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

Toralf ZARNT,* Kurt LANG,† Helmut BURTSCHER† and Gunter FISCHER*‡

*Max-Planck-Gesellschaft, Arbeitsgruppe 'Enzymologie der Peptidbindung', Weinbergweg 16a, 06120 Halle, Germany, and †Boehringer Mannheim G.m.b.H., Biochemical Research Centre Penzberg, Nonnenwald 2, 82377 Penzberg, Germany

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 pipercolinyl bond of the three peptidomacrolides may be responsible for the slow process. There was no indication of a suicide catalysis of this process by FKBP.

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). Furthermore, 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 μ M FKBP12cy in T-lymphocytes (Siekierka et al., 1991) and at least 4 μ M CyP18cy in human erythrocytes (Akagi et al., 1991). As one consequence among others, enzyme inhibition experiments usually performed *in vitro*

at $[E]_0 \ll [I]$ find completely different kinetic conditions within the cell.

Selective, tight-binding inhibitors of the eukaryotic CyP and FKBP 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 molecules contain N-acyl, N-alkyl peptide bonds adjacent to further putative binding sites. These structures may be accompanied by a certain degree of topochemical 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): Ko and Dalvit, 1992; LiCl–THF: Köck et al., 1992; dimethyl sulphoxide (DMSO), CH₃OH and 50% (v/v) CH₃OH/H₂O: Ko and Dalvit, 1992) were determined. When complexed to CyP18cy, CsA is uniformly bound (Fesik et

Abbreviations used: PPIase, peptidylprolyl *cis*–*trans*-isomerase; CyP, cyclophilin; FKBP, FK506-binding protein; Mip, macrophage infectivity potentiator; CsA, cyclosporin A; CyP18cy, human 18 kDa cytosolic cyclophilin; FKBP12cy, human 12 kDa cytosolic FK506-binding protein; FKBP25mem, 25 kDa membrane FK506-binding protein; MeLeu⁹–MeLeu¹⁰, N-methyl-L-leucine⁹–N-methyl-L-leucine¹⁰; Suc, succinyl; pNA, p-nitroanilide; DMSO, dimethyl sulphoxide; THF, tetrahydrofuran.

‡ To whom correspondence should be addressed.

Figure 1 Structures of FK506, ascomycin and rapamycin

al., 1990) but differs strongly in its conformation from any one of the uncomplexed molecules characterized in the structural investigations. Furthermore, in contrast to the homogeneously bound conformation of CsA, structural diversity due to the presence of *cis* and *trans* conformational isomers with respect to the *N*-methyl-L-leucine⁹-*N*-methyl-L-leucine¹⁰ (MeLeu⁹-MeLeu¹⁰) peptide bond was described for most of the CsA solutions (Kessler et al., 1990).

On the basis of this knowledge, the multiphasic time course of the inactivation of CyP18cy by CsA was found to be due to rate-limiting rearrangements of the initial enzyme-inhibitor complex to a tighter one in the final state. In each case only *trans* MeLeu⁹-MeLeu¹⁰ is extracted by the enzyme from the multiple conformational equilibrium present in the assay, because the *cis* isomer has no or reduced affinity for the enzyme (Kofron et al., 1992).

Likewise, structural investigations of FK506 show the α -ketoacyl-pipecolinyl moiety either in *cis* (Tanaka et al., 1987) or, as in chloroform, in a 2:1 ratio of *cis* to *trans* conformation (Karuso et al., 1990). Rapamycin on the contrary prefers the *trans* conformation in the crystal (Swindells et al., 1978). But the FKBP12cy template forces both FK506 and rapamycin to a homogeneously bound conformation with the imidic bond in the *trans* form and a nearly superimposable structure for the common binding region of both of the macrolides (Van Duyne et al., 1991, 1993). Therefore considerable reorganization of the conformation of FK506 upon binding may be inferred from the different structure of FK506 in apolar solvents and in complex with FKBP12cy.

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 [Arg³²]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 PPIase activity of structurally different FKBP. 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 β -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 1200×10^5 Pa followed by precipitation with 5.7% (v/v) polymin P (10% neutralized polymin P solution) while being stirred on ice. The supernatant was incubated with DNAase I (0.1 mg/ml DNAase I (Boehringer Mannheim)/6 mM MgCl₂) for 30 min at 20 °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 PPIase activity were pooled and applied to an Ni²⁺-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 °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 hydroxylapatite 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 ($\epsilon_{280} = 9530 \text{ M}^{-1} \cdot \text{cm}^{-1}$, 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 μM FKBP12cy (determined spectrophotometrically as described above) with FK506 in the range 0–6 μM were prepared and incubated for 60 min before measuring. Saturation was achieved at 4 μM FK506. Both of the methods for determining the concentration of FKBP12cy showed the same result.

The PPIase 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_{obs} of the catalysed process ($k_{\text{obs}} - k_0$)/[FKBP12cy]_{total} = k_{cat}/K_m and was found to be $2.1 \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$ at 10 °C ($2.2 \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$ at 10 °C: Albers et al., 1990; Yang et al., 1993).

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.

PPIase assays

To test the PPIase activity the assay of Fischer et al. (1984) was modified. In a typical kinetic experiment, a stock solution of FKBP12cy (3.44 μ M, 120 μ l) was added to the thermally equilibrated buffer at 10 °C (8400 μ l; 100 mM Tris/HCl, pH 8.0 at 10 °C). Inhibitor dissolved in the respective solvent (2 μ l) was added to final concentrations of 10 nM–40 nM. The components were mixed and subsequently aliquots (710 μ l) of the enzyme–inhibitor mixture were withdrawn at various preincubation times. Residual PPIase activity was assayed after addition of 120 μ l of α -chymotrypsin (0.55 mM) (Boehringer Mannheim) and 30 μ l 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 A_{390} 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).

$$k = k_{\text{unc.}} + 0.5k_{\text{cat.}}/K_m(E_0 - I_0 - K_i + \sqrt{(E_0 - I_0 - K_i)^2 + 4E_0K_i}) \quad (1)$$

This equation includes the condition that the initial concentration of the PPIase substrate in the *cis* conformation is negligible compared with the K_m value and considers the uncatalysed background reaction. E_0 and I_0 are the concentrations of enzyme and inhibitor, and k and $k_{\text{unc.}}$ are the observed first-order rate constants of the progress curves for the catalysed and uncatalysed *cis* to *trans* isomerization of the PPIase substrate. The $k_{\text{cat.}}/K_m$ 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 PPIase activity was determined relative to a control sample treated identically but devoid of inhibitor.

RESULTS

Time-dependent inhibition of FKBP 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 PPIase 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 $k_{\text{obs.}} = (8.6 \pm 0.8) \times 10^{-4} \text{ s}^{-1}$. Considering that the experiments shown in Figure 2 were carried out with $([\text{FKBP}]_{\text{total}} > [\text{FK506}]_{\text{total}}) \gg K_{i,\text{initial}}$, 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 rapamycin (■) 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 μ M) and α -chymotrypsin (77 μ M). The same procedure was carried out for rapamycin at 15 °C [40 nM FKBP12cy, 34 nM 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 μ M) and α -chymotrypsin (77 μ M). Each curve joining the data-points represents a fit according to a first-order reaction. The inset shows the corresponding semilogarithmic plot.

Figure 3 Determination of K_i for FK506 after 1 min (●) and 60 min (○) preincubation with FKBP12cy

Relative PPIase activity versus concentration of FK506. k_i and k_0 represent the first-order rate constants of the progress curve of the PPIase 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-Leu-Pro-Phe-pNA and α -chymotrypsin (final concentrations 73 μ M and 77 μ M, 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.

For FKBP12cy–FK506 the ratio of amplitudes r is 0.75 ($r = \text{percentage}_{\text{fast phase}}/\text{percentage}_{\text{slow phase}}$), simply indicating that the major part of enzyme inhibition occurs in a slow process.

When the inhibition of the PPIase 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 $K_{i,\text{final}} = 0.5 \pm 0.3$ 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_{obs} of $(7.1 \pm 0.7) \times 10^{-4} \text{ s}^{-1}$ 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 FKBP, 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 PPIase 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_{cat}/K_m value of $6.4 \times 10^4 \text{ M}^{-1} \cdot \text{s}^{-1}$ [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_{obs} and ratio of amplitudes r were determined to be

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

FKBP12cy (40 nM) was inhibited by 26 nM FK506 dissolved in their respective solvents and the remaining PPIase activity was measured after different preincubation times at 10 °C.

Solvent of FK506	$10^4 \times k_{\text{obs}}$ (s^{-1})	r^*
50% (v/v) aqueous ethanol	8.6 ± 0.8	0.75
DMSO	7.8 ± 0.5	0.45
THF	7.6 ± 0.6	0.55
0.47 M LiCl in THF	8.6 ± 0.5	0.47

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

$(7.7 \pm 0.9) \times 10^{-4} \text{ s}^{-1}$ 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-enzymic, 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 $k_{\text{obs}} = 8.4 \times 10^{-4} \text{ s}^{-1}$, 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_{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.

Time-dependent inhibition of FKBP by rapamycin

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

Figure 4 Eyring plot for the first-order rate constants of the slow phase of inactivation

The time-dependent inhibition of FKBP12cy with FK506 [dissolved in 50% (v/v) aqueous ethanol] was determined in the range 4.7 °C–20 °C as described in the caption to Figure 2. The activation enthalpy $\Delta H^\ddagger$ was calculated to be $67 \pm 12 \text{ kJ} \cdot \text{mol}^{-1}$.

isomer with respect to the α -ketoacyl-pipecolinyl moiety is mainly in the *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 FKBP12cy 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 PPIase 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 peptidomacrolides. For this purpose k_{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 $67 \pm 12 \text{ kJ} \cdot \text{mol}^{-1}$. This value is located within the range of the uncatalysed rotational barrier of the *cis*–*trans* isomerization of Xaa–Pro bonds (Brandts et al., 1975: $[\Delta H^\ddagger = 84 \pm 12 \text{ kJ} \cdot \text{mol}^{-1}]$; Fischer et al., 1983: $\Delta H^\ddagger_{\text{Gly-Pro-NHNp}} = 66.9 \text{ kJ} \cdot \text{mol}^{-1}$). Since a PPIase is involved in the inactivation reaction, the $\Delta H^\ddagger$ value of the enzyme catalysed prolyl bond-isomerization is also of interest. However the fact that with CyPs and FKBP12cy the magnitude of $\Delta H^\ddagger$ does not exceed $20 \text{ kJ} \cdot \text{mol}^{-1}$, regardless of either k_{cat} (G. Fischer, unpublished work) or k_{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

Table 2 pH dependence of the *cis*–*trans* interconversion of FK506

FKBP12cy (40 nM) was incubated with 26 nM FK506 [dissolved in 50% (v/v) aqueous ethanol] and the remaining activity was measured as a function of time at 10 °C.

pH	$10^4 \times k_{\text{obs}}$ (s^{-1})	r^*
6.0	6.5	0.85
8.0	7.3	0.86
9.5	6.9	0.80

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

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 FKBP12cy 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 peptidomacrolides strongly resemble CsA in the time-course of their interaction with PPIases. However, in contrast to the cyclosporins, for peptidomacrolides detailed knowledge of the conformational heterogeneity in most of the polar solvents is still lacking. For FK506 in CDCl_3 there is a 2:1 excess of the *cis* conformer over the *trans* pipecolinyl bond. For rapamycin, 80% *trans* isomers are present in CDCl_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 α -dicarbonyl derivatives the carbonyl groups usually adopt an anti orientation (Mobilio et al., 1991). The *cis* conformer in CDCl_3 (Karuso et al., 1990) or in the crystal presents the α -dicarbonyl moiety in an approximately orthogonal orientation as can also be found for the *trans* conformer in the FKBP12cy complex. However, the imidic bond itself provides the scaffolding effect for the α -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, *N,N*-dimethylpyruvamide served as the model system to provide parameters for the flexibility of the binding-region of peptidomacrolides (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–CsA complex, but is a non-enzymic event that arises from a reaction of the unbound peptidomacrolides. 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 pipecolinic acid correlates with this finding, because the modified oligopeptides cannot be catalytically utilized by PPIases (G. Fischer, unpublished work).

Among the monomolecular rearrangements possibly occurring in the inhibitor molecules the *cis*–*trans* isomerization of the α -ketoacyl pipecolinyl bond 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 peptidomacrolide, 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 tautomerization does not contribute to slow phases of inhibition. Thus, a minimal kinetic scheme for the inactivation process is shown in eqn. (2):

where k_{ct} and k_{tc} 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 $\text{FK506} \approx \text{ascomycin} > \text{rapamycin}$ was found for the *cis* content, which accords with values in CDCl_3 solution.

The above considerations are emphasized by the time-dependent uptake of $[\text{Arg}^{32}]$ 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 peptidomacrolides with FKBP is caused by the uncatalysed *cis* to *trans* isomerization of the α -ketoacyl pipecolinyl 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 enzymic activity observed concomitantly, should be taken into consideration in biological assays. It is clear that at high intracellular concentrations of FKBP and for assays working in the time-scale of the inactivation process, these effects could have a particular influence.

We gratefully acknowledge for financial support by the Fonds der Chemischen Industrie and the Deutsche Forschungsgemeinschaft (grant number Fi 455/1-1).

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