Tertiary structure of calcineurin B by homology modeling

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The crystal structure of the calcium-binding protein calmodulin is used to model the immunologically important calcineurin subunit B. The rough structure is produced by computer-aided homology modeling. Refinement of this using molecular dynamics leads to a suggested structure which appears to satisfy reasonable hydrophilicity and hydrogen-bonding criteria. In the absence of a crystal structure, the model may prove useful in modeling of its interactions with the phosphatase catalytic subunit calcineurin A, and help to explain the calcium modulation of this protein.

Keywords: calcineurin, protein phosphatase 2B, immunology, protein structure, homology modeling

INTRODUCTION

Studies into the possible sites of action of the immunosuppressants cyclosporin A (CsA), FK506, and rapamycin have made a significant contribution to research in signal transduction in T-lymphocytes, in particular in the relatively unknown pathways within the cytoplasm.[1,2] The discoveries that both cyclosporin A and FK506 form 1:1 complexes with the functionally identical but structurally different peptidyl-prolyl isomerase enzymes cyclophilin[3,4] (CP) and FK506 binding protein (FKBP)[5,6] gave rise to the suggestion that the immunological actions of the two macrocycles might be linked to the inhibition of rotamase involvement in the folding of proteins essential to T-cell activation or proliferation. However, the abundance of different PPIase enzymes and the high potency of CsA and FK506 cast doubt upon this concept. Moreover, rapamycin is structurally similar to FK506 and binds more potently to FKBP, and yet appears to possess inhibitory effects on an alternative pathway of T-cell signaling. This led to the theory that the two complexes FK506-FKBP and rapamycin-FKBP present dissimilar effector regions to different cellular targets.[7]

The recent discovery[8] that CsA-cyclophilin and FK506-FKBP bind competitively to the same cytosolic phosphatase, calcineurin (Cn), supports the cellular target theory. The inhibition of this calmodulin (CaM) dependent enzyme might provide an explanation for the apparently similar modes of action of CsA and FK506, involving the suppression of a calcium-dependent pathway of T-cell activation. The observation that the rapamycin-FKBP complex does not competitively bind to and inhibit calcineurin correlates with its mode of action on a calcium-independent pathway. The X-ray structures of the two complexes have now been determined.[9,10]

Calcineurin[11-17] is a type 2B calcium and calmodulin dependent phosphatase, and consists of two tightly bound subunits, the tertiary structures of which are unknown. It is known to bind other metal ions in vitro. This heterodimer has been implicated in the regulation of T-lymphocytes,[18] and is associated with the cytosolic membrane by the covalent attachment of myristic acid to the amine terminus of the B subunit.[19,20] Limited proteolysis experiments have shown the 61kd A subunit contains the phosphatase catalytic site, an auto-inhibitory domain and a calmodulin-binding site.[21,22] The 19kd calcineurin B shows extensive sequence similarity to calmodulin.[23] Both calcineurin B and calmodulin bind to calcineurin A, calmodulin in a Ca^{2+}-dependent manner, in a 1:1 ratio, though in spite of the strong homology their binding to the A subunit is noncompetitive. Both calmodulin and calcineurin B exert some Ca^{2+}-dependent influence on the activity.[24] Figure 1 shows one possible mode of action of the CP:CsA complex on calcineurin.

Calmodulin is highly conserved between species, consisting of 2 domains linked by a long central helix. Each domain contains 2 calcium-binding sites. Both calcineurin B and calmodulin bind 4 moles of Ca^{2+} per mole, suggesting close structural as well as sequence similarity. This gives rise to the possibility of building a model of calcineurin B by sequence alignment with calmodulin, with a view to comparing their interactions with calcineurin A, the various domains of which have been mapped.

METHODS

The sequence of calcineurin B was aligned with that of calmodulin using the University of Wisconsin Genetics Computer Group program Bestfit.[27,28] The output of Bestfit was slightly modified to allow for conservation of the structure of helical residues, and is shown in Figure 2. The two

Color Plates for this article are on page 45.

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Received 2 February 1992; accepted 4 March 1992

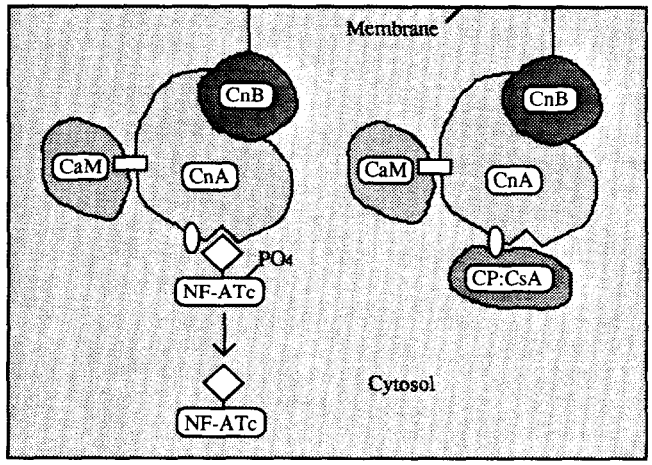

Figure 1. Schematic diagram of the calcineurin/calmodulin catalyzed dephosphorylation of the cytosolic component of the nuclear transport factor (NF-ATc),[25] which may be necessary for transport of this factor to the nucleus. Dephosphorylation appears to be inhibited by the complex between CsA or FK506 and the appropriate immunophilin.[26]

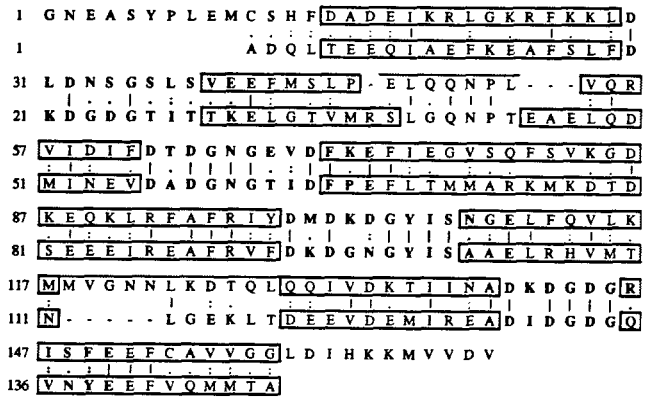

Figure 2. Alignment of calcineurin B sequence (top) with calmodulin. Regions of helical structure are enclosed in solid boxes. The 2 loops requiring loop searching are overscored, and the calcium binding regions are denoted by bold-type residues: | denotes sequence identity, : and . show degrees of conservative mutations. The alignment was obtained with the help of the University of Wisconsin Genetics program Bestfit.[27]

sequences have 35% residue identity, and are, unsurprisingly, especially similar in the regions believed to be the 4 E-F hand Ca^{2+}-binding sites in calmodulin.[29,30] These sites contain 2 helices joined by a loop, in which the bound ion is close to acidic side chains and backbone oxygens. Calcineurin has lower similarity to a related calcium-binding protein, troponin C.[31] Alignment with troponin C requires an insertion of 3 residues in the domain-spanning helix, leading to a change in the relative orientation of the two domains. For these reasons, calmodulin rather than troponin C was used to build calcineurin B.

The crystal structure 3CLN of calmodulin from the Brookhaven databank[32] was used to generate a backbone framework with the program Composer,[33,34] running as part of Sybyl.[35] From this framework, a model of calcineurin B was constructed by mutation of the side chains, and the modification of the two surface loops shown in Figure 2. The tertiary folds of the two calcium-binding sites in each domain were assumed to be conserved, as were the β-sheet hydrogen bonds linking these regions, and the other secondary structure features so far as possible. There were, therefore, two disruptions to the calmodulin structure that needed to be made: a shortening of loop A (calmodulin residues 37–47) and a lengthening of loop B (calmodulin residues 112–117).

For loop A, the preceding helix (helix 2) was shortened by two residues due to the conversion of MET 36 to PRO, which is a common helix-breaking residue. The following helix was shortened by three residues, approximately one turn of the helix, as part of the deletion, so that the conserved L-X-Q-N-P sequence could retain its fold. This loop was moved so as to connect the ends of the chain after the deletions had been made (see Color Plate 1).

For loop B (see Color Plate 2), helical structure was not disrupted, and an insertion of 5 residues was made into the existing loop, with backbone angles for the new residues selected using the Composer loop search routine. Twenty possibilities for each loop were examined and the one with fewest van der Waals clashes and positive phi angles chosen. These angles arose mostly where GLY residue angles were assigned to non-GLY residues—statistically GLY is the only residue with significant occurrence of positive phi, due to the lack of Cβ. Where both loops were connected to the conserved structure, sterically disfavored backbone angles also appeared. These were removed in two ways, leading to two similar structures.

(1) Dynamics was performed on the loop region alone, using the CHARMm program.[36,37]
(2) The angles were rotated manually with the Sybyl modeling package until all phi angles were positive.

Both methods produced structures with no positive phi angles in the loop regions. Interestingly, the modeled calcineurin B has LYS 102, sited in a calcium-binding site, with phi = +91.5°, an angle which is uncommonly observed. The corresponding residue LYS 54 in the first calcium-binding site of parvalbumin,[38] a related protein, has phi = +64.8°, showing that this value in calcineurin B is reasonable, and not a reason to dismiss the model.

The two structures resulting after these changes were refined using the CHARMm program. Firstly a dynamics run of 100 ps at 500 K with harmonic constraints on the backbone and calcium atoms was carried out to reduce bad interactions between buried residues. Then a dynamics run at 300 K with no constraints and a solvated protein was performed to obtain a more realistic structure. The resultant structure was energy minimized, and is shown in Color Plate 3.

DISCUSSION

The hydrogen-bonding and salt bridge pattern was examined in both the model of calcineurin B and the calmodulin

crystal structure. No disulfide bridges or nonsurface salt bridges were found in either. Virtually all residues capable of forming hydrogen bonds in both calmodulin and calcineurin B do so.

The solvent accessibility[39] of the residues in both structures was found. Generally, hydrophobic residues, as expected, are found to be at least partially buried (<80% accessibility) while hydrophilic residues are mainly exposed (>80% accessibility). Most exceptions to this are conserved in both structures. On hydropathic and hydrogen bonding grounds, the built structure is therefore reasonable.

Predictions of secondary structure characteristics were made using the University of Wisconsin Genetics Computer Group Peptidestructure program.[27] The results are shown in Figure 3. The predictions are largely consistent with the model. Helices 1, 2, 4, 5, and 7 are predicted, as are the turns within the predicted calcium binding sites, and the predicted flexibility is greatest in the model regions without regular structure. Residues 80–90 have a high surface probability, supporting the exposed central helix of the model. Sheet prediction does not agree with the model, suggesting that residues 54–61 and 129–139, helices 3 and 6, have high sheet probability. However, to maintain integrity of the calcium binding sites these need to be helical, as they are in calmodulin.

The model conserves the 4 Ca^{2+}-binding sites of calmodulin, as well as the associated local secondary structure. In production of the model it was assumed that the long central domain-spanning helix of the calmodulin crystal structure is conserved in calcineurin B, although the presence of 2 GLY residues in calcineurin B makes this questionable. However, NMR experiments suggest that in solution this helix may not be well defined even in calmodulin.[47]

The hydrophobic patches exposed in each calmodulin domain remain in calcineurin B. Molecules binding to calmodulin typically possess a region which would be capable of forming an amphiphilic helix,[48–51] positively charged on one face. It may be that the hydrophobic face of the helix contacts a hydrophobic patch in calmodulin, but that electrostatic forces encourage the helix to attach to a site near to a predominantly acidic part of calmodulin,[52] although this is not yet certain. There is evidence[53–57] that binding to calmodulin occurs in such a way as to involve the hydrophobic patches in both domains, and it has been suggested that in bound complexes the central helix loses some of its regular structure to allow the two hydrophobic patches to move relative to one another. Amphiphilic helices, which seem to be involved in the binding, may then dock into these exposed areas, although the precise docking mode is not yet certain. If the two domains move upon binding, the precise solution structure of this central region will prove extremely difficult to determine, although in the crystal structure it is possible that packing forces may encourage the helix to

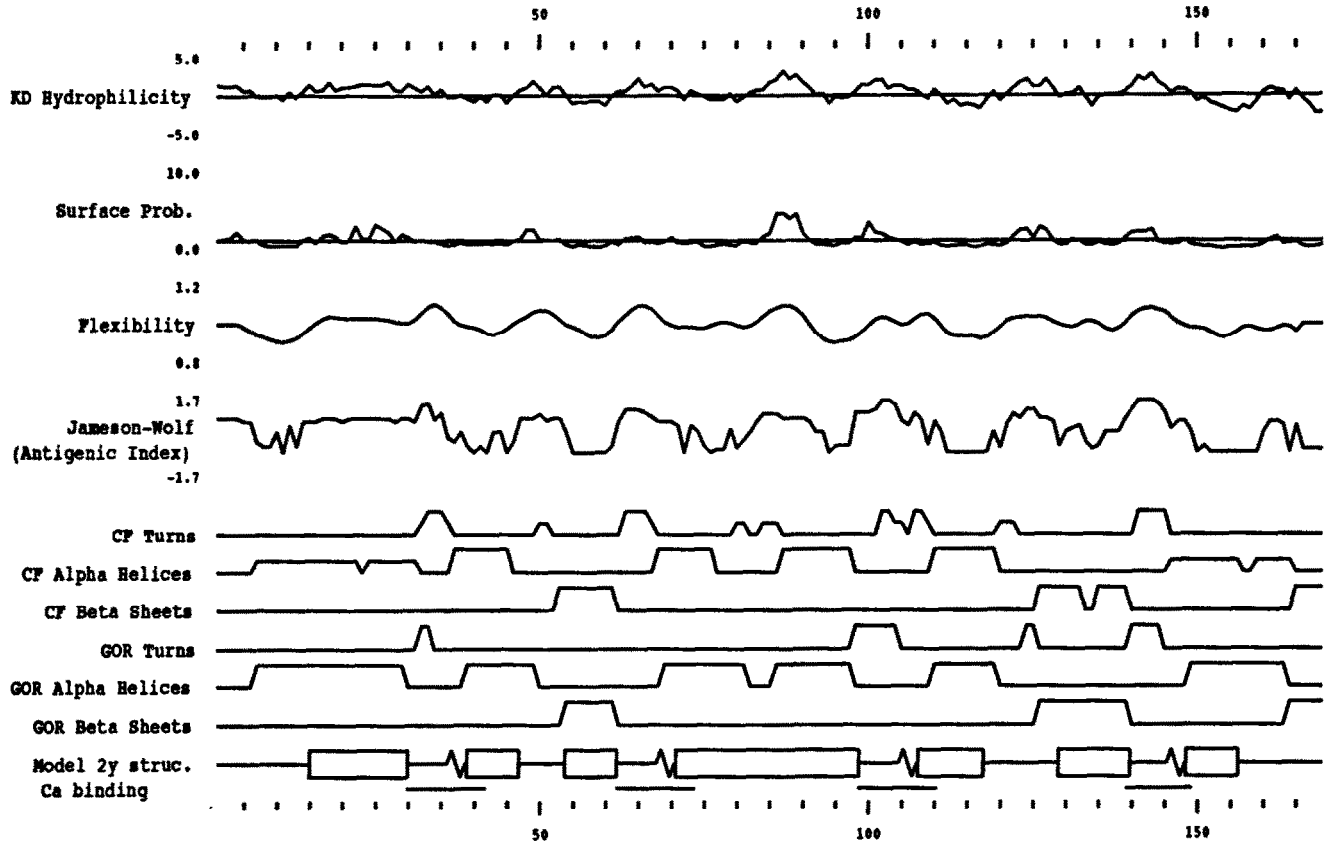

Figure 3. The predicted Kyte–Doolittle hydrophobicity,[40] Emini surface probability,[41,42] Karplus–Schulz flexibility,[43] Jameson–Wolf antigenicity,[44] Chou–Fasman,[45] and Garnier–Osguthorpe–Robson[46] secondary structure prediction is shown above the modeled secondary structure, derived from calmodulin.

form. In calmodulin both hydrophobic patches are accessible, but in the calcineurin B model the C-terminal hydrophobic patch is partially obscured by the longer loop B between the two calcium-binding regions. This may lead to different binding properties.

It has been observed that in many calmodulin-regulated proteins there exists both a calmodulin binding site and a calmodulin-like site. In the absence of calmodulin the calmodulin-like site may dock into the calmodulin binding site, and cause auto-inhibition.[58–60] This is observed experimentally: binding of calmodulin to calcineurin A leads to an increase in activity comparable to that obtained on cleavage of the calcineurin A autoinhibitory domain. The sequence L-G-E-K-L-T-D-E, present in residues 112–117 of calmodulin loop B, but not in that of calcineurin B, is present with minor changes in calcineurin A, as well as in other calmodulin binding proteins (Figure 4). It is noticeable that this loop is in the region of calcineurin B which differs most from calmodulin. If this is part of the autoinhibitory region in calcineurin A, the binding of calmodulin may displace this region on the A subunit, leading to a structural change and the observed increase in activity. This may be extendable to other CaM-binding proteins with autoinhibitory action. We suggest that this loop may act in conjunction with the amphiphilic helix requirement to determine autoinhibition, as well as CaM and CaM-like protein specificity.

The loop A sequence L-X-Q-N-P, which is situated near the calmodulin N-terminal hydrophobic pocket, also occurs in troponin C and calcineurin B. It may be important in determining binding specificity. In calcineurin B, the position of the loop is altered with respect to the ends of the adjacent helices, which may go some way towards explaining the difference between calcineurin B and calmodulin binding sites in calcineurin A.

It is interesting to note the existence of an amphiphilic helix in the model of calcineurin B in residues 15–28. It is not known if this has importance in binding.

So far there has been little experimental work on the determination of the structure of the B subunit of calcineurin. When NMR data on interproton distances emerge it will be possible to comment with more certainty on the accuracy of the present model, but until then it may give some insight into the interactions between the two calcineurin subunits. We are happy to provide coordinates on request.

Name and residue numbers								
Calmodulin (112-119)	L	G	E	K	L	T	D	E
Calcineurin A (384-391)	V	G	E	K	V	T	E	M
MLCK (chicken) (153-160)	V	G	E	K	Q	E	E	E
Ca-transp. ATPase (937-944)	A	G	E	K	F	F	D	I
6-phosphofructokinase (143-150)	K	A	G	K	I	T	D	E
Calcineurin B (123-130)	L	K	D	T	Q	L	Q	Q

Figure 4. Loop B region of calmodulin and the corresponding region of calcineurin B. Also shown are the regions with highest similarity to calmodulin's loop B in 4 calmodulin-binding proteins. It has been proposed that these proteins contain autoinhibitory domains, deactivated in the presence of calmodulin, which would be similar to the calmodulin-binding site, perhaps providing evidence for the importance of the C-terminal loop in binding.

NOTES ADDED IN PROOF

The docking of a peptide to CaM has been studied crystallographically[61] and the proposed mode of binding found to be correct. It is not known whether a large protein is capable of binding in the same way.

The recent identification of actin and Hsp70 as CS-A binding proteins[62] may suggest some structural similarity to CnA. Further work is needed in this area.

ACKNOWLEDGMENTS

This work is supported by a SERC CASE grant with the Oxford Centre for Molecular Studies and Roussel Laboratories Limited.

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