doi:10.1016/j.jmb.2006.08.047 J. Mol. Biol. (2006) 363, 958–976

JMB

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Identification of a Collapsed Intermediate with Non-native Long-range Interactions on the Folding Pathway of a Pair of Fyn SH3 Domain Mutants by NMR Relaxation Dispersion Spectroscopy

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Recent ^{15}N and ^{13}C spin-relaxation dispersion studies of fast-folding mutants of the Fyn SH3 domain have established that folding proceeds through a low-populated on-pathway intermediate (I) where the central β-sheet is at least partially formed, but without interactions between the NH_2- and COOH-terminal β-strands that exist in the folded state (F). Initial studies focused on mutants where Gly48 is replaced; in an effort to establish whether this intermediate is a general feature of Fyn SH3 folding a series of ^{15}N relaxation experiments monitoring the folding of Fyn SH3 mutants N53P/V55L and A39V/N53P/V55L are reported here. For these mutants as well, folding proceeds through an on-pathway intermediate with similar features to those observed for G48M and G48V Fyn SH3 domains. However, the ^{15}N chemical shifts extracted for the intermediate indicate pronounced non-native contacts between the NH_2 and COOH-terminal regions not observed previously. The kinetic parameters extracted for the folding of A39V/N53P/V55L Fyn SH3 from the three-state folding model F↔I↔U are in good agreement with folding and unfolding rates extrapolated to zero denaturant obtained from stopped-flow experiments analyzed in terms of a simplified two-state folding reaction. The folding of the triple mutant was studied over a wide range of temperatures, establishing that there is no difference in heat capacities between F and I states. This confirms a compact folding intermediate structure, which is supported by the ^{15}N chemical shifts of the I state extracted from the dispersion data. The temperature-dependent relaxation data simplifies data analysis because at low temperatures (<25 °C) the unfolded state (U) is negligibly populated relative to I and F. A comparison between parameters extracted at low temperatures where the F↔I exchange model is appropriate with those from the more complex, three-state model at higher temperatures has been used to validate the protocol for analysis of three-site exchange relaxation data.

Keywords: chemical exchange; CPMG NMR relaxation dispersion; Fyn SH3 domain; protein folding; stopped-flow kinetics

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Abbreviations used: CPMG, Carr-Purcell-Meiboom-Gill; HSQC/HMQC, heteronuclear single/multiple quantum coherence; RMSD, root-mean-square deviation; SH3, Src homology 3; TPPI, time-proportional phase incrementation; NOESY, nuclear Overhauser enhancement spectroscopy; TOCSY, total correlated spectroscopy.

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Introduction

An understanding of the process(es) by which a protein folds into its native conformation requires knowledge of the various states that populate its folding pathway(s), their relative stabilities, the kinetics of interconversion between them, as well as their structural properties. In practice such information can be difficult to obtain. Many, if not all, intermediates are populated at very low levels and are transient so that state-of-the-art biophysical techniques that provide detailed information about folded proteins, for example, become less useful. As a result information about these "elusive" excited states is often obtained through indirect studies that invoke changes in variables such as temperature, pressure, denaturant concentration or perturbations in the primary amino acid sequence.[1] One group of proteins whose folding properties have been studied in detail *via* the approaches summarized above is the SH3 domain family.[2–11] SH3 domains are small modules comprised of approximately 60 residues that fold into a five-strand β-sandwich structure[12] (Figure 1). As in the case of many other small protein moieties, folding studies of SH3 domains involving calorimetric, equilibrium and kinetic folding/unfolding experiments are consistent with a two-state folding transition, with little evidence for the formation of partially folded intermediates (reviewed by Capaldi & Radford).[13]

Traditionally, many of the techniques that have been used to study protein folding are based on the use of a single probe or report a single average property that involves many or all residues in the molecule. It is thus difficult to obtain site-specific information over a wide range of sites that can be necessary to detect intermediates in the first place or that is necessary to obtain a complete description of

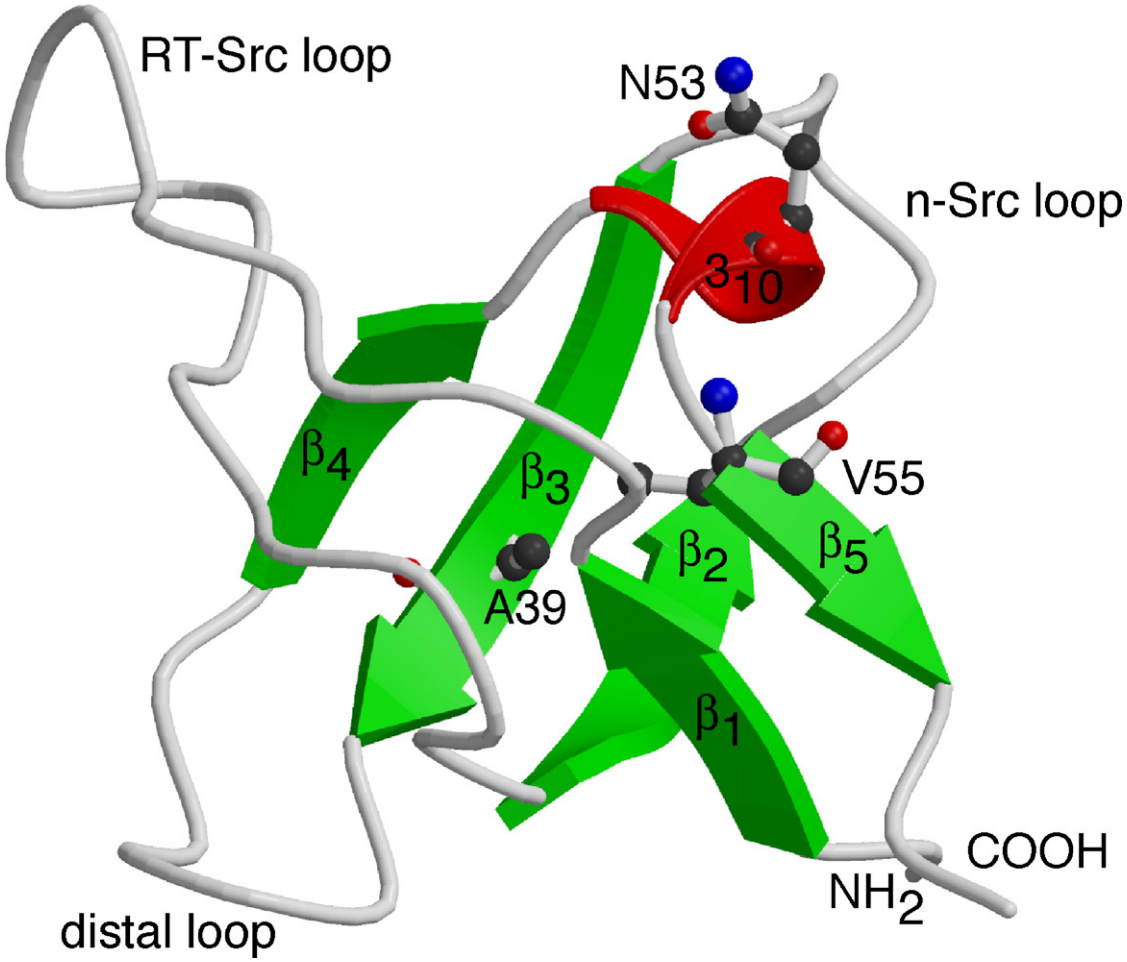

Figure 1. Schematic representation of the secondary structure of the major conformation (SH3-1) of the wild-type *Homo sapiens* Fyn SH3 domain,[12] featuring the characteristic SH3 domain β-sandwich fold formed by the terminal (strands β_1 from Leu3 to Ala6 and β_5 from Val55 to Pro57) and the approximately orthogonal central (strands β_2 from Asp25 to Asn30, β_3 from Trp36 to Ser41, and β_4 from Thr47 to Ile50) β-sheets, along with a helical turn with 3_{10} geometry from Pro51 to Tyr54. The second conformation (SH3-2), which differs in the structure of the n-Src loop region (Leu29 to Asp35), was not found to be significantly populated in solution.[14] The only sequence differences between the WT *H. sapiens* SH3 domain and the WT *Gallus gallus* Fyn SH3 domains studied here are a valine to glutamate (*G. gallus*) substitution at position 5. The residues Ala39, Asn53, and Val55 mutated in this study are shown in ball-and-stick representation. The Figure was drawn with MolScript 2.1.2[51] and rendered with Raster3D 2.7.[52]