Bioorganic & Medicinal Chemistry Letters, Vol.2, No.7, pp. 747-753, 1992
Printed in Great Britain

0960-894X/92 $5.00 + .00

THE CONFORMATION OF CYCLOSPORIN A BOUND TO CYCLOPHILIN IS ALTERED (ONCE AGAIN) FOLLOWING BINDING TO CALCINEURIN: AN ANALYSIS OF RECEPTOR-LIGAND-RECEPTOR INTERACTIONS

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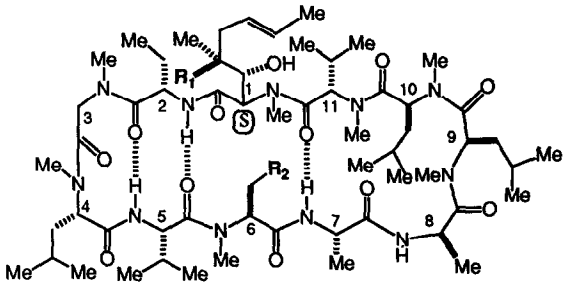
(*Received* 8 *April* 1992)

Abstract Analyses of the complexation of cyclosporin A (CsA) by cyclophilin and the unusual properties of MeBm$_2$t^1-CsA lead us to propose a conformational change upon binding of the cyclophilin-CsA complex to the protein phosphatase, calcineurin.

Structural analyses of natural products bound to their protein receptors can provide a detailed understanding of phenomena ranging from enzymatic catalysis to signal transduction. This is especially true when such analyses are combined with experimental results from other disciplines such as synthetic chemistry and molecular biology. In fact, it may be argued that the power of structure determination resides primarily in its ability to explain experimental data, and in doing so to facilitate the formulation of hypotheses which then can direct further experiments. In this report we describe a proposed model for the interactions in a multimeric complex involving two proteins and a natural product ligand. The model is based on crystallographic and NMR spectroscopic analyses of one of the proteins and its complex with the natural product, and on biochemical and biological data on a synthetic analog of the natural product.



CsA: R_1 = H, R_2 = i-Pr;
MeBm$_2$t^1-CsA: R_1 = Me, R_2 = i-Pr;
MeAla^6-CsA: R_1 = H, R_2 = H.

Figure 1 Structures of CsA, MeBm$_2$t^1-CsA, and MeAla^6-CsA.

The cyclic undecapeptide cyclosporin A (CsA) inhibits signal transduction pathways in cell types including T cells, B cells and mast cells. A variety of evidence has shown the inhibitory properties of CsA are mediated through binding to the soluble protein receptor, and rotamase enzyme, cyclophilin (CyP).[1] That is, the CyP-CsA complex, but not CyP or CsA alone, inhibits signal transduction pathways, and CsA is seen to be a mere prodrug for immunosuppression.[2] Only upon binding to its endogenous cognate immunophilin (FK506 binding protein (FKBP) in the cases of the mechanistically related signaling inhibitors FK506 and rapamycin) is the ac-

tual inhibitory entity formed. Strong evidence for this proposal was recently provided by the finding that the CyP-CsA complex, but not CyP or CsA alone, binds to and inhibits the enzymatic activity of the enzyme calcineurin (CN), a Ca^{2+}-dependent, serine/threonine protein phosphatase, and this argument is strengthened by the additional finding that the FKBP-FK506 complex exhibits similar actions towards CN.[3]

In attempts to understand the actions of CsA, a large number of synthetic analogs have also been studied. In most cases an analog's affinity for CyP was shown to correlate with its immunossupresive activity; however, for a select few ("off-diagonal" analogs[4]) this correlation does not hold.[5] The existence of these off-diagonal analogs led to speculation that CyP may not be the relevant biological receptor for CsA.[5b] The complex hypothesis, though, provides a simple explanation for the seemingly conflicting binding and immunosuppressive properties of these analogs. Put simply, the ability of a CsA analog to elicit an immunosuppressive effect in the earlier studies,[5] or to inhibit signal transduction in a more recent study,[6] is dependent on the ability of the CyP-CsA analog *complex* to interact with its downstream target, CN. We have recently demonstrated that the ability of several CyP-CsA-analog complexes to inhibit the phosphatase activity of CN correlates with the inhibitory potency of the analogs in a signal transduction assay, in spite of the fact that the relative immunosuppressive potencies of these compounds do not correlate with their affinity for CyP.[6] From a thermodynamic viewpoint, the inhibitory properties of a given molecule are dependent on the net binding constant for formation of the ternary complex:

$$[\text{CyP}] + [\text{lig}] \overset{K_1}{\rightleftharpoons} [\text{CyP•lig}] + [\text{CN}] \overset{K_2}{\rightleftharpoons} [\text{Cyp•lig•CN}]$$

(the implicit assumption here that the ligand and CyP alone have negligible affinity for the target has been demonstrated with CN). A ligand with a low association constant in the first reaction may still be an effective inhibitor of signal transduction if it has a high association constant in the second reaction. An example of such a compound is MeBm$_2$t^1-CsA (Figure 1). This molecule's affinity for CyP (K_d = 500 nM) is approximately 100-fold lower than that of CsA (K_d = 6 nM); yet the affinity of the CyP-MeBm$_2$t^1-CsA complex for CN (K_d = 13 nM) is *greater* than that of the CyP-CsA complex (K_d = 40 nM). Furthermore, the relative potency of the two compounds to induce ternary complex formation correlates with their relative activity in direct, cellular signal transduction assays, where MeBm$_2$t^1-CsA is only 10-fold less potent than CsA.[6] In this article we provide a plausible explanation for these properties of MeBm$_2$t^1-CsA based on the bound structure of CsA, and a model of the CyP-CsA complex. The important implication of our hypothesis is that the conformation of CsA bound to CyP, which has been noted to differ markedly from its solution (chloroform) and solid state conformation, appears to undergo yet another change upon formation of the CN-based multimeric complex.

The conformation of CsA bound to CyP has recently been determined by isotope-edited NMR.[7] The structures of unligated CyP and CyP bound to a tetrapeptide have also been determined by x-ray crystallography.[8] The structure of the CyP-CsA complex has not yet been solved, however, intermolecular NOEs between protein and ligand have been observed in ^{15}N- and ^{13}C-edited NOESY spectra of the complex.[9] These interactions have provided the basis for a model of CyP-CsA generated through computational docking of the bound conformation of CsA into the active site of CyP (Figure 2a).[10] In this model, most of the protein-drug contacts involve CsA residues 9→3, the "CyP binding domain", while CsA residues 4→8, the "effector domain," remain largely ex-

posed to solvent and interact directly with CN. The complex is maintained by a number of hydrophobic and hydrogen bonding interactions between CyP and the CsA binding domain.[10] Two interactions relevant to our discussion are hydrogen bonds involving the hydroxyl group of the MeBmt-1 sidechain. The first of these is to the carbonyl oxygen of Asn-102 of CyP, and the second is to the amide NH of Abu-2 of CsA. Note that the existence of these energetically favorable interactions is dependent on the χ_1 torsion angle of the MeBmt-1 sidechain. Rotation about the Cα-Cβ bond would preclude, or at least strain, these hydrogen bonds, and thus would raise the energy of the protein-ligand complex.

Computational addition of a Pro S methyl group at the Cγ position of the MeBmt-1 sidechain, to form MeBm$_2$t^1-CsA, suggests that the bound conformation of this compound must be different from that of CsA. As can be seen in Figure 2b, if the conformation of MeBm$_2$t^1-CsA remains unchanged, this methyl group will be directed into the macrocyclic ring of the molecule, toward the effector domain, resulting in steric interactions with the N-methyl groups of MeLeu-4 (C-C distance < 3 Å) and MeBmt-1 (C-C distance ~ 3 Å).

The conformation of MeBm$_2$t^1-CsA can be changed in two limiting ways to relieve the steric strain introduced by the Pro S methyl group. First, the effector domain may be distorted in such a way as to move the N-methyl group of MeLeu-4 away from the Pro S methyl group (e.g., by tilting the plane of the Sar-3-MeLeu-4 amide bond). Second, the MeBm$_2$t-1 sidechain may be distorted by rotation about the χ_1 torsion (rotation about χ_2 would not effectively relieve steric strain since the Cγ is a quaternary center). Both of these changes would result in a decrease in affinity of the ligand for CyP—the first because it forces the effector domain into an energetically unfavorable conformation (presumably the bound CsA structure represents the most stable conformation of the region), and the second because it strains or eliminates the two hydrogen bonds to the MeBm$_2$t-1 hydroxyl group.

These conformational changes lead to molecular descriptions of two models to explain the anomolously high inhibitory properties of MeBm$_2$t^1-CsA. The first model proposes that the effector domain of MeBm$_2$t^1-CsA is distorted in order to relieve steric strain caused by the methyl-methyl interaction (Figure 2b). The result of this distortion may be an effector domain conformation, in the CyP-MeBm$_2$t^1-CsA complex, that is better suited to interact with CN than is the effector domain of the CyP-CsA complex. Thus, the model implies that the conformation of CsA in the CyP-CsA-CN ternary complex is different from that in the CyP-CsA binary complex, and that the conformation of the MeBm$_2$t^1-CsA effector domain, seen in its binary complex with CyP, closely resembles that of the CsA effector domain in the ternary complex. In terms of energetics, the price of effector domain distortion necessary to form the ternary complex is paid by MeBm$_2$t^1-CsA on binding to CyP, whereas, for CsA it is paid on binding of CyP-CsA to CN. Thus, although the affinities of CsA and MeBm$_2$t^1-CsA for CyP differ by a factor of 100, their net capacities to affect ternary complex formation, which is presumably the true measure of signal inhibition potency, differ by only a factor of 10.

The second model also proposes that CyP-bound MeBm$_2$t^1-CsA is distorted compared to CyP-bound CsA (Figure 2c), but the major distortion here is a χ_1 rotation of the MeBm$_2$t-1 sidechain. In this model, a conformational change in MeBm$_2$t^1-CsA upon formation of the ternary complex partially relieves the offending methyl-methyl interactions, allowing the MeBm$_2$t-1 sidechain to relax into a non-distorted conformation, and thus interact more tightly with CyP. On a molecular level, the steric strain of CyP-bound MeBm$_2$t^1-CsA, due to the

methyl-methyl interaction, would be relieved primarily by rotation about the χ_1 torsion in the MeBm$_2$t-1 sidechain. The loss of the two hydrogen bonds involving the sidechain hydroxyl would then explain the decreased affinity for CyP. Like the first model, the second also proposes that the conformation of the effector domain is changed on binding of the complex to CN. This change could allow the MeBm$_2$t-1 sidechain to assume the conformation seen in CyP-bound CsA. Thus, in the absence of CN, the hydrogen bonds to the MeBm$_2$t-1 sidechain are absent, but in the presence of CN they are regained. Effectively, the interactions of the CyP-MeBm$_2$t^1-CsA complex with CN increase the affinity of MeBm$_2$t^1-CsA for CyP. Again in terms of energetics, although MeBm$_2$t^1-CsA and CsA must both pay the price of effector domain distortion on CN binding, MeBm$_2$t^1-CsA is able to regain some of this energy by assuming a more favorable conformation of the MeBm$_2$t-1 sidechain upon binding to CN.

Although the two models differ in the details of their explanation, both share two important features: (1) the structural roots of the altered properties of MeBm$_2$t^1-CsA are steric interactions between the Pro S methyl group at Cγ of the MeBm$_2$t-1 sidechain and the N-methyl groups of MeLeu-4 and MeBm$_2$t-1, and (2) the conformation of the CsA effector domain is distorted from that seen in the CyP complex on CN binding. Of course, it is likely that the true picture may involve elements of both models.

The models illustrated in Figure 2 can also be used to rationalize the behavior of another off-diagonal CsA analog, MeAla^6-CsA.[5] In this molecule, the MeLeu-6 residue of CsA is replaced by a MeAla residue. MeAla^6-CsA binds tightly to CyP (K_i = 9 nM); however, the resulting CyP-MeAla^6-CsA complex has no measurable affinity for CN, presumably accounting for this analog's the lack of signaling inhibition.[6] Presumably, both CsA and MeAla^6-CsA bind to CyP in similar conformations, with the sidechain of residue-6 exposed to solvent and pointing out of the active site. Thus, this sidechain may be a crucial component of the effector domain, involved in the interaction of the CyP-drug complex with CN. The poor binding of CN to the CyP-MeAla^6-CsA complex, then, is due to the absence of an isopropyl group on this important sidechain. Furthermore, immunosuppressive activity is regained as the length of the sidechain of residue-6 is increased from Ala (-CH_3, 0.4% immunosuppressive activity compared to CsA) to Abu (-CH_2CH_3, 7% immunosuppressive activity) to Norvaline (-$CH_2CH_2CH_3$, 46% immunosuppressive activity).[5,11]

The unbound conformations of CsA and FK506 differ from those of the prodrugs when bound to their cognate immunophilins (an important caution to comparisons of the free and bound conformations of CsA and FK506 is that the structures of the free molecules in *aqueous* solution have not yet been determined experimentally; thus, the differences observed between the free and immunophilin-bound structures do not necessarily reflect effects of binding, but may be due to differences in the conformations of the unbound molecules in aqueous and organic solution). Structural analyses of CyP, CsA, and the CyP-CsA complex lead to the conclusion that the conformation of CsA bound to CyP undergoes yet another change upon binding its second target, CN. The differences observed between free and bound immunophilin ligands served as a reminder of the importance of examining receptor-ligand complexes when contemplating the design of new drugs.[12] Our analysis suggests that this warning is equally applicable to the structure of the CyP-CsA complex, which is in fact the structure of the unbound drug in this remarkable story of receptor-ligand-receptor interactions.[13]

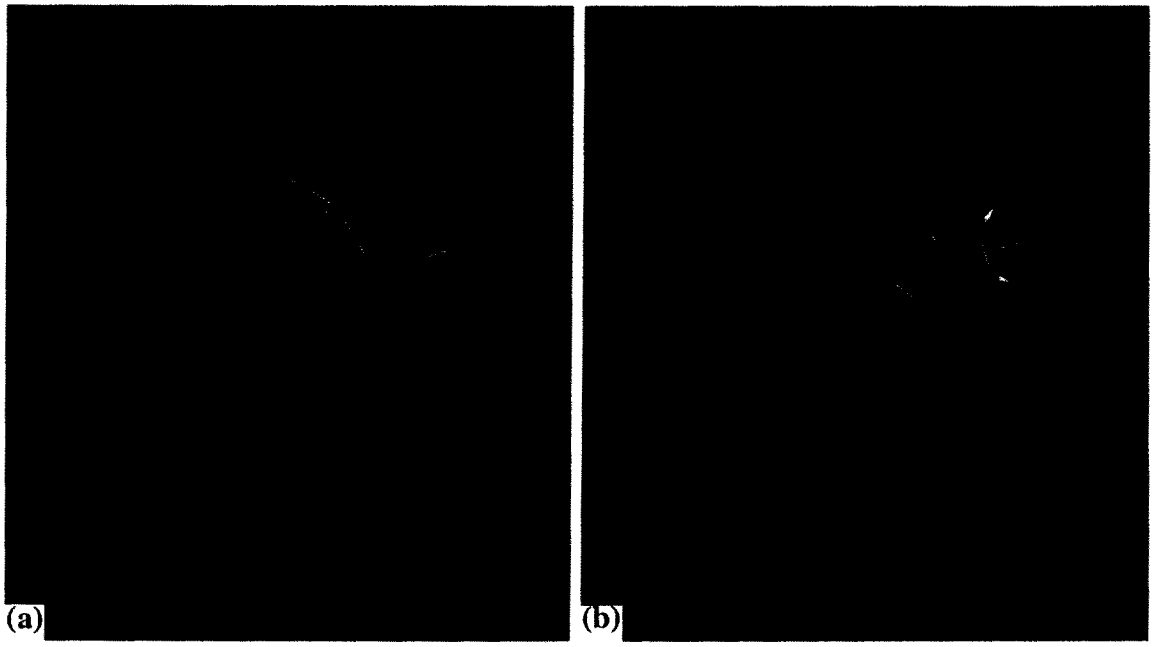


Figure 2 (a) Model of the Cyp (blue) - CsA (red) complex.[10] The white dotted lines indicate the hydrogen bond relay consisting of the NH group of Abu-2, the hydroxyl group of the MeBmt-1 sidechain, and the mainchain CO group of Asn-102. The following atoms of CsA are shown with their van der Waals surfaces: N-Me group of MeLeu-4, N-Me of MeBmt-1 and the γ-H of MeBmt-1. (b) Model of the CyP-CsA complex with the computational addition of a γ-Pro-S methyl group on the sidechain of MeBmt-1. Severe van der Waals contacts between the Pro-S methyl group and N-Me groups of MeLeu-4 and MeBmt-1 preclude $MeBm_2t$-1 from adopting a conformation similar to CsA when bound in the active site of Cyp. Steric strain could be relieved by a distortion of the effector domain so as to move the N-Me group of MeLeu-4 away from the $MeBm_2t$-1 sidechain, as indicated by the double-headed green arrow. (c) The steric strain could also be relieved by rotation about the χ_1 torsion of the MeBmt-1 sidechain, as indicated by the green arrow, resulting in a disruption of the hydrogen bonding network.

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11. It is also interesting to note that β-branching on the sidechain of residue-6 results in a loss in affinity for CyP (both MeVal^6-CsA and MeIle^6-CsA exhibit only 5% of CyP binding compared to CsA).[5] Inspection of the CsA bound conformation indicates that the introduction of a β-methyl group would cause severe steric crowding with the NH amide group of Ala-7. Relief of this strain requires either rotation of the residue-6 sidechain or a rotation of the MeLeu^6-Ala^7 peptide bond. Rotation of the sidechain only introduces equally bad steric interactions with the sidechain of MeLeu-10 and rotation of the 6-7 amide bond would lead to serious distortion of the local backbone conformation. In either case, the introduction of the β-branch precludes the peptide from easily adopting the conformation required for tight binding to CyP.
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13. This work was supported by the National Institute of General Medical Sciences (GM-38627 and GM-40660 to S.L.S.), an NIH Postdoctoral Fellowship (to D.G.A.), an NSERC Postgraduate Scholarship (to P.J.B.), and an American Chemical Society Division of Organic Chemistry Graduate Fellowship sponsored by Merck, Sharp, & Dohme (to M. K. R.)