RESEARCH ARTICLE

Energy-dependent protein folding: modeling how a protein folding machine may work [version 1; peer review: 1 approved, 3 approved with reservations]

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Abstract

Background: Proteins fold robustly and reproducibly *in vivo*, but many cannot fold *in vitro* in isolation from cellular components. Despite the remarkable progress that has been achieved by the artificial intelligence approaches in predicting the protein native conformations, the pathways that lead to such conformations, either *in vitro* or *in vivo*, remain largely unknown. The slow progress in recapitulating protein folding pathways *in silico* may be an indication of the fundamental deficiencies in our understanding of folding as it occurs in nature. Here we consider the possibility that protein folding in living cells may not be driven solely by the decrease in Gibbs free energy and propose that protein folding *in vivo* should be modeled as an active energy-dependent process. The mechanism of action of such a protein folding machine might include direct manipulation of the peptide backbone.

Methods: To show the feasibility of a protein folding machine, we conducted molecular dynamics simulations that were augmented by the application of mechanical force to rotate the C-terminal amino acid while simultaneously limiting the N-terminal amino acid movements.

Results: Remarkably, the addition of this simple manipulation of peptide backbones to the standard molecular dynamics simulation indeed facilitated the formation of native structures in five diverse alpha-helical peptides. Steric clashes that arise in the peptides due to the forced directional rotation resulted in the behavior of the peptide backbone no longer resembling a freely jointed chain.

Conclusions: These simulations show the feasibility of a protein

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folding machine operating under the conditions when the movements of the polypeptide backbone are restricted by applying external forces and constraints. Further investigation is needed to see whether such an effect may play a role during co-translational protein folding *in vivo* and how it can be utilized to facilitate folding of proteins in artificial environments.

Keywords
Protein folding, ribosome function, chaperone, computer modeling, molecular dynamics, energy-dependent protein folding, co-translational protein folding, nascent peptide rotation, peptide backbone manipulation, protein folding machine

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Introduction

Once they are synthesized in a living cell, the majority of proteins rapidly attain their distinctive biologically active three-dimensional structures, called native conformations. These conformations are robustly achieved *in vivo* via a folding process that involves interactions of the folding chain with molecular chaperones and other maturation factors. The folding process often cannot be reproduced *in vitro*, in the absence of chaperones and other cellular components[1–5]. However, some small proteins fold spontaneously *in vitro* in the absence of any other macromolecules[6].

What exactly happens during the folding of a linear polypeptide chain into a native conformation either *in vivo* or *in vitro* remains largely unknown. Despite decades of intense laboratory research, theory development and computer simulations, we still cannot recapitulate complete folding trajectories *in silico*, except for those of a few relatively short polypeptides[7]. Knowledge of the intermediates in the folding pathways and the mechanisms that enable them is essential for determining the points of intervention at which folding and misfolding processes can be altered.

The painfully slow progress in our ability to fold *in silico* all but the shortest polypeptides could be due to the sheer complexity of the system: the number of possible conformations of a polypeptide chain, and the number of interactions between the atoms of all amino acid residues within the polypeptide itself and with the surrounding solvent, are so astronomically high that the existing computational power is not yet sufficient, and might never become sufficient, to capture the folding trajectories for longer proteins[8]. It is also possible, however, that there are fundamental deficiencies in our understanding of folding as it occurs in nature, and progress in recapitulating protein folding pathways requires a more realistic physical model of folding than the one we have been relying upon.

The current dominant model of protein folding was prompted by early observations that some small proteins are able to fold *in vitro* into their native conformations spontaneously, in isolation from other proteins or cellular components (reviewed in 6). These observations gave rise to the thermodynamic hypothesis of protein folding[6,9], which in turn led to the development of the physical model that describes protein folding as a thermodynamically favorable, unassisted process. In a more recent, refined form, this model includes the description of a rugged funnel-shaped energy landscape, in which the various unfolded, unstructured conformations occupy the high-free-energy brim of the funnel[10–13]. As the polypeptide chains fold, they sample conformations with progressively decreasing Gibbs free energy until they reach the native conformation, which is presumed to occupy the global thermodynamic minimum at the bottom of the funnel. The sampling of conformations during the folding process is assumed to occur via random thermal motions[14]. The driving force of protein folding is assumed to be the decrease in free energy to the global minimum.

In summary, the current general physical model of protein folding describes a process that occurs in a closed system in the absence of external sources of energy. It assumes that folding starts from a random, unstructured conformation and proceeds unassisted, with no apparent requirement for the folding chain to interact with other proteins or macromolecular cellular components. This model describes an extremely artificial process that is only likely to occur *in vitro* and has little resemblance to what takes place during the folding of all proteins in the living cell.

In nature, folding of the majority of proteins occurs in the environment of a living cell, which is an open system with a constant flow of energy and shifting chemical composition. In a cell, a polypeptide starts folding while it is still being synthesized on a ribosome, where it occupies a tight space that allows it to adopt only a limited set of conformations. The nascent peptide emerges into a crowded, viscous environment outside of the ribosomal tunnel and interacts with multiple proteins, including chaperones, and with other cellular components, at all stages of folding. In the course of peptide synthesis and co-translational folding, a large amount of energy is released by GTP hydrolysis. This energy is not required for the formation of peptide bonds[15], but may be spent, at least partially, on various motions and adjustments of the ribosomal components, directly affecting the folding environment of the nascent peptide[16–18]. It is difficult to escape the conclusion that protein folding *in vivo* must be described by a physical model that takes into account the interactions of a folding polypeptide chain with its complex dynamic cellular environment.

We have recently proposed that a more realistic physical model of protein folding might be built on the assumption that protein folding *in vivo* is an active, energy-dependent process. In this alternative model, proteins that are not able to fold spontaneously must rely on additional external forces to achieve native conformations[19]. We hypothesized that the mechanism of action of such a protein folding machine might include direct mechanical manipulation of the peptide backbone by the concerted actions of the ribosome and chaperone complexes[20,21]. During translation in the peptidyl transferase center of the ribosome, the 3’ terminus of the tRNA in the A-site swings by nearly 180 degrees in every elongation cycle[22,23]. We hypothesized that this motion might lead to the rotation of the C-terminus of the nascent peptide. Simultaneously, the movements of the N-terminal regions of the nascent peptides may be restricted, first, by occlusions in the ribosome exit tunnel and then by steric capture mediated by the ribosome-associated “nascent chain welcoming committee”, such as the trigger factor in bacteria and the nascent polypeptide-associated complex in archaea and eukaryotes[21]. As a result, the folding polypeptide may experience transient strained conformations with elevated free energy[19].

As the first step in exploring the feasibility of a protein folding machine capable of facilitating the attainment of native structure by mechanical manipulation of the peptide backbone, we performed molecular dynamics simulations augmented by application of torsion to the peptide backbones. During the simulations, the C-termini of various polypeptides were mechanically rotated either clockwise or counterclockwise,

while the motions of their N-termini were restricted. We compared the trajectories of both types of simulations with the folding of the same peptides without the application of torque. In our experiments, directional rotation of the C-terminal amino acids with simultaneous limitation of the movements of the N-termini indeed facilitated the formation of native structures in five diverse alpha-helical peptides.

Methods

The initial stretched structures of peptides (Table 1) with four additional alanine residues, two at each end, were generated using ICM software[24]. These alanines were attached as handles to which the rotation or restraint could be applied directly without affecting the sequence whose folding was investigated, and were not considered in the RMSD calculations. We aligned a peptide along the X-axis and solvated it in a dodecahedron box in the case of the simulations of unassisted folding and triclinic box in all other cases, with minimum distance of 1.5 nm between a peptide and the simulations box. Potassium and sodium ions were added to neutralize the charges in the system. The system was then minimized with the steepest descent algorithm, equilibrated for 100 ps in the NVT ensemble using V-rescale thermostat[25] for temperature coupling, and continued in the NPT ensembles for 1 ns using V-rescale thermostat and Berendsen barostat[26]. After the equilibration, we kept temperature and pressure constant at 300 K and 1 bar respectively, using Nose–Hoover thermostat[27,28] and isotropic Parinello–Rahman barostat[29].

For all simulations, we used the ff14SB force field[30] with the TIP3P water model[31] and ion parameters modified by Joung and Cheatham[32]. Electrostatic interactions were calculated using particle-mesh Ewald (PME) summation[33] with a Fourier grid spacing of 0.135 nm. For non-bonded Coulomb and Lennard-Jones interactions, 1 nm cutoff was used. We constrained the hydrogen bonds with the LINCS algorithm[34] and used a 2-fs integration time step.

To exert an external mechanical torque to the C-termini of the peptides, we adopted the enforced rotation method, originally designed to study rearrangements during the rotation of a folded protein within the F1-ATPase assembly[35], implemented in the GROMACS molecular dynamics package. To this end, we restrained the positions of the O and N atoms of the C-terminal alanine to keep it aligned with the X-axis, about which the rotation was applied. The restraints with a force constant of 10000 kJ/mol*nm^2 were applied only for the YZ-plane, so the C-terminal amino acid could move along the X-axis. In addition, we restrained the O atom of the C-terminal amino acid in the X direction with a force constant of 5 kJ/mol*nm^2 and N and Cα atoms of the N-terminal alanine with a force constant 10000 kJ/mol*nm^2 in all directions. The C-terminal amino acid was rotated using a flexible axis approach (Vflex2) with a reference rotation rate of 60 degrees/ps and a force constant of 1500 kJ/mol*nm^2.

The GROMACS package version 2020.2[36] was used for all simulations and trajectory analyses. The simulations were carried out on CUDA-enabled GPUs with Turing architecture, running Ubuntu 18.04. For visualization of protein structures and trajectories, the programs ICM-Pro 3.9[24] and VMD 1.9.3[37] were used.

Results

We performed atomistic molecular dynamics simulations to study peptide folding under conditions when, throughout the simulation, an external mechanical torque was applied to the C-terminal amino acid of a peptide and the motions of the N-terminal amino acid were restrained (Figure 1). We compared the folding trajectories of the peptide to which a mechanical force was applied to rotate the C-terminal amino acid in one of the two possible directions – either clockwise as in Figure 1, or counterclockwise – with the trajectories for the same peptide which was allowed to fold without any motion restriction or application of any mechanical force (referred to as "unassisted folding" below). As an additional control, we ran a fourth type of simulation, where motion restraints were applied to both ends of each peptide but the torque was omitted. The details of the simulations are described in the Methods section. Each of the four types of simulations were repeated three times, giving 12 simulations for each peptide.

The experiments were run on five peptides that are known to adopt alpha-helical conformations in their folded form (Table 1). Two of these, P1 and P2, have been designed *de novo*, and the other three, P3-P5, are parts of naturally occurring proteins.

Table 1. Peptides used for the molecular dynamics simulations in this study.

Peptide	Peptide description	Sequence	Length, amino acids	PDB ID
P1	Peptide Fs (Folded short), designed de novo	AAAA(AAARA)3	19	n/a
P2	First helix of the three-helix bundle, designed de novo	SWAEFKQRLAAIKTR	15	2A3D
P3	Fragment of the tetramerization domain of potassium channel Kv7.1	HLNLMVRIKELQRRLDQSL	19	6UZZ
P4	Loop and third helix of the villin headpiece fragment HP35	PLWLQQHLLKEKGLF	15	2F4K
P5	Fragment of the coiled-coil region of pyrin	KIQKQLEHLKKLRKSGEEQRS	21	4CD4

The folding of the peptides was monitored by calculating the root mean square deviation (RMSD) distance of the peptide backbone from the native structure of the same fragment determined by X-ray crystallography (peptides P2-P5), or computed ab initio (peptide P1). The results of the simulations for each peptide when folded unassisted in the standard force field, and when an external torque force was added to the field, are presented in Table 2 and Figure 2. All folding trajectories and the additional information on the properties of all simulation boxes are available at Zenodo[38].

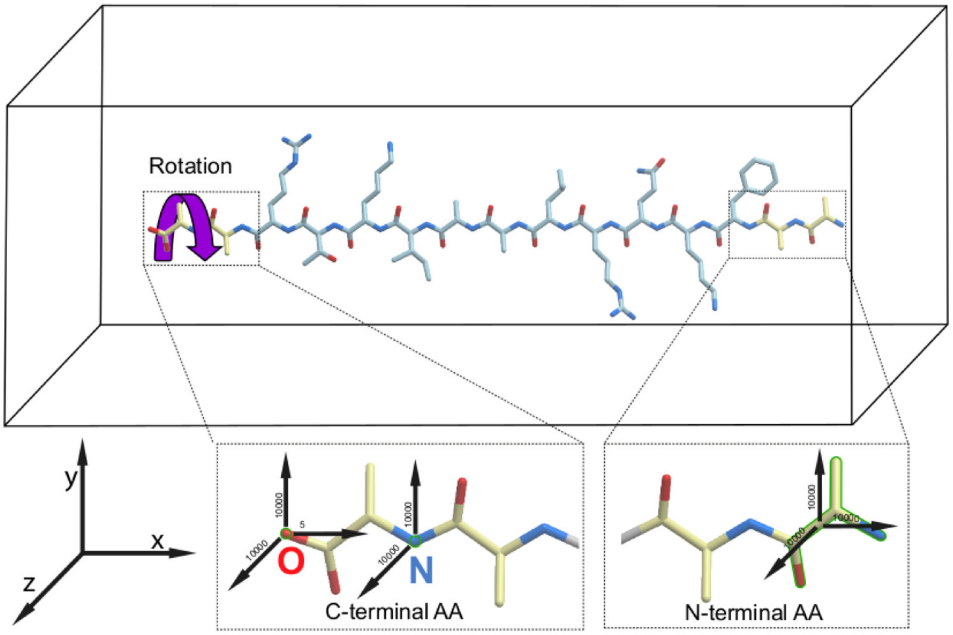

Figure 1. Schematic representation of the energy-dependent peptide folding protocol employed in this study. The force vectors applied to the C- and N-termini of a peptide in the simulation box are shown by black arrows. All force values are in kJ/mol*nm^2. The purple curled arrow indicates the direction of the clockwise rotation of the peptides that resulted in the accelerated productive folding of all peptides to their helical conformations. The restrained groups are shown by green outline.

Table 2. Peptide folding rates in the molecular dynamics simulations. The first number indicates the time (ns) spent before reaching the RMSD of 0.2 nm from the native conformation, and the second number indicates the duration of the experiment. 500/500 and 1500/1500 values indicate that folding was not observed in this simulation.

Peptide	No rotation, no restraints	No rotation, restrained ends	Restrained ends, rotation clockwise	Restrained ends, rotation counterclockwise
P1	500/500 500/500 500/500	500/500 500/500 500/500	22/100 18/100 29/100	500/500 500/500 500/500
P2	1500/1500 1500/1500 1500/1500	1500/1500 1500/1500 1500/1500	160/200 140/200 105/200	1500/1500 1500/1500 1500/1500
P3	1500/1500 1500/1500 1500/1500	1500/1500 1500/1500 1500/1500	105/150 95/150 125/150	1500/1500 1500/1500 1500/1500
P4	1500/1500 545/1500 360/1500	250/1500 1500/1500 1500/1500	130/175 80/175 150/175	1500/1500 1500/1500 1500/1500
P5	1500/1500 1500/1500 1500/1500	1500/1500 1500/1500 1500/1500	230/1500 380/1500 170/1500	1500/1500 1500/1500 1500/1500

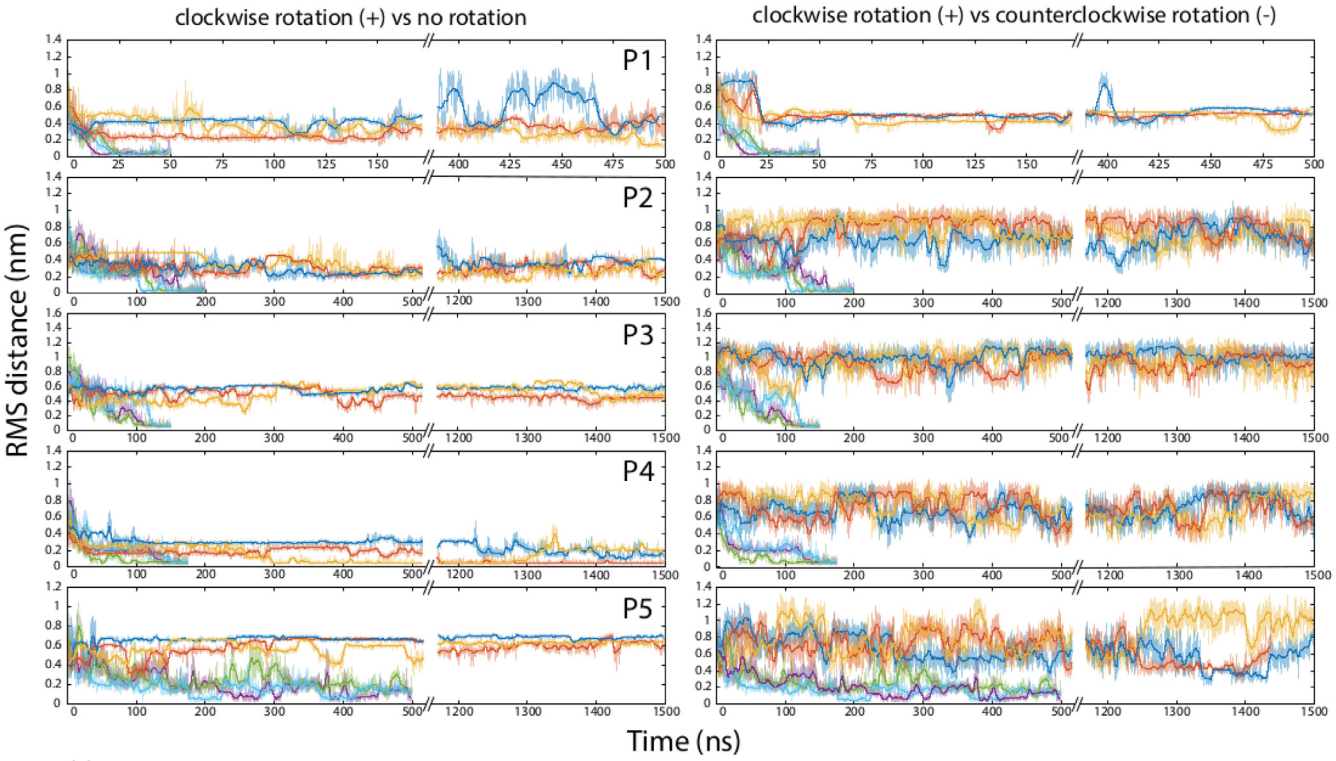

Figure 2. Folding of peptides in the force field with and without an augmentation by the application of external rotation forces to the polypeptide backbone. Each horizontal pane represents molecular dynamics simulations for one peptide, numbered P1 through P5 (Table 1). On the left side, top three curves (dark blue, orange, and yellow) indicate three independent runs for one peptide in the standard force field without externally applied backbone rotation, and the bottom three curves (purple, green, and light blue) indicate three runs in the presence of the clockwise rotational force. On the right side, the bottom three curves are the same as in the corresponding left pane (three runs in the presence of the clockwise rotational force), and the top three curves (dark blue, orange, and yellow) indicate three runs for the same peptide in the presence of the counterclockwise rotational force.

Within our simulation lengths, we observed the completion of unassisted folding into the native-like alpha-helical structure only in some runs for one peptide, P4, which represents the third helix and preceding loop in the villin headpiece domain HP35. Other peptides remained essentially unfolded throughout the 500–1500-nanosecond runs. The peptides also failed to fold when their ends were restricted in mobility but torque was not applied (Table 2). In contrast, when the external torsion force was applied to the C-termini of the peptides in the clockwise direction, as described in Methods and illustrated in Figure 1, peptides P1-P4 all folded into alpha-helical structures and were brought within 0.2 nm RMSD from their native structures in every run, typically within the first 100–200 ns of simulation. These peptides stayed in the native or nearly-native conformations for the remainder of the experiments. Peptide P5 was a special case; similarly to P1-P4, it adopted a compact conformation early in the experiments, but remained only partially folded for the duration of all runs (Figure 2).

For all five peptides, folding was observed when the rotation force was applied to the C-terminal amino acid in the clockwise direction (Figure 1). In contrast, the torque applied to the C-terminus counterclockwise with the same force constant did not facilitate folding of P1-P3 and P5, and may have inhibited folding of P4 (Figure 2).

Discussion and conclusions

To test the idea that inclusion of external forces can improve modeling of protein folding pathways *in silico*, we performed molecular dynamics simulations in which a standard force field was augmented by the application of external mechanical forces to the polypeptide backbone. We compared these simulations to control runs without any additional external forces. The directional rotation of the C-terminal amino acid with simultaneous restriction of the movements of the N-terminal amino acid facilitated the formation of native structures in five diverse alpha-helical peptides, confirming that such constraints can have significant consequences for folding dynamics. Strikingly, application of mechanical force accelerated the folding of P4, a fragment of an on-pathway folding intermediate of the well-studied villin headpiece domain HP35, which is one of the fastest-folding protein domains known[7,39,40]. The several-fold increase in the rate of P4 folding that was achieved in our experiments seems to suggest that the postulated "physical limit of folding" of HP35 as a whole[39,41] could be overcome by a protein folding machine. The other four

peptides in our experiments likewise attained their alpha-helical structure in the presence of the rotating force, but did not reach their native conformations when allowed to fold unassisted, even though we ran the control unassisted simulations for ~10 times longer than the simulations that included the application of the external force (Table 2). Some of those peptides might take a very long time to reach their native conformations without application of an external force, whereas others might never fold unassisted, if their unfolded states are more stable than the folded conformations.

These results are in line with our protein folding machine hypothesis[19]. They also support a hypothetical mechanism through which the machine would directly alter the conformations of proteins by applying mechanical force to the peptide backbone[20,21]. The feasibility of such a mechanism, however, is dependent on whether the torsion applied at one point of a peptide would propagate through the rest of the peptide chain and affect the movements of the distal parts of the peptide. The peptide backbone is often viewed as a freely jointed chain, due to the 360-degrees rotation ability around the phi- and psi-bonds within each amino acid[42]. If the peptides in our simulations were to behave as freely jointed chains, the rotation of a single amino acid at the end of the peptide would not have any appreciable effect on the motions of the rest of the peptide. However, if a mechanical torque were applied to a peptide while it was being folded in a viscous crowded environment (e.g., co-translationally in a living cell), we predicted that the free rotation of the phi- and psi-bonds in the peptide backbone would be hindered enough that escape from the forbidden sections of the Ramachandran plots would become difficult for many residues, and as a result, the entire peptide backbone may experience transient strained conformations. Although our simulation could not account for all the details of the protein folding environment *in vivo*, we were able to devise a set of conditions under which the peptide indeed did not behave as a freely jointed chain. When a force was applied to a single amino acid residue, and the motion of just one other residue at least 15 amino acids apart was restricted, the folding trajectory of the entire peptide was affected dramatically, leading to the rapid attainment of the native helical conformation. Some of the steric hindrances that make this rapid folding possible involve amino acid side chains, and therefore the effect might be sequence-specific. For example, glycine residues are more likely to experience the full 360-degree rotation around the phi- and psi-bonds, relieving the strain in the main chain; this might explain why P5, a peptide with an internal glycine, first acquired and then partially lost its folded conformation in our experiments (Figure 2).

It remains unclear whether our simulation captures the main features of the folding process as it occurs in nature. For example, one of the parameters that differs between our simulations and real co-translational protein folding process is their characteristic times. The rotation of the backbone in our system occurs at the submicrosecond time scale, whereas the addition of amino acids to the nascent peptide is much slower, on the order of subseconds[43–45]. Molecular dynamics simulations have been known to model, at a fast scale, the essential parts of the molecular processes that are much slower when observed with bulk kinetics or single-molecule methods[17,40], but the effect of the rotation rate on the peptide folding trajectory remains to be investigated.

The key feature of the hypothetical mechanism of co-translational protein folding that we simulated is the directional rotation of the peptide backbone. As discussed above, the 3' terminus of the tRNA in the A-site of the ribosome peptidyl transferase center turns by nearly 180 degrees in every translation elongation cycle. Only a 45-degree swing is necessary to achieve the proper stereochemistry of the peptide bond formation[46]; the function of the remaining portion of the turn is unknown, and we have hypothesized that it may be needed to facilitate co-translational folding[20,21]. It is notable, however, that the tRNA within the translating ribosome appears to turn in the counterclockwise direction when looking from the C-terminus of the nascent peptide[22,23]. In contrast, folding of all peptides into the right-handed alpha-helices in our experiments took place only with clockwise rotation of the C-termini (Figure 1 and Figure 2). It remains to be determined what, exactly, happens to the nascent peptide in the peptidyl transferase center and in the ribosome exit tunnel. The nascent peptide might be rotated counterclockwise (in the direction of the tRNA swing), or clockwise (as a result of a gear-like interaction with the tunnel walls), or might not be rotated at all but rearranged in a more complex way, being subject to pushing and pulling forces as well as interactions with the exit tunnel walls and other components of the ribosomal complex.

Regardless of whether the peptide torsion mechanism operates during co-translational folding on the ribosome *in vivo*, we demonstrate that it is possible to facilitate protein folding under conditions when an external mechanical force is applied to the peptide backbone. Importantly, we show that the peptide does not always behave as a freely jointed chain, opening the possibility that *in vivo* the peptide backbone can be manipulated into conformations that cannot be reached without assistance because they are either thermodynamically unstable or kinetically inaccessible. The results of our simulations thus demonstrate the feasibility of a protein folding machine. Some recently published results, including studies of the role of the exit tunnel in nascent chain folding[47–51] and of direct coupling between ATP hydrolysis and protein refolding by the chaperones of the HSP70 family[52–54], may be also interpreted as evidence of protein folding *in vivo* being an active process.

The notion of an active, energy-dependent protein folding mechanisms *in vivo* is better compatible with the current understanding of evolution than the generally accepted, standard thermodynamic hypothesis of protein folding. Although it is accepted that the ability of proteins to attain their native conformations must have evolved by natural selection of sequences that fold quickly and correctly ("evolution solved the protein folding problem"[55]), models of unassisted folding sidestep the fact that ribosomes and translation factors are among the oldest molecular machines shared by all extant cellular life[56], and were present during much of the evolution of proteins and of their folding pathways. The evolutionary

optimization of the tempo and mode of protein folding, for at least 3.5 billion years of biological evolution, has taken place not in dilute solutions of isolated proteins, but in a dynamic environment of living cells with their constant flow of matter and energy. Thus, the ability of any present-day protein to fold in isolation and without assistance is likely to be either an incidental or derived property, not shared by most other proteins. Realistic computational modeling of protein folding must therefore take into account the presence of a multitude of external forces. Further studies should attempt to more closely recreate the conditions of protein folding *in vivo*.

Data availability

Zenodo: Energy-dependent protein folding: modeling how a protein folding machine may work. http://doi.org/10.5281/zenodo.4392959[38]

This project contains the following underlying data:

- trajectories.zip (xtc files of the folding trajectories obtained in the molecular dynamics simulations)
- m2020nepfSF1.pdf (pdf file of the properties of all simulation boxes)

Authors' contributions

Conceptualization: INS; Formal analysis: HKS, INS; Funding acquisition: KBN, INS; Investigation: HKS, KBN, ARM, INS; Methodology: HKS, INS; Visualization: HKS; Writing – original draft preparation: ARM, INS; Writing – review and editing: HKS, KBN, ARM, INS.

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Current Peer Review Status: ✔ ? ? ?

Version 1

Reviewer Report 22 February 2021

https://doi.org/10.5256/f1000research.31167.r78384

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José Arcadio Farías Rico
Systems and Synthetic Biology Laboratory, Center for Genome Sciences from the National Autonomous University of Mexico, Cuernavaca, Mexico

This short MD publication by Sahakyan *et al.*, proposes an interesting alternative to the classic thermodynamic theory of protein folding in which the polypeptide chain acquires its native conformation spontaneously without any external aid. The authors argue that protein folding must be modelled as an active energy dependent process assisted by a hypothetical folding machine (the ribosome) that applies torsion to the polypeptide chain.

They present folding trajectories (up to 1500 ns) of five short peptides (15-21 aa) under four different conditions: a) unassisted folding without restrictions and rotation, b) motions restraints to both ends of the peptide, c) a mechanical force is applied to rotate the C-terminus counterclockwise while the N-terminus is restrained and d) a mechanical force is applied to rotate the C-terminus clockwise while the N-terminus is restrained. Most of the peptides adopt an α helical conformation only in the last condition. The authors suggest that the ribosome, and specially the tRNA molecules moving from the A-site to the P-site, apply rotary forces to the nascent chain during cotranslational folding.

The idea is interesting and the protein folding community would benefit from the indexing of this study. Currently, it is well established that some proteins fold differently while being synthesized by the ribosome than refolded in diluted buffer conditions. The work acceptably adds to these new ideas, the MD experiments are consistent and its properly presented. This study certainly provides food for thought and it does not intend to present a complete picture of the forces acting on the polypeptide chain during cotranslational folding.

The exit tunnel is a complex environment where transient interactions can be stablished between every residue of the growing chain and the macromolecules lining the tunnel. A myriad of factors other than torsion can aid the folding of macromolecules in the tunnel (conformational entropy reduction, transient salt-bridges and Van Der Waals interactions, for instance). Therefore, torsion might play a role but more experiments are needed to fully support this assumption. Also, since the force is applied in the same direction as the alpha helix formation it would definitely accelerate

the folding process.

The weakest point of the publication is the timescale. The rotation (60 degrees/ps) is several orders of magnitud faster than tRNA rotation (1aa/50ms) given a bacterial translation rate of 20aa/s. It would be interesting to see another setup where they rotate the C-terminal residue by 180 degrees only once, and then follow the equilibration of the peptide conformation for some microseconds (helices take microseconds to milliseconds to fold). If the hypothesis is correct one should see the same increased tendency for the peptide to become helical with clockwise rotation compared to counterclockwise as they do under continuous rotation.

Several further studies are possible, such as testing a bigger number of peptides with different helix propensities to draw statistics on how likely is that torque in the chain produce helix formation versus the effect of the simple reduction on the conformational entropy by confining the peptide in a closed environment (as it is the ribosomal tunnel).

Also one could try to perform MD with torsion on small protein domains that have already proven to fold very deep of the ribosomal tunnel. There is a very small domain (29 aa) (ref 47) for which its folding has been tested. How difficult is to test the same conditions used for the peptides with this domain? This domain is almost the same size than the peptides and it is a much better representative of a folded domain.

Is the work clearly and accurately presented and does it cite the current literature?
Yes

Is the study design appropriate and is the work technically sound?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
Not applicable

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
Partly

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Cotranslational protein folding, biophysics, synthetic biology, protein evolution.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Reviewer Report 19 February 2021

https://doi.org/10.5256/f1000research.31167.r78915

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Antonio Trovato
Department of Physics and Astronomy, University of Padova, Padova, Italy

In this short contribution the authors set out to validate, by means of all-atom molecular dynamics simulations in explicit solvent, the idea that the folding of proteins into their biologically relevant native state depends crucially on the presence of the "in vivo" cellular machinery. They have been arguing through several papers, in recent years, that only a minor subset of short proteins conforms to the widely accepted thermodynamic hypothesis (i.e. the native state is the global free energy minimum for a protein in the test tube). In their view, most proteins are not able to fold "in vitro" and instead evolved to be able to fold while they are synthesized at the ribosome. The energy flow provided by the cellular machinery through mechanical manipulations would than make protein folding an activated process "in vivo".

Here, in particular, they consider short peptides (around 20 residues) with helical native states. Their molecular dynamics simulations, 1.5 microseconds long, show that folding to the native state rarely occurs in the absence of any restraint. On the other hand, when restraints on the position of the N-terminal residue and a constant clockwise torque on the C-terminal residue are applied, folding to the native helical state readily occurs for all peptides.

The paper is well written and makes for an interesting read. The authors are very careful in making it clear that any connection between the mechanical restraints used in their simulations and the ones present during co-translational folding is yet to be substantiated. Their findings are in principle interesting since they show how energy pumping through a torque may in fact improve the ability of the folding process to reach a given configuration.

However, the authors should consider carefully the following remarks, which could undermine, to some extent, their conclusions:

1. I think it is important to check whether the helical native states are stable for the considered all-atom force field in the absence of any restraint (e.g. running a microsecond long simulation with the native state as initial condition); if not, failure to fold into the native state could be more simply ascribed to incorrect force field parametrization. I am assuming that the stability of the native state in solution, at least on the microsecond time scale, should be guaranteed also within the unorthodox view advocated by the authors; a comment by them on this point would be useful.

2. Considering only helical native states may bias the conclusion of this study, due to their definite right-handed chirality. Applying a clockwise torque may be effective in biasing any polymer chain towards adopting a right-handed helical configuration. The authors should then consider, at least for future work, to run simulations for peptides forming beta-

hairpins or beta-sheets in their native states.

3. Along the same lines, it had been in fact observed that helical shapes are kinetically favored for growing polymers attached to a moving end[1], even in the absence of an explicit torque. My point is again that what found by the authors could be a generic feature shared by any polymer chain, not a property specific to peptide sequences adopting a helical native state.

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Is the work clearly and accurately presented and does it cite the current literature?
Yes

Is the study design appropriate and is the work technically sound?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
Not applicable

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
Partly

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Computational biophysics; protein folding and aggregation

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Reviewer Report 02 February 2021

https://doi.org/10.5256/f1000research.31167.r77095

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Stephen D. Fried
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In this short article, Sahakyan *et al.* present molecular dynamics (MD) simulations that seek to question the overarching paradigm that protein folding is a predominantly thermodynamically-driven process in which native states are achieved exclusively by identifying states with minimal free energy in the absence of any energy dissipation or physical forces acting on them.

In contrast, by presenting several μs-length trajectories of several short (20mer) alpha-helix forming peptides, the authors show that the ability for the peptide to rapidly adopt a helical conformation is dependent on the application of an externally applied torque, rotating the C-terminus clockwise. The authors draw the connection that the C-terminus of a nascent protein might also experience rotary forces during co-translational folding induced by the movement of tRNA molecules transiting through the A-site and P-site

This article presents an interesting and novel idea that the protein folding community should be aware of, as recent years have seen a growing number of contributions highlighting the potential difference between co-translational folding and 'classical' folding experiments. I would say that rather than 'answering' a problem, it is more an invitation for future work and thought, given the relatively few results that have been reported. That being said, the results presented are intriguing and internally consistent, and deserve the consideration of any worker in the protein folding field interested in understanding how biological folding could differ from the classic scenario of an ergodic search on a constant free energy landscape.

In evaluating this work, I have a number of conceptual comments, suggestions for further study, and recommendations on reproducibility.

Conceptual comments:

- At several points, the authors mention that the 3' terminus of the A-site tRNA undergoes a large-scale (ca. 180˚) rotation, and that this forms the basis of their hypothesis that rotary motions could be transduced to a nascent chain during translation. The motion that the authors are referring to – if I'm not mistaken – is the accommodation of the tRNA from the A/T-hybrid state to the A/A-canonical state upon hydrolysis of GTP by EF-Tu (though they do not mention this process by name). My understanding is that the nature of this large rotation *is* understood; it results from the fact that the presence of EF-Tu precludes the 'top half' of the tRNA from entering the A-site on the 50S subunit whilst the anticodon engages with the decoding center; release of EF-Tu then allows the 3'-end of the A-site tRNA to swing into the PTC. That being said, this large movement would not be experienced by the nascent chain *per se*, because it is occurring on the A-site tRNA (not the P-site tRNA carrying the nascent chain). Hence, the authors may need to clarify what rotational conformational changes are experienced by the P-site tRNA, and provide structural data that directly support this.

- Is the rate of the rotary movement relevant to the timescale of translation? If not, this should be commented on directly, or at least probed by varying its rotational frequency.

Further study:

- Seeing as the larger rotational movement is associated with the A-site tRNA (before its aminoacylated acid is added to the peptidyl chain), the authors may want to use simulation

to directly test their hypothesis that "*this motion might lead to the rotation of the C-terminus of the nascent peptide*." I appreciate the challenge involved in such, as it would surely require performing relatively long simulations on the ribosome. Though this would represent a truly important contribution and should be considered. If simulations of this size/timescale are too infeasible, potentially a careful analysis of the many ribosome structures with P-site tRNA-nascent chains bound could also serve as a reasonable way to interrogate this question.

- The authors seem to assume that all the peptides they studied *could* become alpha helical under the present forcefield, and that this is accelerated by the presence of the external torque. I suppose the first part of this should probably be shown explicitly, perhaps by a free-energy perturbation or umbrella sampling approach, just to show that the minimum free-energy states are in fact what we would think they are.

- What is the mechanism whereby the rotary force induces folding? Careful analysis of the trajectories could probably support or refute the hypothesis that it helps 'seed' the formation of the alpha helix, which then 'zippers' up. (Actually the zippering model for alpha-helix formation is probably very relevant to the authors' discussion, and they might consider seeing if their simulations reproduce the parameters from classical statistical mechanical treatments of the helix-coil problem).

Reproducibility:

- Seeing as performing MD simulations in the presence of external torques is fairly non-standard, the authors may want to provide the actual GROMACS input files for any computational biochemist interested in performing similar simulations.

Is the work clearly and accurately presented and does it cite the current literature?
Yes

Is the study design appropriate and is the work technically sound?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Partly

If applicable, is the statistical analysis and its interpretation appropriate?
Yes

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
Partly

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Protein folding biophysics; proteomics and mass spectrometry.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Reviewer Report 25 January 2021

https://doi.org/10.5256/f1000research.31167.r76648

Lisa J. Lapidus
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This paper presents an intriguing hypothesis, that torque applied by the ribosome at the A-site, actively assists folding. To demonstrate this, the folding of 5 small proteins were simulated from an extended state. When clockwise torque is applied to the C-terminus while constraining the N-terminus (so that the torque is maintained within the backbone), the proteins typically folded within 1.5 microseconds and did not fold within that window without torque, without restraint, and if the torque was applied counter-clockwise.

The results are quite convincing but a few details are missing. Below are some questions whose answers would add to the understanding of the reader:

1. What happens to the backbone after the torque is applied? It seems like the N-terminus is constrained for the entire length of the simulation but this should be made more clear.

2. How does the collapse of the backbone proceed with the constraints compared to without?

3. Do the helical segments form simultaneously or do helices propagate down the length of the chain? Another figure showing native structures of the sequences color coded by when native structure is formed might by useful (or something equivalent).

This paper has an interesting premise with a lot of caveats given the simplified results supporting the conclusion. However, the authors do a good job of addressing some of them. I am not completely convinced that crowded cell conditions could provide the N-terminal constraint required to acquire structure rapidly, but this is a testable result for current co-translational folding simulations. The authors discuss that the torque applied by the ribosome tunnel is a open question and may not be mimicked by the simple torque in the simulations. Another question that could be answered by more realistic simulations is what is the effect of torque at the A-site on the nascent chain outside the ribosome tunnel. While helices can form in the tunnel, there is not a lot of evidence that helices emerge from the tunnel for most sequences. So is the net torque on the last part of the chain before leaving the tunnel what really matters?

Is the work clearly and accurately presented and does it cite the current literature?

Yes

Is the study design appropriate and is the work technically sound?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
Not applicable

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
Partly

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: I study protein folding using ultra-fast experimental methods. I have pioneered the method of measuring intramolecular diffusion of unfolded proteins leading to the estimates of protein folding speed limits.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.