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The Periplasmic *Escherichia coli* Peptidylprolyl *cis,trans*-Isomerase FkpA

II. ISOMERASE-INDEPENDENT CHAPERONE ACTIVITY *IN VITRO**

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We recently identified FkpA by selecting for the increased yield of antibody single-chain Fv (scFv) fragments in phage display, even of those not containing *cis*-prolines. We have now investigated the properties of FkpA *in vitro*. The peptidylprolyl *cis-trans*-isomerase activity of FkpA was found to be among the highest of any such enzyme with a protein substrate, yet FkpA is not able to enhance the proline-limited refolding rate of the disulfide-free hu4D5-8 scFv fragment, probably due to inaccessibility of Pro-L95. Nevertheless, the yield of the soluble and functional scFv fragment was dramatically increased *in vitro* in the presence of FkpA. Similar effects were observed for an scFv fragment devoid of *cis*-prolines. We are thus forced to conclude that the observed folding-assisting function is independent of the isomerase activity of the protein. The beneficial effect of FkpA was found to be due to two components. First, FkpA interacts with early folding intermediates, thus preventing their aggregation. Additionally, it has the ability to reactivate inactive protein, possibly also by binding to a partially unfolded species that may exist in equilibrium with the aggregated form, which may thus be released on a productive pathway. These *in vitro* measurements therefore fully reflect the *in vivo* results from periplasmic overexpression of FkpA.

FkpA is one of three soluble peptidylprolyl *cis,trans*-isomerases (PPIase)[1] in the periplasm of *Escherichia coli*, in addition to PpiA (RotA) and SurA (1). It was first described as being similar to the macrophage infectivity potentiator of *Legionella* species (2). It is homologous to the FK506-binding proteins and was subsequently shown to belong to the σ^E regulon and thus to be inducible under stress conditions (Refs. 3–5; reviewed in Ref. 6). Although the *fkpA* deletion mutant has been shown to be viable (1, 2), FkpA has been proposed to have a general folding-assisting function in the periplasm (Refs. 1 and 5; reviewed in Ref. 6). This idea was based on an *fkpA* deletion causing up-regulation of σ^E and thus stimulation of *degP* transcription and, in addition, FkpA's own induction by extracytoplasmic stress (5) and its ability to restore near-normal σ^E-dependent response when overexpressed (1).

In the accompanying paper (7), FkpA was identified in a selection system as a factor improving the functional expression of antibody scFv fragments in the bacterial periplasm. This was achieved by overexpressing a library of *E. coli* proteins in a phagemid, on which a poorly expressing antibody was encoded for phage display, and panning for functional antibody. *In vivo* overexpression experiments provided further evidence that the yield of functional antibody scFv fragments produced in the periplasm is increased upon coexpression of FkpA. Interestingly, the effect was also observed with antibody fragments devoid of *cis*-prolines (7). In antibody scFv fragments, the variable domain of the heavy chain (V_H) is connected via a linker to the variable domain of the light chain (V_L). Antibody V_H domains have never been found to carry a *cis*-proline in their framework,[2] whereas antibody V_L domains come in two types, κ and λ. κ domains have two *cis*-prolines, at positions L8 and L95 (consensus numbering of Kabat *et al.* (8)). In contrast, λ domains do not carry any *cis*-prolines in the framework in any known structure in the Protein Data Bank.[2]

The process of structure formation during *in vitro* refolding of an scFv fragment has been described in some detail for the scFv fragments McPC603 (9–13) and hu4D5-8 (14).[3] It was found that a state with considerable structure within the domains is formed on the millisecond time scale, regardless of the peptide conformation at the proline residues. However, a complication is introduced by the slow isomerization of the peptide bond preceding Pro-L95, which must be *cis* for the native V_H/V_L interface to form. As a consequence, the final correct docking of V_H and V_L (and thus, the subsequent stabilization of the structure) is limited in rate by this proline *cis,trans*-isomerization (10, 12, 13). In the scFv format, presumably through the increased effective domain concentration mediated by the interdomain peptide linker, the domains have been shown to associate prematurely instead of folding rapidly and independently into native-like structures,[3] thus forming off-pathway intermediates that are potentially prone to aggregation.

We have chosen two different model systems to investigate the effect of FkpA on scFv fragment refolding *in vitro*. The disulfide-free variant (15) of the hu4D5-8 scFv fragment (termed $4D5^{--}$) to indicate the absence of both disulfide bonds (16), an antibody scFv fragment binding the extracellular domain of $p185^{HER2}$, was chosen for its low stability and reduced reversibility of refolding. With refolding yields of 75% at 10 °C under optimal conditions, this scFv fragment leaves room for a possible improvement by FkpA (14). The anti-GCN4 scFv fragment, which binds the transcriptional activator Gcn4p (17), was chosen because it has a V_L domain of the λ type and thus

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[1] The abbreviations used are: PPIase, peptidylprolyl *cis,trans*-isomerase; scFv, single-chain Fv; ELISA, enzyme-linked immunosorbent assay; GdmCl, guanidinium chloride; BSA, bovine serum albumin; FKBP, FK506-binding protein.

[2] A. Honegger, personal communication.

[3] M. Jäger, P. Gehrig, and A. Plückthun, submitted for publication.

no *cis*-prolines in its native structure.[2] Since, however, the wild-type anti-GCN4 fragment displays excellent folding properties, we used a destabilized variant for this study containing the H-R66K mutation, where H refers to the heavy chain (18). Arg at the heavy chain position H66 was shown previously to result in higher protein stability than Lys (19, 20). The obtained mutant is indeed considerably less stable (18) and shows reduced folding reversibility due to aggregation at elevated temperatures. The experiments with the anti-GCN4 (H-R66K) scFv fragment could therefore be carried out at physiologically relevant temperatures (37 °C) under conditions allowing for the observation of an effect of FkpA.

In this study, we have attempted a thorough analysis of the biochemical properties of FkpA *in vitro* and its effect on scFv fragments. We investigated whether FkpA can accelerate the rate of refolding or increase the refolding yield of antibody fragments *in vitro* and whether these two effects are connected. Particularly, we also determined the effect of FkpA on an scFv fragment with a λ domain, devoid of *cis*-prolines. We measured the rate of acceleration in folding and the amount of soluble monomeric as well as functional scFv protein and also tested the dependence on the time of PPIase addition. We came to the conclusion that the effect on increasing the yield is independent of the PPIase activity of the enzyme.

MATERIALS AND METHODS

Protein Expression and Purification—Protein expression and purification of the disulfide-free hu4D5-8 scFv fragment (abbreviated $4D5^{--}$) and the destabilized version of the anti-GCN4 scFv fragment carrying the heavy chain mutation H-R66K were carried out essentially as described (14, 15, 18, 17). FkpA was expressed at 37 °C in *E. coli* JM83, transformed with the phagemid pHB602 or pHB602-His, containing FkpA (without and with the histidine tag) under control of its own promoter. Both phagemids also carried the scFv fragment 4-4-20 under control of the *lac* promoter. The *E. coli* cells were harvested after 5 h of induction of the scFv fragment with isopropyl-β-D-thiogalactopyranoside. For purification of the His-tagged version, the soluble cell extract was purified by immobilized metal ion affinity chromatography at pH 7, followed by S/H cation-exchange chromatography at pH 6. FkpA without a His tag was purified by Q-Sepharose anion exchange chromatography at pH 8, followed by S/H cation-exchange chromatography at pH 6 and gel filtration at pH 7. All ELISA experiments were performed exclusively with FkpA without a His tag, to avoid its interference with the detection system, while the activity was determined for both variants, as were the $4D5^{--}$ fluorescence kinetics of folding, whereas the gel filtration experiments were mostly carried out with the His-tagged version.

FkpA Spectra and Denaturation Curve—Fluorescence measurements were performed with a PTI Alpha Scan spectrofluorometer (Photon Technologies, Inc.) at 20 °C using excitation wavelengths of 295 and 280 nm as indicated and an emission wavelength of 350 nm. The buffer was 50 mM Tris, pH 7.0, and 50 mM NaCl (filtered and degassed). The denaturation curve was prepared by incubating the native protein at the respective GdmCl concentrations at 20 °C overnight. Intensity values have been corrected for the fluorescence of the buffer, and GdmCl concentrations were determined by the refractive index.

RNase T1 Refolding Assay—Wild-type RNase T1 (Sigma) was unfolded by incubating the protein in 5.6 M GdmCl, pH 7, at 10 °C overnight. Refolding was initiated by 80-fold dilution to a final concentration of 0.2 μM in 50 mM Tris, pH 7, 50 mM NaCl, and the indicated concentrations of FkpA at 10 °C. RCM-RNase T1 (S54G/P55N) is a variant in which both disulfide bonds have been reduced and *S*-carboxymethylated. Its refolding has been shown to be a monoexponential process, but the protein requires 2 M NaCl for stability (21, 22). The wild-type protein, which has two slow isomerization-limited refolding phases, does not require 2 M NaCl for stability (23), and the measurement could therefore be carried out in the standard buffer. The folding reaction was followed with a PTI Alpha Scan spectrofluorometer at 323 nm after excitation at 295 nm. Kinetic traces were evaluated with double exponential functions using Kaleidagraph software (Synergy Software, Reading, United Kingdom), and the rates of the faster phase were plotted to determine k_{cat}/K_m.

$4D5^{--}$ scFv Refolding Kinetics—Fluorescence measurements were performed with a PTI Alpha Scan spectrofluorometer at 10 °C using excitation and emission wavelengths of 295 and 326 nm, respectively. The buffer was 50 mM Tris, pH 7.0, and 50 mM NaCl (filtered and degassed). Final protein concentrations were 0.4 μM for both proteins, calculated for a dimer of FkpA. Refolding was initiated by rapid 20-fold dilution of the unfolded $4D5^{--}$ scFv fragment in buffer alone or buffer containing FkpA. Kinetic traces were corrected for the fluorescence of FkpA and evaluated using Kaleidagraph software with single exponential functions.

Analytical Gel Filtration—Analytical gel filtrations were carried out on a SMART system with a Superose 12 column in 50 mM Tris, pH 7.0, and 150 mM NaCl with 0.005% Tween 20. Proteins were injected in a volume of 50 μl at the concentrations indicated in the figure legends. The concentrations given are theoretical concentrations with 100% refolding yield. The samples were passed through a filter with 0.22-μm pore size prior to injection to remove large aggregates and to prevent clogging of the column. The column was calibrated with β-amylase (200 kDa), aldolase (158 kDa), bovine serum albumin (66 kDa), carbonic anhydrase (29 kDa), and cytochrome *c* (12.4 kDa) as molecular mass standards.

Refolding experiments prior to gel filtration were initiated by rapid 20-fold dilution of the unfolded scFv fragments at 10 °C ($4D5^{--}$ scFv) or 37 °C (anti-GCN4 scFv) in buffer alone (50 mM Tris, pH 7.0, and 50 mM NaCl) or buffer containing FkpA (or BSA and PpiA as controls) in equimolar amounts, calculated for a dimer for FkpA. The samples were injected after incubation overnight in the case of the $4D5^{--}$ scFv fragment and after 1–2 h in the case of the anti-GCN4 scFv fragment.

ELISA—ELISA plates (Nunc) were coated at 4 °C overnight with 100 μl of Tris-buffered saline containing either 1.5 μg/ml HER2 extracellular domain (for the $4D5^{--}$ scFv fragment) or BSA-GCN4 conjugate (for the anti-GCN4 scFv fragment) at a dilution of 1:5000 (17). Blocking was in Tris-buffered saline containing 0.005% Tween 20 with 5% milk at room temperature for 1 h. Binding took place in the presence of 3% milk at room temperature for 45 min. Detection was carried out by incubation for 45 min at room temperature with either mouse anti-His tag IgG (anti-GCN4 scFv) or mouse anti-Myc tag IgG ($4D5^{--}$ scFv) and subsequent incubation with a goat anti-mouse IgG-peroxidase conjugate for 45 min. After addition of the substrate (BM Blue POD substrate, soluble; Roche Molecular Biochemicals), the reaction was stopped with 0.1 M HCl, and the plates were measured at 405 nm. The results are averages of at least duplicate measurements per plate that have been reproduced at least twice in independent experiments. In all ELISA experiments, wild-type FkpA without any tags was used.

All native protein was centrifuged at 16,000 × *g* for 20 min at 4 °C before incubation or denaturation. Proteins were denatured by incubation in 3 M GdmCl ($4D5^{--}$ scFv) or 4 M GdmCl (anti-GCN4 scFv), pH 7, at 4 °C overnight. Refolding reactions for ELISA measurement were initiated by 40–60-fold dilution of the unfolded protein to a final concentration of 0.2 μM in 50 mM Tris, pH 7, and 50 mM NaCl at the indicated temperatures and incubated for 5 h or overnight ($4D5^{--}$ scFv) and for 1 h (anti-GCN4 scFv).

Before addition to the ELISA wells, samples were centrifuged at 16,000 × *g* for 20 min at 4 °C to remove aggregates and diluted to the concentrations indicated at 4 °C. Probes with native protein were treated identically. For the controls, FkpA was added after the final centrifugation, before dilution and addition to the ELISA wells. Inhibition was achieved with a 10-fold molar excess of the antigen over the scFv fragment present in the respective dilution.

RESULTS

Characterization of the Oligomeric State and Thermodynamic Stability—FkpA could be obtained in a soluble and functional form in high amounts after expression under the control of its own promoter, as described under "Materials and Methods." A molecular mass of 26,225.4 Da (theoretical mass of 26,223.6 Da) was confirmed by mass spectrometry. In analytical gel filtration, however, FkpA eluted slightly after BSA (66 kDa), more consistent with being a dimer, but not rigorously excluding a trimer (data not shown). A monomer peak was never observed under any condition tested (1–10 μM), nor was the peak shifted to higher values. Native FkpA therefore seems to form dimers, and the monomeric form does not appear to be populated under the conditions used in gel filtration experiments.

FkpA contains only one tryptophan residue in its sequence, which is highly quenched in the native state and becomes solvent-exposed upon unfolding of the protein. Upon excitation

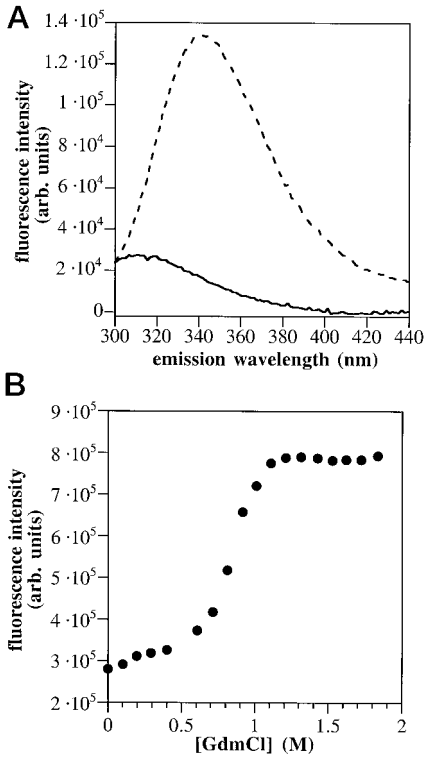

FIG. 1. *A*, fluorescence spectra of native (——) and unfolded (4 M GdmCl) (– – –) FkpA (0.4 μM) upon excitation at 295 nm and 20 °C; *B*, GdmCl denaturation transition measured with 0.4 μM FkpA at 20 °C and 350 nm upon excitation at 280 nm. *arb.*, arbitrary.

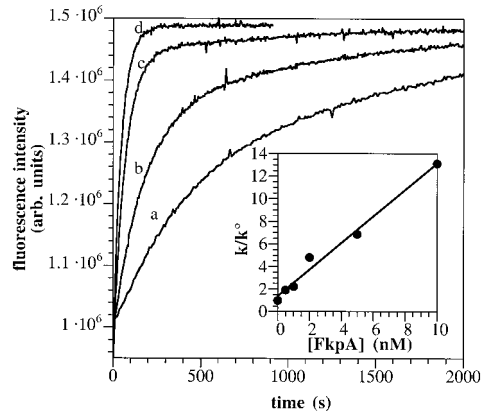

FIG. 2. **Fluorescence traces of refolding of RNase T1 (0.2 μM) at 10 °C starting from equilibrium-denatured protein (*trace a*) in the absence or presence of 0.5 nM (*trace b*), 2 nM (*trace c*), and 5 nM (*trace d*) FkpA (calculated for the dimer).** The intensity at 323 nm was followed. *Inset*, the refolding curves were evaluated with a double exponential fit; and the rates of the faster refolding phase, divided by the uncatalyzed rate, are plotted against the respective FkpA concentration present during the measurement. *arb.*, arbitrary.

at 295 nm, the native protein therefore exhibited very low fluorescence, which increased dramatically upon denaturation, providing a convenient means to follow unfolding transitions (Fig. 1*A*). The unfolding transition (Fig. 1*B*) revealed a rather low thermodynamic stability of FkpA with a midpoint of the equilibrium transition below 1 M GdmCl at 20 °C. No stabilizing effect of increasing protein concentrations could be observed in a 5-fold concentration range (0.4–2 μM), suggesting that the limiting factor is the stability of the monomer, whereas any potential extrinsic contribution of a dimer interface to stability must be small. As it is currently unclear whether the transition is two-state or which molecular changes occur in the sloped pre-transition base line, we do not report ΔG values.

Isomerase Activity—FkpA has long been suggested to possess PPIase activity (2) based on its high similarity to the eukaryotic FK506-binding proteins. Its k_{cat}/K_m was estimated by a protease-coupled assay using chymotrypsin and a peptide substrate to be 9×10^4 M^{-1} s^{-1} (1). Since we noted, however, that FkpA is highly susceptible to very rapid digestion by the protease at the concentrations used in this assay (data not shown), FkpA activity was estimated directly from a protein folding assay. In this assay (24), the enhancement of the proline-limited refolding rate of RNase T1 is observed as a function of enzyme concentration. This assay has been shown in several instances to give a more reliable estimate of the enzymatic activity for those PPIases that are prone to rapid digestion in the standard assay (24, 21).

In the case of FkpA, the faster of the two slow RNase T1 refolding phases was 13-fold accelerated (Fig. 2) when 10 nM FkpA was present, comparable to the 22-fold acceleration observed in the presence of 50 nM Cpr3 (22) in a comparable assay involving RCM-RNase T1 (see "Materials and Methods"). The k_{cat}/K_m was estimated from the slope of the plot in the *inset* of Fig. 2 to be $\sim 4 \times 10^6$ M^{-1} s^{-1} and thus ~2 orders of magnitude higher than the value estimated from the protease-coupled colorimetric assay. FkpA is thus a very efficient isomerase, comparable to other FKBPs (FKBP12 $k_{cat}/K_m = 8 \times 10^5$ M^{-1} s^{-1} (21) and trigger factor $k_{cat}/K_m = 7.4 \times 10^5$ M^{-1} s^{-1} (24)) or the periplasmic *E. coli* cyclophilin PpiA ($k_{cat}/K_m = 6 \times 10^6$ M^{-1} s^{-1} (25)).

Influence on the Proline-limited Refolding Kinetics of the Disulfide-free 4D5^{--} scFv Fragment—Due to the very dramatic effect of coexpression of FkpA on the yield of soluble and active scFv fragments *in vivo* (7), we decided to carry out *in vitro* experiments to further characterize the mode of action of FkpA, focusing on the relationship between its positive effect on *in vivo* folding on the one hand and its peptidylprolyl *cis-,trans*-isomerase activity on the other. Two model systems were used for these investigations: first, the disulfide-free variant of the scFv fragment hu4D5-8 (abbreviated 4D5^{--} to indicate the missing disulfides), and second, a destabilized variant of the anti-GCN4 scFv fragment. The 4D5^{--} scFv fragment was chosen for the reasons that the rate-limiting steps on its folding pathway are well characterized and known to be proline-limited (14)[3] and that it shows a relatively high tendency to aggregate upon refolding, with yields of 75% native protein at the most. Due to the low intrinsic fluorescence of FkpA in its native state compared with the scFv protein (Fig. 3*A*), FkpA does not disturb the fluorescence measurements, even if present in stoichiometric amounts.

As has been described before (14), refolding of the 4D5^{--} scFv fragment is characterized by an initial burst phase. This burst phase is apparent in Fig. 3*B* from the amplitude reached already in the dead time of manual mixing, followed by a much slower conversion to the native state.[3] The initial phase cannot be resolved by stopped-flow mixing techniques and has been shown to involve the formation of an early structured intermediate, indicated by the fast protection of exchangeable protons. As has been shown by short-term unfolding experiments (14),[3] the rate-limiting phase observed by fluorescence spectroscopy (Fig. 3*B*) is a slow proline isomerization-limited folding reaction ($k = 0.001$ s^{-1}). Even in the presence of equimolar amounts of FkpA (0.4 μM, calculated for a dimer) in the refolding buffer, this rate-limiting folding step was not accelerated ($k = 0.001$ s^{-1}). FkpA, although an efficient isomerase, is thus not able to efficiently catalyze the limiting *cis,trans*-isomerization in the refolding of this scFv fragment. This probably reflects the lack of accessibility of the relevant proline in the scFv format, as short-term denaturation experiments

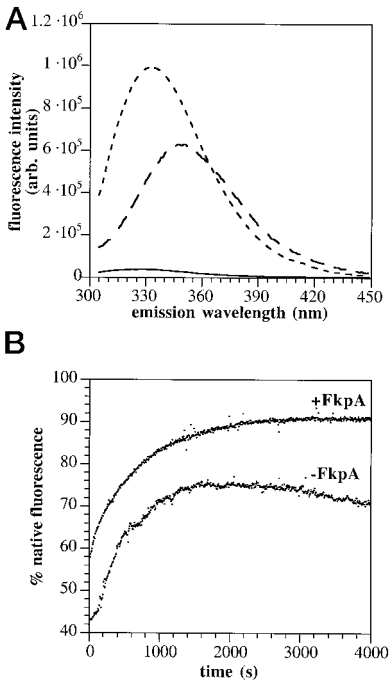

FIG. 3. *A*, fluorescence spectra of native FkpA (0.4 μM; ——), native 4D5⁻ scFv (0.4 μM; - - - -), and unfolded 4D5⁻ scFv (0.4 μM; – – –) upon excitation at 295 nm and 20 °C; *B*, fluorescence traces of refolding of the 4D5⁻ scFv fragment at 10 °C starting from equilibrium-denatured protein in the absence or presence of equimolar amounts of FkpA (calculated for the dimer, both at 0.4 μM) as indicated. The intensity at 326 nm was followed. The value of 100% corresponds to the normalized intensity of native protein at 326 nm, which was obtained by subtracting the fluorescence of the unfolded protein from that of the folded protein. *arb.*, arbitrary.

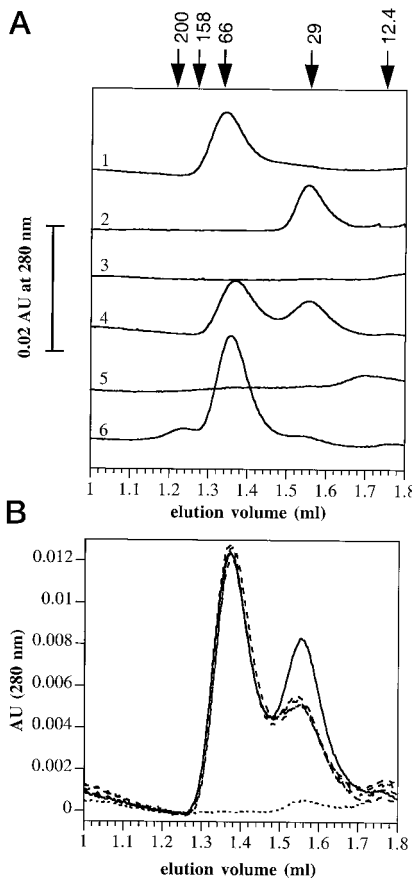

FIG. 4. **Elution profiles of analytical gel filtration runs followed by absorption at 280 nm.** *A*: *trace 1*, FkpA (2 μM); *trace 2*, 4D5⁻⁻ scFv (1 μM); *trace 3*, 4D5⁻ scFv refolded alone (2 μM); *traces 4–6*, 4D5⁻⁻ scFv refolded in the presence of FkpA (both at 2 μM), in the presence of PpiA (2 and 4 μM), and in the presence of BSA (both at 2 μM), respectively. *B*: 4D5⁻⁻ scFv refolded alone (2 μM; - - - -), in the presence of FkpA from the beginning (both at 2 μM; ——), and after addition of FkpA after different times of refolding (1, 2, 5, and 10 min; – – –). Refolding was at 10 °C and pH 7. Samples were passed through a 0.22-μm filter prior to injection. The column was calibrated with β-amylase (200 kDa), aldolase (158 kDa), bovine serum albumin (66 kDa), carbonic anhydrase (29 kDa), and cytochrome *c* (12.4 kDa); and the molecular mass standard *arrows* are valid for both panels. FkpA concentrations are given for dimeric species. *AU*, absorbance units.

clearly do lead to a rate acceleration (14). For comparison, at 10 nM FkpA, the refolding of RNase T1 was already accelerated 13-fold (Fig. 2*A*). An acceleration of the isomerization of Pro-L8, which is not overall limiting in refolding, can, however, not be excluded.

Despite this apparent lack of a catalytic effect on isomerization, aggregation was reduced, and the yield of native protein increased substantially in the presence of FkpA as judged by fluorescence intensity. Some light scattering may contribute to the total intensity of fluorescence measurements in the absence of FkpA, as inferred from the decline of the signal at long times and the small lag phase. The rate should therefore be treated only as an estimate, even though it is very similar to the one obtained in numerous other measurements (14) under slightly different conditions, where aggregation is less severe (data not shown).

Effect of FkpA on the Yield of Soluble and Active 4D5⁻⁻ scFv in Vitro—To further investigate the positive effect of FkpA on the refolding yield of the 4D5⁻⁻ scFv fragment, we carried out gel filtration experiments to determine the amount of soluble monomeric scFv fragment obtained after refolding in the absence and presence of FkpA. As mentioned before, FkpA eluted from the column at an elution volume corresponding roughly to that of BSA under all conditions tested (Fig. 4*A*), whereas the native 4D5⁻⁻ scFv fragment eluted at the volume expected for a monomer, thus allowing clear separation and identification of the two peaks. No formation of stable complexes was observed, neither upon co-incubation nor after refolding of the scFv fragment in the presence of FkpA. This indicates that any interaction must be low affinity and possibly transient in nature.

If refolding of the 4D5⁻⁻ scFv fragment was carried out in the absence of FkpA at the concentrations necessary for gel filtration experiments (2–10 μM), hardly any monomer could be detected; and instead, a broad peak eluted at the exclusion volume of the column, most likely consisting of higher molecular mass aggregates of the scFv protein, which were not removed by the filtration step prior to gel chromatography. In contrast, if FkpA was present in the refolding buffer in stoichiometric amounts (2 μM dimer) from the onset of the reaction, a significant amount of soluble monomeric protein was obtained, whereas the formation of high molecular mass aggregates was not observed (Fig. 4*A*, *trace 4*). FkpA thus has a highly beneficial effect on the yield of soluble monomeric protein. Controls with BSA and PpiA at the same concentrations had no effect on the refolding yield and thus demonstrate that the observed increase in the presence of FkpA is not due to mere unspecific shielding and that it cannot be reproduced by just any other PPIase (Fig. 4*A*, *trace 5*).

To further test the hypothesis that the positive effect on the yield is independent of the prolyl isomerase function of FkpA, we added FkpA at various time points after the onset of the refolding reaction. The result clearly demonstrates that the beneficial effect depends critically on the presence of FkpA at the start of refolding (Fig. 4*B*). If FkpA was added later on during the refolding, most of the effect was lost, regardless if it

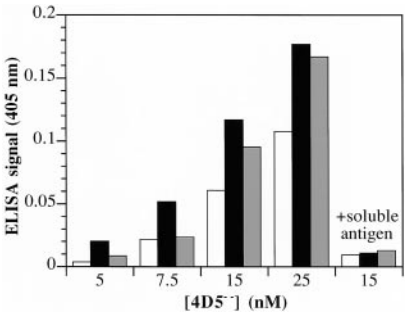

FIG. 5. **Serial dilutions of refolding reactions measured by ELISA as described in detail under "Materials and Methods."** Shown is 4D5^{--} scFv refolded alone (*white bars*), in the presence of equimolar amounts of FkpA present from the beginning (*black bars*), and after addition of equimolar amounts of FkpA after 1 min of refolding (*gray bars*). The final concentrations of 4D5^{--} scFv and FkpA (as dimer) were 0.2 μM. Refolding was at 10 °C and pH 7. The *last three bars* denote an inhibition experiment with soluble antigen, demonstrating that the ELISA signals indicate antigen binding and thus native antibody. *Numbers* below the bars indicate the total 4D5^{--} scFv concentration in the ELISA wells.

was added only 1 min or hours later. This finding is fully consistent with a role of FkpA early during refolding, most likely by interacting with early folding intermediates. It is important to note, however, that the beneficial effect is apparently not lost completely upon late addition of FkpA, as there is still an increase in yield compared with refolding of the scFv fragment alone. Therefore, there appear to be two mechanistic components, a time-dependent one and a persistent one. Although the interaction with early folding intermediates is only possible for a very short time, the other mechanism seems to be effective during the whole duration of incubation. This point will be addressed below.

To further confirm the results obtained by gel filtration experiments and to ensure that the gain in soluble protein is matched by a simultaneous gain in activity, we performed ELISA measurements after refolding in the presence or absence of FkpA. The results clearly demonstrate that FkpA added in stoichiometric amounts (0.2 μM dimer) has the same beneficial effect on the yield of active scFv as it has on the amount of soluble monomeric protein (Fig. 5). It follows that FkpA must indeed increase the yield of correctly folded, monomeric scFv protein. The data also confirm that the larger part of the effect is lost when FkpA is not present from the beginning of the refolding reaction and thus argue again for the independence of the yield increase from the isomerase activity of the protein.

Nevertheless, these experiments also show more clearly than the gel filtration experiments that FkpA still has some beneficial effect, even if added later on, be this 1 min or hours after starting the refolding reaction. Again, this point will be addressed below. Due to the 10-fold lower concentrations used during refolding for ELISA than for the gel filtration experiments, the overall effect is somewhat less dramatic, as the scFv protein is able to refold alone to a larger extent under these conditions. To ensure that the increase in ELISA signal is not due to an unspecific effect during refolding, BSA and PPIase were used as controls, but no effect on scFv yield was found (data not shown). Furthermore, to exclude an unspecific signal due to FkpA itself in ELISA, controls were carried out with FkpA alone as well as with FkpA added either to the native or the refolded scFv fragment after the incubation step, but before addition to the ELISA plate. FkpA alone gave no signal at all, whereas the late addition of FkpA to either native or refolded protein did not change the signal compared with its absence (see below). We can therefore rule out the possibility that FkpA itself might have caused an ELISA signal by binding unspecifically to either the wells or to the scFv fragment.

In summary, FkpA increases the yield of refolding of the 4D5^{--} scFv antibody without having any effect on its rate-limiting proline isomerization. However, as 4D5^{--} scFv carries another *cis*-proline (Pro-L8), there could conceivably be an effect on proline isomerization at this residue that is not rate-limiting, but might influence the yield. We therefore decided to study an scFv fragment that does not have any *cis*-prolines.

Effect of FkpA on the Yield of Soluble and Active anti-GCN4 scFv in Vitro—We chose to investigate the effect of FkpA on the folding of the anti-GCN4 (H-R66K) scFv fragment (18), which, in contrast to the 4D5^{--} scFv fragment, carries a murine λ-chain. The mouse λ locus consists of two closely related genes and one more distantly related gene (26, 27) that lack the *cis*-prolines at positions L8 and L95. They have been crystallized in a number of independent structures. In no case has a *cis*-proline been observed in a λ domain.[2] The refolding of this scFv fragment is therefore not proline-limited, which could additionally be confirmed by preliminary folding experiments.

The anti-GCN4 scFv fragment is much more stable than the disulfide-free 4D5^{--} scFv fragment despite its destabilizing mutation and refolds reversibly to a much greater extent. Refolding experiments with the anti-GCN4 (H-R66K) scFv fragment were therefore performed at 37 °C to approximate better the conditions in a growing *E. coli* cell and to ensure that refolding is not quantitative anyway.

Gel filtration and ELISA experiments clearly demonstrate that the presence of FkpA in the refolding buffer substantially increased the yield of soluble monomeric as well as active protein (Fig. 6, *A* and *B*), thus reproducing the results obtained with the 4D5^{--} scFv fragment. Both types of experiments demonstrate moreover that stoichiometric amounts of FkpA are needed and that the effect reaches saturation only at ~3 μM (FkpA as dimer), which corresponds to a 16-fold molar excess of FkpA over the scFv protein (Fig. 6*C*). These combined findings provide further strong evidence for a chaperone-like effect of FkpA on these scFv fragments that is disconnected from its prolyl isomerase activity.

As in the case of the 4D5^{--} scFv fragment, a large part of the effect was lost if FkpA was added only later during refolding, again pointing toward an interaction with early folding intermediates. The residual increase after late addition of FkpA is, however, proportionally higher than in the case of the 4D5^{--} scFv fragment. This point will be addressed below. Analogous to the experiments described above for the 4D5^{--} scFv fragment, appropriate controls were carried out to exclude the possibility that the observed effect is either unspecific (by verifying the absence of the effect with proteins other than FkpA) or might be caused by a signal coming from FkpA itself in ELISA.

Reactivation Function of FkpA—As already mentioned above, FkpA clearly seems to have an influence on the final yield of soluble and active protein in a refolding mixture, even if added hours later, when the refolding process of the scFv fragment is already finished. The rate-limiting proline phase of the 4D5^{--} scFv fragment has a rate constant of ~0.001 s^{-1} at 10 °C, meaning that the reaction is 75% complete after 1 h at 10 °C. These observations prompted us to investigate the effect of FkpA on native scFv protein upon prolonged co-incubation.

Interestingly, the presence of FkpA in the buffer has a very pronounced effect on the amount of active protein detected in ELISA experiments after incubation (Fig. 7, *A* and *B*). This is true both for the 4D5^{--} scFv and anti-GCN4 scFv fragments and reflects well the increase in activity gained upon delayed addition of FkpA to refolding mixtures, *i.e.* after the critical

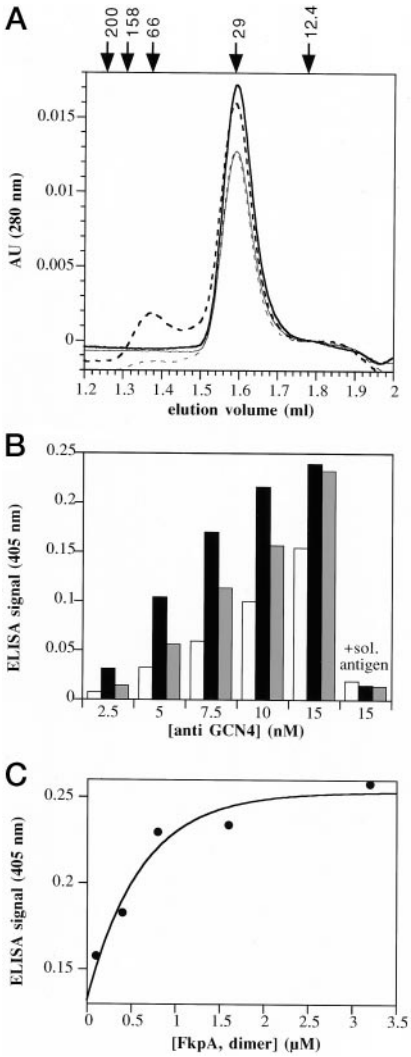

FIG. 6. *A*, elution profiles of analytical gel filtration followed by absorption at 280 nm. Shown are the native anti-GCN4 scFv fragment (2 μM; *thick solid line*) and the anti-GCN4 scFv fragment refolded alone (2 μM; *thin solid line*), in the presence of 0.5 μM FkpA (*thin dashed line*), and in the presence of 2 μM FkpA (*thick dashed line*). *B*, serial dilutions of refolding reactions measured by ELISA as described in detail under "Materials and Methods." Shown is the anti-GCN4 scFv fragment refolded alone (*white bars*), in the presence of equimolar amounts of FkpA present from the beginning (*black bars*), and after addition of equimolar amounts of FkpA after 1 min of refolding (*gray bars*). The final concentrations of anti-GCN4 scFv fragment and FkpA (as dimer) were 0.2 μM. The *last three bars* show the inhibition with soluble (*sol.*) antigen. *Numbers* below the bars indicate the total anti-GCN4 concentration in the ELISA wells. *C*, refolding of the anti-GCN4 scFv fragment in the presence of an increasing molar excess of FkpA as measured by ELISA. Plotted are the ELISA signals at one representative concentration of anti-GCN4 scFv fragment (5 nM) against the FkpA concentration at refolding. Refolding in *A–C* was carried out at 37 °C and pH 7. All concentrations for FkpA are given for the dimer.

initial collapse. This implies a second *in vitro* activity that is typical for chaperones, besides a possible interaction with early folding intermediates. It involves the reactivation of aggregated protein or the maintenance of activity and solubility of native protein, possibly by binding to partially unfolded species present in the equilibrium and thus keeping them in a folding-competent state.

We can exclude a conceivable artifact that would be caused by an ELISA signal that is due only to the *presence* of FkpA in the mixture and not to its *activity* since the control reactions, in which FkpA was added after the incubation time, right before the ELISA measurement, all show no effect of FkpA (Fig. 7, *A* and *B*). It follows that the scFv protein needs to be incubated with FkpA. After co-incubation with FkpA, the activity of the protein appears to exceed the activity before any incubation. This can easily be explained by the purification method. For both scFv fragments, the last step involves an affinity chromatography with acid elution, where the proteins have to unfold partially for a short period of time. Not surprisingly, they are never 100% active afterward, which can be confirmed by a considerable amount of scFv fragment that does not bind to the affinity column upon re-injecting the purified sample (data not shown).

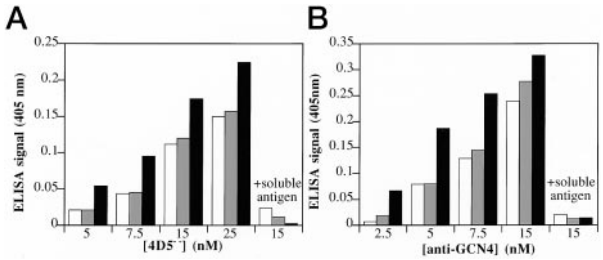

FIG. 7. **Serial dilutions of samples measured by ELISA as described in detail under "Materials and Methods."** *A*, native 4D5^{--} scFv fragment (*white bars*), native 4D5^{--} scFv fragment with equimolar amounts of FkpA added without co-incubation (*gray bars*), and native 4D5^{--} scFv fragment incubated with equimolar amounts of FkpA (*black bars*). Incubation was at 10 °C and pH 7. *B*, same as in *A*, but with the anti-GCN4 scFv fragment and incubation at 37 °C. The *last three* bars show the inhibition with soluble antigen. *Numbers* below the bars indicate the total scFv concentration in the ELISA wells.

To gather further information on the mode of interaction, we repeated the experiments at different temperatures. As shown in Fig. 8*A*, the "reactivation effect" of FkpA on native 4D5^{--} was slightly more pronounced at 20 °C than at 10 °C, whereas slightly more active protein was lost upon incubation at the higher temperature, reflecting perhaps the rather marginal stability of this disulfide-free protein at 20 °C, which might lead to a higher proportion of partially unfolded molecules present in the mixture that can subsequently be recovered by FkpA.

In the case of the anti-GCN4 scFv fragment, on the other hand, no dependence of the amount of active protein on the temperature could be detected in the range from 10 to 37 °C either in the absence or presence of FkpA (data not shown). Due to its significantly higher stability, no activity may be lost, even upon incubation at 37 °C. The increase in activity obtained in the presence of FkpA would then appear to be solely due to reactivation of inactive protein present in the mixture from the start, which might be less stably folded and thus accessible for chaperone interaction.

When the refolding at different temperatures is regarded, we observed in the case of the anti-GCN4 scFv fragment (Fig. 8*B*) a clear requirement for FkpA to be present at the onset of the reaction at 37 °C, whereas there was almost no difference regarding the time point of the addition at 10 °C. This can be explained by considering the excellent refolding properties of this scFv fragment at low temperatures. Under these conditions, off-pathway aggregation during *in vitro* refolding is so little of a problem that the absence or presence of FkpA at the beginning of the reaction does not make any noticeable difference. All the benefit at 10 °C appears to come from rescuing inactive protein present after refolding. The 4D5^{--} scFv fragment, in contrast, benefits from the presence of FkpA from the onset of refolding at 10 °C as much as at 20 °C (data not shown) since the refolding of this protein is only partially reversible even at 10 °C, thus leaving room for improvement.

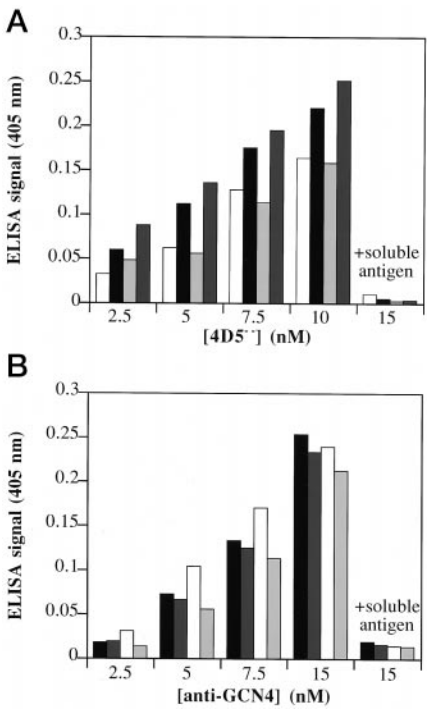

FIG. 8. **Serial dilutions of samples measured by ELISA as described in detail under "Materials and Methods."** *A*, native 4D5^{-} scFv fragment incubated alone (*white* and *light-gray bars*) and in the presence of equimolar amounts of FkpA (*black* and *dark-gray bars*). Incubation was at 10 °C and pH 7 (*white* and *black bars*) and at 20 °C and pH 7 (*light-* and *dark-gray bars*). *B*, anti-GCN4 scFv fragment refolded in the presence of equimolar amounts of FkpA (*black* and *white bars*) and after addition of equimolar amounts of FkpA after 1 min of refolding (*dark-* and *light-gray bars*). For the *black* and *dark-gray bars*, incubation was done at 10 °C; and for the *white* and *light-gray bars*, at 37 °C. The *last four bars* denote the inhibition with soluble antigen. *Numbers* below the bars indicate the total scFv concentration in the ELISA wells.

DISCUSSION

Our *in vitro* measurements of FkpA activity have shown that it is an extremely efficient PPIase, with a k_{cat}/K_m of 4×10^6 M^{-1} s^{-1} and thus comparable to other FKBPs (21, 28). The activities of the other FKBPs and PpiA mentioned here have been determined using peptide substrates; and depending on the protein, higher (trigger factor (24)) or lower (FKBP12 (21)) values were found when measurements were carried out with a folding protein as a substrate. Especially for the trigger factor, the data show that it catalyzes isomerization in a folding protein much more efficiently than in an oligopeptide of comparable local sequence (24). This was found to result predominantly from the tight binding of the trigger factor to the folding protein substrate (28). Given the present data, it is tempting to speculate that the very efficient catalysis of RNase T1 refolding by FkpA may also partly be due to such a binding effect. FkpA is, however, not able to accelerate the rate-limiting proline *cis,trans*-isomerization of scFv fragment hu4D5-8. This must be attributed to the inaccessibility of Pro-L95 in a very early trapped folding intermediate of the scFv fragment (14)[3] since this isomerization can be catalyzed when the same V_L domain is refolded alone.[3]

Nevertheless, *in vivo* overexpression of FkpA, which had initially been selected as a factor increasing the amount of active scFv fragment expressed in the periplasm (7), has a dramatic effect on the expression yield of a number of scFv fragments, among them the 4D5^{-} $^{-}$ scFv fragment, whose rate-limiting proline isomerization is not accelerated by FkpA, and the anti-GCN4 scFv fragment, which carries no *cis*-prolines at all. This prompted us to take a closer look at the molecular mechanisms behind this beneficial effect of FkpA on scFv refolding *in vitro*, with the aim to understand the importance of the prolyl isomerase activity of FkpA in this context and to distinguish it from other possible folding-assisting functions.

In the past years, there has been a controversial discussion as to whether protein-folding catalysts such as prolyl *cis,trans*-isomerases could also exhibit chaperone-like activity independent of or in addition to their catalytic activity (29–31). The definition of a chaperone itself will be, of course, decisive for the inclusion of factors. The most widely accepted definition is a protein that transiently binds and thus stabilizes an otherwise unstable conformer of another protein, thus facilitating folding by preventing misfolding and aggregation (see, for example, Ref. 32). More recently, there have been further examples demonstrating a chaperoning function for classical PPIases, namely Cyp-40 (33), and FKBP52 (34), both proteins from the eukaryotic cytosol. The *E. coli* trigger factor, an isomerase of the FKBP family, has long been suggested to possess additional chaperone activity (reviewed in Ref. 35), which is dependent on the presence of its additional flanking domains (36). FkpA is a member of the FKBP family since it shows 83% sequence identity to the consensus sequence of the FK506-binding domain, derived from an alignment of mammalian, yeast, and bacterial members of this family (1).

Our results clearly show that FkpA assists protein folding of the investigated scFv fragments *in vitro*. As there is no correlation with the acceleration of proline *cis,trans*-isomerization or even the presence of *cis*-prolines, we are forced to conclude that the chaperoning effect of FkpA must be independent of its peptidylprolyl *cis,trans*-isomerase activity. This view is supported by the absence of any *in vivo* or *in vitro* effect of PpiA (RotA).

The observed effect is 2-fold. On the one hand, FkpA appears to be able to interact with early folding intermediates, thus preventing aggregation, provided the scFv protein is prone to such off-pathway reactions under the conditions used. This is clearly demonstrated by the marked dependence of the magnitude of the beneficial effect of FkpA on the time point of its addition during refolding. Recent studies with the 4D5^{-} $^{-}$ scFv fragment (14)[3] have shown that premature interaction between the domains occurs very fast in the dead time of the stopped-flow instrument, and it is possible that such a non-native intermediate binds to FkpA transiently. Stable binding was not detectable.

On the other hand, FkpA increases the activity even when added very late to the folding reaction and, moreover, also when incubated with "native" protein. We interpret this effect as FkpA acting on non-native, possibly aggregated, but soluble scFv fragments, which were also present in our preparation of native scFv protein. They may very likely have formed during acid elution from the affinity columns, and they also occur in equilibrium with the native form at least in the case of the 4D5^{-} $^{-}$ scFv fragment, due to its marginal stability, at temperatures of 20 °C or higher. FkpA can thus be proposed to act also by binding to partially unfolded or aggregated species present at equilibrium, thus preventing their further irreversible aggregation and facilitating their reactivation.

It is tempting to speculate that both activities (early prevention of aggregation and "rescue" of inactive molecules) have the same molecular cause: a transient interaction of non-native intermediates with FkpA. In the one case, they may immediately be released in a form that is productive for further folding, without ever forming an aggregate, thereby increasing the yield of the reaction, provided FkpA is present from the beginning. In the other case, FkpA binds either to the aggregate or to

non-native forms in equilibrium with the aggregated state, which can thus productively fold from the FkpA-bound state. What the exact recognition motif is and whether it is the same in both cases must await further studies.

Based on these findings, the reasons for the remarkably large effect of coexpression of FkpA *in vivo* (7) can now be understood. When quantitating expression of antibody fragments *in vivo*, one cannot distinguish between assistance of refolding and dynamic stabilization of the native protein by a rescue mechanism such that the observed effect will be the sum of the two. Moreover, conditions in the cell are rather on the unfavorable side for folding yields compared with the conditions used in the *in vitro* study due to elevated temperatures and very high concentrations of protein in the cell. A combination of all these factors may well explain the fact that the beneficial effect of FkpA coexpression *in vivo* is 2–3-fold higher than what we observed in ELISA measurements *in vitro*.

Whether the dramatic effect of FkpA observed here *in vitro* and *in vivo* is physiologically relevant for *E. coli* proteins is, however, a difficult question since the maximal effect requires very large concentrations of the protein. In this context, it is interesting to note that some overexpression of FkpA can be achieved under stress conditions, as the gene is at least partially controlled by σ^E (3, 4), possessing an σ^E-activable promoter. It has also been shown that extracytoplasmic defects that increase σ^E activity stimulate *fkpA* transcription to some extent (5). Together with the observation that a null mutation in *fkpA* stimulates transcription of the *degP* locus (which encodes a heat shock-inducible periplasmic protease (Refs. 1 and 2; reviewed in Ref. 6)) and *vice versa*, FkpA has already been suggested to perform a general periplasmic folding function (1, 5). It is therefore conceivable that the biological purpose of FkpA induction under stress conditions may be related to the chaperoning properties reported here, in conjunction with or even independent of its unquestionable PPIase activity. In the periplasm, especially under stress conditions, a "protein buffer" would be needed to which unfolded proteins can transiently bind and from which they can be refolded. We have clearly shown this function for FkpA *in vitro*, and it is attractive to propose that it can fulfill this role *in vivo* as well.

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