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A homodimer represents an active species of the peptidyl-prolyl *cis/trans* isomerase FKBP25mem from *Legionella pneumophila*

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Abstract The molecular mass of the native FK506-binding peptidyl-prolyl *cis/trans* isomerase (PPIase) FKBP25mem from *Legionella pneumophila* (Mip (macrophage infectivity potentiator) protein) was determined by two methods. By gel-permeation chromatography we found no indication of the presence of the monomeric enzyme. However, an oligomeric state with a molecular mass of about 62 kDa was detected. By cross-linking with dimethyl pimelimidate and subsequent SDS-PAGE of either the surface proteins of intact *L. pneumophila* cells or the purified recombinant FKBP25mem in solution, we observed an immunoreactive band indicative of a mass in the dimer range. In contrast to human recombinant FKBP12, the enzymatic activity of *Legionella* FKBP25mem was strongly dependent on the protein concentration, pointing to a dimer as the most active species. However, the inhibition by FK506 yielded a nearly constant value of K_i of about 250 nM when measured in the same range of FKBP25mem concentration. These results may be explained by the fact that monomeric FKBP25mem has little, if any, influence on enzymatic activity when compared with the homodimer.

Key words: Peptidyl-prolyl *cis/trans* isomerase; Mip protein; Oligomerization; *Legionella pneumophila*; Virulence factor; FK506-binding protein; Enzyme kinetics

1. Introduction

Currently, the enzyme class of peptidyl prolyl *cis/trans* isomerases (PPIases, EC 5.2.1.8) can be subdivided into three families lacking convincing amino acid sequence similarity to each other. They are named cyclophilins (Cyp), FK506 binding proteins (FKBPs) (for reviews see [1,2]) and parvulins [3,27]. Cyclophilins and FKBPs are specifically inhibited by cyclosporin A and FK506, respectively. Some FKBPs are proteins of the outer membrane surface of several pathogenic bacteria and are thought to be involved in the survival of these organisms in their eukaryotic host cells (macrophages and monocytes) [4]. The FKBP25mem (Mip (macrophage infectivity potentiator) protein) of *Legionella pneumophila*, the causative agent of the Legionnaire's disease in man, has been considered as a virulence factor because FKPB25mem-negative mutants of *L. pneumophila* showed reduced ability to survive in macrophages [5]. Similar to the Mip-like protein from *Chlamydia trachomatis* [6], FKBP25mem has PPIase activity. It resembles the human FKBP12cy concerning subsite specificity and catalytic efficiency [7,8]. The amino acid sequence of FKBP25mem shows a C-terminal part homologous to the FKBP12 sequence (comprising 107 amino acids) and an N-terminal extension that is 106 residues in length in the mature protein. For the chlamydial homologue of FKBP25mem, the presence of the N-terminal sequence on the outer membrane surface of the microorganism was shown [28].

FKBP25mem was found to be inhibited at FK506 concentrations several hundredfold higher than FKBP12cy [8]. Until recently it was assumed that PPIases exert their catalytic function as monomeric enzymes. Because homodimerization and oligomerization are known to function in controlling signal transduction and receptor activation, experimental data about the exact functional state of certain PPIases would be required.

The involvement of several cyclophilins and FKBPs in stable oligomeric complexes have been reported [9–15]. Association of the p59 protein, the rabbit homologue to human FKBP52, with the heat-shock proteins hsp90 and hsp 70 is suggested to have biological significance because all the components are included in the unactivated, non-DNA binding progesteron receptor complex [10]. Although recombinant FKBP52 possesses PPIase activity [11], information about the enzyme activity after formation of the heterocomplex is lacking. Limited digestion by endoproteinase Lys C of the chicken thymus analogue to FKBP52, the hsp 56, resulted in a 17 kDa fragment comprising the FKBP12-homologous part of the polypeptide chain. This fragment, bearing a 23 aa N-terminal and a 15 aa C-terminal extension to the human FKBP12cy-homologous part was shown to exist as a homodimer when complexed to FK506 [12]. The same type of promotion by FK506 of homodimer formation has been described for the FKBP52 of Jurkat cells [13].

High-affinity binding of FKBP12 was demonstrated for the 565 kDa ryanodine receptor, the subunit of the intracellular Ca^{2+} channel of the sarcoplasmic and endoplasmic reticulum. The positive effect of FKBP12 on the conductance of these channels was clearly demonstrated, indicating that the PPIase may be a normal constituent of the Ca^{2+} channel [16] formed by four ryanodine chains. The effect of this association on the enzymatic constants of FKBP12 has not been measured.

In this study, by analysis of enzyme kinetics and separation techniques, it is shown that, in solutions of the recombinant FKBP25mem, homooligomerization dramatically enhances the enzymatic activity. Furthermore, cross-linking experiments using intact *L. pneumophila* cells point to a dimeric state of the

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authentic enzyme when bound on the outer surface of the bacterial cell wall.

2. Experimental

2.1. Materials

The recombinant FKBP25mem used for the experiments was purified as previously described [7]. This protein showed identical properties compared with the mature, authentic FKBP25mem isolated from *L. pneumophila* cells (N-terminal sequence, substrate specificity, K_i value for inhibition with FK506; pI = 9.85) [7]. Amino acid sequence numbering refers to the full-length protein including the signal sequence. Secondary structure predictions were made using the built-in modules of the HIBIO PROSIS version 7.0 software (Hitachi Software, Brisbane, USA).

2.2. Cross-linking of the surface proteins of intact cells of Legionella pneumophila Philadelphia I

Freshly cultured *L. pneumophila* cells (5×10^{10} cells/ml after harvesting) were washed twice with 50 mM sodium phosphate buffer, pH 9.1, and incubated in a 1 ml sample with the cross-linker dimethyl pimelimidate dihydrochloride (SERVA, Heidelberg) for 1 h at 25°C. The concentration of the cross-linker was varied from 0.4 to 20 mM (final concentration). Best results were achieved using a final concentration of 2 mM dimethyl pimelimidate. After centrifugation of the samples (10 min at 4000 rpm), the sedimented cells were resolved in 50 mM sodium phosphate buffer, pH 9.1 (containing 1% Triton X-100), incubated for 30 min at 25°C and centrifuged again (10 min at 4000 rpm). The supernatant was used for SDS-PAGE and Western immunoblotting. For cross-linking, the purified recombinant FKBP25mem (8.3 μM in 50 mM sodium phosphate buffer, pH 9.1) was incubated with dimethyl pimelimidate dihydrochloride (final concentration 38 mM) for 1 h at 25°C. After adding SDS-PAGE sample buffer in a 2:1 ratio for stopping the reaction, the samples were heated and used for SDS-PAGE and immunoblotting.

2.3. SDS-PAGE and Western immunoblot analysis

Proteins were analysed on 10% SDS-polyacrylamide gels as described [17]. Western immunoblot analysis was performed by semi-dry electroblotting in a graphite chamber [18] using anti-FKBP25mem specific antiserum.

2.4. Determination of PPIase activity and inhibition measurements

PPIase activity was measured according to Fischer et al. [8] using a Hewlett Packard 8452 diode array UV/VIS spectrophotometer. The substrates of the structure succinyl-Ala-Xaa-Pro-Phe-4-nitroanilide (Bachem, Heidelberg) were dissolved in DMSO (40 mg/ml) and diluted 1:10 in 35 mM HEPES buffer, pH 7.8. The dependence of PPIase activity of the enzymes on their concentration was measured using substrates with different amino acid residues at the P_1 position (Xaa). PPIase activity of the FKBP25mem at enzyme concentrations ranging from 1 to 30 nM was determined with the substrate with Xaa = Leu, followed by Xaa = Trp in the range 30–60 nM and Xaa = Ala in the range 60–750 nM. Concentration-dependent PPIase activity of the FKBP12cy was measured using the two substrates with Xaa = Leu at FKBP12cy concentrations ranging from 15 to 22 nM and with Xaa = Ala in the range of FKBP12cy concentrations of 22–220 nM. To determine the Hill coefficient, the dependence of PPIase activity of the recombinant FKBP25mem on the substrate concentration (Suc-Ala-Phe-Pro-Phe-4-nitroanilide) was measured using the method of Kofron et al. [21]. The enzyme concentration in the assay was 35 nM. The partial inhibition of FKBP25mem by trifluoroethanol (Merck, Darmstadt) applied from the stock solution of the substrate into the assay was taken into account.

For inhibition studies, the stock solution of FK506 was prepared in 50% ethanol. At high PPIase concentrations (764 nM), enzyme and inhibitor were preincubated for 1 h before measuring the remaining PPIase activity. At low enzyme concentrations (11 and 31 nM), PPIase activity was monitored using the substrate Suc-Ala-Leu-Pro-Phe-4-nitroanilide; at high enzyme concentrations (764 nM) Suc-Ala-Ala-Pro-Phe-4-nitroanilide was used.

For $[S] \ll K_M$ the *cis*-to-*trans* isomerization catalysed by PPIase of concentration $[E]_0$ follows the kinetics given in eq. 1:

$$k_{obs} = k_0 + \frac{k_{cat}}{K_M}[E]_0;\ k_{obs} = k_0 + k_{enz} \quad (1)$$

where k_{obs} is the observed, first-order rate constant for the enzyme-catalyzed isomerization, and k_0 is the first-order rate constant for uncatalyzed isomerization. Eq. 1 allows the calculation of the specificity constant $(k_{cat}/K_M)_{app}$ with known PPIase concentrations. The apparent $(k_{cat}/K_M)_{app}$ values were calculated using the total enzyme protein concentration in the activity assay.

The protein concentrations were determined spectrophotometrically. Extinction coefficients at 280 nm were derived from the amino acid sequence according to Gill and von Hippel [19].

2.5. Calculation of the dissociation constant K_D

The dissociation constant K_D for the FKBP25mem dimer is

$$K_D = \frac{[M][M]}{[MM]}, \quad (2)$$

where M represents the monomeric and MM the dimeric FKBP25mem.

Eq. 3 describes the PPIase catalysis by monomeric and dimeric FKBP25mem under first-order conditions in the Michaelis–Menten equation characterised by $[S] \ll K_M$:

$$v = \left(\frac{k_{cat,M}}{K_{M,M}}[M] + \frac{k_{cat,MM}}{K_{M,M}}[MM] \right)[S] \quad (3)$$

with

$$E_0 = [M] + 2[MM] \quad (4)$$

Using eqs. 2–4, the rate equation for PPIase catalysis by monomeric and dimeric FKBP25mem was derived as described [20] and used for fitting the experimental data.

2.6. Determination of the molecular mass

A sample of 80 μg protein in 35 mM HEPES buffer, pH 7.8, was transferred into a volatile solvent by means of narrowbore reverse-phase HPLC using a 50 × 3 mm C_4 column equilibrated with 0.1% aqueous trifluoroacetic acid. Using a gradient of 0 to 60% acetonitrile in 0.1% trifluoroacetic acid at a flow rate of 1 ml/min, the protein was eluted within 12 min.

Molecular mass was determined by electrospray mass spectrometry on a VG Bio-Q (Fisons instruments) using 5 μl of the HPLC-purified protein solution according to Rahfeld et al. [3].

For determining the molecular mass of the protein using gel-filtration, the pure proteins were applied to a Superdex 75 (HR 10/30) gel-filtration column (Pharmacia, Uppsala), equilibrated with 10 mM HEPES buffer, pH 7.8, containing 1.5 mM $MgCl_2$, 150 mM KCl and 0.5 mM dithiothreitol. The column was calibrated with molecular mass markers (Boehringer, Mannheim) in the range 12.5–68.0 kDa.

3. Results and discussion

Previously, we reported that native polyacrylamide gel-electrophoresis of recombinant FKBP25mem at pH 4.1 fails to detect a protein band in the molecular mass range of monomeric enzyme [7]. The authentic enzymes purified from two strains (*L. pneumophila* Philadelphia I and U21S6) showed the same behaviour. These results indicated an oligomeric protein under these acidic conditions because the protein band migrated closely to the 45 kDa molecular mass marker. Since the enzymatic activity of PPIases is low under acidic conditions the question arose whether under more optimal conditions for catalysis oligomerization may play a role as well.

Electrospray mass spectrometry of the recombinant FKBP25mem used for the following experiments was performed to ensure that the molecular mass derived from the amino acid sequence agrees with the isolated protein. The spectra indicated a molecular mass of 22,840 ± 2.29 or about 1 Da smaller than the molecular mass calculated for the mature

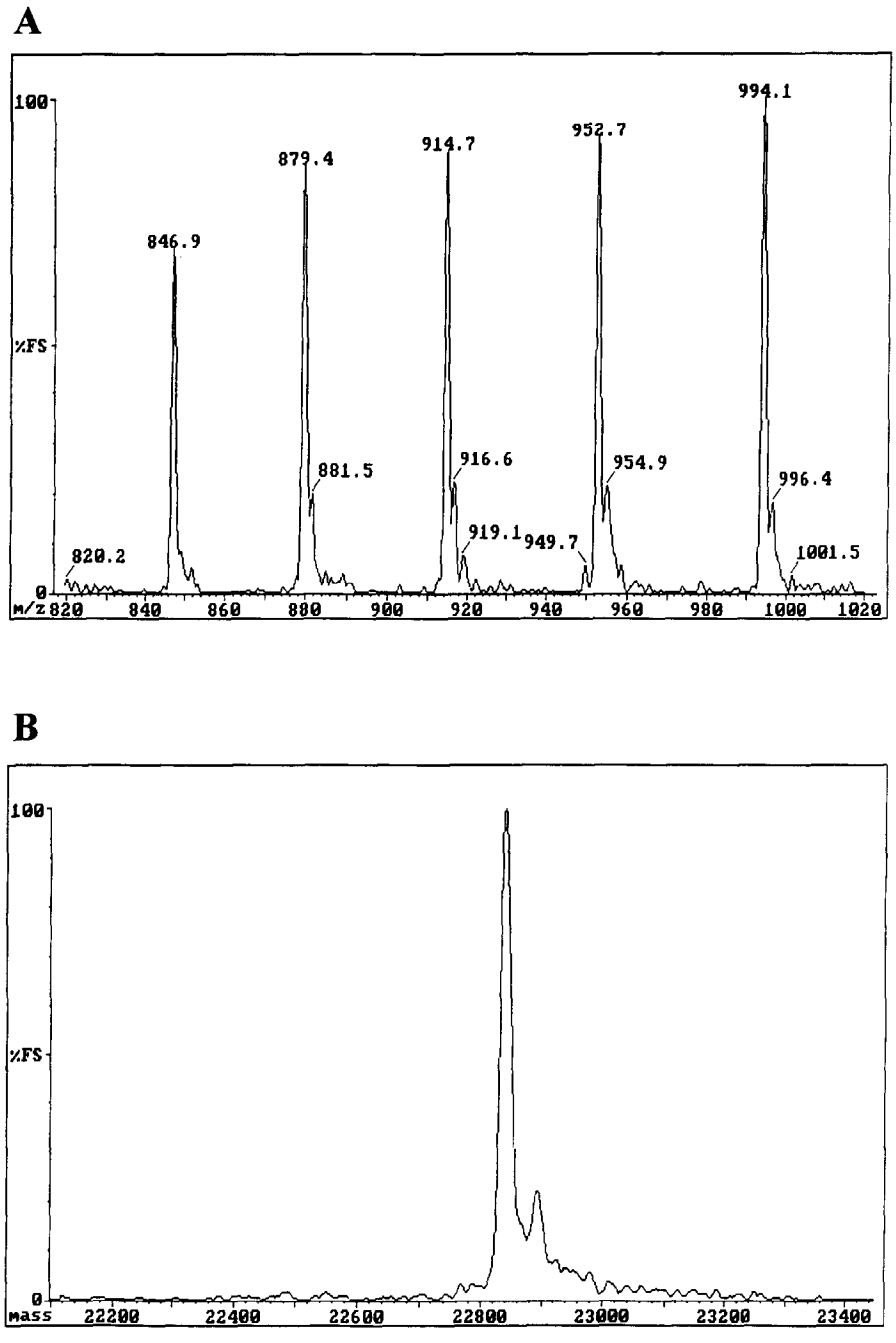

Fig. 1. Electrospray ionisation mass spectrum of the recombinant *L. pneumophila* FKBP25mem. The spectrum (B) was derived from deconvolution of 5 peaks from the mass/charge spectrum (A).

FKBP25mem (22,841.84 Da; Fig. 1). Thus, the enzyme represented the unmodified protein chain and exhibited the monomeric state under the conditions of electrospray mass spectrometry. The minor peak with a mass difference of 53 Da to the mature FKBP25mem was assigned as a metal ion-derived artifact of the HPLC procedure.

Furthermore, in a typical gel-filtration experiment with Superdex 75 beads at pH 7.8, a stock solution of 100.0 μM recombinant FKBP25mem from *L. pneumophila* was applied to the column. The protein eluted in 3 fractions. The highest enzyme concentration obtained in the eluate was 7.0 μM. Obviously, at these concentrations the experiment did not reveal a significant signal at the position of the monomeric enzyme, but the protein eluted much earlier at a position of about 62 kDa (Fig. 2).

In a third approach, we cross-linked the surface proteins of intact *L. pneumophila* cells and detected cross-linked FKBP25mem by Western immunoblotting. Along with the monomeric band of FKBP25mem at 24 kDa, and two cross-reactivities at 47 and 70 kDa already present before cross-linking, the cross-linked sample exhibited only dimers of the FKBP25mem at about 50 kDa, while cross-linked trimers or tetramers could not be detected (Fig. 3). Even a 10-fold increase in the concentration of the cross-linking reagent did not result

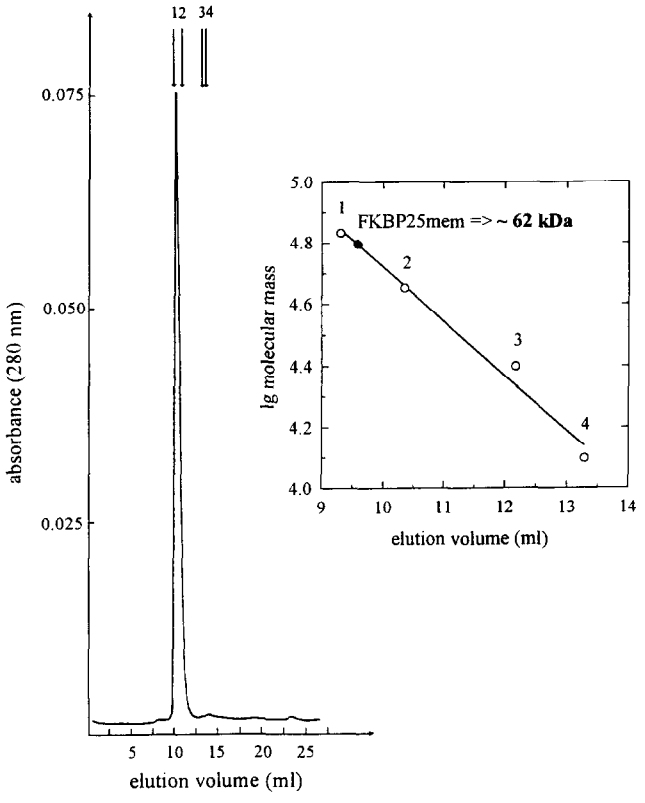

Fig. 2. Gel-filtration of pure FKBP25mem (100 μM) on a Superdex 75 (HR 10/30) column equilibrated with 10 mM HEPES buffer, pH 7.8, containing 1.5 mM $MgCl_2$, 150 mM KCl and 0.5 mM dithioerythritol. The arrows indicate the retention times of the molecular mass markers used for calibration (1, bovine albumin (68,000 Da); 2, ovalbumin (45,000 Da); 3, chymotrypsinogen A (25,000 Da); 4, cytochrome *c* (12,500 Da)). The inserted graph shows the calibration curve.

in the disappearance of all monomeric FKBP25mem. Similarly, cross-reactive bands with a higher molecular mass could not be observed. A reference experiment cross-linking the purified recombinant FKBP25mem in solution also showed dimerization in that the same band at 50 kDa, even in rather dilute solution, was detected (data not shown).

Next, it was asked if the oligomerization, which occurred under the optimal conditions for PPIase catalysis, would have any influence on enzyme kinetics. For this purpose the *cis*-to-*trans* interconversion of substrates of the type Suc-Ala-Xaa-Pro-Phe-4-nitroanilide was measured at various FKBP25mem concentrations. $(k_{cat}/K_M)_{app}$ values were chosen as a measurement of the catalytic activity. To check the method, the human recombinant FKBP12cy that never gave any indication for oligomerization in gel-filtration experiments was included. The range of protein concentration available to our kinetic method was 2 orders of magnitude for FKBP25mem. Highest measurable concentrations were 0.2 and 0.8 μM for FKBP12cy and FKBP25mem, respectively. As it is clearly depicted in Fig. 4, $(k_{cat}/K_M)_{app}$ for FKBP25mem is highly dependent on the enzyme concentration and shows a considerable drop at low enzyme concentrations. As expected for a stoichiometric oligomerization reaction there is a plateau region at the high concentration range.

For FKBP12 any dependence of activity on enzyme concentration could not be observed (Fig. 4).

From the results of the native polyacrylamide gel-electrophoresis and the cross-linking experiments the existence of a homodimer of FKBP25mem was taken into considerations. Constructing the Hill plot using Suc-Ala-Phe-Pro-Phe-4-nitroanilide as substrate and FKBP25mem concentrations located in the beginning of this plateau region yielded a linear correlation (correlation coefficient $r^2 = 0.99$) with a Hill coefficient $n_H = 0.99$ indicating the lack of cooperativity for the two-site enzyme. Therefore, the corresponding eq. 3 for the description of the dependence in Fig. 3 contains two terms which account for the activities of the monomeric and the dimeric enzyme. The dimerization model presents two independent active sites in the enzyme molecule. Non-linear fitting of the experimental data with eqs. 3 and 4 could successfully describe the data and allow an estimation of the kinetic parameters. The curve fitting procedure gave for the monomeric FKBP25mem $(k_{cat}/K_m)_M = 5.3 \pm 6.5 \times 10^4\ M^{-1} \cdot s^{-1}$, and for the dimer $(k_{cat}/K_m)MM = 2.2 \pm 0.06 \times 10^6\ M^{-1} \cdot s^{-1}$.

Furthermore, the dissociation constant of the dimer was found to be in the nanomolar range (9.9 ± 4.6 nM), indicating a rather high binding energy for the polypeptide chains. This tight association may be responsible for the lack of monomeric species in the gel-filtration experiments. Since FKBP25mem does not contain any cysteine residue and the dimer formation was a reversible reaction, non covalent forces must be accounted for the association process. The calculated (k_{cat}/K_m) values for the two enzyme forms led to the assumption that the dimer is at least 40-fold higher in activity than the monomer. Currently, the possibiltiy cannot be excluded that the monomer is entirely inactive as an enzyme. As documented in Table 1,

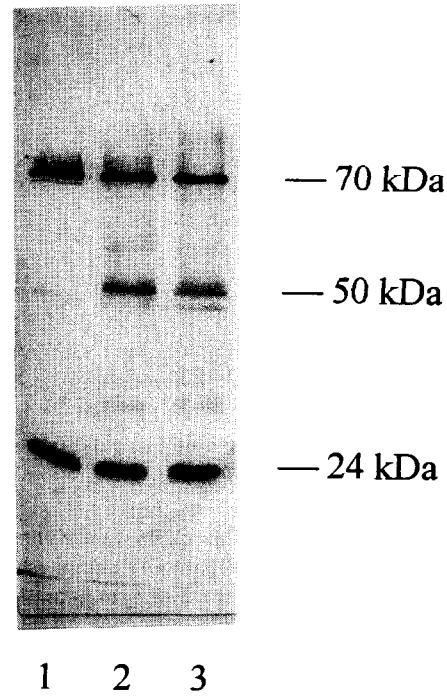

Fig. 3. Western immunoblot of the cross-linked surface proteins of intact *L. pneumophila* Philadelphia I cells using anti-FKBP25mem specific antiserum. Lane 1 shows the non-cross-linked sample with the FKBP25mem at 24 kDa and two cross-reactivities of unknown origin at 48 and 70 kDa; lanes 2 and 3 represent cross-linked samples showing the additional immunoreactive band at 50 kDa representing the cross-linked dimeric FKBP25mem.

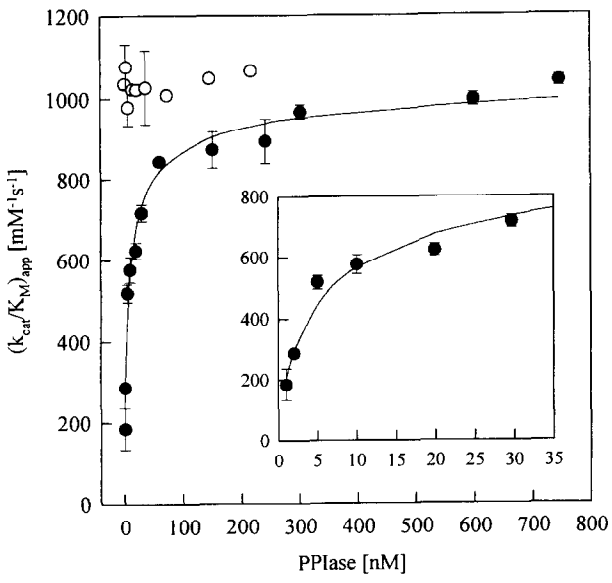

Fig. 4. Dependence of the apparent $(k_{cat}/K_M)_{app}$ values of the human FKBP12cy (○) and the *L. pneumophila* FKBP25mem (●) on their respective concentrations. The drawn line represents the fit of the experimental data after the derived rate equation (see eqs. 2–4) giving for the monomeric FKBP25mem $(k_{cat}/K_m)_M = 5.3 \pm 6.5 \times 10^4$ M$^{-1}\cdot$s^{-1} and for the dimer $(k_{cat}/K_m)MM = 2.2 \pm 0.06 \times 10^6$ M$^{-1}\cdot$s^{-1} and a K_D value of 9.9 ± 4.6 nM. The insert magnifies the low concentration region of FKBP25mem.

in contrast to the enzymatic activity, the inhibition of FKBP25mem with FK506, a competitive inhibitor for all FKBPs, was independent of enzyme concentration within the limits of error. Obviously, this would be the case if the monomeric FKBP25mem is inactive as an enzyme concomitant with the loss of FK506 binding ability. However, if the monomer is assumed to bind to FK506 regardless of its PPIase activity, interpretation of the data in Table 1 becomes more difficult. However, the K_i values are precise enough to detect an about 1.3-fold higher K_i value for the lowest enzyme concentration used compared with the highest one. Because this enhancement of K_i could not be found, a potential PPIase activity of the monomer must be connected with weak FK506 binding properties.

The different behaviour of FKBP12 and FKBP25mem concerning the tendency to form oligomers during this event may be located either in the non-conserved part of the FKBP12 homologous stretch of FKBP25mem or in the N-terminal extension. The decrease in enzymatic activity after formation of the monomer may be located in the same sequence regions.

Secondary structure prediction by the Chou, Fasman and Rose algorithm [22,23] suggested a long α-helix, including the residues 34–70, that builds up a typical amphiphilic helical structure in the graphical display, based on the Schiffer–Edmundson helical wheel representation [24]. Long terminal helices are known to mediate dimerization by the formation of two antiparallel helices packs. For example, this type of arrangement could be obtained in the crystals of the dimeric λ repressor fragment [25]. On the other hand, the C-terminal part has 55% homology to FKBP12 and is thought to be responsible for enzymatic activity. All the amino acids located at a 5.5 Å distance from the atoms of FK506 bound in the FKBP12–FK506 complex are fully conserved in the amino acid sequence of FKBP25mem [1]. This observation usually would point to a fully active monomer because the active site might be unperturbed in FKBP25mem. However, initial experiments with the C-terminal fragment of the FKBP25mem (starting from residue 107 of the mature protein) cloned as a fusion protein with glutathione-*S*-transferase revealed that for the fusion protein, PPIase activity was lacking. For example, the fusion protein of glutathione-*S*-transferase with the a full-length FKBP52 homologue, as well as the FKBP12-homologous domain of this protein, revealed high k_{cat}/K_M values for the PPIase activity [26]. Moreover, the *Neisseria meningitidis* FKBP fused to maltose-binding protein could probably fold as an independent domain, because it was able to express both enzyme activity and FK506 inhibition. Thus, our data suggest that the FKBP12-homologous domain of FKBP25 does not express significant PPIase activity by itself. Restructuring of the active center after dimerization must occur to explain the enhancement in catalytic power.

We conclude that even at low protein concentrations *L. pneumophila* FKBP25mem largely occurs as a dimer in solution. The same form seems to exist on the surface of the bacterial cell. From these kinetic investigations the monomer was found to express rather low, if any, PPIase activity when compared to the dimeric state. It remains to be cetermined whether the activity-related oligomerization of the virulence factor, FKBP25mem, has any impact on the impairment of the defense system of human host cells.

Table 1
Apparent inhibition constant $K_{i,app}$ for the inhibition of the PPIase activity of the FKBP25mem by FK506 at different enzyme concentrations

Concentration of FKBP25mem (nM)	$K_{i,app}$ (nM)
11	244 ± 17
31	223 ± 9
764	270 ± 77

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