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Conformational Exchange Is Critical for the Productivity of an Oxidative Folding Intermediate with Buried Free Cysteines

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Much has been learned about the folding of proteins from comparative studies of the folding of proteins that are related in sequence and structure. Observation of the effects of mutations helps account for sequence-specific properties and large variations in folding rates observed in homologous proteins, which are not explained by structure-derived descriptions. The folding kinetics of variants of a β-stranded protein, toxin α from *Naja nigricollis*, depends on the length of their loop lk1. These proteins, named Tox60, Tox61, and Tox62, contain four disulfide bonds. We show that their oxidative refolding pathways are similar. Differences in these pathways are restricted to the last step of the reaction, that is, the closure of the last disulfide. At this step, two species of three-disulfide intermediates are observed: intermediate C lacking the B3 disulfide and intermediate D lacking the B2 disulfide. Surprisingly, D is the most productive intermediate for Tox61 despite the low accessibility of its free cysteines. However, in the case of Tox62, its conversion efficiency drops by 2 orders of magnitude and C becomes the most productive intermediate. NMR was used in order to study the structural dynamics of each of these intermediates. Both three-disulfide intermediates of Tox61 exist in two forms, exchanging on the 1- to 100-ms scale. One of these forms is structurally very close to the native Tox61, whereas the other is always significantly more flexible on a picosecond-to-nanosecond timescale. On the other hand, in the case of Tox62, the three-disulfide intermediates only show a native-like structure. The higher conformational heterogeneity of Tox61 intermediate D allows an increased accessibility of its free cysteines to oxidative agents, which explains its faster native disulfide formation. Thus, residue deletion in loop lk1 probably abrogates stabilizing intramolecular interactions, creates conformational heterogeneity, and increases the folding rate of Tox60 and Tox61 compared to Tox62.

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Abbreviations used: 2D, two-dimensional; 3D, three-dimensional; COSY, correlation spectroscopy; EDTA, ethylenediaminetetraacetic acid; GSH, reduced glutathione; HSQC, heteronuclear single quantum coherence; NOE, nuclear Overhauser enhancement; NOESY, nuclear Overhauser enhancement spectroscopy; PDB, Protein Data Bank; ROESY, rotating-frame Overhauser enhancement spectroscopy; TFA, trifluoroacetic acid; TOCSY, total correlation spectroscopy.

Introduction

Understanding the relationship between the amino acid sequence and the three-dimensional (3D) structure of a native protein is a fundamental challenge with important practical applications. 3D structure prediction from its sequence would open the way to rapid interpretation of the wealth of genomic data obtained from large-scale sequencing. Such knowledge would also help optimize recombinant protein techniques rationally.[1,2] Even if great progress has been made regarding 3D structure prediction,[3,4] the kinetics by which a protein reaches its native state remains unclear. It is now widely accepted that protein folds through several possible pathways that define the folding landscape.[5] While some proteins have a smooth landscape, suggesting a two-state mechanism, others are better defined by a rugged terrain that betrays the existence of metastable intermediates along their folding pathways.[6] This is particularly true for disulfide-containing proteins. Oxidative folding pathways for many such proteins have been accurately described.[7–10] The reaction produces a large number of intermediates containing one or more disulfides, native or not. The folding landscape then gets continuously simpler by oxidation of cysteines and/or disulfide exchange until the native state is arrived at.[11] In most cases, closure of the last disulfide bridge is the rate-limiting step.[12]

Structures of several disulfide intermediates have been solved in order to highlight the molecular details of their oxidative folding kinetics.[13–18] These structures display a much more native-like structure than expected,[12,19,20] which raises the following question: why do native conformations with spatially closed free cysteines display such a poor ability to form their lacking disulfide bridges? The most accepted hypothesis suggests that the free cysteines find themselves buried inside a hydrophobic protein core with poor access to oxidative agents.[21] It is assumed that these "locking-in" intermediates have to be shuffled into productive ones that, in turn, could be oxidized into the native form. In this regard, their structural fluctuations should expose their disulfide bonds in concert with their thiol groups, leading to reshuffling rather than oxidation. By contrast, structural fluctuations of productive intermediates should expose the thiol groups while keeping the disulfide bonds buried. Analysis of the dynamic behavior of oxidative folding intermediates would supply valuable information on the structural mechanisms involved in the oxidation of disulfide bridges. An extensive analysis of the dynamics of the conformational ensemble of an early single-disulfide variant of BPTI was carried out,[22] and more recently, the dynamics of the wild-type onconase and its three-disulfide intermediate mutated in the folding initiation site was described.[23] In this article, we report the NMR study of the dynamics of late-folding intermediates of three homologous small disulfide-rich toxins with very different folding rates, in order to describe the final and rate-limiting step of their oxidative folding.

Toxin α from *Naja nigricollis* belongs to the family of curaremimetic snake toxins and behaves as a potent inhibitor of muscular nicotinic acetylcholine receptors. Its 3D structure exhibits three adjacent loops emerging from a hydrophobic palm, which includes four disulfide bonds (Fig. 1). Such a fold is shared by a large family of snake toxins called the three-finger protein family. In a previous work, the oxidative folding kinetic rates of nine snake neurotoxins belonging to this family were determined, showing that the longer the loop between β1 and β2 (lk1) was, the slower the secondary structures appeared.[24] As these molecules exhibit several differences throughout their sequences, a direct correlation between the length of this loop and the folding rate could not be demonstrated. In order to test this hypothesis, Ruoppolo *et al.* produced mutants of toxin α containing four to six residues in the loop between β1 and β2: Tox60, Tox61 (wt), and Tox62, respectively.[25] They confirmed that the

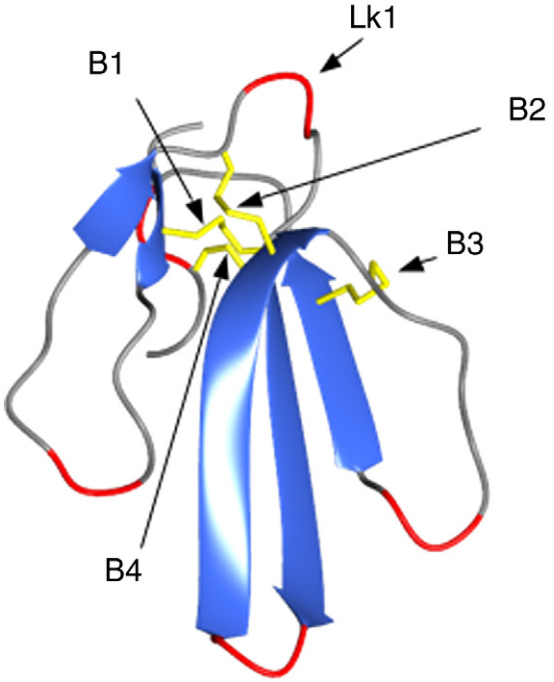

Fig. 1. Ribbon structure of toxin α from *N. nigricollis* (PDB ID: 1IQ9), showing structural determinants of three-fingered proteins. β-Strands are in blue, β-turns are in red, and disulfide bridges are in yellow. B1 to B4 correspond to Cys3–23, Cys17–40, Cys42–53, and Cys54–59 bridges, respectively. Lk1 indicates the turn joining the first and second loops.