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High Enzymatic Activity and Chaperone Function are Mechanistically Related Features of the Dimeric *E. coli* Peptidyl-prolyl-isomerase FkpA

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We have recently described the existence of a chaperone activity for the dimeric peptidyl-prolyl *cis/trans* isomerase FkpA from the periplasm of *Escherichia coli* that is independent of its isomerase activity. We have now investigated the molecular mechansim of these two activities *in vitro* in greater detail. The isomerase activity with a protein substrate (RNaseT1) is characterized by a 100-fold higher k_{cat}/K_M value than with a short tetrapeptide substrate. This enhanced activity with a protein is due to an increased affinity towards the protein substrate mediated by a polypeptide-binding site that is distinct from the active site. The chaperone activity is also mediated by interaction of folding and unfolding intermediates with a binding site that is most likely identical to the polypeptide-binding site which enhances catalysis. Both activities are thus mechanistically related, being based on the transient interaction with this high-affinity polypeptide-binding site. Only the isomerase activity, but not the chaperone activity, with the substrate citrate synthase can be inhibited by FK520. Experiments with the isolated domains of FkpA imply that both the isomerase and the chaperone site are located on the highly conserved FKBP domain. The additional amino-terminal domain mediates the dimerization and thus places the two active sites of the FKBP domains in juxtaposition, such that they can simultaneously interact with a protein, and this is required for full catalytic activity.

Keywords: peptidyl-prolyl-*cis/trans*-isomerase; chaperone; protein folding; aggregation; FKPB

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Introduction

Folding in the cell has usually been thought to be assisted by two separate classes of proteins. On the one hand different kinds of molecular chaperones have been discovered that act by preventing off-pathway reactions, which would otherwise lead to aggregation.[1] On the other hand, so-called ''folding catalysts'' have been described that catalyze rate-limiting steps in protein folding. Besides the formation and isomerization of disulfide bonds,[2] the major chemical rate-limiting step in protein folding is the *cis/trans* isomerization of peptidyl-bonds N-terminal to proline. Enzymes which catalyze this prolyl isomerization occur in all forms of life, from primates to archaea.[3–5] They were found to belong to three families, named cyclophilins (Cyp), FK506-binding proteins (FKBPs), and parvulins. Because of their inhibition by immunosuppressant drugs (cyclosporin A or FK506), the first two families are also called immunophilins. Despite their ubiquitous occurrence, however, most of these enzymes are not essential under normal growth conditions. In yeast, for example, 12 peptidyl-prolyl-*cis/trans* isomerases (PPIases) have

Abbreviations used: CS, citrate synthase; Cyp, cyclophilin; DTNB, dithionitrobenzoic acid; ELISA, enzyme-linked immunosorbent assay; FKBP, FK506-binding protein; GdmCl, guanidine hydrochloride; MIP, macrophage infectivity potentiator; OAA, oxaloacetate; PPIase, peptidyl-prolyl *cis-trans* isomerase; POD, horseradish peroxidase; RCM-la, reduced and *S*-carboxymethylated bovine α-lactalbumin; RCM-RNaseT1, reduced and *S*-carboxymethylated S54G/P55N-variant of ribonuclease T1; scFv, single-chain antibody fragment, consisting of the variable domains of the heavy and the light chain connected by a peptide linker; V_H, variable domain of the heavy chain of an antibody; V_L, variable domain of the light chain of an antibody; BSA, bovine serum albumin; wt, wild-type.

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been knocked out with only a subtle effect on the phenotype,[6] implying that they can be functionally replaced by other proteins.

In vitro, the PPIases have initially been characterized by their ability to catalyze peptide bond rotation in a short tetrapeptide model substrate.[7,8] As judged by this assay, their activities differ widely, with second-order rate constants k_{cat}/K_M ranging from 10^3 M^{-1} s^{-1} up to almost the diffusion limit.

The FKBPs are a family of PPIases that have been named due to their ability to bind the inhibitor FK506, a macrolide isolated from *Streptomyces tsukubaensis*.[9] This inhibitor was initially discovered for its immunosupressive effect, and since then several analogs have been isolated from different *Streptomyces* strains. FK520, the analogue used in this study, was isolated from *Streptomyces hygroscopicus*.[10,11] It differs from FK506 only by one propenyl moiety that is replaced by an ethyl group in a position which does not interact with FKBPs. Their potential role as a target for immunosuppressants is also the main reason for the sustained interest
in this class of proteins (for a review, see Fruman *et al.*[12]).

FkpA was originally discovered as a periplasmic *Escherichia coli* homolog of the MIP-like FK506-binding proteins.[13] The macrophage infectivity potentiators (MIP), in turn, have been described in *Legionella* species to facilitate the invasion of the host cells by these intracellular parasites, and were found to be dimeric FKBPs with an additional N-terminal domain.[14] FkpA also consists of a highly conserved FKBP-domain, which is about 50 % identical to this domain in members of the MIP-family and an additional N-terminal domain, which is homologous to the respective domain of the MIP-proteins and some other two-domain FKBPs. A largely helical structure has been predicted for this N-terminal domain, its function, however, remained unknown. Recently, we have shown that FkpA, a dimer under native conditions, is a very efficient proline *cis/trans* isomerization catalyst with a folding protein as substrate and that it possesses an independent chaperone-like activity,[15] similar to other members of the FKBP-family.[16,17] We have now investigated the molecular mechanism of these apparently separate activities of FkpA in detail, to gain a better understanding of its function *in vivo*.

Results

High isomerase activity with protein substrates mediated by tight substrate binding

Recently, we described the high PPIase activity of FkpA with protein substrates. This was demonstrated in a protein folding assay measuring the catalysis of wild-type (wt) RNaseT1 refolding.[15] A 13-fold acceleration of the prolyl isomerization-limited folding rate was observed in the presence of 10 nM FkpA, whereas the homologous human FKBP12, used as reference, showed no effect at this concentration (Figure 1(a)). The k_{cat}/K_M value of 4×10^6 M^{-1} s^{-1}, determined for the FkpA dimer, was much higher than the one which had been estimated before[18] for FkpA from a protease-

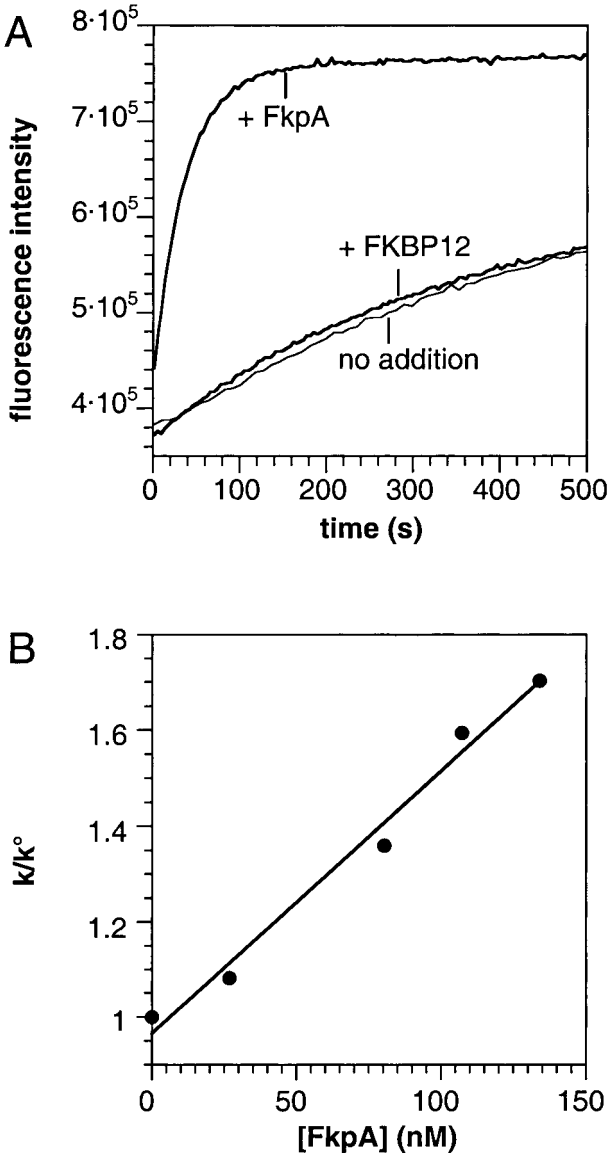

Figure 1. (a) Fluorescence traces of RNaseT1 refolding (0.4 μM) at 10 °C, starting from equilibrium-denatured protein and in the presence of 10 nM FkpA or 10 nM human FKBP12, as indicated. The intensity was followed at 323 nm upon excitation at 295 nm. (b) Catalysis of the isomerization of a tetrapeptide (Suc-Ala-Ala-Pro-Phe-NA). Kinetic traces were evaluated with a single exponential, and the ratios of the rates of catalyzed isomerization, divided by the uncatalyzed rate, are plotted against the respective FkpA concentration present during the measurement. From the slope of the line a value of 5×10^4 M^{-1} s^{-1} can be estimated for k_{cat}/K_M.

coupled colorimetric assay with a peptide substrate (9×10^4 M^{-1} s^{-1}).

Since we observed rapid degradation of FkpA in the presence of the chymotrypsin concentrations employed in the protease-coupled peptide assay, implying that rapid digestion within the assay might be the reason for the low catalytic activity, we have now reexamined the isomerase activity of FkpA with a tetrapeptide using a photometric assay that can be carried out in the absence of any protease.[19] From the slope of the plot shown in Figure 1(b) k_{cat}/K_M was estimated to be 5×10^4 $M^{-1}s^{-1}$. This corresponds roughly to the value that had been obtained previously in the protease-coupled assay and is clearly much lower than the k_{cat}/K_M value of 8.7×10^5 M^{-1} s^{-1} that we obtained for human FKBP12 (data not shown), measured as a control. The latter constant corresponds well to the previously measured value for this enzyme (8.1×10^5 M^{-1} s^{-120}). It is thus clear that FkpA has indeed a rather low k_{cat}/K_M value with a tetrapeptide as substrate, 17-fold lower than the homologous hFKBP12, and that this is not an artefact of its own susceptibility to the protease. This stands in marked contrast to its almost 100-fold higher catalytic activity with a folding protein, which hFKBP12 in turn catalyzes very inefficiently.

To understand the basis of this enhanced catalytic activity with a folding protein, compared to a short tetrapeptide, we determined the kinetic parameters K_M and k_{cat} of the catalysis of FkpA with RNaseT1 wt and a mutant. In these experiments the concentration of FkpA was kept constant at 10 nM, and the substrate RNaseT1 concentration was varied between 0.05 and 2 μM (wt) or 0.05 and 6 μM (mutant), while care was taken to ensure identical final refolding conditions. Since uncatalyzed refolding occurs in parallel with the catalyzed reaction, the amplitude of the uncatalyzed reaction dominates the observed kinetics at high substrate concentrations, thus complicating the analysis. The measured kinetic traces were, therefore, analyzed by numerical iteration and global fit to the progress curves with the program DynaFit,[21] based on a model that accounts for the presence of the uncatalyzed reaction (see Experimental Procedures), whose rate had been determined independently and was held constant in the fitting procedure.

Since the refolding kinetics of the wt RNaseT1 are very complex, with three different proline-limited reactions all contributing to the faster phase observed in fluorescence measurements[22,23] and an additional slow proline-limited phase, the determination of single K_M and k_{cat} values is not possible, strictly speaking. The isomerization reactions in RNaseT1 wt can be catalyzed to different extents, however, and isomerases also affect which branch of the folding pathway the slow-folding molecules choose, resulting in dramatic changes in the amplitudes of the different slow refolding reactions.[23] Since only the faster phase of proline-limited RNaseT1 wt-folding is catalyzed well by FkpA (Figure 2(a)), while the amplitude of the slower phase decreases dramatically, we believe that the values determined from analysis of the faster phase are still provide a good estimate for the catalytic constants of FkpA, albeit averaged for a small

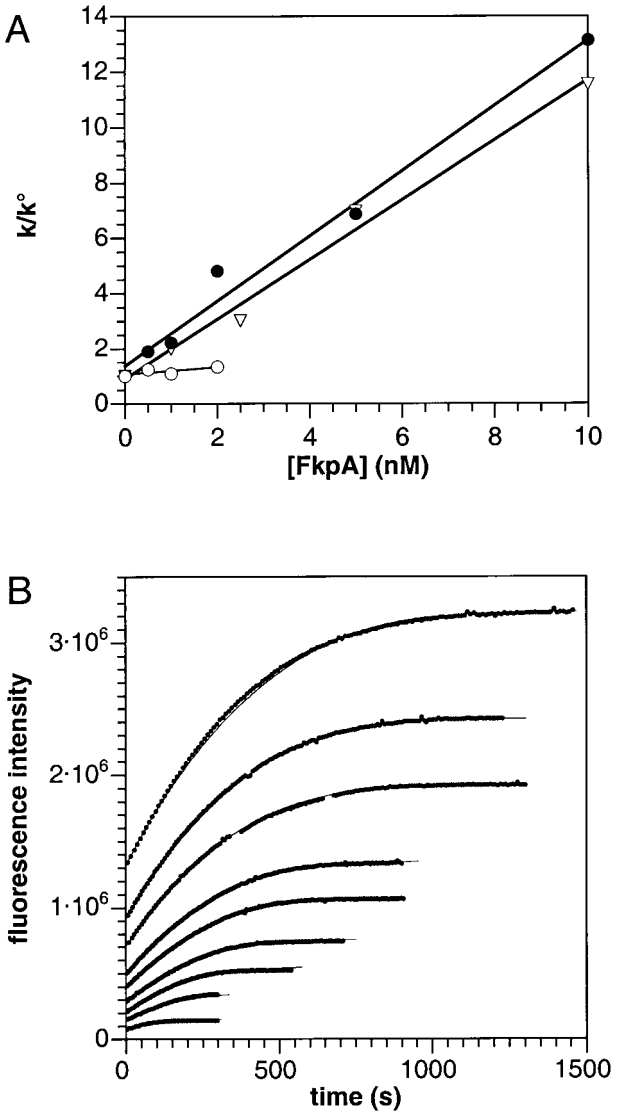

Figure 2. (a) Catalysis of (▽) RCM-RNaseT1 refolding and wt RNaseT1 refolding, (●) fast phase and (○) slow phase at 10 °C. The substrate RNaseT1 concentrations was 0.5 μM. The fluorescence traces were analyzed by single (RCM-RNaseT1) or double (wt) exponential fits and the ratios of the rates of catalyzed folding, divided by the uncatalyzed rate, are plotted against the respective FkpA concentration present during the measurement. (b) Catalysis of RCM-RNaseT1 refolding by 5 nM FkpA at 10 °C. The substrate RNaseT1 concentrations varied between 0.05 μM to 6 μM. The fluorescence traces were evaluated by global fitting with the DynaFit software (see Experimental Procedures). The continuous lines represent the obtained gobal fit yielding values of 0.8 s^{-1} for k_{cat} and 0.3 μM for K_M.

number of structurally closely related RNaseT1 wt folding intermediates as substrates. A mathematically correct analysis of the data is, on the other hand, possible with a mutant of RNaseT1 as substrate, which lacks one of the two *cis*-prolines (P55N) and is additionally reduced and carboxymethylated to prevent disulfide-bond formation (RCM-RNaseT1). Its folding mechanism is simple and well characterized[24,25] and it has been used as a model substrate for PPIases before.[16,26] The tradeoff for this substrate is, however, that it requires 2 M NaCl for stability and that the experimental conditions are, therefore, not physiological, and it appears that product dissociation becomes rate limiting (see below). We therefore measured the effect of FkpA on the folding of both wt RNaseT1 and RCM-RNaseT1.

The analysis of the data obtained for the wt RNaseT1 yields values of 0.5 μM for K_M and 1.6 s^{-1} for k_{cat} (data not shown). The ratio of these two values (3.2×10^6 M^{-1} s^{-1}) is in very good agreement with the composite estimate for k_{cat}/K_M (4×10^6 M^{-1} s^{-1}), obtained initially, thus providing additional evidence for the validity of the Michaelis-Menten equation for the description of the FkpA-catalyzed RNaseT1 wt folding. Analysis of the mutant RCM-RNaseT1 data shown in Figure 2(b) yields values of 0.3 μM for K_M and 0.5 s^{-1} for k_{cat}, which are very similar to those of wt. The submicromolar K_M value clearly shows that the high value of k_{cat}/K_M of FkpA with these folding proteins is due to strong substrate binding. In contrast, a K_M value of 730 μM has been estimated for the affinity of a tetrapeptide substrate to FkpA in a very recent publication,[27] supporting this conclusion. Undoubtedly, every substrate protein will have a somewhat different K_M value, including the periplasmic *E. coli* proteins constituting the natural substrates.

It is interesting to note that the catalysis of the RCM-RNaseT1 is not more efficient than that of the wt RNaseT1, even though substrate binding is apparently even stronger. RCM-RNaseT1 is known to form no stable structured folding intermediates in contrast to the wt protein,[28] and this may be the reason for the tighter binding to FkpA. The lower k_{cat} value may indicate that release of the substrate rather than bond rotation has become rate-limiting.

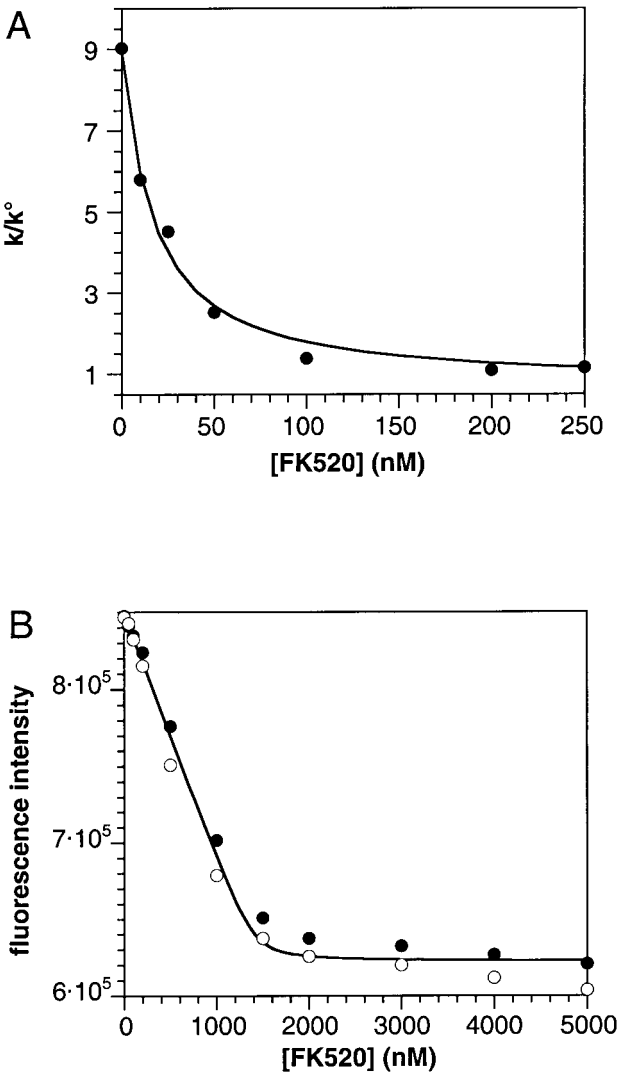

Figure 3. Inhibition of the catalytic activity with wt RNaseT1 as substrate at 10 °C by FK520 (10 nM FkpA dimer). Kinetic traces were analyzed by double exponential fits. The obtained rates for the faster reaction were divided by the uncatalyzed rate and plotted. The fit yields a K_i value of 10 nM. (b) Fluorescence titration of FkpA (1 μM, dimer) with FK520 at (●) 25 °C and (○) 38 °C, followed at 307 nm upon excitation at 280 nm. The fit yields an active site concentration of about 1.6 μM, but since the FkpA concentration is much higher than K_D in this experiment, K_D cannot be determined.

FK520 binds with nanomolar affinity to both active sites of a dimeric FkpA

The FKB-proteins owe their name to the fact that they bind the inhibitor FK506 more or less tightly in the active site. FK520 is essentially identical with FK506,[9] differing only by possessing an ethyl group that does not participate in the interaction[29] instead of a propenyl group in FK506. To determine the binding constant of FkpA to FK520[11] we performed inhibition measurements with the wt RNaseT1 as a substrate. Catalysis of RNaseT1 wt refolding at 10 °C (Figure 3(a)) is fully inhibited with 100 nM FK520 and the fit yields a K_i value of 10 nM. Binding of the inhibitor is not disturbed by an increase of the assay temperature up to 25 °C. Measurements at even higher temperatures are, however, not possible with this folding assay. The K_i obtained is in good agreement with the K_i of about 30 nM for FK506, which has been reported very recently for FkpA based on a tetrapeptide assay.[27]

We have shown before that FkpA is exclusively dimeric under all conditions tested in solution,[15] such that there are two active sites per native dimer. In order to confirm that both active sites on the dimer are accessible to the inhibitor, we carried out fluorescence titration experiments (Figure 3(b)). The titrations were performed at 25 °C as well as at 38 °C, to establish whether inhibitor binding also occurs at the elevated temperatures used in chaperone experiments (see below). When 1 μM dimeric FkpA, a much higher concentration than the K_i determined from kinetic inhibition, is titrated with FK520, the stoichiometry of binding can be determined. An active site concentration of about 1.6 μM is obtained at both temperatures (fit in Figure 3(b)). The somewhat reduced concentration of active sites compared to the expected number of 2 μM probably arises from the prolonged pre-incubation in the cuvette, which is necessary to prevent titration artefacts due to fluorescence decrease caused by some absorption to the cuvette. Importantly, the results clearly show that binding of the inhibitor to the catalytic site occurs at all temperatures from 10 °C to 38 °C.

RCM α-lactalbumin competes for protein substrate binding only

In order to determine whether the low K_M value for RNaseT1 is due to an avidity effect of binding to both active sites on the FkpA dimer, or rather mediated by binding to a site distinct from the active site, we performed inhibition studies with reduced and carboxymethylated bovine α-lactalbumin (RCM-la). RCM-la is denatured and unfolded under physiological conditions and, most importantly, it remains soluble and unfolded under the conditions used in the assay. RCM-la has been shown before to be a substrate for GroEL [30] and to compete with RNaseT1 for binding to the trigger factor.[26]

RCM-la inhibits the catalysis of RNaseT1 refolding at micromolar concentrations (Figure 4(a)). The inhibition follows a saturation curve, and the measured rate of refolding of RNaseT1 in the presence of high inhibitor concentrations approaches the rate of uncatalyzed refolding. Inhibition is, however, never complete and this might be due to the fact that the inhibitor blocks only the additional polypeptide-binding site, thus increasing the K_M, but not the catalytic site itself. Half-maximal inhibition was observed at 3 μM RCM-la. The catalysis of the isomerization of the tetrapeptide, on the other hand, is not inhibited at all, even in the presence of 170 μM RCM-la, a 850-fold excess over FkpA (Figure 4(b)). These results suggest that RCM-la does not block the active sites of FkpA, since the catalysis of the tetrapeptide is not influenced. The inhibitory effect on the catalysis of the folding protein must thus be explained by a competition of RCM-la with RNaseT1 for binding. Since the actual catalysis of the tetrapeptide substrate is undisturbed, the binding of RCM-la must occur to a site on FkpA that is distinct from the active site, but can of course be adjacent to it.

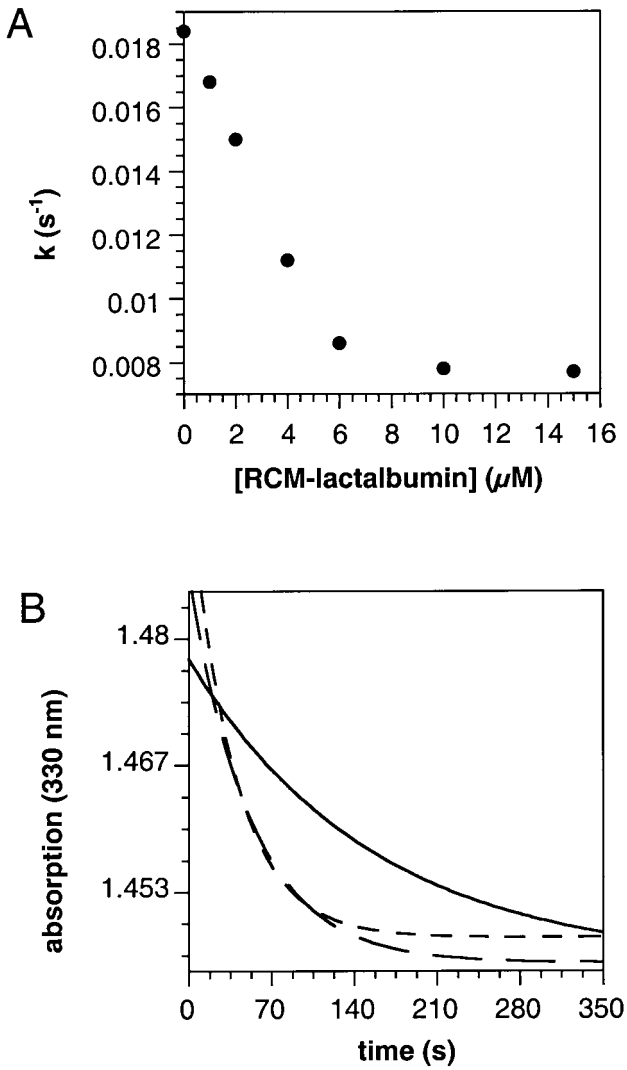

Figure 4. (a) Inhibition of the FkpA-catalyzed wt RNaseT1 folding (10 nM FkpA, dimer; 0.4 μM RNaseT1) by RCM-α-lactalbumin at 10 °C. Kinetic traces were analyzed by double exponential fits and the faster rate constants are plotted as a function of the RCM-α-lactalbumin concentration. (b) Lack of inhibition of FkpA-catalyzed tetrapeptide isomerization by RCM-la. Kinetic traces of tetrapeptide isomerization at 10 °C, followed at 330 nm; uncatalyzed reaction (—), in the presence of 0.1 μM FkpA-dimer (– –) and in the presence of 0.1 μM FkpA-dimer and 170 μM RCM-la (- - -).

It is tempting to speculate that the additional binding capacity for polypeptides may also be the basis for the chaperone activity of FkpA that has been shown to exist independently of its isomerase activity.[15,31] In order to investigate this hypothesis and to gain a better understanding of the mode of polypeptide binding, we carried out experiments

with a chaperone model substrate that is well characterized and allows testing for chaperone activity in the presence of the active site inhibitor FK520.

FkpA prevents aggregation of unfolding CS intermediates

Experiments addressing the chaperone activity in the presence of the active site inhibitor FK520 could not be carried out with the substrate used before, a single-chain fragment of an antibody,[15] due to severe aggregation of the scFv in the refolding reaction caused by the presence of the inhibitor FK520 alone, a large hydrophobic macrolide. This may be a general problem when probing chaperone activity with refolding proteins in the presence of FK506-derivatives. We have therefore chosen to investigate the chaperone activity of FkpA further, making use of a well-described chaperone substrate, citrate synthase (CS). Native CS is known to lose activity and subsequently aggregate upon incubation at elevated temperatures.[32] In the course of thermal unfolding, two native-like intermediates are populated transiently that can be reactivated by the addition of oxaloacetate (OAA). The action of different families of chaperones on CS unfolding has been described in considerable detail.[17,33]

Upon coincubation with CS at 43 °C, FkpA is able to delay and reduce light scattering. At an eightfold molar excess of FkpA (0.6 μM) over CS, no visible aggregation, detectable by light scattering, occurs over the time of the experiment (Figure 5(a)). Bovine serum albumin (BSA) at the same concentration does not have any inhibitory effect on CS-aggregation. FkpA is thus able to recognize and bind non-native protein efficiently. In suppressing CS-aggregation, it is as effective as the Hsp90-associated FKBP52[17] or as Hsp90 itself.[33] Besides preventing CS aggregation, FkpA can also apparently dissolve existing aggregates, as indicated by the decrease in light scattering, when FkpA is added later on, after the onset of aggregation (Figure 5(b)).

FkpA binds reversibly to early unfolding intermediates of CS

Different families of chaperones have been shown to bind different types of folding intermediates, and they are also distinct in their binding mode. Binding can be either weak and thus transient, as in the case of Hsp90, or strong and thus essentially permanent, hence requiring further factors for release. In order to obtain additional information about the molecular mechanism of the chaperone activity of FkpA, we have investigated its influence on the CS-inactivation kinetics.

CS inactivation in the absence of chaperones occurs with an apparent rate of 0.0093 s^{-1} at 43 °C (Figure 5(c)), as has been demonstrated before.[33] FkpA at 0.6 μM (eightfold molar excess) slows the

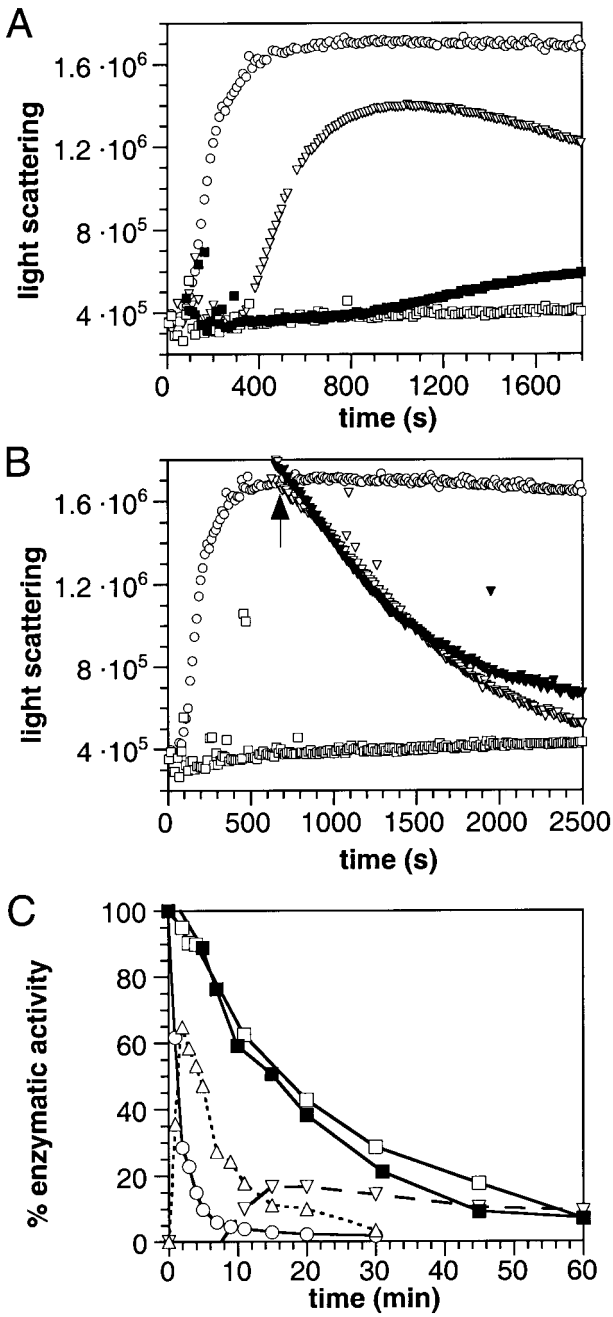

Figure 5. Influence of FkpA on thermal unfolding and aggregation of citrate synthase (CS, 0.075 μM) at 43 °C. All CS and FkpA concentrations are given for the dimer. (a) Aggregation observed by light scattering at 500 nm. CS alone (○), in the presence of 0.3 (▽) and 0.6 μM (□) FkpA and in the presence 0.6 μM FkpA preincubated with 6 μM FK520 (■). (b) Aggregation observed by light scattering at 500 nm. CS alone (○), in the presence of 0.6 μM FkpA (□), 0.6 μM FkpA added after a 600 second delay (arrow) (▽), and 0.6 μM FkpA preincubated with 6 μM FK520, added after a 600 second delay (arrow) (▼). (c) CS inactivation at 43 °C, followed by measuring the enzymatic activity of CS at 25 °C. CS alone (○), in the presence 0.6 μM FkpA (□) and in the presence 0.6 μM FkpA preincubated with 60 μM FK520 (■). The amount of reactivatable intermediates present during unfolding of CS alone (△) and CS + 0.6 μM FkpA (▽) is given by the difference of the activity after and before reactivation with oxaloacetate.

inactivation-kinetics of CS by a factor of 11, with 40 % active molecules still present after an incubation period of 20 minutes at 43 °C (Figure 5(c)). It is thus more than twice as effective as a stabilizing agent than yeast Hsp90, which yields 20 % active CS after incubation for the same period with the same molar excess of chaperone, slowing down the inactivation process by a factor of 5.[33]

Upon inactivation of CS, intermediate states are populated transiently that can be reactivated by the addition of OAA, which binds to the enzyme and stabilizes the native state. In the absence of chaperones the reactivatable intermediates reach a maximum at around two minutes and then disappear within 20 minutes (Figure 5(c)). FkpA prevents significant accumulation of the reactivatable unfolding intermediates within the first few minutes and instead leads to an increased persistance of the native state at this time. In the presence of FkpA, such intermediates are found at later time points than in its absence, with 10 % still present after 60 minutes of incubation at 43 °C (Figure 5(c)). This behavior is reminiscent of Hsp90[33] and indicates that FkpA is able to interact transiently with early, native-like CS-unfolding intermediates, and release them in a productive fashion, such that they can be reactivated. Irreversible binding to FkpA, on the other hand, would not be expected to slow the inactivation kinetics, nor should reactivation be possible, and it thus does not seem to occur under these conditions.

Binding of CS-unfolding intermediates is not influenced by inhibition of isomerase activity

Preincubation of FkpA with a tenfold molar excess (6 μM) of the inhibitor FK520 only has a very marginal influence, both on its ability to slow down the aggregation kinetics of CS (Figure 5(a)) and on its ability to dissolve preexisting aggregates (Figure 5(a) and (b), compare corresponding open and closed symbols). The slight increase in aggregation might even be an effect of FK520 itself on aggregation, rather than an effect due to inhibition of FkpA. This view is supported by the fact that the kinetics of loss of enzymatic activity, decelerated in the presence of FkpA, are not influenced at all by the additional presence of the inhibitor (Figure 5(c)) even at a 100-fold excess (60 μM). At these inhibitor concentrations both active sites of the FkpA dimer are fully inhibited, and binding has been shown to occur despite the elevated temperature (Figure 3(a) and (b)).

Obstruction of the active site therefore does not interfere with the chaperone effect of FkpA on CS under unfolding conditions. This implies that the binding of unfolding intermediates of CS to FkpA occurs at a second site, distinct from the isomerase active site and separated widely enough from it to remain undisturbed by the presence of the inhibitor. These results correlate with the inhibition data described above and support the hypothesis of a second non-specific polypeptide binding site, in addition to the active site. It mediates chaperone activity on the one hand, and on the other hand is responsible for the high catalytic activity with folding proteins by lowering K_M.

Both activities reside primarily in the FKBP-domain

To identify the location of the additional binding activity of FkpA, we expressed the two domains constituting the monomer separately and investigated their activity. The domain boundaries were deduced from a homology model† with the crystal structure of hFKBP12. We defined as the N-terminal domain residues 1 to 122 and as the FKBP-domain residues 122 to 245 of the mature wt FkpA protein.

Both domains could be expressed in soluble form in large amounts, both in the peri- and in the cytoplasm. The N-terminal, non-FKBP domain was found to be monomeric in solution, but it formed tetramers after prolonged storage at high concentrations. The C-terminal FKBP-domain showed an even more pronounced tendency to oligomerize. This was unexpected due to the high degree of homology of the FKBP-domain with other monomeric FKBPs (47 % identity with human FKBP12). Upon expression of the FKBP-domain under control of a strong promotor in the cytoplasm, formation of stable tetramers was observed in gel filtration, and no concentration dependence was found over the fivefold concentration range tested (data not shown). The interactions in these tetrameric species are probably non-native, since they were devoid of any activity, i.e. isomerase or chaperone.

When the FKBP-domain was expressed under the control of a weaker promotor and exported to the periplasm, an equilibrium mixture of different oligomeric forms and the monomer (about 50 %) could be isolated. This mixture, although inactive in the RNaseT1 folding assay (see Figure 7(a)), retained tenfold reduced isomerase activity with the tetrapeptide substrate (data not shown), compared to an equimolar concentration of the wt active site. This could, however, be an underestimate, since we do not know what percentage of the monomeric species is active. The N-terminal domain, on the other hand, showed no activity in either of the two isomerase assays. Mixing of the two domains could not reconstitute the catalytic activity with the folding protein, nor did it increase the activity of the isolated FKBP-domain towards the tetrapeptide. These findings are paralleled by the inability of the two domains to form the native homodimer, as judged by gel filtration experiments.

The chaperone activity of the isolated domains was quantified by two independent methods. On the one hand, prevention of CS aggregation at

† Guido Capitani, personal communication.

43 °C was measured as described above, and on the other the increase in refolding yield of the antibody scFv fragment 4D5^{--} scFv was determined, as described in detail elsewhere.[15] The two minus signs describe the absence of both disulfide bonds. In both assays the isolated FKBP-domain seems to be highly active. CS aggregation is delayed considerably and reduced to a high extent by the presence of an eightfold molar excess of the FKBP-domain (Figure 6(a)). The N-terminal domain has only a small effect on CS aggregation. A combination of both domains, only increases the effect marginally, compared to the FKBP domain alone.

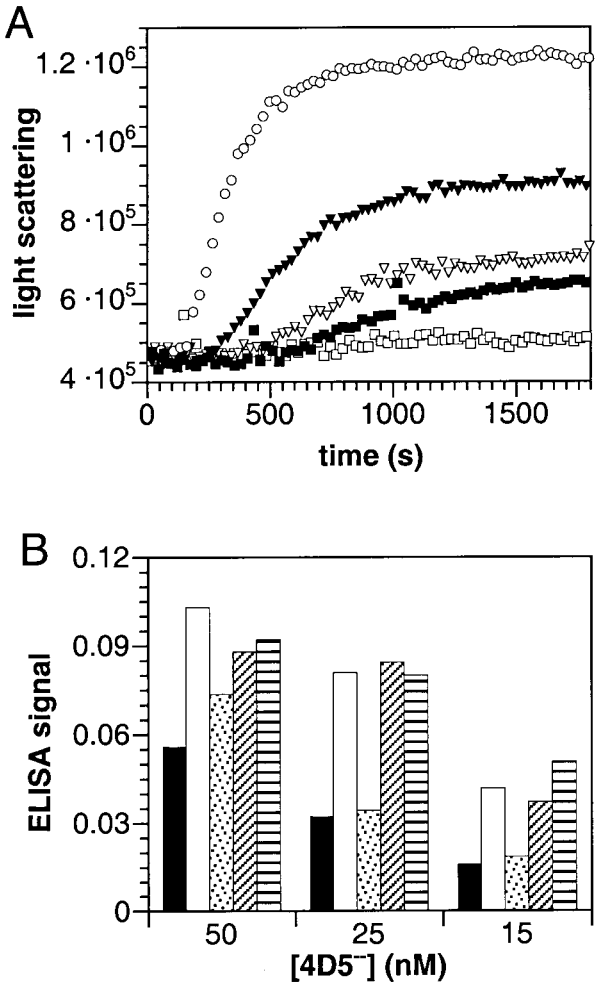

Figure 6. Chaperone function of FkpA domains. (a) Thermal aggregation of citrate synthase (CS, 0.075 μM) at 43 °C, observed by light scattering at 500 nm. CS in the presence of 0.6 μM BSA (○), in the presence of 0.6 μM FkpA-dimer (□), 0.6 μM N-terminal domain (▼), 0.6 μM FKBP-domain (▽) and a mixture of both (each 0.6 μM) (■). (b) Refolding of antibody scFv fragment. Serial dilutions of samples measured by ELISA to determine the activity of the 4D5^{--} scFv refolded at 10 °C alone (0.2 μM, black bars), in the presence of 0.4 μM FkpA-dimer (white), N-terminal domain, (dotted), FKBP-domain (diagonal lines) and a mixture of both (horizontal lines). Numbers below the bars indicate the calculated total scFv concentration in the ELISA wells.

Since the experiments with thermal unfolding of CS have to be carried out at elevated temperature, where the stability of the isolated domains might be affected, we additionally performed refolding experiments with the 4D5^{--} scFv at 10 °C, followed by ELISA quantification of the refolding yield. While the N-terminal domain has no influence on the yield of active scFv, the presence of the FKBP domain alone accounts for the entire chaperone activity of the wt FkpA in this assay (Figure 6(b)).

Both activities, i.e. isomerase and chaperone, thus seem to be located essentially in the FKBP domain. Although the isolated FKBP domain is reduced in its catalytic *cis-trans* isomerase activity, it retains almost full chaperone activity. Covalent linking of the domains and correct dimerization, mediated by the N-terminal domain, on the other hand, seems to be required for full isomerase activity with protein substrates and it may also enhance chaperone activity.

As already mentioned above, these findings are rather surprising considering the high degree of homology that the catalytic domain of FkpA shares with other FKBPs that are, however, monomeric and show no chaperone activity. The identity on the protein level to human FKBP12 is as high as 47 %. Even higher scores are observed with FKBP domains from other two-domain proteins, which are known to be dimeric in solution and have already been suggested to form a subfamily of FKB-proteins.[13] The closest relative (60 % identity) is the *H. influenzae* homolog FkpY,[34] while FKBP22 from *E. coli*[35] has 52 % identity and the MIP-proteins from different *Legionella* species[36] around 48 %. The identity of the FkpA N-terminal domain is, however, never higher than 25 % with any of the MIPs or FKBP22, implying that the need for conservation in this part may be rather low.

FkpY from *Haemophilus influenzae* has identical characteristics

FkpY from *H. influenzae* is the closest homolog to FkpA found in the database, with an overall identity of 46 %, with 60 % in the FKBP domain and 34 % in the N-terminal domain. FkpY is shortened with respect to FkpA, by 11 amino acid residues at the N terminus and by 16 amino acid residues at the C terminus. Interestingly, FkpA is proteolytically cleaved upon prolonged storage at both the N and the C terminus at sites close to these positions, indicating that the termini might be less well structured than the rest of the FkpA protein. Both the shortened FkpA mutant corresponding in length to FkpY, and FkpY itself have essentially the same characteristics as wt FkpA. Both proteins are exclusively dimeric in solution and their stability towards denaturant is identical with that of the wt FkpA (data not shown). Both catalyze the refolding of RNaseT1 efficiently, with only slight variations in activity compared to FkpA wt (Figure 7(a)). Both proteins also display chaper-

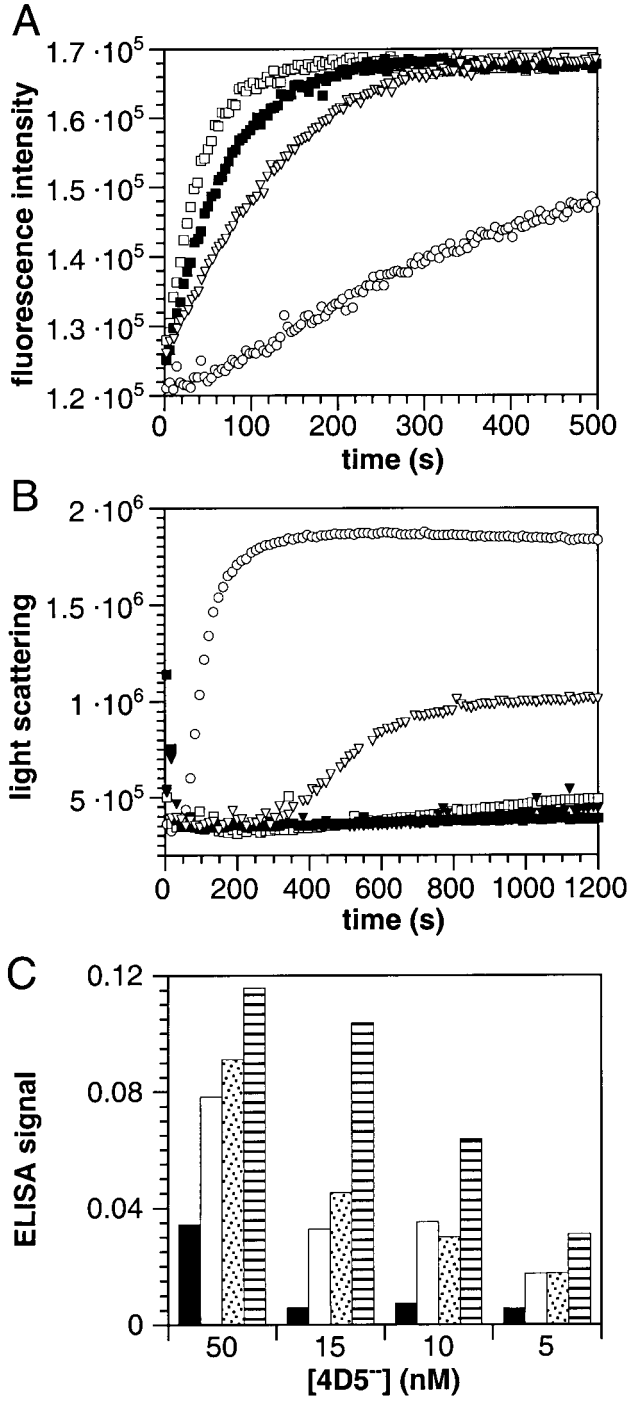

Figure 7. Comparison of FkpA, FkpY and a shortened form of FkpA. (a) Fluorescence traces of refolding of RNaseT1 (0.4 μM) at 10 °C, starting from equilibrium denatured protein in the presence of 10 nM FKBP-domain (○), 10 nM FkpA wt (□), 10 nM shortened FkpA mutant (■) and 10 nM FkpY (▽). The intensity was followed at 323 nm upon excitation at 295 nm. (b) Thermal aggregation of citrate synthase (CS, 0.075 μM) at 43 °C, observed by light scattering at 500 nm. CS alone (○), in the presence of 0.6 μM FkpA □), 0.6 μM shortened FkpA mutant (■), 0.6 μM FkpY (▽) and 1.2 μM FkpY (▼). (c) Serial dilutions of samples measured by ELISA to determine the activity of the 4D5^{--} scFv refolded at 10 °C alone (0.2 μM, black bars), in the presence of 0.4 μM FkpA wt (white), shortened FkpA mutant (dotted) and FkpY (horizontal lines). Numbers below the bars indicate the total calculated scFv concentration in the ELISA wells. All enzyme concentrations are given for dimers.

one activity. In the case of the shortened FkpA mutant, it is unchanged compared to that of the wild-type (Figure 7(b) and (c)). FkpY, on the other hand, has a different activity pattern for the two chaperone substrates investigated. While it is less active than FkpA in the CS aggregation assay (Figure 7(b)), its effectiveness in increasing the scFv refolding yield is higher (Figure 7(c)). This could be due to a different amino acid composition of the hypothetical polypeptide binding site. In light of the almost identical activities *in vitro*, the less stringent conservation of the N-terminal domain may be indicative for a more structural function of this part, as has already been suggested above.

Discussion

From the results described above, it is clear that the high enzymatic activity of FkpA with folding proteins and its independent chaperone function have the same basis. On the one hand, a low K_M value for the folding protein substrate increases the k_{cat}/K_M value for the catalyzed reaction. Reversible binding of refolding and unfolding intermediates, on the other hand, mediates the chaperone effect. In both cases, binding of the polypeptide occurs at a second, probably rather unspecific binding site. This polypeptide-binding site must be distinct from the active site, even through it may be adjacent, based on the fact that binding of CS unfolding intermediates is undisturbed by the presence of the active site inhibitor FK520 and, conversely, that protein binding, but not peptide catalysis can be competed for by an unfolded protein (RCM-la). Moreover, the affinity of the peptide-binding site towards specific substrates seems to differ between homologous proteins, although the active site is highly conserved, and all FKBPs are known to have the same characteristic substrate specificity with regard to tetrapeptide substrates for the active site.[37] It is, however, clear that in the case of rather unstructured substrates, especially those that are subject to both catalytic and chaperone activity, both active sites may well contribute to substrate binding, as well as to the observed chaperone effect, rendering an attempted disentangling of the contributions more difficult.

Surprisingly, both functions of FkpA, isomerase and chaperone, appear to be located in the FKBP-domain and may be adjacent, as both need to contribute to binding the same protein molecule. This was inferred from the observation that the isolated FKBP-domain retains the binding capacity for folding intermediates *in vitro*, demonstrated by the chaperone assays with unfolding CS and refolding

scFv. This is in apparent contradiction with observations made *in vivo* by Arié *et al.*[27] very recently, with a deletion mutant of FkpA lacking the greater part of the N-terminal domain. The authors find that the deletion mutant of FkpA has no chaperone effect on their substrate protein MalE31 upon coexpression *in vivo* and conclude that the chaperone activity hence requires the N-terminal domain. We observe, however, that the isolated FKBP domain shows a high tendency to oligomerize upon strong overexpression *in vivo* and to form stable, but inactive tetramers. This result was at first unexpected but it is in accordance with recent findings which suggest that dimerization involving the active sites and hence leading to inactivation may be a latent property of the FKBP fold.[38]

A closer look at the homology within the subfamily of dimeric FKBPs may further support our argument for a more structural function of the N-terminal domain of these FKBPs, securing the dimerization, orientation and stability of the FKBP domain. While the homology in the FKBP domain of this subfamily is very high, not only within the active site, the homology in the additional N-terminal domain is rather low, mostly below 25%. From these sequence comparisons, it had already been concluded that any enzymatic or binding function of these domains cannot be conserved.[35] In the example of FkpY, the identity to FkpA in the FKBP domain is 60%, which corresponds to the average identity observed between orthologous pairs in a recent comparison of the *E. coli* and *H. influenzae* genomes.[39] The percent identity of the N-terminal domain of FkpY to FkpA (34%), on the other hand, lies below the lower limit found for the majority of the orthologs. It is therefore conceivable that the function of this additional domain is merely the formation of the correct quartenary structure, particularly to secure the relative distance and orientation of the FKBP-domains, with no requirement for binding or catalysis. The evolutionary pressure on this domain would thus be on maintaining the structure, and many sequences may fulfill this function.

The current working model for FkpA and similar dimeric FKBPs can be summarized as follows (Figure 8). Each FKBP domain contains an active site catalyzing proline residue *cis/trans* isomerization, and a separate protein binding site, which may, however, overlap with the active site. A denatured protein such as RCM-la can bind to the protein binding site, without necessarily engaging in the active site, thereby not inhibiting the PPIase activity with a short peptide substrate. Catalysis of a protein substrate is, however, severely impaired by the presence of such a denatured protein, since it competes for the additional protein binding site that is at the basis of the high catalytic activity of FkpA with protein substrates. Conversely, FK520 inhibits isomerase activity with the protein and the peptide substrate. Since the chaperone activity is mainly mediated by the additional polypetide-binding site, it is not necessarily affected by the inhibitor binding to the active sites. In cases, however, where the chaperone substrate is mostly unfolded and/or is also a substrate for isomeriza-

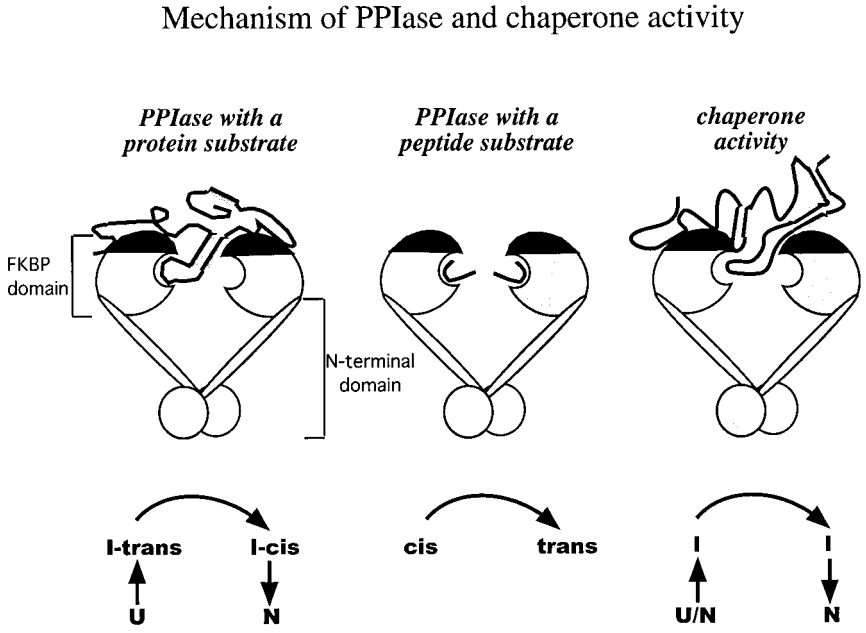

Figure 8. Model for the prolyl-isomerase and chaperone activities of the FkpA dimer. The protein folding and unfolding intermediates interact with a polypeptide-binding site (black), distinct from the active site (indicated as a cavity), and both are located on the FKBP-domain. Two tetrapeptides can be bound and isomerized simultaneously by the FkpA dimer, but only one protein molecule. In the chaperone activity on non-*cis*-proline substrates (right), binding to the active site may occur but appears to be non-essential. For more explanations see text.

tion catalysis, the interaction with the catalytic site may well contribute to the overall binding. The N-terminal domain of FkpA then has an entirely structural function, keeping the FKBP-domains in an orientation, such that a protein substrate can bind to both domains at the same time, i.e. that they face each other. While the chaperone activity can also be mediated by the isolated FKBP domain, dimerization to the correct native homodimer of the two domain protein probably enhances chaperone function through an avidity effect and it is a prerequisite for the efficient catalysis of protein folding. The recently solved crystal structure of the FkpA-homolog MIP from *L. pneumophila* (R. Hilgenfeld *et al*., unpublished data; ribbon structure at http://www.imb-jena.de/www_sbx/projects/sbx_mip.html) supports such an interpretation, since it shows that the two FKBP-domains are juxtaposed in such a way that they can interact with the same substrate, the precise orientation being determined by the long, potentially flexible helix within this N-terminal domain.

Well adapted, highly evolved enzymes usually have maximized turnover at the substrate concentrations they encounter within the cell. This requires a high turnover together with relatively weak substrate binding. By this criterion FkpA, like other FKBPs,[26] does not appear to have evolved towards the expected properties for efficiently catalyzing protein substrates. Rather than by a high turnover, catalysis is enhanced through tight substrate binding, mediated by a second site. In light of the chaperone activity observed for FkpA and other FKB-proteins, this may indicate that folding catalysts, such as PPIases and disulfide isomerases, may have been subject to a twofold selective pressure, especially in such cellular compartments as the periplasm of bacteria, where no dedicated chaperone network seems to exist. More precisely, the presence of an additional high-affinity polypeptide-binding site may provide a gratuitous advantage, apart from increasing catalytic activity, compared to a protein with high turnover and low substrate affinity. The additional, independent chaperone function mediated by such a polypeptide-binding site could constitute an important selective advantage, especially in the periplasm of *E. coli*. The absence of a classical chaperone network in the periplasm and the number of periplasmic folding catalysts for which an additional, independent chaperone activity has been proposed[15,31,40–42] seem to support this hypothesis. It is therefore conceivable that the subfamily of two-domain, dimeric FKBPs, among them FkpA, FkpY, and the MIP proteins, may fulfill a twofold function *in vivo* that comprises proline isomerization on the one hand and, independent from this activity, chaperone function on the other.

The fact that both features seem to be located within the conserved FKBP-domain may shed a new light on the entire family of FKPBs that are generally believed to be much poorer catalysts (10 to 20-fold) than the cyclophilins.[3] At the same time, the data might facilitate understanding of the puzzling fact that hardly any of the many PPIases is essential. Yeast, for example, is viable when all of its four FKBPs and eight cyclophilins are deleted.[6] If the *in vivo* function of at least a subset of them consists to a high extent in the prevention of aggregation through their chaperone activity, which will be independent of proline isomerization in many cases, then it is clear that several other proteins could potentially replace them. In the periplasm of *E. coli*, the PPIases FkpA[15,31] and SurA[40] and the disulfide-isomerases DsbG[42] and DsbC[41] are four examples of such proteins, for which an additional chaperone activity has been described. It thus appears that a number of proteins, whose activity involves transient binding to polypeptides, have evolved binding sites which give them a chaperone function, even independent of the original catalytic activity.

Experimental Procedures

Protein expression and purification

Expression and purification of the disulfide-free hu4D5-8 antibody scFv fragment (abbreviated 4D5^{--}), where the two minus signs denote the absence of both disulfide bonds and of FkpA with and without His-tag were carried out as described.[15,43] The FkpA ORF contains a signal sequence of 25 amino acid residues, followed by the mature protein of 245 amino acid residues. The FkpA construct termed ''shortened FkpA'' is missing the first 9 N-terminal and 18 C-terminal amino acid residues of the mature protein. The N-terminal domain construct encompasses amino acid residues 1 to 122 of the mature wt FkpA, the construct of the C-terminal domain starts at amino acid residues 122. FkpY from a *H. influenzae*,[34] clone obtained from the TIGR/ATCC was also investigated. All four sequences were cloned without signal sequence into the vector pTFT74 with C-terminal His-tag,[44] which places them under the control of the phage T7-promotor for cytoplasmic expression. Expression was carried out at 25 °C in *E. coli* BL21 with induction by IPTG overnight. The domains were additionally subcloned into the vector pAK400[45] for periplasmic expression with the *pelB* signal sequence, and expressed under control of the *lac* promotor at 25 °C. For purification, the soluble cell extracts were passed over an immobilized metal ion affinity chromatography (IMAC) column at pH 7, followed by S/H-cation exchange chromatography at pH 6 for the FkpA mutant, FkpY and the FKBP domain, while for the N-terminal both columns were run at pH 7.5. Protein concentrations were determined using absorption coefficients.[46] FkpA and FkpY concentrations given refer to the molar concentrations of dimers.

Yeast citrate synthase was a kind gift from Dr Peter Lindner, purified as described.[47] Human FKBP12 was a kind gift from Dr Heinz Gehring, FK520 was a gift from Novartis (Basel), and the antigen HER2 extracellular domain was a gift from Genentech (South San Francisco). The S54G/P55N-variant of RNaseT1 from *Aspergillus oryzae* was a gift from Raimund Maier and Professor Franz X. Schmid (University of Bayreath). Its reduction and carboxymethylation was carried out as described.[28] Wild-type RNaseT1 from *Aspergillus oryzae* and the car-

boxymethylated bovine α-lactalbumin were both obtained from Sigma.

Analytical gel filtration

Analytical gel filtration experiments were carried out on a SMART system with a Superose-12 column in 50 mM Tris (pH 7.0), 150 mM NaCl, 0.005 % Tween-20.

Fluorescence titration

Fluorescence measurements were performed with a PTI Alpha Scan spectrofluorimeter (Photon Technologies Inc.) at 25 and 38 °C, as indicated, using excitation and emission wavelengths of 280 nm and 307 nm, respectively. The buffer was 50 mM Tris (pH 7.0), 50 mM NaCl (filtered, degassed). FkpA at 1 μM (dimer) was incubated with increasing amounts of the inhibitor FK520 before measurements were taken, and the obtained fluorescence signal was corrected for the fluorescence of the buffer and the dilution effect. Before measurements were started, the protein solution was preincubated in the cuvette to minimize artefacts due to fluorescence decrease caused by absorption of FkpA to the cuvette-walls during the titration experiments. The binding curve obtained was analyzed using Kaleidagraph (Synergy Software, Reading, UK) by fitting to a quadratic equation, as described,[48] assuming two identical binding sites.

RNaseT1-refolding assay

The refolding assays with the wild-type (wt) RNaseT1 were carried out essentially as described.[15] In experiments with the carboxymethylated S54G/P55N-variant (RCM-RNaseT1), the protein was unfolded in 100 mM Tris (pH 8) and the refolding buffer contained additionally 2 M NaCl for stability. The salt concentration had no effect on the catalytic activity of FkpA. The final FkpA-dimer concentration was either 5 or 10 nM, as indicated. For the determination of Michaelis-Menten parameters, the RNaseT1 concentration was varied from 0.05 to 2 μM (wt) or 6 μM (RCM-RNaseT1). The kinetic traces obtained were analyzed by global fitting with numeric iteration using the software DynaFit[21] in a model that accounted for the presence of the uncatalyzed reaction, described by the following reaction mechanism:

$$\mathrm{FkpA} + \mathrm{RNaseT1_U} \overset{K_M}{\Longleftrightarrow} \mathrm{FkpA \cdot RNaseT1_U} \qquad (1)$$

$$\mathrm{FkpA \cdot RNaseT1_U} \xrightarrow{k_{cat}} \mathrm{FkpA} + \mathrm{RNaseT1_N} \qquad (2)$$

$$\mathrm{RNaseT1_U} \xrightarrow{k_0} \mathrm{RNaseT1_N} \qquad (3)$$

K_M is treated as an equilibrium constant, k_{cat} and k_0 are first-order rate constants and k_0 is the rate of the uncatalyzed folding reaction. The subscripts U and N refer to the unfolded and native state, respectively. Fit parameters were K_M and k_{cat}, while the uncatalyzed rate was determined separately and held constant, and folding reactions were considered to be irreversible under the conditions used.

The final concentration used in the inhibition measurements was 0.4 μM wt RNaseT1. Kinetic traces were evaluated with double exponential functions using Kaleidagraph and the rates of the faster phases were plotted against the inhibitor concentration. Control experiments (data not shown) showed that neither FK520 nor RCM-la had by themselves any effect on the rate or the yield of RNaseT1 refolding under the conditions used.

Protease-free assay for peptidyl prolyl isomerization

The improved assay conditions introduced by Kofron *et al.*[49] were used in an assay carried out in the absence of protease, as described initially by Janowski *et al.*[19] The tetrapeptide had the sequence succinyl-Ala-Ala-Pro-Phe-nitroanilide and was used at a final concentration of 120 μM. Measurements were carried out at 10 °C in plastic cuvettes, and the decrease in absorbance was followed at 330 nm for six minutes. Kinetic traces were analyzed by a non-linear least-squares fit to a single exponential function using Kaleidagraph, and an initial estimate for k_{cat}/K_M was obtained from the slope of a plot of the rate versus the enzyme concentration used. Human FKBP12 was used as a control.

Citrate synthase aggregation

Citrate synthase (CS) aggregation was followed by light scattering measurements with a PTI Alpha Scan spectrofluorimeter (Photon Technologies Inc.) at 43 °C, using an excitation and emission wavelength of 500 nm. The final CS concentration was 0.075 μM in 35 mM Hepes (pH 7.8) (filtered, degassed). Concentrations of FkpA, either present from the start or added later on, were as indicated.

Citrate synthase inactivation

CS at a concentration of 0.075 μM in 35 mM Hepes (pH 7.8) was incubated at 43 °C, alone or in the presence of FkpA and FK520, as indicated. Inactivation and reactivation were followed by measuring activity at the respective time points. Activity measurements were carried out at 25 °C in an assay containing 200 μM oxaloacetate (OAA), 100 μM DNTB and 100 μM acetyl-CoA in 200 mM Tris (pH 8), freshly mixed before each measurement. Measurements were carried out in plastic cuvettes by following the increase of absorbance at 412 nm, and the activity was determined from the linear part of the progress curve, in relation to the activity of native CS, which was set to 100 %. The enzymatic reaction was started by the addition of 50 μl of CS, which had been incubated at 43 °C for a given period of time, to a total volume of 1 ml. At the same time point a second aliquot of 43 °C incubated CS was taken, but its reactivation was initiated by the addition of OAA to a final concentration of 1 mM. The activity of the reactivated samples was measured as described above, following incubation at 25 °C for one hour. The amount of reactivatable intermediate present after incubation at 43 °C for a given period of time was determined from the difference of the activity with and without reactivation.

ELISA

ELISA experiments to determine the refolding yield of the 4D5^{--} scFv antibody fragment were carried out as described.[15] Briefly, the scFv was refolded overnight from 3 M GdmCl at 10 °C in the presence of chaperones as indicated and the activity was quantified by ELISA. For the ELISA, plates were coated with the antigen, the

HER2 extracellular domain. The refolded scFv bound to the plate was quantified by an anti-myc-tag IgG from mouse, followed by a goat-anti-mouse-IgG-POD for detection. The results are averages of at least duplicate measurements that have been reproduced at least twice in independent experiments.

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