

Protein oligomerization confers several structural and functional advantages with respect to monomeric proteins [38]. Monomer–monomer interactions in small homodimers, as well as the modularity that distinguishes large protein complexes, are mostly characterized by a global structural symmetry of the spatial subunit orientation, which is guaranteed by the interface contacts [1,39]. The analysis of such quaternary interactions over thousands of crystallographic structures has demonstrated that symmetry has played a major role in the evolution of homo-oligomers [40,41]. On the other hand, asymmetric structural configurations of oligomers naturally arise upon ligand binding [42] and in allosteric regulation [2] and, more in general, to entropy decrease during catalytic events [42]. An asymmetric “dynamic” behavior in homo-oligomers was introduced for the first time in 2003 by Ma and co-workers, arising from the *in silico*

trajectories of lactose repressor protein MD [43]. It was found that the monomer–monomer interface plays a major role in propagating asymmetric motion of domains belonging to different clusters of the protein structure [43]. In the case of TRAF2-C, the presence of an odd number of subunits and the different correlated motions at the monomers interfaces has revealed another kind of dynamic asymmetry, that is, the clustering in a 2:1 configuration, in which, alternately, two subunits preferentially interact with each other [17,44]. As shown in Figure 9, the MD simulation of TRAF2-C suggests that this behavior leads to a disordered state at the level of the subunit interface. Such an effect may be important in the control of the protein oligomeric state, for instance, initiating the monomerization process, which, in turn, depends the protein capacity to bind membranes [15]. In this regard, it is important to note that the N-terminal segment of the more independent monomer loses its contacts with the tails of the other two subunits (Figures 4 and 5). This finding is in line with MD simulations performed on a single protein chain [14], which demonstrated how the N-terminal section of TRAF2-C becomes less stable and more flexible upon monomerization. This study demonstrates that a longer tail impairs the 2:1 dynamic clusterization (Figure 8), stabilizing the N-terminal domain of TRAF2-plus (Figure 3). In particular, the PCA of motion (Figure 5 and supplementary materials) suggests that the movements of both head and tail of TRAF2-plus recall the dynamics of two rigid bodies (a flat disk and a long rod, respectively), hinged through a very short, flexible loop that includes Thr 349, Tyr 350, Asp 351, and Gly 342. The physical size of coiled-coil motifs plays a crucial role in proteins: such domains are highly conserved and mainly used as spacers and scaffolds [45].

In the case of TRAF2, a more unexpected result of this study concerns the influence that the length of the coiled-coil section has on the dynamics of the globular protein heads, where the receptor binding sites are located. The simulation demonstrates that in TRAF2-plus, the shape of the external protein surface is better maintained with respect to what happens in TRAF2-C (Figure 7). Such an effect depends on the fact that the three protein heads in TRAF2-plus are grouped together in a single cluster (Figure 8), characterized by ordered subunit interfaces (Figure 9). The tension arising from monomers approaching and distancing in TRAF2-C is, instead, the source of an interface more disordered state, which produces a mechanical stress, strongly influencing the roughness of the protein shape (Figure 7). Docking models of receptor peptides to a simulated structure of TRAF2-C at 37°C [17] have suggested that the protein binding to the receptor might not necessarily occur in a symmetric way, as instead observed in the crystallographic models [7]. It follows that the coiled-coil length might influence not only the protein stability but also one of its biological functions, such as membrane binding. It must be recalled that in the specific case of TRAF2, the coiled-coil section has another important function, that is, the asymmetric recruitment and binding of cIAPs [11], forming an heterotrimeric complex (2:1) with TRAF1 [10]. The crucial role of cIAPs in blocking cell death [46] makes the study of TRAF2–TRAF1–cIAPs interaction mandatory for the design of anticancer drugs [12,47]. The way in which such association occurs depends on the protein oligomeric state, namely, on those quaternary interactions at the subunit interface that the length of the coiled-coil section of TRAF2 seems to efficiently regulate (Figure 9).

In conclusion, thanks to their peculiar, mushroom-shaped three-dimensional structure, the members of the TRAF family can interact with a heterogeneous variety of other proteins, using both the globular, β -sandwich heads and the long coiled-coil tail [4]. For such a reason, TRAF malfunctioning is related to cancer and severe inflammatory diseases [5]. The non-trivial control that the TRAF2 tail has on the dynamics of the globular heads suggests that defects in the coiled-coil-section might compromise not only cIAPs binding but also the correct quaternary structure on which all the other protein functions depend.

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Conflict of interest: The authors state no conflict of interest.

Data availability statement: The datasets generated during and/or analyzed during this study are available from the corresponding author on a reasonable request.

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