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A ribosome-associated peptidyl-prolyl *cis/trans* isomerase identified as the trigger factor

Gerlind Stoller, K.Peter Rücknagel, Knud H.Nierhaus[1], Franz X.Schmid[2], Gunter Fischer[3] and Jens-U.Rahfeld

Max-Planck-Gesellschaft, Arbeitsgruppe 'Enzymologie der Peptidbindung', Weinbergweg 16a, D-06120 Halle/S., [1]Max-Planck-Institut für Molekulare Genetik, Ihnestraße 73, D-14195 Berlin and [2]Laboratorium für Biochemie, Universität Bayreuth, D-95440 Bayreuth, Germany

[3]Corresponding author

Peptidyl-prolyl *cis/trans* isomerases (PPIases) are enzymes that catalyse protein folding both *in vitro* and *in vivo*. We isolated a peptidyl-prolyl *cis/trans* isomerase (PPIase) which is specifically associated with the 50S subunit of the *Escherichia coli* ribosome. This association was abolished by adding at least 1.5 M LiCl. Sequencing the N-terminal amino acids in addition to three proteolytic fragments totalling 62 amino acids revealed that this PPIase is identical to the *E.coli* trigger factor. A comparison of the amino acid sequence of trigger factor with those of other PPIase families shows little similarities, suggesting that trigger factor may represent an additional family of PPIases. Trigger factor was purified to homogeneity on a preparative scale from *E.coli* and its enzymatic properties were studied. In its activity towards oligopeptide substrates, the trigger factor resembles the FK506-binding proteins (FKBPs). Additionally, the pattern of subsite specificities with respect to the amino acid preceding proline in Suc-Ala-Xaa-Pro-Phe-4-nitroanilides is reminiscent of FKBPs. However, the PPIase activity of the trigger factor was not inhibited by either FK506 or by cyclosporin A at concentrations up to 100 μM. *In vitro*, the trigger factor catalysed the proline-limited refolding of a variant of RNase T1 much better than all other PPIases that have been examined so far.

Keywords: *Escherichia coli*/folding helper enzyme/peptidyl-prolyl *cis/trans* isomerase/protein folding/trigger factor

Introduction

In contrast to the *in vitro* refolding of denatured proteins, investigations of *de novo* protein folding have to take into account effects of the cellular environment on dynamics and equilibria of molecular steps like bond rotations, oligomerization and disulfide bridging. It is now clear that cellular components provide numerous interactions with a potential to influence both the pathway and the rate of folding (Gething and Sambrook, 1992; Jaenicke, 1993).

The *cis/trans* isomerization of prolyl peptide bonds (-Xaa-Pro-) is a slow molecular step during the *in vitro* refolding because non-native prolyl bond conformations occur in the denatured state of a protein (Brandts *et al.*, 1975; Schmid, 1986a, 1993). About 5.1% of all prolyl bonds are *cis* in native proteins when calculated by published methods (Stewart *et al.*, 1990; MacArthur and Thornton, 1991) from an updated version of the Brookhaven database. Many globular proteins contain at least one *cis* prolyl residue in the native state, and their refolding has to involve slow *trans* to *cis* isomerizations.

However, we have to consider that biosynthetic folding starts from different preconditions. Studies concerning the stereochemical control of transpeptidation at the ribosome showed that the spatial arrangement of peptide bonds of the nascent polypeptide chain probably fits to an *all trans* conformation (Lim and Spirin, 1986).

Furthermore, the rate of cellular protein folding appears to be enhanced when compared with that of the refolding of denatured proteins measured by regaining the activity and other methods (Fedorov and Baldwin, 1995). An extrapolation of the *in vitro* folding kinetics to the conditions of co-translational or post-translational protein folding can be performed. The rate of cellular folding reactions which must be limited by prolyl bond isomerization in some cases suggested a decrease in the activation energy of this conformational interconversion within the cell (Wetlaufer, 1985).

In view of these results, different effects may contribute to a lowering of the rotational barrier of prolyl peptide bonds during biosynthetic folding. Most of these effects should be detectable in *in vitro* refolding assays. In folding intermediates of RNase A, a marked increase in the rate of the -Tyr-Pro^{93}- isomerization has been detected when compared with unstructured peptides (Schmid, 1986b). Moreover, intramolecular assistance of *cis/trans* isomerization has been suggested for side chains of amino acids which are distant in sequence but in proximity to the nitrogen atom of the prolyl bond in a folding intermediate (Texter *et al.*, 1992). However, *cis/trans* isomerization is not necessarily accelerated by chain structuring in polypeptides (Kiefhaber *et al.*, 1992). Transfer of the prolyl peptide bonds from aqueous solution into the membrane lipid environment is another factor which may contribute to stabilization of the less polar transition state of isomerization. This effect has been simulated by measurement of *cis/trans* isomerization rates in non-aqueous solutions, but only a moderate enhancement of the rates below a factor of 100 was seen (Eberhardt *et al.*, 1992; Radzicka *et al.*, 1992).

Among the various factors so far described, enzymatic catalysis by peptidyl-prolyl *cis/trans* isomerases (PPIases, EC no. 5.2.1.8) offers the most attractive way to accelerate folding reactions which are limited in rate by the isomerization of a particular prolyl bond (Fischer *et al.*, 1984; Schmid, 1993).

Currently, the class of PPIases is divided into three unrelated families: the cyclophilins, the FK506-binding

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proteins (FKBPs) (both reviewed by Fischer, 1994) and the parvulins (Rahfeld *et al.*, 1994a; Rudd *et al.*, 1995). Despite their ubiquitous occurrence and high intracellular concentration, evidence for the involvement of PPIases in cellular protein folding is still scare (Schmid, 1993). For example, a lag is produced for the folding of transferrin in hep G2 cells in the presence of cyclosporin A which specifically inhibits the PPIase activity of cyclophilins (Lodish and Kong, 1991). Further evidence was obtained for the involvement of both cyclophilins and FKBPs in the maturation of two bacterial luciferases which were translated *in vitro* in a reticulocyte lysate system (Kruse *et al.*, 1995).

The refolding of newly imported precursor proteins in the mitochondrial matrix compartment is a protein folding event within the cell, but occurring after the synthesis on the ribosome. Recent studies have shown that the folding of such imported proteins is accelerated by mitochondrial cyclophilins by virtue of their PPIase activity (Matouschek *et al.*, 1995; Rassow *et al.*, 1995).

Here we asked whether PPIases exist close to the site of protein synthesis, i.e. at or near the ribosome of *Escherichia coli*. In *E.coli*, two cyclophilins and a single parvulin have been found to date, and there was no indication for an association of these PPIases with ribosomes (Liu and Walsh, 1990; Hayano *et al.*, 1991; Compton *et al.*, 1992; Rahfeld *et al.*, 1994a). Consequently, we investigated whether PPIase activity co-purifies with ribosomes, and we could indeed detect PPIase activity on ribosomes. This activity was specifically associated with the 50S subunit, which carries the peptidyltransferase centre.

Partial amino acid sequencing revealed that the ribosome-bound PPIase was identical with the already known trigger factor (Crooke and Wickner, 1987). Owing to the lack of similarity with the sequences of known PPIases, the trigger factor represents a new family, the fourth one of this class of enzymes. Furthermore, we describe an isolation procedure and some enzymatic properties of this novel enzyme.

Results

Localization of PPIase activity on ribosomal particles

Samples of crude 70S, tightly coupled 70S ribosomes, 50S and 30S subunits from *E.coli* were prepared and applied to a sensitive PPIase assay utilizing isomer-specific proteolysis by chymotrypsin. In certain cases, the measurements were carried out with several PPIase substrates (Suc-Ala-Xaa-Pro-Phe-NH-Np; Xaa = Phe, Leu, Ala) to obtain information regarding the nature of the PPIase detected.

A considerable PPIase activity was found in all fractions assayed in the course of the purification of ribosomes with Suc-Ala-Phe-Pro-Phe-NH-Np, except for samples of the 30S subunits. Crude 70S ribosomes showed a much higher PPIase activity than the tightly coupled ribosomes. Besides a putative PPIase specifically present on ribosomes, this might be caused by the known PPIases abundant in the *E.coli* cytoplasm which could still contaminate the preparation. Furthermore, a comparison of the specific activities associated with coupled 70S [75 arbitrary units (AU)/nmol particle] and with 50S subunit (1.4 AU/nmol particle) revealed a 50-fold higher value of the activity for the 70S particles. In order to check whether this activity also originates from known *E.coli* PPIases, the enzymatic activity of the particles towards the extended set of substrates was measured (Table I). Neither known *E.coli* PPIases nor any mixture of these enzymes could explain the pattern of subsite specificities detected in the ribosomal particles. However, a high level of similarity was obtained for 70S and 50S particles. This provides the first strong evidence for a novel PPIase of *E.coli* that was responsible for the isomerase activity bound to ribosomal particles.

Table I. Substrate specificity of the ribosomal PPIase compared to the known PPIases of *E.coli* (Cyp18cy, Cyp21peri and parvulin) toward Suc-Ala-Xaa-Pro-Phe-NH-Np

Xaa	Ribosomal PPIase		*E.coli* Cyp18cy[a]	*E.coli* Cyp21peri[a]	Parvulin[b]
	70S	50S			
Phe	100	100	100	100	100
Ala	21	22	400	345	60
Leu	64	67	150	120	150

Measurements were carried out in 0.035 M HEPES (pH 7.8) at 10°C. The ratios of specificity constants are shown by setting the value of the substrate Suc-Ala-Phe-Pro-Phe-NH-Np to 100%. Data were taken from [a]Compton *et al.* (1992); [b]Rahfeld *et al.* (1994a).

Ribosome-bound PPIase is the trigger factor

Ribosomal 50S subunits (1160 A_{260} units/ml) were diluted in a buffer containing 2 M LiCl, 0.01 M $MgCl_2$, 0.5 mM EDTA, 1 mM dithiothreitol (DTT), 0.02 M HEPES (pH 7.5) and incubated for 5 h at 0°C. Then, a sample of 50 µl was subjected to size-exclusion chromatography on a calibrated Superdex 75 column. PPIase activity was measured in each fraction using the substrate Suc-Ala-Phe-Pro-Phe-NH-Np. The activity was found in fractions that corresponded to an approximate molecular mass of 67 kDa (Figure 1A). A sample of the subsequently purified 67 kDa protein (see below) coincided with the peak of activity found after the dissociation from the 50S subunit (Figure 1B). Other components of the 50S particle appeared mainly in the exclusion volume.

The fact that the PPIase activity eluted with a molecular mass notably larger than the largest protein of the 50S subunit (L2, M_r = 29 730 Da; Wittmann-Liebold, 1986) prompted us to identify this protein by SDS–PAGE in total protein extracts of ribosomes and ribosomal subunits. The protein pattern of 70S ribosomes, 50S and 30S subunits together with that of total cell extract and post-ribosomal S100 supernatant is shown in Figure 2A.

Only three proteins could be detected at the high molecular mass range of the 70S sample (lane 5) with apparent molecular masses of 67, 60 and 59 kDa. The largest protein extractable from 30S subunits is S1 which co-migrates with 67 kDa proteins, but has a molecular mass of only 61 159 Da (Wittmann-Liebold, 1986). The protein of apparent mol. wt 60 kDa observed in lane 5 (70S) had a high intensity within proteins extracted from the 30S subunit (lane 7) which is devoid of any PPIase activity. Within the expected range of 30–67 kDa, there was only a single protein band in the 50S sample (lane 6)

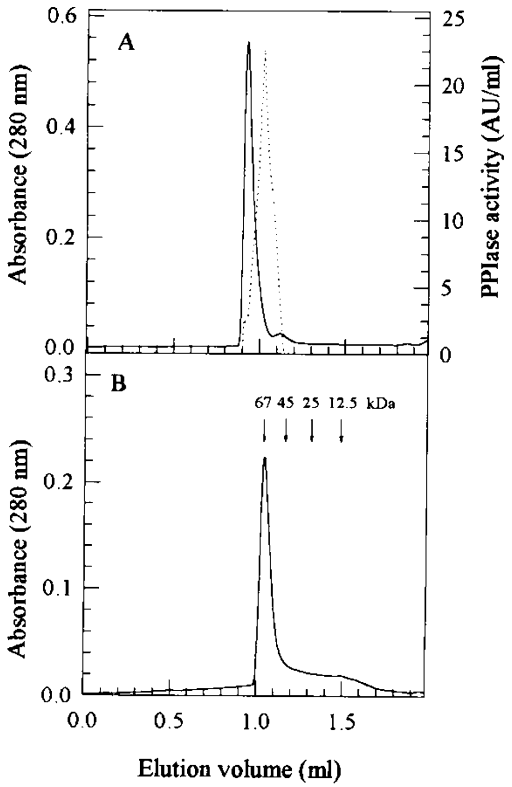


Fig. 1. Gel filtration of 50S subunits. (**A**) The 50S subunits (773 A_{260} units/ml) were pre-incubated in 2 M LiCl, 0.02 M HEPES, 0.01 M manganese chloride, 0.5 mM EDTA, 1 mM DTT (pH 7.5) at 0°C for 5 h. A diluted sample (200-fold) of 50 µl was applied to a Superdex 75 PC 3.2/30 column equilibrated with the same buffer. For the runs a flow rate of 40 µl/min was used. The temperature was maintained at 4°C. The shown PPIase activity was calculated for the solution of the 50S subunits in the LiCl-containing mixture. It is indicated as a dotted line using AU/ml. (**B**) A sample of the purified 59 kDa protein (1.8 µg) was treated as described above and loaded onto the column. Calibration was performed under the same running conditions using four proteins (bovine serum albumin, 67 kDa; ovalbumin, 45 kDa; chymotrypsinogen A, 25 kDa; cytochrome c, 12.5 kDa).

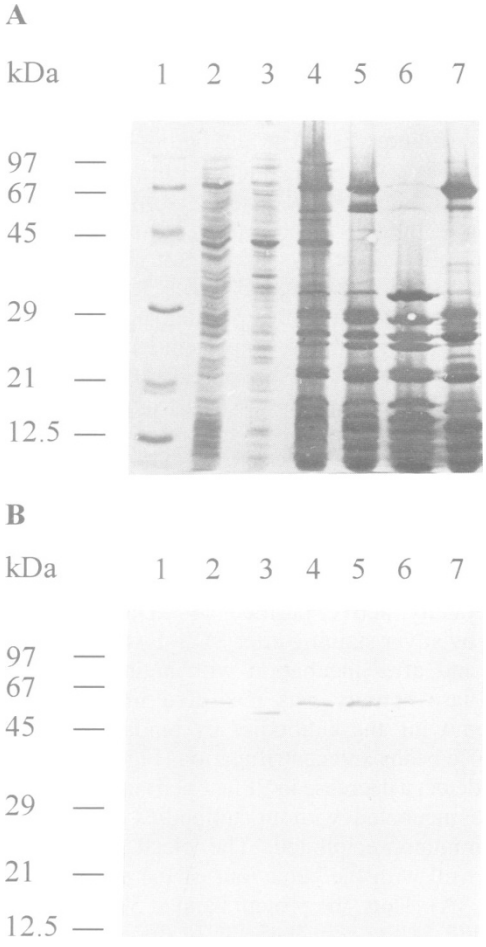


Fig. 2. Electrophoretic analysis of the distribution of the 59 kDa protein among ribosomal subunits. (**A**) Amido black staining of the NC membrane. (**B**) Immunoblot. A polyclonal antiserum raised against two truncated forms of the 59 kDa protein (lacking either 46 or 58 amino acid residues subsequent to the N-terminus) was used for the primary incubation step. A peroxidase-conjugated goat anti-rabbit IgG was used for the second incubation step. Development was carried out using a buffer containing 0.018% 4-chloro-1-naphthol (w/v), 0.024% hydrogen peroxide (v/v), 0.15 M NaCl, 0.05 M Tris–HCl (pH 6.0). Lane 1, molecular weight markers. Lane 2, proteins of the *E.coli* homogenate (5 µg). Lane 3, proteins of the S100 supernatant (5 µg). Lanes 4–7, equal amounts of ribosomal fractions (2.7 A_{260} units); 4, crude 70S; 5, tightly coupled 70S; 6, 50S subunits; 7, 30S subunits.

with an apparent molecular mass of 59 kDa. This band had a much lower intensity in the 50S (lane 6) than in the 70S sample (lane 5). This observation agrees well with the decrease in PPIase activity when going from 70S to 50S particles. This indicated that the 59 kDa band in SDS–PAGE might be the novel PPIase which showed a relative molecular mass of ~67 kDa in gel filtration. After electroblotting of the 59 kDa protein from SDS–polyacrylamide gel to a Selex 20 membrane, the first 20 amino acids of the protein at the N-terminus were sequenced. Comparison of this sequence, MQVSVETTQ-GLGRRVTITIA, with those present in the combined protein database (Pir, Mipsown, Patchx, Swissprot) by FASTA revealed identity to the first 20 amino acids of the trigger factor from *E.coli* (Guthrie and Wickner, 1990). The relative molecular mass of the trigger factor (432 amino acids) was calculated to be 48 023 Da and roughly agrees with the value estimated by SDS–PAGE. Molecular mass determination by electrospray mass spectrometry yielded 48 228 ± 20 Da, possibly indicating a yet unidentified post-translational modification. In several preparations, two smaller proteins of 54 and 50 kDa estimated by SDS–PAGE also displayed PPIase activity. After separation by reversed-phase HPLC, truncated polypeptide chains of the trigger factor emerged from amino acid sequencing. The respective sequences GKVPMNIVAQR-YGASVRQD and GASVRQDVLGDLMSRNFID suggested that the trigger factor was apparently cleaved proteolytically at positions 47 and 59, possibly during the purification procedure. As was already found for the N-terminal part, complete identity to the trigger factor also existed for the internal sequences.

The trigger factor shares only 18.2 and 28.3% overall identity with the conserved region of the amino acid sequences of cyclophilins and FKBPs, respectively (calculated with the BESTFIT alignment program). The corresponding value found with the 10.1 kDa *E.coli* parvulin is 21.1%. Values could be obtained only by the extensive use of sequence gaps. Trigger factor also does not contain any of the short sequence motifs typical for each other

family of PPIases (Trandinh *et al.*, 1992; Rahfeld *et al.*, 1994b).

Western blot analysis was carried out to check and confirm the distribution of trigger factor among ribosomal subunits (Figure 2B). The antibodies used for the first incubation step were raised against the truncated forms of trigger factor mentioned above. Using these polyclonal antibodies, the 59 kDa protein could be specifically detected in *E.coli* cell lysates (Figure 2B, lane 2), in 70S and 50S particles (Figure 2B, lanes 5 and 6), as well as in fractions containing the purified protein (Figure 3B, lane 2). In these samples, no protein band at a lower mol. wt than 59 kDa could be observed giving a positive reaction to these antibodies. Thus, trigger factor could be detected in all fractions except that derived from 30S subunits. Therefore, the results of Western blotting are in excellent agreement with the distribution of PPIase activity among the ribosomal subunits.

The identity of trigger factor as the PPIase was further proved by immunoprecipitation. Fractions containing the enzymatically active purified 59 kDa protein, as was proved by silver staining after SDS–PAGE, were analysed before and after incubation with antibodies (Figure 3). The PPIase activity was measured in the supernatants after removing the antibodies by binding on Protein A–Sepharose beads and centrifugation (Figure 3C). We could indeed detect a decrease in PPIase activity when comparing samples incubated without antibodies and samples that were immunoprecipitated. The electrophoretic analysis agreed well with the behaviour of the enzymatic activity (Figure 3A). Thus, the protein band at 59 kDa disappeared depending on the concentration of the antibodies and the intensity corresponds to the value found for PPIase activity.

LiCl-mediated dissociation of 50S subunits

Lill *et al.* (1988) demonstrated that the trigger factor can be dissociated from the ribosome by incubating the particles at a concentration of 1.5 M LiCl. The questions were addressed whether or not trigger factor represents a unique example for PPIases on ribosomes, and whether the trigger factor and PPIase activity are extracted in parallel from the 50S subunit. For these investigations, 50S subunits (2.4 μM) were submitted to various concentrations of LiCl and incubated for 5 h on ice. The dissociated proteins were separated from the residual ribosomal cores by ultracentrifugation. The supernatants (10 ml each) and the pellets redissolved in 0.5 ml of HEPES buffer were collected and the PPIase activities were determined. For this purpose, portions of 3 ml of the supernatants were desalted and concentrated 10-fold using ultrafiltration on Centricon tubes with an exclusion size of 3500 Da. The results of the experiments are summarized in Figure 4. The PPIase activity is indeed released from the 50S subunit by LiCl. It increases in the soluble fractions up to 1.5 M LiCl, whereas the PPIase activity decreases in a reciprocal fashion in the pellets (Figure 4A). As indicated by Western blot analysis, the distribution of the protein band of the trigger factor was similar to the pattern of activities determined in the soluble and the sediment fractions. Thus, the intensity of the 59 kDa protein band of trigger factor increases from 0 to 1.5 M in the samples of the soluble fractions (Figure 4B). Because of the monophasic increase in activity (Figure 4A) of the supernatant fraction and the very low proportion of remaining activity at high salt concentration with the pellet, an additional PPIase attached to the 50S subunit seems improbable.

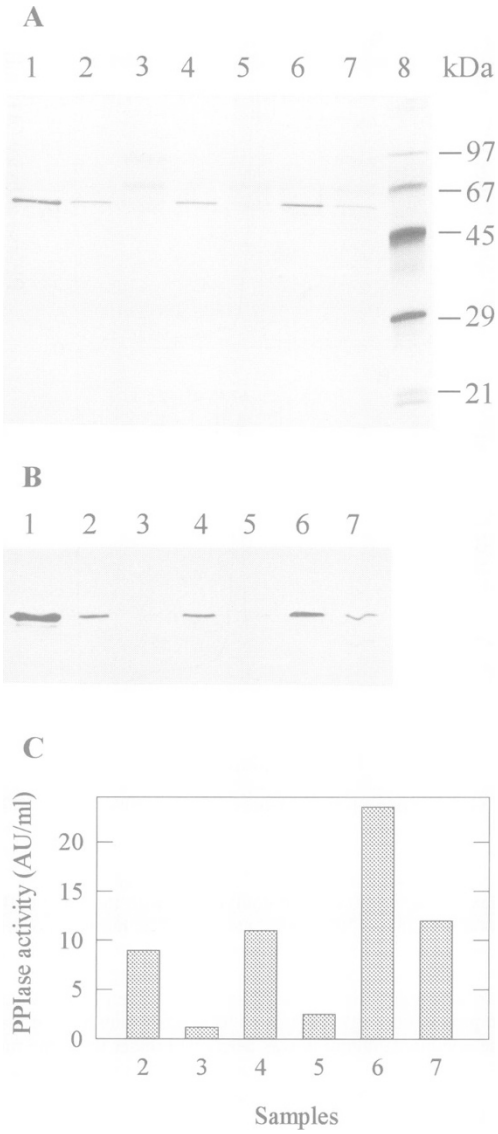


Fig. 3. Immunoprecipitation of the purified trigger factor. Samples of trigger factor were incubated with polyclonal antibodies for 2 h at 4°C. Swollen Protein A–Sepharose was then added and incubated for 1.5 h. The supernatants of the subsequent centrifugation were used for analysis by silver staining after SDS–PAGE (**A**), immunoblotting (**B**) and PPIase measurements (**C**). For comparison, control samples of trigger factor at the different concentrations were treated equally, but without adding antibodies. Lane 1, trigger factor. Lane 2, control without antibodies (34 nM trigger factor). Lane 3, 34 nM trigger factor and 240 nM polyclonal IgG. Lane 4, control (45 nM trigger factor). Lane 5, 45 nM trigger factor and 160 nM IgG. Lane 6, control (87 nM trigger factor). Lane 7, 87 nM trigger factor and 150 nM IgG. Lane 8, molecular weight markers. The samples in (C) were numbered according to the lanes in (A) and (B).

Purification of trigger factor from cell extracts

The PPIase activity of the trigger factor was used to develop a purification protocol that enables us to isolate ~1 mg of homogeneous protein from a 3 l overnight culture of *E.coli* cells (see Table II). The purification procedure includes anion-exchange chromatography steps

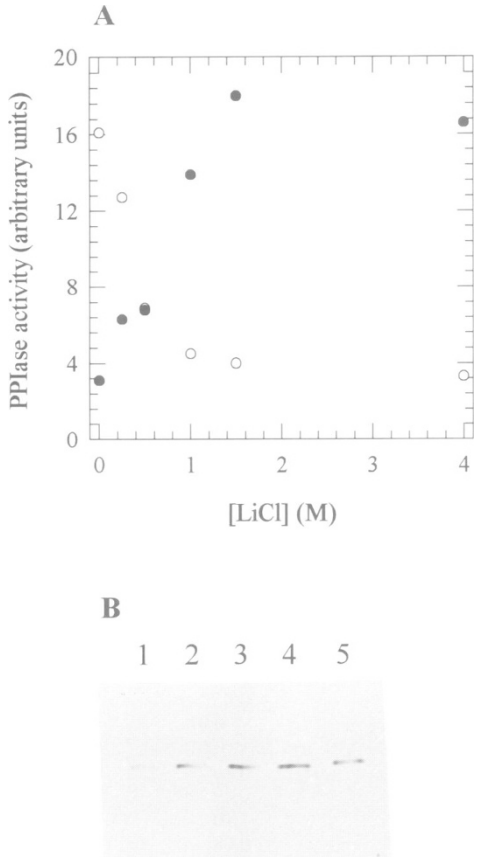


Fig. 4. Dissociation of the ribosomal 50S subunit by LiCl at increasing concentrations. The PPIase activity was determined using the substrate Suc-Ala-Phe-Pro-Phe-NH-Np under standard conditions [0.035 M HEPES buffer (pH 7.8), 10°C]. (**A**) The 50S subunits (2.4 μM) were incubated at various concentrations of LiCl for 5 h and then centrifuged. The values refer to the total enzymatic activity in the soluble fractions (supernatants of 10 ml) and in the pellets (0.5 ml after dissolving in buffer), respectively. (●) enzymatic activity of the supernatants and (○) of the sediments. Samples of 200 μl of the supernatants were used for Western blot analysis (**B**) after dialysis and lyophilization. Lane 1, 0 M LiCl; lane 2, 0.25 M LiCl; lane 3, 0.5 M LiCl; lane 4, 1.0 M LiCl; lane 5, 1.5 M LiCl.

using Fractogel EMD-650(M), immobilized metal chelate affinity chromatography (IMAC) on Chelating Sepharose with immobilized Ni^{2+} ions, hydrophobic interaction chromatography (HIC) on Phenyl-Sepharose and dye ligand chromatography on a Fractogel TSK AF-Blue. The cytosolic cyclophilin (Cyp18cy) dominates in the first purification steps because it accounts for >90% of the total PPIase activity of the *E.coli* cell (Compton *et al.*, 1992). This enzyme reacted quite similar to the trigger factor in the most chromatographic steps (e.g. the pI values are 4.6 for the trigger factor and 4.8 for the Cyp18cy). The other PPIases, parvulin and periplasmic cyclophilin (Cyp21peri), could be separated by the first anion-exchange chromatography. Cyp18cy could largely be removed from the trigger factor by HIC. However, a complete separation was not obtained although the trigger factor adsorbs more strongly to the hydrophobic matrix than Cyp18cy. Inhibition with cyclosporin A and Western blot analyses with antibodies raised against a peptide consensus sequence of cyclophilins were used to identify and to discard fractions contaminated with Cyp18cy. Thus,

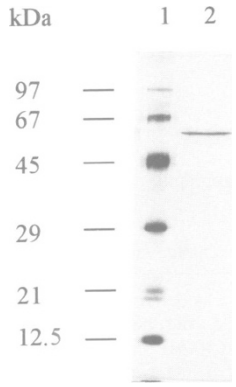


Fig. 5. SDS–PAGE (12.5%) of purified trigger factor. The polyacrylamide gel was silver stained. Lane 1, molecular weight markers. Lane 2, purified trigger factor (0.6 μg).

trigger factor could be completely separated from Cyp18cy, but at the expense of the yield of trigger factor protein. Final purification was performed by dye ligand chromatography and anion-exchange chromatography, leading to homogeneous protein as indicated by silver staining after SDS–PAGE (Figure 5).

The trigger factor resembles FKBP in its subsite specificity but is insensitive toward FK506

The role of the P_1 subsite of substrates [for subsite nomenclature, see Schechter and Berger (1966) and Harrison and Stein (1990)] in catalysis was probed by determining the apparent specificity constants k_{cat}/K_m for 10 different oligopeptide substrates of the sequence Suc-Ala-Xaa-Pro-Phe-NH-Np. As shown in Table III, the specific activities of the trigger factor were found to be ranging on the lower end of the scale when compared with those of the other PPIases from *E.coli* or FKBP25mem from *Legionella pneumophila*. The latter is a prokaryotic member of the FKBPs that represents a typical FK506-binding PPIase with respect to its subsite specificity (Fischer *et al.*, 1992). However, the measured values represent the lower limit of activity because a method for active site titration of the trigger factor is not yet known. Obviously, the pattern of specificity of the trigger factor is reminiscent of FKBP25mem with a remarkable exclusion of Ile preceding proline in the substrates. Clearly, hydrophobic side chains are preferred in the P_1 position and a particular preference is observed for Phe in this position. The ratios of k_{cat}/K_m values are most meaningful to differentiate into enzyme families by activity. As was exemplified for substrates changed from Phe to Ala in the P_1 position, the ratio is 4.6 for the trigger factor, but was found to be 0.3 and 16.7 for cyclophilins and parvulin, respectively.

Many members of the two subgroups, the cyclophilins and the FKBPs, are inhibited by cyclosporin A and FK506, respectively, in nanomolar concentrations. For parvulin, there was no evidence for inhibition by either cyclosporin A or FK506 up to a concentration of 5 μM (Rahfeld *et al.*, 1994a). To clarify further whether the trigger factor establishes a novel family of PPIases, the effect of concentrations up to 100 μM FK506 and cyclosporin A was studied. The PPIase activity of the trigger factor is not affected by FK506 and cyclosporin A. This result

Table II. Purification scheme leading to homogeneous trigger factor

Purification step	Total protein (mg)	Total PPIase activity[a] (AU)	Specific PPIase activity[a] (AU/mg)	Purification	Recovery (%)
Crude extract	930	225 000	242	1.0	100.0
Anion-exchange chromatography[b]	205	25 400	124	0.5	11.3
IMAC[c]	90	16 500	183	0.8	7.3
HIC[d]	4	8 600	2 150	8.8	3.8
Dye ligand[e] and anion-exchange chromatography[b]	1.1	4 400	4 400	18.2	1.8

[a]The enzymatic activity was determined toward Suc-Ala-Phe-Pro-Phe-NH-Np. The PPIase activity accounts for the sum of activity of *E.coli* PPIases. Trigger factor is separated after the HIC step using Phenyl-Sepharose.
[b]Fractogel EMD DEAE-650(M).
[c]Chelating Sepharose.
[d]Phenyl-Sepharose.
[e]Fractogel TSK AF-Blue.

Table III. Substrate specificity of trigger factor toward substrates of the structure Suc-Ala-Xaa-Pro-Phe-NH-Np compared with *E.coli* Cyp18cy, *E.coli* Cyp18peri, *E.coli* parvulin and *L.pneumophila* FKBP25mem

Xaa	Trigger factor k_{cat}/K_m ($\mu M^{-1}s^{-1}$)	*E.coli* Cyp18cy[a] k_{cat}/K_m ($\mu M^{-1}s^{-1}$) (at 15°C)	*E.coli* Cyp21peri[a] k_{cat}/K_m ($\mu M^{-1}s^{-1}$) (at 15°C)	Parvulin[b] k_{cat}/K_m ($\mu M^{-1}s^{-1}$)	*L.pneumophila* FKBP25mem[c] k_{cat}/K_m ($\mu M^{-1}s^{-1}$)
Ala	0.16	67.4	57.1	8.5	0.05
Glu	< 0.01	16.8	9.1	2.1	< 0.01
Gly	< 0.01	18.2	17.8	< 0.01	< 0.01
His	0.21	10.7	4.0	4.8	0.16
Ile	0.02	n.d.	n.d.	5.6	0.29
Leu	0.43	23.4	27.4	16.9	1.10
Lys	0.17	3.3	5.6	4.2	0.08
Phe	0.74	19.5	14.3	14.1	0.90
Trp	0.07	n.d.	n.d.	6.2	0.29
Val	0.04	n.d.	n.d.	4.5	0.08

Measurements were carried out in 0.035 M HEPES (pH 7.8) at 10°C. Data were taken from [a]Compton *et al.* (1992); [b]Rahfeld *et al.* (1994a); [c]Ludwig *et al.* (1994).
n.d., not determined.

confirms that the trigger factor is distinct from all known PPIases.

We noted that truncated forms of the trigger factor described above displayed an ~1.6-fold higher catalytic activity than the intact trigger factor. The pattern of the subsite specificities was not changed for the truncated forms.

Trigger factor is an extremely effective catalyst in refolding of a RNase T1 derivative

The slow refolding reactions of RNase T1 are limited in rate by the *trans* to *cis* isomerization of -Tyr-Pro[39]- and -Ser-Pro[55]-, and these reactions have often been used to assess the efficiency of PPIases as catalysts of protein folding (Schmid, 1993). To probe the trigger factor as folding catalyst, we used a mutagenized variant of this protein, S-carboxymethylated S54G/P55N-RNase T1, which offers a simplified refolding kinetics. This variant of RNase T1 contains a single *cis* proline (Pro[39]) only, and its unfolding and refolding can be studied simply in the absence of denaturants by varying the NaCl concentration (Mücke and Schmid, 1994). Refolding in the presence of 2 M NaCl is catalysed by the cytosolic Cyp18 from *E.coli* (Mücke and Schmid, 1994) and by other PPIases (Ch.Scholz and F.X.Schmid, unpublished).

Catalysis of the Pro[39]-limited folding of the RNase T1 variant is extremely efficient. Figure 6 shows that this

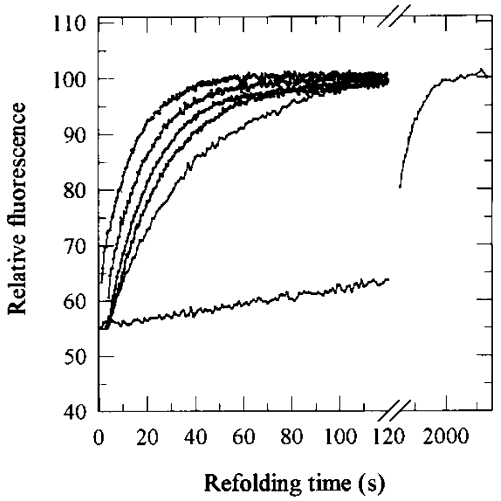


Fig. 6. Catalysis of refolding of RCM S54G/P55N RNase T1 by trigger factor. Refolding was measured by the increase in fluorescence at 320 nm after an excitation at 268 nm at 15°C. The relative fluorescence at 320 nm is shown as a function of the refolding time. The final value is adjusted to 100%. The refolding reaction was started by a 40-fold dilution of RCM S54G/P55N RNase T1 [in 0.1 M Tris–HCl (pH 8.0)] to a final concentration of 0.85 μM in the same buffer containing 2 M NaCl. For enzymatic catalysis, refolding was measured in the presence of (from bottom to the top) 0, 88, 177, 265, 350 and 530 nM of trigger factor. The time constants of refolding were (in the same order) 625, 35, 24, 20, 16 and 14 s.

Table IV. Subsite specificity of trigger factor and rhCyp18cy toward substrates of the structure Suc-Ala-Xaa-Pro-Gln-NH-Np and Suc-Ala-Xaa-Pro-Phe-NH-Np

Peptide substrate	$k_{cis\rightarrow trans}$ (s^{-1})	*cis* content (%)	Trigger factor k_{cat}/K_m ($\mu M^{-1}s^{-1}$)	rhCyp18cy k_{cat}/K_m ($\mu M^{-1}s^{-1}$)	Trigger factor k_{cat}/K_m ($\mu M^{-1}s^{-1}$)[a]
Suc-Ala-Phe-Pro-Phe-NH-Np	4.2	14.9	0.74	6.90	2.70
Suc-Ala-Leu-Pro-Phe-NH-Np	5.5	7.0	0.32	n.d.	2.00
Suc-Ala-Phe-Pro-Gln-NH-Np	4.7	12.6	0.42	5.50	1.60
Suc-Ala-Tyr-Pro-Gln-NH-Np	4.6	13.8	0.12	4.90	0.50

PPIase measurements were carried out in 0.035 M HEPES (pH 7.8) at 10°C.
[a]Conditions of these measurements were 0.1 M Tris–HCl, 2 M NaCl (pH 8.0), 15°C, which were used in the refolding experiments.
n.d., not determined.

refolding reaction is 18-fold accelerated when 88 nM trigger factor is present. The same concentration of trigger factor would lead to a 16-fold acceleration in an oligopeptide containing the -Leu-Pro-Phe- sequence and to a 6-fold acceleration in the -Tyr-Pro-Gln- sequence under identical experimental conditions. While cyclophilins display minor sensitivity toward the amino acids flanking proline in substrates, trigger factor can easily differentiate between very similar residues like Phe and Tyr. We therefore reasoned that oligopeptide-4-nitroanilides with Tyr preceding Pro may serve as model systems taking into account subsite effects in refolding catalysis (Table IV). In the model peptide, the Gln residue placed C-terminally to proline does not completely fit the properties of His^{40} in the -Tyr^{38}-Pro^{39}-His^{40}- region of RNase T1, but must be chosen for synthetic purposes. However, as was shown by Dayhoff and colleagues (George *et al.*, 1990) both residues, His and Gln, have a certain degree of similarity.

Taken together, the quantitative evaluation of folding catalysis indicates that the trigger factor catalyses prolyl isomerization in a folding protein much more efficiently than in an oligopeptide of comparable local sequence.

Discussion

Currently, PPIases are divided into three structurally unrelated subgroups: the cyclophilins, the FKBPs and the parvulins. Cyclophilins and FKBPs occur in great molecular diversity in almost every organism that has been analysed (Trandinh *et al.*, 1992; Fischer, 1994). In addition to extracellular and cytosolic forms, proteins of the PPIase families were found in the lumen of the endoplasmatic reticulum (Colley *et al.*, 1991), associated with membranes, in the Golgi apparatus and as constituents of several hetero-oligomeric complexes (Galat, 1993; Fischer, 1994). Since the *trans* to *cis* isomerization of prolyl peptide bonds is a slow and rate-limiting step of folding reactions *in vitro*, and probably also *in vivo*, *de novo* protein folding should benefit from a PPIase localized close to the site of protein synthesis. However, evidence for PPIases associated with the ribosomes was still lacking.

In this report, we present data about the existence of such a ribosome-bound PPIase. The amino acid sequence as well as enzyme inhibition are unrelated to those of other PPIases. The enzyme was first detected in a particle fraction resulting from zonal centrifugation at 40 000 *g* of crude extracts derived from *E.coli* cells and could be purified to apparent homogeneity. It binds exclusively to the large subunit of functional ribosomes known to contain the peptidyltransferase centre to cover the exit for the nascent polypeptide chain. Based on Edman degradation of both the complete enzyme and two N-terminally truncated species, the identity of the 59 kDa band in SDS–PAGE with the already known trigger factor was demonstrated. Correlation of PPIase activity and the 59 kDa protein was further proved by immunoprecipitation. This protein was discovered earlier by its ability to bind and stabilize the precursor form of the outer membrane protein A (proOmpA) in a membrane assembly-competent form *in vitro* (Crooke and Wickner, 1987; Crooke *et al.*, 1988a,b; Lecker *et al.*, 1989).

The trigger factor was quantitatively lost from the particle by incubation of ribosomes with 1.5 M LiCl, as originally monitored by its ability to promote proOmpA translocation (Lill *et al.*, 1988). Therefore, the protein was considered to be a peripheral component of 50S subunits. For this particle, the observed partitioning of PPIase activity during fractionation by increasing concentrations of LiCl agrees very well with the known behaviour of the trigger factor. However, the specific PPIase activity associated with the 50S subunit is much lower than that bound to 70S particles. This could be caused by a partial loss of trigger factor protein during the isolation of 50S subunits from tightly coupled 70S ribosomes, according to the relatively high dissociation constant of 0.3 μM (Lill *et al.*, 1988) or, alternatively, by an enhanced enzymatic activity due to the interaction with other components of the 70S ribosome.

The observed enzymatic activities of the trigger factor measured with a set of tetrapeptide-4-nitroanilides are in line with the assumption of FKBP-like preferences for the P_1 subsite of the substrate. We have also noted that the activity and the subsite specificity of the trigger factor do not change strongly when 59 amino acids are cleaved from its N-terminal end. In addition, cyclosporin A and FK506 were applied to clarify that trigger factor is distinct from cyclophilins and FKBPs, respectively.

Relatively high values (25–50 μM) for inhibitory constants have been found for cyclophilins from *E.coli* (Hayano *et al.*, 1991; Compton *et al.*, 1992). The differences to those of eukaryotic origin are much lower for bacterial FKBPs. For example, the inhibition constants of FKBPs from various streptomycetes are in the range of 30–60 nM (Pahl and Keller, 1992) and the K_i value for the *L.pneumophila* FKBP25mem (Mip) is 244 nM (Schmidt *et al.*, 1994). Incubation of trigger factor with either FK506 or cyclosporin A up to 100 μM effector in the assay did not reduce its enzymatic activity. This finding

implies that in contrast to many other PPIases, this enzyme does not belong to the immunophilins (Schreiber, 1991).

Catalytic amounts of PPIases are able to accelerate slow steps of protein folding *in vitro*, but acceleration factors are often small (Lang *et al.*, 1987; Tropschug *et al.*, 1990; Schönbrunner *et al.*, 1991; Kern *et al.*, 1995). Slow folding steps are often monitored as late events at the refolding of proteins diluted out from the denaturing solvent. Under strongly native refolding conditions, native-like secondary structure can form prior to prolyl isomerization and limit the access of PPIases to the prolyl bonds in the refolding protein (for a review, see Schmid, 1992). The RCM-(S54G/P55N) variant of RNase T1 has a very low stability and therefore Pro^{39} remains accessible during its refolding and is well catalysed by PPIases. The efficiency of trigger factor is exceptionally high. The acceleration factor interpolated to the enzyme concentration of 10 nM was found as a 4-fold increase in the slow refolding reaction of RCM S54G/P55N RNase T1. As much as 120 nM of the cytoplasmic *E.coli* cyclophilin are necessary to accomplish the same acceleration factor. Until now, this enzyme was considered to be a particular effective catalyst of proline-limited folding reactions (Schmid *et al.*, 1993).

This high power of the trigger factor as a folding catalyst becomes more remarkable considering the Tyr–Pro bond isomerizing during the folding of the RNase T1 derivative and the fairly low activity exhibited by trigger factor towards this bond within oligopeptides (Table IV).

Our results are not necessarily related to the *in vivo* function of the PPIase activity of the trigger factor. Originally thought to be involved in maintaining the translocation competence of proOmpA, it later became clear that the protein is not required for this function. Genetically engineered *E.coli* cells either overproducing or depleted of the protein displayed a filamentous phenotype. It was concluded that trigger factor serves as a chaperone for proteins involved in cell division (Guthrie and Wickner, 1990).

There is some evidence that folding helpers such as molecular chaperones bind to the nascent polypeptide chain co-translationally (Frydman *et al.*, 1994; Kudlicki *et al.*, 1994; Wiedmann *et al.*, 1994), and they are supposed to prevent aggregation and misfolding of the nascent proteins. The occurrence of a PPIase with a very high activity as folding catalyst at the ribosomal 50S subunit indicates that *de novo* protein folding may be an actively catalysed process.

Materials and methods

Materials

Subtilisin Carlsberg (type VIII bacterial, Lot 72H0115) was from Sigma (Deisenhofen, Germany) and bovine pancreatic α-chymotrypsin was from Merck (Darmstadt, Germany). The substrates (Suc-Ala-Xaa-Pro-Phe-NH-Np) were purchased from Bachem (Heidelberg, Germany). The substrates Suc-Ala-Xaa-Pro-Gln-NH-Np (Xaa = Phe, Tyr) were obtained by solid-phase peptide synthesis and characterized by ^{1}H NMR spectroscopy and electrospray mass spectrometry (M.Schutkowski, unpublished results). Fractogel EMD DEAE-650(M) and Fractogel TSK AF-Blue were from Merck (Darmstadt, Germany). Peroxidase-conjugated goat anti-rabbit IgGs were obtained from Sigma (Deisenhofen, Germany). Chelating Sepharose Fast Flow and Phenyl-Sepharose CL-4B were from Pharmacia (Uppsala, Sweden). Molecular weight markers, nitrocellulose NC45 and buffers (HEPES, Tris) were from SERVA (Heidelberg, Germany). Polyclonal antiserum against trigger factor was obtained from rabbit after immunization with 0.85 mg of trigger factor fragments (pab productions, Herbertshausen). The purified proteins (42.0 and 41.6 kDa) were desalted by HPLC as described and lyophilized.

Isolation of trigger factor from cell extracts

Cells of *E.coli* strain BL21 were harvested from a 3 l overnight culture using a Beckman centrifuge (6300 *g*, 4°C, 10 min). The cell paste (10 *g* wet weight) was washed with 200 ml of 0.02 M HEPES (pH 7.0), 0.15 M NaCl and centrifuged as described above. The cells were suspended in 80 ml of the same buffer and passed through a French press (SLM Aminco, Buettelborn, Germany). Cell debris were removed by ultracentrifugation for 40 min at 95 800 *g* (Beckman, 4°C). The supernatant was pooled and diluted using an equal volume of 0.02 M HEPES (pH 7.0). This solution was applied to a Fractogel EMD DEAE-650(M) column (2.5×20 cm) equilibrated in 0.02 M HEPES (pH 7.0). Using a 0–1 M NaCl linear gradient (500 ml total), trigger factor was eluted together with two other PPIases of *E.coli*. Enzymatically active fractions (at ~280 mM NaCl) were loaded onto a Chelating Sepharose column (1.6×16 cm) with immobilized Ni^{2+} ions. The column was equilibrated in 0.02 M HEPES (pH 7.0), 0.3 M NaCl. Trigger factor was contained in the flow-through fractions. To these fractions with PPIase activity was added ammonium sulfate to a total amount of 20% (w/v). After stirring for 1 h, the solution was applied to a Phenyl-Sepharose CL-4B column (1×8 cm) equilibrated in 0.01 M HEPES (pH 7.5), 0.15 M KCl, 20% ammonium sulfate (w/v). The elution of bound proteins was carried out by a linear gradient from 0 to 20% glycerine, 0 to 0.5% CHAPS crossing a linear gradient decreasing from 20 to 0% ammonium sulfate (80 ml total). Trigger factor was recovered from the second enzymatically active peak fractions. These samples were pooled and dialysed against 4×3 l portions of 0.01 mM HEPES (pH 7.0) for 4 h. The dialysate was loaded onto a Fractogel TSK AF-Blue column equilibrated in the same buffer. The flow-through fractions of this column were applied to a Fractogel EMD DEAE-650(M) column. The trigger factor was eluted by a linear gradient of 0–1 M KCl (80 ml total). Trigger factor was purified to homogeneity by this procedure, as verified by silver staining after SDS–PAGE.

Treatment of the ribosomal 50S subunit with LiCl

Ribosomes were prepared according to Rheinberger *et al.* (1988). For splitting experiments, the method of Homann and Nierhaus (1971) was slightly modified. Subunits (50S) were diluted to salt solutions of 0, 0.25, 0.5, 1.0, 1.5 and 4 M LiCl in buffer (0.01 M Tris, 0.01 M manganese acetate, 5 mM EDTA) to give a final concentration of 2 μM. After being shaken for 5 h at 0°C, the ribosomal cores were pelleted by ultracentrifugation (40 000 r.p.m., 4°C) in a Beckman 45 Ti rotor for 5 h. The obtained supernatants (10 ml) were portioned and shock frozen by rapid pipetting into liquid nitrogen. The pellets were dissolved in 0.5 ml of 0.02 M HEPES (pH 7.5) at 7°C, 4 mM manganese acetate, 0.4 M ammonium chloride, 4 mM EDTA, 4 mM 2-mercaptoethanol and frozen as described above. Prior to the determination of enzymatic activity, LiCl was diluted out from the supernatants (aliquots of 3 ml) with buffer [0.2 M HEPES (pH 7.5) at 7°C, 4 mM manganese chloride, 0.4 M potassium chloride] using Centricon tubes (exclusion size of 3500 Da, Amicon). Since preliminary examinations have revealed that high concentrations of LiCl (≥1.0 M) lead to a slight decrease of the activity of purified trigger factor, removing the LiCl prior to measuring was recommended. For the measurements of PPIase activity, the substrate Suc-Ala-Phe-Pro-Phe-NH-Np was used. For Western blot analysis, 200 μl samples of the supernatants were dialysed, lyophilized and redissolved in SDS sample buffer.

Gel filtration after 50S subunit dissociation

Gel filtration was performed using a SMART system from Pharmacia (Uppsala, Sweden). A sample of 50S particles (1160 A_{260}/ml) was diluted in a buffer containing 0.02 M HEPES, 2 M LiCl, 0.01 mM manganese chloride, 1 mM DTT, 0.5 mM EDTA (pH 7.5) to give a final concentration of 773 A_{260}/ml. After incubating for 5 h at 0°C, 50 μl of the solution were applied to a Superdex 75 PC 3.2/30 column. The flow rate was 40 μl/min. Calibration was carried out using proteins with mol. wts within the range of 67 000–12 500 Da (bovine serum albumin, ovalbumin, chymotrypsinogen A, cytochrome c; SERVA, Heidelberg). The temperature was maintained at 4°C during all runs. Fractions of 30 μl were collected and analysed using the substrate Suc-Ala-Phe-Pro-Phe-NH-Np in the PPIase assay.

Refolding kinetics

Unfolded RCM S54G/P55N RNase T1 was prepared as described by Mücke and Schmid (1994). The measurements were carried out on a

Hitachi F4010 fluorescence spectrophotometer. The final concentration of RCM S54G/P55N RNaseT1 was 0.85 μM. Refolding was initiated by 40-fold dilution of the unfolded protein [in 0.1 M Tris–HCl (pH 8.0)] to folding conditions of 2 M NaCl and 0.1 M Tris–HCl (pH 8.0) at 15°C. For enzymatic catalysis of the slow refolding reaction, various concentrations of trigger factor in the range 88–530 nM were added. After the mixing time of 2 s, the kinetics were followed by the increase in tryptophan fluorescence at 320 nm (5 nm band width) after excitation at 268 nm (1.5 nm band width). The temperature was maintained at 15 ± 0.1°C during the time of measurements. All solutions were thermostatted at 15°C. The observed kinetic data were fitted to a mono-exponential function using the software Sigma Plot (VER 1.02) from Jandel Scientific (Erkrath, Germany).

Assay of PPIase activity, substrate specificity and inhibitory studies

PPIase activity was determined using the protease-coupled assay as described by Fischer *et al.* (1992). Measurements were carried out in 0.035 M HEPES buffer (pH 7.8) at 10°C using a Hewlett Packard 8452 diode array UV/VIS spectrophotometer. For proteolytic cleavage of Suc-Ala-Xaa-Pro-Phe-NH-Np (Xaa = Phe, Leu, Ala, Val, Ile, Gly, Trp, His, Lys, Glu), α-chymotrypsin (0.08 mg/ml) was used. In the case of substrates of the structure Suc-Ala-Xaa-Pro-Gln-NH-Np, subtilisin at a final concentration of 0.4 mg/ml was used. Stock solutions of the substrates (10 mg/ml) were prepared in dimethylsulfoxide (DMSO). The kinetics were initiated by adding 3 μl of the substrate solution to the reacting mixture. Under these conditions ($[S] << K_m$), the observed kinetics can be described by the first-order rate equation $v = k_{obs}[cis]$; $k_{obs} = k_0 + k_{enz}$ and $k_{obs} = k_0 + k_{cat}/K_m\ [E]_0$, respectively, where $[cis]$ is the time-dependent concentration of the *cis* conformer, k_0 is the rate constant of the uncatalysed *cis* to *trans* interconversion and k_{obs} is the observed first-order rate constant in the case of PPIase catalysis. The value of the specificity constant k_{cat}/K_m was calculated from the relationship $k_{enz}/[E]_0 = k_{cat}/K_m$ in which $[E]_0 = [PPIase]_{total}$. This value was determined using the total enzyme protein concentration assuming that all PPIase molecules are enzymatically active. For comparing PPIase activities of crude protein fractions, the arbitrary unit AU = $(k_{obs}/k_0) - 1$ was used. When assaying PPIase activity of ribosomal fractions, attention was given to a pre-reaction between the components of the test mixture (e.g. buffer, α-chymotrypsin and the ribosomal sample) which interferes with the determination of PPIase activity. To suppress this pre-reaction, the reaction mixture was incubated for 5 min at 10°C prior to substrate injection.

For inhibitory measurements, FK506 and cyclosporin A at final concentrations of 100 μM were used. Stock solutions were prepared in 50% ethanol (v/v). To check the enzymatic activity without the inhibitory molecules, an equal volume of 50% ethanol (v/v) was added to the reaction mixture.

Determination of the N-terminal amino acid sequence

Proteins of the 70S ribosomes were electrophoretically separated and blotted to a Selex 20 membrane (Schleicher and Schuell). The blotting procedure was performed according to Kyhse-Anderson (1984). The membrane was treated with methanol for 1 min before blotting. The transfer was done in 0.05 M sodium borate buffer (pH 9.0) containing 20% methanol (v/v) at 3.5 mA/cm^2 for 45 min. The membrane was stained with Coomassie Blue which was stopped by washing with water. The protein was obtained by cutting out the corresponding piece (band of 59 kDa) of the membrane. Sequencing was carried out using an Applied Biosystems 476 A gas-phase sequencer.

Electrophoretically pure trigger factor and truncated forms were sequenced after desalting through reversed-phase HPLC (Shimadzu LC-10A) using a Nucleosil 500-5 C_3-PPN column (125×4 mm, guard column 11 mm) from Macherey-Nagel (Düren, Germany). The HPLC was performed with a flow rate of 1 ml/min at 40°C. Portions of 1 ml were loaded onto the column equilibrated with 0.1% aqueous trifluoroacetic acid in 1% acetonitrile (v/v). The concentration of acetonitrile was raised to 35% (v/v) within 10 s. The protein was eluted running a gradient of acetonitrile from 35 to 60% (v/v) within 25 min. Elution was monitored at wavelengths of 215 and 280 nm.

Immunoblotting and immunoprecipitation

The purchased polyclonal antiserum was heated to 56°C for 30 min and clarified by centrifugation (20 min, 14 000 r.p.m.). Further purification was achieved by performing affinity chromatography using a Protein A–Sepharose CL-4B column (Pharmacia, Uppsala, Sweden) following the protocol of the manufacturer. The final titre of the antiserum for immunoblotting was 1:500. For development of the blots, a peroxidase-conjugated goat anti-rabbit IgG was used as second antibody. Western blotting was performed according to Kyhse-Anderson (1984). Transfer of proteins from SDS slab gels (12.5%) to nitrocellulose was done by semi-dry electroblotting using the Fast Blot B32 chamber from Biometra (Göttingen, Germany). Amido black staining of the NC membrane was performed with a solution of Amido black in 1% acetic acid. Prior to immunoprecipitation, the antiserum was purified by affinity chromatography using trigger factor-linked Affi-15 gel (Bio-Rad, München, Germany). The procedure of antigen coupling (using ~1 mg of trigger factor fragments) and subsequently purification of the antiserum was carried out following the protocol of the manufacturer. For immunoprecipitation, different concentrations of purified trigger factor were used, reaching the final (after adding the Protein A beads) trigger factor:IgG ratios 1:7, 1:3.5 and 1:1.7. The mixtures were shaken for 2 h at 4°C. Swollen Protein A–Sepharose beads [0.025 M HEPES, 0.075 M NaCl, 0.1% Tween 20 (pH 7.5)] were then added and the suspensions were incubated for 1.5 h at 4°C under vigorous shaking. After subsequent centrifugation (15 s, 10 000 *g*), the supernatants were analysed by SDS–PAGE and silver staining, immunoblotting and measurements of PPIase activity. For each concentration of trigger factor, a control sample was treated in the same way, but adding buffer instead of antibodies.

Other methods

Concentrations of crude protein fractions were determined following the procedure of Bradford (1976) using bovine serum albumin as standard. The protein concentration of purified trigger factor was determined spectrophotometrically. The molar extinction coefficient at 280 nm (ε = 15 930/M/cm) was derived from primary structure according to Gill and von Hippel (1989).

The concentration of ribosomal samples was determined spectrophotometrically using the relationships 1 A_{260} = 36 pmol of 50S subunits and 1 A_{260} = 24 pmol of tightly coupled ribosomes.

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