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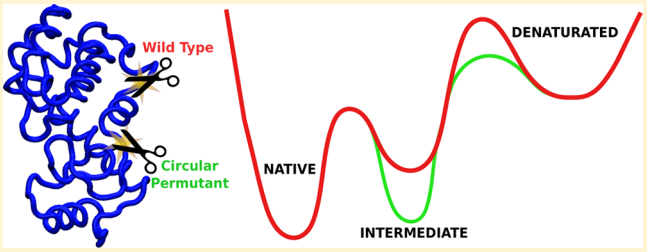
Modulation of a Protein Free-Energy Landscape by Circular Permutation

Gaël Radou,[†] Marta Enciso,[†,‡] Sergei Krivov,[†] and Emanuele Paci*[,†]

[†]Astbury Centre for Structural Molecular Biology, University of Leeds, Leeds LS2 9JT, United Kingdom

Supporting Information

ABSTRACT: Circular permutations usually retain the native structure and function of a protein while inevitably perturbing its folding dynamics. By using simulations with a structure-based model and a rigorous methodology to determine free-energy surfaces from trajectories, we evaluate the effect of a circular permutation on the free-energy landscape of the protein T4 lysozyme. We observe changes which, although subtle, largely affect the cooperativity between the two subdomains. Such a change in cooperativity has been previously experimentally observed and recently also characterized using single molecule optical tweezers and the Crooks relation. The free-energy landscapes show that both the wild type and circular permutant have an on-pathway intermediate, previously experimentally characterized, in which one of the subdomains is completely formed. The landscapes, however, differ in the position of the rate-limiting step for folding, which occurs before the intermediate in the wild type and after in the circular permutant. This shift of transition state explains the observed change in the cooperativity. The underlying free-energy landscape thus provides a microscopic description of the folding dynamics and the connection between circular permutation and the loss of cooperativity experimentally observed.



A circular permutation is a rearrangement of the connectivity of a protein obtained by linking the N- and C-termini of a protein with a peptide linker and creating new termini elsewhere. Circular permutations can occur naturally and are an important mechanism through which evolution has used stable folds to create new ones.[1] Circular permutations can also be introduced by protein engineering, for example, to manipulate protein scaffolds, improve catalytic activity and modulate affinity.[2] Circular permutations selectively perturb the folding dynamics without affecting the native structure. For this reason, they have been used to probe the effect of chain connectivity on the folding mechanism of proteins.[3−5]

Here, we focus on the lysozyme from phage T4 (Figure 1), which is a 164-residue protein with two structural subdomains connected by a long α-helix. The α/β N-domain is continuous (residues 13−59), whereas the all-α C-domain is discontinuous because it also contains the re-entrant N-terminal helix (residues 1−12) that forms the domain with residues 60−164. The folding of T4 lysozyme has been extensively investigated experimentally.[7−12] The specific role of its discontinuous subdomain has been experimentally probed by studying circular permutants of the protein.[13−17] These studies showed that T4 lysozyme folds through an on-pathway intermediate that occurs after the rate-limiting step. A two-domain protein with discontinuous subdomains is an example of a naturally occurring circular permutation, very likely introduced through evolution to improve the folding property of the protein, while preserving structure and function. In fact, the presence of discontinuous subdomains among proteins has been suggested as one strategy to enhance subdomain coupling and, thus, folding cooperativity.[18] Recently, this change in cooperativity between subdomains has been further demonstrated, using a combination of optical tweezers and protein engineering: in a circular permutant in which the contiguity of two subdomains in the sequence is re-established, there is loss of cooperativity and coupling between subdomains.[18]

The description of the folding mechanism of a protein is challenging because of the large dimensionality of the problem and its stochasticity. In principle, folding can be described as diffusion on a free-energy landscape.[19−21] The question we here try to answer is, how does a circular permutation affect the free-energy landscape of a protein? The free-energy landscape is not directly accessible by experiment because of the limited spatial and time resolution. Techniques such as φ-value analysis[22] have been exploited to infer the effects of circular permutations on the folding mechanism.[5] Such experiments provide insight at residue level on the folding pathway but do not clarify the effect of the circular permutation on the free-energy landscape.

Atomistic simulation, unlike any experimental technique, can provide information at ångstrom space resolution and femtosecond time resolution on the sequence of events that take place during a folding event.[23] Simulation is also the only practical way to determine directly intricate details about a free-energy landscape. Simulation results depend on the model and force field used. The most accurate force fields are also

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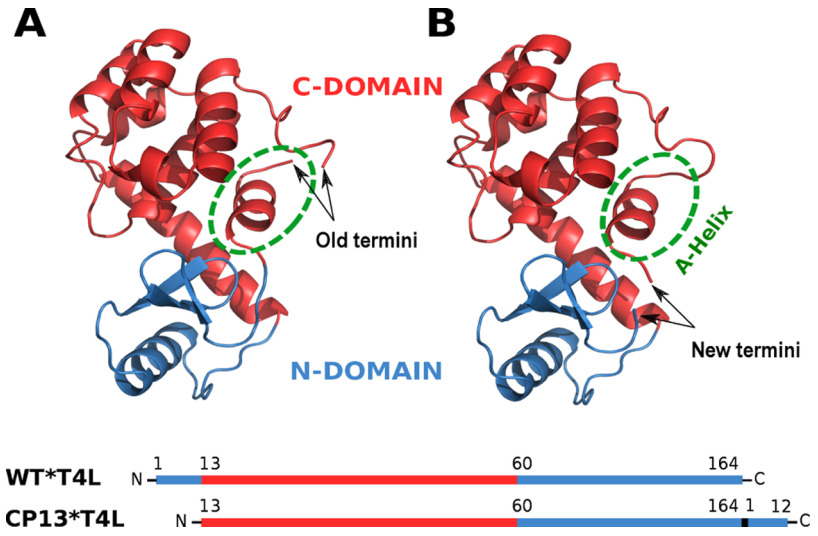

Figure 1. Structure of the cysteine free WT*T4L (A) and CP13*T4L (B). For both species, the C-domain is on the top and the N-domain is on the bottom. The C-terminal part of each subdomain is shown in red; the N-terminal part is in blue. Thus, the A-helix (green dashed circle), which encompasses residues 1−12 in WT*T4L, is displayed in red for WT*T4L and CP13*T4L but is linked to the N-domain in A although it is no longer in B. The respective discontinuity and continuity of WT*T4L and CP13*T4L are clear when one observes their sequences (bottom). Protein structures (PDB ID: 3DKE) have been rendered with Pymol.[6]

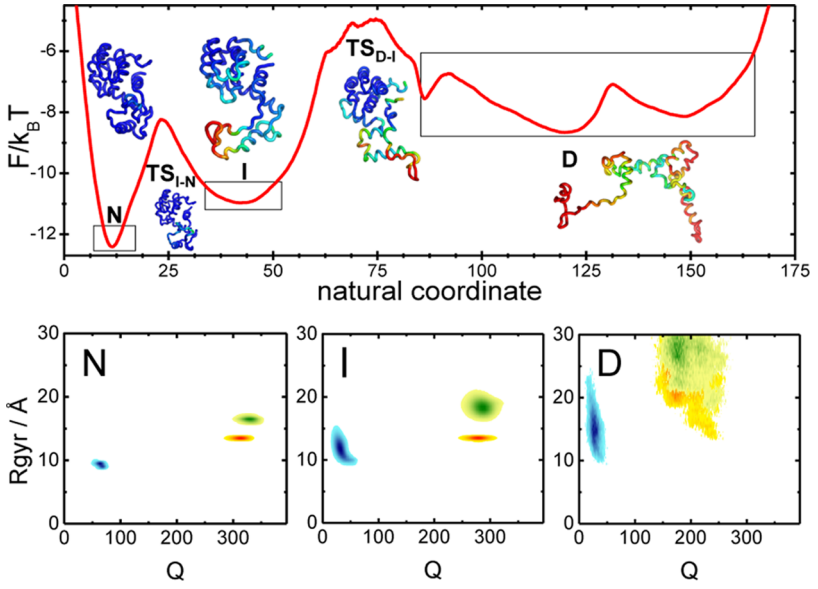

Figure 2. FEP of WT*T4L along the optimal reaction coordinate. The different basins (native, intermediate, and denatured) and transition states are shown as structures representative of the corresponding ensemble and colored in a blue to red scale according to increasing B-factor values. A projection of each basin onto the radius of gyration and number of native contacts is shown for the native basin (N), the intermediate basin (I), and the denatured basin (D) for the whole protein (greens), N-domain (blues), and C-domain (reds); darker colors mean higher probability. In the native basin (N), both subdomains and the whole protein are completely formed; the intermediate basin (I) has a folded C-domain and an unfolded N-domain; in the denatured basin (D), both subdomains are unfolded.

computationally more demanding, and equilibrium folding simulations can only be performed for small fast-folding proteins using purpose-designed computers.[24] Coarse-grained or simplified models, such as minimally frustrated, structure-based ones, turn out to be valuable when accurate sampling of the conformation space accessible is required.[25] Any attempt to characterize the folding free-energy landscape of a protein requires accurate sampling of the slowest event, which consists in the crossing of the major free energy barrier between the native conformation and denatured ones. Thus, unless one is looking at small and fast-folding proteins,[24] coarse-grained, structure-based models, such as the one used here, are for the time being unavoidable. The availability of experimental high-resolution measurements to validate the results from such simplified models becomes necessary if the conclusions are to be generalized to the real system being modeled.

Only a small number of simulation studies have focused on the effect of circular permutations on folding dynamics, and even fewer have addressed the effects of such perturbation on the free-energy landscape. In most cases, simplified models of proteins, such as lattice models, have been used.[26] Itoh et al. applied an Ising model and analyzed the free-energy landscape using the number of native contacts as a reaction coordinate.[27]

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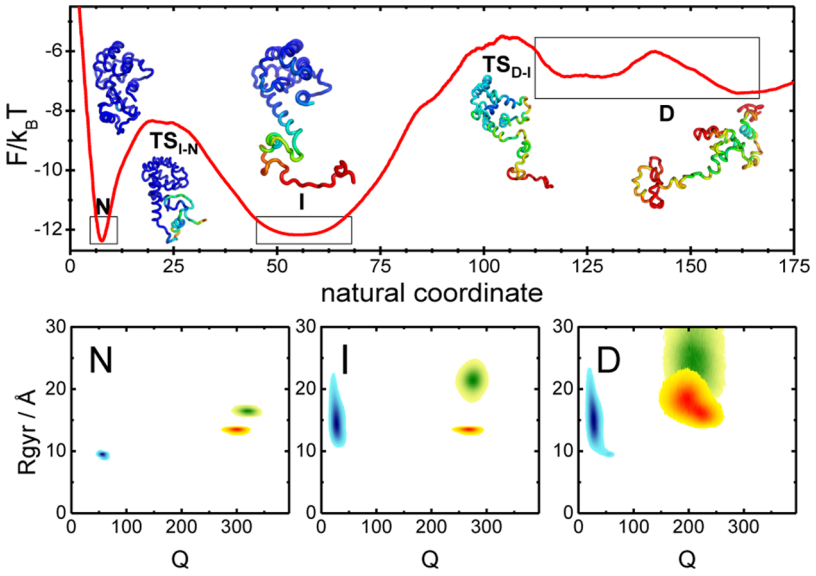

Figure 3. FEP of CP13*T4L along the natural optimal reaction coordinate and representation of the different basins (native, intermediate, and denatured); see caption to Figure 2, where the analogous result for WT*T4L is shown. It appears clearly that the rate-limiting step for folding corresponds to overcoming the large barrier between I and N. Despite this capital difference, the features of the various states are remarkably similar; in particular, in I, the C-domain is formed, and the N-domain is not. Interestingly, the N-domain is more disordered than in the case of WT*T4L as a consequence because of the lack of the docked re-entrant helix.

Here, we use molecular dynamics simulations with a structure-based, minimally frustrated model to determine the free-energy landscape of both wild type (WT*T4L (Figure 1A)) and a circular permutant (CP13*T4L (Figure 1B)) of a cysteine-free variant of T4 lysozyme.[28] Good agreement with experimental measurements justifies the use of a structure-based model in this specific study. Key to our approach is the use of a method[29−32] to determine low dimensional projection of the free energy that is rigorous and general and that provides results that are easily interpretable.

The free-energy profile (FEP) of WT*T4L shown in Figure 2 provides a description of the main features of WT*T4L folding. Three main basins are clearly identifiable: the denatured, the intermediate, and the native basins (labeled D, I, and N respectively). The kinetics can be modeled as

$$D \rightleftharpoons I \rightleftharpoons N$$

Structures belonging to the denatured basin are highly diverse and lack secondary structure. Further on the folding pathway, the protein encounters an intermediate state, which has been experimentally observed.[12,14,33] This intermediate presents an average radius of gyration of 18.7 Å, as compared with 16.6 Å for the native state and 26 Å for the denatured state. Projections of the native and intermediate basins onto plots of the radius of gyration versus number of native contacts (Figure 2) shows that the intermediate exhibits a well-folded C-domain, whereas the N-domain is mostly unfolded. This feature tallies with the intermediate observed experimentally.[33]

The transition between states D and I is the rate-limiting step of the folding landscape as it corresponds to the highest free-energy barrier (about 4 k_BT). As a consequence, the crossing of the transition state TS_{D-I} is the rate-limiting step in the folding reaction, with a mean first passage time (MFPT) of 7.4 μs. The present result agrees with the experimental finding that places the formation of the intermediate species after the rate-limiting step.[12,14,33] An important feature of the transition state TS_{D-I} is the native-like placement of the A-helix and the whole of the C-domain.[17,18,33] The height of the barrier between D and I can be ascribed at least in part to the loss in entropy upon the docking of the A-helix to the C-domain. After the formation of the intermediate, we find an additional transition state (TS_{I-N}), characterized by a smaller free-energy barrier (about 2.5 k_BT), between the intermediate and the native state. This relatively faster step (MFPT 2.3 μs) corresponds to the structuring of the N-domain that completes the folding of the protein.

To test the role of discontinuous subdomains in folding dynamics, we have performed analogous simulations for the circular permutant CP13*T4L, in which the A-helix is covalently attached to the C-domain. The resulting FEP is shown in Figure 3. The free-energy landscape of CP13*T4L is qualitatively similar to that of the wild type enzyme, with three distinguishable basins (D, I, and N) and two transition states (TS_{D-I} and TS_{I-N}). The observation that the circular permutant folds in a manner similar to the wild-type is in agreement with experimental evidence.[14,33,34]

Despite the apparent similarity in the folding landscape of the wild type and the circular permutant T4 lysozyme, the folding mechanism has changed. One interesting observation comes from structural indicators such as the radius of gyration and number of native contacts of the three identified states of the FEP. The three lower panels of Figure 2 and Figure 3 show the distributions of such properties for the three states of each variant. In the native state (N), both the radius of gyration and the number of contacts are narrowly distributed; on the contrary, in the denatured state (D), they are broadly distributed, and similarly so in the wild-type and the circular permutant. In the intermediate (I), the C-terminal domain has a native-like radius of gyration and number of contacts in both variants; the N-terminal domain, instead, is considerably disordered, as previously inferred from native state hydrogen exchange.[35] In the intermediate of the circular permutant, the radius of gyration and number of native contacts of the N-terminal domain vary as broadly as in the denatured state. In the wild-type, the N-terminal domain is also considerably

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disordered in the intermediate, but less than in the denatured state because the folding of the C-domain includes the A-helix and the latter keeps the N-terminus anchored to the folded C-domain.

These considerations provide a microscopic explanation of the shift in the rate-limiting step upon circular permutation. In the circular permutant, although the D → I transition involves the ordering of the C-domain, it does not involve a large loss of entropy, as in the wild type, where the folding of the C-domain restrains the dynamics of the N-domain through the A-helix. A relatively more stable state, I, for the circular permutant results in an higher free-energy barrier between I and N (MFPT ≈ 22.2 μs); the barrier between D and I (MFPT ≈ 2.7 μs) is much lower. This results in the movement of the main free energy barrier relative to that observed for the wild type, that now is located at the I → N transition and involves the formation of the N-domain and the achievement of the correct overall topology.

To quantify the cooperativity between the subdomains, we have used quasiharmonic principal component analysis to extract the essential dynamics space of each subdomain for both WT*T4L and CP13*T4L.[36] The cross-correlation between the projection of the individual subdomains' trajectories over the lowest frequency mode is much larger for the WT*T4L (0.6) compared with CP13*T4L (0.02). This means that slow motions are correlated between subdomains for the wild-type and not for the circular permutant. These findings are consistent with the experimental evidence of the two-subdomain cooperativity during the folding of the wild-type T4 lysozyme[10,11,13,14] and the loss of coupling in the circular permutant.[14,15,17,18,33]

We thus conclude that the construction of the circular permutant of T4 lysozyme has a major effect on the folding cooperativity of this protein. Although there is broad experimental evidence of the relation between circular permutation and interdomain cooperativity, the present simulations show that this arises from a subtle change in the folding landscape. The source of this cooperativity is found in the transition state. Structurally, it *enforces* the communication between the two subdomains through the docking of the A-helix to the rest of the C-domain. In the wild type protein, since the formation of the transition state is an early event in the folding pathway, this connection between subdomains (i.e., cooperativity) is present throughout the folding process.

The structure-based potential that makes possible the thorough sampling of the folding process turns out to be a good choice. Important features of the system (such as the existence and essential characteristics of the intermediate) are in excellent agreement with the experiment.[14,15,18,33] Here, we want to stress that the features of the model system and their relation with the experimental properties of T4L could not have been extracted from the simulation without the specific algorithm we used to construct the optimal reaction coordinate and the corresponding free-energy landscape. An optimal reaction coordinate not only preserves the diffusive dynamics of the system[29−32] but also provides an intuitive representation of the results of the simulation. The approach uses no adjustable parameters apart from those used in the optimization of the reaction coordinate and analyzes the free-energy landscape with any number of transition states (see the Supporting Information). The good agreement between the dynamics on the profile and the unprojected dynamics (i.e., the MFPTs computed from diffusion on the profile and directly from the trajectories agree) confirm the robustness of choice of reaction coordinate. Alternative approaches (for example, the method proposed by Best and Hummer[37]) provide similar results in the case of a single transition state but fail when multiple transitions are present, as in the case studied here.

The approach used to determine free energy landscapes from molecular dynamics trajectories is rigorous and here generalized to nonequilibrium trajectories. For the specific case of T4 lysozyme, we observe that a circular permutant in which the two domains are continuous folds less cooperatively, as experimentally previously shown. This change is reflected by a subtle change in the free energy landscape, that is, the shift of the rate-limiting barrier after an intermediate state; the latter, although present in both species, is relatively more populated in the circular permutant.

For the two discontinuous domain protein dihydropholate reductases, it has been shown that several circular permutants, including one that makes the two domains continuous,[3,38] preserve the folding ability of the protein. The effect of a circular permutant on the folding mechanism of ∼100 amino acid single-domain protein S6[4] has shown how the folding ability of a protein can be preserved while the folding mechanism can be dramatically changed; for protein S6, as for T4 lysozyme investigated in the present work, the wild type proteins fold more cooperatively than the respective circular permutants.

It seems plausible, as suggested by Shank et al.,[18] that proteins have likely evolved to select topologies that allow for more cooperative folding to avoid kinetic trapping and misfolding. In multidomain proteins, where individual domains independently evolved join together to perform new functions,[39] coupling between domains through circular permutation may be an evolutionary strategy to improve their folding. Indeed, it has been shown that circular permutations play an important role in protein evolution,[40] particularly for multidomain proteins.[41]

For the specific case of T4 lysozyme, we have shown that the radical change in topology induced by a circular permutation that turns the two domains from continuous to discontinuous, does not involve a complete change of the free energy landscape. Although this may not be true in general, it appears that enhanced cooperativity through domain coupling may be obtained by preserving the essential features of the free-energy landscape of an ancestor protein where the subdomains were continuous.

■ ASSOCIATED CONTENT

Supporting Information

Details on the simulations and on the method used to determine the optimal reaction coordinate. This material is available free of charge via the Internet at http://pubs.acs.org/.

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: e.paci@leeds.ac.uk.

Present Address

‡(M. Enciso) Department of Mathematics and Computer Science, Freie Universitaet Berlin, Germany

Notes

The authors declare no competing financial interest.

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