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Structural Analysis of Protein Folding by the Long-chain Archaeal Chaperone FKBP26

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Summary

In the cell, protein folding is mediated by folding catalysts and chaperones. The two functions are often linked, especially when the catalytic module forms part of a multidomain protein, as in *Methanococcus jannaschii* peptidyl-prolyl cis/trans isomerase (PPIase) FKBP26. Here we show that FKBP26 chaperone activity requires both a 50-residue insertion in the catalytic FKBP domain, also called ‘Insert-in-Flap’ or IF domain, and also an 80-residue C-terminal domain. We determined FKBP26 structures from four crystal forms and analyzed chaperone domains in light of their ability to mediate protein-protein interactions. FKBP26 is a crescent-shaped homodimer. We reason that folding proteins are bound inside the large crescent cleft, thus enabling their access to inward-facing PPIase catalytic sites and ipsilateral chaperone domain surfaces. As these chaperone surfaces participate extensively in crystal lattice contacts, we speculate that the observed lattice contacts reflect a proclivity for protein associations and represent substrate interactions by FKBP26 chaperone domains. Finally, we find that FKBP26 is an exceptionally flexible molecule, suggesting a mechanism for non-specific substrate recognition.

Keywords

crystal structure; FKBP; PPIase; prolyl isomerase; protein-protein interactions

Introduction

Protein folding in the cell is an enormously complicated and error-prone process that requires the help of a wide range of folding helpers, including molecular chaperones and folding catalysts¹. Chaperones can prevent the formation of protein aggregates in the cell by binding to misfolded proteins and by promoting the folded state² and folding catalysts can

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Accession codes

Coordinate and structure factor accession numbers (Protein Data Bank, RCSB): 3PR9, 3PRA, 3PRB and 3PRD, for hexagonal FKBP26-150, monoclinic FKBP26-150, orthorhombic FKBP26-231 and tetragonal FKBP26-231, respectively, as is also indicated in Tables 1 and 2.

accelerate rate-limiting chemical steps in the folding process³. Peptidyl-prolyl *cis/trans* isomerization is such a rate-limiting step in protein folding and peptidyl-prolyl *cis/trans* isomerases (PPIases), including cyclophilins, parvulins and FK506-binding proteins (FKBPs)^{4,5} can catalyze this step. FKBPs also function as chaperones especially when the catalytic, FK506-binding module is coupled to additional domains, as in the bacterial chaperone trigger factor (TF)^{6,7}.

FKBPs are widely distributed in both eukarya and bacteria and two distinct types have been identified in archaea, a short- and a long-type FKBP, with molecular masses of 17–18 kDa and 26–30 kDa, respectively^{8,9}. Both consist of an FK506-binding domain and a small ‘Insert-in-Flap’ (IF) domain^{8,10}, and both have the ability to refold denatured proteins *in vitro* and to suppress protein aggregation *in vivo*^{11–15}. Long-type FKBPs have an additional C-terminal domain of as yet unknown function. Chaperones comprising FKBP and IF domains are additionally found in bacteria and in some fungi and plants, and insertion of a bacterial IF domain into human FKBP12 converts it into a powerful chaperone^{16,17}.

The hyperthermophilic archaeon *Methanococcus jannaschii* has FKBP chaperones of both types. Both its short-type FKBP18 and its long-type FKBP26 proteins have chaperone-like protein-folding activity *in vitro* as seen by recovery of enzymatic activity from chemically unfolded citrate synthase; however, whereas the PPIase activity of FKBP18 is comparable to that of other short-type FKBPs and of human FKBP12, the activity of FKBP26 is much lower (< 1/1000 in catalytic efficiency on oligopeptide substrates)¹³. Both proteins are also produced *in vivo* in *M. jannaschii* cells; however, FKBP18 is expressed preferentially at growth temperatures below 80°C while FKBP26 expression increases with growth temperature up to 90°C¹³. It has been suggested that a PPIase is not needed by *M. jannaschii* when at high growth temperatures because spontaneous peptidyl-prolyl isomerase activity is facilitated under these conditions.

Because chaperones are ubiquitous and germane to many cellular processes, they have been widely studied, yet the molecular basis of substrate recognition remains poorly understood with some exceptions^{18–22}. Here, we have analyzed the structure and chaperone function of long-type *M. jannaschii* FKBP26 (FKBP26). Using domain deletion mutants, we show that FKBP26 chaperone activity is associated with ‘Insert-in-Flap’ IF and the C-terminal domains. We also report four different crystal structures including two crystal structures of the full-length protein and two crystal structures of a truncated form that lacks the C-terminal domain and corresponds structurally to the short-type form. FKBP26 is a crescent-shaped, homodimeric protein, where the C-terminal domain mediates dimerization. The different crystallographic structures reveal an exceptionally flexible molecule with a proclivity for protein interactions, as, respectively, interdomain dispositions vary greatly and crystal lattice contacts are exceptionally large. We propose that structural plasticity effected by interdomain flexion supports non-specific associations and that crystal packing interactions involving the ‘Insert-in-Flap’ domain and surfaces exposed by truncation exemplify biologically relevant associations of chaperone domains with non-native surfaces.

Results

Construct design and protein characterization

To analyze the functions of individual FKBP26 domains, we generated a number of different constructs (Fig. 1a). Construct 231 corresponds to the full-length protein, fragment 231-ΔIF designates deletion of the ‘Insert-in-Flap’ (residues 83-134), fragment 150 lacks the C-terminal domain (residues 151-231) and fragment 150-ΔIF lacks both ‘Insert-in-Flap’ and C-terminal domain (residues 83-134 and 151-231, respectively). Thus, fragment 150-ΔIF

corresponds to the FKBP domain alone and fragment 150 corresponds to a short-type FKBP. All constructs were expressed in *Escherichia coli* and purified to homogeneity.

Size exclusion chromatography, performed as a last step of purification, showed that the C-terminal domain is a dimerization domain. Consistent with sizes predicted from the four constructs (Fig. 1a), the two proteins that include the C-terminal domain elute as dimers whereas the two lacking it elute as monomers.

Folding activity

To determine the contribution of individual FKBP26 domains to chaperone activity, we tested the different constructs in a well established *in vitro* chaperone assay using denatured hen egg white lysozyme as a substrate^{23,24}. Chemically denatured lysozyme aggregated rapidly upon 100-fold dilution, but stoichiometric amounts of FKBP26 efficiently prevented lysozyme aggregation, as no light-scattering signal at 360 nm was observed (Fig. 1b). Fragments that comprise the FKBP domain together with either CTD or IF (231- Δ IF and 150) prevented aggregation in a concentration-dependent manner as well, but in contrast to full-length FKBP26 (231), 2- and 5-fold-higher concentrations, respectively, were required for 50% suppression of lysozyme aggregation (Fig. 1c). The construct lacking both CTD and IF domains (150- Δ IF) was inactive in the refolding assay, suggesting that the FKBP domain alone does not contribute significantly to FKBP26 chaperone activity toward chemically denatured lysozyme (Fig. 1c).

Crystallization and structure determination

We determined the structure of FKBP26 in four different crystal forms: as the full-length polypeptide in crystal lattices P2₁2₁2₁ and I4₁22, as well as a C-terminal deletion (fragment 150) in crystal lattices P3₁2₁ and P2₁. Crystal parameters, diffraction data statistics and refinement statistics are given in Tables 1 and 2. Crystals of full-length FKBP26 grow readily under many different buffer conditions but diffract only to about 10 Å Bragg spacings for all conditions tested. However, we devised dehydration protocols that resulted in a dramatic improvement in diffraction quality when using orthorhombic crystals grown in 15% PMME550, 0.1 M NaCl and 0.1 M bicine (pH 9.0). Tetragonal crystals also diffracted to higher resolution after dehydration, but improvements were much less dramatic.

We solved the crystallographic structure of the orthorhombic form by multiple wavelength anomalous dispersion (MAD) using selenomethionyl protein²⁵ using MAD data collected to 2.9 Å spacings, and we refined the structure at 2.2 Å resolution using native data and non-crystallographic symmetry averaging. Tetragonal crystals diffracted anisotropically to 3.3 Å Bragg spacings (Table 1). An electron density map from selenomethionyl MAD data was interpretable for the overall fold, but inadequate for detailed modelling; however, a full atomic model could be refined after positioning high-resolution models into the appropriate experimental electron density followed. Hexagonal and monoclinic crystals of C-terminally truncated FKBP26 diffracted to 1.95 Å and 2.4 Å, respectively, and we solved these structures by molecular replacement. All structures with exception of the tetragonal form are well defined throughout and the stereochemistry is in excellent agreement with expected values (Table 2).

FKBP26 structure

FKBP26 is a three-domain protein that consists of an N-terminal FKBP domain, an intermediate domain (IF) and a C-terminal dimerization domain (CTD, Fig. 2a). The structures of full-length FKBP26 are similar in both orthorhombic and tetragonal lattices, except for conformational flexion at domain junctions (as noted below). We describe only the orthorhombic structure because of its higher resolution. The FKBP domain is composed

of a four-stranded antiparallel β -sheet flanked by two α -helices (Fig. 2c). The topology of this FKBP domain can be described in the spatial order of its β -strands as N-terminus- $\beta 4$ - $\beta 5_a$ - $\alpha 2$ - $\beta 5_b$ - $\alpha 1$ - $\beta 2$ -IF- $\beta 3$ -C-terminus. This is, in essence, a canonical FK506 binding protein that lacks an N-terminal β -strand ($\beta 1$) as observed in human FKBP12 (hsFKBP12)²⁶, but includes an α -helical region ($\alpha 2$) inserted into a bulge that breaks up strand $\beta 5$ (Figs. 2c,f).

The structured IF insertion replaces a loop that connects $\beta 2$ and $\beta 3$ in most FKBP (Figs. 2a,b,c,f). This 'Insert-in-Flap', also present in the short-type archaeal FKBP¹⁰, resembles a fractured four-stranded β -barrel (also 'incomplete barrel'²⁷) where the first and second strands are connected by a helix that partially covers the fracture (Fig. 2b). In addition to IF domains of the structurally characterized SlyD and archaeal short-type FKBP^{10,27}, IF is similar to one domain of the Mg^{2+}/Co^{2+} efflux protein CorC (Fig. 2e, PDBID: 2R8D).

CTD has an α/β -sandwich structure composed of three α -helices and a three-stranded, mixed-orientation β -sheet (Fig. 2d) determined here for the first time. A CTD-like structure is also found in the phylogenetically conserved NifU proteins (HIRIP5 in mouse and humans), which are thought to provide a molecular scaffold for [Fe-S] cluster formation (Fig. 2g, PDBID: 1TH5²⁸). Dimerization of FKBP26 is achieved through inter-subunit antiparallel pairing of the third β -strands of the two CTDs. As a result the CTD dimer forms a continuous, six-stranded mixed β -sheet (Fig. 2d). The dimerization interface is significant (buried area at interface 1885 Å²)²⁹ and well packed (shape complementarity value 0.75), as corresponds to packing within a protein core.

Overall, the FKBP26 homodimer is shaped like a crescent with a wide and deep crescent cleft (approximately 35 Å by 35 Å in cross section). The apices are formed by apposed IF domains and the base by the CTD dimer (Figs. 2a,3a). Both IF and CTD domains contribute disproportionately large areas to the inward surface of the crescent, while FKPB only presents a smaller area that includes the PPIase active site. The shape of the FKBP26 dimer is reminiscent of other chaperones, including heat shock proteins 90 (HSP90) and 40 (HSP40).

Surface properties

Molecular surfaces corresponding to each of the FKBP26 domains are clearly delineated; individual domains form distinct units (Fig. 3a). The inward surface of the crescent is mostly basic but also includes significant hydrophobic regions (Figs. 3b,c). The charge distribution of the outward surface on the other hand appears to be random and includes positively and negatively charged patches. By domain, IF is particularly basic while the FKBP domain and CTD are less so (Figs. 3b,c). Hydrophobic surface patches, which consist of vicinal carbon atoms, are mostly located on the inward surface, with smaller patches covering CTD and significantly larger patches covering IF (Fig. 3c).

Conformational variability

The structures of the three FKBP26 domains as determined in four different crystal forms are preserved within domains (with some small adaptations in IF); however, relative domain orientations reveal an intrinsic conformational variability due to rigid-body movements between domains (Fig. 4). IF orientations relative to the FKBP domain vary and domain movements at this juncture are substantial ($X=15.1^\circ$, $t_x=-1.8\text{\AA}$, Fig. 4a). Relating to CTD, the superposition transformations needed at junctures from CTD to the FKBP domain from the orthorhombic and tetragonal crystals are especially large ($X=22.1^\circ$, $t_x=0.8\text{\AA}$, Fig. 4b).

Crystal lattice contacts

The lattice contacts found in crystals of FKBP26 and its truncated forms include exceptionally large interfacial contact areas that engage the inward surface of the FKBP crescent. This is most strikingly seen in the orthorhombic lattice, wherein the IF domain at each apex of the dimer has a lattice-related FKBP26 dimer inverted over it such that each IF apex is making contacts with the inward-facing CTD surfaces at the base of another crescent (Fig. 5a). Whereas typical lattice contacts in protein crystals are smaller than those in biologically relevant protein-protein interactions ($> 800 \text{ \AA}^2$)³⁰ often much smaller, the interface between each protomer of the FKBP26 dimer in the orthorhombic lattice and its lattice-mate from another dimer is relatively large (buried surface area $\approx 2400 \text{ \AA}^2$). Indeed, these inter-locking lattice interactions are more extensive (albeit less intimately complementary) than those in the dimer interface ($1885 \text{ \AA}^2$) (Figs. 2a,d,3).

Both crystals of the CTD-truncated protein, FKBP26-150 (Fig. 5b), also feature large lattice-contact interfaces involving inward-facing surfaces of the FKBP crescent. These interactions include an FKBP domain surface that is natively buried against CTD but exposed here by virtue of the CTD truncation (Fig. 5c). This surface, highly hydrophobic as expected for a natively buried interface (Fig. 5d), is almost completely covered by IF in two lattices where it shields the same exposed atoms in two distinct ways (Figs. 5e,f,6). The IF surface also features substantial hydrophobic patches (Fig. 5d), which become buried into lattice contacts for the truncated crystals (Figs. 5e&f, 6b&c) as they are also for the full-length orthorhombic crystal (Fig. 5g, 6c).

IF-domain residues involved in lattice contacts in the different crystals are all largely found in two areas of its inward facing and apex surfaces (Fig. 6a). These surfaces include residues 89–99 ($\beta 1$ - $\alpha 1$) and 110–115 (loop linking $\beta 2$ and $\beta 3$) (Fig. 2b). In the monoclinic crystal, IF covers the core of non-natively exposed hydrophobic FKBP domain residues (buried surface area $\approx 1820 \text{ \AA}^2$) mainly by using amino acid residues that are scattered throughout the IF sequence but cluster at the base of its inward surface. Contacting IF residues form part of a hydrophobic gap exposed by the fracture in the β -barrel (Figs. 5d,e,6a,b). Strictly hydrophobic IF residues at this interface include the partially exposed Ile91, Ile114 and Phe130, and aliphatic components of some hydrophilic side chains also contribute (Lys89) (Fig. 6b). In the hexagonal crystal, IF covers a similar and overlapping group of non-natively exposed FKBP domain atoms (buried surface area $\approx 1950 \text{ \AA}^2$); however, IF contacting residues cluster on the lateral and apical surfaces formed by $\beta 1$ and $\alpha 1$ instead (Figs. 2b,6c). Strictly hydrophobic residues are less prevalent in this interaction and include Ile91 and Pro92, while hydrophilic side chains, including Lys89, Glu95 and Lys98, also form hydrogen bonds with FKBP domain backbone carbonyl and amide atoms (Figs. 5d,f, 6c).

In the orthorhombic crystal, IF contacts are scattered throughout but there are especially large contributions from the apical surface. Strictly hydrophobic residues at this interface include Pro92, Leu93, Ile114, and Pro115. Additionally, several hydrophilic and polar side chains, including Lys87, Ser94, Thr97, Lys98 and Lys102, contribute aliphatic components, while some also form hydrogen bonds (Figs. 5d,g,6d). Residues on CTD that contact IF converge spatially on two protruding ridges formed by each one of two large helices (Fig. 6e). Contacting side chains are mostly polar or neutral (Tyr163, Asn192, Thr195, Ala196, Ala199) and contribute both hydrophobic interactions and hydrogen bonds.

Discussion

FKBP chaperones and several other folding catalysts are both enzymes and chaperones, and all are multi-domain proteins^{11,13–15,27,31–33}. The archaeal long-type FKBP26 of *M.*

jannaschii is such a multi-functional folding catalyst; it consists of three domains, an N-terminal FKBP, an 'Insert-in-Flap' (IF) and a C-terminal dimerization (CTD) domain (Fig. 1a). The prolyl isomerase activity of FKBP chaperones is localized to the FKBP domain, and this domain alone has little if any chaperone activity, as found for human FKBP12¹⁶ and here for Δ IF/ Δ CTD FKBP26 (Fig. 1c). Substantial chaperone activity is acquired only through added domains, as in trigger factor^{6,7} or in the IF-FKBP chaperones^{8,9}. This acquisition of chaperone activity was illustrated elegantly through an engineered deletion of the IF domain from a SlyD chaperone and its parallel insertion into human FKBP12¹⁶. These added domains not only engage substrates to facilitate folding, however; by recruiting protein substrates in this way, they also markedly enhance the effective PPIase efficiency for these substrates while leaving intrinsic efficiency unchanged as measured for oligopeptide substrates¹⁶. Moreover, chaperone efficiency can also be enhanced for proline-limited folding¹⁶.

FKBP26 and other archaeal, long-type FKBP are special, however, in that they have very low intrinsic PPIase activity, perhaps reflecting the high rate of spontaneous proline isomerization at ~90°C in the normal environment of *M. jannaschii*¹³. The low intrinsic PPIase activity of these proteins seems to derive from a Phe→Tyr substitution at positions homologous to Phe99 in human FKBP12, Tyr141 in *M. jannaschii* FKBP26. The analogous F99Y mutation in human FKBP12 and at the homologous positions in *E. coli* trigger factor³⁴ and short-type FKBP17 from *M. thermolithotrophicus*³⁵ each reduce PPIase activity to low levels, often near zero. It appears that evolution has adapted the FKBP enzyme into a chaperone by domain embellishments; and then, when not needed, to have abandoned the enzyme role while retaining the chaperone framework.

FKBP26 is a homodimeric protein. Its structure resembles a crescent with IF forming its apices and the CTD dimer its base (Figs. 2a,3a,7a). We hypothesize that FKBP26 cradles folding proteins within the large crescent cleft, whereby the folding substrate protein would have access to the inward facing PPIase catalytic sites (Fig. 7a). An analogous binding mode has been suggested for other folding catalysts^{36–38}. Such a binding mode would also position substrate proteins in close proximity to inward facing surfaces on IF and CTD (Fig. 3,7a), suggesting that these surfaces mediate protein folding^{16,17,27}. We observe an analogous, inward facing orientation of the PPIase active site and binding of the substrate to ipsilateral surfaces, in the structure of substrate bound chaperone TF¹⁹.

Although protein-protein interfaces at lattice contacts in crystals are generally non-specific and not reflective of cellular functions, in certain cases they may provide genuine indications of biologically relevant interactions³⁹. For instance, for the FKBP chaperone TF we showed previously that TF-substrate contacts and TF-TF lattice contacts in crystals of the substrate free form are strongly correlated^{19,40,41}. The very cleft-like TF surfaces that interact with the ribosomal S7 substrate also interact with TF itself in apo crystals. Following from this observation, we surmise that the substantial self-associations of FKBP26 in crystal lattices could reflect the kind of non-specific associations that the chaperone may have with diverse substrates. We therefore analyzed FKBP26 crystal lattice interfaces involving inner surfaces of IF and CTD in the FKBP crescent, as both domains mediate folding (Fig. 1)^{12,16,17,27,35}. We note especially those interactions involving IF and the surface on FKBP that is exposed by truncation of CTD (Figs. 5,6). We find that IF masks this non-native, hydrophobic FKBP surface in two substantial yet distinct ways, either as it forms a hydrophobic, cup-like shield over or as it provides a scaffold for the non-natively exposed amino acids (Figs. 6b,c). As IF contacts many natively buried residues that are nearby in structure but not necessarily in sequence, we propose that FKBP26, like TF, can recognize three-dimensional motifs as found in advanced folding states, thereby masking exposed non-native, aggregation-prone surfaces. Importantly, the ability of the IF

chaperone domain to recognize the same surface in completely different ways shows how the chameleon-like properties of chaperones allow these to interact flexibly with a large number of completely unrelated substrates^{22,42,43}.

The arrangement of domains in the FKBP26 homodimer is likely very dynamic; this is evidenced by substantial variability at CTD-to-FKBP and at FKBP-to-IF junctions (Fig. 4a,b), indicative of rigid-body motions. NMR structures of IF-FKBP chaperones in solution show a similar flexibility between IF and FKBP domains^{10,44}. Interdomain flexibility appears to be a common property in molecular chaperones^{19,36,38}. This implies that some chaperones, like FKBP26 and TF, have access to an ensemble of energetically equivalent, isomeric 'native states' that are separated by low energy barriers (Fig. 7b,c)⁴⁵. Such isomeric plasticity could enable the chaperone to bind different protein ligands, as one protein ligand with a particular structure may preferentially associate with a particular chaperone conformer, whereas another protein ligand with a different structure may bind an alternate chaperone conformation with the equilibrium adjusting itself in favor of the appropriate chaperone conformer (Fig. 7c)⁴⁶. We propose that the observed structural plasticity of FKBP26 permits adaptation of its binding cleft to accommodate and bind diverse protein ligands. Access to multiple conformations also allows for multiple modes of chaperone action that are dictated, ultimately, by the folding protein, not the folding helper; the chameleon adapts to its environment.

Experimental procedures

Protein expression and purification

M. jannaschii FKBP26 (SwissProt Q58235) was PCR-amplified from genomic DNA (ATCC) and fragments encoding full length protein (231: amino acid residues 1-231) and domain deleted mutants (231-ΔIF: 1-231Δ83-G-A-G-134; 150: 1-150; 150-ΔIF: 1-150Δ83-G-A-G-134), were cloned into pet24d+ (Novagen) by standard molecular biology techniques. Transformed *E. coli* BL21(DE3) were grown at 37°C and protein expression was induced for 3h with Isopropyl-β-D-thio-galactoside (IPTG). Cells were harvested by centrifugation, resuspended and frozen for storage. Thawed cells were lysed by sonication and centrifuged. The supernatant was loaded onto a HiTrap (GE Healthcare Life Sciences) S Column equilibrated with buffer (10 mM Tris (pH 7.5), 100 mM NaCl). Proteins were eluted with a linear gradient of 100–500 mM NaCl. Peak fractions were dialyzed and loaded onto a HiTrap Q Column equilibrated with buffer (10 mM Tris (pH 7.5), 100 mM NaCl) and eluted with a linear gradient of 100–500 mM NaCl. Pooled fractions were concentrated and purified with Superdex 200 gel filtration. Selenomethionyl (SeMet) FKBP26 was produced as above by following a non-auxotrophic expression protocol.

Lysozyme refolding

Lyophilized hen egg white lysozyme (Sigma) was dissolved to a final concentration of 1000 μM in denaturing buffer (20 mM Tris-HCl (pH 8.0), 100 mM NaCl, 6 M guanidine hydrochloride (GuHCl) and 100 mM DTT). The solution was incubated at 37 °C for 2 h after mixing. Individual samples of denatured and reduced lysozyme were briefly stored at 4 °C and subsequently diluted 100-fold into buffer (0.1 M Tris-HCl (pH 8.0), 100mM NaCl) ± FKBP26 constructs at different concentrations. Mixtures were vortexed for 5 seconds, immediately centrifuged at 20,000 × g for 30 seconds and loaded onto a FluoroMax-3 fluorescence spectrometer (Horiba Jobin Yvon). Lysozyme aggregation was measured by light scattering at 360 nm. Experiments were performed in triplicate and were highly reproducible.

Crystallization and data collection

Orthorhombic crystals ($P2_12_12_1$) were grown at 20 °C in hanging drops by mixing 2 μ l protein with 2 μ l reservoir buffer (15–20% (w/v) PMME550, 0.1 M bicine (pH 9.0), 0.08 M NaCl). Tetragonal crystals ($I4_122$) were grown at 20 °C in hanging drops by mixing 8 μ l protein with 2 μ l reservoir buffer (1.4–1.6 M $(\text{NH}_4)_2\text{SO}_4$, 0.1 M Tris (pH 8.5), 2% Glycerol). Crystals diffracted to ~ 10 Å Bragg spacings with synchrotron radiation (at 293 K and 100 K). Orthorhombic crystals were reproducibly dehydrated by adding 20 μ l reservoir buffer to the crystal drop and equilibrating one hour over saturated $(\text{NH}_4)_2\text{SO}_4$. Crystals remained stable, were frozen at 100 K in nitrogen gas and 2.2 Å data were collected. Tetragonal crystals were dehydrated by adding 10 μ l of cryobuffer (1.4–1.6 M $(\text{NH}_4)_2\text{SO}_4$, 0.1 M Tris (pH 8.5), 10% glycerol) and equilibrating over saturated $(\text{NH}_4)_2\text{SO}_4$. Dehydrated crystals were frozen at 100 K in nitrogen gas and anisotropic 3.6 Å data were collected. Hexagonal crystals ($P3_121$) of the C-terminally truncated FKBP26 (150) were grown in hanging drops by mixing 2 μ l protein with 2 μ l reservoir buffer (1.7 M sodium malonate (pH 8.0), 0.1 M Tris (pH 8.4)). Crystals were transferred into cryobuffer (2.3 M sodium malonate (pH 8.0), 0.1 M Tris (pH 8.4), 10% glycerol) and frozen at 100 K in nitrogen gas for data collection. Monoclinic crystals ($P2_1$) were grown in hanging drops by mixing 2 μ l protein with 2 μ l reservoir buffer containing 26% PMME2K, 0.1M HEPES (pH 7.25) and 0.2 M $(\text{NH}_4)_2\text{SO}_4$. Crystals were transferred into cryobuffer (crystallization buffer and 20% glycerol) and frozen at 100 K in nitrogen gas. All X-ray data were collected at the National Synchrotron Light Source, beamline X4A.

Structure determinations

The structure of full-length FKBP26 (231) was solved in two space groups by multiple wavelength anomalous diffraction (MAD)²⁵. Coordinates for 8 of 10 selenium atoms were located from 2.9 Å MAD data from the orthorhombic crystal (two molecules per asymmetric unit) with SOLVE⁴⁷. Density modification, phase extension, two-fold averaging and model building at 2.2 Å resolution using RESOLVE⁴⁷ produced an excellent electron-density map with clearly interpretable atomic details and a model comprising 195 alanine residues (out of 462 residues). Additional amino acid residues were placed and sequences were assigned manually. The completed model was subjected to several cycles of rebuilding and refinement.

The tetragonal ($I4_122$) FKBP26 structure was solved from anisotropic 3.6 Å MAD data. Density modification produced a clear outline of the molecule with some interpretable secondary structure for FKBP and CTD; however, atomic details were absent. One copy from the orthorhombic crystal was manually placed in experimental density and domains were adjusted with rigid body refinement. The structure was refined at 3.3 Å using phased simulated annealing and TLS.

Truncated FKBP26 (FKBP-150) in the hexagonal space group ($P3_121$) was determined by molecular replacement using CNS. A clear solution for one molecule per asymmetric unit was obtained with CNS using FKBP from the orthorhombic crystal. IF was positioned manually followed by rigid body fitting. The complete model was subjected to model update (ARP/wARP) and manual rebuilding. Successive models were fitted into simulated annealing omit maps and refined to 1.95 Å resolution.

The structure of monoclinic FKBP26-150 ($P2_1$) was determined by molecular replacement using CNS. A clear solution for two molecules per asymmetric unit was obtained with CNS with refined FKBP26-150 from the hexagonal crystal. The model was subjected to rigid body fitting, model update, manual fitting and refinement at 2.4 Å resolution. O⁴⁸ was used for model manipulations and rebuilding, ARP/wARP⁴⁹ for model update, CNS⁵⁰ for

simulated annealing, simulated annealing omit map calculations and phased refinement and REFMAC⁵¹ for TLS⁵² refinement. FKBP26 domains were defined as TLS groups (FKBP: 1–85&135–150, IF: 86–134 and CTD: 151–224.)

Figures showing crystallographic models and surfaces were generated with POVSCRIPT⁵³, GRASP⁵⁴ and RASTER3D/RENDER⁵⁵ Structures were superimposed with CCP4⁵⁶ programs TOPP⁵⁷ and SUPERPOSE⁵⁸ and values for t_x and X were obtained with TOSS⁵⁹ The program QUILT⁶⁰ was used to identify hydrophobic patches.

Abbreviations

PPIase	peptidyl-prolyl <i>cis/trans</i> isomerase
FKBP	FK506-binding protein
FKBP26	26kDa <i>Methanococcus jannaschii</i> FKBP
TF	trigger factor
IF	Insert-in-Flap domain
CTD	C-terminal domain

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Figure 1. Construct design and refolding activity

(a) Schematic representation of *M. jannaschii* FKBP26 and constructs used in this study. 231 (wild type FKBP26) corresponds to amino acid residues 1–231; 231-ΔIF corresponds to residues 1–231Δ83-G-A-G-134; 150 corresponds to residues 1–150; 150-ΔIF corresponds to residues 1–150Δ83-G-A-G-134. (b) Aggregation of denatured hen egg white lysozyme after 100-fold dilution into non-denaturing buffer can be detected by an increase in light scattering at 360 nm (final lysozyme concentration 10 μM, solid line). FKBP26 inhibits lysozyme aggregation in a concentration dependent manner. Addition of 10 μM wild-type FKBP26 (231, solid triangles) completely inhibits lysozyme aggregation. (c) Refolding activities of FKBP26 fragments in comparison to wild-type FKBP26 (231, solid triangles).

Addition of 10 μM 231- ΔIF (crosses) or 50 μM 150 (solid circles) significantly inhibits aggregation, whereas addition of 10 or 50 μM 150- ΔIF (diamonds) does not.

Figure 2. Structure of FKBP26

(a) Ribbon diagram of FKBP26 based on the 2.2 Å resolution orthorhombic crystal structure. The FK506 binding protein domain (FKBP, residues 1-83 and 134-150) is colored green; the ‘Insert-in-Flap’ domain (IF, residues 84-133) is colored blue; the C-terminal domain (CTD, residues 151-231) is colored red. Orthogonal views rotated about the horizontal axis are shown. The two-fold dimerization axis (non-crystallographic symmetry) is represented by a vertical arrow and by the black eye-shaped symbol. FKBP26 forms a homodimer in the shape of a crescent. IF forms its apices and CTD its base. (b) Stereo diagram of IF in blue. (c) Stereo diagram of FKBP in green. (d) Stereo diagram of CTD in red, its homodimerization partner is shown in grey. Secondary structure elements are labeled throughout. (e) IF structural homolog corC (PDBID: 2R8D). Matching secondary structure elements are colored correspondingly. Unrelated segments are colored yellow. (f) FKBP structural homolog hsFKBP12 (PDBID: 1FKK). Matching secondary structure elements are colored correspondingly. Differing segments are colored yellow. (g) CTD structural homolog NifU (PDBID: 1TH5). Matching secondary structure elements are colored correspondingly.

Figure 3. Surface properties

(a) Molecular surface of FKBP26 based on the 2.2 Å resolution orthorhombic crystal structure and colored by domains. FKBP domain is colored green; IF is colored blue; CTD is colored red. (b) Electrostatic surface potential of FKBP26 domains calculated with the program *GRASP*. The concave surface of the homodimer is basic (blue). (c) Hydrophobic surface patches of FKBP26 as identified with the program *QUILT* are shown in light blue. The most prominent hydrophobic patches (light blue) are localized on the concave surface of the homodimer. Orthogonal views rotated about the horizontal axis are shown throughout. Orientations are as in figure 2a.

Figure 4. Conformational variability

(a) Stereo diagram showing superposition of FKBP from all FKBP26 structures reveals appreciable flexibility at the FKBP-IF junction. **(b)** Stereo diagram showing superposition of CTD from the orthorhombic and tetragonal structures reveals extraordinary flexibility at CTD-FKBP junction. Two structures in the hexagonal form solved independently are colored red and green; two structures in the monoclinic form and related by non-crystallographic symmetry are colored gold and khaki; two structures in the orthorhombic form and related by non-crystallographic symmetry are colored blue and cyan; the structure in the tetragonal form is colored purple.

Figure 5. Molecular surfaces and crystal lattice contacts

(a) Lattice contacts associated with full-length FKBP26 in the orthorhombic crystal. The molecular surface from a full dimer on a non-crystallographic diad is shown with lattice mates related by crystallographic symmetry drawn as dark grey Ca traces. Surfaces that are in contact with lattice partners from IF are colored blue and those from CTD or FKBP are colored gold. The orientation at left is as in Fig. 2a, and that at right is rotated about the horizontal axis as indicated. (b) Ribbon diagram of the FKBP26-150 fragment based on the 1.95 Å resolution hexagonal crystal structure. FKBP is colored green; IF is colored blue. (c) Molecular surface of FKBP26-150 showing intramolecular contacts with CTD. Red areas show FKBP residues in the hexagonal crystal that would contact CTD in the orthorhombic structure. (d) Molecular surface of FKBP26-150 showing hydrophobic surface patches. Hydrophobic patches as identified with the program *QUILT* for the hexagonal crystal structure are shown in light blue. Atoms that are exposed by truncation of CTD are predominantly non-polar (compare with 5c). (e) Lattice contacts to inward-facing surfaces of the FKBP dimer as found in the monoclinic crystal form. The molecular surface for FKBP-150 is colored for IF (gold) and FKBP (blue) residues in contact with lattice partners (Ca traces in dark grey). The orientation at left has the FKBP domain positioned as in 5b-d, and an orthogonal view rotated about the vertical axis is on the right. FKBP atoms that are exposed by truncation of CTD are covered by IF. (f) Lattice contacts to inward-facing surfaces in the hexagonal form. A similar set of FKBP atoms (colored gold) is covered by IF in both the hexagonal and monoclinic crystals, but implicated IF residues (colored blue) are distinct. Orientations and colorings are as in 5e. (g) Lattice contacts to inward-facing surfaces in the orthorhombic form. For visual simplicity, only residues 1–150 are shown here. Orientations and colorings are as in 5e.

Figure 6. Molecular basis for IF contacts

(a) Left panel, molecular surface of IF as determined in the hexagonal crystal form. Surfaces from residues that form crystal lattice contacts are colored according to the crystal lattice: gold in the monoclinic crystal, maroon in the hexagonal crystal, green in the orthorhombic crystal, and cyan for residues that make contacts in more than one lattice. Right panel, stereo diagram showing superposition and interfacing amino acids of IF as found in the three crystal lattices, gold shows IF from the monoclinic, maroon from the hexagonal and green from the orthorhombic crystal. **(b)** The left panel shows the molecular surface of IF as found in the monoclinic crystal and oriented as in a). Colored surfaces are directly involved in lattice contacts. Gold highlights atoms that uniquely contribute to the monoclinic lattice and cyan shows atoms that contribute to this and additional lattices. The right panel shows a stereo diagram with a trace of IF and contacting side chains in the front and the molecular surface of FKBP in the back. Gold colored surfaces interface with IF. **(c)** The left panel shows the molecular surface of IF as found in the hexagonal crystal and oriented as in a). Red highlights atoms that uniquely contribute to the hexagonal lattice and cyan shows atoms that contribute to this and additional lattices. The right panel shows a stereo diagram with a trace of IF and contacting side chains in the front and the molecular surface of FKBP in the back. Red surfaces interface with IF. **(d)** The left panel shows the molecular surface of IF as found in the orthorhombic crystal and oriented as in a). Green highlights atoms that uniquely contribute to the orthorhombic lattice and cyan shows atoms that contribute to this and additional lattices. The right panel shows a stereo diagram with a trace of IF and contacting side chains in the front and the molecular surface of FKBP and CTD in the back. Green surfaces interface with IF. **(e)** Orthogonal views relative to **d**. Molecules are rotated about the horizontal axis. The left panel shows the molecular surface of CTD as found in the orthorhombic crystal. Green highlights atoms that contact IF. The right panel shows a stereo diagram with a trace of CTD and contacting side chains in the front and the molecular surface of FKBP and IF in the back. Green and cyan surfaces interface with CTD. **(a–e)** Left and right side panels are related by a 180° rotation about the vertical axis throughout the figure.

Figure 7. Model for FKBP26 function

(a) Schematic diagram of FKBP26 colored by domains. A substrate (represented by the grey shape) could bind within the crescent-cleft, positioning itself close to inward facing PPIase catalytic sites and to inward facing chaperone domain surfaces on IF and CTD. PPIase catalytic sites are labeled and suggested chaperone surfaces are highlighted by the molecular surface representation and by the yellow border. (b,c) Schematic diagram of different folding landscapes and their relations to binding mechanisms. H stands for enthalpy and ΔS stands for change in entropy. (b) A folding funnel with a distinct free energy minimum represents a stable protein with one discrete 'native-state' conformation and specific binding. (c) A folding funnel with a wide and jagged bottom represents a flexible protein with multiple practicable 'native-state' conformations and non-specific binding. Schematic diagrams of FKBP26 colored by domains are shown. Domain orientations vary and may adapt to bind different substrates, represented by the colored shapes.

Table 1

Data collection, phasing and refinement statistics for MAD (SeMet) structures

Data collection		Orthorhombic (231)	Orthorhombic (231, SeMet)	Tetragonal (231, SeMet)
Space group		P2 ₁ 2 ₁ 2 ₁	P2 ₁ 2 ₁ 2 ₁	14 ₂ 22
Cell dimensions				
<i>a</i> , <i>b</i> , <i>c</i> (Å)		55.53, 65.20, 148.62	54.79, 65.37, 149.53	137.95, 137.95, 114.57
<i>Z</i> _u		2	2	1
Solvent content (%)		52	52	76
		Peak	Inflection	Remote
Wavelength		0.9790	0.9792	0.9795
Bragg spacings (Å)		20.0-2.8	20.0-2.8	25.0-3.6
<i>R</i> _{sym}		0.06	0.049	0.039
// <i>σ</i>		24.7 (2.93)	34.5	35.0
Completeness %		91.6 (71.2)	84.4	83.4
Redundancy		2.9 (2.5)	4.8	5.0
Refinement				
Bragg spacings (Å)		2.20		3.30
No. reflections		24476		7776
<i>R</i> _{work} / <i>R</i> _{free}		0.227/0.274		0.314/0.327
No. atoms				
Protein		3524		1767
Water		179		n.a.
<i>B</i> -factors				
Protein		40.3		140.3
Water		47.6		n.a.
R.m.s deviations				
Bond lengths (Å)		0.013		0.009
Bond angles (°)		1.283		1.056
PDB codes		3PRB		3PRD

* Values in parentheses are for highest-resolution shell.

Table 2

Data collection and refinement statistics (molecular replacement)

	Hexagonal (150)	Monoclinic (150)
Data collection		
Space group	P3 ₁ 21	P2 ₁
Cell dimensions		
a, b, c (Å)	41.39, 41.39, 178.42	45.98, 67.89, 54.47
α, β, γ (°)	90, 90, 120	90, 95.28, 90
Z_a	1	2
Solvent content (%)	51	50
Bragg spacings (Å)	30.0-1.95 (1.98-1.95)*	30.0-2.4 (2.44-2.4)*
R_{sym}	0.053 (0.035)	0.055 (0.206)
// σ	23.2 (19.1)	27.4 (6.8)
Completeness (%)	87.7 (75.1)	99.5 (100.0)
Redundancy	2.0 (1.7)	3.7 (3.7)
Refinement		
Bragg spacings (Å)	19.84-1.95	19.76-2.40
No. reflections	11600	12405
$R_{\text{work}} / R_{\text{free}}$	0.209 / 0.268	0.234 / 0.291
No. atoms		
Protein	1164	2343
Water	344	154
B -factors		
Protein	17.05	49.33
Water	38.03	52.67
R.m.s. deviations		
Bond lengths (Å)	0.008	0.010
Bond angles (°)	1.100	1.227
PDB codes	3PR9	3PRA

* Values in parentheses are for highest-resolution shell.