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ARCHAEAL PEPTIDYL PROLYL *CIS-TRANS* ISOMERASES (PPIases) UPDATE 2004

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1. ABSTRACT

PPIases are ubiquitous in living organisms. While three families of PPIases, cyclophilin (CyP), FK506 binding protein (FKBP) and parvulin (Pvn), have been studied in detail in Eukarya and Bacteria (eubacteria), little is known about archaeal PPIases. Among 13 cyclophilins found in Archaea, only *Halobacterium* cyclophilin (HbsCyP19) has been characterized. This is a cyclosporin A (CsA) sensitive CyP with a molecular weight of 19.4 kDa. The PPIase activity and CsA sensitivity of HbsCyP19 is higher at higher salt concentration in the medium. No parvulin except a homolog in *Cenarchaeum symbiosum* has been found in Archaea. Two types of FKBPs, 26-30 kDa long-type and 17-18 kDa short-type FKBP, have been found in Archaea. Up to date, 12 short-type FKBPs and 18 long-type FKBPs have been known. The short-type FKBPs and N-terminal sequences of the long-type FKBPs are similar to each other and show homology to human FKBP12 (HsFKBP12). However, they have two insertion sequences in the regions corresponding to bulge and flap loops of HsFKBP12. The long-type archaeal FKBPs have additional ca. 100 amino-acid sequences at their C-terminal regions. A short-type archaeal FKBP from *Methanothermococcus thermolithotrophicus* has not only a PPIase activity but also a chaperone-like activity, which includes protein refolding and aggregation suppressing activities with regard to protein folding intermediates. Mutational analysis revealed that this chaperone-like activity was independent of the PPIase activity, and that the insertion sequence in the region corresponding to the flap seemed to be important. Three-dimensional structure of this FKBP showed that the insertion in the flap makes a domain which has a hydrophobic surface. Coexpression of aggregation prone proteins with these archaeal FKBPs were shown to improve their expression in soluble fraction in *Escherichia coli.* Fusion protein of the archaeal FKBP and an aggregation prone protein also show improved expression of the latter in *E. coli*.

2. OVERVIEW OF PPIASE

2.1. History of PPIase discovery

Among peptide bonds in the protein, rotation of prolyl imide bond is slow and is the rate-limiting step in the folding process of proteins. An enzyme which catalyses this slow rotation was found and called peptidyl prolyl *cis-trans* isomerase (PPIase) (1). In 1989, porcine kidney PPIase was purified and its gene was cloned. It was shown to have the same amino acid sequence with that of bovine

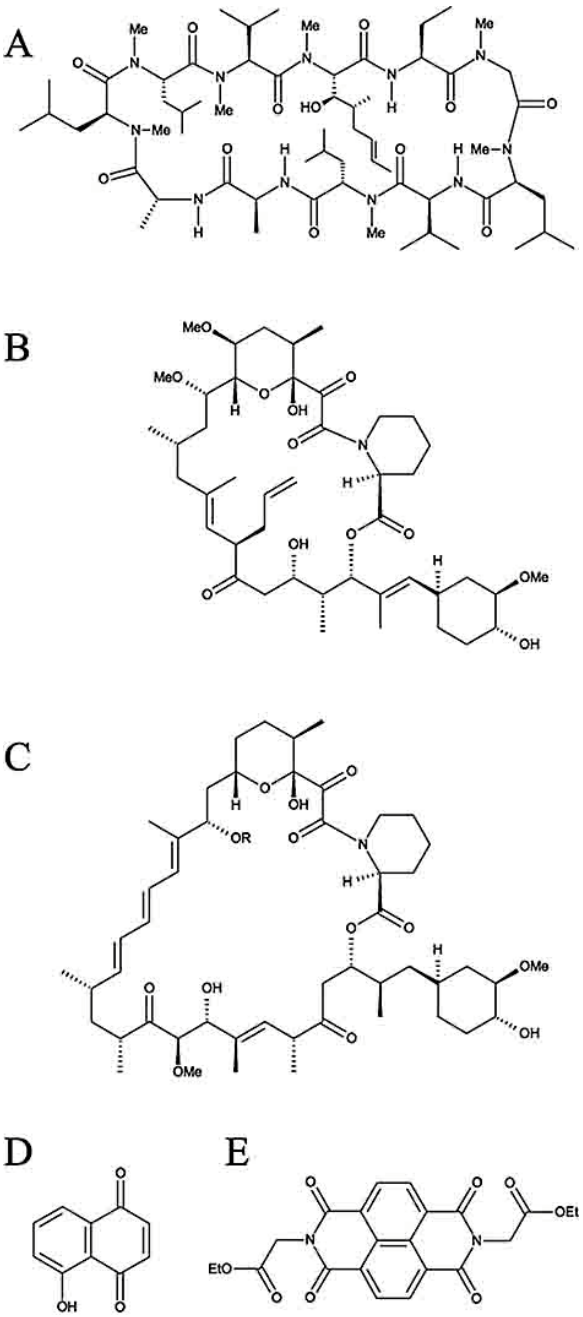

Figure 1. Chemical structures of PPIase inhibitors. (A) cyclosporin A, (B) FK506, (C) rapamycin, (D) juglone, (E) PiB.

cyclophilin (CyP), a target protein for an immunosuppressant, cyclosporin A (CsA) (2, 3). Soon after this discovery, a target protein for FK506 (FKBP, FK506 binding protein), a macrolide immunosuppressant, was also shown to be a PPIase, while amino-acid sequence homology between them was low (4). Another macrolide immunosuppressant, rapamycin (Rap) also binds to FKBP (5). In 1994, the third family of PPIase, parvulin (Pvn), was discovered in *Escherichia coli* (6). This PPIase has low homology to CyPs and FKBPs, and insensitive to either CsA or FK506. The PPIase is also referred to as rotamase or protein foldase. Because CyPs and FKBPs bind the corresponding immunosuppressants, they are also called immunophilins. Various PPIases in these three families have been reported and characterized. Usually more than one gene in each of these families are found in eukaryotic and prokaryotic cells. Even a parasitic bacterium, *Mycoplasma genitalium,* with the smallest known genome of 0.58 Mbp has a gene for a FKBP homolog, trigger factor (7, 8). These observations suggest that PPIase plays an essential role in the living cell. However, mutational analysis has revealed that in yeast all of the 8 CyP and 4 FKBP genes are dispensable, indicating that they are not essential for the growth (9).

Function of PPIase is still enigmatic. While PPIases have been studied in Eukarya and Bacteria, little is known about PPIases in Archaea. Many members of Archaea live in extreme environments with high temperature, high salt concentration and/or extremely anaerobic conditions. Studies on archaeal PPIases may show the new function of PPIase in the life in extreme environments. Since a variety of PPIases have been reported with different names, it is useful to name them with species name, type of PPIase and molecular weight in kDa. For example, *E. coli* SlyD (10) is called EcFKBP20 (*E. coli* FKBP with molecular weight of 20 kDa).

2.2. Three families of PPIase

Three PPIase families have been reported. Cyclophilins (CyP) are PPIases sensitive to CsA (figure 1) or homologs to the prototype CyP, mammalian CyP18. The smallest human CyP is HsCyP18 with molecular weight of 18 kDa (11) and the largest one is a giant nucleopore protein, HsCyP358 (Nup358 or nucleoporin), with molecular weight of 358 kDa (12). Despite of the wide variety of their molecular weights, most of them have only one PPIase domain. Most small CyPs in Eukarya have high binding affinity to CsA; *i.e.*, IC_{50} of HsCyP18 against CsA is approximately 6 nM (13). Bacterial CyPs have lower affinities for CsA; two CyPs from *E. coli* are insensitive to CsA (14) but *Bacillus subtilis* BsCyP17 is moderately sensitive (IC_{50}=120 nM) (15). Crystallographic analysis has revealed that human HsCyP18 has eight antiparallel beta-strands forming a beta-barrel structure and two alpha-helices (16). 3D structures of bacterial and eukaryotic CyPs including HsCyP18 are available in Protein Data Bank (PDB: http://www.rcsb.org/pdb/).

Cyclophilins have been found in multicellular eukaryotes (animals and plants), unicellular eukaryotes, and bacteria except some parasitic bacteria such as *M. genitalium* (7) and some thermophilic bacteria, *Aquifex aeolicus* and *Thermotoga maritima* (17, 18; http://www.ncbi.nlm.nih.gov/cgi-bin/COG/palox?COG0652). Some viruses, such as HIV, contain the CyP of host cell in their virion which is important for starting reproduction after infection to the target cells (19). Large CyPs, which are involved in the interaction with various cellular components, have multi-domain structures to execute their function. Human HsCyP40, which is a component of heterocomplex of steroid receptor and HSP90, has three units of TPR (tetratrico-pepetide-repeat) domain and

calmodulin binding motif at its C-terminus (20). The TPR is a degenerate 34 amino-acid motif involved in various protein-protein interactions and found in the three domains of living organisms, Eukarya, Bacteria and Archaea (21). The giant human HsCyP358 have a leucine rich region, eight zinc finger domains and RanBP (Ras related nuclear protein binding protein) homologous domains in addition to a cyclophilin domain (12).

PPIases with high binding affinity for FK506 (figure 1) and their homologous proteins are called FK506 binding protein (FKBP). The prototype of this group is human FKBP12 (HsFKBP12) (22). Three-dimensional structure of HsFKBP12 has been resolved by X-ray crystallography (23) and NMR spectroscopy (24, 25), and is available at PDB (http://www.rcsb.org/pdb/) as well as many bacterial and eukaryotic FKBPs. HsFKBP12 has five antiparallel beta-strands wrapping around a short alpha-helix. Like large CyPs, large FKBPs have multi-domain structures but frequently they contain multi-FKBP domains while large CyPs have only a single CyP domain. HsFKBP52 is a component of a heterocomplex of HSP90-steroid hormone receptor and has two FKBP-, three TPR- and a calmodulin binding-domain (26). An *E. coli* FKBP, SlyD (EcFKBP20), which is required for the sensitivity to a phage, phiX174, is inhibited by Ni^{2+} and is composed of two domains, a N-terminal FKBP domain and a histidine rich C-terminal domain (27).

Trigger factor (EcFKBP48) was originally found as chaperone (28, 29) and later it was revealed to be a member of bacterial FKBP homologs (30). Trigger factor has low homology to other FKBPs without detectable binding affinity to FK506 or Rapamycin (31). It binds to the 50S ribosomal subunit in the vicinity of peptide tunnel in the ribosome and is thought to participate in folding of nascent proteins (32, 33). Trigger factor has high PPIase activity and is composed of three domains, middle FKBP domain and flanking N- and C-terminal domains. While the truncated middle FKBP domain has high PPIase activity for short peptide substrates, both N- and C-terminal domains are required for high PPIase activity for protein substrates (34). 3D structure of FKBP domain of trigger factor from *M. genitalium* (MgFKBP51) is similar to that of HsFKBP12, except the shorter bulge and flap loops (35). Although the trigger factor itself is not essential for survival of *E. coli*, its depletion in the DnaK lacking mutant is lethal, resulting in massive aggregation of cytosolic proteins (36, 37). The similar double mutant does not affect the growth of *B. subtilis* but suffers the survival at high temperature (38). DnaK and trigger factor are mechanistically distinct but probably cooperate to promote proper folding of a variety of proteins, based on their overlap in substrate specificities (39). Genes for the trigger factor have been found in the genomes of many bacteria including two hyperthermophilic bacteria, *A. aeolicus* and *T. maritima* (17, 18; http://www.ncbi.nlm.nih.gov/cgi-bin/COG/palox?COG0544).

The third family of PPIase is the Parvulin (Pvn: from Parvulus, very small in Latin) family. The prototype of this family was found as the smallest *E. coli* PPIase with no significant homology with the other two families of PPIases (6). Parvulins have no affinity to the immunosuppressant, FK506 or CsA. Larger parvulins have been reported in Bacteria and Eukarya. SurA (EcPvn47) is a periplasmic *E. coli* parvulin with two parvulin domains which is thought to be involved in the folding of the outer membrane protein, porin (40). Eukaryotic members of this family, human Pin1 (HsPvn18) and yeast ESS1 (ScPvn19) are essential for cell cycle progression (41, 42). Pin1/ESS1 and related larger parvulins have a WW domain, which contains 38-40 amino-acid residues in a triple-stranded antiparallel beta-sheet with two highly conserved tryptophan residues and functions as a phosphoserine and phosphothreonine binding module in protein-protein interaction of the signal transduction (43). PPIase domain of HsPvn18 recognizes phosphoserine and phosphothreonine before proline residue and catalyzes the isomerization of these peptide bonds (44, 45). Two specific irreversible inhibitors of PPIase activity, juglone (46) and PiB (47), have been reported for this family (figure 1). Genome sequence analyses have revealed that hyperthermophilic bacteria, *T. maritima* and *A. aeolicus* have two and three parvulin homologs in each of their genomes, respectively (17, 18; http://www.ncbi.nlm.nih.gov/cgi-bin/COG/palox?COG0760).

Several 3D structures of parvulin PPIase domains from human, *Arabidopsis thaliana* and *E. coli* have been resolved (http://www.rcsb.org/pdb/). The structure of parvulin PPIase domain consists of four-stranded antiparallel beta-sheet and four alpha-helices. Despite the lack of homology in primary sequence, the core beta-sheet and one of the alpha-helices in parvulin structure resemble the FKBP fold (44).

2.3. Mechanism of immunosuppression by cyclosporin A, FK506 and rapamycin

Inhibition of PPIase activity is not important in the immunosuppressive action of CsA or FK506. Instead, the ligand-PPIase complexes act as inhibitors for signal transduction pathways in the immunological reactions. CsA-cyclophilin complex or FK506-FKBP complex binds and inhibits a protein phosphatase, calcineulin which is involved in the signal transduction to produce a T-cell growth factor, interleukin (IL)-2 (for review, see 48).

Interestingly, rapamycin (Rap) which also binds to FKBP mediates immunosuppression by a different mechanism. The Rap-FKBP complex binds to a protein kinase, TOR (Target of Rapamycin) in yeast or mTOR (mammalian TOR) (for review, see 49). This protein regulates the activity of p70S6 kinase, which is a key enzyme involved in cell-cycle signal transductions. Consequently, the Rap-FKBP complex inhibits IL-2 stimulated growth of T-cells.

2.4. Assay methods for PPIase activity

A chymotrypsin (or trypsin) coupled assay method for PPIase activity was first invented by Fischer *et al.* in 1984 and is widely used (1, 50). Chymotrypsin cleaves only the *trans*-form of the Xaa-Pro bond (where Xaa is the preceding amino acid) of a small model peptide such as N-succinyl-Ala-Xaa-Pro-Phe-*p*-nitroanilide (N-suc-Ala-Xaa-Pro-Phe-pNA). In aqueous solution, 90% of Xaa-Pro bond of this molecule is in *trans*-conformation. After

addition of excess amount of alpha-chymotrypsin, the *trans* form of Xaa-Pro bond is cleaved instantaneously. The hydrolysis rate of the remaining 10% Xaa-Pro bond is limited by its *cis* to *trans* isomerization. Thus, the *cis-trans* isomerization rate of the model peptide is measured by the release of *p*-nitroanilide by absorption spectroscopy.

A chymotrypsin-free spectrophotometric assay was developed by Janowski (51). In the mixture of trifluoroethanol and LiCl, the percentage of *cis* conformation of the Xaa-Pro bond of a small peptide N-suc-Ala-Xaa-Pro-Phe-pNA increases to 70%. After a solvent jump from this solution to aqueous buffer, *cis-trans* isomerization occurs. *Cis-trans* isomerization is measured by the small difference in absorbance between the *cis* and *trans* forms of the prolyl imide bond in the model peptide. This method is useful to detect the PPIase activity of yeast mitochondrial cyclophilin, Cpr3 (ScCyp20), whose rotamase activity cannot be measured by the conventional chymotrypsin coupled assay (52). NMR spectroscopy is also employed to directly measure the interconversion rate of *cis* and *trans* forms of peptides (53, 54). If the conventional method does not work in some conditions, the other methods might help.

To study the PPIase activity in proteins, ribonuclease T1 (RNase T1) is a suitable model, because it is completely refoldable and its refolding is rate-limited by prolyl isomerization (55). RNase T1 is a small peptide of 104 amino acids containing two disulfide bonds (Cys2-Cys10 and Cys6-Cys103), and two *cis* peptidyl prolyl bonds (Tyr38-Pro39 and Ser54-Pro55). The refolding of 8 M urea-denatured RNase T1 proceeds in a biphasic fashion because of these two *trans* to *cis* prolyl isomerizations. Mücke and Schmid (56, 57) improved this model protein by reduction of its two disulfide bonds and subsequent carboxymethylation. The resulting reduced and carboxymethylated (RCM)-RNase T1 is unfolded in 0.1 M Tris/HCl (pH 8.0), and complete refolding is induced by the addition of 1-2 M NaCl. The RCM form of the S54G/P55N variant of RNase T1 is a suitable substrate for studying PPIase activity in protein folding. Only a single *trans-cis* isomerization of Pro39 is involved in its folding. This folding system does not require denaturants, such as guanidine hydrochloride or urea which often affect the activities of PPIases even at low concentration.

2.5. PPIase in *in vivo* protein folding

Although a yeast mutant lacking all the cyclophilins and FKBPs has been shown to be viable (9), there has been ample evidence showing that PPIase is involved in protein folding *in vivo*. Cyclosporin A (CsA) partially inhibits collagen triple-helix formation in chick embryo and human fibroblasts (58). A fruit fly mutant lacking the eye-specific ninaA (DmCyP26) gene has a defect in vision with reduced amount of rhodopsin (59). This cyclophilin was shown to form a complex with a rhodopsin, Rh1, *in vivo* (60). When the *Drosophila* ninaA gene was introduced into an insect cell line Sf9 and expressed, folding of a foreign protein expressed by recombinant baculovirus was improved (61). In *Saccharomyces cerevisiae*, accumulation of unfolded protein in the endoplasmic reticulum (ER) induced upregulation of ScFKBP13 (62). A periplasmic parvulin of *E. coli*, SurA (EcPvn47), was shown to be involved in the folding and assembly of outer membrane protein, porin (40).

In protein folding in living cells, PPIase may collaborate with other chaperones. PPIase is reported to enhance oxidative folding of RNase T1 by protein disulfide isomerase (63). In mouse fibroblast ER and Golgi, CyPB (MmCyP20) and Hsp47 form a complex with a newly synthesized procollagen I and are involved in its folding (64). In *E. coli* cells, trigger factor (EcFKBP48) cooperate with DnaK in nascent protein folding (36, 37).

2.6. PPIase in *in vitro* protein folding

PPIases increase the refolding rate of RNase T1 and its reduced and carboxymethylated derivative RCM-RNase T1, which are completely refoldable (34, 63, 65).

In addition to this PPIase activity, some PPIases have been suggested to have a chaperone-like activity which prevents aggregation of protein folding intermediates and increases the yield of refolded protein. Porcine kidney CyP (SsCyP18) and N-terminal cyclophilin-homologous domain of NK-TR (tumor recognition protein in natural killer cells, HsCyP150) have chaperone-like activity in addition to the PPIase activity (66, 67). *E. coli* FkpA (EcFKBP29) also shows both activities (68). On the other hand, Kern *et al.* showed that human CyP (HsCyP18), porcine SsCyP18 and human HsFKBP12 accelerate the speed of refolding of human carbonic anhydrase II but did not prevent its aggregation during refolding (69). Human HsCyP18, HsFKBP12 and bovine serum albumin (BSA) were reported to increase the yield of refolding of antibody Fab fragment *in vitro* even in the presence of the inhibitor CsA (70). This suggested that this effect might be a result of nonspecific protein-protein interaction because they were insensitive to the inhibitor and because BSA also showed the similar effect. These somewhat contradictory results have stranded the chaperone-like activity of PPIase in an enigmatic question. In the meanwhile, more PPIases have been reported to have chaperone activity, such as rabbit FKBP52 (OcFKBP52; 71), wheat FKBP73 (TaFKBP73; 72), *E. coli* trigger factor (EcFKBP48; 30, 31), and *E. coli* SurA (EcPvn47; 73). These are large multi-domain PPIases and their chaperone activities reside in the domains other than their PPIase domains or, at least, their PPIase domains are not responsible to their chaperone activities (72-75).

2.7. Other functions of PPIases

At least one PPIase is found in any one of organisms so far studied. Large variety has been found in the PPIases so far reported. They are probably participated in various biological processes. Some of their possible functions are shown in table 1.

3. STRUCTURE AND FUNCTION OF ARCHAEAL PPIASES

3.1. PPIases in Archaea

The domain Archaea includes microorganisms

living under extreme conditions, hyperthermophiles, thermophiles, halophiles and methanogens. Recent genome analyses have revealed many PPIase homologs in archaea. Cyclophilin gene has been found in eight out of eighteen completely analyzed archaeal genomes (table 2). At least one FKBP homolog gene has been found in any archaeal genomes analyzed (table 3). So far, 13 cyclophilin homologs and 30 FKBP homologs have been found in domain Archaea either by genome analyses or by molecular cloning. Hyperthermophilic bacteria, *Thermotoga maritima* and *Aquifex aeolicus* which are phylogenetically closest to archaea, have a trigger factor and two parvulin homologs (17, 18). However, the gene for trigger factor or its homolog has not been found in archaeal genomes (76). Only one archaeal parvulin gene has been found in partially analyzed genome sequence of a symbiotic archaeum *Cenarchaeum symbiosum* (77).

Several molecular chaperones, trigger factor, DnaK, DnaJ, GrpE and GroEs (GroEL and its cofactor, GroES, group I chaperonin) cooperate in the protein folding in the bacterial cytosol (36, 37, 78, 79). HSP60-HSP10 (group I chaperonin) and HSP70 (DnaK homolog) cooperate in mitochondria (81). In eukaryotic cytosol, nascent chain-associated complex (NAC), HSP70, HSP40 (DnaJ homolog), prefoldin and CCT (group II chaperonin) cooperate (80). The genes encoding group II chaperonin are found in all of the archaeal genomes studied. Surprisingly, three *Methanosarcina* species have both group I and group II chaperonins (82, 83, JGI, http://genome.jgi-psf.org/draft_microbes/metba/metba.home.html). While genes encoding DnaK, DnaJ or GrpE, have been found in mesophilic and thermophilic archaea (82-88), they have not been found in the genomes of hyperthermophilic archaea (89-96) except DnaK in *Methanopyrus kandleri* (97). *E. coli* DnaK have been revealed to accelerate *cis-trans* isomerization of nonprolyl bonds (98), but the isomerase activity of DnaK from archaea remains unknown. Trigger factor has not been found in archaea as noted above. NAC is a ribosome-associated heterodimeric complex in eukaryote without PPIase domain (99) but the functional similarity of NAC to trigger factor has been suggested (100). While beta chain of NAC is not found in archaea, all archaeal genomes analyzed other than that of *Sulfolobus solfataricus* contains one gene encoding protein homologous to alpha chain of NAC (82-87, 90-97; http://www.ncbi.nlm.nih.gov/cgi-bin/COG/palox?COG1308). *S. solfataricus* lacks alpha NAC as well as beta NAC (89).

3.2. Structure of archaeal PPIases

3.2.1. Cyclophilins in archaea

A cyclophilin (CyP) was purified from a halophilic archaeum, *Halobacterium salinarum* (formerly called *H. cutirubrum*). This cyclophilin (HbsCyP19) was sensitive to cyclosporin A (CsA) with the IC_{50} value of 15 nM in the presence of 2.9 M KCl (101). While this is the only archaeal CyP characterized so far, genes encoding CyP-like proteins have been found in the genomes of a thermophilic methanogen, *M. thermautotrophicus* (85), a halophilic archaeum, *Halobacterium* sp. NRC-1 (84), an acidophilic archaeum, *Ferroplasma acidarmanus* (JGI, http://genome.jgi-psf.org/draft_microbes/ferac/ferac.home.html) and three mesophilic methanogens: *Methanosarcina barkeri* (JGI, http://genome.jgi-psf.org/draft_microbes/metba/metba.home.html), *M. mazei* (82) and *M. acetivorans* (83) (table 2). No cyclophilin gene or its homolog has been found in complete genome sequences of most of hyperthermophilic archaea, *Archaeoglobus fulgidus* (94), *Pyrococcus horikoshii* (96) and *Aeropyrum pernix* (92).

The alignment of amino-acid sequences of archaeal short CyPs with those of other eukaryotic and bacterial CyPs is shown in figure 2. The amino-acid sequence of HbsCyP19 is 52.9% identical to that of 15 kDa CyP from *M. thermautotrophicus* (MbtCyP15) and 40-45% to those of eukaryotes and *Bacillus subtilis* (BsCyP15) with high CsA-sensitivity, but 27% identical to those of CsA-insensitive CyPs from *Escherichia coli* (102). HbsCyP19 and CyP from *Halobacterium* sp. NRC-1 was reported to have a unique 23-amino-acids insertion sequence that showed no homology to other CyPs from bacteria and eukaryotes (102). However, this insertion sequence is not found in MbtCyP15 or other archaeal CyPs. It may be involved in halotolerance of HbsCyP19. While amino-acid sequence homology between HbsCyP19 and MbtCyP15 is higher than those between HbsCyP19 and other CyPs, MbtCyP15 is more similar to BsCyP15. No distinctive feature is found in archaeal CyPs.

A tryptophan residue corresponding to Trp121 in HsCyP18 is conserved in many eukaryotic CyPs and has been shown to be important for CsA sensitivity (103). In CsA-insensitive CyPs from *E. coli*, this residue is substituted with phenylalanin. CsA sensitive HbsCyP19 has histidine residue at this site. Corresponding histidine residue is also found in BsCyP15. Because these CyPs are sensitive to CsA, replacement of tryptophan with histidine does not affect the CsA sensitivity. On the other hand, it is substituted with tyrosine in other four archaeal CyPs from genera *Methanosarcina* and *Ferroplasma* (figure 2). Tyrosine residue at this site has been found in CYP-4 isoforms from nematodes (104), which are members in the divergent 60 kDa CyP group found in nematodes. 59 kDa human CyP localized in nucleus also has tyrosine at this site (105). The CsA sensitivity of these CyPs and the role of the tyrosine residue are unknown.

Genes for 60 kDa CyP-homolog with three-time repeats of CyP-like sequences have been found in the genomes of methanogens, *M. kandleri* (97), *Methanocaldococcus jannaschii* (91), *M. thermautotrophicus* (85), *M. barkeri* (JGI, http://genome.jgi-psf.org/draft_microbes/metba/metba.home.html), *M. mazei* (82) and *M. acetivorans* (83) (http://www.ncbi.nlm.nih.gov/COG/new/release/cow.cgi?cog=COG4070). Among them, *M. kandleri* and *M. jannaschii* are hyperthermophiles. It may be noteworthy that intracellular K^+ ion concentration of *M. kandleri* is 3 M which is comparable to that of *Halobacterium* (97). These methanogen CyP genes with the repeats show low homology to other CyPs. Moreover, they do not possess

Table 1. Possible functions of PPIases

Function	PPIase	Cells/Organisms	References
Cell cycle progression	Pin1/ESS1 (HsPvn18/ScPvn19)	Eukaryotic cells	42
Restoration of Alzheimer's disease associated phosphorylated tau to interact with microtubules	Pin 1 (HsPvn18)	Human	141
Association with hormone receptors	FKBP52/CyP40	Mammals	142
Apoptosis	Cyclophilins A-C, D	Eukaryotic cells	143, 144
Mitochondrial membrane permeabilization to calcium ion	Cyclophilin D	Eukaryotic cells	144
Calcium ion release from sarcoplasmic reticulum	FKBP12	Mammals	145
Nuclease activity	Cyclophilins A-C	Eukaryotic cells	143
Nucleopore formation and protein transport to nucleus	RanBP2, Nup358	Mammals	12, 146
Protein synthesis and folding of nascent proteins	trigger factor	Bacteria	31, 37
Virulence factor in pathogenic bacteria	Mip[1]	Pathogenic bacteria	147, 148
Possible involvement in parasitism	Cyclophilins	Parasites	149
Virus particle formation and virulence in virus	Cyclophilin A	Virus	150
Nerve regeneration	FKBP52	Mammals	151
Protection against oxidative stress	Cyclophilin A	Mammalian cells	152
Vision (correct folding of rhodopsin)	NinaA (DmCyP26)	Fruit fly	59
Male fertility and fidelity of homologous chromosome pairing	Fkbp6	Mouse	153

1, macrophage infectivity potentiator.

Table 2. Cyclophilins in Archaea

CyPs	Sources	AA[1]	MW(kDa)[2]	PI[2]	IC_{50}	References
MsmCyP17	*Methanosarcina mazei* Goe1	155	17.1	6.06	NR[3]	82
MsaCyP16	*Methanosarcina acetivorans* str. C2A	147	16.2	5.31	NR[3]	83
MsbCyP18	*Methanosarcina barkeri*	162	17.8	6.91	NR[3]	JGI[4]
FpaCyP17	*Ferroplasma acidarmanus*	151	16.7	9.27	NR[3]	JGI[4]
HbNCyP20	*Halobacterium* sp. NRC-1	189	20.5	4.20	NR[3]	84
HbsCyP19	*Halobacterium salinarum*	180	19.4	4.09	15 nM (at 2.9 M KCl), 140 nM (at 1.4 M KCl)	101, 102
MbtCyP15	*Methanothermobacter thermautotrophcus*	141	15.4	5.49	NR[3]	85
MsmCyP58	*Methanosarcina mazei* Goe1	525	58.1	5.30	NR[3]	82
MsaCyP58	*Methanosarcina acetivorans* str. C2A	525	58.3	4.88	NR[3]	83
MsbCyP57	*Methanosarcina barkeri*	518	57.0	4.92	NR[3]	JGI[4]
McjCyP56	*Methanocaldococcus jannaschii*	498	56.3	5.52	NR[3]	91
MbtCyP59	*Methanothermobacter thermautotrophicus*	522	58.5	4.95	NR[3]	85
MpkCyP58	*Methanopyrus kandleri* AV19	511	57.6	4.64	NR[3]	97

1, amino acid residues; 2, calculated using Compute pI/Mw tool at ExPASy molecular biology server (154, http://www.expasy.org/tools/pi_tool.html); 3, not reported; 4, the draft genome sequences are available at JGI web site (http://www.jgi.doe.gov/).

highly conserved arginine residue (Arg55 in HsCyP18) which has been proposed to be an active residue for PPIase activity (106, 107). Further investigations are necessary to discuss the function of these archaeal CyP homologs.

3.2.2. FKBPs in archaea

The short-type and the long-type FKBPs have been reported from Archaea. The molecular masses are approximately 17-18 and 26-30 kDa, respectively (82-87, 89-97, 108-110; table 3). Both a short-type and a long-type archaeal FKBP genes have been found in the genome of *Methanocaldococcus jannaschii* (91). Three *Methanosarcina* species contain genes for three short-type FKBPs and one long-type FKBP (82, 83; JGI, http://genome.jgi-psf.org/draft_microbes/metba/metba.home.html). On the other hand, only one FKBP gene encoding a long-type FKBP was found in the other archaea whose complete genomes have been analyzed. We isolated genes encoding a short-type FKBP from *Methanothermococcus thermolithotrophicus* (108) and *Thermococcus* sp. KS-1 (109). It is not clear whether they have the long-type FKBP, or not.

The alignment of the amino-acid sequences of archaeal FKBPs with those of eukaryotic and bacterial FKBPs are shown in figure 3. The alignment shows that the archaeal short-type FKBPs are similar to HsFKBP12 in amino acid sequence, and the archaeal long-type FKBP has an N-terminal sequence homologous to short-type FKBP with a 100-amino-acids surplus region at the C-terminus.

Table 3. FKBPs from Archaea

FKBPs	Sources	AA[1]	MW(kDa)[2]	PI[2]	IC_{50}	References
MsbFKBP21	*Methanosarcina barkeri*	192	20.9	4.71	NR[3]	JGI[4]
MsmFKBP17.96	*Methanosarcina mazei* Goe1	166	18.0	4.21	NR[3]	82
MsaFKBP19	*Methanosarcina acetivorans* str. C2A	181	19.4	4.44	NR[3]	83
MsbFKBP18	*Methanosarcina barkeri*	156	17.6	5.22	NR[3]	JGI[4]
MsmFKBP18.2	*Methanosarcina mazei* Goe1	160	18.2	5.57	NR[3]	82
MsaFKBP18.2	*Methanosarcina acetivorans* str. C2A	162	18.2	4.81	NR[3]	83
MsbFKBP19	*Methanosarcina barkeri*	169	18.8	4.48	NR[3]	JGI[4]
MsmFKBP18.03	*Methanosarcina mazei* Goe1	163	18.0	4.40	NR[3]	82
MsaFKBP17.8	*Methanosarcina acetivorans* str. C2A	160	17.8	4.39	NR[3]	83
TcFKBP18	*Thermococcus* sp. KS1	159	17.6	4.32	7 μM	109
MtFKBP17	*Methanothermococcus thermolithotrophicus*	154	17.2	4.25	250 nM	108
MjFKBP18	*Methanocaldococcus jannaschii*	157	17.7	4.44	170 nM	91, 140
MjFKBP26	*Methanocaldococcus jannaschii*	231	25.9	6.69	IC_{70}=20 μM	91, 140
FpaFKBP30	*Ferroplasma acidarmanus*	257	29.7	5.20	NR[3]	JGI[4]
TpvFKBP30	*Thermoplasma volcanium*	268	30.4	5.74	NR[3]	86
TpaFKBP30	*Thermoplasma acidophilum*	261	29.9	5.18	NR[3]	87
AppFKBP30	*Aeropyrum pernix*	268	29.6	4.93	NR[3]	92
HbsFKBP33	*Halobacterium salinarum* *Halobacterium* sp. NRC-1	303	33.3	3.55	insensitive	110 84
AfFKBP29	*Archaeoglobus fulgidus* DSM 4304	253	29.0	4.71	NR[3]	94
MsbFKBP28	*Methanosarcina barkeri*	254	28.1	4.58	NR[3]	JGI[4]
MsmFKBP28	*Methanosarcina mazei* Goe1	250	27.6	4.62	NR[3]	82
MsaFKBP28	*Methanosarcina acetivorans* str. C2A	255	28.2	4.59	NR[3]	83
PbaFKBP26	*Pyrobaculum aerophilum*	229	25.5	5.46	NR[3]	93
MpkFKBP24	*Methanopyrus kandleri* AV19	219	24.2	4.45	NR[3]	97
SlsFKBP27	*Sulfolobus solfataricus*	235	26.7	6.66	NR[3]	89
SltFKBP27	*Sulfolobus tokodaii*	233	26.7	6.25	NR[3]	90
MbtFKBP28	*Methanothermobacter thermautotrophicus*	250	28.3	4.70	insensitive	85, 126
PfFKBP29	*Pyrococcus furiosus* DSM 3638	258	29.1	5.03	NR[3]	95
PhFKBP29	*Pyrococcus horikoshii*	257	29.0	5.07	IC_{75}=20 μM	96, 129
PaFKBP30	*Pyrococcus abysii*	266	30.0	5.18	NR[3]	Genoscope[5]

1, amino acid residues; 2, calculated using Compute pI/Mw tool at ExPASy molecular biology server (154, http://www.expasy.org/tools/pi_tool.html); 3, not reported; 4, the draft genome sequences are available at JGI web site (http://www.jgi.doe.gov/); 5, the draft genome sequence is available at Genoscope web site (http://www.genoscope.cns.fr/).

This C-terminal region shows no homology to the sequences in database. The secondary structures of HsFKBP12 are arranged in the order: (N-terminus)-beta 1-beta 4-beta 5a-beta 5b-alpha-beta 2-beta 3-(C-terminus). Between beta 2 and beta 3 strands, a surface loop called "flap" exists, and in the middle of beta 5 strand, an intervening sequence called "bulge" splits beta 5 strand into beta 5a and beta 5b (23, 30). The alignment analysis revealed that the archaeal FKBP has a 12- or 13-amino acids insertion sequence in the bulge region of HsFKBP12, and a 41- to 49-amino acids insertion sequence in the flap region of HsFKBP12. *E. coli* SlyD (EcFKBP20) and SlpA (EcFKBP16) also has an insertion sequence homologous to the archaeal insertion in the flap region, but they do not have insertion sequence in the bulge region (figure 3). Interestingly, there is another group of bacterial FKBPs without both of the insertion sequences. This group involves *Legionella pneumophila* Mip (LpFKBP25) and *E. coli* FkpA (EcFKBP29).

3.2.3. 3D structure of archaeal FKBP

Recently, 3D structure of MtFKBP17 has been solved by NMR (111, 112). Figure 4 shows the 3-D structure of MtFKPB17 and HsFKBP12 with positions of the insertions. MtFKBP17 is composed of two domains. One is a typical PPIase domain of FKBP family. The insertion in the bulge loop is included in the PPIase domain. The other domain is formed by the 44-amino acids insertion in the flap loop; this domain is named as IF (the Insert in the Flap) domain. The PPIase domain of MtFKBP17 consists of a four-stranded antiparallel beta-sheet accompanied by two alpha-helices with a topology of beta 4-beta 5a-alpha 2-beta 5b-alpha 1-beta 2-beta 3. MtFKBP17 lacks an N-terminal beta-strand (beta 1) observed in HsFKBP12, but other secondary structures of HsFKBP12 are conserved. The unique alpha 2-helix is located in the insertion in the bulge loop between beta 5a and beta 5b. The side chains on alpha 2-helix form a hydrophobic cluster together with the side chains on the beta-sheet and stabilize beta 5a-strand. The IF domain consists of a four-stranded beta-sheet and an alpha-helix. No similar fold to the IF domain is found in the atomic coordinates deposited in PDB. The surface of IF domain is highly hydrophobic.

The number of beta-strands in the PPIase domain of FKBPs varies from four to six. HsFKBP12 has a five-stranded beta-sheet (23). Yeast FKBP12 (ScFKBP12; 113),

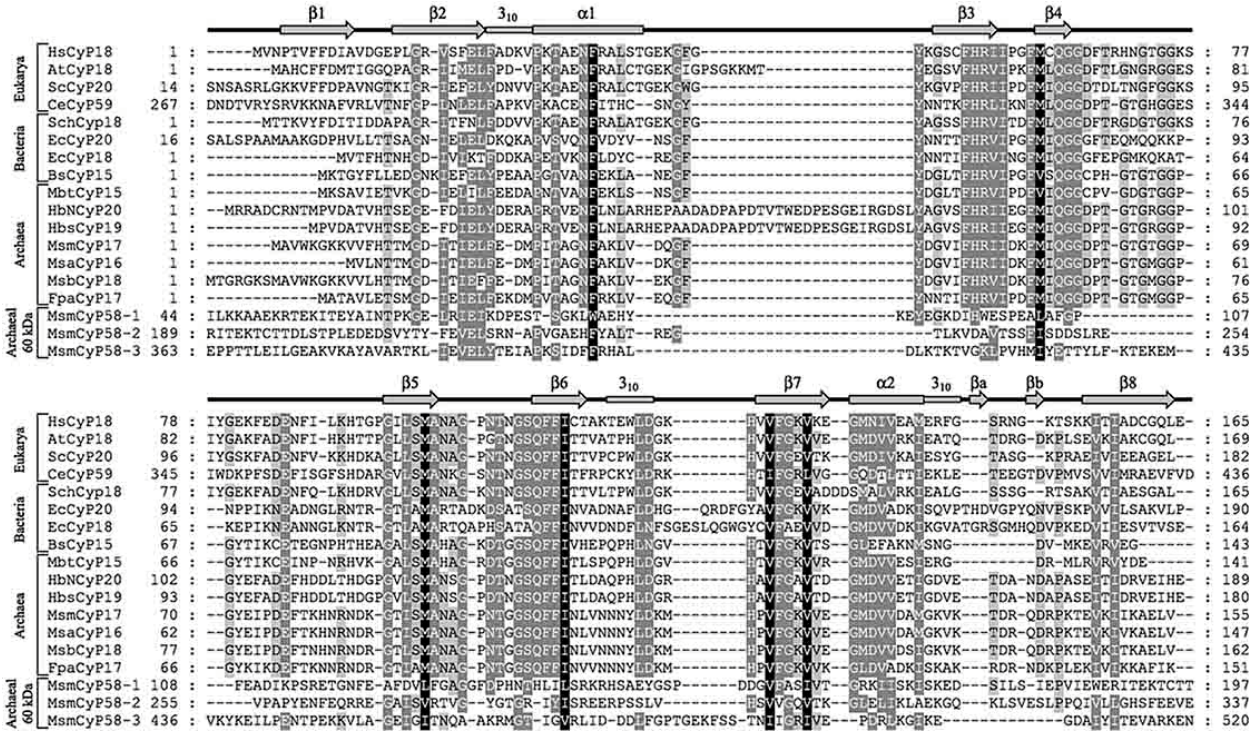

Figure 2. Alignment of archaeal CyP amino acid sequences with those of other bacterial and eukaryotic CyPs. Secondary structure elements reported for HsCyP18 or EcCyP18 are shown above the aligned sequences. For the abbreviations for archaeal CyPs, see table 2. Source of other CyPs are; HsCyP18 (human CyP A); AtCyP18 (*Arabidopsis thaliana*); ScCyP20 (*S. cerevisiae* CYP3); CeCyP59 (*Caenorhabditis elegans* CYP-4); SchCyP18 (*Streptomyces chrysomallus*); EcCyP20 (*E. coli* CyP a); EcCyP18 (*E. coli* CyP b); BsCyP15 (*B. subtilis*).

rabbit FKBP52 (OcFKBP52; 114), and bacterial Mip-type FKBP from *Legionella pneumophila* (LpFKBP25; 115) have an extra beta-strand at the N-terminus (figure 5). On the other hand, human FKBP13 (HsFKBP13; 116) and trigger factor from *M. genitalium* (MgFKBP51; 35) has a four-stranded beta-sheet. They have N-terminal segment corresponding to beta 1-strand, but these regions were thought to be disordered. MtFKBP17 also has a four-stranded beta-sheet and lacks beta 1-strand because the corresponding sequence is absent in MtFKBP17 (112). Thus, a four-stranded beta-sheet and an alpha-helix (beta 4-beta 5a-beta 5b-alpha-beta 2-beta 3) would form a minimal structural frame required for the FKBP fold.

The sequences of insertion in the flap of archaeal FKBPs and bacterial SlyD/SlpA-type FKBPs show significant homology to each others and these FKBPs are expected to have IF domain. On the other hand, Eukaryotic FKBPs, bacterial Mip/FkpA-type FKBPs and trigger factors do not possess this insertion sequence for the IF domain. Thus FKBP is classified by the presence or absense of IF domain. Because trigger factors show low homology to other FKBPs, there are three groups of FKBP; 1) FKBPs without IF domain in eukarya and bacteria, 2) FKBPs with IF domain in archaea and bacteria and 3) trigger factors without IF domain in bacteria. Some bacteria such as *Chlamydia pneumoniae* has only group 1 FKBP (117) while *Helicobacter pylori* has only group 2 FKBP (118). *E. coli* possesses both types of FKBP (119) and *M. genitalium* lacks both FKBPs (7). In contrast, all reported bacteria have group 3 FKBP, trigger factor, which is unique to bacteria.

3.3. PPIase activity of the short-type archaeal FKBPs

The PPIase activities of archaeal FKBPs and other PPIases against tetrapeptide substrate are shown in table 4. The catalytic efficiencies (k_{cat}/K_M) for N-suc-Ala-Leu-Pro-Phe-pNA of MtFKBP17 and that of *Thermococcus* short-type FKBP (TcFKBP18) are 350 $mM^{-1}S^{-1}$ (108, 109). These values are similar to those of *E. coli* trigger factor (EcFKBP48; 430 $mM^{-1}S^{-1}$) (31) and human HsFKBP12 (640 $mM^{-1}S^{-1}$) (120), and significantly lower than those of *E. coli* EcCyP18 (23400 $mM^{-1}S^{-1}$) (121) and bovine BtCyP18 (2700 $mM^{-1}S^{-1}$) (120). The PPIase activities of MtFKBP17 and TcFKBP18 are inhibited by FK506 with IC_{50} values of 250 nM and 7 μM, respectively (108, 109).

Generally, cyclophilins prefer N-suc-Ala-Ala-Pro-Phe-pNA to N-suc-Ala-Leu-Pro-Phe-pNA, while the reverse is true for FKBPs (table 4). The Leu /Ala values, the ratio of k_{cat}/K_M for N-suc-Ala-Leu-Pro-Phe-pNA versus that for N-suc-Ala-Ala-Pro-Phe-pNA, of thermophilic archaeal FKBPs are 1.2 to 1.85, respectively, while those of other FKBPs are 2.69 to 22.0 (table 4). This may indicate that archaeal FKBPs have broad substrate specificity. The 3D structure of MtFKBP17 revealed that the small substrate-binding pockets of MtFKBP17 would be able to fit both leucine and alanine residues preceding to proline, whereas the pocket of HsFKBP12 would be too big to fit alanine (112).

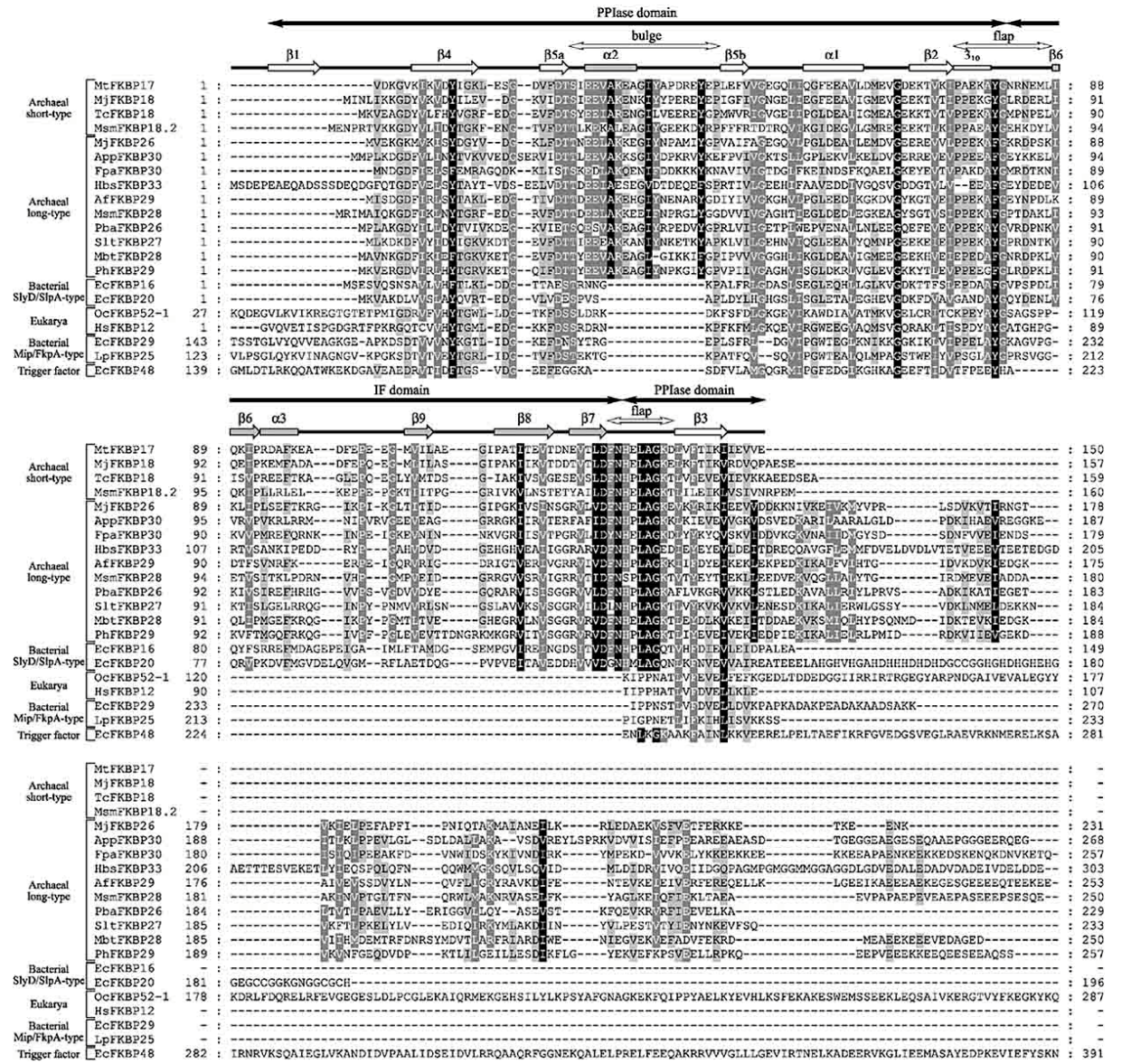

Figure 3. Alignment of archaeal FKBP amino acid sequences with those of other bacterial and eukaryotic FKBPs. Secondary structure is shown above the alignments. Secondary structure elements of HsFKBP12 are colored gray and those found only in MtFKBP17 are colored white. The position of flap and bulge loops, and the domain structure of MtFKBP17 are shown above the secondary structure. For the abbreviations for archaeal FKBPs, see table 3. Source of other FKBPs are; EcFKBP16 (*E. coli* SlpA); EcFKBP20 (*E. coli* SlyD); OcFKBP52-1 (first FKBP domain of rabbit FKBP52); HsFKBP12 (human FKBP12); EcFKBP29 (*E. coli* FkpA); EcFKBP22, (*E coli* Mip-like FKBP); EcFKBP48 (*E. coli* trigger factor).

At higher temperature, it is expected that the spontaneous rotation rate of peptidyl-prolyl imide bond increases. With increasing temperature (15-35°C), the difference between the rate constant of spontaneous peptidyl-prolyl isomerization of tetrapeptide and that of the MtFKBP17-catalyzed isomerization became smaller (108) (figure 6). This suggests that PPIase activity at least for small peptides is less important at higher temperature.

3.4. Chaperone-like activity of the short-type archaeal FKBP

Two different model substrate proteins, ribonuclease T1 (RNase T1) and rhodanese, have been used to study the protein folding activity of a short-type archaeal FKBP (122). RNase T1 is completely refoldable and has two *cis* peptidyl-prolyl bonds (Tyr38-Pro39 and Ser54-Pro55) (55). On the other hand, folding intermediates of rhodanese tend to aggregate and thus rhodanese is convenient to assess the activity of chaperones by measuring their capacity to prevent aggregation of folding intermediates and to increase the yield of properly folded rhodanese. *Escherichia coli* GroE binds to folding intermediates of rhodanese and releases them in an ATP-dependent fashion (123, 124).

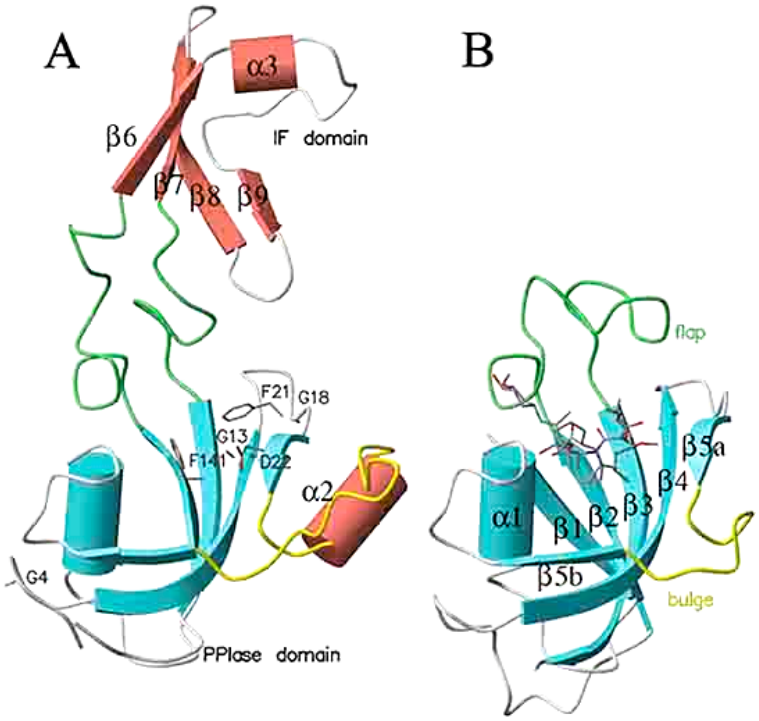

Figure 4. Three dimensional structure of *Methanothermococcus thermolithotrophicus* MtFKBP17 (A) and human HsFKBP12 (B). FK506 molecule bound to HsFKBP12 is shown in stick model. The bulge and flap loops of HsFKBP12 and corresponding loops in MtFKBP17 are shown in yellow and green, respectively. The secondary structures found in HsFKBP12 are colored blue and those found only in MtFKBP17 are colored red. Positions of amino-acid substitutions in MtFKBP17 mutants are also shown. FK-dB is a deletion mutant lacking the insertion sequence in the region of bulge. FK-dF is the deletion mutant lacking the insertion sequence in the flap region and FK-dBF is the deletion mutant lacking both of these insertion sequences (122). Reprinted from ref. 112 with permission from Elsevier.

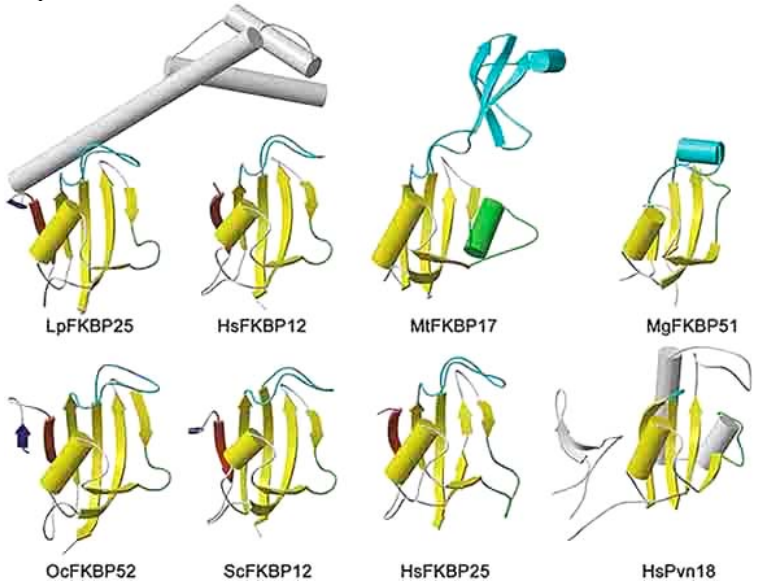

Figure 5. Structural comparison of proteins with the FKBP fold. The common secondary structure elements are colored yellow. Extra beta-strand at the N-terminus and beta 1-strand are colored blue and red, respectively. The flap and bulge regions are colored light blue and green, respectively. The other structural elements, N-terminal dimerization domain of LpFKBP25, N-terminal WW domain of HsPvn18, and two alpha-helices between beta 4 and beta 5b of HsPvn18 are shown in gray. Atomic coordinates are obtained from PDB: LpFKBP25 (*L. pneumophila* Mip), 1FD9; HsFKBP12, 1FKF; MtFKBP17, 1IX5; MgFKBP51 (*M. genitalium* trigger factor), 1HXY; OcFKBP52 (first FKBP domain of rabbit FKBP52), 1ROT; ScFKBP12 (Yeast FKBP1), 1YAT; HsFKBP25 (human FKBP25), 1PBK; HsPvn18 (human Pin1), 1PIN. Reprinted from ref. 112 with permission from Elsevier.

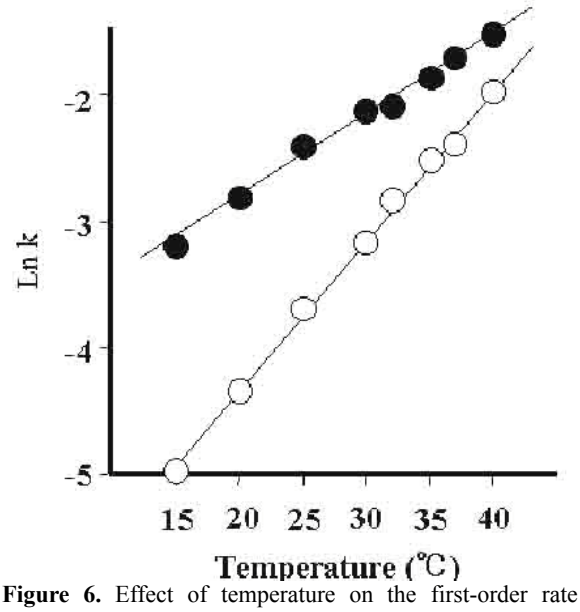

Figure 6. Effect of temperature on the first-order rate constant (K) of *cis-trans* isomerization measured using tetrapeptide substrate and chymotrypsin coupled assay. Open circles, absence of PPIase; closed circles, presence of MtFKBP17 (108).

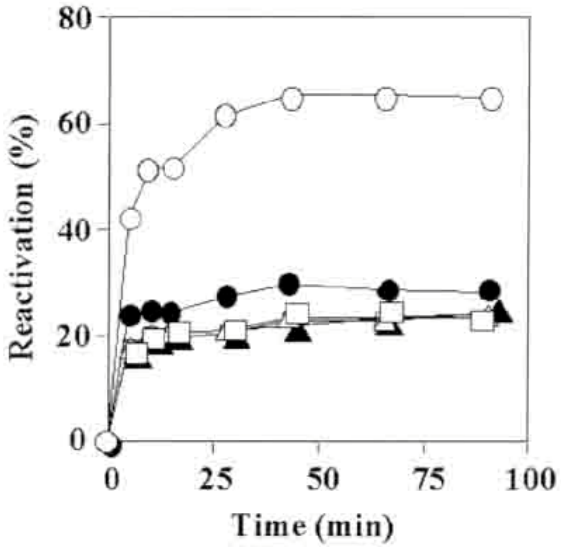

Figure 7. Effect of PPIases on the refolding of denatured rhodanese. The 6 M guanidine hydrochloride-denatured rhodanese was diluted 60 fold at time 0 min. with the refolding buffer (10 mM DTT, 50 mM Na-thiosulfate, and 50 mM K-phosphate (pH 7.8) containing PPIase at 25°C. Open squares, absence of PPIase; open circles, 5 μM recombinant MtFKBP17; closed circles, 5 μM recombinant MtFKBP17 with 20 μM FK506; closed triangles, 5 μM recombinant human HsFKBP12; open triangles, 5 μM bovine BtCyP18 (122).

MtFKBP17 accelerates the refolding of 8 M urea-denatured RNase T1 in a dose-dependent fashion. This PPIase activity of MtFKBP17 is completely inhibited by FK506 (122). MtFKBP17 protects aggregation of folding intermediates and elevated the final recovery of rhodanese refolding in dose-dependent fashion (figure 7; 122). This is called chaperone-like activity of MtFKBP17 and is partially inhibited by FK506. Neither human HsFKBP12 nor bovine BtCyP18 exhibits chaperone-like activity in rhodanese refolding. The k_{cat}/K_M values (PPIase activity) of HsFKBP12, BtCyP18, and the recombinant MtFKBP17 for N-suc-Ala-Leu-Pro-Phe-pNA are 0.64 (120), 2.7 (120), and 0.96 (122) $\mu M^{-1}S^{-1}$, respectively. These comparable PPIase activities raise the question whether the PPIase activity of MtFKBP17 contributes the chaperone-like activity or not.

3.5. Deletion analysis of the short-type FKBP from *M. thermolithotrophicus*

The role of the insertion sequences of a short-type archaeal FKBP, MtFKBP17, in protein folding has been analyzed by making deletion mutants (122) (figure 4). Catalytic efficiency (k_{cat}/K_M) of PPIase activity of the mutant with deletion of the bulge insertion (FK-dB), or both bulge and flap insertions (FK-dBF), was dramatically reduced to 0.4 and 0.6% as compared with the wild type MtFKBP17 (FK-W), respectively. The PPIase activity of the mutant lacking the flap insertion (FK-dF) was undetectable. The far-UV circular dichroism (CD) spectral analysis revealed that the both bulge and flap insertions are important for a proper conformation of MtFKBP17 (122). While the secondary structure of FK-dB was shown to be changed, 62% of chaperone-like activity of the wild type remained (table 5). On the other hand, FK-dF that has an almost intact secondary structure exhibited little chaperone-like and undetectable PPIase activities. This suggests that the flap insertion sequence is important for the chaperone-like activity of MtFKBP17, and that the contribution of PPIase activity to chaperone-like activity is low.

3.6. Is chaperone activity of the short-type archaeal FKBP independent of PPIase activity ?

The contribution of MtFKBP17 PPIase activity to chaperone-like activity has been estimated by analyzing amino-acid substitution mutants with reduced PPIase activities (figure 4; 122). The CD spectra of the substitution mutants, F21Y/D22V, G4R/F21Y/D22V, G18R/F21Y/D22V, and F141Y, indicated that the secondary structure was intact in these mutants (122). While the double mutant, F21Y/D22V exhibited 1.3% of the PPIase activity of wild-type (FK-W), it exhibited chaperone-like activity comparable to that of FK-W (table 6; 122). This indicates that Phe21 and Asp22 are important for PPIase activity, but not for chaperone-like activity. The triple mutants, both G4R/F21Y/D22V and G18R/F21Y/D22V also exhibited little PPIase activity, but their chaperone-like activities remained almost intact. While PPIase activity of F21Y/D22V was lower than that of F141Y which has a dramatically reduced chaperone-like activity, it showed intact chaperone activity. The amino-acid residue Phe141 is probably important not only for PPIase but also for chaperone-like activity. These mutation analyses indicate that chaperone-like activity of MtFKBP17 is independent of PPIase activity. *Thermococcus* short-type FKBP (TcFKBP18) has been shown to exert its chaperone like activity by hydrophobic interaction with the target

Table 4. Catalytic efficiencies of PPIases against N-suc-Ala-Xaa-Pro-Phe-pNA (k_{cat}/K_M ($mM^{-1}s^{-1}$))[1]

Xaa	FKBPs					CyPs	
	Methanothermo-coccus	*Thermococcus*	*Methanothermo-bacter*	*Escherichia*	Human	*Escherichia*	Bovine
	MtFKBP17	TcFKBP18	MbtFKBP28	trigger factor	HsFKBP12	EcCyP18	BtCyP18
Leu	350	350	0.74	430	640	23400	2700
Ala	200	290	0.4	160	53	67400	3180
Leu/Ala[2]	1.75	1.21	1.85	2.69	12.8	0.35	0.71
Reference	76	77	82	23	79	80	79

1, PPIase activities were measured by the chymotrypsin-coupled assay at 10 or 15°C; 2, Ratio of k_{cat}/K_M for N-suc-Ala-Leu-Pro-Phe-pNA to that for N-suc-Ala-Ala-Pro-Phe-pNA

Table 5. Deletion analysis of MTFK (MtFKBP17)

MTFKs	PPIase activity[1]	Chaperone-like activity[2]
	k_{cat}/K_M ($\mu M^{-1}s^{-1}$)	% of rhodanese activity[3]
Wild type (FK-W)	0.96 (100%)[4]	64.2+/-2.8 (100%)[4]
FK-W + FK506	0 (0%)[4]	30.0+/-0.7 (16%)[4]
none[5]	0 (0%)[4]	14.1+/-0.4 (0%)[4]
FK-dB	0.004 (0.4%)[4]	45.3+/-1.7 (62%)[4]
FK-dF	undetectable[6] (0%)[4]	14.5+/-0.4 (0.8%)[4]
FK-dBF	0.006 (0.6%)[4]	15.4+/-0.7 (2.6%)[4]

1, PPIase activity was assayed at 25°C by the chymotrypsin-coupled assay in 50 mM K-phosphate buffer (pH 7.5); 2, For chaperone activity, the denatured rhodanese (37.8 μM) was 60-fold diluted in the presence of 5.0 μM mutants, or FK-W in 50 mM K-phosphate buffer (pH 7.8) containing 10 mM DTT and 50 mM Na thiosulfate at 35°C; 3, Recovered rhodanese activity after 60 min refolding in the presence of FKBPs. Means +/- S.D. (n=3); 4, percentage in parenthesis indicates relative yield of refolded rhodanese to that by wild type (FK-W); 5, Spontaneous refolding of rhodanese without PPIase; 6, No PPIase activity was detected with 5 μM mutant MtFKBP17.

Table 6. PPIase and chaperone-like activities of amino-acid substitution mutants of MtFKBP17 from *M. thermolithotrophicus*

MTFKs	PPIase activity	Chaperone-like activity[2]
	[k_{cat}/K_M ($\mu M^{-1}s^{-1}$)][1]	[% of rhodanese activity][3]
rMtFKBP17[4]	1.0 (100%)[5]	66.7 +/- 3.3 (100%)[5]
FK-dB	0.005 (0.5%)[5]	49.3 +/- 3.4 (65%)[5]
F141Y	0.072 (7.2%)[5]	20.6 +/- 1.6 (8.3%)[5]
F21Y/D22V	0.013 (1.3%)[5]	67.4 +/- 4.1 (101%)[5]
G4R/F21Y/D22V	0.009 (1.0%)[5]	62.7 +/- 3.3 (92%)[5]
G13R/F21Y/D22V	0.006 (0.6%)[5]	32.4 +/- 2.6 (32%)[5]
G18R/F21Y/D22V	0.014 (1.4%)[5]	66.8 +/- 2.5 (100%)[5]
None[6]	n.m.[7]	16.4 +/- 1.5 (0%)[5]

1, PPIase activity was assayed at 25°C by chymotrypsin-coupled assay in 50 mM K-phosphate buffer (pH 7.5); 2, For chaperone activity, the denatured rhodanese (37.8 μM) was diluted 60-fold in the presence of 5.0 μM mutants or wild type recombinant MtFKBP17 in 50 mM K-phosphate buffer (pH 7.8) containing 10 mM DTT and 50 mM Na thiosulfate at 35°C; 3, Recovered rhodanese activity after 60 min refolding in the presence of FKBPs. Mean +/- S.D. (n=3); 4, recombinant wild type MtFKBP17; 5, percentage in the parenthesis indicates relative folding yield to that by wild type (rMtFKBP17); 6, Spontaneous refolding of rhodanese without PPIase; 7, Not measured.

protein (125). At higher concentration of TcFKBP18, more than one of its molecules are thought to bind to the unfolded target protein. When refolding of the target protein proceeds, the number of bound TcFKBP18 decreases (125). The 3D structure of MtFKBP17 suggests that the hydrophobic surface of the IF domain probably plays an important role for the chaperone-like activity (figure 4; 112).

3.7. The long-type archaeal FKBP

The archaeal long-type FKBPs with molecular mass of about 30 kDa have a PPIase domain and an IF domain similar to archaeal short-type FKBPs. Additionally, the long-type FKBPs have surplus 100 amino-acid residues at their C-terminal region. A long-type archaeal FKBP, MbtFKBP28 from *Methanothermobacter thermautotrophicus*, has been shown to have a weak PPIase activity (table 4), which is insensitive to FK506 (126). Another long-type FKBP, AfFKBP29 from a hyperthermophilic sulfate reducer, *Archaeoglobus fulgidus*, also exhibited little PPIase activity (Ideno *et al.*

unpublished data). The substitution of Phe99 with tyrosine in human HsFKBP12 (127), and the corresponding substitutions in *E. coli* trigger factor (EcFKBP48; 127) and MtFKBP17 (122), significantly reduce their PPIase activities. In the protein sequences of MbtFKBP28 and other archaeal long-type FKBPs, the residues corresponding to Phe99 in HsFKBP12 are substituted with tyrosine (figure 3). This may be the cause of low PPIase activity in MbtFKBP28. Most of hyperthermophilic or thermophilic archaea have no cyclophilin but long-type FKBP. These archaeal long-type FKBPs may have higher activity at higher temperature and/or may have some other functions in addition to the PPIase activity.

MbtFKBP28 was recently shown to prevent aggregation of folding intermediates of 8 M urea-denatured rhodanese in a dose dependent-fashion (126). The C-terminal domain of MbtFKBP28 has high content of acidic amino-acid residues (29.0%). The previous studies have revealed that the acidic region of the C-termini of human cyclophilin 40 kDa and HsFKBP52 are important for the interaction with HSP90 (128). The acidic amino acid-rich C-terminal region of MbtFKBP28 may be important for the suppression of protein aggregation (126). Other archaeal long-type FKBPs also have acidic amino acid-rich region (figure 3). This suggests that the aggregation-suppressing activity may be a common feature of archaeal long-type FKBPs.

3.8 Application of archaeal FKBP to foreign protein expression system in bacteria

Short-type archaeal FKBPs have both PPIase and the chaperone-like activities (122, 125). Although long-type archaeal FKBPs have weaker PPIase activities, they also prevent protein aggregation (129). These suggest that they may prevent inclusion body formation when foreign aggregation prone protein is expressed in bacteria, such as *E. coli*. When the recombinant Fab fragment of the anti-hen egg lysozyme antibody was co-expressed with a long-type FKBP, PhFKBP29, in *E. coli*, the final yield of the soluble Fab fragment with its antibody specificity increased significantly (129). The final yield was even higher when the target protein was fused with the short-type FKBP, TcFKBP18 (130). These indicate that those FKBPs from thermophilic archaea improve foreign protein expression in a mesophilic bacterium, *E. coli*.

4. PERSPECTIVES

In vivo-function of archaeal PPIases is yet enigmatic. In high temperature environments, where hyperthermophiles and thermophiles thrive, PPIase activity may be less important because of high spontaneous rotation rate of the peptidyl-prolyl bond. Besides the PPIase activity, the hyperthermophilic and thermophilic archaeal FKBPs have chaperone-like activity or aggregation-suppression activity that may be more important at high temperature. In addition, most hyperthermophilic archaea lack some chaperones like DnaK, DnaJ and GrpE (131) and cyclophilin type PPIase (see the section 3.1). This suggest that these hyperthermophilic archaea do not need these protein folding factors, or that the fewer kinds of protein folding factors are multifunctional and fulfill the requirements for the protein folding in the cells of hyperthermophilic archaea, or that they have unknown new protein folding factors. To understand the *in vivo*-function of archaeal PPIases, the following questions ought to be answered.

In the first place, a question arises whether PPIases, CyP and/or FKBP, are essential for their growth or not. In yeast, all the CyP and FKBP genes except ESS1 (ScPvn19) are dispensable (9, 41, 42). Gene manipulation techniques for hyperthermophilic archaea are required to answer this question, especially considering that for these archaea genetic analysis is not yet fully developed (132).

In *Escherichia coli*, trigger factor (EcFKBP48) is reported to associate with ribosomes (133) and collaborate with DnaK in nascent protein folding (37). Hyperthermophilic archaea lack both the factor and the chaperone in their genomes. While alpha NAC gene is found in most archaea, beta NAC is not found and the function of NAC in archaea is unclear (see the section 3.1). It is pertinent to ask whether archaeal FKBP functions as a trigger factor. To answer this question, the *in vitro* translation technique developed for *Sulfolobus solfataricus* (134) may be useful.

Some enzymes from hyperthermophiles have unique properties that manifest themselves in the face of heat treatment. When *Pyrococcus* glutamate dehydrogenase is expressed in *E. coli* as a recombinant protein, its activity is elevated by heat treatment (135). The heat treatment is thought to affect the monomer structure and facilitate hexamer formation (136). This may indicate that these proteins are in a transient metastable state at lower temperature and require heat energy to take a proper structure. It would be interesting whether chaperones or PPIases are involved in this transition from the intermediate or metastable state to a properly-folded stable enzyme.

Expression of yeast CyP1 (ScCyP17) is heat inducible but that of FKBP1 (ScFKBP12) is not (137). Disruption of the CyP1 gene decreases the survival at high temperature. Expression of the *Vicia faba* (horsebean) FKBP (VfFKBP13) is also heat inducible (138). On the other hand, *E. coli* trigger factor (EcFKBP48) is a cold-shock protein and is thought to contribute to viability at low temperature (139). Cellular contents of two short-type archaeal FKBPS, TcFKBP18 and MjFKBP18, were shown to decrease at higher temperatures but increase at lower temperatures (125, 140). Cellular contents of long-type FKBPs did not change (140). These may indicate that the short-type FKBP is a cold-shock protein and function at lower temperatures.

Many PPIases are known to bind to other proteins to form a heterocomplex. Human FKBP52 (HsFKBP52) and HsCyP40 bind to steroid receptor (20, 26). No WW-, TRP-, or leucine rich-domain has been found in archaeal FKBPs or CyPs. However, it is possible that the PPIase bind to other cellular proteins to form a heterocomplex. Cross-linking experiments may answer this question.

Although some archaea, especially hyperthermophilic archaea, lack some chaperones, they have group II chaperonin, small heat shock protein and prefoldin (for review see 88). It is still a question whether archaeal PPIase (CyP or FKBP) collaborate with these protein folding factors in protein folding.

What is the functional differentiation between CyPs and FKBPs in archaeal cells? Most hyperthermophilic archaea so far studied have only one PPIase (FKBP). However, *M. thermoautotrophicum* have both CyP and FKBP, and *M. jannaschii* have two types of FKBPs. Some mesophilic methanogens have four FKBPs and two cyclophilins (82, 83). Functional differentiation between these PPIases remains to be elucidated.

Some trials to use archaeal FKBPs to improve foreign protein expression system in *E. coli* have been shown to be effective. The application of these FKBPs seems to be practical. Further studies are required for practical applications of these FKBPs.

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Abbreviations: 3D: three-dimensional; BSA: bovine serum albumin; CCT: cytosolic chaperonin containing TCP-1; CD: circular dichroism; CsA: cyclosporin A; CyP: cyclophilin; ER: endoplasmic reticulum; FK-dB: MtFKBP17 mutant with deletion of the bulge insertion; FK-dBF MtFKBP17 mutant with deletion of the bulge and the flap insertions; FK-dF: MtFKBP17 mutant with deletion of the flap insertion; FK-W: wild type MtFKBP17; FKBP: FK506 binding protein; HSP: heat shock protein; IL: interleukin; NAC: nascent chain-associated complex; NK-TR: tumor recognition protein in natural killer cells; pNA: *p*-nitroanilide; PPIase: peptidyl prolyl *cis-trans* isomerase; Pvn: parvulin; RanBP: Ras related nuclear protein binding protein; Rap: rapamycin; RCM-RNase T1: reduced and carboxymethylated RNase T1; RNase T1: Ribonuclease T1; S.D.; standard deviation; suc: succinyl; TOR: target of rapamycin; TPR: tetratrico-peptide-repeat.

Key Words: PPIase, FKBP, cyclophilin, protein folding, chaperone, Archaea, Review

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