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The *E. coli* thioredoxin folding mechanism: The key role of the C-terminal helix

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ABSTRACT

In this work, the unfolding mechanism of oxidized *Escherichia coli* thioredoxin (EcTRX) was investigated experimentally and computationally. We characterized seven point mutants distributed along the C-terminal α-helix (CTH) and the preceding loop. The mutations destabilized the protein against global unfolding while leaving the native structure unchanged. Global analysis of the unfolding kinetics of all variants revealed a linear unfolding route with a high-energy on-pathway intermediate state flanked by two transition state ensembles *TSE*1 and *TSE*2. The experiments show that CTH is mainly unfolded in *TSE*1 and the intermediate and becomes structured in *TSE*2. Structure-based molecular dynamics are in agreement with these experiments and provide protein-wide structural information on transient states. In our model, EcTRX folding starts with structure formation in the β-sheet, while the protein helices coalesce later. As a whole, our results indicate that the CTH is a critical module in the folding process, restraining a heterogeneous intermediate ensemble into a biologically active native state and providing the native protein with thermodynamic and kinetic stability.

1. Introduction

Protein folding involves the coordination of a myriad of interaction events. Intra- and intermolecular contacts establish a cooperative and plastic interaction network that determines protein topology, stability, dynamics, and biological activity.

Thioredoxin (TRX) is a key protein in the archaea, bacteria, and eukaryote domains. TRX, TRX reductase and the co-enzyme NADPH are components of the TRX system that controls the global protein dithiol/disulfide balance in the cell [1,2] and are involved in a large number of biochemical processes [3]. The role of TRX as an oxidoreductase enzyme is crucial in the peroxide detoxification process, as a partner of thiol peroxidases [1]. TRX, the physiological electron donor of methionine sulfoxide reductases, acts in the reversible reduction of the oxidized methionine as well [4]. Along with the ribonucleotide reductase enzyme, both TRX and glutaredoxin are involved in the reduction of ribonucleotides to desoxiribonucleotides [3]. TRX also acts by controlling the redox state of transcription factors [5,6].

Escherichia coli TRX (EcTRX) has been extensively used in protein engineering to investigate the bases of protein stability, folding and protein function [7–17]. Full-length EcTRX is a small (108 residues) globular and monomeric protein with a characteristic α/β topology, and its structure has been already investigated by both NMR [18–21] and X-ray crystallography [22] (Fig. 1). The structural features and the knowledge gained by several research groups make EcTRX a very interesting model to try to obtain a complete picture of the protein folding process. The active site of EcTRX is characterized by the presence of the –WCGPC– catalytic motif, which is characteristic of the TRX family, and the reversible oxidation of these Cys residues forms an –S–S– bond.

Both chemical [23] and temperature [24–26] equilibrium unfolding experiments indicate that EcTRX, a thermodynamically stable protein (~8 kcal mol^{-1}, in the case of the oxidized form), behaves as a two-state folder. The EcTRX folding kinetics was also studied [27–29]. Folding traces of oxidized EcTRX show high complexity, and at least five multiple phases are necessary to describe the process (followed by Trp fluorescence). Among them, a burst phase is observed, characterized by a time constant smaller than the observation dead time (2.5 ms), which, more likely, corresponds to the initial hydrophobic collapse [30]. In addition, the slowest phase arises from the *trans* to *cis* isomerization

Abbreviations: CD, circular dichroism; CTH, C-terminal α-helix; DLS, dynamic light scattering; ESI-MS, electrospray ionization mass spectrometry; GdmCl, guanidinium chloride; HB, hydrogen bond; I^* and I^*-sim, high energy intermediate state inferred from experiments and simulations, respectively; MALS, multiangle light scattering; MDS, molecular dynamics simulation; *N*, the native state; NMR, nuclear magnetic resonance; R_S, the hydrodynamic radius; SASA, solvent accessible surface area; SEC, size exclusion chromatography; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; *TSE*, the transition state ensemble; TRX, thioredoxin; EcTRX, TRX from *E. coli*; *U*, the unfolded state

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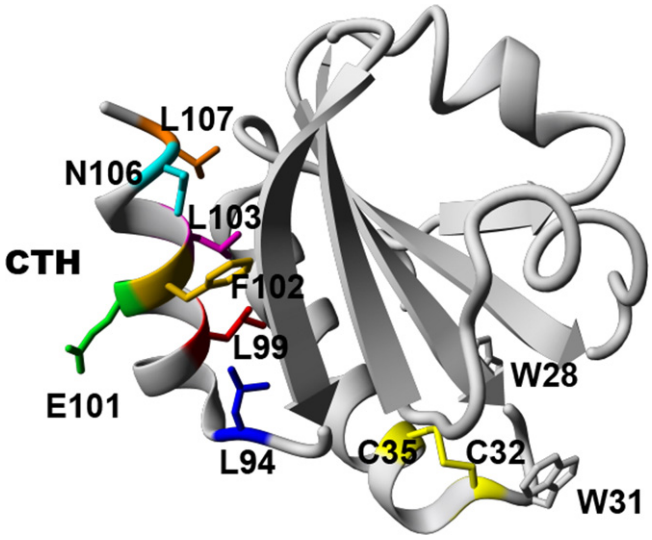

Fig. 1. Ribbon diagram of EcTRX. Residues that were mutated in this work are shown in sticks. The disulfide bridge (residues 31–35) is in yellow. In addition, the side chains of W28 and W31 are shown in gray. The model was generated with YASARA view software [84] and the PDB ID: 2TRX.

of Pro76. On the other hand, the unfolding of EcTRX may be described by a single exponential. The rate of unfolding for wild-type EcTRX in water, *i.e.*, extrapolated to zero urea concentration, was estimated as 9.5×10^{-8} s^{-1}, which yields a half-life time in water of around four months [31]. This fact supports the idea that EcTRX is also a kinetically stable protein [31].

In addition, there is invaluable thermodynamic and kinetic information for an extended list of EcTRX point mutants [13,14,26,31–33]. Godoy-Ruiz and coworkers have found, based on results from urea-induced folded/unfolded experiments, that there is a large fraction of residues that occupy unstructured regions in the EcTRX *TSE*, yielding a high energy barrier, presumably as the result of the evolution towards a highly kinetically stable conformation [31]. Remarkably, Hamid Wani and coworkers [34] showed that the unfolding of EcTRX includes an intermediate state when the reaction is performed at pH 3.0. They suggest that at pH 7.0, the energy of this intermediate state is too high for it to be detected. Thus, how the transition state ensembles (*TSE*) are organized seems to be an open question.

Experiments between different couples of EcTRX fragments point to the plasticity of the core of this protein [35–40] and to the relevance of C-terminal α-helix (CTH) on the consolidation of the native state [41,42]. Experiments involving fragment TRX1–93 showed that the absence of the CTH (residues 94–108) results in the stabilization of a premolten globule-like state, with only a residual secondary structure and without signatures of a persistent tertiary structure. In particular, complementation between fragment TRX1–93 and peptide TRX94–108 indicated that interactions mediated by apolar residues of CTH strongly contribute to the secondary structure propensity of this secondary structure element [43] and simultaneously stabilize the tertiary structure of EcTRX native-like complex [44]. Interestingly, it was suggested that the alteration of the C-terminal region of the protein may considerably affect folding kinetics [30,45], thus indicating that this region plays a key role, not only contributing to the native state stability, but also acting on the stabilization of *TSE*.

As previously described [41,43], helix α5 consists of a set of spatially aligned buried apolar residues L99, F102, L103 and L107 that establishes contact among them (apparently stabilizing the α-helical structure of the last 15 residues of the protein, as predicted by the AGADIR algorithm [46–49], Table S1) and also with residues of helix α3 and residues from the β-sheet (tertiary interactions). In particular, L99 is highly conserved and the apolar residues Ile, Leu and Val are very frequently found in position 103 along the TRX family (L103 in the *E. coli* sequence), suggestive of the key role these residues exhibit in the EcTRX structure (a sequence logo for the CTH is shown in Fig. S1). In addition, L94, a less conserved residue, is located in the connector between CTH and the last strand, establishing contacts with L99 that could also be relevant in contributing to the native stability of EcTRX. The side chain of residue N106 establishes an intra-helical hydrogen bond (HB) with the carbonyl oxygen of F102 and one tertiary hydrogen bond between the carbonyl oxygen of N106 and the amine group of K82. Finally, E101 is close in space to the charged groups of residues K96, K100, and D104 from the CTH. To evaluate the role in the unfolding mechanism of some interactions established by key residues of the CTH (helix α5) in the native state of EcTRX, a series of point mutants was designed (Fig. 1). We chose to mutate L94, L99, F102, L103, N106 and L107 to alanine, and E101 to glycine. We expected that these mutations would destabilize the native state of EcTRX relative to the unfolded state because of the truncation of the side chains forming stabilizing interactions. Mutation E101G should further destabilize the native state because of the higher entropic cost of restricting the conformation of a glycine to the α-helical region.

In this work we investigated experimentally and computationally the role of CTH in the EcTRX unfolding mechanism. Based on the observation and analysis of non-linear rate profiles (kinks or rollovers) in the unfolding arm of EcTRX *chevron plot* [50,51], equilibrium unfolding experiments and structure-based simulation results, we suggest that the EcTRX folding/unfolding mechanism includes an on-pathway high-energy intermediate state. In addition, we characterized the involvement of CTH in the energetics of the transition-state ensembles. Finally, based on molecular dynamics simulation (MDS) results, we described the folding landscape and the conformational ensembles across the folding process.

2. Materials and methods

2.1. Subcloning, expression and purification of EcTRX mutants

EcTRX mutants were obtained by PCR-based site-directed mutagenesis. Expression and purification were carried out as previously described in Santos et al. [41,44]. Briefly, transformed *E. coli* BL21 (DE3) cells were grown in a Luria–Bertani medium at 37 °C to $OD_{600nm} = 0.9–1.0$. Finally, overexpression of each EcTRX variant (3 h, 37 °C, 250 rpm) was induced using 1.0 mM IPTG. For the protein purification, cells were disrupted in 20 mM Tris–HCl, pH 7.0, and centrifuged at 6000 rpm. The isolated fluid was loaded onto a DE52 Sepharose column equilibrated with 20 mM Tris–HCl, pH 7.0. Elution was performed with increasing concentrations of NaCl up to 1.0 M. Fractions containing EcTRX (evaluated by SDS-PAGE and UV absorption) were loaded onto a preparative Sephadex G-100 chromatography (SEC, 93 cm × 2.7 cm) column, previously equilibrated with 10 mM Tris–HCl and 100 mM NaCl, pH 7.0. Then, pure EcTRX fractions were pooled and extensively dialyzed against distilled water. Finally, the protein was lyophilized and preserved at −20 °C.

2.2. Mass spectrometry

Protein samples were analyzed by RP-HPLC–MS using a 1.0 mm × 30 mm Vydac C8 column, operating at 40 μL min^{-1}, connected to a Surveyor HPLC System on-line with an LCQ Duo (ESI ion trap) mass spectrometer (Thermo Fisher, San José, CA, USA). Samples were eluted using a 15 min gradient from 10% to 100% solvent B (solvent A: 2% acetic acid, 2% ACN; solvent B: 2% acetic acid, 96% ACN). Protein characterization was performed by full scan 300–2000 amu and deconvoluted by the XCalibur software.