Substrate Specificity for the Human Rotamase FKBP: A View of FK506 and Rapamycin as Leucine-(Twisted amide)-Proline Mimics

Mark W. Albers,[1a,b] Christopher T. Walsh,[1a] and Stuart L. Schreiber*,[1b]

Department of Chemistry, Harvard University, Cambridge, Massachusetts 02138, and Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, Massachusetts 02115

Received May 14, 1990

Summary: The temperature dependence and the substrate specificity at the P1 site of the human rotamase enzyme FKBP were examined and support a view of the rotamase inhibitors FK506 and rapamycin as peptidomimetics of a leucine-(twisted amide)-proline dipeptide.

FKBP is a cytosolic protein that binds the potent immunosuppressant agents FK506 (1) and rapamycin (2) (Figure 1) with high affinity.[2] Like the cyclosporin A (3) binding protein, cyclophilin, FKBP catalyzes the interconversion of cis and trans rotamers of peptidyl-prolyl amide bonds of peptide substrates.[2] Both FK506 and rapamycin are potent inhibitors of the rotamase activity of FKBP but not of cyclophilin; likewise, cyclosporin A does not inhibit FKBP, yet is a potent inhibitor of cyclophilin. The therapeutic value of these compounds and their potent inhibition of the strikingly similar enzymatic activity of their target proteins has spurred an interest in the chemical mechanism of amide rotation by these enzymes and the mechanism of inhibition by these drugs. Although the first mechanistic studies on cyclophilin, which involved modification of the sulfhydryl groups and the determination of deuterium isotope effects, suggested a hemithioorthoamide intermediate,[3] recent investigations have provided evidence inconsistent with a mechanism for rotamase catalysis involving a tetrahedral intermediate.[4] Instead a mechanism has been proposed for cyclophilin based on the stabilization of a twisted amide intermediate (Figure 2, part A).[4]

In order to address the mechanism of amide rotation by FKBP, temperature-dependence studies were performed (vide infra) which indicate a high entropic contribution and a low enthalpic contribution to the activation barrier for the FKBP-catalyzed reaction. In addition, kinetic isotope studies exhibited a β-deuterium isotope effect ($k_H/k_D > 1$) for the FKBP-catalyzed reaction (data not shown). Both studies implicate the stabilization of a twisted amide intermediate as the mechanism of amide rotation for FKBP.

This mechanistic reasoning coupled with a ^{13}C NMR study[5] of the complex of a synthetic FK506 molecule with recombinant human FKBP (rhFKBP) has led to a proposal for the structural basis of FK506 and rapamycin inhibition of FKBP: allylic strain considerations explain the perpendicular relation of the α-keto amide dicarbonyl function in these agents, which mimic the geometry of the twisted amide transition state of a peptidyl-prolyl substrate (Figure 2, parts B-D).[6] This view of FK506 and rapamycin as twisted amide surrogates led to the question of whether their structures could reveal additional information about the nature of molecular recognition between FKBP and its substrates. Using the twisted amide surrogate as a frame of reference, the structures of FK506 and rapamycin were traced in both the "N-terminal" and the "C-terminal" direction.[7]

Tracing the structures in the "N-terminal" direction, a ketal hydroxyl group precedes the α-keto carbonyl and may mimic the amino group of the preceding amino acid in the native peptide substrate (Figure 3). Moreover, following the structure back along the "side chain" leads to a branched methine with methyl and methylene appendages. Taking into consideration the distance between the nitrogen atom and the α-keto carbonyl carbon (2.47 Å)[6] compared with a typical amide bond length (1.33 Å),[8] one can project that leucine, isoleucine, valine, or threonine may fit in the P1 pocket at the binding site of FKBP and therefore predict that the tetrapeptides with these residues at P1 should be superior substrates.[9]

Table I. Kinetic Constants and Catalytic Efficiencies of Various Peptide Substrates for the Rotamase Activity of Human FKBP

peptide[a]	$10^{-3}k_{obs}$,[b] s^{-1}	$10^{-3}k_{unc}$,[b] s^{-1}	$10^{-3}k_{enz}$,[c] s^{-1}	k_{enz}/k_{unc}	k_{cat}/K_m,[d] $\mu M^{-1}\ s^{-1}$
ALPF	99 ± 8	9.7 ± 0.2	89	9.2	2.2
AIPF	66 ± 1	6.5 ± 0.1	59	9.1	1.5
AnlePF[e]	68 ± 4	9.9 ± 0.2	58	5.9	1.5
AFPF	58 ± 7	5.9 ± 0.1	50	8.5	1.3
AVPF	33 ± 2	4.8 ± 0.1	28	5.8	0.70
AWPF	15.5 ± 0.3	3.6 ± 0.1	12	3.3	0.30
AAPF	18.5 ± 0.4	10.1 ± 0.1	8.4	0.83	0.21
AKPF	15.5 ± 0.5	9.7 ± 0.2	5.9	0.61	0.15
AQPF	10.9 ± 0.4	5.7 ± 0.1	4.9	0.86	0.12
AHPF	11.0 ± 0.2	6.8 ± 0.3	4.2	0.62	0.10
AEPF	5.0 ± 0.2	4.3 ± 0.4	0.75	0.17	0.019
AGPF	7.7 ± 0.2	6.9 ± 0.1	0.70	0.10	0.017

[a] Actual peptide structure: succinyl-alanyl-(varied amino acid)-prolyl-phenylalanyl-*p*-nitroanilide. [b] Values given as mean ± standard error of mean. Observed rate constants based on six or seven measurements and uncatalyzed rate constants based on four measurements. [c] $k_{enz} = k_{obs} - k_{unc}$. [d] Since $[S] \ll K_m$, the Michaelis-Menten equation simplifies to a first-order equation. Therefore, $k_{obs} = k_{unc} + (k_{cat}/K_m)[FKBP] \rightarrow k_{cat}/K_m = (k_{obs} - k_{unc})/[FKBP]$. [e] nle = norleucine.

(1) (a) Department of Biological Chemistry and Molecular Pharmacology. (b) Department of Chemistry.

(2) (a) Harding, M. W.; Galat, A.; Uehling, D. E.; Schreiber, S. L. *Nature* **1989**, *341*, 758. (b) Siekierka, J. J.; Hung, S. H. Y.; Poe, M.; Lin, C. S.; Sigal, N. H. *Nature* **1989**, *341*, 755.

(3) Fischer, G.; Wittmann-Liebold, B.; Lang, K.; Kiefhaber, T.; Schmid, F. X. *Nature* **1989**, *337*, 476.

(4) (a) Liu, J.; Albers, M. W.; Chen, C. M.; Schreiber, S. L.; Walsh, C. T. *Proc. Natl. Acad. Sci. U.S.A.* **1990**, *87*, 2304. (b) Harrison, R. K.; Stein, R. L. *Biochemistry* **1990**, *29*, 1684.

(5) (a) Rosen, M. K.; Standaert, R. F.; Galat, A.; Nakatsuka, M.; Schreiber, S. L. *Science* **1990**, *248*, 863. (b) Standaert, R. F.; Galat, A.; Verdine, G. L.; Schreiber, S. L. *Nature*, in press.

(6) For the crystal structure of FK506, see: (a) Tanaka, H.; Kuroda, A.; Marusawa, H. H.; Hatanaka, H.; Kino, T.; Goto, T.; Hashimoto, M.; Taga, T. *J. Am. Chem. Soc.* **1987**, *109*, 5031. For the crystal structure of rapamycin, see: (b) Swindells, D. C. N.; White, P. S.; Findlay, J. A. *Can. J. Chem.* **1978**, *56*, 2491. For the solution structure of rapamycin, see: (c) Findlay, J. A.; Radics, L. *Can. J. Chem.* **1980**, *56*, 591.

(7) Studies investigating the role of the "C-terminal" structure will be reported separately (unpublished results, H. Fretz and S.L.S.).

(8) Ramachandran, G. N.; Kolaskar, A. S.; Ramakrishnan, K.; Sasisekharan, V. *Biochim. Biophys. Acta* **1974**, *359*, 298.

(9) We have adopted the nomenclature proposed for protease substrates and enzymes (Berger, A.; Schecter, I. *Phil. Trans. R. Soc.* **1970**, *B257*, 249) to describe peptide substrates of and binding sites on rotamase enzymes. The peptide bond which is rotated is defined as the P1-P1′ bond and the adjacent residues are numbered sequentially in both directions, i.e. (...-P3-P2-P1-P1′-P2′-P3′-...).

0022-3263/90/1955-4984$02.50/0

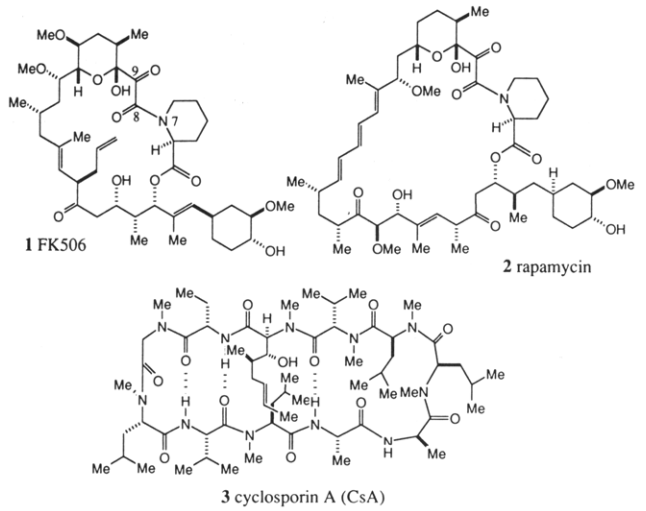


Figure 1. Structures of the immunosuppressive agents FK506, rapamycin, and cyclosporin A.

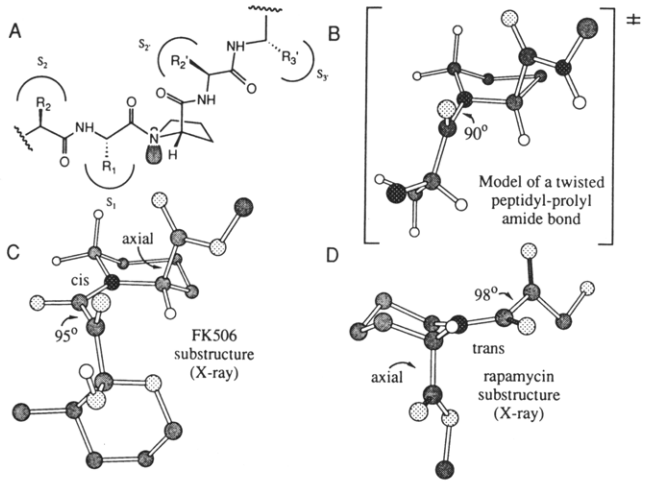


Figure 2. (A, B) Transition-state structure that is stabilized by rotamase, (C) partial structure of FK506, and (D) rapamycin illustrating perpendicular dicarbonyl.

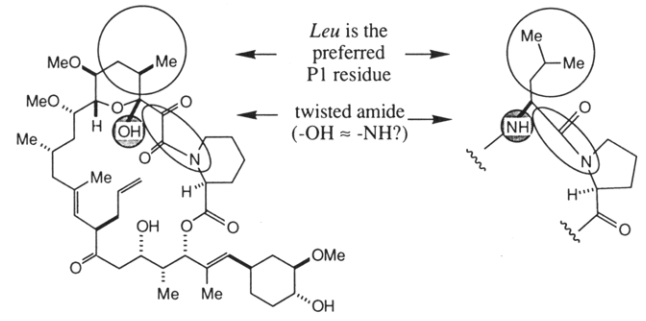


Figure 3. A view of FK506 as a Leu–(twisted amide)–Pro peptidomimetic.

We have tested these ideas by measuring the rate of amide rotation catalyzed by pure rhFKBP for a series of peptide substrates (BACHEM, Basel, Switzerland) of the general structure succinyl-alanyl-*X*-prolyl-phenylalanyl-*p*-nitroanilide (AXPF); the substrates differ only in the identity of the amino acid preceding the proline moiety.[10]

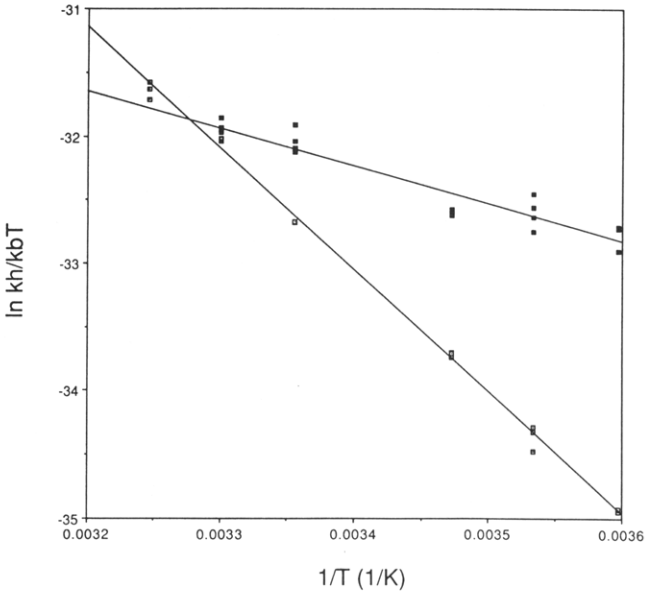


Figure 4. Eyring plot of FKBP-catalyzed and uncatalyzed processes. Experimental conditions are outlined in the text. (□) Uncatalyzed process. (■) Catalyzed process.

A modified version of the in vitro chymotrypsin-coupled rotamase assay of Fischer[11] in which chymotrypsin cleaves the chromogen *p*-nitroaniline from the remainder of the peptide in a conformationally dependent manner was used. The observed rates for the catalyzed and uncatalyzed reactions ± standard error of the mean as well as the rate constants for the FKBP-catalyzed reactions ($k_{obs} - k_{unc}$) are listed in Table I for each substrate examined. From these data k_{cat}/K_m (catalytic efficiency) can be calculated for each substrate and these values are listed in Table I as well.

The peptide with leucine in the P1 position was used to conduct temperature-dependence studies. Rates were determined between 5 °C and 30 °C using 20 nM rhFKBP and 50 μM peptide substrate (Figure 4). From the slope and intercept, $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ can be determined, respectively.[12] For the FKBP-catalyzed reaction $\Delta H^{\ddagger}$ = 5.85 kcal/mol and $\Delta S^{\ddagger}$ = −43.95 eu. However, for the uncatalyzed reaction, $\Delta H^{\ddagger}$ = 18.9 kcal/mol and $\Delta S^{\ddagger}$ = −1.16 eu. The striking difference in the temperature-dependence profiles indicate that the barriers to amide rotation with and without FKBP are distinct. The large entropic and small enthalpic contribution to $\Delta G^{\ddagger}$ in the catalyzed reaction suggests that the rate-determining step is physical rather than chemical and is consistent with a mechanism of selectively stabilizing a twisted amide, i.e. the binding energy is used principally to compensate for the loss of amide resonance.

Two trends from the data in Table I are evident: the P1 amino acids with hydrophobic side chains are better substrates than their charged counterparts, and branching in the side chain seems to be readily accommodated. The polar and charged P1 amino acid substrates all have catalytic efficiencies at least 1 order of magnitude less than the leucine and isoleucine substrates and as is evident from the ratios of the enzymatic rate to the uncatalyzed rate some of these substrates are barely catalyzed by FKBP. No trends are evident among the uncatalyzed and cata-

(10) During the preparation of this manuscript, an article appeared (Harrison, R. K.; Stein, R. L. *Biochemistry* **1990**, *29*, 3813) which investigated the substrate specificity of native FKBP using a subset of the panel of peptides examined in this paper. The results reported herein are consistent with and extend the original findings of these workers.

(11) (a) Fischer, G.; Bang, H.; Berger, E.; Schellenberger, A. *Biochim. Biophys. Acta* **1984**, *791*, 87. (b) Fischer, G.; Bang, H.; Mech, C. *Biomed. Biochim. Acta* **1984**, *43*, 1101.

(12) Segel, I. H. *Enzyme Kinetics*; John Wiley: New York, 1975; p 791.

lyzed rates for the peptides, indicating that FKBP is exercising specificity as a catalyst and not nonspecifically enhancing the uncatalyzed process for each peptide. Moreover, there is no evident trend among the enzymatic rates and the equilibrium constant of the two rotamers for each peptide (data not shown), indicating that the differences in enzymatic rates are not simply a reflection of the relative abundance of the cis peptide.

The rank order of this series of peptides is fully consistent with the aforementioned hypothesis regarding the nature of binding of FKBP by FK506 and rapamycin as twisted amide surrogates. The fact that predictions made from the structures of these inhibitors about the substrate specificity of the rotamase activity were upheld argues that the binding site of FK506 and rapamycin and the active site of the rotamase activity of FKBP are identical, i.e., FK506 and rapamycin are competitive inhibitors of the rotamase activity of FKBP—an issue which cannot be addressed directly due to limitations of the current assay. These results corroborate our view of FK506 and rapamycin as transition-state analogues for amide rotation by FKBP and extend the region of molecular recognition into the S1 site of FKBP. We conclude that the potent inhibition (e.g., K_i(rapamycin) = 2.0×10^{-10} M)[13] of the rotamase activity by these structures is in part due to the complementary topographical relationship between the binding site of FKBP and the immunosuppressive agents FK506 and rapamycin, which act as leucine–(twisted amide)–proline surrogates (Figure 3).

Acknowledgment. This research was supported by grants from the NIGMS (S.L.S., C.T.W.). M.W.A. is a Howard Hughes Medical Institute Predoctoral Fellow.

(13) Bierer, B. E.; Mattila, P. S.; Standaert, R. F.; Burakoff; S. J.; Crabtree, G.; Schreiber, S. L., submitted.

Asymmetric Cyclopropanation of 1-Alkenylboronic Esters and Its Application to the Synthesis of Optically Active Cyclopropanols

Toshiro Imai,* Hiroshi Mineta, and Shinya Nishida

Department of Chemistry, Faculty of Science, Hokkaido University, Sapporo 060, Japan

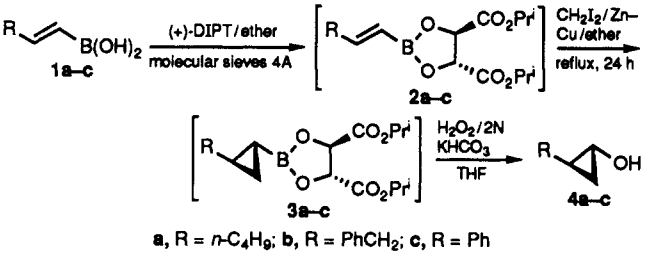
Received June 20, 1990

Summary: The first asymmetric cyclopropanation of 1-alkenylboronic esters was realized by diastereofacial selective Simmons–Smith reaction of the esters modified by enantiomerically pure diols such as tetramethyltartaramide. Subsequent oxidation of the resulting cyclopropylboronates gave optically active 2-substituted cyclopropanols in 73–94% ee. These reactive compounds possess high synthetic potential.

We report herein the asymmetric cyclopropanation of 1-alkenylboronic esters to the corresponding cyclopropylboronic esters, which, on subsequent oxidation, afford 2-substituted cyclopropanols of high optical purity.[1,2] To our knowledge, this is the first example of diastereofacial selection in enantiomerically pure 1-alkenylboronic esters.[3] Since the optically active cyclopropylboronic esters as well as the resultant cyclopropanols[4] could serve as versatile intermediates in enantioselective synthesis, the present, simple method for preparing these compounds should be of considerable value in synthetic organic chemistry.

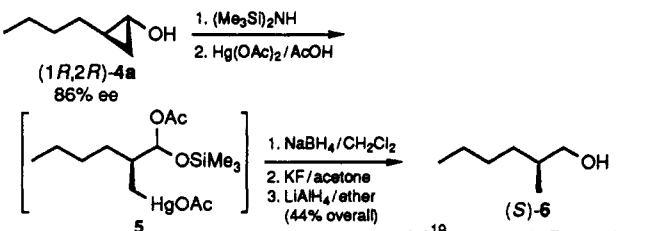
Recently, two new methods for preparing optically active cyclopropanols by asymmetric cyclopropanation of enol ether derivatives have appeared.[5,6] Although one of them has achieved a very high level of asymmetric induction, the types of cyclopropanols prepared are rather limited (mainly bicyclic tertiary alcohols).[5b] Our new method enables access to a different type of cyclopropanol (secondary ones bearing a vicinal substituent). These methods may, thus, be complementary for preparing this important class of compounds with various substitution patterns.

Scheme I



a, R = *n*-C_4H_9; **b**, R = $PhCH_2$; **c**, R = Ph

Scheme II



(1) A part of this study was presented in the 58th Annual Meeting of the Chemical Society of Japan, April 1–4, 1989, Abstracts-II, p 2004.

(2) With regard to the cyclopropanation of 1-alkenylboronic esters, the reaction with diazoalkanes in the presence of a rhodium catalyst has been reported very recently, but this work was not concerned with asymmetric induction: Fontani, P.; Carboni, B.; Vaultier, M.; Carrie, R. *Tetrahedron Lett.* **1989**, *30*, 4815.

(3) For a review on the asymmetric synthesis with boronic esters, see: Matteson, D. S. *Acc. Chem. Res.* **1988**, *21*, 294.

(4) (a) DePuy, C. H. *Acc. Chem. Res.* **1968**, *1*, 33. (b) Gibson, D. H.; DePuy, C. H. *Chem. Rev.* **1974**, *74*, 605. (c) Murai, S.; Ryu, I.; Sonoda, N. *J. Organomet. Chem.* **1983**, *250*, 121. (d) Weber, W. P. *Silicon Reagents for Organic Synthesis*; Springer-Verlag: Berlin, 1983; Chapter 14, p 235.

(5) (a) Sugimura, T.; Futagawa, T.; Tai, A. *Tetrahedron Lett.* **1988**, *29*, 5775. (b) Sugimura, T.; Futagawa, T.; Yoshikawa, M.; Tai, A *Ibid.* **1989**, *30*, 3807.

(6) Kunz, T.; Reissig, H.-U. *Tetrahedron Lett.* **1989**, *30*, 2079.

0022-3263/90/1955-4986$02.50/0