Time-dependent inhibition of peptidylprolyl cis–trans-isomerases by FK506
is probably due to cis–trans isomerization of the inhibitor’s imide bond

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Free in solution, the immunosuppressive compounds cyclosporin A (CsA), FK506, ascomycin and rapamycin are present in many solvents in various slowly interconverting conformations. Together with their cellular receptor proteins, cyclophilin (CyP) and FK506-binding protein (FKBP), however, these inhibitors have been shown to have a homogeneous conformation. The existence of a slow cis–trans interconversion of an imidic bond in the inhibitor molecule during the course of the formation of the CsA–CyP18cy complex (where CyP18cy is human 18 kDa cytosolic CyP) prompted us to investigate the reaction of the peptidomacrolides FK506, ascomycin and rapamycin with two specific binding-proteins in more detail. Since formation of the FK506–FKBP complex results in the inhibition of the peptidylprolyl cis–trans-isomerase activity of the binding protein, we used the enzyme’s decrease in enzymic activity to monitor binding of the inhibitors to their enzyme targets. For FK506, the kinetics of inhibition of human 12 kDa cytosolic FKBP (FKBP12cy) were clearly dependent on time. Subsequent to a rapid inactivation reaction, not resolved in its kinetics due to manual mixing, a slow dominant first-order inactivation process with a relaxation time of 1163 s at 10 °C was observed. Concomitantly the $K_i$ value of the slow phase dropped 2.6-fold within the first 60 min of incubation. Using the FKBP12cy homologue 25 kDa membrane FKBP (FKBP25mem), a bacterial peptidylprolyl cis–trans-isomerase, the rate and amplitudes of the inhibition reactions were very similar to FKBP12cy. On the other hand, the kinetics and amplitudes of the inhibition of FKBP12cy varied significantly if rapamycin was used as an inhibitor instead of FK506. Owing to reduced conformation transition in rapamycin upon binding to FKBP12cy, the slow phase during inhibition was significantly decreased in amplitude. A likely reason for this became apparent when the activation-enthalpy and the pH-dependence of the rate constants of the slow phase were determined. We conclude that the cis to trans interconversion of the peptidyl bond of the three peptidomacrolides may be responsible for the slow process. There was no indication of a suicide catalysis of this process by FKBPs.

INTRODUCTION

Peptidylprolyl cis–trans-isomerases (PPIases, EC 5.2.1.8) very efficiently catalyse the reversible cis–trans interconversion of a number of imidic peptide bonds arranged within an oligopeptide chain in either direction. The enzymes act specifically on proline-containing stretches in polypeptides, which results in an acceleration of protein folding in refolding experiments under suitable conditions (Schmid, 1993). Thus they represent an important functional group in the rapidly growing class of folding helper-proteins for protein folding reactions. The two structurally unrelated families of PPIases known to date, the cyclophilins (CyP) and the FK506-binding proteins (FKBPs), are ubiquitously distributed and highly conserved in a core region of the amino acid sequence during evolution (for a recent review see Galat, 1993). Thus the cytosolic members, human 18 kDa cytosolic CyP (CyP18cy) and human 12 kDa cytosolic FKBP (FKBP12cy) are highly abundant within cells and tissues, e.g. about 20 μM FKBP12cy in T-lymphocytes (Siekierka et al., 1991) and at least 4 μM CyP18cy in human erythrocytes (Akagi et al., 1991). As one consequence among others, enzyme inhibition experiments usually performed in vitro at [E]₀ ≪ [I] find completely different kinetic conditions within the cell.

Selective, tight-binding inhibitors of the eukaryotic CyP and FKBPs are the clinically important, immunosuppressive compounds cyclosporin A (CsA) as well as rapamycin and FK506, respectively. Cross-inhibition does not occur. (The structures of FK506, ascomycin and rapamycin are shown in Figure 1.)

Both types of inhibitor molecule contain N-acyl, N-alkyl peptide bonds adjacent to further putative binding sites. These structures may be accompanied by a certain degree of topo-chemical equivalence to natural, proline-containing substrates not hitherto identified. Since it became clear that the suppression of the T-cell answer after stimulation may arise from the chemical properties of the immunosuppressant–enzyme complexes, detailed knowledge of the course of binding of the inhibitors to PPIases is required.

The structures of CsA in the crystal (Loosli et al., 1985) and in solution (CDCl₃; Kessler et al., 1985, 1990; C₆H₆; Kessler et al., 1985; tetrahydrofuran (THF); Kessler et al., 1985; tetrahydrofuran (THF); Ko and Dalvit, 1992, LiCl–THF; Kock et al., 1992; dimethyl sulfoxide (DMSO), CH₂OH and 50% (v/v) CH₂OH/H₂O: Ko and Dalvit, 1992) were determined. When complexed to CyP18cy, CsA is uniformly bound (Fesik et
However, there was no evidence of a slow kinetic phase during the course of FKBP12cy's inhibition by FK506 (Harrison and Stein, 1990; Spencer et al., 1993). Recently, Petros et al. (1993) have proved, by n.m.r. spectroscopy, a time-dependent binding of $[\text{Arg}]$ ascomycin, a water-soluble FK506-derivative, that is caused by the cis to trans isomerization of the unbound macrolide. This association reaction was not accelerated by FKBP12cy.

Here we demonstrate for the first time that FK506, ascomycin and rapamycin cause a time-dependent inhibition of PPLase activity of structurally different FKBP s. The multiphasic kinetics of inhibition are solely determined by the conformational parameters of the peptidomacrolides.

**MATERIALS AND METHODS**

**Enzymes**

Recombinant human FKBP12cy was expressed at high levels in *Escherichia coli* (19% of total soluble protein) as a fusion protein with an N-terminal extension consisting of the first eight N-terminal amino acids of the $\beta$-galactosidase, followed by four histidines and an enterokinase cleaving-site for complete elimination of the fusion peptide. The cells (83 g) were resuspended in 330 ml of buffer (20 mM Hepes/1 mM EDTA/1 mM dithioerythritol, pH 7.5) and disrupted at 1200 $\times$ 10$^3$ Pa followed by precipitation with 5.7% (v/v) polyvinyl P (10% neutralized polyvinyl P solution) while being stirred on ice. The supernatant was incubated with DNAase I (0.1 mg/ml DNAase I (Boehringer Mannheim)/6 mM MgCl$_2$) for 30 min at 20 $^\circ$C followed by precipitation with 80% ammonium sulphate. The dissolved pellet was applied to a size-exclusion column (Sephacryl S-200 HR, 5 cm $\times$ 150 cm, 20 mM Hepes/0.2 M NaCl, pH 7.5, 190 ml/h). Fractions with PPLase activity were pooled and applied to an Ni$^{2+}$-chelating-Sepharose column (5 $\times$ 4.6 cm, equilibrated in 20 mM Hepes, pH 7.5, 4 ml/min). A 1800 ml linear gradient of histidine (0–10 mM) in buffer was used to elute the protein. The yield of purified fusion-protein was 708 mg (1.47 mg/ml). An aliquot of 50 mg of fusion-protein was cleaved by enterokinase (Boehringer Mannheim) (100:1, w/w) for 72 h at 20 $^\circ$C in 20 mM Hepes, pH 7.5. The reaction was stopped by dialysis against 20 mM sodium phosphate, pH 6.5, and native FKBP12cy without N-terminal fusion-peptide was obtained by negative-elution on a hydroxyapatite column (3 cm $\times$ 5.6 cm) equilibrated in 20 mM sodium phosphate, pH 6.5. Purified FKBP12cy (25 mg) was analysed by SDS/PAGE, reverse-phase h.p.l.c. and automatic N-terminal sequencing. The protein was more than 99% pure and showed a homogeneous N-terminus starting with Met-Gly-Val-Gln-Val.

The concentration of the mature FKBP12cy was determined spectrophotometrically ($\epsilon_{360} = 9530$ M$^{-1}$cm$^{-1}$), calculated from the amino acid sequence (Gill and von Hippel, 1989) and confirmed by a titration of FK506 binding-sites monitored by fluorescence. Several mixtures of 4 $\mu$M FKBP12cy (determined spectrophotometrically as described above) with FK506 in the range 0–6 $\mu$M were prepared and incubated for 60 min before measuring. Saturation was achieved at 4 $\mu$M FK506. Both of the methods for determining the concentration of FKBP12cy showed the same result.

The PPLase activity of the recombinant protein was assayed as described below using the peptide Suc-Ala-Leu-Pro-Phe-pNA (where Suc = succinyl and pNA = p-nitroanilide). The $k_{cat}/K_{m}$ value was calculated from the first-order rate constant $k_{cat}$ of the catalysed process ($k_{cat} - k_{res}$)/[FKBP12cy]$_{bound} = k_{cat}/K_{m}$ and was found to be $2.1 \times 10^4$ M$^{-1}$s$^{-1}$ at 10 $^\circ$C (2.2 $\times 10^4$ M$^{-1}$s$^{-1}$ at 10 $^\circ$C: Albers et al., 1990; Yang et al., 1993).

**Figure 1 Structures of FK506, ascomycin and rapamycin**
Recombinant 25 kDa membrane FKBP (FKBP25mem) [macrophage infectivity potentiator (Mip protein)] from Legionella pneumophila Philadelphia I was a gift from B. Schmidt (MPG, Halle, Germany).

Solvents, inhibitors and substrates

Anhydrous THF and LiCl were obtained from Aldrich (Steinheim, Germany). LiCl was additionally dried by heating to 150 °C under vacuum for 5 h. All other materials were of reagent grade and were used without further purification. FK506, rapamycin and ascomycin were kindly provided by Dr. A. Lawen, Monash University, Clayton, Australia. Stock solutions of FK506 (0.04 mM–2 mM) were prepared in 50 % (v/v) aqueous ethanol, anhydrous DMSO, anhydrous THF and 0.47 M LiCl in THF. The solution of LiCl–THF was made under an argon atmosphere. The peptide substrates Suc-Ala-Xaa-Pro-Phe-pNA (Xaa = Leu, Phe) were purchased from Bachem (Heidelberg, Germany) and Suc-Ala-Ala-Pro-Phe-pNA was obtained from Boehringer Mannheim and used without further purification. Peptide substrate (14 mg/ml) were dissolved in DMSO and diluted 1:10 in assay buffer (1.4 mg/ml) before use to decrease the final concentration of DMSO in the assay.

PPlase assays

To test the PPlase activity the assay of Fischer et al. (1984) was modified. In a typical kinetic experiment, a stock solution of FKBP12cy (3.44 μM, 120 μl) was added to the thermally equilibrated buffer at 10 °C (8400 μl; 100 mM Tris/HCl, pH 8.0 at 10 °C). Inhibitor dissolved in the respective solvent (2 μl) was added to final concentrations of 10 mM–40 mM. The components were mixed and subsequently aliquoted (710 μl) of the enzyme–inhibitor mixture were withdrawn at various preincubation times. Residual PPlase activity was assayed after addition of 120 μl of α-chymotrypsin (0.55 mM) (Boehringer Mannheim) and 30 μl of peptide substrate (1.4 mg/ml).

First data-points for residual activity are available after 1 min because the dead-time of the experiment is the sum of mixing the solution in the preincubation mixture and the dead-time of the subsequent enzyme assay. The reaction progress was monitored for <3 min by sampling the increase in A405 that accompanies the hydrolysis of the anilide bond, using a Hewlett-Packard 8452A diodearray spectrophotometer equipped with a thermostated cell-holder. The spectrophotometer was interfaced to a model 300 Hewlett-Packard Chemstation computer. Sampled absorbance data-points (typically 50–100 data-pairs) were fitted according to a first-order rate law using Hewlett-Packard kinetic software. Only traces showing strict first-order dependence were used.

The calculation of the K_i values was carried out by non-linear fitting of the data according to a competitive tight-binding equation (eqn. 1) (Morrison and Walsh, 1988) using Sigma Plot software (Jandel Scientific).

\[
k = k_{unc} + 0.5 k_{cat}/K_m (E_o - I_o - K_i + \sqrt{(E_o - I_o - K_i)^2 + 4E_o K_i})
\]

This equation includes the condition that the initial concentration of the PPlase substrate in the cis conformation is negligible compared with the \(K_m\) value and considers the uncatalysed background reaction. \(E_o\) and \(I_o\) are the concentrations of enzyme and inhibitor, and \(k\) and \(k_{unc}\) are the observed first-order rate constants of the progress curves for the catalysed and uncatalysed cis to trans isomerization of the PPlase substrate. The \(k_{unc}/K_m\) value was determined as described above. The FK506 concentration is based on the determination by weight and is related to both inactive and active conformers.

To determine the pH-dependence of the time-dependent inhibition, buffers were kept at constant ionic strength (Park et al., 1992).

All data-points are averaged from three independent measurements. Residual PPlase activity was determined relative to a control sample treated identically but devoid of inhibitor.

RESULTS

Time-dependent inhibition of FKBP12cy by FK506 and ascomycin

The tight-binding inhibitor FK506 and the FKBP12cy are known to form a reversible, stoichiometric complex (1:1) which results in inactivation of the PPlase activity characterized by a \(K_i\) of 1.7 nM (Harrison and Stein, 1990). FKBP's loss of enzymic activity was therefore used to investigate the kinetics of the assembly of the protein with peptidomacrolides. An excess of 50 mM FKBP12cy was incubated with 26 nM FK506 delivered from a stock solution in ethanol/water (1:1, v/v). The remaining activity of the enzyme was discontinuously determined depending on incubation time, monitoring the cis to trans interconversion of a proline-containing oligopeptide in the assay. The resulting time-course of inhibition of recombinant human FKBP12cy by FK506 is shown in Figure 2. Two distinct kinetic phases can be clearly resolved. The first, fast phase is finished within the dead-time of the experiment. The second phase is characterized by a slow increase of inhibition that is finished after approximately 60 min at 10 °C. The data represent a strict first-order reaction with a rate constant \(k_{obs} = (8.6 \pm 0.8) \times 10^{-4} s^{-1}\). Considering that the experiments shown in Figure 2 were carried out with ([FKBP12cy]_{initial} - [FK506]) \(\approx K_i\) the remaining activity after the inactivation fades out is simply a function of stoichiometry. However, the ratio of the kinetic phases provides information on functional effects on the pathway of inactivation.

Figure 2 Time-dependent inhibition of FKBP12cy by FK506 (○) and of FKBP25mem by FK506 (□). FKBP12cy (40 nM) and 26 nM FK506 (dissolved in 50% (v/v) aqueous ethanol) were coincubated in 0.1 M Tris/HCl, pH 8.0 (10 °C) before determining the remaining activity with Suc-Ala-Leu-Pro-Phe-pNA (73 μM) and α-chymotrypsin (77 μM). The same procedure was carried out for rapamycin at 15 °C (40 mM FKBP12cy, 34 mM rapamycin dissolved in 50% (v/v) aqueous ethanol). FKBP25mem (900 nM) and 900 nM FK506 (dissolved in 50% (v/v) aqueous ethanol) were coincubated in 0.1 M Tris/HCl, pH 8.0 (10 °C) before determining the remaining activity with Suc-Ala-Ala-Pro-Phe-pNA (73 μM) and α-chymotrypsin (77 μM). Each curve joining the data-points represents a fit according to a first-order reaction. The inset shows the corresponding semilogarithmic plot.
For FKBP12cy-FK506 the ratio of amplitudes \( r \) is 0.75 \( (r = \text{percentage fast phase/percentage slow phase}) \), simply indicating that the major part of enzyme inhibition occurs in a slow process.

When the inhibition of the PPlase activity of FKBP12cy increases during preincubation in the presence of FK506 then this should result in a decrease in the inhibition constant \( K_i \). As illustrated in Figure 3, prolongation of the incubation time to 60 min leads to enhanced potency of the inhibitor. The initial \( K_i \) value decreases from 1.3 ± 0.5 nM to \( K_{i_{\text{final}}} = 0.5 ± 0.3 \) nM after 60 min preincubation.

In a similar kinetic experiment we used ascomycin instead of FK506 as an inhibitor of FKBP12cy. This homologue of FK506 is nearly as potent as the parent compound in immunosuppression (Organ et al., 1993) and does not significantly differ from FK506 when bound to FKBP12cy (Petros et al., 1991). By incubating 30 nM ascomycin [delivered from a stock solution of 50% (v/v) aqueous ethanol] with 40 nM FKBP we could obtain a \( k_{\text{obs}} \) of (7.1 ± 0.7) × 10^{-4} s^{-1} concomitant with \( r = 0.76 \). Despite the slightly higher \( K_i \) value for the ascomycin–FKBP12cy interaction (Kawai et al., 1993) the kinetic pattern strongly resembles that of FK506.

To examine whether the slow phase of inhibition and the tightening of the complex is due to reshuffling within the enzyme–inhibitor molecule, the inactivation of a homologous FKBP was investigated. The mature membrane-bound FKBP25mem, the Mip protein of \( L. \) pneumophila, consists of an N-terminal extension of 106 amino acids and a C-terminal domain that is 55.2% similar to FKBP12cy ( Hacker et al., 1993). With a \( K_i \) value considerably higher than eukaryotic FKBPs, the kinetic pattern of inhibition should be expected to be influenced through the changed primary structure of the protein as far as the enzyme is involved in the slow reaction of inhibition. However, results of the experiment are inconsistent with this assumption. Because of the high PPlase activity of 900 nM recombinant FKBP25mem necessary for this experiment, we have to utilize Suc-Ala-Ala-Pro-Phe-pNA, a peptide substrate with a relatively low \( k_{\text{max}}/K_m \) value of 6.4 × 10^4 M^{-1} s^{-1} [B. Schmidt (MPG, Halle, Germany), personal communication]. As shown in Figure 2 the inhibition of FKBP25mem by FK506 was rather similar to the human enzyme. The first-order rate constant \( k_{\text{obs}} \) and ratio of amplitudes \( r \) were determined to be (7.7 ± 0.9) × 10^{-4} s^{-1} and 0.91, respectively. Obviously the slow phase of inhibition is independent of the enzyme and is caused solely by a structural change within the inhibitor.

Further support for an enzyme-independent reaction pathway of the slow inactivation process was obtained by investigating the dependence of inhibition kinetics upon varying enzyme and ligand concentrations. If the FK506 interconversion is a non-enzymatic, monomolecular process, then the first-order rate constant of the slow reaction should be independent of the enzyme and inhibitor concentrations. Neither changes of the FKBP12cy concentration in the range 20 nM–40 nM in the preincubation mixture nor changes of the FK506 concentration (13 nM–40 nM) yielded any systematic deviation from the mean value of \( k_{\text{obs}} = 8.4 × 10^{-4} \) s^{-1}, and the ratio of amplitudes \( r \) fluctuated within the limit or error \( (r = 0.72) \).

For CsA it was shown that solvents have great influence on the type and quantity of conformers in solution (Kofron et al., 1992; Kessler et al., 1985, 1990; Ko and Dalvit, 1992; Köck et al., 1992). Delivering CsA from stock solutions prepared in various solvents leads to considerable differences in the kinetic pattern of the inhibition. To probe for similar effects of the FK506–FKBP12cy reaction we investigated the inhibition kinetics of FK506 dissolved in 50% (v/v) aqueous ethanol, DMSO, THF and 0.47 M LiCl–THF before injection into the aqueous assay mixture. Non-aqueous solutions of Li^+ salts are known to affect specifically the thermodynamics of cis-trans isomerism of imide bonds (Kofron et al., 1991, 1992; Köck et al., 1992). Obviously, for FK506 the observed inhibition pattern is similar in each case and \( k_{\text{obs}} \) does not vary greatly (Table 1). The ratio of the amplitude \( r \) is more sensitive but still tends to remain within the limits of error. A convincing Li^+ perturbation of the amplitude of the phases is lacking. Recently published n.m.r. experiments with FK506 have shown conformational changes induced by complexation with LiCl in THF. However, these conformational changes could not be characterized in detail (Seebach et al., 1994). The identical amplitudes of the slow phases in the absence and presence of LiCl presumably indicate that the conformational changes detected in the n.m.r. experiments were due to Li^+ perturbation of the inhibitor’s imide bond.

**Table 1** Kinetic parameters of the inhibition of FKBP12cy by FK506 from various stock solutions

<table>
<thead>
<tr>
<th>Solvent of FK506</th>
<th>( 10^6 \times K_{\text{obs}} ) ( (s^{-1}) )</th>
<th>( r )</th>
</tr>
</thead>
<tbody>
<tr>
<td>50% (v/v) aqueous ethanol</td>
<td>8.6 ± 0.8</td>
<td>0.75</td>
</tr>
<tr>
<td>DMSO</td>
<td>7.8 ± 0.5</td>
<td>0.45</td>
</tr>
<tr>
<td>THF</td>
<td>7.6 ± 0.6</td>
<td>0.55</td>
</tr>
<tr>
<td>0.47 M LiCl in THF</td>
<td>8.6 ± 0.5</td>
<td>0.47</td>
</tr>
</tbody>
</table>

* Ratio of amplitudes \( r (r = \text{percentage fast phase/percentage slow phase}) \).

**Figure 3** Determination of \( K_i \) for FK506 after 1 min \((\bullet)\) and 60 min \((\bigcirc)\) preincubation with FKBP12cy

Relative PPlase activity versus concentration of FK506. \( k_i \) and \( k_{\text{obs}} \) represent the first-order rate constants of the progress curve of the PPlase assay both with and without inhibitor. FKBP12cy (40 nM) was incubated with 0–128 nM of FK506 [dissolved in 50% (v/v) aqueous ethanol] in 0.1 M Tris/HCl, pH 8 (10 °C) before adding Suc-Ala-Ala-Pro-Phe-pNA and α-chymotrypsin (final concentrations 73 μM and 77 μM, respectively). The data points were fitted and the \( K_i \) values calculated according to the equation of Morrison and Walsh (1988) for competitive tight-binding inhibition.

Although structurally related to FK506 in the FKBP12cy-binding region, rapamycin differs from the structure of FK506 in several important aspects. Even in the crystal (Swindells et al., 1978) and in DMSO solution (Kessler et al., 1993) the structure of rapamycin is virtually identical with the conformation bound to FKBP12cy. Unlike unbound FK506, the predominant rapamycin...
isomer with respect to the $\alpha$-ketoacyl-pipecolinyl moiety is mainly in the \textit{trans} form, rendering rearrangement unnecessary when binding occurs. Furthermore, rapamycin does not form tautomers after transfer into different solvent conditions as was shown for FK506 (Namiki et al., 1993). Since the rate of this tautomerization approaches a time-scale related to those obtained for the slow phase of the inactivation of FKBP12, it was advisable to check the importance of this effect with regard to the kinetic pattern of inhibition.

Like FK506, rapamycin inhibits FKBP12cy in a two-phase process (Figure 2). For practical reasons, the inactivation reaction was carried out at 15 °C. At 10 °C the plateau region of inhibition had not yet been achieved after 120 min, which led to irregularities due to the loss of PPiPase activity by adsorption on the surface of the reaction vessel, indicated by the reference reaction lacking inhibitor. Under these conditions we determined $k_{\text{obs}} = (6.1 \pm 0.9) \times 10^{-4} \text{s}^{-1}$ and $r = 3.6$, corresponding to a predominance of the high-affinity binding conformation of rapamycin in the organic stock solution.

Temperature- and pH-dependence of the slow inhibition

Measuring the value of the activation enthalpy $\Delta H^\ddagger$ may offer, by comparison, additional insights into the molecular process involved in the time-mediated improvement of the inhibitory potency of the peptidomacroldes. For this purpose $k_{\text{obs}}$ for the time-dependent inhibition of FKBP12cy by FK506 was measured in the range 4.7 °C–20 °C. After calculating the parameters of the Eyring plot (Figure 4), $\Delta H^\ddagger$ was found to be 67 ± 12 kJ·mol⁻¹. This value is located within the range of the uncatalysed rotational barrier of the \textit{cis-trans} isomerisation of Xaa-Pro bonds (Brandts et al., 1975: $\Delta H^\ddagger = 84 \pm 12$ kJ·mol⁻¹; Fischer et al., 1983: $\Delta H^\ddagger_{\text{Gly,Pro-SH (SH)}} = 66.9$ kJ·mol⁻¹). Since a PPiPase is involved in the inactivation reaction, the $\Delta H^\ddagger$ value of the enzyme catalysed prolyl bond-isomerisation is also of interest. However the fact that with CyPs and FKBP12cy the magnitude of $\Delta H^\ddagger$ does not exceed 20 kJ·mol⁻¹, regardless of either $k_{\text{cat}}$ (G. Fischer, unpublished work) or $k_{\text{cat}}/K_m$ (Stein, 1993), was investigated.

The reversible hydration or the formation and decomposition of hemiacetals thought to be involved in the tautomerization of FK506 (Namiki et al., 1993) are usually subject to general acid–base catalysis (Jencks, 1969). pH-dependent rate constants would therefore be indicative of proton transfer reactions on the reaction pathway. We examined the kinetics of the FK506–FKBP12cy interaction as a function of pH in the range 6.0–9.5 at 10 °C. In fact, pH had no influence on inhibition kinetics (Table 2). Therefore, any tautomerization or hydration/dehydration within the tricarbonyl stretch of the peptidomacrolides can be ruled out as being the source of the slow inactivation process.

### DISCUSSION

Contrary to present knowledge, by detailed analysis of the kinetics of inhibition of FKBP56 by FK506, ascomycin and rapamycin, we were able to detect a rather slow phase of inactivation upon preincubation of the reactants. In all cases the inhibition clearly occurs in two phases, a rather slow first-order process dominating in its amplitude subsequent to a fast reaction of inactivation still remaining unresolved in its time-course. Additionally, the increase of the inhibitory potency of FK506 during preincubation results in a 2.6-fold decrease in the inhibition constant $K_i$ in comparison to the initial value. This may explain the variance in the $K_i$ values reported, ranging from 1.7 nM initial values (Harrison and Stein, 1990) to 0.4 nM (Yang et al., 1993) after a few minutes (not clearly defined) of preincubation.

As a consequence of the experiments it became clear that the peptidomacroldes strongly resemble CsA in the time-course of their interaction with PPiPases. However, in contrast to the cyclosporins, for peptidomacroldes detailed knowledge of the conformation heterogeneity in most of the polar solvents is still lacking. For FK506 in CDC$_3$ there is a 2:1 excess of the \textit{cis} conformer over the \textit{trans} peptidomacrold bond. For rapamycin, 80% \textit{trans} isomers are present in CDC$_3$, increasing in DMSO to 90% (Kessler et al., 1993). Low solubility prevents n.m.r. spectroscopic investigations in aqueous systems. Besides amide bond rotation, a further conformational peculiarity of the FK506 region interacting with the active site of FKBP12cy might be important. In unconstrained $\alpha$-dicarbonyl derivatives the carboxyl groups usually adopt an anti orientation (Mobilo et al., 1991). The \textit{cis} conformer in CDC$_3$ (Karuso et al., 1990) or in the crystal presents the $\alpha$-dicarbonyl moiety in an approximately orthogonal orientation as can also be found for the \textit{trans} conformer in the FKBP12cy complex. However, the imidic bond itself provides the scaffolding effect for the $\alpha$-ketoamide moiety, rendering it unnecessary for strain to be applied from either the protein or the macrocycles. On the HF/6-31G(d) computational level, \textit{N,N}-dimethylpyruvamide served as the model system to provide parameters for the flexibility of the binding-region of peptidomacroldes (Bach et al., 1993). Under vacuum it predicts com-
parably high torsional barriers for both OC–CO and C–N bond rotation.

From the results presented above, it seems likely that the slow inactivation process, although monomolecular in its kinetics, does not occur on the enzyme-inhibitor molecule, as is thought to be the case in the Cyp18cy–CypA complex, but is a non-enzymic event that arises from a reaction of the unbound peptidomacroolides. Consequently, a sort of suicide inhibition characterized by the trapping of an intermediate of the catalytic pathway is less probable. Substitution of proline in oligopeptide substrates by peptidic acid correlates with this finding, because the modified oligopeptides cannot be catalytically utilized by PPlases (G. Fischer, unpublished work).

Among the monomolecular rearrangements possibly occurring in the inhibitor molecules the cis–trans isomerization of the α-ketoacyl peptidic group provides a convincing candidate for this process. Both the measured activation enthalpy, which is typical for prolyl-peptide bond isomerization, and the knowledge about the conformation of the bound peptidomacroolide, provide convincing evidence that the OC–CO rotation should not be involved in the slow inactivation. It was also evident from the pH-independence of the kinetics and the rapamycin experiment that tautomeration does not contribute to slow phases of inhibition. Thus, a minimal kinetic scheme for the inactivation process is shown in eqn. (2):

\[
FK506_{\text{cis}} + K_{\text{gb}} \rightarrow FKBP/FK506_{\text{trans}}
\]

where \( k_{\text{gs}} \) and \( k_{\text{ct}} \) are the first order rate constants for cis–trans and trans–cis isomerization respectively.

In this case the amplitude ratio \( r \) can be directly assigned to the equilibrium constant of isomerization which thus becomes available for aqueous solution. By this criterion the order FK506 ≈ ascomycin > rapamycin was found for the cis content, which accords with values in CDCl₃ solution.

The above considerations are emphasized by the time-dependent uptake of [Arg²⁹]ascomycin by FKBP12cy from an aqueous solution, since the addition of FKBP12cy selectively depletes the trans conformer. The rate constant for the cis to trans interconversion was estimated to be \( 2.4 \times 10^{-4} \text{ s}^{-1} \) at 12 °C (Petros et al., 1993), which is slightly lower compared with the same parameter of unsubstituted ascomycin and FK506 given above.

In conclusion, there is ample evidence that the biphasic inhibition process monitored during the interaction of peptidomacroolides with FKBP's is caused by the uncatalysed cis to trans isomerization of the α-ketoacyl peptidic bond of the unbound molecules. There was no sign of a reshuffling of the bound trans isomer to a more bioactive conformation. However, both processes, the time-dependent formation of an increasing amount of the complex and the slow decrease of residual enzyme activity observed concomitantly, should be taken into consideration in biological assays. It is clear that at high intracellular concentrations of FKBP's and for assays working in the time-scale of the inactivation process, these effects could have a particular influence.

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