FK506-binding protein of the hyperthermophilic archaeum, *Thermococcus* sp. KS-1, a cold-shock-inducible peptidyl-prolyl *cis–trans* isomerase with activities to trap and refold denatured proteins

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The FK506 (tacrolimus)-binding protein (FKBP) type peptidyl-prolyl *cis–trans* isomerase (PPIase) in the hyperthermophilic archaeum *Thermococcus* sp. KS-1 was shown to be induced by temperature downshift to growth temperatures lower than the optimum. This PPIase (TcFKBP18) showed chaperone-like protein refolding activity in addition to PPIase activity *in vitro*. It refolded unfolded citrate synthase (CS) and increased the yield of the refolded protein. At a molar ratio of 15:1 ([TcFKBP18] to [CS]) in the refolding mixture, the recovered yield of folded CS was maximal at 62 %, whereas that of spontaneous refolding was 11 %. Increasing FKBP above a 15:1 ratio decreased the final yield, whereas the aggregation of unfolded CS was suppressed. A cross-linking analysis showed the formation of a complex between TcFKBP18 and unfolded CS (1:1 complex) at molar ratios of 3:1 to 15:1. However, molar ratios of 15:1 or 60:1 induced the binding of multiple FKBP molecules to an unfolded CS molecule (multimeric complex). Disrupting hydrophobic interaction by adding ethylene glycol at a molar ratio of 60:1 ([TcFKBP18] to [CS]) suppressed the formation of this multimeric complex, simultaneously enhancing CS refolding. FK506 also suppressed the formation of the multimeric complex while increasing the chaperone-like activity. These results suggest that the hydrophobic region of TcFKBP18, probably the FK506-binding pocket, was important for the interaction with unfolded proteins. No cross-linked product was detected between TcFKBP18 and native dimeric CS. TcFKBP18 probably traps the unfolded protein, then refolds and releases it in a native form. This FKBP might be important at growth temperatures lower than the optimum in *Thermococcus* sp. KS-1 cells.

Key words: cold shock protein, complex formation, molecular chaperone, protein folding.

INTRODUCTION

Peptidyl-prolyl *cis–trans* isomerase (PPIase), protein disulphide isomerase and various molecular chaperones have been identified as mediators of protein folding and assembly *in vivo* [1]. PPIases are classified into three distinct families according to their sensitivity to inhibitors [2–4]. FK506 (tacrolimus)-binding proteins (FKBPs) are sensitive to the immunosuppressant FK506. Cyclophilins are sensitive to another immunosuppressant, cyclosporin A. Parvulins are insensitive to either FK506 or cyclosporin A. The proteins of these three families have little similarity in their amino acid sequences. PPIases catalyse the *cis–trans* isomerization of the proline imide bond in polypeptides, which is the rate-limiting step in protein folding, and consequently accelerate protein folding [5].

Genome sequence analysis of hyperthermophilic archaea have revealed that they have a relatively small number of protein folding mediators: PPIase, chaperonin, prefoldin and small heat shock protein [6–9]. They have no cyclophilin gene but have one or two FKBP genes as PPIases in their genomes. Two types of FKBP have been reported from archaea, one short-type FKBPs (17–18 kDa) and the other long-type FKBPs (26–30 kDa) [10]. The short-type FKBPs possess comparable PPIase activity to those of other bacterial FKBPs at 15 °C [11,12], whereas the activity of the long-type FKBPs from *Methanobacterium thermoautotrophicum* and from *Halobacterium cutirubrum* is low [13,14]. The short-type FKBP from *Methanococcus thermolithotrophicus* (MtFKBP17) has not only PPIase activity but also chaperone-like activity to refold unfolded proteins *in vitro* [15]. In contrast, the long-type FKBP from *M. thermoautotrophicum*, MbtFKBP28, whose N-terminal domain resembles that of MtFKBP17, has weaker chaperone-like activity [13]. It is not yet clear whether or not other archaeal short-type FKBPs, whose structures are essentially the same as that of MtFKBP17, also have this chaperone-like activity. Although the chaperone-like activity of MtFKBP17 has been shown to be attributed to the insertion sequence (44 amino acids) in the region corresponding to the 'flap' of human 12 kDa FKBP (HsFKBP12) [15], little information is available on the reaction mechanism for this chaperone-like activity. It has been reported that the 48 kDa *Escherichia coli* trigger factor and 52 kDa human FKBP also have chaperone-like protein-folding activity [16–19]. The trigger factor, which is a ribosome-associating FKBP, is expressed upon cold shock and enhances the viability of *E. coli* at low temperatures [20,21]. In contrast with this, 52 kDa human FKBP is a heat-shock protein [22]. It is interesting to clarify whether or not archaeal FKBPs are temperature-stress-inducible proteins. We studied the effect of growth temperature on the expression of a short-type FKBP from *Thermococcus* sp. KS-1 (TcFKBP18) in cells of the sulphur-dependent hyperthermophilic archaeum

Abbreviations used: CS, citrate synthase; FKBP, FK506-binding protein; HsFKBP12, human 12 kDa FKBP; MtFKBP17, FKBP from *Methanococcus thermolithotrophicus*; PPIase, peptidyl-prolyl *cis–trans* isomerase; Sulfo-SANPAH, *N*-sulphosuccinimidyl-6-(4′-azido-2′-nitrophenylamino)hexanoate; TcFKBP18, FKBP from *Thermococcus* sp. KS-1.

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Thermococcus sp. KS-1. To understand the mechanism for its chaperone-like activity, we also investigated the interaction between TcFKBP18 and the substrate protein *in vitro*.

EXPERIMENTAL

Materials

Citrate synthase (CS) (from *Thermoplasma acidophilum*, no. C9454), RNase T1 (from *Aspergillus oryzae*, no. R7384) HsFKBP12 (no. F5398) were purchased from Sigma Co. (St Louis, MO, U.S.A). FK506 was presented by Fujisawa Pharmaceutical. Co. (Osaka, Japan). FK506 was dissolved in ethanol and stored at −20 °C until being used. Recombinant TcFKBP18 was prepared as reported previously [12]. *N*-Sulphosuccinimidyl-6-(4′-azido-2′-nitrophenylamino)hexanoate (Sulfo-SANPAH) was purchased from Pierce Co. (Rockford, IL, U.S.A.). Rabbit antiserum against TcFKBP18 was prepared by Takara Shuzo Co. (Kyoto, Japan). The protein concentration was determined by the Bradford dye-binding method [23] with a protein assay kit (Bio-Rad, Hercules, CA, U.S.A.), with BSA as the standard.

Western blot analysis of TcFKBP18 in the cell lysate

Thermococcus sp. KS-1 was grown anaerobically in a medium (pH 6.8) containing 0.13 % yeast extract, 0.13 % Bacto-peptone, 0.09 % L-cystine, 0.5 % (v/v) Wolf's trace minerals solution [24], 0.26 ml/l of a 0.2 % resazurin solution, 0.2 % sulphur powder and 75 % (v/v) sea water [25]. The cells were grown in 1 litre of this medium at 60–95 °C for 6–24 h. The cell density was determined by the Acridine Orange direct count method with filtered sea water containing 0.5 % (v/v) glutaraldehyde and 0.1 % Acridine Orange. The cells were harvested at the stationary phase by centrifugation (12600 ***g*** for 10 min), resuspended in 25 mM Hepes/KOH, pH 6.8, containing 1 mM EDTA and sonicated for 3 min on ice. After centrifugation to remove the cell debris, the cell lysate (30 μg of soluble protein) was subjected to SDS/PAGE and blotted to a PVDF membrane (Bio-Rad). TcFKBP18 was detected with rabbit anti-TcFKBP18 serum. After being washed with PBS [140 mM NaCl/8.1 mM Na_2HPO_4/2.7 mM KCl/1.5 mM KH_2PO_4 (pH 7.2)], the bound antibody was detected by the horseradish peroxidase-conjugated anti-rabbit goat antibody (ICN Biomedicals, Costa Mesa, CA, U.S.A.), using 3,3′-diaminobenzidine,4HCl as a substrate. The band densities were estimated with Bio-Rad Multi-Analyst software.

PPIase activity of the *Thermococcus* sp. KS-1 cell lysate

The PPIase activity of the *Thermococcus* sp. KS-1 cell lysate was measured by a chymotrypsin-coupled assay with the oligopeptide *N*-succinyl-Ala-Leu-Pro-Phe-*p*-nitroanilide as substrate [26,27]. The reaction mixture (2 ml) contained 20 μM oligopeptide and 1 mg of protein from the cell lysate, which had been prepared from cells grown at 60 or 85 °C, in 100 mM sodium phosphate (pH 7.8). The reaction was started by adding 10 μM chymotrypsin after preincubation at 15 °C for 2 min. The reaction was monitored with a spectrophotometer (Jasco V-560; Nippon Bunko Co., Tokyo, Japan) at 15 °C for 3 min by the increase in A_{390} corresponding to the release of *p*-nitroanilide. The arbitrary unit of PPIase activity, U_p, was calculated from $U_p = (k_p - k_n)/k_n$, where k_p and k_n are the first-order rate constants for *p*-nitroanilide release in the presence and in the absence of the cell lysate respectively [11]. The concentration of TcFKBP18 in *Thermococcus* sp. KS-1 cells was estimated from the specific activity (U_p/mg of protein) of recombinant TcFKBP18.

Cold shock treatment and Northern blot analysis of TcFKBP18 mRNA

A 2 litre bottle of the culture of *Thermococcus* sp. KS-1 in the exponential phase at 85 °C was cooled to 60 °C in a water bath and incubated for a further 1–3 h. The cells were harvested from the 2 litre culture by centrifugation at 6500 ***g*** for 15 min. Total RNA was extracted by the guanidinium thiocyanate method with a phenol-free total RNA isolation kit (Ambion, Austin, TX, U.S.A.). The probe for mRNA of TcFKBP18 was produced by PCR with a primer set of 5′-terminal primer (5′-ATGAAA-GTTGAAGCTGGTGATTATGTTCTC-3′) and 3′-terminal primer (5′-CTAAGCTTCTGAGTCCTCTTCGGC-3′), from the expression plasmid pEFE1-3 [12] as the template. The probe was labelled with digoxigenin-dUTP by a PCR digoxigenin probe synthesis kit (Boehringer Mannheim, Germany). Total RNA (8 μg) was applied to a 1 % (w/v) agarose gel containing 2.2 M formaldehyde; after electrophoresis it was transferred to a nylon membrane (Hybond N^+; Amersham, Little Chalfont, Bucks., U.K.). Prehybridization and hybridization for a Northern blot analysis were performed at 68 °C; the membrane was washed with SSC buffer [1.7 mM sodium chloride/1.7 mM sodium citrate (pH 7.0)] containing 0.1 % SDS at 68 °C, in accordance with the supplier's instructions. The hybridized probe was detected with a digoxigenin nucleic acid detection kit (Boehringer Mannheim). The band density was estimated with Bio-Rad Multi-Analyst software.

Refolding of chemically unfolded CS

CS from *T. acidophilum* was unfolded by incubation at 50 °C for 30 min in a 25 mM sodium phosphate buffer, pH 7.0, containing 6 M guanidinium chloride and 5 mM dithiothreitol. Refolding was initiated by 60-fold dilution with 25 mM sodium phosphate buffer, pH 7.0, in the presence of 0–50 μM TcFKBP18 or 5–20 μM RNase T1 at 50 °C. To compare the chaperone-like activity of TcFKBP18 with those of mesophilic HsFKBP12 and BSA, CS refolding was performed at 30 °C. The final concentration of CS was 0.33 μM. After an appropriate period of incubation, the CS activity in the folding mixture was immediately measured as described previously ($n = 2$ or 3) [28,29]. The effects of ethylene glycol, which disrupts hydrophobic interaction [19,30], and of FK506 on the CS refolding were also investigated in the presence of 5 or 20 μM TcFKBP18 at 50 °C. Chaperone-like activity is expressed as a percentage of the native CS activity.

Effect of TcFKBP18 on the aggregation of chemically unfolded CS

CS unfolded by 6 M guanidinium chloride was diluted 60-fold with a 25 mM sodium phosphate buffer, pH 7.0, in the presence of 0–50 μM TcFKBP18 at 50 °C; the aggregation of unfolded CS was monitored by light scattering at 320 nm with a Jasco-777 model fluorescence spectrophotometer (Nippon Bunko) in a final volume of 1.5 ml. The final concentration of CS was 0.33 μM. RNase T1 (5 μM) was used as a negative control.

Cross-linking between TcFKBP18 and CS

The heterobifunctional cross-linker Sulfo-SANPAH, which possesses a sulphonated *N*-hydroxysuccinimido active ester and a photoactivatable aryl azide, was used in accordance with the supplier's instructions (Pierce Co.). TcFKBP18 (2 ml; 4.8 mg/ml) was reacted with 9.8 mg of Sulfo-SANPAH in PBS. After incubation for 3 h at room temperature, free Sulfo-SANPAH was removed by centrifugation and gel-filtration chromatography in a Sephadex G-25M column (9.1 ml bed volume, 5 cm bed height; Amersham Pharmacia Biotech, Uppsala, Sweden)

with a PBS as the mobile phase. Native or chemically unfolded CS was diluted 60-fold to 1 μM with 60 μl of PBS containing Sulfo-SANPAH-bound TcFKBP18 (0–60 μM). The solution was incubated for 2 min at 50 °C and then activated photochemically with a UV (312 nm) lamp (VL-6LM; Vilber Lourmat, Marne la Vallée, France) at a distance of 5 cm for 15 min to link Sulfo-SANPAH-TcFKBP18 and the associated protein through the photoactivated aryl azide moiety. The reaction mixture was subjected to SDS/PAGE [16% (w/v) gel] and stained with Coomassie Brilliant Blue R250. The effects of FK506 (20 or 200 μM) and ethylene glycol (20%, v/v) on cross-linking between TcFKBP18 and CS were also investigated.

RESULTS

Expression of TcFKBP18 in *Thermococcus* sp. KS-1 cells

TcFKBP18 in *Thermococcus* sp. KS-1 cells was specifically detected by a Western blot analysis with the anti-TcFKBP18 serum. The cellular content of TcFKBP18 was found to be higher at lower growth temperatures, particularly in the range 60–65 °C (Figure 1). The band densities of TcFKBP18 in the cell lysates grown at 60 and 65 °C were respectively 14-fold and 8-fold higher than the TcFKBP18 band density of cells grown at 75 °C.

PPIase activities in the cell lysates of *Thermococcus* sp. KS-1

Soluble protein (1 mg) in the cells grown at 60 °C showed approx. 3.6 U_p of PPIase activity; however, that grown at 85 °C showed only 0.44 U_p of PPIase activity. This PPIase activity was inhibited by more than 95% by 20 μM FK506. This indicates that most of the PPIase activity of the cell lysate was derived from the FKBP type of PPIase, probably from TcFKBP18. Recombinant TcFKBP18 (2 pmol; 35 ng) showed 0.032 U_p of activity. On the basis of this specific activity, the concentration of TcFKBP18 in the cells grown at 60 °C was estimated to be 3.9 μg (0.22 nmol)/mg of soluble protein, and that grown at 85 °C was 0.48 μg (0.027 nmol)/mg of soluble protein.

Induction of TcFKBP18 expression by cold shock

Messenger RNA of TcFKBP18 of approx. 0.7 kb was detected by the Northern blot analysis of total RNA from *Thermococcus* sp. KS-1. The intensity of the band corresponding to the mRNA was maximal 1 h after the cold shock, increasing to 17-fold higher than that before the cold shock. After a further 1 or 2 h of incubation, the band density gradually decreased to 27% and 11% of the maximum value respectively.

Effect of TcFKBP18 on the refolding of unfolded CS

In the spontaneous refolding reaction of 0.33 μM CS without TcFKBP18, the final recovery of refolded CS was approx. 11±0.6% (mean±S.D.) after incubation for 50 min at 50 °C (Figure 2A). The recovery of CS was elevated by TcFKBP18 in a dose-dependent manner at molar ratios from 1.5:1 to 15:1 ([TcFKBP18] to [CS]). This recovery was the maximum of 62±4% (mean±S.D.) at a molar ratio of 15:1, and decreased with further increasing ratios of FKBP to the substrate. In the presence of 20 or 50 μM TcFKBP18 ([TcFKBP18] to [CS] 60:1 or 150:1), the final yields were suppressed to 8±3% (mean±S.D.) and zero respectively. RNaseT1 (5 and 20 μM) ([RNaseT1] to [CS] 15:1 and 60:1), as negative control, did not significantly affect the CS refolding. The time-course profile showed that CS refolding with 0–20 μM of TcFKBP18 reached a plateau within 40 min (Figure 2B). At 30 °C the yield of spontaneous CS refolding was 48±10% (mean±S.D.), which was higher than that at 50 °C. HsFKBP12 (5 and 40 μM) and 5 μM BSA did not show any significant effects on CS refolding (46±5%, 55±9% and 54±2% respectively). In contrast, the yield obtained by 5 μM TcFKBP18 was significantly higher (72±4%) than that of spontaneous folding or those obtained by human PPIase or BSA (t test, $P < 0.02$) and was suppressed by 40 μM TcFKBP18 to 10±0.8%. These results indicate that

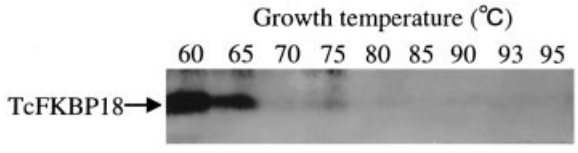


Figure 1 Expression of TcFKBP18 in *Thermococcus* sp. KS-1 cells

TcFKBP18 was detected by a Western blot analysis of the cell lysates. Cell lysate (30 μg) was analysed with the anti-TcFKBP18 rabbit antiserum.

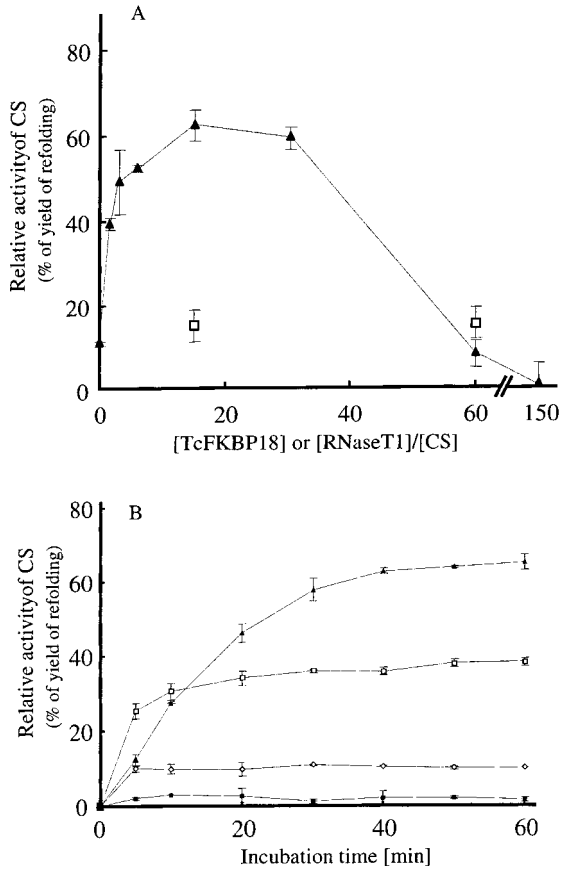


Figure 2 Refolding of chemically unfolded CS mediated by TcFKBP18

Refolding of unfolded CS was initiated by dilution with sodium phosphate buffer, pH 7.0, in the presence of TcFKBP18 (0–50 μM). The final concentration of CS was 0.33 μM. The activity of native CS is defined as 100%. Error bars indicate S. D. (**A**) Final yield of reactivated CS after 50 min of incubation at 50 °C. Symbols: ▲, refolding with 0, 0.5, 1, 2, 5, 10, 20 or 50 μM TcFKBP18; □, refolding with 5 or 20 μM RNase T1 as a negative control. (**B**) Time-course profiles of the unfolded CS refolding reaction in the presence of 0 (◇), 0.5 (□), 5 (▲) and 20 μM (■) TcFKBP18 at 50 °C.

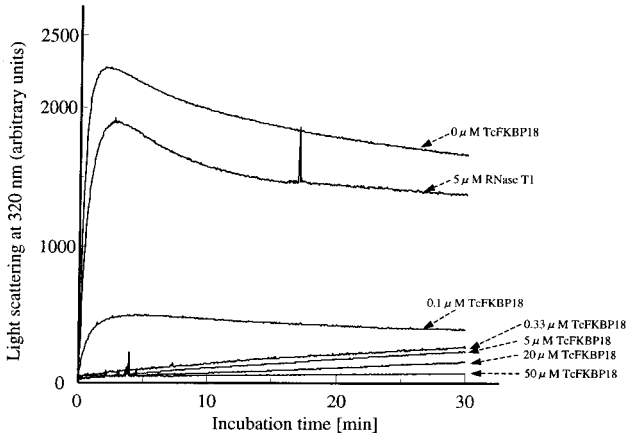


Figure 3 Effect of TcFKBP18 on the aggregation of unfolded CS

The aggregation of unfolded CS was monitored continuously at 50 °C by light scattering at 320 nm. Unfolded CS was diluted with sodium phosphate buffer, pH 7.0, containing TcFKBP18 (0–50 μM) under the same conditions as those described in the legend to Figure 2.

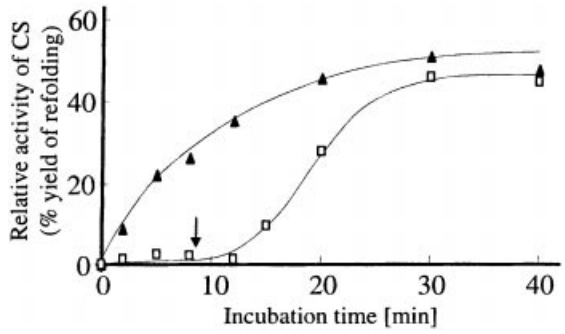


Figure 4 Effect of 20 μM FK506 on the refolding of unfolded CS mediated by 20 μM TcFKBP18

The CS refolding reaction was performed as described in the legend to Figure 2, except for the presence of 2% (v/v) ethanol used in the solution of FK506 to inhibit the activity of FKBP. The CS concentration was 0.33 μM in the reaction mixture. Symbols: ▲, 20 μM FK506 was added in advance; □, 20 μM FK506 was added at the point marked by the arrow (8 min after starting the reaction).

these effects of TcFKBP18 on CS refolding were independent of its PPIase activity.

Effect of TcFKBP18 on CS aggregation

In the CS refolding mixture, unfolded CS rapidly aggregated spontaneously (Figure 3). The intensity of light scattering reached a maximum 2–3 min after dilution and then decreased gradually. This aggregation was effectively suppressed by 0.1–50 μM TcFKBP18 ([TcFKBP18] to [CS] 0.3/1 to 150:1) in a dose-dependent manner and was completely suppressed at 50 μM. However, 5 μM RNase T1 only partly suppressed the CS aggregation.

Effect of ethylene glycol on CS refolding mediated by TcFKBP18

In the presence of 20 μM TcFKBP18 ([TcFKBP18] to [CS] 60:1), the refolded yield of unfolded CS was increased by the addition of ethylene glycol. Whereas the value was $11 \pm 2\%$ without ethylene glycol, it increased with increasing ethylene glycol concentration and reached a plateau at approx. 5% ($66 \pm 6\%$ with 5% ethylene glycol and $61 \pm 2\%$ with 20% ethylene glycol). In contrast, the refolded CS yield mediated by 5 μM TcFKBP18 ([TcFKBP18] to [CS] 15:1) was not significantly affected by ethylene glycol concentrations from 0% (yield $55 \pm 4\%$) to 20% ethylene glycol (yield $51 \pm 5\%$).

Effect of FK506 on CS refolding mediated by TcFKBP18

FK506 lowered the final yield of refolded CS mediated by 5 μM TcFKBP18 ([TcFKBP18] to [CS] 15:1) from $47 \pm 2\%$ (without FK506) to $22 \pm 2\%$ (200 μM FK506) in a dose-dependent manner. The yield without FK506, $47 \pm 2\%$, was significantly lower than that of the similar experiment shown in Figure 2(A) ($62 \pm 4\%$; t test, $P < 0.05$) because 2% (v/v) ethanol, which was the solvent of FK506, was present in the reaction mixture. In contrast, in the presence of a higher concentration (20 μM) of TcFKBP18 ([TcFKBP18] to [CS] 60:1), the yield increased with increasing concentration of FK506 from $1 \pm 0.6\%$ (without FK506) to the maximum of $54 \pm 4\%$ (with 20 μM FK506). However, the yield decreased in a dose-dependent manner with further increases in concentration of FK506 (20–200 μM). The yield with 200 μM FK506 was $32 \pm 0.6\%$ in the presence of 20 μM TcFKBP18.

When FK506 was added at 20 μM to the reaction mixture containing TcFKBP18 and unfolded CS at a ratio of 60:1, in which CS refolding was suppressed to approx. 2%, CS activity gradually recovered and reached a plateau within 30 min of the addition of FK506 (Figure 4). These results indicated that the suppression of CS refolding at a high concentration of TcFKBP18 was due to the reversible binding of TcFKBP18 to unfolded CS and that the interruption of this binding by FK506 resulted in an increase in the final yield of CS refolding.

Binding of TcFKBP18 to unfolded CS

The interaction between TcFKBP18 and CS was analysed by cross-linking with Sulfo-SANPAH followed by SDS/PAGE (Figure 5). In the reaction between a low concentration of TcFKBP18 (3 μM) and 1 μM unfolded CS, a 66 kDa band corresponding to a complex between a TcFKBP18 and unfolded CS (1:1 complex) was observed (Figure 5, lane 4). Without unfolded CS, this band was not present (Figure 5, lane 2). In the reaction between a higher concentration of TcFKBP18 (15 or 60 μM) and 1 μM unfolded CS, a smeared band with a molecular mass range of 94–204 kDa appeared, while the band density of the CS monomer decreased (Figure 5, lanes 5 and 6). This is thought to correspond to the cross-linked complexes between one unfolded CS molecule and more than one molecule of TcFKBP18. No cross-linked product was detected in the reaction mixture containing 60 μM TcFKBP18 and 1 μM native dimeric CS (Figure 5, lane 7).

In the reaction between a high concentration of TcFKBP18 (15 or 60 μM) and 1 μM unfolded CS, the smeared band disappeared with the addition of 20% ethylene glycol, whereas the bands corresponding to the CS monomer and 1:1 ([TcFKBP18] to [CS]) complex remained (Figure 5, lanes 8 and 9). In the presence of 20 μM FK506 with 60 μM TcFKBP18, several bands in the range of 79–128 kDa corresponding to 2:1–6:1 ([TcFKBP18] to [CS]) complexes appeared in addition to the CS monomer and 1:1 ([TcFKBP18] to [CS]) complex bands (Figure 5, lane 10). A further addition of FK506 (200 μM) resulted in an increase in the band density of the 1:1 complex,

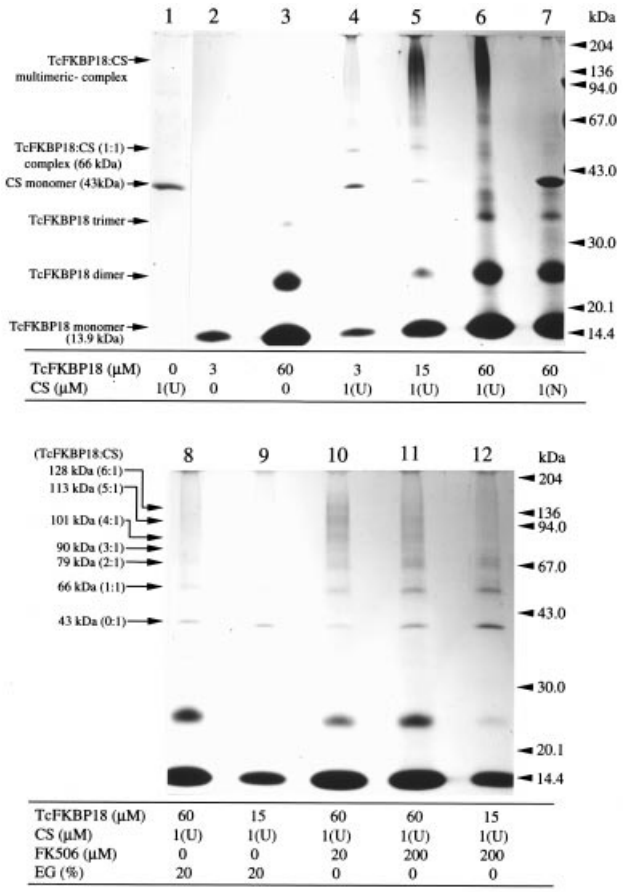


Figure 5 Interaction between TcFKBP18 and unfolded CS

TcFKBP18 (0–60 μM) labelled with Sulfo-SANPAH was incubated with 1 μM native dimeric (N) or unfolded CS (U). After photoactivation with UV, the cross-linked proteins were analysed by SDS/PAGE [16% (v/v) gel, stained with Coomassie Brilliant Blue R250]. Lane 1, 1 μM unfolded CS without TcFKBP18; lanes 2 and 3, 3 or 60 μM TcFKBP18 without CS respectively; lanes 4–6, 3, 15 or 60 μM TcFKBP18 with 1 μM unfolded CS respectively; lane 7, 60 μM TcFKBP18 with 1 μM native dimeric CS; lanes 8 and 9, reactions between 60 or 15 μM TcFKBP18 and 1 μM unfolded CS in the presence of 20% ethylene glycol (EG); lanes 10 and 11, reactions between 60 μM TcFKBP18 and 1 μM unfolded CS in the presence of 20 or 200 μM FK506; lane 12, reaction between 15 μM TcFKBP18 and 1 μM unfolded CS with 200 μM FK506. The positions of molecular mass markers are indicated at the right.

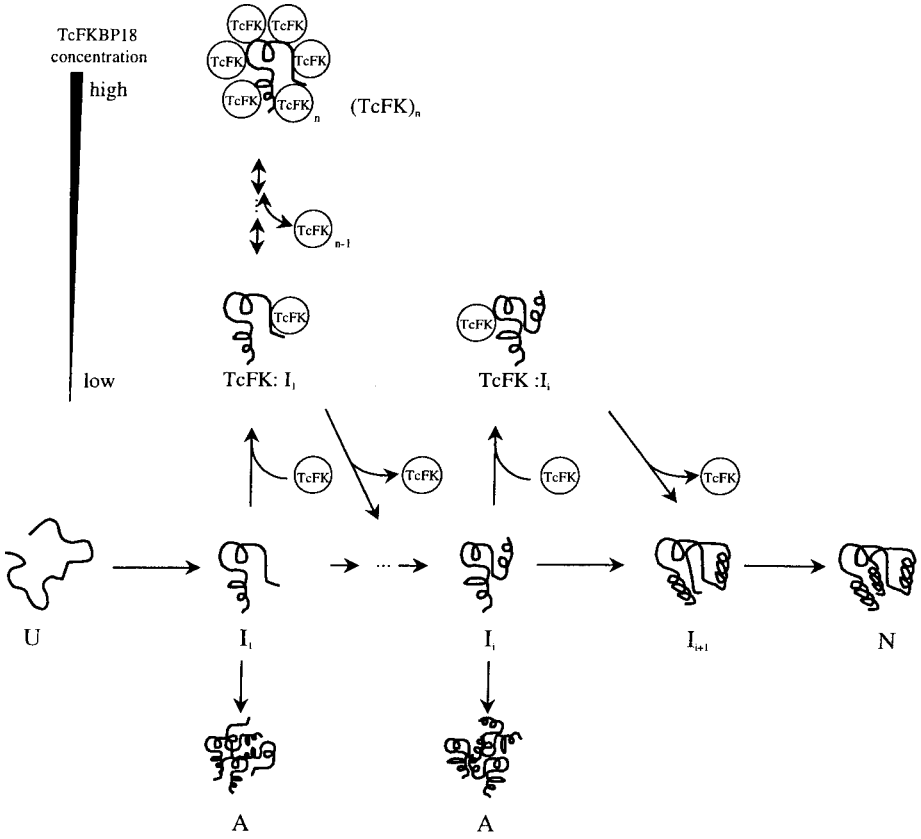
whereas the band density of higher-molecular-mass complexes decreased (Figure 5, lane 11). In the reaction between 15 μM TcFKBP18 and 1 μM unfolded CS, the density of the 66–79 kDa bands corresponding to 1:1 to 2:1 complexes and the CS monomer was increased by the addition of 200 μM FK506, whereas that of the smeared band was decreased (Figure 5, lane 12). In the absence of CS but 60 μM TcFKBP18, a few bands corresponding to the dimer and/or trimer of FKBP were observed (Figure 5, lane 3); these bands were also detected in lanes 5–8 and 10–12. Without TcFKBP18, only a band corresponding to the CS monomer was observed (Figure 5, lane 1).

DISCUSSION

The archaeal short-type FKBP from *M. thermolithotrophicus*, MtFKBP17, possesses not only PPIase activity but also chaperone-like activity to refold unfolded proteins *in vitro* [15]. In contrast, the PPIase and chaperone-like activities of the archaeal long-type FKBP from *M. thermoautotrophicum*, MbtFKBP28, have been shown to be weaker [13]. In the present study, TcFKBP18 was also found to have chaperone-like activity in addition to PPIase activity *in vitro* [12] (Figure 2). Although TcFKBP18 increased the yield of refolded CS in a molar ratio range between 1.5:1 and 15:1 ([TcFKBP18] to [CS]) in a dose-dependent manner, the yield decreased at higher FKBP ratios and was almost zero at a molar ratio of 150:1 (Figure 2A). Because no CS aggregation was apparent at higher TcFKBP18 ratios (Figure 3), it is suggested that the lower yield was the result of trapping unfolded CS by the high concentration of TcFKBP18. The cross-linking analysis indicates that TcFKBP18 and unfolded CS interacted in a 1:1 fashion at a low concentration of TcFKBP18; however, at higher concentrations of TcFKBP18 more than one molecule of TcFKBP18 associated with an unfolded CS molecule (multimeric complex) (Figure 5), resulting in the suppression of refolding (Figure 2A). Although the formation of multimeric complexes was suppressed by disrupting the hydrophobic interaction with ethylene glycol (Figure 5), the 1:1 ([TcFKBP18] to [CS]) complex remained with a higher final yield of refolded CS. TcFKBP18 did not interact with the native dimeric form of CS but did so with the unfolded form. TcFKBP18 probably interacted with the extended hydrophobic region of the unfolded protein. There might be stronger and weaker hydrophobic interactions between TcFKBP18 and the intermediate. The formation of a 1:1 ([TcFKBP18] to [CS]) complex was attributed to the probably stronger interaction that occurred between TcFKBP18 and the highly hydrophobic site of unfolded CS, whereas the multimeric complexes formation was attributed to the weak interaction that was disrupted by adding ethylene glycol. A weak interaction might exist between TcFKBP18 and some lower hydrophobic regions of unfolded CS or of the partly refolded CS.

FK506 inhibited the chaperone-like activity of TcFKBP18. It has been reported that the inner surface of the FK506-binding pocket is hydrophobic [31]. This hydrophobic region was probably necessary for the chaperone-like activity and association with the unfolded protein. The 1:1 complex formation seems to be important for exerting the chaperone-like activity. However, whereas 1:1 complex formation was increased by adding FK506 at a molar ratio of 15:1 ([TcFKBP18] to [CS]) (Figure 5), the yield of refolded CS was decreased. This suggests that some region of TcFKBP18 other than its FK506-binding pocket also associated with the unfolded CS. Furutani et al. [11,15] have indicated that chaperone-like activity of MtFKBP17 is attributed to the archaea-specific insertion sequence in the flap region. It is possible that both this insertion sequence and the FK506-binding site might be necessary for TcFKBP18 to interact with the unfolded protein and to exert chaperone-like activity.

A possible mechanism for the chaperone-like activity of TcFKBP18 is proposed in Scheme 1. Spontaneous protein folding occurs from the unfolded state (U) to the native product (N) via several different intermediate states ($I_1 - I_{i+1}$). The intermediates tend to form inactive aggregates (A). TcFKBP18 and folding intermediate (I_1–I_i) associate in a 1:1 ratio at low concentrations of FKBP. The final yield of folded CS was not 100%; some CS aggregation was apparent (Figures 2 and 3). The intermediate probably repeats a cyclic interaction of binding to and releasing from TcFKBP18. During this cyclic reaction, a fraction of the free folding intermediates might interact with each other to form inactive aggregates. The refolding of unfolded protein might occur by binding and releasing processes and the protein gradually refolds. As this folding progresses, the exposed hydrophobic surface of the folding intermediate is reduced (from I_1 to I_{i+1}): the smaller the hydrophobic surface, the lower the interaction between TcFKBP18 and the folding intermediate (I_{i+1} or N).



Scheme 1 Proposed mechanism for the chaperone-like activity of TcFKBP18

Unfolded protein (U) spontaneously refolds to the native form (N) through a series of intermediates (I_1 to I_{i+1}). Protein folding proceeds from left to right. The intermediates (I_1 to I_i) tend to form irreversible aggregates (A).

Trapped unfolded protein was refolded and released as a native form. At a high concentration of TcFKBP18, one molecule of the folding intermediate and several molecules of FKBP form multimeric complexes by hydrophobic interaction [$(TcFK)_n$ to I_1], where n is the number of TcFKBP18 molecules binding to the intermediate. The 1:1 complex might be a stable intermediate in chaperone-like protein folding by TcFKBP18; dissociation of this complex might be a key step to the native form.

The *E. coli* 48 kDa trigger factor has been reported as a cold-shock-inducible FKBP and has been shown to enhance cell viability at low temperature [21]. The trigger factor is suggested to interact with newly synthesized proteins at the ribosome [32]. The trigger factor is composed of three domains: the FKBP domain and N-terminal and C-terminal domains [33]. The N-terminal and/or C-terminal domains are important for binding to unfolded proteins [17]. Huang et al. have recently revealed [19] that the *E. coli* trigger factor has chaperone-like protein folding activity and that this activity is suppressed at a high concentration of trigger factor. They have suggested that the trigger factor forms complexes with the refolding intermediates and refolds it to the native state by a repeated binding and releasing cycle [19]. No similar protein to the trigger factor has been found in archaea. Because TcFKBP18 was induced by a temperature downshift to a growth temperature lower than the optimum (Figures 1) and showed chaperone-like protein folding activity, it might have functions similar to those of the trigger factor in protein folding. This similarity suggests that TcFKBP18 might function in protein folding at an early stage of protein synthesis. It has been reported that spontaneous isomerization of the peptidyl-prolyl bond is facilitated by increasing temperature and that PPIase activity might be required more at lower temperatures [11,34]. TcFKBP18 might be important in prolyl *cis–trans* isomerization of proteins at temperatures lower than the optimum.

Little information is available on the co-operation between protein folding mediators in the cells of hyperthermophilic archaea. In *E. coli*, although the expressions of the trigger factor and of chaperonin are differently regulated [21], they have been reported to co-operate with each other in protein folding [35]. *Thermococcus* sp. KS-1 possesses two kinds of chaperonin subunit, α and β, which form a hetero-oligomer [36,37]. The subunit composition changes with growth temperature, the α-subunit content being higher at low temperature, whereas the β-subunit content is higher at higher temperature [37]. It would be interesting to study the co-operation between TcFKBP18 and α-chaperonin in protein folding. Because the genomes of hyperthermophilic archaea have a small number of known protein folding mediators, these mediators might be multifunctional. TcFKBP18 might function in the folding of newly synthesized protein associating with a ribosome similar to *E. coli* trigger factor, in addition to that of the cytosolic PPIase.

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