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Substrate Specificities of the Peptidyl Prolyl Cis–Trans Isomerase Activities of Cyclophilin and FK-506 Binding Protein: Evidence for the Existence of a Family of Distinct Enzymes

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ABSTRACT: Substrate specificities, as reflected in k_c/K_m, were determined for the peptidyl prolyl cis–trans isomerase activities of cyclophilin and the FK-506 binding protein (FKBP). The substrates investigated were peptides of the general structure Suc-Ala-Xaa-Pro-Phe-*p*-nitroanilide, where Xaa = Gly, Ala, Val, Leu, Phe, His, Lys, on Glu. While k_c/K_m for cyclophilin-catalyzed isomerization shows little dependence on Xaa, k_c/K_m values for FKBP-catalyzed isomerization display a marked dependence on Xaa and vary over 3 orders of magnitude. An important outcome of this work is the discovery that Suc-Ala-Leu-Pro-Phe-pNA is a reactive substrate for FKBP (k_c/K_m = 640 000 M^{-1} s^{-1}). This substrate can be used with FKBP concentrations that are low enough to allow, for the first time, accurate determinations of K_i values for tight-binding inhibitors of FKBP. Using this new assay, we found that FK-506 inhibits FKBP with K_i = 1.7 ± 0.6 nM. The results of this work support the hypothesis that cyclophilin and FKBP are members of a family of peptidyl prolyl cis–trans isomerases and that the members of this family possess distinct substrate specificities that allow them to play diverse physiologic roles.

Cyclosporin A and FK-506 are structurally unrelated immunosuppressive drugs (Sawada et al., 1987) whose mechanisms of action are thought to involve the initial binding to two distinct intracellular proteins, cyclophilin and FKBP,[1] respectively (Warty et al., 1988; Siekierka et al., 1989a). Significantly, both of these proteins possess peptidyl prolyl cis–trans isomerase activity (Fischer et al., 1989; Takahashi et al., 1989; Harding et al., 1989; Siekierka et al., 1989). Furthermore, the PPI activities of cyclophilin and FKBP can be inhibited by their respective ligands with no cross-inhibition (Fischer et al., 1989; Takahashi et al., 1989; Harding et al., 1989; Siekierka et al., 1989).

The chemical reaction that cyclophilin and FKBP catalyze, prolyl cis–trans isomerization, is a facile process: at 37 °C, the first-order rate constant for spontaneous cis to trans isomerization of the PPI substrate, Suc-Ala-Ala-*cis*-Pro-Phe-pNA, is about 0.15 s^{-1} (Harrison & Stein, 1990). This translates into a reaction half-time of less than 5 s. An obvious issue of some concern is whether cyclophilin and FKBP can effect rate accelerations that are of any real physiologic importance. For example, to realize only a 100-fold rate acceleration over background, a cyclophilin concentration of 5 μM would have to be attained.[2] An even higher concentration would be needed for the less efficient FKBP: given the difference in specific activity between cyclophilin and FKBP[3]

[1] Abbreviations: CsA, cyclosporin A; FKBP, FK-506 binding protein; α-CT, α-chymotrypsin; PPI, peptidyl prolyl cis–trans isomerase; Suc-Ala-Ala-Pro-Phe-pNA, *N*-carboxypropionyl-Ala-Ala-Pro-Phe-*p*-nitroanilide.

[2] The calculation of this enzyme concentration is based on the expression $100k_u = (k_c/K_m)$[cyclophilin], where k_u is the first-order rate constant for uncatalyzed isomerization. Upon rearrangement, this expression becomes [cyclophilin] = $100k_u/(k_c/K_m)$. Given that k_c/K_m = 3 μM^{-1} s^{-1} at 37 °C [Table I and the temperature independence of k_c/K_m as demonstrated by Harrison and Stein (1990)] and that k_u is 0.15 s^{-1} at 37 °C, we can calculate [cyclophilin] = 100(0.15 s^{-1})/3 μM^{-1} s^{-1} = 5 μM.

[3] Schreiber and his co-workers reported that they used an FKBP concentration of 0.67 nM in their standard assay of PPI activity (Harding et al., 1989). This concentration is about 1000-fold lower than the concentration used by Siekierka et al. (1989b) and us in the same assay. This discrepancy has now been resolved by recent personal communications with Dr. Schreiber in which he indicated that a typographical error was made in their publication (Harding et al., 1989): 0.67 μM, not 0.67 nM, FKBP was used. Thus, data from all three laboratories can be used to calculate the same approximate k_c/K_m value of 50 μM^{-1} s^{-1} for the FKBP-catalyzed cis to trans isomerization of Suc-Ala-Ala-*cis*-Pro-Phe-pNA at 10 °C.

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(Siekierka et al., 1989b), an FKBP concentration of 0.3 mM would be needed for the same 100-fold rate acceleration.

These large PPI concentrations raise the following question: How can cyclophilin and FKBP, with their apparent crucial roles in immunoregulation, produce such poor catalytic accelerations? To answer this, one can argue for high intracellular concentrations of PPI. Or, one can propose the existence of critical prolyl isomerizations that need only a minimum of acceleration. However, neither of these solutions is satisfying; one is still left with the feeling that the cellular cost of synthesizing such high concentrations of these enzymes is hardly worth their modest levels of catalysis. A resolution to this paradox might be found by exploring the substrate specificities of these enzymes. Recall that the concentrations of PPI that we calculated were based on kinetic data for the reaction of Suc-Ala-Ala-Pro-Phe-pNA, the only PPI substrate for which kinetic data exist. Clearly, if specific substrates exist for these enzymes, more impressive catalytic accelerations might be attainable at more reasonable concentrations of enzyme.

We now report an initial study of the substrate specificities of the PPI activities of cyclophilin and FKBP. To probe the specificity of these enzymes toward small peptide substrates, we determined k_c/K_m values for the PPI-catalyzed cis to trans isomerization of substrates of the general structure Suc-Ala-Xaa-Pro-Phe-pNA, where Xaa = Gly, Ala, Val, Leu, Phe, His, Lys, or Glu. We found that while cyclophilin does not discriminate among these substrates, FKBP is keenly sensitive to Xaa. The results of this work support the hypothesis that cyclophilin and FKBP are members of a family of peptidyl prolyl cis–trans isomerases and that the members of this family possess distinct substrate specificities that allow them to play diverse physiologic roles.

An important practical outcome of this study is the discovery of an FKBP substrate, Suc-Ala-Leu-Pro-Phe-pNA, that is 12-times more active than the standard PPI substrate, Suc-Ala-Ala-Pro-Phe-pNA. With this new substrate, we can use concentrations of FKBP that are low enough to allow accurate determinations of K_i values for tight-binding inhibitors.

Materials and Methods

General. Buffer salts were from Sigma Chemical Co. Water was distilled and passed through a deionizer. FKBP and cyclophilin were purified according to published procedures (Harding et al., 1986; Siekierka et al., 1989b) and provided to us by Dr. John Siekierka of the Department of Immunology Research, Merck Research Laboratories. CsA and FK-506 were provided by Dr. Matt Wyvratt of the Department of Medicinal Chemistry, Merck Research Laboratories.

Substrates. Substrates of structure Suc-Ala-Xaa-Pro-Phe-pNA, where Xaa is Gly, Ala, Val, Leu, Phe, His, Lys, or Glu, were prepared by BACHEM, Switzerland; 2.2 mM stock solutions of substrates were prepared in DMSO and diluted into buffer to concentrations between 70 and 75 μM.

Kinetic Methods (Harrison & Stein, 1990). In a typical kinetic experiment, 2.900 mL of a 70–75 μM stock solution of Suc-Ala-Xaa-Pro-Phe-pNA in buffer and 0 or 0.015 mL of a stock solution of PPI were added to a 4-mL disposable polystyrene cuvette, and the cuvette was placed in the jacketed cell holder of an Aviv Model 14DS spectrophotometer. Constant temperature was maintained within the cuvette by water circulated from a Brinkmann RM6 water bath. Temperature variation during a kinetic run was less than 0.02 °C. After the reaction solution had reached thermal equilibrium ($\geq$15 min), we started the reaction by the addition of 0.100 mL of a 2.24 mM stock solution of α-CT in 1 mM HCl. Reaction progress was monitored by the absorbance change at 390 nm ($\Delta\epsilon$ = 13400) that accompanies the hydrolysis of the anilide bond and the release of *p*-nitroaniline product. For each kinetic run, between 300 and 1000 data points, corresponding to (time, OD_{390}) pairs, were collected by an AT&T PC 6300 microcomputer interfaced to the spectrophotometer. Since these reactions were all conducted at a concentration of Suc-Ala-Xaa-*cis*-Pro-Phe-pNA much less than its K_m for PPI, the progress curves, after the initial rapid α-CT-catalyzed consumption of Suc-Ala-Xaa-*trans*-Pro-Phe-pNA, were first-order in substrate and could be fit to a simple first-order rate law. The analysis program was written by Dr. Phil Huskey (Chemistry Department, Rutgers University, Newark, NJ).

Results

Assay of Peptidyl Prolyl Cis–Trans Isomerization. The assay of prolyl isomerization has been described previously (Fischer et al., 1984; Harrison & Stein, 1990). Briefly, the assay relies on the inability of α-CT to hydrolyze Suc-Ala-Xaa-*cis,trans*-Pro-Phe-pNA when the Xaa–Pro bond is in its *cis* conformation. Thus, if a sufficiently high concentration of α-CT is added to an equilibrated solution of Suc-Ala-Xaa-Pro-Phe-pNA, Suc-Ala-Xaa-*trans*-Pro-Phe-pNA will be rapidly hydrolyzed to Suc-Ala-Xaa-*trans*-Pro-Phe and *p*-nitroaniline before the remaining Suc-Ala-Xaa-*cis*-Pro-Phe-pNA returns to its cis–trans equilibrium. In typical assays, the α-CT concentration is between 50 and 100 μM, and the initial burst of Suc-Ala-Xaa-*trans*-Pro-Phe-pNA hydrolysis takes less than 10 s. The hydrolysis of the remaining Suc-Ala-Xaa-*cis*-Pro-Phe-pNA is rate limited by its conversion to Suc-Ala-Xaa-*trans*-Pro-Phe-pNA.

In the presence of PPI, the following kinetic expression holds:

$$k_{obs} = k_u + \frac{k_c}{K_m}[PPI] \quad (1)$$

where k_{obs} is the observed, first-order rate constant for isomerization, k_u is the first-order rate constant for uncatalyzed, spontaneous isomerization, and $(k_c/K_m)[PPI]$ is the pseudo-first-order rate constant for enzyme-catalyzed isomerization. Thus, k_c/K_m can be calculated according to a rearranged form of eq 1:

$$\frac{k_c}{K_m} = \frac{k_{obs} - k_u}{[PPI]} \quad (2)$$

We see then that calculation of accurate k_c/K_m values requires accurately determined PPI concentrations. As we will see below, PPI concentrations can be determined from titration of cyclophilin with CsA and FKBP with FK-506.

k_c/K_m Values for PPI Catalysis. Using the general assay methods described above, we determined k_c/K_m values for the cyclophilin- and FKBP-catalyzed cis to trans isomerization of substrates of structure Suc-Ala-Xaa-Pro-Phe-pNA. These data are summarized in Table I and indicate different substrate specificities for the two enzymes. The most reactive substrate toward cyclophilin, Suc-Ala-Ala-Pro-Phe-pNA, has a k_c/K_m value (3.2×10^6 M^{-1} s^{-1}) that is only 5 times greater than the k_c/K_m value for the least reactive substrate, Suc-Ala-His-Pro-Phe-pNA ($k_c/K_m = 6.0 \times 10^5$ M^{-1} s^{-1}). In contrast, the most reactive substrate toward FKBP, Suc-Ala-Leu-Pro-Phe-pNA, has a k_c/K_m value (6.4×10^5 M^{-1} s^{-1}) that is 3 orders of magnitude greater than that of the least reactive substrate, Suc-Ala-Glu-Pro-Phe-pNA ($k_c/K_m = 6 \times 10^2$ M^{-1} s^{-1}). Thus, while cyclophilin has a broad specificity toward

Table I: Specificity of Peptidyl Prolyl Cis–Trans Isomerases toward Peptide Substrates of the Structure Suc-Ala-Xaa-Pro-Phe-pNA[a]

Xaa	K_{eq}[c]	k_u (10^{-3} s^{-1})	k_c/K_m (mM^{-1} s^{-1})[b]	
			cyclophilin	FKBP
Gly	0.204 ± 0.016	7.1 ± 0.8	1190 ± 120	1.2 ± 1.1
Ala	0.103 ± 0.040	9.4 ± 1.2	3180 ± 880	53 ± 3
Val	0.071 ± 0.007	3.8 ± 0.7	3180 ± 530	170 ± 8
Leu	0.097 ± 0.014	7.6 ± 0.7	2700 ± 250	640 ± 48
Phe	0.235 ± 0.016	5.3 ± 0.3	1390 ± 108	620 ± 140
His	0.135 ± 0.001	6.2 ± 0.7	600 ± 30	28 ± 1
Lys	0.060 ± 0.004	9.1 ± 0.8	920 ± 90	28 ± 0.5
Glu	0.091 ± 0.001	3.8 ± 0.5	2140 ± 330	0.6 ± 0.5

[a] Reactions were conducted at pH 7.8 in buffered solutions containing 50 mM HEPES. Final concentrations in reaction solutions wer [CT] = 74 μM and [Suc-Ala-Xaa-Pro-Phe-pNA] = 70 μM. Temperature = 10 °C. [b] Values of k_c/K_m were calculated according to the expression $k_c/K_m = (k_{obs} - k_u)/[E]$, where k_{obs} is the observed first-order rate constants determined in the presence of enzyme, k_i is the rate constant for nonenzymatic isomerization, and [E] is the concentration of cyclophin or FKBP. [cyclophilin] = 0.010 μM, by titration with CsA; [FKBP] = 0.33 μM, by titration with FK-506. Error limits are standard deviations of the means of three or four determinations. [c] K_{eq} = [cis]/[trans].

these peptides, FKBP has a narrow specificity with a preference for hydrophobic residues at P_1.[4]

Values of K_{eq} and k_u are also summarized in Table I. Neither of these parameters correlates with k_c/K_m values for either cyclophilin- or FKBP-catalyzed reactions. Such correlations would be expected if enzymic reactivity paralleled inherent chemical reactivity.

Determination of K_i Values for the Tight-Binding Inhibition of PPI. K_i values for the inhibition of cyclophin by CsA and FKBP by FK-506 were determined from the dependence of k_{obs} on inhibitor concentration. For cyclophilin, k_{obs} values were determined with the standard PPI substrate, Suc-Ala-Ala-Pro-Phe-pNA, while for FKBP Suc-Ala-Leu-Pro-Phe-pNA was used. For both K_i determinations, the concentration of protein was adjusted such that $k_{control}$ was $4k_u$, where $k_{control}$ is the observed rate constant in the presence of enzyme and in the absence of inhibitor.

The data are shown in Figure 1 along with the best-fit lines calculated by a nonlinear regression analysis of the data according to the equation of Williams and Morrison (1979) for tight-binding inhibition.[5] From this analysis, we obtain both K_i and $[E]_0$. For the experiments measuring the inhibition of cyclophilin by CsA, we calculate K_i = 5.6 ± 0.5 nM and $[E]_0$ = 9.9 ± 1.1 nM, while in our experiments with FKBP and FK-506 K_i = 1.7 ± 0.6 nM and $[E]_0$ = 50 ± 2 nM. Since these K_i values were determined at $[S]_0 \ll K_m$, they are true dissociation constants for the E·I complex.

Discussion

Inhibition of the PPI Activities of Cyclophilin and FK-506 Binding Protein. An important outcome of this work is our finding that Suc-Ala-Leu-Pro-Phe-pNA is 12 times more reactive toward FKBP than the substrate that is commonly used to measure PPI activity, Suc-Ala-Ala-Pro-Phe-pNA. With this new substrate, K_i values for tight-binding inhibitors of FKBP can be more accurately determined since much less enzyme can now be used in inhibition assays. Thus, we are able to determine that FK-506 inhibits FKBP with a K_i value of 1.7 nM and that inhibition is complete (i.e., $k_{inhib}/k_{control}$ approaches 0 at high [FK-506], see Figure 1B) and not time dependent (data not shown). This K_i value is similar to the K_d value of 0.8 nM found by Siekierka et al. (1989b) for the competitive displacement of [^{3}H]dihydroFK-506 from FKBP by unlabeled dihydroFK-506 but disagrees with the results of Harding et al. (1989), who report that FK-506 is a partial inhibitor of the PPI activity of FKBP with a K_i of 50 nM. This anomalous value may have resulted from the large FKBP concentration that Harding and his co-workers had to use in their assays[3] due to the low reactivity of Suc-Ala-Ala-Pro-Phe-pNA toward FKBP (see Table I).

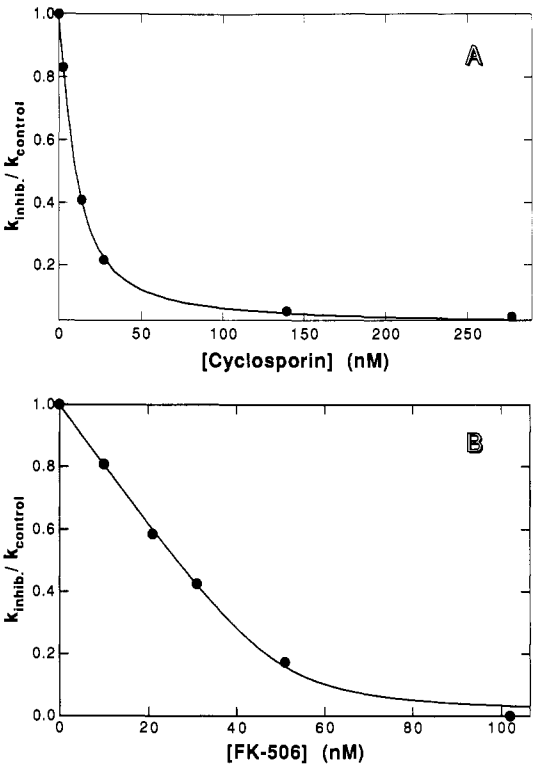

FIGURE 1: (A) Inhibition of the PPI activity of cyclophilin by CsA. (B) Inhibition of the PPI activity of FKBP by FK-506. See text for details.

The inhibition of the PPI activity of cyclophilin by CsA is complete and characterized by a K_i of 5.6 nM. This result is similar to that of Fischer et al. (1989b), who found a K_i of 2.6 nM.

Substrate Specificity of Cyclophilin and FK-506 Binding Protein. The results of Table I reveal a dramatic difference in substrate specificity between cyclophilin and FKBP. While cyclophilin has a broad specificity and cannot discriminate among P_1 amino acid residues, FKBP has a narrow specificity and is acutely sensitive to the identity of the residue at P_1.

We believe that the mechanistic origin of these unique specificities lies in the way in which these enzymes interact with their substrates and probably does not involve a fundamental difference in active site chemistry. For cyclophilin, there is no obvious correlation between k_c/K_m and any structural feature of the P_1 amino acid residue. This suggests that the extended active site of this enzyme does not a have a well-defined S_1 subsite. However, for FKBP, we see a strong positive correlation between k_c/K_m and hydrophobicity of the P_1 amino acid side chain. This correlation is demonstrated in Figure 2 for un-ionizable side chains where we plot $\Delta\Delta G^*$

[4] For reference to amino acid residues of PPI substrates and their corresponding enzyme subsites, we will adopt the nomenclature system of Schecter and Berger (1967) that is commonly used in protease chemistry. According to this system, if isomerization occurs at the P_1–$P_{1'}$ bond and $P_{1'}$ is Pro, the amino acid residues of the peptide substrate are named P_n-...P_3-P_2-P_1-$P_{1'}$-$P_{2'}$-$P_{3'}$-...-$P_{n'}$, while the corresponding enzyme subsites are named S_n...S_3-S_2-S_1-$S_{1'}$-$S_{2'}$-$S_{3'}$-...-$S_{n'}$.

[5] The program that we used to fit the tight-binding inhibition data was written by Nancy Thornberry of the Department of Enzymology, Merck Research Laboratories.

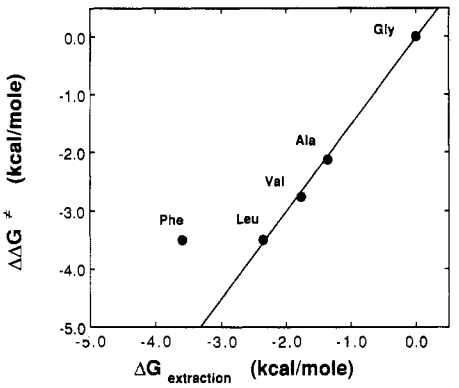

FIGURE 2: Correlation of $\Delta\Delta G^{\ddagger}$ for the FKBP-catalyzed isomerization of Suc-Ala-Xaa-Pro-Phe-pNA with ΔG for the extraction of the side chain of Xaa from water into 1-octanol. $\Delta\Delta G^{\ddagger}$ is calculated as $\Delta G^{\ddagger}_{Xaa} - \Delta G^{\ddagger}_{Gly}$. The linear regression best-fit line was calculated and drawn with the data point for Suc-Ala-Phe-Pro-Phe-pNA excluded.

vs $\Delta G_{extraction}$, where $\Delta\Delta G^{\ddagger}$ is calculated as $\Delta G^{\ddagger}_{Xaa} - \Delta G^{\ddagger}_{Gly}$ (or RT ln $[(k_c/K_m)_{Gly}/(k_c/K_m)_{Xaa}]$; T = 283 °C) and $\Delta G_{extraction}$ is the free-energy change for the extraction of the amino acid side chain from water into 1-octanol (Hansch & Coats, 1970). The slope of this line, 1.51 ± 0.03 (r^2 = 0.998), indicates that the active site of FKBP, in the rate-limiting transition state, is 1.5 times more hydrophobic than 1-octanol.

This correlation explains the P_1 specificity of FKBP in that it suggests that the S_1 subsite of FKBP is hydrophobic. Therefore, the enzyme is able to establish strong transition state interactions only with substrates that have hydrophobic P_1 side chains. Similar correlations have been observed for the P_1 specificity of α-CT (Dorovskaya et al., 1972) and the $P_{1'}$ specificity of thermolysin (Pank et al., 1982; Izquierdo and Stein, unpublished results). X-ray crystallographic data are available for these two proteases and support the existence of hydrophobic, S_1 "pockets" (Steitz & Shulman, 1982; Kester & Matthews, 1977).

It is interesting that this correlation predicts a k_c/K_m value of 2.2 × 10^7 M^{-1} s^{-1} for the FKBP-catalyzed isomerization of Suc-Ala-Phe-Pro-Phe-pNA. This value is about 7 times greater than experiment (3.2 × 10^6 M^{-1} s^{-1}). We can offer two explanations for this substrate being an outlier: (1) P_1 specificity is determined not only by hydrophobity but also by the size and/or shape of the P_1 residue. For this explanation to hold, we must also propose that Phe is too large to fit into the S_1 pocket. (2) The observed k_c/K_m value of 3.2 × 10^6 M^{-1} s^{-1} is near the upper limit for bimolecular reaction of FKBP and tetrapeptides. The data that we currently have do not allow us to distinguish between these two mechanistic alternatives.

Conclusions. Several investigators have suggested that cyclophilin and FKBP may be members of a family of peptidyl prolyl cis–trans isomerases (Harding et al., 1989; Siekierka et al., 1989) and, in particular, that lymphocytes contain specific substrates or unique forms of PPI that can account for the selective sensitivity of this cell type to CsA and FK-506 (Siekierka et al., 1989). The results of this study indicate that cyclophilin and FKBP have dramatically different substrate specificities and, therefore, support this hypothesis. Furthermore, these results suggest that selective inhibitors can be designed for cyclophilin, FKBP, and other members of this class of enzyme.

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