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Structure Report

3D domain swapping in a chimeric c-Src SH3 domain takes place through two hinge loops

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ABSTRACT

In the Src Homology 3 domain (SH3) the RT and n-Src loops form a pocket that accounts for the specificity and affinity in binding of proline rich motifs (PRMs), while the distal and diverging turns play a key role in the folding of the protein. We have solved the structure of a chimeric mutant c-Src-SH3 domain where specific residues at the RT- and n-Src-loops have been replaced by those present in the corresponding Abl-SH3 domain. Crystals of the chimeric protein show a single molecule in the asymmetric unit, which appears in an unfolded-like structure that upon generation of the symmetry related molecules reveals the presence of a domain swapped dimer where both, RT- and n-Src loops, act as hinge loops. In contrast, the fold of the diverging type II β-turn and the distal loop are well conserved. Our results are the first evidence for the presence of a structured diverging type II β-turn in an unfolded-like intermediate of the c-Src-SH3 domain, which can be stabilized by interactions from the β-strands of the same polypeptide chain or from a neighboring one. Futhermore, this crystallographic structure opens a unique opportunity to study the effect of the amino acid sequence of the hinge loops on the 3D domain swapping process of c-Src-SH3.

1. Introduction

Since the first studies performed by Anfinsen on ribonuclease (Anfinsen et al., 1961) the literature is full of studies on how the protein sequence may drive the native structure of proteins, the local conformational behavior and mediates the hydrophobobic effect, and other intramolecular interacions (Wako and Blundell, 1994). It is also well known that the β-strand by itself is a much less stable secondary structure element than the α-helix due to the absence of intrinsic hydrogen bonding on its formation. This extended conformation of the polypeptide chain is only stable when incorporated into a β-sheet, where hydrogen bonds are formed between peptide groups in adjacent β-strands. Interestingly, β-strands are common in amyloid like structures, which are an apparent violation of the one fold per sequence paradigm as stated in Anfinsen's thermodynamic hypothesis (Greenwald and Riek, 2012).

3D domain swapping is a phenomenon that occurs when two or more molecules of a partially unfolded protomer form homodimers, or higher-order oligomers, by exchanging protein domains (Jaskolski, 2001; Rousseau et al., 2012). So far, over 50 proteins have been documented to undergo 3D domain swapping (Jaskolski, 2012), but the diversity of their sequences and secondary structures suggests that almost any protein may oligomerize via this mechanism. In this way, the swapped-oligomers appear as an alternative way of folding of the protomer, which could be driven by a lower energy of the self-associated species, as compared with the single folded chain, or alternatively, by the faster formation of the oligomer and its later stabilization. The energetic of any folded globular protein in its native state is essentially kept by a combination of entropic and enthalpic contributions where most of the hydrophobic residues are buried in the protein core and polar residues are mainly located on the surface. When a partially unfolded protomer exposes part of its hydrophobic core to the solvent, the formation of the swapped-oligomer may take place. Most of the new swap domain-core interactions are already present in the hydrophobic core of the protomer and, in most cases, the secondary structure elements forming it are conserved (Yang et al., 2005). On the other hand, major changes are observed in the hinge loops, which are exposed to the solvent and composed mainly by polar residues. The reason why

Abbreviations: c-Src-SH3, Src-homology 3 domain of c-Src tyrosine kinase; Abl-SH3, Src-homology 3 domain of the Abl tyrosine kinase; DLS, dynamic light scattering; PRM, proline rich motif.

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these loops change their preferred conformation to an open-like conformation to allow the 3D domain swapping remains unclear.

Src-homology region 3 (SH3) domains are protein modules of about 60 residues that mediate interaction with proline-rich motifs (PRMs). The overall structure of these domains is defined by a β-barrel sandwich, which consists of five β-strands arranged as two tightly packed anti-parallel β sheets. The loops connecting strands β1–β2, β2–β3 and β3–β4 are referred to as the RT loop, the n-Src loop and the distal loop, respectively; strands β4–β5 are separated by a 3_{10} helix. A structural comparison of different SH3 domains reveals that the hydrophobic core of these domains is highly conserved. However, the sequences and conformations of their loops show a large variability, which is related with the affinity and specifity of each different SH3 domain. As way of example, the c-Src-SH3 and the Abl-SH3 domains share a 42% sequence similarity, with the most significant differences occurring in the loop regions. In the Src and family-related tyrosine kinases, the binding of PRMs is driven by the presence of a negatively charged residue at the RT-loop which interacts with a positively charged residue flanking the consensus motif PxxP (Feng et al., 1995). This is not the case in the Abl-SH3 domain, where an exhaustive analysis indicates that this domain prefers a flanking sequence without charged residues (Pisabarro and Serrano, 1996).

To date only four SH3 domains have been described to form domain-swapped oligomers: Eps8 (Kishan et al., 1997), p47phox (Yuzawa et al., 2004), the c-terminal SH3 domain of CRKL (Harkiolaki et al., 2006) and Gln128Arg mutant of the c-Src-SH3 (Camara-Artigas et al., 2009). The 3D domain swapping in p47phox-SH3 takes place through a conformational change in the distal loop. In the case of Eps8-SH3 and c-Src-SH3, the n-Src loop acts as a hinge region, although their intertwined dimers show different orientations between the swapped chains. However, the c-Src-SH3 domain is especially interesting as subject of study because molecular dynamics analysis predict the presence of an intertwined dimer as a precursor of amyloid formation (Ding et al., 2002). Interestingly, these studies were performed before the crystallographic structure of the intertwined dimer of the Gln128Arg mutant of this protein was obtained.

Here we describe the crystallographic structure of a chimeric c-Src-SH3 domain, c-Src-[RT/n-Src-Abl]-SH3 domain (SRC-2X). In this construct, residues Glu93, Ser94, Arg95 and Thr96 in the RT loop of c-Src-SH3 domain were replaced by Val, Ala, Ser and Gly as in the Abl-SH3 domain, and residues Asn112, Asn113, Thr114 and Glu115 in the n-Src loop of c-Src-SH3 were replaced by Gly, Tyr, Asn and His as in the Abl-SH3 domain. The initial purpose of this chimera design was to examine whether the binding determinants of the c-Src-SH3 domain can be exchanged by those in the Abl-SH3 domain by changing the residues involved in the binding at the RT and n-Src loop. Unexpectedly, this chimera crystallizes as a domain swapped dimer where both, RT and n-Src loops, act as hinge loops. The swapped element is an internal loop and this type of domain swapping falls in the "loop swap" subcategory (Janowski et al., 2001; Zerovnik et al., 2011). In this work, we have performed a structural analysis of the chimeric SH3 domain to better understand the principles which drive the formation of intertwined dimers in the c-Src-SH3 domain.

2. Materials and methods

2.1. Cloning, expression and purification of chicken c-Src-SH3 domain

The plasmids containing the WT c-Src-SH3 and the chimeric SRC-2X domains were generous gifts from Dr. E. Freire (Johns Hopkins University, USA) and Dr. I. Luque (Granada University, Spain), respectively. The chimeric mutant SRC-2X was created using the pET15b plasmid containing the chicken c-Src-SH3 domain gene as a template. In this multiple mutant, residues Glu93, Ser94, Arg95 and Thr96 in the RT loop of c-Src-SH3 domain were replaced by Val, Ala, Ser and Gly as in the Abl-SH3 domain, and residues Asn112, Asn113, Thr114 and Glu115 in the n-Src loop of c-Src-SH3 were replaced by Gly, Tyr, Asn and His as in the Abl-SH3 domain. The gene coding for SRC-2X with an N-terminal 6x-His-tag and an engineered thrombin cleavage site was overexpressed using the *Escherichia coli* BL21 (DE3) strain (Novagen). The protein was purified following a standard protocol. Briefly, harvested cells were suspended in 50 mM sodium phosphate pH 8.0, 300 mM sodium chloride (column buffer: CB) and then disrupted using a French press cell. Cell lysate was centrifuged at 36,200×*g* for 60 min at 4 °C. The supernatant was loaded on a 5 mL of Ni–NTA resin (Clontech Lab. Inc.) previously equilibrated with 5 column volumes of CB and subsequently washed with CB, CB + 20 mM imidazole, and CB + 50 mM imidazole. SRC-2X was finally eluted with CB containing 500 mM imidazole. In order to remove the imidazole from the solution, the fractions containing the protein were combined and dialyzed extensively against 50 mM sodium phosphate pH 8.0, 300 mM sodium chloride. Protein was then subjected to a limited proteolysis stage using thrombin in an approximate ratio of 50:1 at room temperature for 4 h to remove the 6x-His-tag from the N-terminus. In a second Ni affinity chromatography step, SRC-2X without tag was eluted in the absence of imidazole. The protein was further purified by size exclusion chromatography (SEC) using a Superdex-75 gel filtration column (GE Healthcare) previously equilibrated in the same dialysis buffer as above. The major elution peak was collected and concentrated up to 2–5 $mg{\cdot}mL^{-1}$ and stored at −80 °C. All eluted fractions from both purification steps were analyzed for purity by SDS–PAGE. Protein concentration was determined by absorbance at 280 nm using an extinction coefficient of 16,500 $M^{-1}\,cm^{-1}$, calculated using the Gill and von Hippel's equation (Gill and von Hippel, 1989).

2.2. Dynamic Light Scattering (DLS)

DLS experiments were performed in a Zetasizer nano instrument (Malvern Instrument Ltd, United Kingdom) and the data were analyzed with Zetasizer software (Malvern Instrument Ltd, United Kingdom). The samples at a protein concentration between 0.1 and 25 $mg{\cdot}mL^{-1}$ were introduced in a 12 μL quartz thermostated sample cuvette (low-volume quartz batch cuvette, Malvern). DLS measurements were performed in 100 mM sodium acetate (pH 4.0) at 25 °C. This pH was selected to reproduce the conditions used to obtain the protein crystals. The measurements were performed after 120 s of thermal equilibration, using 9 runs per measurement, 10 s per run, with 4 s delay between each measurement. Each DLS reported value is the average of 15 measurements. Before performing the measurements, all the protein samples were filtered through 0.2 μm IC Millex-LG (Millipore) filters and centrifuged for 45 min at 20,000×*g*, 4 °C. Immediately before measurements, the protein solutions were sonicated for 1 min to remove air bubbles.

2.3. Protein crystallization

Crystals of the chimeric SRC-2X mutant were obtained by the vapor diffusion technique using a hanging drop setup. Briefly, droplets were prepared by mixing 3 μL of chimeric protein (10 $mg{\cdot}mL^{-1}$ in 10 mM Tris buffer pH 8.0) with 3 μL of reservoir solution. The mixture was vapor equilibrated against 1 mL of reservoir solution. The best crystals were grown in 1.5 M ammonium sulfate, 10% glycerol, 5% PEG 300, 0.01 M non-detergent sulfobetaine 201 (NDBS-201), 0.1 M sodium acetate pH 4.0. Crystals typically appear within 2 weeks. For X-ray data collection, the crystals