Conformation of an FK506 Analog in Aqueous Solution Is Similar to the FKBP-Bound Conformation of FK506

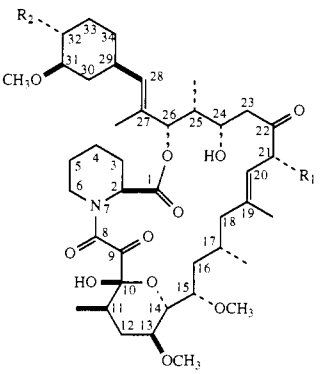
Andrew M. Petros, Jay R. Luly, Heng Liang, and Stephen W. Fesik*

Contribution from the Pharmaceutical Discovery Division, Abbott Laboratories, Abbott Park, Illinois 60064

Received May 28, 1993®

Abstract: The conformation of a water-soluble analog of the immunosuppressant FK506 was determined in aqueous solution using NMR spectroscopy. The three-dimensional structures for both the 7,8-*cis* and *trans* isomers of [32-Arg]ascomycin which are present in a 1:1 ratio were found to be quite different from FK506 in the crystal state or in chloroform solution but more closely resemble the structure of FK506 when bound to the FK506-binding protein (FKBP). These results suggest that the FKBP-bound conformation of FK506 largely preexists in aqueous solution and is not induced by the protein as previously postulated. In addition, using uniformly ^{15}N-^{13}C-labeled FKBP and isotope-filtering NMR techniques, it is shown that FKBP binds exclusively the *trans* form of [32-Arg]ascomycin and does not catalyze the *cis*/*trans* interconversion of the inhibitor.

FK506 is a potent immunosuppressant which binds tightly (Kd ~ 0.4 nm) to the FK506-binding protein (FKBP) and inhibits its peptidyl-prolyl *cis*/*trans* isomerase activity.[1-3] The FK506/FKBP complex inhibits the calcium-dependent phosphatase calcineurin,[4] leading to suppression of the T-cell-mediated immune response. Recently, it was shown that the conformation of FK506 and a structural analog, ascomycin (Figure 1), when bound to FKBP was very different from the conformation of uncomplexed FK506 in the solid state and in chloroform solution.[5-8] Similarly, the conformation of the undecapeptide immunosuppressant cyclosporin A (CsA) when bound to its target protein, cyclophilin, was also very different from the X-ray and chloroform solution conformation of uncomplexed CsA.[9-12] These results provided a serious challenge to the traditional "lock and key" notion of protein/ligand interactions and suggested that FK506 and CsA undergo substantial distortions upon binding to FKBP and cyclophilin, respectively.[13,14] Further support for this hypothesis was derived from molecular dynamics simulations of these molecules in an aqueous environment in which both CsA and FK506 did not drift far from the uncomplexed conformations,[15,16] suggesting that the proteins were required to "induce" the proper binding conformation. These results also questioned the use of the uncomplexed conformations of these immunosuppressants for designing improved drug molecules. However, these conclusions were based on the uncomplexed conformations of these molecules determined in the solid state or in chloroform solution which could be markedly influenced by crystal contacts or hydrophobic solvent effects. Indeed, based on an X-ray crystal structure of a CsA/Fab complex[17] and kinetic evidence,[18] it has been suggested that the bound conformation of CsA must preexist in aqueous solution to at least some extent. However, three-dimensional structures of CsA and FK506 in aqueous solution have not been reported due to their poor water solubility.



	R_1	R_2
FK506	$CH_2CH{=}CH_2$	OH
Ascomycin	CH_2CH_3	OH
[32-Arg]ascomycin	CH_2CH_3	$OOCCH(NH_3^+)(CH_2)_3NHC(NH_2)_2^+$

Figure 1. Strucures of FK506, ascomycin, and [32-Arg]ascomycin.

In this paper we present the three-dimensional structure of a water-soluble FK506 analog, [32-Arg]ascomycin (Figure 1), in aqueous solution which exists in two equally populated forms, containing either a *cis* or *trans* 7,8 amide bond. Like FK506 and ascomycin, this analog is potently immunosuppressive and has the same affinity for FKBP (unpublished observations). In order

* Author to whom correspondence should be addressed.

® Abstract published in *Advance ACS Abstracts*, October 1, 1993.

(1) Siekierka, J. J.; Hung, S. H. Y.; Poe, M.; Lin, C. S.; Sigal, N. H. *Nature* **1989**, *341*, 755.
(2) Harding, M. W.; Galat, A.; Uehling, D. E.; Schreiber, S. L. *Nature* **1989**, *341*, 758.
(3) Bierer, B. E.; Mattila, P. S.; Standaertet, R. F.; Herzenberg, L. A.; Burakoff, S. J.; Crabtree, G.; Schreiber, S. L. *Proc. Natl. Acad. Sci. U.S.A.* **1990**, *87*, 9231.
(4) Liu, J.; Farmer, J. D., Jr.; Lane, W. S.; Friedman, J.; Weissman, I.; Schreiber, S. L. *Cell* **1991**, *66*, 807.
(5) Van Duyne, G. D.; Standaert, R. F.; Karplus, P. A.; Schreiber, S. L.; Clardy, J. *Science* **1991**, *252*, 839.
(6) Petros, A. M.; Gampe, R. T., Jr.; Gemmecker, G.; Neri, P. N.; Holzman, T. F.; Edalji, R.; Hochlowski, J.; Jackson, M.; McAlpine, J.; Luly, J. R.; Pilot-Matias, T.; Pratt, S.; Fesik, S. W. *J. Med. Chem.* **1991**, *34*, 2925.
(7) Taga, T.; Tanaka, H.; Goto, T.; Tada, S. *Acta Crystallogr.* **1987**, *C43*, 751.
(8) Mierke, D. F.; Schmeider, P.; Karuso, P.; Kessler, H. *Helv. Chim. Acta* **1991**, *74*, 1027.
(9) Weber, C.; Wider, C.; von Freyberg, B.; Traber, R.; Braum, W.; Widmer, H.; Wüthrich, K. *Biochemistry* **1991**, *30*, 6563.
(10) Fesik, S. W.; Gampe, R. T., Jr.; Eaton, H. L.; Gemmecker, G.; Olejniczak, E. T.; Neri, P.; Holzman, T. F.; Egan, D. A.; Edalji, R.; Simmer, R.; Helfrich, R.; Hochlowski, J.; Jackson, M. *Biochemistry* **1991**, *30*, 6574.
(11) Kessler, H.; Köck, M.; Wein, T.; Gehrke, M. *Helv. Chim. Acta* **1990**, *73*, 1818.
(12) Hsu, V. L.; Armitage, I. M. *Biochemistry* **1992**, *31*, 12778.
(13) Wüthrich, K.; von Freyberg, B.; Weber, C.; Wider, G.; Traber, R.; Widmer, H.; Braun, W. *Science* **1991**, *254*, 953.
(14) Jorgensen, W. L. *Science* **1991**, *254*, 954.
(15) Lautz, J.; Kessler, H.; van Gunsteren, W. F.; Weber, H. P.; Wenger, R. M. *Biopolymers* **1990**, *29*, 1669.
(16) Pranata, J.; Jorgensen, W. L. *J. Am. Chem. Soc.* **1991**, *113*, 9483.
(17) Altschuh, D.; Vix, O.; Rees, B.; Thierry, J. *Science* **1992**, *256*, 92.
(18) Kofron, J. L.; Kuzmic, P.; Kishore, V.; Gemmecker, G.; Fesik, S. W.; Rich, D. H. *J. Am. Chem. Soc.* **1992**, *114*, 2670.

0002-7863/93/1515-9920$04.00/0

Table I. Structural Statistics for *cis*- and *trans*-[32-Arg]Ascomycin

	cis-[32-Arg]ascomycin		*trans*-[32-Arg]ascomycin	
	SAi	AVm	SAi	AVm
rms dev from distance restraints (Å)[a]	0.014 ± 0.003	0.004	0.012 ± 0.003	0.009
rms dev from idealized geometry				
bonds (Å)	0.0038 ± 0.0002	0.0036	0.0039 ± 0.0003	0.0036
angles (deg)	0.59 ± 0.05	0.54	0.57 ± 0.03	0.53
impropers (deg)	1.99 ± 0.02	1.96	2.01 ± 0.02	1.98
conformational energy				
E_{NOE}[b] (kcal/mol)	0.67 ± 0.23	0.66	0.49 ± 0.26	0.24
$E_{L\text{-}J}$[c] (kcal/mol)	−15.9 ± 3.4	−24.2	−15.5 ± 3.3	−18.8
atomic rms diff for heavy atoms (Å)[d]	SAi vs AV	1.10 ± 0.19	SAi vs AV	0.97 ± 0.09
	SAi vs AVm	1.33 ± 0.62	SAi vs AVm	1.26 ± 0.38
	AV vs AVm	0.95	AV vs AVm	0.88

[a] SAi is the average over the final 25 structures; AV is the average structure; AVm is the average structure obtained after restrained energy minimization of AV. [b] E_{NOE} is the energy contribution from the square-well NOE potential using a force constant of 50 kcal/(mol·Å^2). [c] $E_{L\text{-}J}$ is the Lennard-Jones van der Waals energy calculated using the CHARMM function. [d] Superposition was performed on only the heavy atoms of the ascomycin portion of the molecule, i.e. the arginine side chain was excluded.

to address the question of whether or not a conformational change of the ligand occurs upon complex formation, the uncomplexed conformation of this analog in aqueous solution is compared to the conformation of ascomycin when bound to FKBP. NMR data obtained for the analog bound to FKBP indicate that it adopts a very similar conformation to that of FKBP-bound ascomycin. In addition, the uptake of [32-Arg]ascomycin by FKBP as a function of time was measured in order to determine the preference, if any, for binding to the *cis* or *trans* isomer and whether FKBP, a peptidyl-prolyl *cis*/*trans* isomerase, can catalyze the interconversion between these two forms.

Materials and Methods

Preparation of [32-Arg]Ascomycin. [32-Arg]Ascomycin was prepared by treating ascomycin (0.42 g, 0.53 mmol) in DMF (1.0 mL) with tri-Cbz-L-arginine *p*-nitrophenyl ester (0.53 mmol) and 4-(dimethylamino)-pyridine (0.26 mmol). After being stirred at room temperature for 5 days, the reaction mixture was diluted with ethyl acetate and washed sequentially with 0.2 M H_3PO_4, aqueous $NaHCO_3$, and brine. The organic phase was dried, concentrated, and purified by RP-HPLC (41.1 mm ID, Dynamax-60A, 8 μm phenyl, 10% CH_3OH in H_2O/acetonitrile gradient) to isolate the 32-tri-Cbz-Arg ester of ascomycin (0.41 g) and unreacted starting material (0.09 g). This product (0.4 g) and 10% Pd/C (0.08 g) in methanol (15.0 mL) were hydrogenated for 20 min. After filtration, the solvent was evaporated, and purification was done by RP-HPLC (41.4 mm ID, Dynamax-60A C18, H_2O/acetonitrile gradient with 0.1% TFA) to give 0.11 g of [32-Arg]ascomycin, $(M + K)^+ = 986$.

Preparation of ^{15}N,^{13}C-FKBP. Recombinant human FKBP-12 was cloned from a Jurkat T cell cDNA library and expressed in *E. coli* using the pKK233-2 vector containing a trc promoter as described elsewhere.[19] Uniformly ^{15}N,^{13}C-labeled FKBP was prepared by growing the FKBP-producing cells on minimal media containing ^{13}C-labeled acetate and ^{15}N-labeled ammonium chloride. The protein was purified from these cells using ion-exchange and size-exclusion chromatography.[19]

NMR Spectroscopy. All 2D spectra were recorded on a Bruker AMX600 spectrometer using a 1 mM sample of [32-Arg]ascomycin in D_2O buffer (50 mM potassium phosphate, 100 mM sodium chloride, pH 6.5). TOCSY spectra were obtained with mixing times of 16 and 48 ms and NOESY spectra were obtained with mixing times of 300 and 600 ms. Spectra were collected as $256(t_1) \times 1048(t_2)$ complex points with a sweep width of 10 000 Hz in both dimensions. Data were processed with in-house-written software on Silicon Graphics Computers. The final data size after zero-filling was $1024(\omega_1) \times 4096(\omega_2)$ complex points for each spectrum. One-dimensional isotope-filtered spectra[20,21] were recorded on a Bruker AMX500 spectrometer.

Structure Calculations. Distance geometry/simulated annealing calculations were carried out with the program XPLOR/DG.[22] Initial structures were embedded into Cartesian coordinate space followed by 200 steps of Powell restrained energy minimization to remove bad van der Waals contacts. During this minimization, and throughout the entire simulated annealing protocol, the NOE force constant was maintained at 50 kcal·mol^{-1}·Å^2, and electrostatic terms were excluded. The minimization step was followed by 7.5 ps of molecular dynamics (time step of 3 fs) at 2000 K during which the van der Waals force constant was decreased from its initial value of 20 kcal·mol^{-1}·Å^{-2} to a value of 0.003 kcal·mol^{-1}·Å^{-2} while increasing all other force constants (bond, angle, etc.). The structures were then cooled from 2000 to 100 K in steps of 50 K. Each step of the cooling process consisted of 1.25 ps of restrained molecular dynamics (time step of 5 fs). The van der Waals force constant was increased at each step by multiplying the previous value by 1.28 until a final value of 4.0 kcal·mol^{-1}·Å^{-2} was obtained. The van der Waals radius was decreased stepwise to a final values of 0.8 times the value used in CHARMM.[23] In the last stage of the refinement, the structures were subjected to 1000 steps of Powell restrained energy minimization using the full CHARMM Lennard-Jones potential.

Results and Discussion

Conformation of [32-Arg]Ascomycin in Aqueous Solution. Complete proton resonance assignments were obtained for the two sets of signals corresponding to the *cis* and *trans* isomers of [32-Arg]ascomycin from analysis of 2D total correlation spectroscopy (TOCSY) and nuclear Overhauser effect (NOE) data. The signals corresponding to the *cis* isomer were easily distinguished from those of the *trans* by comparing the chemical shifts of the protons at the 2 and 6 postions (Figure 1) which are diagnostic of the conformation of the amide bond.[8] From the 2D NOE data, 105 distance restraints were derived for the *cis* isomer, and 96 were derived for the *trans* isomer. These distance restraints were classified as either strong, medium, or weak corresponding to distances of 1.8–2.8, 1.8–3.4, and 1.8–4.4 Å, respectively, and pseudoatom corrections were applied to all distances involving methyl protons or non-stereospecifically assigned methylene protons.[24] For each isomer, 100 structures were calculated using a distance geometry/simulated annealing protocol with the program XPLOR/DG.[22] As shown in Table I, the 25 lowest-energy structures exhibit only small deviations from idealized covalent geometry and show good nonbonded contacts.

Figure 2A depicts the average, energy-minimized structure of the *trans* form of [32-Arg]ascomycin in aqueous solution (black) superimposed on the chloroform solution conformation of the *trans* form of FK506 (red). The three-dimensional structures of these molecules obtained in the two different solvents are clearly very different (RMSD = 2.8 Å). As shown in Figure 2B, the conformation of *trans*-[32-Arg]ascomycin in aqueous solution more closely resembles (RMSD = 1.5 Å) the FKBP-bound

(19) Edalji, R.; Pilot-Matias, T. J.; Pratt, S. D.; Egan, D. A.; Severin, J. M.; Gubbins, E. G.; Smith, H.; Park, C. H.; Petros, A. M.; Fesik, S. W.; Luly, J. R.; Burres, N. S.; Holzman, T. F. *J. Protein Chem.* **1992**, *11*, 213.
(20) Gemmecker, G.; Olejniczak, E. T.; Fesik, S. W. *J. Magn. Reson.* **1992**, *96*, 199.
(21) Petros, A. M.; Kawai, M.; Luly, J. R.; Fesik, S. W. *FEBS Lett.* **1992**, *308*, 309.
(22) Kuszewski, J.; Nigles, M.; Brunger, A. T. *J. Biomol. NMR* **1992**, *2*, 33.
(23) Brooks, B. R.; Bruccoleri, R. E.; Olafson, B. D.; States, D. J.; Swaminathan, S.; Karplus, M. *J. Comput. Chem.* **1983**, *4*, 187.
(24) Wüthrich, K.; Billiter, M.; Braun, W. *J. Mol. Biol.* **1983**, *169*, 949.

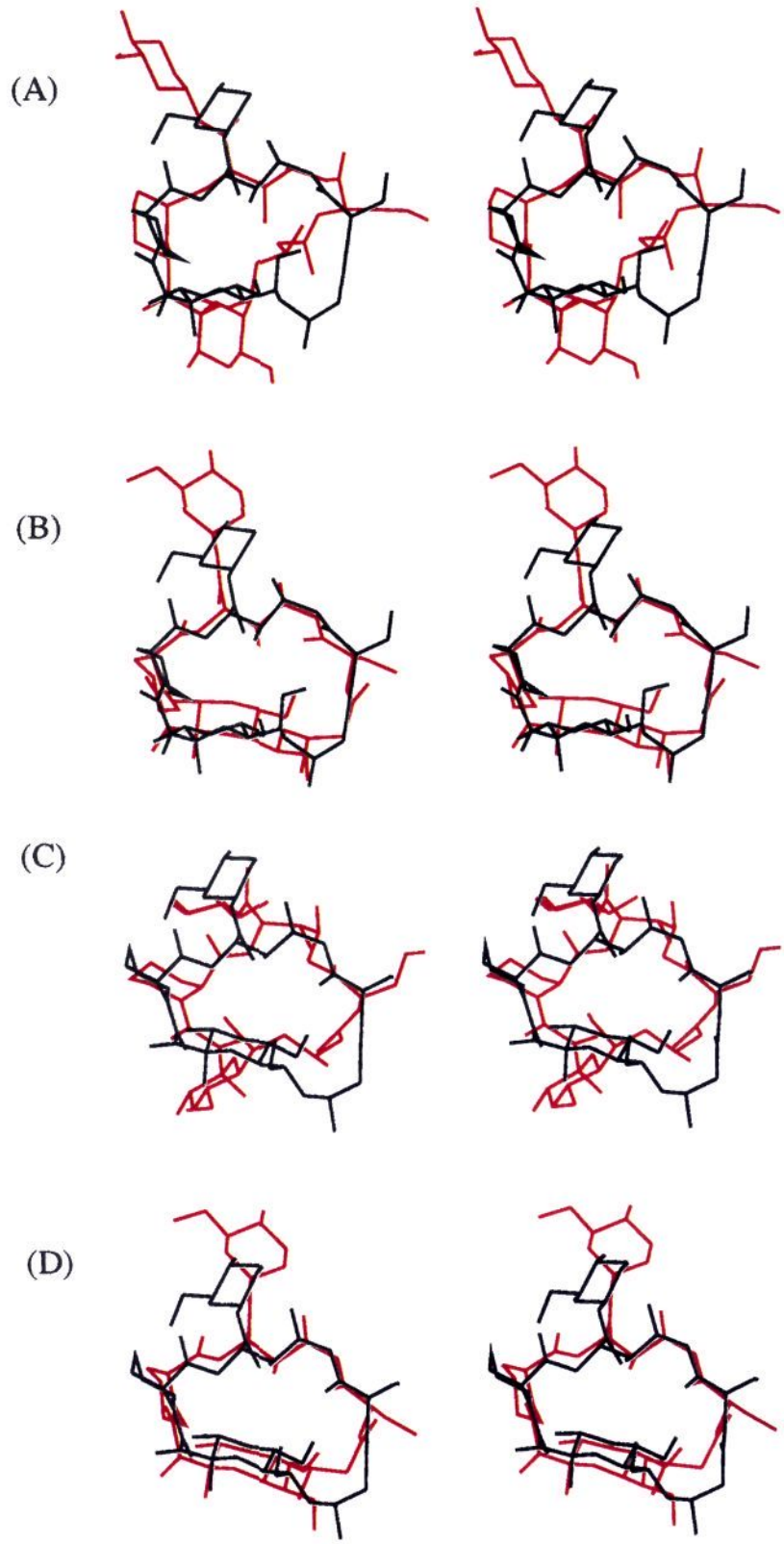


Figure 2. (A) Superposition of the average NMR structure of *trans*-[32-Arg]ascomycin in water (black) on *trans*-FK506 in chloroform (red); root-mean-square deviation (RMSD) of common heavy atoms is 2.8 Å. For clarity, the arginine residue of [32-Arg]ascomycin is not shown. (B) Superposition of the average NMR structure of *trans*-[32-Arg]ascomycin in water on ascomyin bound to FKBP; RMSD = 1.5 Å. (C) Superposition of the average NMR structure of *cis*-[32-Arg]ascomycin in water (black) on *cis*-FK506 in chloroform (red); RMSD = 2.7 Å. (D) Superposition of the average NMR structure of *cis*-[32-Arg]ascomycin in water on ascomycin bound to FKBP; RMSD = 1.4 Å.

conformation of ascomycin. Analogously, the conformation of the *cis* isomer of [32-Arg]ascomycin in aqueous solution (Figure 2C,D, black) is dissimilar (RMSD = 2.7 Å) to the *cis* isomer (Figure 2C, red) of FK506 in chloroform solution and in the solid state[7] (RMSD = 2.8 Å), but more closely resembles (RMSD = 1.4 Å) the conformation of ascomycin when bound to FKBP (Figure 2D, red). These results indicate that significant conformational alterations of FK506 are not required for FKBP

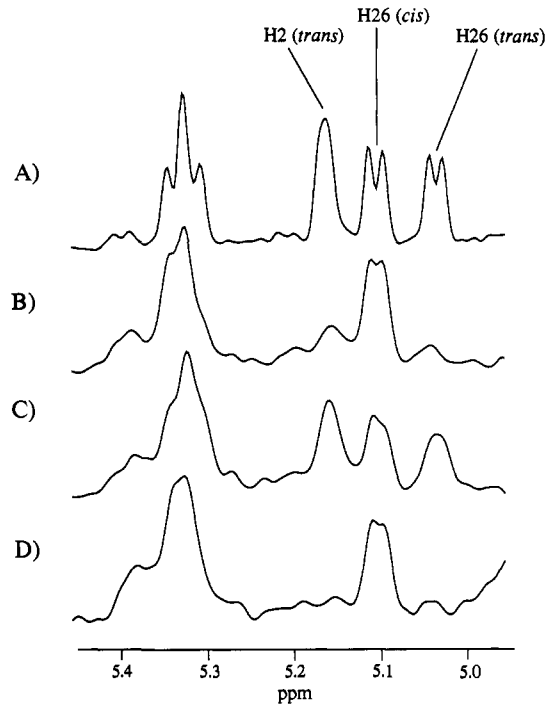


Figure 3. (A) Downfield region of a 500-MHz proton spectrum of [32-Arg]ascomycin in D_2O buffer, pH 6.5, 12° C. (B) Downfield region of a ^{13}C-filtered proton spectrum of [32-Arg]ascomycin recorded 3.8 min after addition of 0.5 mole equiv of uniformly ^{15}N,^{13}C-labeled FKBP. (C) Same as "B" except after 90.6 min. (D) Downfield region of a ^{13}C-filtered proton spectrum of [32-Arg]ascomycin recorded 3.6 min after addition of a slight molar excess of uniformly ^{15}N,^{13}C-labeled FKBP. To facilitate accurate intensity measurement, spectra recorded in the presence of FKBP were sensitivity enhanced by Lorentz–Gaussian multiplication.

complex formation and contradict earlier suggestions[14] that were based on a comparison between the conformations of FKBP-bound FK506 and uncomplexed FK506 in the solid state or in chloroform solution. In a hydrophobic solvent such as chloroform, the pyranose ring and the methyl group at the 27 position of FK506 are pointing away from the macrocyclic ring for both *cis* and *trans* isomers (Figure 2A,C). In contrast, for [32-Arg]-ascomycin in aqueous solution, these groups have collapsed inward forming a more compact macrocyclic ring which is maintained upon binding to FKBP (Figure 2B,D, red). As previously suggested for CsA,[25] such a hydrophobic collapse induced by an aqueous environment may play a key role in determining the bioactive conformations of hydrophobic molecules. The uncomplexed conformation of a molecule in the solid state or in a hydrophobic solvent may be very different from the bioactive conformation and should not generally be used as a guide for designing new drug molecules or interpreting structure/activity relationships. Furthermore, the fact that the bioactive conformation of CsA or FK506 could not be found using molecular dynamics even when performed in water[15,16] highlights the importance of experimentally determining the conformation of the ligand when bound to its target site.

FKBP Binding of [32-Arg]Ascomycin and Effects of *Cis*/*Trans* Isomerization. It is interesting to note that even though FK506 exclusively contains a *trans* 7,8 amide bond when bound to FKBP, both the *cis* and *trans* isomers of [32-Arg]ascomycin are similar and resemble the bound conformation of FK506. Is there a preferential uptake of the *cis* or *trans* isomer by FKBP? We addressed this question by recording a series of ^{13}C-filtered NMR spectra[20,21] of [32-Arg]ascomycin as a function of time after the addition of 0.5 mole equiv of uniformly ^{15}N- and ^{13}C-labeled FKBP in which the resonances corresponding to the labeled protein are suppressed. In the ^{13}C-filtered spectrum recorded 3.8 min

(25) Wiley, R. A.; Rich, D. H. *Med. Res. Rev.* **1993**, *13*, 327.

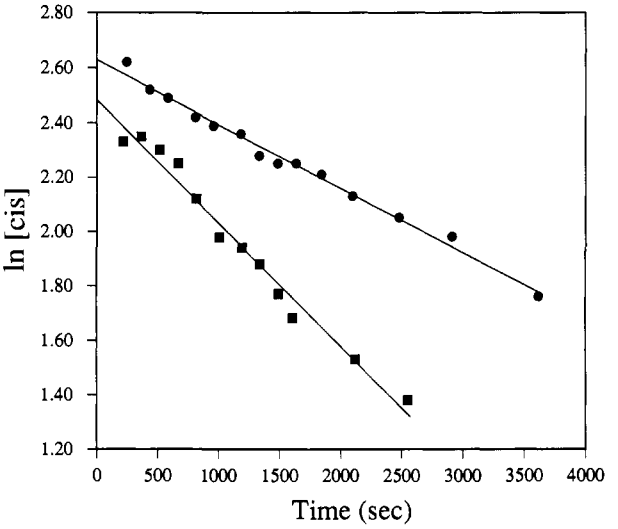


Figure 4. First-order rate plots for the disappearance of the *cis* form of [32-Arg]ascomycin after addition of 0.5 mole equiv (●) and a slight molar excess (■) of FKBP.

after addition of FKBP (Figure 3B), the NMR signals of the *trans* form of free [32-Arg]ascomycin are selectively decreased in intensity compared to these signals in the spectrum recorded in the absence of FKBP (Figure 3A). These results indicate that the *trans* isomer is preferentially binding to FKBP. After approximately 90 min, the thermodynamic *cis*/*trans* equilibrium is reestablished (Figure 3C). By following the decrease in intensity of the H26(*cis*) resonance as a function of time, the rate constant, k_c, for the *cis* to *trans* conversion was determined[26] to be 2.4×10^{-4} s^{-1}. Similar values have been obtained for the uncatalyzed *cis* to *trans* conversion of various proline-containing peptides.[27,28] This suggests that although FK506 has been postulated to mimic a peptide substrate for FKBP,[29] the enzyme does not catalyze the *cis*/*trans* isomerization of the ligand. Further evidence for this was obtained by comparing the rate of *cis*/*trans* isomerization in the presence of 0.5 mole equiv of FKBP to that in the presence of excess FKBP. When excess labeled FKBP was added to a solution of [32-Arg]ascomycin, the *trans* form was again selectively depleted (Figure 3D). Since in this case there is an excess amount of FKBP to bind the *trans* molecules as they are formed, no signal from the *trans* form of uncomplexed [32-Arg]-ascomycin appears in the spectrum, and the intensity of the NMR signals corresponding to the *cis* form now decreases with time. After approximately 45 min, all NMR signals corresponding to uncomplexed [32-Arg]ascomycin disappear. First-order rate plots for the disappearance of the *cis* form with time following addition of both 0.5 equiv and excess FKBP are shown in Figure 4. As can be seen by comparing the slope of the lines in this figure, the rate for *cis* to *trans* conversion upon addition of excess FKBP is approximately twice that obtained upon addition of 0.5 equiv of protein.[30] These results further support the notion that FKBP binds only the *trans* form of [32-Arg]ascomycin, and does not induce the *cis* to *trans* interconversion of the 7,8 amide bond.

(26) The rate equation for the disappearance of the *cis* form of [32-Arg]-ascomycin after addition of 0.5 mole equiv of FKBP is as follows: $-d[cis]/dt = k_c[cis] - k_t[trans]$, where k_c and k_t are the rate constants for the conversion of *cis* to *trans* and *trans* to *cis*, respectively. Since at equilibrium the *cis*/*trans* ratio is 1:1, it follows that k_c must equal k_t. The rate equation can therefore be simplified to $-d[cis]/dt = 2k_c[cis] - k_c[cis_0]$, where cis_0 is the initial concentration of the *cis* form. The rate constant, k_c, can be obtained from an exponential fit of the concentration of the *cis* form versus time.

(27) Harrison, R. K.; Stein, R. L. *J. Am. Chem. Soc.* **1992**, *114*, 3464.

(28) Grathwohl, C.; Wüthrich, K. *Biopolymers* **1981**, *20*, 2623.

(29) Albers, M. A.; Walsh, C. T.; Schreiber, S. L. *J. Org. Chem.* **1990**, *55*, 4984.

(30) If we assume that FKBP binds only the *trans* form of [32-Arg]-ascomycin, then the rate of disappearance of the *cis* form after addition of excess FKBP follows simple first-order kinetics: $-d[cis]/dt = k_c[cis]$, since there is now no competing back reaction. Thus the expected rate is double that obtained for addition of 0.5 mole equiv FKBP.

This specificity of FKBP for binding the *trans* form of [32-Arg]-ascomycin can be explained, at least in part, by the formation of a hydrogen bond between the C8 carbonyl oxygen of FK506 and the hydroxyl group of Tyr82[5] which could not be formed in the *cis* conformation. Furthermore, these results indicate that in aqueous solution (unlike in the solid state or in chloroform solution) half of the molecules of [32-Arg]ascomycin preexist in the bioactive conformation—poised for binding to FKBP. The other half need to undergo a conformational change from a *cis* to a *trans* 7,8 amide bond to allow binding to FKBP; however, this conformational change is not induced by the protein.

Acknowledgment. We thank Drs. Thomas Perun and George Carter for their support and encouragement. We also thank Dr. Horst Kessler (Technische Universtät München) for giving us the coordinates for the *cis* and *trans* forms for FK506 determined by NMR in chloroform solution and Dr. Daniel H. Rich (University of Wisconsin) for supplying us with a preprint of his manuscript (ref 25).