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A conserved tryptophan (W91) at the barrel-lid junction modulates the packing and stability of Kunitz (STI) family of inhibitors

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ABSTRACT

β-trefoil fold, consisting of a six stranded β-barrel capped at one end by a lid comprising of another six β-strands, is one of the most important folds among proteins. Important classes of proteins like Interleukins (ILs), Fibroblast Growth Factors (FGFs), Kunitz (STI) family of inhibitors etc. belong to this fold. Their core is packed by hydrophobic residues contributed by the 6 stranded β-barrel and three β-hairpins that make essential contacts with each other and keep the protein in 'topologically minimal frustrated state'. A complete database analysis of the core residues of the β-trefoil fold proteins presented here identified a conserved tryptophan (W91) residue in the Kunitz (STI) family of inhibitors that projects from the lid and interacts with the bottom layer residues of the barrel. This kind of interactions is unique in Kunitz (STI) family because no other families of β-trefoil fold have such a shear sized residue at the barrel lid junction; suggesting its possible importance in packing and stability. We took WCI as a representative of this family and prepared four cavity creating mutants W91F-WCI, W91M-WCI, W91I-WCI & W91A-WCI. CD experiments show that the secondary structure of the mutants remains indistinguishable with the wild type. Crystal structures of the mutants W91F-WCI, W91M-WCI & W91A-WCI also show the same feature. However, slight readjustments of the side chains around the site of mutation have been observed so as to minimize the cavity created due to mutation. Comparative stability of these mutants, estimated using heat denaturation CD spectroscopy, indicates that stability of the mutants inversely correlates with the size of the cavity inside the core. Interestingly, although we mutated at the core, mutants show varying susceptibility against tryptic digestion that grossly follow their instability determined by CD. Our findings suggest that the W91 residue plays an important role in determining the stability and packing of the core of WCI.

1. Introduction

Structural studies with globular proteins have identified an enormous number of uniquely different proteins that are classified under one of the ten fundamental folds [1]. The majority of these superfolds contain a highly symmetric tertiary structure; postulated to evolve via gene duplication and fusion events [2]. β-trefoil fold represents a distinct and important class of superfold consisting of proteins having a wide spectrum of functionalities, which include human fibroblast growth factors, interleukins-1, clostridium neurotoxins, mannose receptors, Kunitz (STI) protease inhibitors, etc. [3–6]. A detailed study of the geometry and architecture of the β-trefoil fold was done by Chothia and his co-workers [7] indicating that they have an all-β structure with structural architecture comprising of three repeating units (Supplementary Fig. 1). Each repeating unit is made up of four β strands with connecting loops of varying size that stack together to form a six stranded β-barrel and a lid composed of three β-hairpins that cap one hollow end of the barrel (Supplementary Fig. 1) [7]. Despite the retention of symmetric tertiary structure, the proteins of β-trefoil family have diverged considerably in terms of their primary sequence [8]. The presence of a pseudo three-fold symmetry suggests that this fold has arisen from the triplication and evolution of gene from one trefoil [9]. Feng et al. have shown that proteins belonging to β-trefoil fold have certain conserved residues which are distributed symmetrically in the structure and explained how these symmetric structures have emerged from apparently asymmetric sequences [10], which is important to understand the evolution and folding pathway of these beta-trefoil fold proteins.

The packing of the core of the β-trefoil fold proteins drew considerable attention in terms of their stability and folding. The 'core' of the barrel is packed by 15–18 hydrophobic residues, contributed by the six stranded β-barrel and three β-hairpins, which make essential contacts with each other and keep the protein in 'topologically minimal frustrated state' [11]. Packing of these hydrophobic residues leaves

Abbreviations: WCI, winged bean chymotrypsin inhibitor; ETI, *Erythrina caffra* trypsin inhibitor; STI, soybean trypsin inhibitor; BPTI, bovine pancreatic trypsin inhibitor; ETI^{L}-WCI^{S}, chimera having loop of ETI on the scaffold of WCI; PDB, Protein Data Bank; SDM, site directed mutagenesis; RING, Residue Interaction Network Generator; CD, circular dichroism

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'cavities' of various sizes at the core of the protein and debate has arisen about the significance of these cavities towards the stability and folding, as 'cavities' inside a protein are destabilizing [12,13]. In general β-trefoil proteins are 'slow folders' despite having a relatively low contact order [14]. Considerable work has been done regarding the involvement of 'core residues' in the stability, packing and folding in case of proteins belonging to FGF and interleukin family. Interleukin family contains three symmetrically placed phenylalanine residues that stitch the core residues of the barrel with the lid and mutation of these residues affects the formation of both native and unfolded states [15]. Brych et al. described a combination mutant of FGF-1, involving five positions within the core that substantially increased the threefold symmetric constraint of the primary structure. They have also shown the presence of a highly conserved methionine 67 residue among the family that appeared intolerant to mutation [16]. Structural analysis suggested that the local packing environment of position 67 involves two regions of apparent insertions that distorted the tertiary structure symmetry inherent in β-trefoil architecture.

Members of Kunitz (STI) family also belong to the β-trefoil fold superfamily and display an extremely high plasticity regarding their interacting partners [17]. The special feature of this family of proteins, compared to FGF and Interleukins, is their surface which is dominated by loops of varying sizes, conformations and sequence that covers >60% of their total amino acids. The six-stranded β-barrel capped with six-stranded lid folded around a hydrophobic core acts as a stable scaffold that harbors loops of varying sizes dominating their surface. Although these loops originate from different subdomains they have very weak sequence constraints — providing a platform for its functional diversity [17]. Although extensive structural and inhibitory studies have been reported for the Kunitz (STI) family [18–23], very little is known about their core packing compared to the FGF and interleukin family. As several crystal structures having β-trefoil fold are available in Protein Data Bank (PDB) we felt it necessary to revisit their hydrophobic core with special emphasis on Kunitz (STI) family. β-trefoil fold proteins were identified in PDB, extracted and superposed on Winged-bean chymotrypsin inhibitor (WCI; PDB code: 1EYL) [24], one of the well studied members of Kunitz (STI) family, using FATCAT server [25]. Residues constituting their hydrophobic core were identified (Table 1) and the three layers of arrangement inside the barrel, as documented by Murzin, are depicted in Fig. 1a. Out of these three layers; the surface exposed top layer is constituted mainly by hydrophilic and polar residues (like Arg, Ser, His, Gly etc.). The tightly packed middle layer is populated mainly by aromatic and large aliphatic amino acid residues (Fig. 1b). The bottom layer is made up of amino acid residues strictly hydrophobic in nature (Fig. 1c). One side of the bottom layer interacts with the middle layer, while the other side interacts with residues coming from the lid (green and red sticks) (Fig. 1d). A closer scrutiny of the arrangement of six residues in the middle and bottom layer reveals that they form two triangles. An inner triangle is made up of three closely interacting residues (Fig. 1 b, c; orange and blue) and an outer triangle (Fig. 1 b, c; yellow and gray) where three other residues remain in a non-interacting position.

Kunitz (STI) inhibitors distinguish themselves from other β-trefoil proteins by having a conserved Trp(W91) residue projected from the lid (Table 1) that packs with the bottom layer residues. Considering its conserved nature, crucial strategic location, sheer size and contact area we felt that this W91 residue could be a key player in the stability and packing of Kunitz (STI) family of inhibitors. We prepared four mutants of WCI (Viz, W91F-WCI-WCI, W91M-WCI, W91I-WCI and W91A-WCI) to deliberately create cavities of varying sizes at the core to investigate how tolerant the W91 position is in terms of structural flexibility, local and global stability. CD spectroscopy of the mutants and crystal structure of W91F-WCI, W91M-WCI and W91A-WCI shows strict preservation of the secondary structure with the wild type indicating that cavity creation led to minimal perturbation of the structure of the folded state. However, small readjustments of the hydrophobic core residues have been observed, which are centered on the site of mutation and directed so as to minimize the cavity volume as much as possible thereby indicating

Table 1
Propensity table of residues forming the hydrophobic core of β- barrel fold proteins of different families.

		β1				β4			β5			β8			β9			β12							
Protein Family	PDB ID	16	18	20	58	60	62	73	75	77	117	119	121	128	130	132	170	172	174	31	45	79	91	102	148
	1EYL	G	Y	L	I	I	S	S	V	L	F	F	K	H	Y	L	L	L	K	I	V	F	W	V	I
	1TIE	G	Y	L	I	I	S	D	V	I	F	F	Q	H	Y	L	V	L	K	V	V	F	W	V	I
	1AVU	G	Y	I	I	I	S	H	L	L	F	L	R	N	Y	L	V	F	K	I	V	F	W	V	I
Kunitz	1R8N	S	Y	V	V	F	S	T	L	I	F	I	K		Y	L	I	F	K	L	I	F	W	V	I
(STI)	1R80	G	Y	I	V	F	A	M	L	I	F	I	u		Y	D	V	I	K	V	V	V	W	V	I
	2G02	A	Y	L	V	F	S	Y	L	I	F	V	K		Y	I	F	I	K	L	V	F	W	V	L
	2GZB	A	Y	L	V	F	T	F	L	I	F	V	K		Y	I	F	I	K	L	V	F	W	V	L
	3S8K	G	Y	V	I	F	A	V	L	L	F	I	K		Y	F	F	F	K	L	L	F	W	V	V
Interleukins																									
	2MIB	L	Y	L	F	M	F	I	V	L	F	K	Q	S	V	F	F	M	S	L	L	L	L	L	F
	1MD6	L	F	M	I	V	P	S	V	L	F	V	N		S	F	F	F	Q	L	L	V	L	L	C
	2NVH	L	C	L	F	M	F	I	V	L	F	K	N	N	L	L	F	M	F	L	L	Q	L	L	I
Ricin -B																									
	2ZQN		F	L	W	T	Q		V	I	W	V	G		-	I	F	I	Q	L	I	S	L	V	L
	1KNL	K	-	I	W	A	N		L	L	W	L	S		-	V	W	R	T	L	L	V	L	V	L
FGF s																									
	2P23	R	R	L	L	I	A	A	V	V	F	Q	I	N	N	Y	F	P	L	L	V	G	L	M	V
	2FGF	K	K	L	L	L	A	Q	V	V	F	Q	L	N	N	Y	F	P	S	L	V	G	L	L	V
	1G82	R	R	L	L	I	I	Q	F	V	F	Q	F	N	N	Y	F	P	P	L	I	G	L	L	V
	1BAS	K	K	L	L	L	A	Q	V	I	F	Q	L	N	N	Y	F	P	S	L	V	G	L	L	V
	1BFB	R	K	L	L	L	A	A	V	I	F	Q	L	N	Y	N	F	P	S	L	V	G	L	L	V
Others																									
	1WD3		V	L	W	L			G	I	F	P	A		T	-	F	I		I	V	S	I	L	F
	1YBI		C	I	W	I	Y		Y	F	W	I	P		Y	I		I		V	V	N	L	V	L

Color code: Green represents the residues from the lid, 'chocolate' represents residues from the top layer, 'yellow and orange' correspond to the outer and inner triangle of middle layer, and 'blue and sky-blue' designate the inner and outer triangle of bottom layer.