An 11.8 kDa proteolytic fragment of the *E. coli* trigger factor represents the domain carrying the peptidyl-prolyl *cis/trans* isomerase activity

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Abstract **The 48 kDa trigger factor (TF) of *E. coli* was shown to be a peptidyl-prolyl *cis/trans* isomerase (PPIase). Its location on a ribosomal particle is unique among the PPIases described so far, and suggests a role in de novo protein folding. The trigger factor was investigated with regard to a domain carrying the catalytic activity. An enzymatically active fragment could be isolated after proteolysis by subtilisin. The resulting polypeptide was analysed by N-terminal sequencing and MALDI-TOF mass spectrometry revealing an 11.8 kDa fragment of TF encompassing the amino acid residues Arg-145 to Glu-251. The nucleotide sequence encoding the amino acid residues Met-140 to Ala-250 of the TF was cloned into vector pQE32. After expression in *E. coli* the resulting His-tagged polypeptide was isolated on an Ni^{2+}-NTA column. Subsequent digestion with subtilisin and anion-exchange chromatography yielded a TF fragment encompassing amino acids Gln-148 to Thr-249. This fragment may represent the catalytic core of TF since PPIase activity with a specificity constant k_{cat}/K_m of 1.3 μM^{-1} s^{-1} could be demonstrated when using Suc-Ala-Phe-Pro-Phe-NH-Np as a substrate. Moreover, as was observed for the complete, authentic TF the PPIase activity of the fragment was not inhibited by the peptidomacrolide FK506.**

Key words: Limited proteolysis; Peptidyl-prolyl *cis/trans* isomerase; Trigger factor; *E. coli*

1. Introduction

The trigger factor protein was originally found assisting in the maintenance of the translocation-competent conformation of the precursor protein proOmpA (outer membrane protein A) in vitro [1]. It has been demonstrated that TF has a binding affinity to this protein when going from a denaturant like urea to renaturation conditions by either dilution or dialysis [2]. Therefore, stoichiometric complexes of TF and proOmpA could be isolated [2,3]. Using genetically engineered strains of *E. coli* either over- or underproducing TF, however, the membrane transport of proOmpA was not affected [4]. Further, TF was detected at the 70S ribosome dissociating in a salt-dependent manner [5]. The binding was proved exclusively for the 50S subunit [5,6] known for covering the exit domain of the nascent polypeptide chain.

Recently, it could be demonstrated that TF belongs to the peptidyl-prolyl *cis/trans* isomerases (PPIases) [6]. PPIases catalyse the *cis/trans* isomerization of Xaa-Pro peptide bonds in oligopeptide derivatives [7] and proteins [8], thereby accelerating conformational interconversions of segments of the polypeptide backbone. They are currently divided into the unrelated families of cyclophilins, FK506-binding proteins [9] and the parvulins [10,11]. The cyclophilins and FKBPs are characterised through their binding to cyclosporin A and FK506, respectively, thereby inhibiting their PPIase activity. The PPIase activity of TF was not affected at all by these effectors but its subsite specificity was found to be reminiscent of FKBPs [6]. A certain degree of sequence homology of an inner area of TF to FKBPs has also been reported [12].

Moreover, TF was successfully crosslinked to the nascent chains of either presecretory or non-signal sequence bearing proteins [13]. This association of TF to the nascent chains was no longer stable under high salt/puromycin conditions indicating a binding affinity to the translating ribosome.

Interestingly, TF was also described to be involved in protein degradation in complex with GroEL/GroES [14]. The degradation of an artificial protein was retarded in TF reduced cells while in TF overproducing cells the entire full-length model protein was hardly detectable because of its rapid degradation by proteases. Thus, TF could be a rate-limiting component in the degradation of abnormal proteins.

TF accelerates effectively the *trans* to *cis* isomerization of a model protein [6]. As was calculated via published methods about 5% of all peptidyl-prolyl bonds known in the spatial structure are in the *cis* conformation in native proteins [15,16]. The *trans* to *cis* isomerization is often the rate limiting step during the refolding of polypeptides provided that there is a *cis* prolyl residue in the native state [8,17,18]. The PPIases have been proved to catalyse these slow folding steps in vitro using several substrate proteins [8]. For cellular folding only sparse evidence for an involvement of PPIases has been given. Thus, a role of cyclophilins and FKBPs in maturation of two bacterial luciferases after their translation in a reticulocyte lysate in vitro was demonstrated [19]. Using cyclosporin A, the involvement of cyclophilins in proper folding of transferrin in hep G2 cells [20] and more recently the contribution of a mitochondrial cyclophilin in folding of newly imported precursor proteins [21,22] could be shown. The occurrence of TF at the *E. coli* ribosome could be an indication of the involvement of PPIases in de novo folding of proteins. However, it remains an open question whether the high catalytic activity of TF toward proteinaceous substrates is brought about by utilising more extended subsite interactions. In this respect the 12 kDa human cytosolic FKBP12 represents the minimal catalytic core of the FKBP-like PPIases. The additional contacting sites may be provided by amino acid regions flanking the putative catalytic domain of TF. Alternatively, the large number of nonconserved amino acids in the FKBP-like re-

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Abbreviations: TF, trigger factor; PPIase, peptidyl-prolyl *cis/trans* isomerase; PMSF, phenylmethylsulfonyl fluoride; BSA, bovine serum albumin; -NH-Np, 4-nitroanilide; Suc, succinyl; FKBP, FK506-binding protein.

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gion may easily cause substantial alterations in subsite specificity.

In this study, we isolated a fragment of TF subsequent to limited digestion of the native protein with subtilisin. Evidence is provided by N-terminal sequencing, molecular mass determination and overexpression that this fragment represents the PPIase active core of TF. The secondary structures of the authentic TF and the catalytically active fragment of TF were compared by CD spectroscopy.

2. Materials and methods

2.1. Materials

Subtilisin Carlsberg (type VIII bacterial, lot 72H0115) and thrombin from bovine plasma (lot 36F9472) were from Sigma (Deisenhofen, Germany). The substrates (Suc-Ala-Xaa-Pro-Phe-NH-Np, Suc-Ala-Ala-Pro-Arg-NH-Np) were purchased from Bachem (Heidelberg, Germany). Peroxidase-conjugated goat anti-rabbit IgG was obtained from Sigma (Deisenhofen, Germany). Molecular mass markers, nitrocellulose NC45 and buffers (HEPES, Tris) were from Serva (Heidelberg, Germany). Polyclonal antiserum against trigger factor was obtained from rabbit after immunisation with trigger factor fragments (Pab Productions, Herbertshausen, Germany). Trigger factor was isolated from *E. coli* cell extracts as described [6]. The 70S ribosomes were a kind gift from K. Nierhaus. Restriction endonucleases, RNase A, lysozyme and T4 DNA ligase were purchased from Boehringer Mannheim (Germany). SureClone Ligation Kit, ^{T7}Sequencing Kit and oligonucleotides were obtained from Pharmacia Biotech (Freiburg, Germany). Sequencing-grade [^{35}S]dATP was from Amersham (Braunschweig, Germany). PrimeZyme polymerase was purchased from Biometra (Göttingen, Germany).

2.2. Protein analysis

N-terminal sequencing was carried out using an Applied Biosystems 476 A gas-phase sequencer after desalting of the sample through reversed-phase HPLC (Shimadzu LC-10A) on a Nucleosil 500-5 C_3-PPN column (125×4 mm, guard column 11×4 mm) from Macherey-Nagel (Düren, Germany). After gel filtration enzymatically active fractions were applied to the column equilibrated with 0.1% aqueous trifluoroacetic acid in 1% acetonitrile (v/v). The fragment of TF was eluted after 19 min running a gradient from 20 to 60% acetonitrile in 0.1% trifluoroacetic acid within 40 min at 40°C. The flow rate was 1 ml/min. Elution was monitored at wavelengths of 215 and 280 nm.

The molecular mass of the fragment was determined using a MALDI-TOF Bruker Reflex mass spectrometer (Bremen, Germany) equipped with an N_2-UV laser (337 nm). The matrix was sinapic acid. For calibration cytochrome *c* (bovine heart) was used as internal standard.

Circular dichroism was measured using a Jasco J-710 spectropolarimeter (Tokyo, Japan). Far-UV measurements were carried out in a thermostatted 0.1 cm quartz cell using 0.005 M sodium phosphate (pH 7.0) at 25°C. The spectra were recorded 8 times and averaged. The protein concentrations were 8.2 and 25.1 μM for authentic TF and the fragment of TF, respectively. Data were analysed using the software from Jasco (Tokyo, Japan). Molar ellipticity was calculated using the mean residue molar concentration of TF and its active fragment.

SDS-PAGE (15%) and immunoblotting was performed as previously described [6].

Protein concentration was determined spectrophotometrically using the molar extinction coefficients at 280 nm (15930 M^{-1} cm^{-1} for TF and 6970 M^{-1} cm^{-1} for TF fragment) which were calculated from the primary structure according to Gill and Von Hippel [23].

2.3. Limited proteolysis of TF and isolation of the PPIase active fragment

Trigger factor (4 μM) was incubated with subtilisin (0.15 μM) for 8 h at 4°C. Proteolysis was stopped by adding 1 mM PMSF. For the following separation the SMART System from Pharmacia (Uppsala, Sweden) was used. A 50 μl sample was applied to a Superdex 75 PC3.2/30 column (Pharmacia, Uppsala, Sweden) equilibrated with 0.01 M HEPES (pH 7.5), 0.15 M KCl, 0.015 M $MgCl_2$. A flow rate of 40 μl/min was used. Fractions of 40 μl were collected. PPIase activity was determined with *N*-succinyl-Ala-Phe-Pro-Phe-*p*-nitroanilide as substrate using samples of 20 μl of each fraction. Calibration was performed under the same running conditions using molecular weight marker proteins from Boehringer Mannheim (Germany). These proteins were bovine serum albumin (68 kDa), ovalbumin (45 kDa), chymotrypsinogen A (25 kDa) and cytochrome *c* (12.5 kDa). The exclusion limit was determined using catalase (240 kDa). The temperature was maintained at 25°C during all runs.

2.4. Molecular cloning, overexpression and isolation of the trigger factor fragment

The complete *tig* gene [4] was amplified by PCR using the chromosomal DNA of the *E. coli* K12 strain DH5α and two primers corresponding to the 5′ and 3′ regions of the *tig* gene (5′-CAGTGGATCCCCATGCAAGTTTCAGTTGAAACC-3′ and 5′-CCTGGCGTCGACGGGCCTTTGTGCG-3′). The resulting PCR product was ligated into vector pUC18 and transformed into *E. coli* strain K12 DH5α. One positive clone was obtained designated as TTtig1. After digestion of the TTtig1 plasmid DNA with *Sph*I and *Pst*I a fragment of 330 bp containing the sequence coding for the amino acids 140–250 of TF was extracted from agarose gel using the Sephaglas BandPrep Kit (Pharmacia, Freiburg, Germany) and ligated into pQE32 (Qiagen, Hilden, Germany). The resulting plasmid was transformed into *E. coli* strain K12 M15 (pREP4). Recombinants were screened by restriction analysis and controlled by sequencing according to the procedure of the manufacturer. The expressed protein contained an oligohistidine tag (6×His) at its N-terminus followed by Gly, Ile, Arg and the chosen sequence of TF including the originally found cleaving site for subtilisin (Leu-144–Arg-145). There were four amino acids at the C-terminus that do not belong to the TF fragment isolated by digestion. For the recombinant protein they were also found to be cleaved off after digestion.

After induction by IPTG, cells of 1 l culture of the overexpressing strain K12 M15 were grown for 5 h at 37°C. Cells were harvested by centrifugation (6000×*g*, 4°C, 10 min) and washed with 0.025 M phosphate buffer (pH 8.0), 0.15 M NaCl. The cells were resuspended in 0.05 M phosphate (pH 8.0), 0.3 M NaCl and passed through a French press (SLM Aminco, Buettelborn, Germany). Cell debris were removed by ultracentrifugation for 40 min at 95800×*g* (Beckman, 4°C). The supernatant was pooled and applied to an Ni^{2+}-NTA column (1×2 cm, Qiagen, Hilden, Germany) equilibrated in the same buffer. Unspecifically bound material was washed off by 0.05 M phosphate (pH 6.0), 0.3 M NaCl, 10% glycerine (v/v). Bound protein was eluted by a gradient of 0–0.5 M imidazole (60 ml total). Further purification of the enzymatically active fractions was achieved by gel filtration. After concentration a 1 ml sample was applied to a Superdex 75 16/60 HiLoad FPLC column (Pharmacia, Uppsala, Sweden) equilibrated with 0.01 M HEPES (pH 7.5), 0.15 M KCl, 0.015 M $MgCl_2$. The trigger factor derivative eluted at a flow rate of 0.8 ml/min was over 95% homogeneous as verified by SDS-PAGE and Coomassie staining. The obtained purified protein was cleaved by subtilisin (2.3 μM) within 1 min giving the final trigger factor fragment. Separation of this TF fragment from the protease was achieved by anion-exchange chromatography. The protein was loaded onto a Fractogel EMD DEAE-650(M) column (1×5 cm, Merck, Darmstadt, Germany) equilibrated with 0.01 M HEPES (pH 7.5). Using a 0–1 M KCl linear gradient (80 ml total) the PPIase active fragment was eluted at 0.25 M KCl while the protease was in the flow through fractions.

2.5. Assay of PPIase activity and inhibitory studies

PPIase activity of TF was determined using the protease-coupled assay as described previously [6,24]. Conditions of isomer specific proteolysis for substrates of the structure Suc-Ala-Xaa-Pro-Phe-NH-Np were reached using subtilisin at the final concentration of 2.3 μM.

Inhibition studies using FK506 were carried out as described [6]. The rapamycin stock solution was prepared in 50% ethanol. In the case of a prolonged incubation of TF with the helper protease in the assay (when measuring low PPIase activities, for example), thrombin (final concentration 460 NIH/ml) as isomer specific protease and Suc-Ala-Ala-Pro-Arg-NH-Np as substrate were used. In the coupled assay thrombin was proved to be proteolytically inactive towards TF.

3. Results

3.1. Limited digestion of TF and isolation of an enzymatically active TF fragment

As could be detected by Western blotting standard preparations of TF sometimes showed a small amount of proteinaceous impurity migrating in the SDS gel at about 13 kDa (compare with lane 2, Fig. 1A). Limited digestion of authentic TF with subtilisin generated a polypeptide with a similar molecular mass on SDS-PAGE. However, the resulting digests were not homogeneous with respect to the product composition when analysed by SDS-PAGE and Western blotting. Thus, the 13 kDa protein was the smallest of the derivatives ranging in molecular mass from 13 to 59 kDa. During a defined time of proteolysis the PPIase activity did not decrease but the authentic TF band disappeared and smaller cross-reacting protein bands became visible instead.

When subjecting to a high concentration of subtilisin (2.3 μM) which is sufficient for conditions of isomer specific proteolysis used in the PPIase assay, TF was readily digested to the 13 kDa fragment within the mixing time (10 s). The remaining PPIase activity was not affected by subtilisin tested up to 30 min. In contrast, the ribosome bound form of TF was stable at least for 8 min when employing 70S particles to the same proteolysis conditions as was proved by Western blotting (Fig. 1B). Using thrombin as helper enzyme, unbound TF was not cleaved and displayed PPIase activity in a range comparable to that of the small fragment.

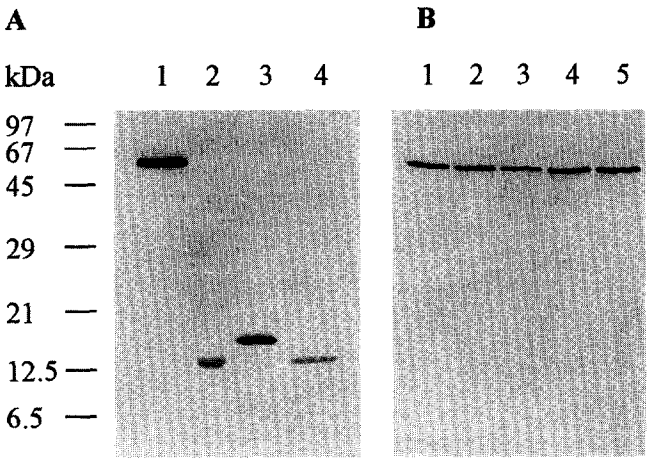

Fig. 1. Western blot analysis of trigger factor and its truncated variants. (A) Full length TF (lane 1) was employed to limited digestion by subtilisin and the PPIase active fragment was isolated (lane 2). Cloning and overexpression of the corresponding fragment carrying a histidine tag was carried out. The recombinant fragment was enriched by an Ni^{2+}-NTA column (lane 3). After a following gel filtration step the purified protein was cleaved by subtilisin (2.3 μM, 1 min). The final product was separated from subtilisin by analytical gel filtration using the SMART system (lane 4). (B) Limited digestion of 70S ribosomes by subtilisin. The 70S particles ($37A_{260}$/ml) were incubated with subtilisin (2.3 μM) for various times. The reaction was stopped by PMSF (1 mM). Aliquots of 30 μl were used for the SDS-PAGE and Western blotting. Heated SDS sample buffer was added and the sample was left at 95°C for 5 min. The incubation times of 70S with subtilisin were: dead time of mixing (about 5 s, lane 2), 4 min (lane 3) and 8 min (lane 4). Lanes 1 and 5 are controls of 70S incubated with PMSF treated subtilisin. A polyclonal antibody raised against trigger factor fragments (lacking either 46 or 58 amino acid residues subsequent to the N-terminus) was used for the primary incubation step.

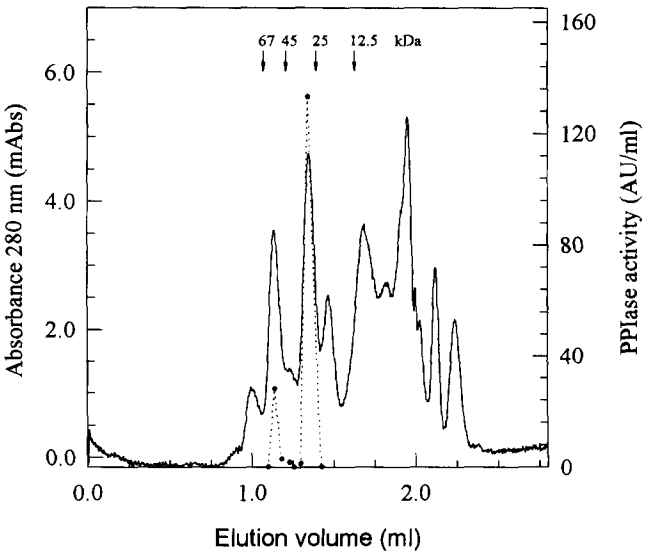

Fig. 2. Gel filtration after cleavage of authentic trigger factor by subtilisin. Trigger factor (4 μM) was incubated with subtilisin (0.15 μM) for 8 h at 4°C. An 50 μl sample was then applied to a Superdex 75 PC 3.2/30 column equilibrated with 0.01 M HEPES (pH 7.5), 0.15 M KCl, 0.015 M $MgCl_2$. A flow rate of 40 μl/min was used. After an eluted volume of 1 ml fractions of 40 μl were collected. PPIase activity was determined using samples of 20 μl of each fraction. It is shown as a dotted line using arbitrary units (AU) per ml. Calibration was performed under the same running conditions using bovine serum albumin (67 kDa), ovalbumin (45 kDa), chymotrypsinogen A (25 kDa) and cytochrome *c* (12.5 kDa).

To gain more information about the site of PPIase activity within the TF we isolated the short TF fragment. In Fig. 1A a Western blot of the authentic TF and the fragment after its isolation is shown (lanes 1,2). Isolation of the fragment was carried out following limited proteolysis of TF (4 μM) by subtilisin (0.15 μM) for 8 h at 4°C. After stopping the reaction by PMSF the mixture was separated by gel filtration (Fig. 2). PPIase activity was detected in protein fractions co-eluting with molecular masses of 56 and 29 kDa. The fractions harbouring the main activity (29 kDa proteins, 13 kDa in SDS-polyacrylamide gel) were subjected to reversed-phase HPLC. Only a single peak was detected. The N-terminus of this fragment was homogeneous in automatic Edman degradation and was found to start with the amino acid sequence RKQQATWKEKDGAVEAEDRVTIDFTGSVDG. This sequence is identical to an internal region of TF following Leu-144. Among several attempts this result was reproducible. Analysis by SDS-PAGE and Western blotting revealed a molecular mass of 13 kDa (Fig. 1A, lane 2). For more detailed information the molecular mass was determined by MALDI-TOF mass spectrometry (Fig. 3). The experimental value of 11 845.2 Da perfectly matched a region of TF which encompasses residues 145–251 exhibiting a calculated molecular mass of 11 846.6 Da.

Using HPLC for analysing the proteolysis products of TF, another fragment migrating at about 14 kDa on SDS-PAGE was also obtained besides some higher molecular mass products (23 and 24 kDa in SDS-polyacrylamide gel). The N-terminal sequence of both the 23 and 24 kDa products which were largely separated by HPLC was again RKQQA as was found

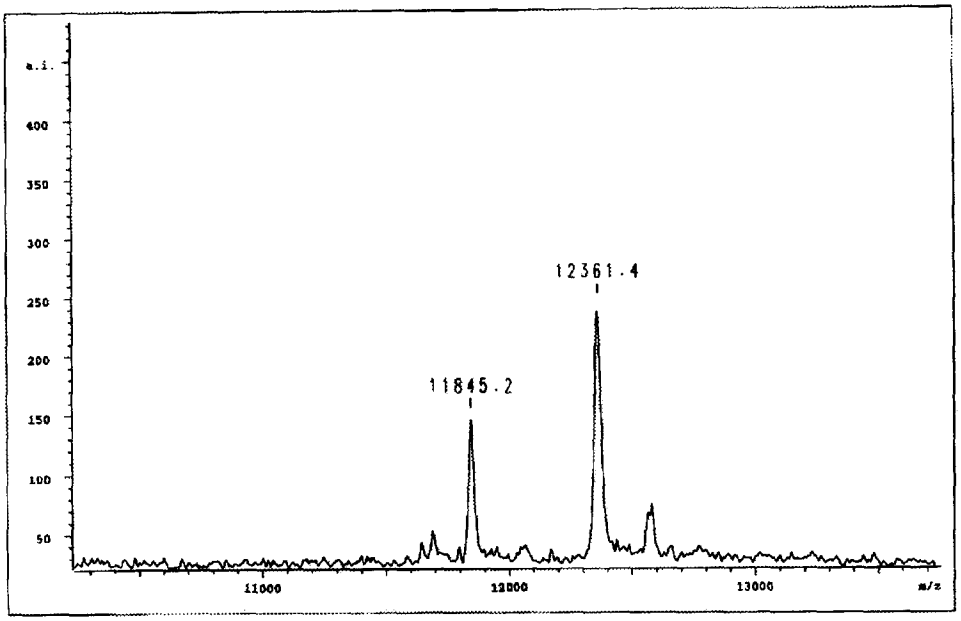

Fig. 3. MALDI-TOF mass spectrum of the purified TF fragment generated by subtilisin cleavage of authentic TF. Cytochrome *c* (molecular mass 12361.4 Da) was used as internal standard.

for the small active fragment. The amino acid sequence of the 14 kDa product started with MQVSVE which is identical to the N-terminus of the authentic TF. This N-terminal fragment was not obtained by gel filtration whereas the 23 and 24 kDa products were co-eluted with a molecular mass of 56 kDa displaying the minor PPIase activity seen in Fig. 2.

3.2. *Molecular cloning, overexpression and isolation of the enzymatically active domain of TF*

The PPIase active domain of TF was cloned and expressed in *E. coli*. Overexpression was controlled by SDS-PAGE and Western blotting (not shown). In addition to an N-terminal histidine tag the construct encompassed residues Met-140 to Ala-250 of TF. The cleaving site for subtilisin found via the experiments described above (Leu-144–Arg-145) was maintained within the expressed fragment. Thus, after purification by an Ni^{2+}NTA column the enriched protein (Fig. 1A, lane 3) was subjected to digestion by subtilisin (2.3 μM) for 1 min. The proteolysis provided an uniform product with regard to the recombinant fragment as monitored by SDS-PAGE (not shown). Larger amounts of the resulting protein were further purified as described in section 2. An analytical sample was applied to gel filtration providing a homogeneous protein (Fig. 1A, lane 4). The protein was eluted with a molecular mass of 29 kDa as was observed for the authentic fragment of TF. However, the molecular mass determined by MALDI mass spectrometry provided a value of 11235.4 Da which is somewhat lower than that of the authentic fragment. N-terminal sequencing revealed the amino acids QATWKEKDG. The molecular mass matched the fragment Gln-148 to Thr-249 of TF. In contrast to native TF, other cleaving sites must be accessible for subtilisin within the constructed protein.

3.3. *Enzymatic and structural properties*

In the PPIase assay using tetrapeptide-4-nitroanilides as substrates the recombinant PPIase domain of TF showed few if any changes in its enzymatic efficiency in comparison to authentic TF. The apparent value of k_{cat}/K_m was determined to be 1.3 μM^{-1} s^{-1} for the recombinant 11.2 kDa protein using Suc-Ala-Phe-Pro-Phe-NH-Np as substrate. Thus, the PPIase domain retains the magnitude of activity usually found for FKBPs. For the amino acid sequence region encompassing the PPIase domain a certain degree of homology with FKBPs was shown [12]. However, in contrast to other FKBPs authentic TF as well as the 11.8 kDa domain were not inhibited by the peptidomacrolide FK506, even when

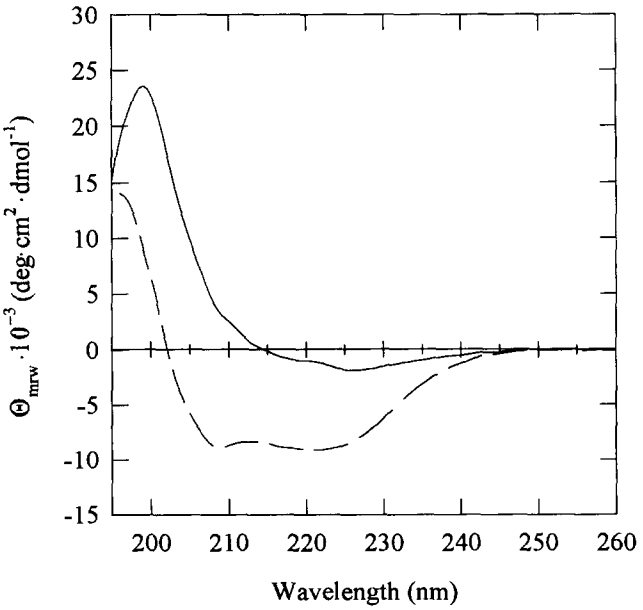

Fig. 4. CD spectra of authentic TF (– –) and the recombinant PPIase domain of TF (—) in the far UV region. The mean residue molecular ellipticity is shown. The protein concentrations were 8.2 μM for the whole TF and 25.1 μM in the case of the fragment. CD measurements were carried out in 5 mM sodium phosphate (pH 7.0) using 0.1 cm cells.

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                                          * *         **
hFKBP12  1    GVQVETISP GDGRTFP.KR GQTCVVHYTG MLEDGKKFDS SRDRNKPFKF 48
TF       141 LDTLRKQQAT WKEKDGAVEA EDRVTIDFTG SV.DGEEFEG GKA..SDFVL 187

                 **   *                    *         *
hFKBP12  49  MLGKQEVIRG WEEGVAQMSV GQRAKLTISP DYAYGATGHP GIIPPHATLV 98
TF       188 AMGQGRMIPG FEDGIKGHKA GEEFTIDVTF PEEYHAENL. ....KGKAAK 232

             *
hFKBP12  99  FDVELLKLE                                            107
TF       233 FAINLKKVEE RELPELTAEF IKRFGVEDGS VEGLRAEVRK NMERELRAPS 282
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Fig. 5. Partial sequence of TF in comparison to human FKBP12 [12,38]. The amino acyl residues enclosed to the PPIase active fragment are underlined. Asterisks mark residues of hFKBP12 which are involved in FK506 binding [39]. Bold letters represent residues which are perfectly conserved among FKBPs [39].

assayed at micromolar concentrations of inhibitor. FKBPs are also sensitive to the structurally related macrolide rapamycin over the nanomolar range. However, TF was also not affected by rapamycin as assessed up to 3 μM.

The secondary structures of the authentic TF and the PPIase-domain were compared by far UV-CD spectroscopy (Fig. 4). As could be derived from the secondary structure prediction algorithm [25], the complete protein contains considerable more α-helix in contrast to the fragment whereas the spectrum of the active domain shows a higher content of β-sheet.

4. Discussion

Recently, some more data have been provided about the trigger factor originally discovered through its binding to a precursor protein and implication in membrane translocation. The intrinsic PPIase activity of this protein taken together with its presence at the ribosome and nascent polypeptide chains, respectively, is most attractive in the view of the de novo folding.

Here we could establish a domain-like organization of TF, and were able to isolate the PPIase domain of the protein.

A certain degree of homology to FKBPs has been reported for the domain of TF encompassing residues 142–241 [12]. Indeed, when TF which is 432 amino acids in length was subjected to digestion by subtilisin, a single remaining active fragment was generated. It matches the sequence region thought to express similarity to FKBPs (Fig. 5). This isolated inner fragment of TF consisting of 107 amino acyl residues is comparable in length to many FKBPs, for example the human FKBP12 which also extends over 107 amino acids. Additionally, the expressed recombinant form was as active as the authentic fragment but consists of only 102 amino acyl residues. Both the authentic and recombinant domain of TF showed a positive reaction with our anti-TF antibodies in Western blot experiments. The antibodies were originally produced against truncated forms of TF lacking either 46 or 58 amino acids subsequent to the N-terminus. However, in comparison to the complete TF the antigen-antibody interaction appeared rather weak in the case of the TF domain.

FKBPs are named due to their high affinity binding to FK506. However, neither the authentic TF nor the active domain was sensitive to FK506 and rapamycin, even when the inhibitors were applied at high concentrations. Some amino acid residues of the FK506 binding region of FKBP12 are conserved in the primary structure of TF but many are altered to closely related residues [12]. Site-directed mutagenesis has been carried out with hFKBP12 in order to define the residues participating in macrolide binding [26,27]. Thus, some mutations led to minor changes of the inhibitory constants of the protein but, as exemplified in the case of the highly conserved Asp-37 (numbered according to hFKBP12, see Fig. 5), substitution through Val revealed a substantial increase in the K_i value from 0.6 nM to 350 nM [27]. In contrast to other FKBPs the TF of *E. coli* and the recently described TF of *Campylobacter jejuni* [28] contain a glutamate residue at this position. For the TF of *C. jejuni*, however, data about PPIase activity and possibly FK506 binding have not been available until now. The sequences of TFs from two other organisms identified by their sequence homology to *E. coli* TF also contain at this position (Asp-37 numbered according to hFKBP12) a glutamate residue [29,30].

There are some larger, multidomain FKBPs and also cyclophilins found as constituents in high molecular mass steroid receptor complexes [31,32]. The FKBP52 (also known as HSP56, p59, FKBP59 or HBI) (reviewed in [33]) contains three domains which are homologous to the FKBP12 [34]. When chicken thymus HSP56, an analogue of the FKBP52, was digested by endoproteinase Lys C an active FK506 binding domain of 17 kDa was obtained [35].

The stable active TF fragment generated by subtilisin treatment supports the idea of a domain structure of TF which was recently predicted using computer methods [12]. The linkages between protein domains are often particularly susceptible to proteolysis. Subtilisin is known as a protease having a broad subsite specificity [36,37]. Therefore, it is comprehensible that proteins possibly are readily digested when employed in subtilisin treatment. In the case of TF at least the central domain remained after digestion by subtilisin while other domains were probably proteolysed. The observation of an N-terminal fragment of TF of 14 kDa by separating the proteolysis products using HPLC still confirms the hypothesis of a domain-like structure of TF.

As was estimated from far UV-CD data the secondary structures of both the authentic TF and its active domain appeared completely different. The authentic TF contains considerable more α-helix. A relatively high content of α-helix within the entire TF was previously predicted from primary structure for the N-terminal domain of TF [12].

However, experimental evidence for a role of domains flanking PPIase cores is still scarce even if this type of en-

zymes dominates the PPIase families [9]. The isolated TF domains may provide an example where the functional analysis can be accomplished.

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