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Design of tryptophan-containing mutants of the symmetrical Pizza protein for biophysical studies

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ABSTRACT

β-propeller proteins are highly symmetrical, being composed of a repeated motif with four anti-parallel β-sheets arranged around a central axis. Recently we designed the first completely symmetrical β-propeller protein, Pizza6, consisting of six identical tandem repeats. Pizza6 is expected to prove a useful building block for bionanotechnology, and also a tool to investigate the folding and evolution of β-propeller proteins. Folding studies are made difficult by the high stability and the lack of buried Trp residues to act as monitor fluorophores, so we have designed and characterized several Trp-containing Pizza6 derivatives. In total four proteins were designed, of which three could be purified and characterized. Crystal structures confirm these mutant proteins maintain the expected structure, and a clear redshift of Trp fluorescence emission could be observed upon denaturation. Among the derivative proteins, Pizza6-AYW appears to be the most suitable model protein for future folding/unfolding kinetics studies as it has a comparable stability as natural β-propeller proteins.

1. Introduction

The β-propeller domain is one of the most common protein architectures observed in nature; it is frequently involved in mediating protein-protein interactions, but also found among enzymes [1,2]. The number of blades in a single domain varies from 4 to 12, with 7-bladed protein being most commonly observed [3]. Each blade consists of four antiparallel β-strands. The domain is typically stabilized by a so-called velcro-strap in which the N-terminal residues complete the C-terminal blade. β-propellers are thought to have arisen from smaller fragments via duplication and fusion events, to create highly symmetrical evolutionary intermediates. Genetic drift, and the development of particular functions under evolutionary pressure, subsequently broke the sequence symmetry, so that the overall symmetry of these ancestral forms is more strongly reflected by present-day β-propeller protein structures than amino acid sequences. In 2014, we reverse-engineered this process to create the perfectly 6-fold symmetrical protein Pizza6 using sequential and structural information of the extracellular domain of *Mycobacterium tuberculosis* PknD (1RWL) [4,5]. Expression of derivatives with a different number of tandem repeats ranging from 2 to 10 repeats resulted in proteins that reassemble into oligomeric proteins with a fixed number of monomers. This controllable self-assembly is highly promising for the development of protein building blocks for bionanotechnology. The first application of Pizza protein was the redesign of the protein to biomineralize the smallest nanocrystal reported to date consisting of 7 Cd^{2+} and 12 Cl^{-} ions between two Pizza proteins, showing a high future potential for symmetric designer proteins to template nanoparticles [6,7].

Another application of symmetric monomeric proteins may be to study protein evolution and folding. To date, very few folding studies of β-propeller proteins have been reported [8]. Pizza proteins are a highly suitable model protein to observe folding processes in propeller proteins generally, as the internal symmetry eliminates the amino acid complexity. The group of Blaber has utilized a symmetrical designer protein named Symfoil, derived from FGF1, to investigate the structure evolution and folding kinetics of the trefoil fold [9–11]. Commonly-used methods for the study of protein folding and unfolding kinetics include circular

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dichroism (CD), nuclear magnetic resonance (NMR) and fluorescence spectroscopy [12–16]. Following the red shift of tryptophan fluorescence emission (λ_{max} increasing from about 330 nm to near 350 nm) upon unfolding is a popular technique because it is highly sensitive to the environmental changes of the probe side-chain. It offers a high signal-to-noise ratio at low concentration, and tryptophan is generally buried, so its environment reflects the overall condition of the protein [15]. The current Pizza proteins however do not contain tryptophan, and are too stable to unfold except under extremely harsh conditions. Therefore, we set out to design symmetrical Pizza mutants that contain buried tryptophan residues, and which exhibit stability similar to natural β-propeller proteins.

In this paper, we report the design and validation of several tryptophan-containing Pizza proteins. The crystal structures and unfolding analyses of these proteins are presented.

2. Materials and methods

2.1. Protein preparation

The genes for each of the tryptophan containing derivatives Pizza6-AYW/SYW/AFW/SFW were ordered as a synthetic DNA fragment (gBlocks, IDT) and cloned into the pET28b (Novagen) vector using the *Nde*I and *Xho*I sites, such that the expressed protein carries an N-terminal histidine tag removable by thrombin. All plasmids were transformed into *E. coli* BL21 (DE3) cells. They were expressed and purified as described [4]. The purified protein was judged to be over 95% pure by SDS-PAGE. Size exclusion column (SEC) purification was performed by Superdex200 increase 10/300 GL column (GE Healthcare) with a 20 mM HEPES pH 8.0 and 200 mM NaCl buffer.

2.2. Crystallization

All protein samples were dialyzed against 20 mM HEPES pH 8.0, concentrated to 10 mg/mL and subjected to crystal screening in sitting-drop 96-well plates using sparse matrix kits (Hampton) at 18 °C. Crystallization conditions were optimized using the hanging drop vapour diffusion method. The Pizza6-AYW crystals were grown using 0.1 M MMT (DL-malic acid:MES:Tris base at 1:2:2 ratio) pH 5.0, 30% (w/v) PEG1500. The Pizza6-SYW crystals were grown using 0.2 M calcium chloride, 15% (w/v) PEG6000, and 0.1 M sodium acetate pH 5.0. The Pizza6-SFW crystals were grown using 0.25 M calcium chloride 15% (w/v) PEG6000, and 0.1 M MES pH 6.0. All crystals were flash frozen using 10–25% glycerol as cryo-protectant.

2.3. Data collection and structure determination

All X-ray diffraction data were collected at the XRD1 beamline of the Elettra synchrotron Trieste (Trieste, Italy) and I04 beam-line of the Diamond Light Source (Oxfordshire, UK) using a DECTRIS PILATUS 2 M and a DECTRIS PILATUS 6M-F detector. All diffraction data were processed with XDS and AIMLESS (CCP4 Software Suite) [17–19]. All structures were determined by molecular replacement with the program PHASER [20] using the original Pizza6 structure (PDB entry 3WW9). Refinement was carried out manually by COOT and PHENIX.REFINE [21,22]. The final structures were validated with MolProbity [23]. Data collection and refinement statistics are shown in Supp. Table 1. The coordinates and X-ray data are deposited with entry codes 6F0Q (Pizza6-AYW), 6F0S (Pizza6-SYW), and 6F0T (Pizza6-SFW).

2.4. Circular dichroism spectroscopy

CD-spectroscopy was performed using a JASCO J-1500 instrument. Protein samples were diluted to 0.1 mg/mL in 20 mM phosphate buffer at pH 7.6 using a 1 mm path quartz cuvette at 20 °C. Protein melting was followed by monitoring the absorption at 218 nm over 0.2 °C steps between 0 and 95 °C using a 2 mm quartz cuvette at a concentration of 0.25 mg/mL protein in the same buffer.

2.5. Tryptophan fluorescence measurements

Tryptophan fluorescence measurements were performed in 96-well microplates (Greiner) on a Safire2 (TECAN). To measure folding and unfolding, the proteins were diluted to 50 μM using 20 mM HEPES buffer at pH 8.0 at 4 °C (folding) or 6 M guanidium chloride (GdnHCl) at room temperature and incubated overnight.

2.6. Isothermal equilibrium denaturation (IED)

IED experiments with GdnHCl were carried out using CD and Trp fluorescence. Protein samples with OD^{280} of 0.3 were equilibrated over 3 days at 20 °C against 20 mM phosphate buffer at pH 7.6 containing different concentrations of GdnHCl. GdnHCl concentration was increased in steps of 0.2 M between buffers. CD was measured at 218 nm using a 1 mm path quartz cuvette holding 0.3 mg/mL protein samples. Trp-containing Pizza proteins were also measured with Trp fluorescence using a plate reader (Tecan, Safire2). 280 nm was used as excitation wavelength and emission was collected at 330 and 350 nm. The data were fitted to a two-state model (folded/unfolded) [24] with six parameters; Gibbs free energy difference (ΔG°), sensitivity towards denaturation (m), the intercepts and the slopes of the folded (F) and unfolded (U) transition baselines (γ^0_F, γ^0_U, m_F, m_U) for all proteins.

3. Results

3.1. Designing Trp mutants on Pizza protein

To identify a suitable location for introducing buried Trp residues, all 40 residues of the tandem repeat motif were sequentially mutated *in silico*. The model employed, called Pizza6-S, is the original Pizza6 structure (PDB 3WW9) with an additional N16S mutation in every repeat. Only two internal residues in the Pizza blade can be replaced by Trp without causing severe steric hindrance, Phe 9 and Tyr 27. Several surface residues can be replaced with Trp, but were not selected since they are unlikely to result in a large shift of the Trp fluorescence signal upon unfolding of the protein. Mutants were created in which either all the Phe or all the Tyr residues of Pizza6-S were replaced with Trp, giving Pizza6-AFW and Pizza6-AYW respectively. Additional mutants were made in which the Phe or Tyr residue of only every second domain was mutated, and called Pizza6-SFW and Pizza6-SYW respectively (Fig. 1).

3.2. Protein expression and purification

The AYW, SYW and SFW Pizza6 variants all expressed well and could be purified readily with yields over 40 mg/L culture, similar to the original Pizza6 protein (Supp. Figure 1A) [4,6]. However, Pizza6-AFW formed inclusion bodies and was not purified. The SEC and CD results indicated that the soluble proteins were monomeric and correctly folded (Supp.Figure.1B, Fig. 2A). The natural protein used as a template in the design of Pizza, an extracellular domain of *Mycobacterium tuberculosis* PknD (PDB 1RWL) was also purified and found to be monodisperse in solution (Supp Figure 1).

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