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Specific Interaction between Bovine Cyclophilin A and Synthetic Analogues of Cyclolinopeptide A

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Like cyclosporin A, cyclolinopeptide A binds specifically bovine cyclophilin A, inhibiting its peptidyl-prolyl *cis-trans* isomerase activity. We describe here the protein interaction with several synthetic analogues of cyclolinopeptide A, which are either homodetic or disulphide bridged heterodetic cyclopeptides characterized by different ring dimensions, in terms of dissociation and inhibition constants evaluated by fluorescence and inhibition of the enzyme activity, respectively. Dissociation constants from fluorescence experiments are practically identical and about 20-fold lower than for cyclosporin A. On the other hand, inhibition constants differ from compound to compound and are higher than for cyclosporin A. This result is therefore difficult to rationalize, but we would suggest decoupling between binding and inhibitory ability of cyclopeptides. The Pro[1] residue of cyclolinopeptide A seems to play a fundamental role in determining the inhibition of the rotamase activity of cyclophilin A, as the homodetic analogue lacking this residue does not show any inhibitory ability. Similarly, heterodetic analogues with a ring size smaller than 7 residues do not display inhibition. We presume that the sequence -Pro-Pro-Phe-Phe- and a ring size of 8 residues for homodetic cyclic peptides could be used as starting points in the targeted synthesis of cyclopeptides able to bind both cyclosporin A and calcineurin. The only peptide showing similar values of the dissociation and inhibition constant is cyclolinopeptide A. This compound can be considered a novel model for the molecular design of immunosuppressant drugs.

Key words: cyclosporin A, cyclopeptides, cyclolinopeptide A, fluorimetric binding constants, rotamase activity, PPIase inhibition constants.

The natural peptide cyclosporin A (CsA) (*1*) and the macrolide FK506 (*2*) are well known immunosuppressive drugs used in the treatment of autoimmune diseases and in the prevention of transplanted organ rejection (*3, 4*). CsA and FK506 bind cyclophilin (Cyp) (*5*) and FK-506 binding protein (FKBP) (*6, 7*), respectively. These proteins belong to two phylogenetically distinct superfamilies with peptidyl-prolyl *cis-trans* isomerase activity. The members of these families are therefore named PPIases or rotamases (*8-13*). The complexes Cyp-CsA and FK506-FKBP interact specifically with the Ca^{2+}-calmodulin dependent serine/threonine phosphatase calcineurin (CN) (*14-16*). These ternary complexes inhibit an early step in the T-lymphocyte activation pathway responsible for the immune response (*17*). The molecular basis of immunosuppressant-PPIase binding has been investigated, both in solution and in the solid state, by means of NMR (*18, 19*) and X-ray crystallography (*20-23*). At present efforts are being made to design CsA and FK506 analogues with the aim of synthesizing drugs that display similar *in vivo* activity but less toxic side effects.

We have recently reported that the natural cyclic peptide cyclolinopeptide A [cyclo(-Pro[1]-Pro[2]-Phe[3]-Phe[4]-Leu[5]-Ile[6]-Ile[7]-Leu[8]-Val[9]-)] (CLA) binds specifically bovine cyclophilin A (CypA), inhibiting its PPIase activity (*24*). The X-ray structure of this cyclic compound shows a Pro[1]-Pro[2] *cis* peptide bond (*25*). We have shown (*26*) that CLA is able to assume a *trans* conformation around the Pro[1]-Pro[2] residues in the presence of Ba^{2+}. We have also hypothesized that the Val[9]-Pro[1]-Pro[2]-Phe[3] moiety might be involved in the binding to CypA (*24*). However, CLA does not interact with FKBP or show any immunosuppressive activity. This feature is presumably ascribable to the remarkable structural differences with the CsA domain involved in the binding to CN. We describe here the interaction between CypA and several synthetic analogues of CLA, which are either homodetic or heterodetic cyclopeptides with different ring size. The focus of the present article is on the amino acid sequence involved in the binding to CypA, which was

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Abbreviations: Ac_6c, 1-amino-1-cyclohexane-carboxylic acid; Aib, α-aminoisobutyric acid; Boc, *N-tert*-butoxy-carbonyl; CLA, cyclolinopeptide A; CN, calcineurin; CsA, cyclosporin A; CypA, cyclophilin A; DMSO, dimethylsulfoxide; FKBP, FK-506 binding protein; PPIase, peptidyl-prolyl *cis-trans* isomerase.

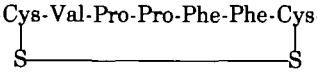
monitored through the fluorescence due to the unique protein tryptophan. Circular dichroism experiments and spectrophotometric assays of PPIase activity inhibition were also performed in order to ascertain whether CLA could constitute a convenient basis for planning peptidomimetic compounds to be used as potential immunosuppressant drugs.

MATERIALS AND METHODS

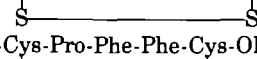
Bovine CypA—The enzyme from calf thymus was purified according to Gallo *et al.* (*24*) to yield a protein sample with specific activity of 84 U_I/mg. The purity of this sample was checked by SDS-PAGE, reversed phase (RP) HPLC, and sequence analysis. The enzyme was used in spectroscopic experiments and activity assays without any further manipulation.

CLA Analogues—The following peptides were used throughout the experiments:

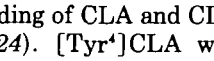
1) NH_2-Phe-Phe-βAla-Pro-βAla-COOH — Test peptide
2) c-(Pro-Pro-Phe-Phe-Leu-Ile-Ile-Leu-Val) — CLA
3) c-(Pro-Pro-Phe-Phe-Aib-Aib-Ile-D-Ala-Val) — CLAIB
4) c-(Pro-Pro-Phe-Phe-Ac_6c^5-Ile-D-Ala-Val) — [Ac_6c^5]CLA
5) c-(Ala-Pro-Phe-Phe-Leu-Ile-Ile-Leu-Val) — [Ala^1]CLA
6) c-(Pro-Ala-Phe-Phe-Leu-Ile-Ile-Leu-Val) — [Ala^2]CLA
7) c-(Pro-Pro-Ala-Phe-Leu-Ile-Ile-Leu-Val) — [Ala^3]CLA
8) c-(Pro-Pro-Phe-Ala-Leu-Ile-Ile-Leu-Val) — [Ala^4]CLA
9) c-(Pro-Pro-Phe-Tyr-Leu-Ile-Ile-Leu-Val) — [Tyr^4]CLA
10) Boc-Cys-Val-Pro-Pro-Phe-Phe-Cys-OMe — Cys^7-CLA



11) Boc-Cys-Pro-Pro-Phe-Phe-Cys-OMe — Cys^6-CLA



12) Boc-Cys-Pro-Phe-Phe-Cys-OMe — Cys^5-CLA



The binding of CLA and CLAIB has been described previously (*24*). [Tyr^4]CLA was a kind gift from Dr. I.Z. Siemion of Warsaw University. All other peptides were synthesized and characterized in our laboratory (*27-29*). The synthetic test peptide NH_2-Phe-Phe-βAla-Pro-βAla-COOH was used to verify how a linear peptide affects fluorescence spectra. This compound is a PPIase substrate.

Fluorescence—Fluorescence emission spectra of CypA were recorded at room temperature on a Perkin Elmer MPF 66B model spectrofluorometer equipped with a thermostatable cell holder and a Grant model LTD 6 water circulating bath to control the temperature. Spectra were scanned from 310 to 420 nm with excitation at either 295 or 289 nm to minimize energy transfer between tyrosine and tryptophan. Other experimental settings were: scan speed, 120 nm/min; time response, 2 s; bandwidths, 5 nm. To ensure fluorescence linearity the protein concentration was taken in the range 470-980 nM ($A_{280} \leq 0.008$). Titrations were performed by adding small aliquots of either CLA or one of its analogues dissolved in 10 mM HEPES/1 mM EDTA buffer at pH 7.5. After each addition the solution was allowed to stand for a few minutes until equilibrium was reached, as judged by the time constancy of the fluorescence signal. Usually, this happened within 5 min after addition of the peptide. Then, the fluorescence spectrum was recorded. Stock solutions of all peptides (0.4-2 mM), including the linear test peptide NH_2-Phe-Phe-βAla-Pro-βAla-COOH, were prepared using spectroscopic grade ethanol (Aldrich). Fluorescence titration curves were drawn to represent the relative change of the fluorescence at 339 nm, $\nu = (F_0 - F)/F_0$, as a function of the logarithm of the total peptide concentration. Assuming a 1:1 peptide: protein complex, the apparent dissociation constant ($K_{d,app}$) can be estimated as half the total peptide concentration at $\nu = 0.5$.

Rotamase Activity Assay—The PPIase activity was monitored by a slightly modified version of the coupled assay described by Fischer *et al.* (*9*). In the rate determining step the *cis*→*trans* isomerization of the substrate peptide *N*-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide around the Ala^2-Pro^3 peptide bond takes place. Then, the Phe^4-*p*-nitroanilide moiety of the *trans* conformer is quickly hydrolyzed by the addition of chymotrypsin. The reaction can be therefore monitored by the absorbance of *p*-nitroaniline at 390 nm, which displays a linear time dependence within 1-2 min after the addition of chymotrypsin to the assay mixture. In a typical experiment at 20°C the substrate peptide was dissolved in 100 mM Tris- HCl/0.2% DMSO buffer, pH 7.5, to a final concentration of 194 μM (0.9 ml final volume). This solution was allowed to reach thermal equilibrium (~10 min). Then CypA was added to a final concentration of 49 nM, as judged by the protein absorbance using $\varepsilon_{280} = 8{,}100\ M^{-1} \cdot cm^{-1}$ (*24*), and allowed to catalyze the *cis*→*trans* isomerization for 1 min. After this time, the hydrolysis of the substrate peptide was started by adding 1 μg of chymotrypsin (Sigma, Italy) from a 0.05 mg/ml stock solution in 1 mM HCl and monitored by absorbance at 390 nm. The inhibition of the rotamase activity was assayed under the same experimental conditions by adding small aliquots of each peptide (0-9,800 nM) to a pre-incubated solution containing the substrate peptide and CypA. After peptide addition, the mixture was allowed to stand until equilibrium was established, which happened within 5 min (see fluorescence experiments). Then chymotrypsin was added, determining the fast hydrolysis of the substrate peptide. The initial rate of the reaction was a linear function of time, which occurrs under steady-state conditions (*30*). As the concentration of peptide required to cause inhibition was comparable to the enzyme concentration, the treatment of data was accomplished by the theory for tight binding inhibition (*30, 31*). Residual activity percentages (average of 5 experiments) were therefore used to evaluate apparent inhibition constants by the equation

$$\frac{V_I}{V_0} = \frac{1}{2E_t}\left[\sqrt{(K_{I,app} + I_t - E_t)^2 + 4K_{I,app}E_t} - (K_{I,app} + I_t - E_t)\right] \quad (1)$$

This equation is a general steady-state rate equation that describes reversible tight binding inhibition irrespective of the type of inhibition (*30, 31*). Here V_I and V_0 are the enzymatic rates in the presence and absence of inhibitor, respectively, I_t and E_t are the total inhibitor and enzyme concentrations, respectively, and $K_{I,app}$ represents the apparent inhibition constant.

Circular Dichroism—CD spectra were recorded in molar ellipticity units at 25°C on a JASCO 500-A spectropolarimeter, using 0.1 cm quartz cells (Hellma). Scansion of spectra was performed from 200 to 250 nm, using a scan speed of 10 nm/min, a time constant of 8 s, and a step resolution of 0.2 nm. CypA solutions with a concentration

ranging from 0.5 to 0.93 mg/ml ($0.23 \leq A_{280} \leq 0.42$) in 10 mM HEPES buffer at pH 7.4 were titrated with increasing amounts of CLA, Cys^7-CLA, or Cys^6-CLA. Small aliquots of each peptide were added from 2 mM stock solutions in spectroscopic grade ethanol. After each addition three spectra were recorded, corrected for blank, and averaged. Then the protein secondary structure was estimated according to Chang *et al.* (*32*).

RESULTS

Preliminary fluorescence titrations were performed using the synthetic test peptide NH_2-Phe-Phe-βAla-Pro-βAla-COOH, in order to characterize the interaction of the enzyme with a linear peptide acting as a substrate. This peptide induces a red shift of the fluorescence emission maximum of CypA from 339 to 363 nm (Fig. 1), which is ascribable to the water exposure of the unique protein Trp and is typical of the interaction of the protein with a substrate molecule. It has been reported (*33, 34*) that the activation mechanism of PPIase requires the presence of water molecules in the catalytic site, where the Trp of CypA is located. The fluorescence behavior of the CLA-CypA binding is totally different. In this case no shift of the emission maximum takes place. Moreover, there is a decrease of the fluorescence emission, which reaches a constant value when the peptide:protein molar ratio is approximately 1:1 (*24*).

Most of the studied cyclopeptides behave like CLA, as they did not induce any shift of the emission maximum of the protein fluorescence spectrum. As an example, the dependence of the fractional fluorescence change (ν) on the concentration of some analogues is shown in Fig. 2. The apparent dissociation constant, $K_{d,app}$, of the peptide-protein complex can be calculated as half the total ligand concentration at $\nu = 0.5$. Values for all peptides examined as well as for CsA, CLA, and CLAIB are summarized in Table I. All the homodetic analogues, except for [Ala^1]CLA, and the heterodetic Cys^7-CLA bind CypA tightly, with $K_{d,app}$ values lower than that of CsA and close to that of CLA. The fluorescence behavior of [Ala^1]CLA seems particularly interesting. This peptide is the first of a series ([Ala^1]CLA through [Ala^4]CLA) of analogues synthesized by substituting in turn one residue of the sequence Pro^1-Pro^2-Phe^3-Phe^4 of CLA with Ala. The substitution of Pro^2, Phe^3, and Phe^4 ([Ala^2]CLA through [Ala^4]CLA) does not affect the ability of the peptide to bind CypA. On the other hand, [Ala^1]CLA shifts the emission maximum of CypA from 339 nm to 345 nm. A similar shift takes place for the heterodetic Cys^6-CLA. These peptides behave therefore like the linear test peptide, suggesting that the Pro^1 residue of CLA could play a fundamental role in determining the inhibition of the rotamase activity of CypA. Although the Pro^1 residue is present in the sequence of the heterodetic analogue Cys^6-CLA, this peptide induces an appreciable red shift of the fluorescence spectrum of CypA from 339 to 355 nm (data not shown), displaying a behavior similar to that of [Ala^1]CLA and Cys^5-CLA. However, the ability of Cys^7-CLA to bind CypA even more tightly ($K_{d,app} = 2.9 \pm 0.6$ nM) than other peptides suggests that the ring size also

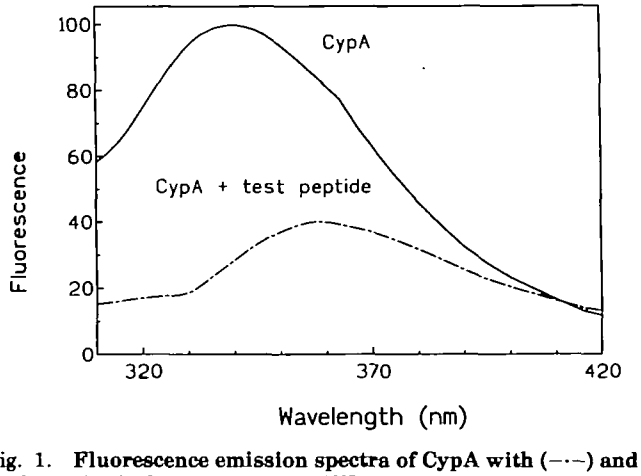


Fig. 1. **Fluorescence emission spectra of CypA with (---) and without (—) the test peptide NH_2-Phe-Phe-βAla-Pro-βAla-COOH.** Spectra excited at 289 nm were scanned from 310 to 420 nm with a scan speed of 120 nm/min, a time response of 2 s, and bandwidths of 5 nm. The protein concentration was in the range 470-980 nM in 10 mM HEPES/1 mM EDTA buffer at pH 7.5. The test peptide was dissolved in the same buffer solution.

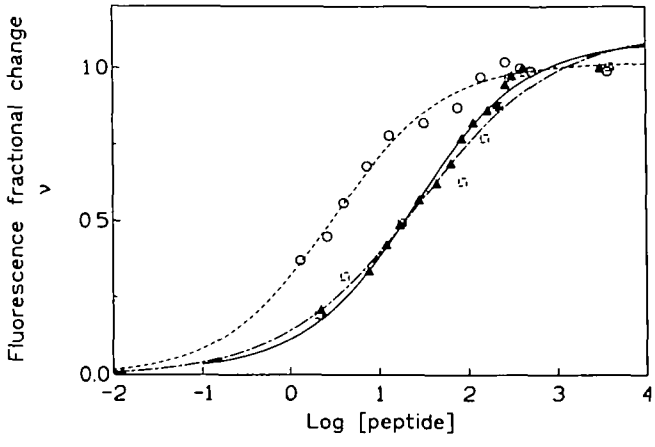


Fig. 2. **CypA binding curves of CLA and some heterodetic analogues: (▲) CLA, (□) [Ala^1]CLA, (○) Cys^7-CLA.** The protein concentration was in the range 470-980 nM in 10 mM HEPES/1 mM EDTA buffer at pH 7.5. Titrations were performed by adding small aliquots of either CLA or one of its analogues dissolved in the same buffer solution. The fluorescence intensity was recorded at 339 nm. Other instrumental settings were the same as in Fig. 1.

TABLE I. **Values of $K_{d,app}$ estimated by fluorimetric titrations.**

Peptide	$K_{d,app}$ (nM ± SD)
Test peptide	—[a]
CsA	205.9 ± 0.5
CLA	12.5 ± 0.6
CLAIB	12.7 ± 1.9
[Ac$_6$c^5]CLA	12.0 ± 1.8
[Ala^1]CLA	—[a]
[Ala^2]CLA	13.6 ± 4.4
[Ala^3]CLA	13.2 ± 3.3
[Ala^4]CLA	12.8 ± 3.5
[Tyr^4]CLA	7.0 ± 2.6
Cys^7-CLA	2.9 ± 0.6
Cys^6-CLA	—[a]
Cys^5-CLA	—[a]

[a]The behavior of these peptides is similar to that of the substrate peptide. The protein emission maximum is red-shifted as a consequence of peptide addition.

TABLE II. **Values of $K_{i,app}$ estimated by inhibition assays.**

Peptide	[Peptide]:[CypA]	Residual activity (%)	$K_{i,app}$ (nM ± SD)
Test peptide	200:1	105	—[a]
CsA	20:1	16.2	9.9 ± 2.2
CLA	20:1	9.7	6.6 ± 2.6
CLAIB	20:1	41.7	96 ± 28
[Ac_6c^5]CLA	100:1	46.8	3,124 ± 653
[Ala^1]CLA	200:1	122	—[a]
[Ala^2]CLA	45:1	30.4	802 ± 87
[Ala^3]CLA	220:1	59.9	10,838 ± 2,280
[Ala^4]CLA	180:1	17.0	2,932 ± 445
[Tyr^4]CLA	100:1	91.3	63,483 ± 10,807
Cys^7-CLA	100:1	21.2	926 ± 277
Cys^6-CLA	100:1	131.7	—[a]
Cys^5-CLA	100:1	129.4	—[a]

[a]The behavior of these peptides is similar to that of the substrate peptide. The protein emission maximum is red-shifted as a consequence of peptide addition.

plays a role in determining the kind of interaction between protein and peptides. Owing to solubility limitations, PPIase inhibition assays were performed using a different molar excess for each peptide. Therefore, peptide:protein molar ratios ranged from 45:1 to 1,000:1 for the least ([Ala^2]CLA) and the most soluble ([Ac_6c^5]CLA) compound, respectively. The inhibition of the PPIase activity is characterized by inhibition constants that span a quite large interval of values (Table II). As observed for dissociation constants, [Ala^1]CLA, Cys^6-CLA, and Cys^5-CLA show a peculiar behavior, because they do not inhibit the rotamase activity at all. As regards other peptides, Cys^7-CLA and [Ala^2]CLA show the lowest inhibition constant ($K_{i,app}$ = 926 ± 277 and 802 ± 87 nM, respectively), after CsA, CLA, and CLAIB. Also, the substitution of Phe^4 with Ala does not seem to be as important as the substitution with Tyr. [Ac_6c^5]CLA and [Ala^4]CLA show almost equal inhibition constants ($K_{i,app}$ = 3,124 ± 653 and 2,932 ± 445 nM, respectively), in spite of the fact that the structure of [Ac_6c^5]CLA is strongly constrained (*35*). On the other hand, [Tyr^4]CLA has the highest inhibition constant among the peptides examined ($K_{i,app}$ = 63,483 ± 10,807 nM).

The last set of experiments involved circular dichroism studies of the protein interaction with the heterodetic peptides Cys^7-CLA and Cys^6-CLA. Even if the dichroic activity of the protein decreased, and small alterations of the spectrum were observed, the relative secondary structure amounts remained approximately constant. As in the case of CsA, CLA, and CLAIB (*24*), heterodetic peptides do not seem to determine appreciable structural rearrangements of CypA (Table III). Due to the lack of X-ray data on bovine CypA, we have compared the CD secondary structure estimates for this protein with those obtained by X-ray on human CypA (*36*). As can be appreciated from Table III, these estimates are nearly coincident and presumably reflect the very high sequence similarity between the two proteins.

TABLE III. **Effect of heterodetic peptides on the secondary structure of CypA.**

	Protein secondary structure			
	α-helix (%)	β-sheet + β-turn (%)	Random (%)	[peptide]:[protein]
CypA	24	41	35	—
hCypA[a]	21	43	36	—
Cys^7-CLA/CypA	22	43	35	2:1
Cys^6-CLA/CypA	25	43	32	2:1

[a]From Ref. *36*.

DISCUSSION

The most important observation to emerge from Tables I and II is that the dissociation constants for all the synthetic analogues are virtually identical to each other, all being about 20-fold lower than CsA. The inhibition constants, on the other hand, which also reflect dissociation of the enzyme-inhibitor complex, show that all the peptides except CLA are poorer inhibitors of the PPIase activity than would be expected on the basis of the dissociation constants. Even CsA displays such a dichotomy between dissociation and inhibition, but with the reversed trend. It is important to stress that the reliability of our data appears to be proved by independent determinations of $K_{d,app}$ and $K_{i,app}$ for CsA. For this compound the fluorimetric dissociation constant was found to be 200 nM (*37*), which well compares with the estimate of 206 nM in Table I. Similarly, the known inhibition constant for CsA binding to bovine CypA, evaluated by enzyme kinetics, is 6.9 nM (*38*), which is in good agreement with the value of 10 nM in Table II. These findings suggest decoupling of the interactions leading to binding and inhibition. It appears, therefore, that the catalytic pocket could be not entirely coincident with the binding site. This point has also been addressed by other authors (*31, 39*). We believe that it deserves further study.

As far as the structure of the peptide-protein complex is concerned, a hypothetical model can be built using the X-ray structure of the binding site of recombinant human (rh) CypA when complexed by CsA (*20*) and the all *trans* structure of CLA (*26*). This choice is dictated by the fact that the X-ray structure of bovine CypA as well as that of protein bound CLA are not available. According to this procedure, Fig. 3 shows that the Pro^2-Phe^3-Phe^4 residues of CLA interact with rhCypA through three direct hydrogen bonds. These involve the $N^{\epsilon 1}$ of Trp^{121} and the C′O of Pro^2, the N^{ϵ} of Arg^{55} and the C′O of Phe^3, and the $N^{\epsilon 2}$ of Gln^{63} and the C′O of Phe^4. In addition, a few hydrophobic contacts between rhCypA and the all *trans* CLA structure further stabilize the proposed complex, involving the CypA Phe^{60}, Met^{61}, Ala^{103}, Leu^{112}, Phe^{113}, Thr^{119} residues and the Val^9-Pro^1-Pro^2-Phe^3-Phe^4 tract of CLA. In particular, it can be noticed that the Phe^4 side chain of CLA is buried inside a hydrophobic pocket of CypA, interacting with Phe^{60} and Phe^{113}. The replacement of Phe^4 with residues characterized by side chains with different steric hindrance ([Ala^4]-CLA) and/or carrying hydrophylic groups ([Tyr^4]CLA) can destabilize the peptide/protein complex (see Table II). As regards Pro^1, which is not directly involved in the interaction with the protein surface (see Fig. 3), it is likely that this residue is necessary to render the *trans* conformation of the peptide suitable for insertion into the binding site. Furthermore, the Pro^1 replacement ([Ala^1]CLA) or deletion (Cys^5-CLA) causes a lack of inhibitory ability. In conclusion, the comparison between the CsA/rhCypA complex and the hypothetical CLA/CypA complex shows that the Val^9-Phe^4 segment of CLA is topologically similar to Ala^7-$MeVal^{11}$ of CsA, and that a minimum ring size of 8 residues for homodetic cyclic peptides is needed for the right orien-

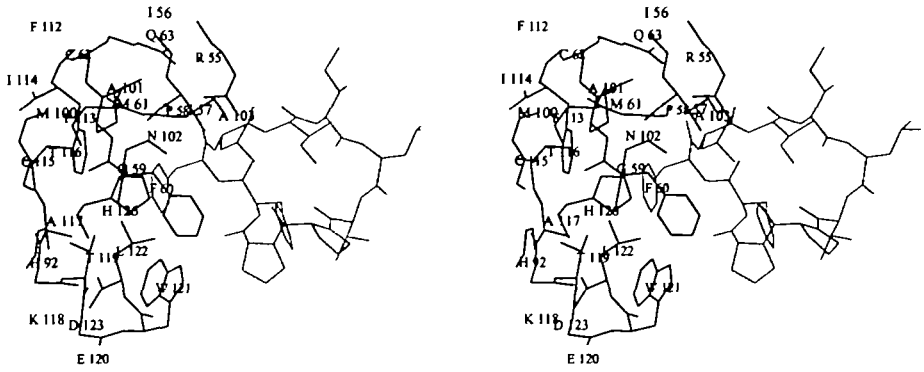


Fig. 3. **Stereodrawing detail of the binding site of the proposed CypA/CLA complex structure.** The all *trans* CLA molecule is drawn in dotted lines. The CypA residues in the binding site are marked with single letter code.

tation of the Pro^2-Phe^4 tract in the binding site. Despite the topological similarities between all *trans* CLA and CsA, there is no analogy between the two cyclic molecules in the residues that are presumed to interact with CN.

CONCLUSION

We have studied the interaction between CypA and synthetic analogues of CLA in order to understand the minimal amino acid sequence responsible for complex formation. Among the peptides studied, CLA, CLAIB, [Ac$_6$c^5]CLA, and Cys^7-CLA have the common sequence Val-Pro-Pro-Phe-Phe. The homodetic Ala-analogue series provided evidence that the substitution of the Pro residue in position 2 and the Phe residues in position 3 or 4 does not substantially affect binding to the enzyme, but it does influence the inhibition constants. On the other hand, in [Ala^1]CLA the substitution of Ala for the Pro^1 residue was efficacious in modifying both the fluorescence properties and the inhibitory ability. This happens also for the heterodetic analogue Cys^5-CLA, which not only lacks the Pro^1 residue but also has a ring size of five residues. This peptide does not inhibit the rotamase activity at all. The lack of inhibitory ability of the heterodetic Cys^6-CLA is exclusively attributable to a ring size smaller than 7 residues. On the basis of the tentative model for the CLA/CypA complex, we presume that the sequence -Pro-Pro-Phe-Phe and a ring size of 8 residues for homodetic cyclic peptides could be used as starting points in the targeted synthesis of cyclopeptides able to bind both CypA and CN. However, while all of the CLA-related peptides show almost equal $K_{d,app}$, the $K_{i,app}$ values are very different, and the effect of substitutions is difficult to rationalise. This could mean that the binding ability of peptides and their inhibitory power are at least in part decoupled, as even in CsA these properties do not seem to be related. Work is in progress on this aspect, and new peptides are being planned in our laboratory, with the aim of discovering novel immunosuppressant molecules.

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REFERENCES

1. Borel, J.F., Feurer, C., Gubler, H.U., and Staelin, H. (1976) Biological effects of cyclosporin A: a new antilymphocytic agent. *Agents Actions* **6**, 468-475
2. Kino, T., Hatanaka, H., Hashimoto, M., Nishiyama, M., Goto, T., Okuhara, M., Kohsaka, M., Aoki, H., and Imanaka, H. (1987) FK-506, a novel immunosuppressant isolated from a Streptomyces. I. Fermentation, isolation, and physico-chemical and biological characteristics. *J. Antibiot. (Tokyo)* **40**, 1249-1255
3. Borel, J.F. (1989) Pharmacology of cyclosporine (Sandimmune). 4. Pharmacological properties *in vivo*. *Pharmacol. Rev.* **41**, 259-370
4. Di Padova, F.E. (1994) Pharmacology of CsA and FK-506. *Perspect. Drug Discov. Design* **2**, 49-56
5. Handschumacher, R.E., Harding, M.W., Rice, J., Drugge, R.J., and Speicher, D.W. (1984) Cyclophilin: a specific cytosolic binding protein for cyclosporin A. *Science* **226**, 544-547
6. Harding, M.W., Galat, A., Uehling, D.E., and Schreiber, S.L. (1989) A receptor for the immunosuppressant FK506 is a *cis-trans* peptidyl-prolyl isomerase. *Nature* **341**, 758-760
7. Siekierka, J.J., Hung, S.H.Y., Poe, M., Lin, S.C., and Sigal, N.H. (1989) A cytosolic binding protein for the immunosuppressant FK506 has peptidyl-prolyl isomerase activity but is distinct from cyclophilin. *Nature* **341**, 755-757
8. Harding, M.W., Handschumacher, R.E., and Speicher, D.W. (1986) Isolation and amino acid sequence of cyclophilin. *J. Biol. Chem.* **261**, 8547-8555
9. Fischer, G., Wittmann-Liebold, B., Lang, K., Kiefhaber, T., and Schmid, F. (1989) Cyclophilin and peptidyl-prolyl *cis-trans* isomerase are probably identical proteins. *Nature* **337**, 476-478
10. Takahashi, N., Hayano, T., and Suzuki, M. (1989) Peptidyl-prolyl *cis-trans* isomerase is the cyclosporin A-binding protein cyclophilin. *Nature* **337**, 473-475
11. Albers, M.W., Walsh, C.T., and Schreiber, S.L. (1990) Substrate specificity for the human rotamase FKBP. A view of FK506 and rapamycin as leucine (twisted amide) proline mimics. *J. Org. Chem.* **55**, 4984-4986
12. Harrison, R.K. and Stein, R.L. (1990) Mechanistic studies of peptidyl prolyl *cis-trans* isomerase: evidence for catalysis by distortion. *Biochemistry* **29**, 1684-1689
13. Harrison, R.K. and Stein, R.L. (1992) Mechanistic studies of enzymic and nonenzymic prolyl *cis-trans* isomerization. *J. Am Chem. Soc.* **114**, 3464-3471
14. Liu, J., Farmer, J.D., Lane, W.S., Friedman, J., Weissman, D., and Schreiber, S.L. (1991) Calcineurin is a common target of cyclophilin-cyclosporin A and FKBP-FK506 complexes. *Cell* **66**, 807-815
15. O'Keefe, S.J., Tamura, J., Kincaid, R.L., Tocci, M.J., and O'Neill, E.A. (1992) FK-506- and CsA-sensitive activation of the interleukin-2 promoter by calcineurin. *Nature* **357**, 692-694
16. Clipstone, N.A. and Crabtree, G.R. (1992) Identification of calcineurin as a key signalling enzyme in T-lymphocyte activa-

tion. *Nature* **357**, 695-697
17. Schreiber, S.L. and Crabtree, G.R. (1992) The mechanism of action of cyclosporin A and FK506. *Immunol. Today* **13**, 136-142
18. Theriault, Y., Logan, T.M., Meadows, M., Yu, L., Olejniczak, E. T., Holzman, T.F., Simmer, R.L., and Fesik, S.W. (1993) Solution structure of the cyclosporin A/cyclophilin complex by NMR. *Nature* **361**, 88-91
19. Michnick, S.W., Rosen, M.K., Wandless, T.J., Karplus, M., and Schreiber, S.L. (1991) Solution structure of FKBP, a rotamase enzyme and receptor for FK506 and rapamycin. *Science* **252**, 836-839
20. Ke, H., Mayrose, D., Belshaw, P.J., Alberg, D.G., Schreiber, S.L., Chang, Z.Y., Etzkorn, F.A., Ho, S., and Walsh, C.T. (1994) Crystal structures of cyclophilin A complexed with cyclosporin A and *N*-methyl-4-[(*E*)-2-butenyl]-4,4-dimethylthreonine cyclosporin A. *Structure* **1**, 33-44
21. Mikol, V., Kallen, J., Pflügl, G., and Walkinshaw, M.D. (1993) X-ray structure of a monomeric cyclophilin A-cyclosporin A crystal complex at 2.1 Å resolution. *J. Mol. Biol.* **234**, 1119-1130
22. Pflügl, G., Kallen, J., Schirmer, T., Jansonius, J.N., Zurini, M., and Walkinshaw, M.D. (1993) X-ray structure of a decameric cyclophilin-cyclosporin crystal complex. *Nature* **361**, 91-94
23. Van Duyne, G.D., Standaert, R.F., Karplus, P.A., Schreiber, S.L., and Clardy, J. (1993) Atomic structures of the human immunophilin FKBP-12 complexes with FK506 and rapamycin. *J. Mol. Biol.* **229**, 105-124
24. Gallo, P., Saviano, M., Rossi, F., Pavone, V., Pedone, C., Ragone, R., Stiuso, P., and Colonna, G. (1995) Specific interaction between cyclophilin and cyclic peptides. *Biopolymers* **36**, 273-281
25. Di Blasio, B., Rossi, F., Benedetti, E., Pavone, V., Pedone, C., Temussi, P.A., Zanotti, G., and Tancredi, T. (1989) Solid state and solution conformation of cyclolinopeptide A. *J. Am. Chem. Soc.* **111**, 9089-9098
26. Tancredi, T., Benedetti, E., Grimaldi, M., Pedone, C., Rossi, F., Saviano, M., Temussi, P.A., and Zanotti, G. (1991) Ion binding of cyclolinopeptide A: an NMR and CD conformational study. *Biopolymers* **31**, 761-767
27. Zanotti, G., Rossi, F., Tancredi, T., Benedetti, E., Pedone, C., and Temussi, P.A. (1989) Ala analogues of the cyclolinopeptide A. *Biopolymers* **28**, 371-383
28. Rossi, F., Saviano, M., Di Blasio, B., Zanotti, G., Maione, A., Tancredi, T., and Pedone, C. (1994) Bioactive peptides: solid state, solution and molecular dynamics studies of a cyclolinopeptide A-related cystinyl cyclopentapeptide. *Biopolymers* **34**, 273-284
29. Zanotti, G., Maione, A., Rossi, F., Saviano, M., Pedone, C., and Tancredi, T. (1993) Bioactive peptides: conformational study of a cystinyl cycloheptapeptide in its free and calcium complexed forms. *Biopolymers* **33**, 1083-1091
30. Williams, J.W. and Morrison, J.F. (1979) The kinetics of reversible tight-binding inhibition. *Methods Enzymol.* **63**, 437-467
31. Bossard, M.J., Koser, P.L., Brandt, M., Bergsma, D.J., and Levy, M.A. (1991) A single Trp^{121} to Ala^{121} mutation in human cyclophilin alters cyclosporin A affinity and peptidyl-prolyl isomerase activity. *Biochem. Biophys. Res. Commun.* **176**, 1142-1148
32. Chang, C.T., Wu, C.-S.C., and Yang, J.T. (1978) Circular dichroic analysis of protein conformation: inclusion of the β-turns. *Anal. Biochem.* **91**, 13-31
33. Heald, S.L., Harding, M.W., Handschumacher, R.E., and Armitage, I.M. (1990) ^{1}H NMR studies on bovine cyclophilin: preliminary structural characterization of its complex with cyclosporin A. *Biochemistry* **29**, 4466-4478
34. Kallen, J. and Walkinshaw, M.D. (1992) The X-ray structure of a tetrapeptide bound to the active site of human cyclophilin A. *FEBS Lett.* **300**, 286-290
35. Mazzeo, M., Isernia, C., Rossi, F., Saviano, M., Pedone, C., Paolillo, L., Benedetti, E., and Pavone, V. (1995) Conformational behaviour of a cyclolinopeptide A analogue: two-dimensional NMR study of cyclo(Pro^1-Pro-Phe-Phe-Ac6c-Ile-Ala-Val^8). *J. Pept. Sci.* **1**, 330-340
36. Ke, H. (1992) Similarities and differences between human cyclophilin A and other beta-barrel structures. Structural refinement at 1.63 Å resolution. *J. Mol. Biol.* **228**, 539-550
37. Liu, J., Albers, M.W., Chen, C.-M., Schreiber, S.L., and Walsh, C.T. (1990) Cloning, expression, and purification of human cyclophilin in *Escherichia coli* and assessment of the catalytic role of cysteines by site-directed mutagenesis. *Proc. Natl. Acad. Sci. USA* **87**, 2304-2308
38. Inoue, T., Yoshida, Y., Isaka, Y., and Tagawa, K. (1993) Isolation of mitochondrial cyclophilin from bovine heart. *Biochem. Biophys. Res. Commun.* **190**, 857-863
39. Liu, J., Chen, C.-M., and Walsh, C.T. (1991) Human and *Escherichia coli* cyclophilins: sensitivity to inhibition by the immunosuppressant cyclosporin A correlates with a specific tryptophan residue. *Biochemistry* **30**, 2306-2310