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Molecular modelling of malaria calmodulin suggests that it is not a suitable target for novel antimalarials

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[Plates 1 and 2]

SUMMARY

The recent cloning and sequencing of many calmodulin genes permits alignment of DNA and protein sequences, as well as structural comparison based on homology modelling. The crystal structure of calmodulin places the four Ca^{2+}-binding domains in a dumbbell-like configuration, with a large hydrophobic cleft in each half of the molecule. Calmodulin from *Plasmodium falciparum* has a high level of sequence identity (89%) with its mammalian counterpart. However, a lower degree of sequence conservation is observed among calmodulins from other lower eukaryotes. Potentially important differences in calmodulin sequences involve amino acids with side-chains forming the hydrophobic clefts as well as in the central helix; these differences could alter interactions with small hydrophobic molecules such as chloroquine and with enzymes modulated by calmodulin. Our modelling studies suggest that neither of the antimalarials examined (chloroquine and quinine) bind tightly to calmodulin. We conclude that the differences between host and parasite calmodulins are insufficient to merit this protein being chosen as a realistic target for antimalarial drug design. By contrast, our sequence comparisons reveal that the fungal calmodulins are significantly divergent from those of higher eukaryotes suggesting that at least in these species, calmodulin might be a target for novel antimycotic drugs.

1. INTRODUCTION

The human malaria parasite, *Plasmodium falciparum*, a unicellular protozoan, is still a major cause of disease in the developing world. A conservative estimate of the number of malaria cases is 200 million. In Africa alone it probably accounts for the deaths of a million children a year. The resurgence of malaria as a worldwide clinical problem is due both to a breakdown in vector control and to a rapid rise of drug resistance by the parasite, notably to pyrimethamine and chloroquine. Hence, novel antimalarials need to be designed, preferably using rational approaches based on the structural information of parasite proteins which have functional significance.

The mechanism underlying resistance to pyrimethamine has been determined by the cloning and sequencing of the dihydrofolate reductase-thymidylate synthetase (DHFR-TS) gene from resistant and sensitive isolates of *P. falciparum*. Resistance is associated with point mutations; for example, the substitution of asparagine for serine or threonine at position 108 (Peterson *et al.* 1988; Cowman *et al.* 1988; Snewin *et al.* 1989). Different point mutations are linked with resistance to proguanil (Foote *et al.* 1990*a*; Peterson *et al.* 1990). All these mutations have occurred at sites bordering the active site of the enzyme, which is also the predicted binding site of the drug. It is probable that these mutations protect the parasite by altering the affinity of the enzyme for its natural substrate and for pyrimethamine.

Several hypotheses have been put forward (Krogstad *et al.* 1987; Warhurst 1988; Meshnick 1990) to explain the mechanism for chloroquine resistance in malaria. These include alterations in the expression and gene copy number of the multidrug resistance gene (Pfmdr-1) as well as a possible role for calmodulin. Evidence for the presence of an 'mdr type phenomenon' comes from the observation that calcium antagonists such as verapamil can reverse chloroquine resistance *in vitro* (Martin *et al.* 1987). Two mdr-like genes have been cloned from *P. falciparum* (Wilson *et al.* 1989; Foote *et al.* 1989). The first, Pfmdr-1, is located on chromosome 5 and shares about 55% homology with human mdr-1 (Foote *et al.* 1989). There are some data to suggest that this gene is amplified or overexpressed in certain chloroquine resistant lines (Foote *et al.* 1989; 1990*b*). Wellems *et al.* (1990), who performed a genetic cross between chloroquine-sensitive and chloroquine-resistant lines, have not shown such a correlation. Pfmdr-2 has been mapped to chromosome 14 and shows no correlation with chloroquine resistance (Wellems *et al.* 1990). Very recent genetic evidence has mapped chloroquine resistance to a segment of chromosome 7 (Wellems *et al.* 1991). The most convincing target for chloroquine

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is the haem polymerase (Slater & Cerami 1992). This enzyme found in trophozoites, is responsible for detoxifying the haem moiety, ferriprotoporphyrin IX, produced from haemoglobin breakdown in the food vacuole. Chloroquine resistance is unrelated to this enzyme but probably due to transport processes which maintain drug levels below those which would inhibit the polymerase (Krogstad *et al.* 1987). It is not known whether the action of chloroquine is only limited to this haem polymerase.

The recent cloning and sequencing of the calmodulin gene from *P. falciparum* (Robson & Jennings 1991) has allowed us to see if this molecule could be used to design novel antimalarials. Calmodulin is highly conserved throughout evolution; the most amino acid changes are found among the lower eukaryotes. It is a small acidic protein (usually 148 amino acids long), that belongs to a family of homologous proteins that bind calcium through similar structural domains. The three-dimensional crystal structure (Babu *et al.* 1988) for mammalian calmodulin shows that the molecule is dumbbell-shaped, the two lobes each containing two Ca^{2+}-binding domains, are connected by a flexible central α-helix. When calmodulin binds Ca^{2+}, a very large hydrophobic cleft is formed in each half of the molecule, and these probably represent the sites of interaction of the many pharmacological agents known to bind calmodulin e.g. chloroquine, as well as the site of interaction of calmodulin with the wide variety of enzymes which it regulates. This is supported by experimental evidence showing that calmodulin binds two moles of trifluoperazine (Shimuzu & Hatano 1983; Shimuzu *et al.* 1984), the protein was originally purified using phenothiazine affinity chromatography (Jamieson & Vanaman 1979) and that trifluoperazine competitively inhibits calmodulin acivation of guanylate cyclase (Nagao *et al.* 1981). We have compared the amino acid sequences of known calmodulins to establish evolutionary relationships and performed model building studies based on the known crystal structure of mammalian calmodulin to understand the significance of amino acid sequence changes in relationship to the hydrophobic clefts.

2. MATERIALS AND METHODS

(a) *Sequence alignments*

Calmodulin sequences were taken from the Swissprot database (release 18 (5/91)), the EMBL update (release 27 (5/91) and EMNEW. The alignments were done using the 'AMPS' (alignment of multiple protein sequences) package of computer programmes (Barton & Sternberg 1987) and compared by the conventional dynamic programming method of pairwise alignment based on the algorithm of Needleman & Wunsch (1970).

To perform these comparisons we have made certain assumptions as some of the sequences have been derived directly whereas others have not. The early data came from protein sequencing. Wheat calmodulin appears to have an extra amino acid between residues 8 and 9 (Toda *et al.* 1985), barley calmodulin does not (Ling & Zielinski 1989). The former is based on available protein sequence and the latter is deduced from the DNA sequence. Consequently, we have chosen to remove the extra 'N' from wheat. Based on DNA sequence data, *Saccharomyces cerevisiae* has lost an amino acid at position 130 and again at 148 (Davis *et al.* 1986). Amino terminal extensions are found in *Dictyostelium* (Marshak *et al.* 1984) *Chlamydomonas* (Zimmer *et al.* 1988) and *Schizosaccharomyces pombe* (Takeda & Yamamoto 1987), carboxy terminal extensions are seen only in *Dictyostelium* (Marshak *et al.* 1984; Goldhagen & Clarke 1986) and *Chlamydomonas* (Zimmer *et al.* 1988). The available sequence for *Dictyostelium* is a combination of incomplete protein and DNA sequences. The sequence for *Chlamydomonas* calmodulin is from the complete DNA sequence and the same is true for *S. pombe*. 'Z' occurs in some of the early protein sequences, namely in spinach (Lukas *et al.* 1984) and in *Dictyostelium* (Marshak *et al.* 1984). We have completed the sequences of Arb A and Arb B based on the discussion in the paper reporting their existence (Hardy *et al.* 1988). We have not included any of the human and rat pseudogenes in these analyses or squidulin or the calmodulin-like sequence from *Caenorhabditis elegans*.

The programme 'Protpars' from the 'Phylip' package of programmes (version 2.8), provided by Dr J. Felsenstein, was used to infer evolutionary relationships.

(b) *Molecular modelling studies*

To investigate the nature of the interaction of calmodulin antagonists with the hydrophobic pockets of Ca^{2+}-activated calmodulin we have performed molecular modelling studies based on the structural data of Babu *et al.* (1988). From the amino acid sequence alignment (table 1) we judged that the tertiary structure of calmodulin is conserved. It has been observed that during the process of evolution, tertiary structure is more conserved than the amino acid sequence and the number of stable folds is limited to each level in the hierarchy of protein structures (Rossmann *et al.* 1974; Phillips *et al.* 1983; Sali & Blundell 1990). Thus, in the absence of three-dimensional structures for *P. falciparum*, *Trypanosoma brucei* and *S. cerevisiae* calmodulins, the high degree of sequence identity enabled us to predict the structures with confidence, based on the refined three-dimensional structure of mammalian calmodulin (Babu *et al.* 1988). The methodologies used for modelling the three structures were similar. Using the 'maximum overlap approach' (MOP) of Hermans & McQueen (1974), the mammalian calmodulin was modified to accommodate the three calmodulin amino acid sequences by changing the side chains that differ in the three molecules and by rearranging the main chain to accommodate deletions. Conserved side chains were placed in the interior of the molecule, and care was taken to orient changed side chains so that they occupied nearly the same positions of the corresponding side chains in mammalian calmodulin.

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The first four and the last amino acid residues are poorly defined in the mammalian calmodulin crystal structure and were not included in this study. In *S. cerevisiae* there is a deletion at position 130 (Davis *et al.* 1986) which occurs in the fourth Ca^{2+}-binding site of mammalian and other known calmodulins. This region was modelled using a database search as described by Jones & Thirup (1986), based on the fragment matching method used in modelling homologous proteins. The possible helix-loop-helix structures for this region in the model structure for *S. cerevisiae* were individually examined on the graphics machine. The loop in this part of the molecule is similar to the corresponding loop in mammalian calmodulin. The resultant model structures were subjected to energy minimization *in vacuo* using GROMOS library of computer programmes (Aqvist *et al.* 1985) with the standard potentials as described by van Gunsteren & Karplus (1982). All bond lengths and bond angles were optimized to fit the potentials used in the energy minimization. Electrostatic charges, including the calcium ion contributions, were not considered in the calculations. Calcium ligand distances within the four loops were idealized. Convergence was achieved after 750 cycles of conjugate gradient energy minimization in steps of 0.01 Kcal mol^{-1}. All the three model structures were reexamined on the graphics machine.

Certain pharmacological reagents prevent the Ca^{2+}-dependent activation of enzymes by calmodulin, these include two antipsychotics, trifluoperazine (TFP) and chlorpromazine (CPZ). The classical features of this class of drugs include two or three aromatic rings, with a positively charged side chain amino group which is at least four atoms away from the ring structure (Weiss *et al.* 1982). Two antimalarials, quinacrine and chloroquine have similar structural features. Consequently, we modelled these four drugs with calmodulin from *P. falciparum*. The drug molecules were chosen from the Cambridge database and fitted into the hydrophobic clefts of the calmodulin molecule. This exercise was based on the available knowledge of previous drug binding studies using: (i) a predicted model of TFP bound calmodulin derived from troponin C (Strynadka & James 1986); and (ii) the preliminary structure of TFP-calmodulin complex (Babu *et al.* 1988).

All the model building was done using the computer graphics programme package FRODO (Jones 1978, 1985) as implemented on the Evans and Sutherland PS390 colour graphics by J. W. Pflugrath, M. Saper, R. Hubbard and P. R. Evans connected to a Vax 3100 computer system.

(*c*) *Parasites*

The clone T9/96 of *P. falciparum*, was grown *in vitro* using the method described by Trager & Jensen (1976). Synchrony was maintained by sorbitol lysis (Lambros & Vanderberg 1979) and Percoll layering (Howard & Reese 1984).

(*d*) *Drugs*

Calmodulin antagonists, *N*-(4-aminobutyl)-2-naphthalene-sulphonamide (W12) and *N*-(4-aminobutyl)-5-chloro-2-naphthalene-sulphonamide (W13), were purchased from Seikagaku Kogyo Co. Ltd, Japan. Trifluoperazine (10-[3-(4-methyl-1-piperazinyl)propyl]-2-trifluoromethyl-phenothiazine)dihydrochloride (TFP), chlorpromazine (CPZ) (2-chloro-10-[3-dimethyl amino propyl] phenothiazine) hydrochloride and verapamil were purchased from Sigma Ltd., Poole, Dorset. All the drugs were initially dissolved in distilled water, sterilized by passage through a 0.22 μm Millex filter and diluted in culture medium when required. Stock solutions were stored aliquoted at −20°C.

(*e*) *In vitro assays*

An erythrocyte suspension of parasitized cells (10% by volume) was dispensed in triplicate into the wells of microtitre plates. An equal volume of complete medium was added containing the various drugs at twice the required final concentration. Culture medium alone was added to the controls. The viability of the cultures was assessed by morphology. This procedure was found to be more suitable than measurements of uptake of nucleic acid precursor as we were able to follow any changes in morphology both of the parasite and of the red cell. Routinely 2000 red blood cells were assessed for the presence of parasites. The 50% inhibitory concentrations (IC_{50}) were obtained by graphic interpolation of concentration–response curves. All experiments were repeated at least once.

Experiments were designed to see whether the calmodulin antagonists were mediating their effects through the host red-cell or parasite calmodulin and at what stage in the erythrocytic life-cycle of the parasite. Parasite maturation was assessed indirectly using an invasion assay as follows: ring-stage parasites were incubated for 24 h in the presence of the different inhibitors prior to washing three times in RPMI-1640 and being returned to culture for a further 36 h. For the red-cell pretreatment assays, red blood cells were incubated at 37°C in the presence of complete medium containing inhibitors, prior to being washed three times in RPMI-1640 and used as targets in a parasite invasion assay.

3. RESULTS

(*a*) *Comparison of amino acid sequences of different calmodulins*

A multiple sequence alignment of calmodulin amino acid sequences is shown in table 1. The levels of sequence identity are given in table 2. The highest homology exists among the vertebrates. Lower eukaryotes, which include the fungi and the protozoa, show much lower levels of homology. The most diverse sequences appear to be those for the two yeasts *S. cerevisiae* (Davis *et al.* 1986) and *S. pombe* (Takeda & Yamamoto 1987), and *Candida albicans* (Saporito & Sypherd 1991). Such differences in levels of homology, may reflect the diversity of calmodulin function in the vertebrates.

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Table 1. *Multiple sequence alignment of calmodulin proteins*

(The numbers down the left hand side refer to the references in the table footnote and apply to table 2 as well. H, T and B refer to names of secondary structure elements as observed in the mammalian calmodulin crystal structure (Babu *et al.* 1988). Structure: secondary structure according to DSSP (Kabsch & Sander 1983), H = helix (α-helix or 3_{10}-helix), E = β-strand and T = turn, dashes refer to disordered structure. Water: solvent accessibility of a residue in rat calmodulin, calculated from the crystal structure, in units of the estimated number of water molecules in contact with this residue, equivalent to solvent-accessible surface area in units of 10Å^2; residues with 'water' = 0 are completely in the protein interior, 'water' = * are highly accessible. The four EF-hands are highlighted with asterisks, the calcium ligands in the four Ca^{2+}-binding sites are marked with the letter 'C'.)

			H1	T1 B1	H2		H3 T2	B2 H4	
		----HHHHHH	HHHHHHHHHT	TTT EEEHH	HHHHHHHH	HHHHHH	HHHHHTTTT	EEEHHHHHH	HHHHHHH
	structure water	----****74	*5**417*51	**6*33130*	220603*9*7	**28*7*07*	609*329*6*	3314481146	544**9*
	residue number	1	11	21	31	41	51	61	71
	EF hand		*	**********	*		*****	*******	
	calcium ligands		C	C C C	C		C C C	C C	
19	rat	ADQLTEEQIA	EFKEAFSLFD	KDGDGTITTK	ELGTVMRSLG	QNPTEAELQD	MINEVDADGN	GTIDFPEFLT	MMARKMK
31	*Tetrahymena*	ADQLTEEQIA	EFKEAFSLFD	KDGDGTITTK	ELGTVMRSLG	QNPTEAELQD	MINEVDADGN	GTIDFPEFLS	LMARKMK
24	*Paramecium*	AQELTEEQIA	EFKEAFALFD	KDGDGTITTK	ELGTVMRSLG	QNPTEAELQD	MINEVDADGN	GTIDFPEFLS	LMARKMK
34	wheat	ADQLTDEQIA	EFKEAFSLFD	KDGDGCITTK	ELGTVMRSLG	QNPTEAELQD	MINEVDADGN	GTIDFPEFLN	LMARKMK
2	alfalfa	ADQLTDEQIS	EFKEAFSLFD	KDGDGCITTK	ELGTVMRSLG	QNPTEAELQD	MINEVDADGN	GTIDFPEFLN	LMARKMK
16	barley	ADQLTDDQIA	EFKEAFSLFD	KDGDGCITTK	ELGTVMRSLG	QNPTEAELQD	MINEVDADGN	GTIDFPEFLN	LMARKMK
30	spinach	AZZLTDEQIA	EFKEAFSLFD	KDGDGCITTK	ELGTVMRSLG	QNPTEAELQD	MINEVDADGN	GTIDFPEFLN	LMARKMK
29	potato	AEQLTEEQIA	EFKEAFSLFD	KDGDGCITTK	ELGTVMRSLG	QNPTEAELQD	MISEADADQN	GTIDFPEFLN	LMARKMK
33	*T. cruzi*	ADQLSNEQIS	EFKEAFSLFD	KDGDGTITTK	ELGTVMRSLG	QNPTEAELQD	MINEVDQDGS	GTIDFPEFLT	LMARKMQ
32	*T. brucei*	ADQLSNEQIS	EFKEAFSLFD	KDGDGTITTK	ELGTVMRSLG	QNPTEAELQD	MINEVDQDGS	GTIDFPEFLT	LMARKMQ
15	*Euglena*	AEALTHEQIA	EFKEAFSLFD	KDGDGTITTK	ELGTVMRSLG	QNPTEAELQD	MINEVDQDGS	GTIDFPEFLT	LMSRKMH
23	*Renilla*	ADQLTEEQIA	EFKEAFSLFD	KDGDGTITTK	ELGTVMRSLG	QNPTEAELQD	MINEVDADGD	GTIDFPEFLT	MMARKMK
22	*Metridium*	ADQLTEEQIA	EFKEAFSLFD	KDGDGTITTK	ELGTVMRSLG	QNPTEAELQD	MINEVDADGD	GTIDFPEFLT	MMARKMK
25	scallop	ADQLTEEQIA	EFKEAFSLFD	KDGDGTITTK	ELGTVMRSLG	QNPTEAELQD	MINEVDADGD	GTIDFPEFLT	MMARKMK
1	*Aplysia*	ADQLTEEQIA	EFKEAFSLFD	KDGDGTITTK	ELGTVMRSLG	QNPTEAELQD	MINEVDADGD	GTIDFPEFLT	MMARKMK
27	sea squirt	ADQLTEEQIA	EFKEAFSLFD	KDGDGTITTK	ELGTVMRSLG	QNPTEAELQD	MINEVDADGD	GTIDFPEFLT	MMARKMK
12	*Drosophila*	ADQLTEEQIA	EFKEAFSLFD	KDGDGTITTK	ELGTVMRSLG	QNPTEAELQD	MINEVDADGN	GTIDFPEFLT	MMARKMK
13	locust	ADQLTEEQIA	EFKEAFSLFD	KDGDGTITTK	ELGTVMRSLG	QNPTEAELQD	MINEVDADGN	GTIDFPEFLT	MMARKMK
8	*Arbacia* B	ADQLTEEQIA	EFKEAFSLFD	KDGDGTITTK	ELGTVMRSLG	QNPTEAELQD	MINEVDADGN	GTIDFPEFLT	MMARKMK
4	Salmon	ADQLTEEQIA	EFKEAFSLFD	KDGDGTITTK	ELGTVMRSLG	QNPTEAELQD	MINEVDADGN	GTIDFPEFLT	MMARKMK
3	*Xenopus*	ADQLTEEQIA	EFKEAFSLFD	KDGDGTITTK	ELGTVMRSLG	QNPTEAELQD	MINEVDADGN	GTIDFPEFLT	MMARKMK
5	*Arbacia* A	ADQLTEEQIA	EFKEAFSLFD	KDGDGTITTK	ELGTVMRSLG	QNPTEAELQD	MINEVDADGN	GTIDFPEFLT	MMARKMK
17	human	ADQLTEEQIA	EFKEAFSLFD	KDGDGTITTK	ELGTVMRSLG	QNPTEAELQD	MINEVDADGN	GTIDFPEFLT	MMARKMK
18	rabbit	ADQLTEEQIA	EFKEAFSLFD	KDGDGTITTK	ELGTVMRSLG	QNPTEAELQD	MINEVDADGN	GTIDFPEFLT	MMARKMK
20	cow	ADQLTEEQIA	EFKEAFSLFD	KDGDGTITTK	ELGTVMRSLG	QNPTEAELQD	MINEVDADGN	GTIDFPEFLT	MMARKMK
21	chicken	ADQLTEEQIA	EFKEAFSLFD	KDGDGTITTK	ELGTVMRSLG	QNPTEAELQD	MINEVDADGN	GTIDFPEFLT	MMARKMK
14	eel	ADQLTEEQIA	EFKEAFSLFD	KDGDGTITTK	ELGTVMRSLG	QNPTEAELQD	MINEVDADGN	GTIDFPEFLT	MMAKKMK
26	*Pleurotus*	ADQLSEEQIS	EFKEAFSLFD	KDGDGTITTK	ELGTVMRSLG	QNPTEAELQD	MINEVDADGN	GTIDFPEFLT	MMARKMR
6	*P. falciparum*	ADKLTEEQIS	EFKEAFSLFD	KDGDGTITTK	ELGTVMRSLG	QNPTEAELQD	MINEIDTDGN	GTIDFPEFLT	LMARKLK
11	*Dictyostelium*	ASSZZLTEEQIA	EFKEAFSLFD	KDGDGSITTK	ELGTVMRSLG	QNPTEAELQD	MINEVDADGN	GNIDFPEFLT	MMARKMQ
10	*Chlamydomonas*	AANTEQLTEEQIA	EFKEAFALFD	KDGDGTITTK	ELGTVMRSLG	QNPTEAELQD	MISEVDADGN	GTIDFPEFLM	LMARKMK
7	*Achlya*	ADQLTEEQIA	EFKEAGSLFD	KDGDGTITTK	ELGTVMRSVG	QNPTEAELQD	MINEVDADGN	GTIDFPEFLT	MMARKMK
9	*Aspergillus*	ADSLTEEQVS	EYKEAFSLFD	KDGDGQITTK	ELGTVMRSLG	QNPSESELQD	MINEVDADNN	GTIDFPEFLT	MMARKMK
28	*S. pombe*	TTRNLTDEQIA	EFREAFSLFD	RDQDGNITSN	ELGVVMRSLG	QSPTAAELQD	MINEVDADGN	GTIDFTEFLT	MMARKMK
35	*S. cerevisiae*	SSNLTEEQIA	EFKEAFALFD	KDNNGSISSS	ELATVMRSLG	LSPSEAEVND	LMNEIDVDGN	HQIEFSEFLA	LMSRQLK
36	tomato	AEQLTEEQIA	EFKEAFSLFD	KDGDGCITTK	ELGTVMRSLG	QNPTEAELQD	MISEVDADQN	GTIDFPEFLN	LMARKMK
37	*Candida*	AEKLSEQQIA	EFKEAFSLFD	KDSDGKITTK	ELGTVMRSLG	QNPSESELTD	MINEVDVNSD	GSIDFPEFLT	MMARKMK

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Table 1. (*Continued*)

			H4	T3 B3	H5		H6	T4 B4	H7	
		HHH	HHHHHHHHHH	HHTTTT EE	EHHHHHHHHH	H HHH	HHHHHHHHTT	TT EEEHHH	HHHHHH-	
	structure water	*59	67**3**42*	*31*977390	32622**146	**6**389*9	06*80**13*	96646135*0	1388*9*-	
	residue number		81	91	101	111	121	131	141 148	
	EF hand			********	****		**	**********		
	calcium ligands			C C C C	C		C	C C C C		
19	rat	DTD	SEEEIREAFR	VFDKDGNGYI	SAAELRHVMT	NLGEKLTDEE	VDEMIREADI	DGDGQVNYEE	FVQMMTAK	
31	*Tetrahymena*	DTD	TEEELIEAFK	VFDRDDNDLI	SAAELRHVMT	NLGEKLTDEE	VDEMIREADI	DGDGHINYEE	FVRMMMAK	
24	*Paramecium*	EQD	SEEELIEAFK	VFDRDGNGLI	SAAELRHVMT	NLGEKLTDDE	VDEMIREADI	DGDGHINYEE	FVRMMVSK	
34	wheat	DTD	SEEELKEAFR	VFDKDQDGFI	SAAELRHVMT	NLGEKLTDEE	VDEMIREADV	DGDGQINYEE	FVKVMMAK	
2	alfalfa	DTD	SEEELKEAFR	VFDKDQNGFI	SAAELRHVMT	NLGEKLTDEE	VDEMIREADV	DGDGQINYEE	FVKVMAAK	
16	barley	DTD	SEEELKEAFR	VFDKDQNGFI	SAAELRHVMT	NLGEKLTDEE	VDEMIREADV	DGDGQINYEE	FVKVMMAK	
30	spinach	DTD	SEEELKEAFR	VFDKDQNGFI	SAAELRHVMT	NLGEKLTDEE	VDEMIREADV	DGDGQINYEE	FVKVMMAK	
29	potato	DTD	SEEELKEAFK	VFDKDQNGFI	SAAELRHVMT	NLGEKLTDEE	VDEMIREADI	DGDGQVNYEE	FVRMMLAK	
33	*T. cruzi*	DSD	SEEEIKEAFR	VFDKDGNGFI	SAAELRHVMT	NLGEKLTDEE	VDEMIREADV	DGDGQINYEE	FVKMMMSK	
32	*T. brucei*	DSD	SEEEIKEAFR	VFDKDGNGFI	SAAELRHIMT	NLGEKLTDEE	VDEMIREADV	DGDGQINYEE	FVKMMMSK	
15	*Euglena*	DTD	TEEEIKEAFR	VFDKDGNGFI	SAAELRHVMT	NLGEKLTDEE	VDEMIREADV	DGDGQINYEE	FVKMMMSK	
23	*Renilla*	DTD	SEEEIREAFR	VFDKDGDGFI	SAAELRHVMT	NLGEKLTDEE	VDEMIREADI	DGDGQVNYEE	FVKMMTSK	
22	*Metridium*	DTD	SEEEIREAFR	VFDKDGDGFI	SAAELRHVMT	NLGEKLTDEE	VDEMIREADI	DGDGQVNYEE	FVKMMTSK	
25	scallop	DTD	SEEEIREAFR	VFDKDGDGFI	SAAELRHVMT	NLGEKLTDEE	VDEMIREADI	DGDGQVNYEE	FVTMMTSK	
1	*Aplysia*	DTD	SEEEIREAFR	VFDKDGDGFI	SAAELRHVMT	NLGEKLTDEE	VDEMIREADI	DGDGQVNYEE	FVTMMTSK	
27	sea squirt	DTD	SEEEIREAFR	VFDKDGNGFI	SAAELRHVMT	NLGEKLTDEE	VDEMIREADI	DGDGQVNYEE	FVTMMTSK	
12	*Drosophila*	DTD	SEEEIREAFR	VFDKDGNGFI	SAAELRHVMT	NLGEKLTDEE	VDEMIREADI	DGDGQVNYEE	FVTMMTSK	
13	locust	DTD	SEEEIREAFR	VFDKDGNGFI	SAAELRHVMT	NLGEKLTDEE	VDEMIREADI	DGDGQVNYEE	FVTMMTSK	
8	*Arbacia* B	ETD	SEEEIREAFR	VFDKDGNGFI	SAAELRHVMT	NLGEKLTDEE	VDEMIREADI	DGDGQVNYEE	FVAMMTSK	
4	salmon	DTD	SEEEIREAFR	VFDKDGNGYI	SAAELRHVMT	NLGEKLTDEE	VDEMIREADI	DGDGQVNYEE	FVQMMTAK	
3	*Xenopus*	DTD	SEEEIREAFR	VFDKDGNGYI	SAAELRHVMT	NLGEKLTDEE	VDEMIREADI	DGDGQVNYEE	FVQMMTAK	
5	*Arbacia* A	DTD	SEEEIREAFR	VFDKDGNGYI	SAAELRHVMT	NLGEKLTDEE	VDEMIREADI	DGDGQVNYEE	FVQMMTAK	
17	human	DTD	SEEEIREAFR	VFDKDGNGYI	SAAELRHVMT	NLGEKLTDEE	VDEMIREADI	DGDGQVNYEE	FVQMMTAK	
18	rabbit	DTD	SEEEIREAFR	VFDKDGNGYI	SAAELRHVMT	NLGEKLTDEE	VDEMIREADI	DGDGQVNYEE	FVQMMTAK	
20	cow	DTD	SEEEIREAFR	VFDKDGNGYI	SAAELRHVMT	NLGEKLTDEE	VDEMIREADI	DGDGQVNYEE	FVQMMTAK	
21	chicken	DTD	SEEEIREAFR	VFDKDGNGYI	SAAELRHVMT	NLGEKLTDEE	VDEMIREADI	DGDGQVNYEE	FVQMMTAK	
14	eel	DTD	SEEEIREAFR	VFDKDGNGYI	SAAELRHVMT	NLGEKLTDEE	VDEMIREADI	DGDGQVNYEE	FVQMMTAK	
26	*Pleurotus*	DTD	SEEEIKEAFK	VFDKDGNGYI	SAAELRHVMT	NLGEKLTDNE	VDEMIREADI	DGDGQINYEE	FVKMMLSK	
6	*P. falciparum*	DTD	TEEELIEAFR	VFDRDGDGYI	SADELRHVMT	NLGEKLTNEE	VDEMIREADI	DGDGQINYEE	FVKMMIAK	
11	*Dictyostelium*	DTD	TEEEIREAFK	VFDKDGNGYI	SAAELRHVMT	SLGEKLTNEE	VDEMIREADL	DGDGQVNYDE	FVKMMIVRN	
10	*Chlamydomonas*	ETD	HEDELREAFK	VFDKDGNGFI	SAAELRHVMT	NLGEKLSEEE	VDEMIREADV	DGDGQVNYEE	FVRMMTSGAT	DDKDKKGHK
7	*Achlya*	DTD	SEEEILEAFQ	GFDKDGNGFI	SAAELRHMMT	NLGEKLTDEE	VDEMIREADI	DGDGQINYEE	FVKMMMSK	
9	*Aspergillus*	DTD	SEEEIREAFK	VFDRDNNGFI	SAAELRHVMT	SIGEKLTDDE	VDEMIREADQ	DGDGRIDYNE	FVQLMMQK	
28	*S. pombe*	DTD	NEEEVREAFK	VFDKDGNGYI	TVEELTHVLT	SLGERLSQEE	VADMIREADT	DGDGVINYEE	FSRVISSK	
35	S. cerevisiae	SND	SEQELLEAFK	VFDKNGDGLI	SAAELKHVLT	SIGEKLTDAE	VDDMLREVS	DGSGEINIQQ	FAALLSK	
36	tomato	DTD	SEEELKEAFK	VFDKDQNGFI	SAAELRHVMT	NLGEKLTDEE	VDEMIREADI	DGDGQVNYEE	FVRMMLAK	
37	*Candida*	DTD	SEAEIAEAFK	VFDRNGDGKI	SAAELRHLLT	SIGEKLSDAD	VDQMIKEADT	NNDGEIDIQE	FTLLLAAK	

1. Swanson *et al.* (1990); 2. Barnett & Long (1990); 3. Chien & Dawid (1984); 4. Yazawa *et al.* (1985); 5. Hardy *et al.* (1988); 6. Robson & Jennings (1991); 7. Le John (1989); 8. Hardy *et al.* (1988); 9. Rasmussen *et al.* (1990); 10. Zimmer *et al.* (1988); 11. Marshak *et al.* (1984); Goldhagen & Clarke (1986); 12. Smith *et al.* (1987); 13. Toda (1988); 14. Lagace *et al.* (1983); 15. Toda *et al.* (1988); 16. Ling & Zielinski (1989); 17. Fischer *et al.* (1988); Wawrzynczak & Perham (1984); SenGupta *et al.* (1987); Sasagawa *et al.* (1982); 18. Grand *et al.* (1981); 19. Nojima *et al.* (1987); 20. Watterson *et al.* (1980); 21. Putkey *et al.* (1983); 22. Yazawa & Yagi (1980); 23. Jamieson *et al.* (1980); 24. Schaefer *et al.* (1987); 25. Toda *et al.* (1981); 26. Toda *et al.* (1987); 27. Yazawa *et al.* (1988); 28. Takeda & Yamamoto (1987); 29. Jena *et al.*, (1989); 30. Lukas *et al.* (1984); 31. Yazawa *et al.* (1981); Hinrichsens *et al.* (1990); 32. Tschudi *et al.* (1985); 33. Chung & Swindle (1990); 34. Toda *et al.* (1985); 35. Davis *et al.* (1986); 36. Colwell *et al.* (1991); 37. Saporito & Sypherd (1991).

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Table 2. *Degree of sequence identity among different calmodulins*

(The numbers along the top axis refer to the references for the sequences which are listed in the footnote to table 1.)

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36
1	*Aplysia*																																				
2	alfalfa	89.2																																			
3	*Xenopus*	96.6	90.5																																		
4	salmon	96.6	90.5	100																																	
5	*Arbacia* A	96.6	90.5	100	100																																
6	*P. falciparum*	87.8	87.8	89.2	89.2	89.2																															
7	*Achlya*	92.6	89.2	92.6	92.6	92.6	85.8																														
8	*Arbacia* B	97.3	89.9	97.3	97.3	97.3	87.2	93.2																													
9	*Aspergillus*	83.1	83.1	84.5	84.5	84.5	79.1	82.4	83.8																												
10	*Chlamydomonas*	87.8	85.1	87.8	87.8	87.8	81.8	84.5	89.9	77																											
11	*Dictyostelium*	87.2	83.1	89.2	89.2	89.2	84.5	85.1	87.8	80.4	82.8																										
12	*Drosophila*	98.7	90.5	98	98	98	87.8	93.9	98.7	84.5	89.2	88.5																									
13	locust	98.7	90.5	98	98	98	87.8	93.9	98.7	84.5	89.2	88.5	100																								
14	electric eel	96	89.9	99.3	99.3	99.3	88.5	91.9	96.6	83.8	87.2	88.5	97.3	97.3																							
15	*Euglena*	89.9	89.9	89.2	89.2	89.2	86.5	89.2	89.9	79.7	85.1	85.1	90.5	90.5	88.5																						
16	barley	89.2	98.7	90.5	90.5	90.5	86.5	89.2	89.9	81.8	85.1	83.1	90.5	90.5	89.9	89.9																					
17	human	96.6	90.5	100	100	100	89.2	92.6	97.3	84.5	87.8	89.2	98	98	99.3	89.2	90.5																				
18	rabbit	96.6	90.5	100	100	100	89.2	92.6	97.3	84.5	87.8	89.2	98	98	99.3	89.2	90.5	100																			
19	rat	96.6	90.5	100	100	100	89.2	92.6	97.3	84.5	87.8	89.2	98	98	99.3	89.2	90.5	100	100																	
20	cow	96.6	90.5	100	100	100	89.2	92.6	97.3	84.5	87.8	89.2	98	98	99.3	89.2	90.5	100	100	100																
21	chicken	96.6	90.5	100	100	100	89.2	92.6	97.3	84.5	87.8	89.2	98	98	99.3	89.2	90.5	100	100	100	100															
22	*Metridium*	99.3	89.9	96.6	96.6	96.6	88.5	93.2	97.3	83.1	87.8	87.8	98	98	96	90.5	89.9	96.6	96.6	96.6	96.6	96.6														
23	*Renilla*	99.3	89.9	96.6	96.6	96.6	88.5	93.2	97.3	83.1	87.8	87.8	98	98	96	90.5	89.9	96.6	96.6	96.6	96.6	96.6	100													
24	*Paramecium*	87.2	85.8	87.8	87.8	87.8	85.8	86.5	89.2	81.1	87.2	81.8	88.5	88.5	87.2	85.1	85.8	87.8	87.8	87.8	87.8	87.8	87.2	87.2												
25	scallop	100	89.2	96.6	96.6	96.6	87.8	92.6	97.3	83.1	87.8	87.2	98.7	98.7	96	89.9	89.2	96.6	96.6	96.6	96.6	96.6	99.3	99.3	87.2											
26	*Pleurotus*	91.9	89.9	93.2	93.2	93.2	87.8	91.9	92.6	83.8	84.5	87.2	93.2	93.2	92.6	89.2	88.5	93.2	93.2	93.2	93.2	93.2	92.6	92.6	87.8	91.9										
27	sea squirt	99.3	89.9	97.3	97.3	97.3	87.2	93.2	98	83.8	88.5	87.8	99.3	99.3	96.6	90.5	89.9	97.3	97.3	97.3	97.3	97.3	98.7	98.7	87.8	99.3	92.6									
28	*S. pombe*	73.7	73	75	75	75	70.3	72.3	74.3	68.2	72.5	73.2	75	75	74.3	71.6	73	75	75	75	75	75	73.7	73.7	71.6	73.7	73.7	74.3								
29	potato	89.2	91.9	90.5	90.5	90.5	85.1	87.2	89.9	80.4	88.5	83.8	90.5	90.5	89.9	86.5	91.9	90.5	90.5	90.5	90.5	90.5	89.2	89.2	87.2	89.2	88.5	89.9	71							
30	spinach	88.5	98	89.9	89.9	89.9	86.5	88.5	89.2	81.8	85.1	85.1	89.9	89.9	89.2	90.5	98	89.9	89.9	89.9	89.9	89.9	89.2	89.2	86.5	88.5	87.8	89.2	73.7	91.9						
31	*Tetrahymena*	88.5	89.9	90.5	90.5	90.5	89.2	88.5	89.2	82.4	85.8	83.8	89.9	89.9	89.9	87.2	89.9	90.5	90.5	90.5	90.5	90.5	88.5	88.5	92.6	88.5	88.5	89.2	72.3	89.9	89.2					
32	*T. brucei*	89.9	91.2	89.2	89.2	89.2	85.8	89.9	89.9	80.4	83.1	83.1	90.5	90.5	88.5	93.2	89.9	89.2	89.2	89.2	89.2	89.2	90.5	90.5	84.5	89.9	91.9	90.5	69.6	85.1	89.2	85.8				
33	*T. cruzi*	90.5	91.9	89.9	89.9	89.9	86.5	89.9	90.5	81.1	83.8	83.8	91.2	91.2	89.2	93.9	90.5	89.9	89.9	89.9	89.9	89.9	91.2	91.2	85.1	90.5	92.6	91.2	70.3	85.8	89.9	86.5	99.3			
34	wheat	90.5	98.7	90.5	90.5	90.5	87.8	89.2	89.9	81.8	85.1	83.1	90.5	90.5	89.9	89.9	98.7	90.5	90.5	90.5	90.5	90.5	91.2	91.2	85.8	90.5	88.5	89.9	73	91.9	98	89.9	89.9	90.5		
35	*S. cerevisiae*	59.6	60.3	59.6	59.6	59.6	60.3	59.6	60.3	58.2	59.6	60.3	59.6	59.6	58.9	60.3	60.3	59.6	59.6	59.6	59.6	59.6	59.6	59.6	63	59.6	61	58.9	56.9	61	61	61	58.2	58.9	61.6	
36	tomato	89.9	92.6	91.2	91.2	91.2	85.1	87.8	90.5	81.1	89.2	84.5	91.2	91.2	90.5	87.1	92.6	91.2	91.2	91.2	91.2	91.2	89.9	89.9	87.8	89.9	89.2	90.5	71.6	99.3	92.6	90.5	85.8	86.5	92.6	61
37	*Candida*	71.6	67.6	71	71	71	68.2	68.9	69.6	73.7	65.5	68.2	70.3	70.3	70.3	68.2	68.2	71	71	71	71	71	71.6	71.6	68.2	71.6	71	71	61.5	68.2	68.2	68.9	68.2	68.2	68.9	60.3	68.9

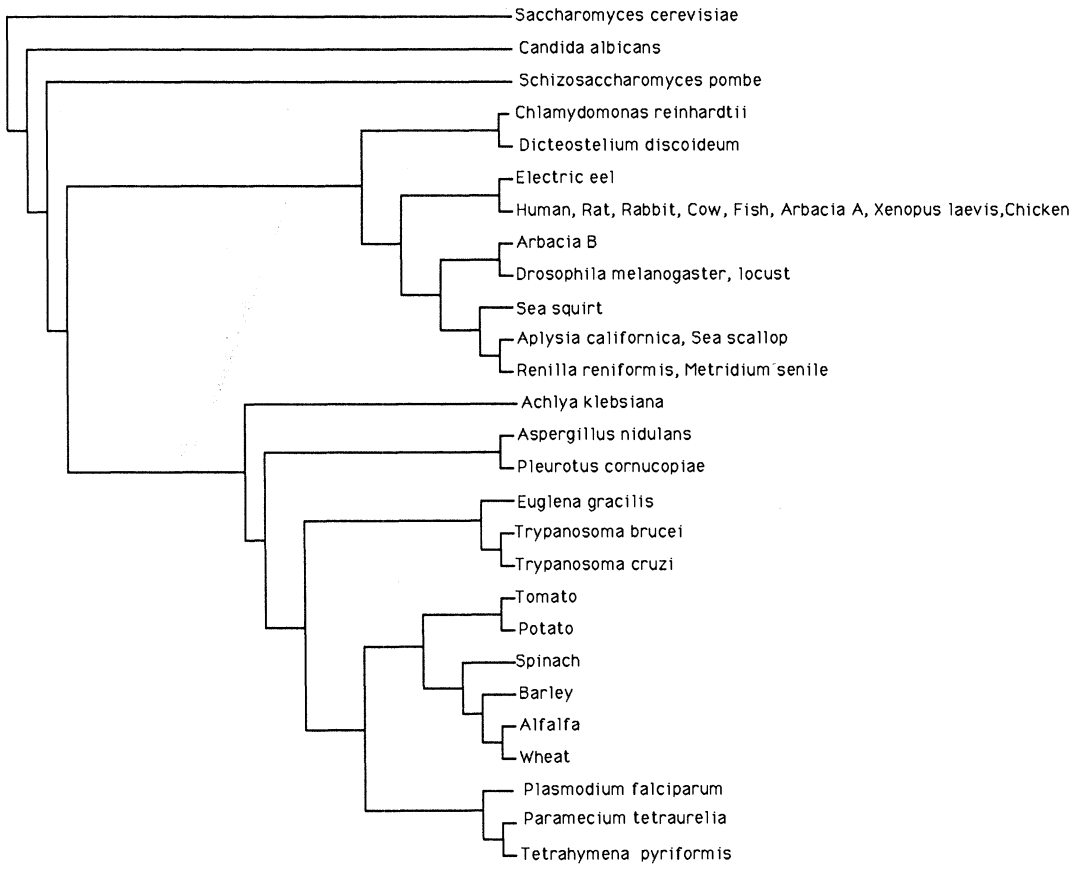

Figure 1. One of the phenograms illustrating the relationships between the different calmodulin sequences. The references for these sequences are given in the footnote to table 1. The order for the plants (spinach, wheat, barley and alfalfa) occurs in 35% (eight out of 23) of the trees, the next most preferred order occurs in four out of 23. The remaining permutations occur in only one or two trees. The order presented in this phenogram for the fungi occurs in 61% (14 out of 23) of the trees, the variation in order involving *Achlya*, *Pleurotus* and *Aspergillus*, and whether the branch order is as shown or reversed. In one of the other trees all the fungi were grouped at the top of the phenogram, before the branch with the animal and plant kingdoms. There is a little variation in the preferred branching among the invertebrates, caused by the sequences for *Drosophila* and *Arbacia* B. The preferred order is shown in this figure and occurs in 17 out of 23 (74%) of the trees.

Across species, there is amino acid sequence conservation and a total of 50 out of 148 residues are invariant (table 1). Of the amino acid changes, 33 occur only in a single organism, and of these 14 are specific to *S. cerevisiae*. This therefore increases the overall sequence identity to 83 out of 148. Where DNA sequence is known, codon usage is characteristic of the given organism.

X-ray crystallographic studies of Babu *et al.* (1988) show that mammalian calmodulin has four domains. Comparison of the various calmodulin sequences corresponding to domain 1 (residues 1–42), shows that this is the most conserved, 42.9%. Domain 3 (residues 76–115) is the next most conserved, 37.5%; and this is not surprising as it is believed to have been derived from domain 1 when there was a ancestral duplication of the gene for a primordial Ca^{2+}-binding protein (Watterson *et al.* 1980). Domains 2 and 4 (residues 43–75 and 116–148 respectively) are less conserved, 33.3% and 18.2% respectively. The drop in homology in domain 4 is due to the sequence changes found in *C. albicans* (Saporito & Sypherd 1991), without the inclusion of this sequence the degree of sequence identity between the different organisms would be 30.3%. The degree of sequence identity in the central helix (residues 65–92) is 42.9%. Certain sequence changes appear to be specific to particular classes of organism, for example cysteine at position 26 in the plants and aspartate at position 126 in the two yeasts.

(*b*) *Evolutionary relationships*

The alignment in table 1 was used to generate an unrooted phylogenetic tree using the maximum parsimony approach (see Felsenstein 1988), which is based on the algorithms of Eck & Dayhoff (1966) and Fitch (1971). Using the sequence for *S. cerevisiae* as the outgroup, 23 trees were generated involving 345 steps. A typical phenogram is shown in figure 1. The overall features of the trees were similar, the differences were caused by the preferred order among the plants, some of the fungi and among the invertebrates. It is difficult to interpret the significance of such details as this is dependent upon the quality of the input sequences.

Table 3. *Changes in the amino acid residues involved in forming the hydrophobic clefts in different calmodulins*

N-terminal half	organism A	B	C	D	E	F	G	H	I	J	K	L	C-terminal half	organism A	B	C	D	E	F	G	H	I	J	K	L
Phe 19													Phe 92													
Ile 27													Ile 100													
Leu 32													Leu 105													
Val 35													Val 108					Ile				Met		Leu		
Met 36													Met 109						Leu					Leu	Leu	
Pro 43													Leu 116													
Leu 48												Val	Val 121													
Met 51												Leu	Met 124													(Ala)
Ile 52												Met	Ile 125												Leu	
Val 55												Ile	Ala 128												Val	
Ile 63				Ile			Ala						Val 136	Ile		Ile	Ile	Ile	Ile		Ile	Ile	Ile	Ile	Asn	(Ile)
Phe 68													Phe 141													(Ala)
Met 71	Leu		Leu		Leu	Leu		Leu	Leu			Leu	Met 144						Val		Val		Leu	Leu	Leu	(Leu)
Met 72													Met 145						Ile					Leu	Leu	(Ser)

A = *Tetrahymena*, *Euglena*, *Paramecium*, *Trypanosoma cruzi*; B = *Chlamydomonas reinhardtii*, *tomato*; C = *Pleurotus cornucopiae*; D = *Plasmodium falciparum*; E = *Trypanosoma brucei*; F = *Schizosaccharomyces pombe*. G = potato; H = alfalfa, barley, wheat, spinach; I = *Achlya klebsiana*; J = *Aspergillus nidulans*; K = *Candida albicans*; L = *Saccharomyces cerevisiae* (the amino acid residue in brackets refers to the next residue in sequence if the deletion is compensated for).

This approach examines the amino acid sequence changes that occur between sequences from different organisms and makes certain that the necessary nucleotide changes are consistent with the genetic code. For example, at the amino acid level, *P. falciparum* shows 89.2% sequence identity with the vertebrates, but is rooted with paramecium and tetrahymena with which it shows 89.2% and 85.8% sequence identity, respectively. Paramecium and tetrahymena show 92.6% identity. The preferred order of branching differs from that of Johnson & Baverstock (1989) who constructed various phylogenetic trees based on the sequence of small subunit ribosomal RNAs. The significance of this probably relates to the different methods used in interpreting the data as well as the quality of the input sequence.

(*c*) *The significance of amino acid sequence changes within the molecule*

Based on the crystal structure of mammalian calmodulin determined at 2.2Å† resolution by Babu *et al.* (1988), the molecule has been described as being dumbbell shaped and prominent secondary structural features include four Ca^{2+}-binding loops (EF hands; Kretsinger & Nockolds 1973) typical of Ca^{2+}-binding proteins. Calmodulin interacts with small hydrophobic molecules such as the phenothiazines and naphthalene sulphonamides (LaPorte *et al.* 1980; Tanaka & Hidaka 1980; Tanaka *et al.* 1982; Strynadka & James 1988). On binding Ca^{2+}, calmodulin undergoes significant conformational changes (Cohen & Klee 1988), generating a hydrophobic cleft at either end of the molecule (figure 2, plate 1), with approximate dimensions of 10Å × 12Å × 9Å, which are the proposed sites of interaction for these molecules. The central helix linking the two Ca^{2+}-binding domains is also the site of interaction between calmodulin and its target enzymes or structural proteins. The high affinity for Ca^{2+} and the resulting conformational changes are responsible for the transmission of the Ca^{2+} signal by binding to and activating numerous enzymes central to cellular regulation (Cohen & Klee 1988). Table 3 shows the changes in the amino acid residues involved in forming these hydrophobic clefts. Based on sequence homology, most changes in the cleft regions have occurred in the two yeasts. Vertebrate calmodulin is involved in many interactions with other proteins so that amino acid sequence changes cannot be tolerated. Between these two ends of the spectrum are the parasites *P. falciparum* and *T. brucei*, plants and free-living organisms, euglena and paramecium.

Amino acid sequence comparisons indicate that these significant differences in the hydrophobic cleft regions of calmodulin deserved further investigation. We have performed malarial invasion assays using known calmodulin antagonists as well as molecular modelling studies of these compounds in the hydrophobic clefts of *P. falciparum* and *T. brucei* calmodulins

† 1 Å = 10^{-10} m = 10^{-1} nm.

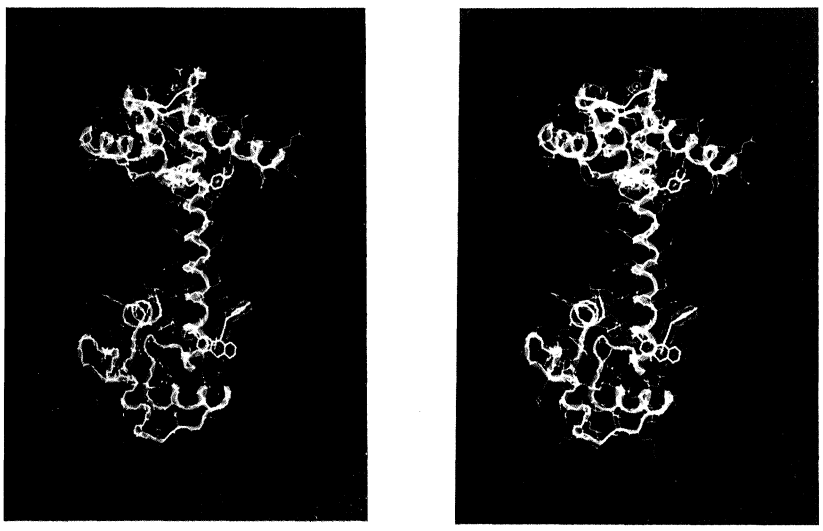

Figure 2. Stereo picture of *P. falciparum* calmodulin. Residues 5–147 are included. The backbone structure is highlighted in orange, the side chains are in pale blue with the calcium ions in the four binding sites being in dark blue. TFP(red) is located in the two plausible drug-binding pockets.

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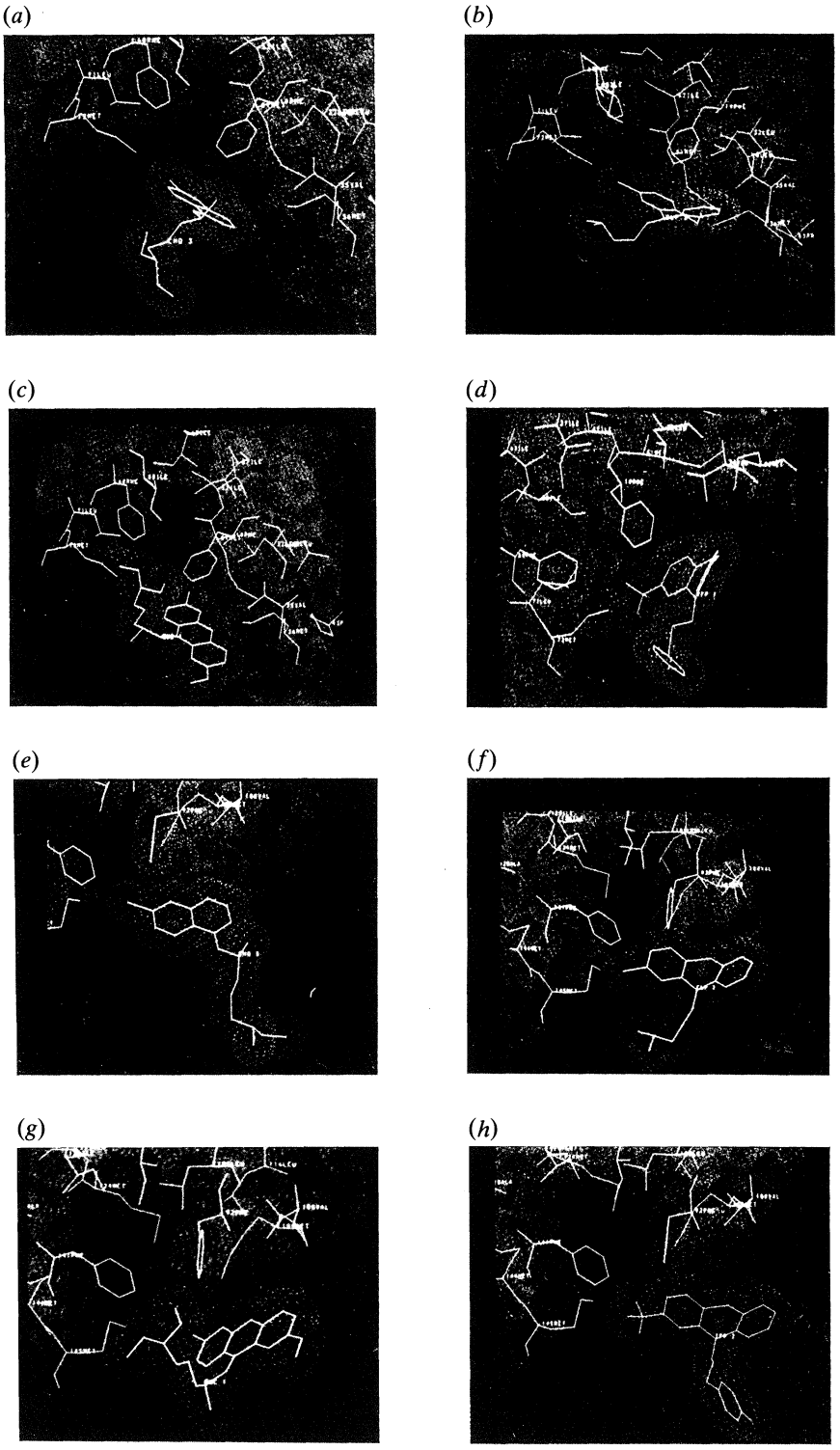

Figure 5. Drug binding in the amino-terminal cleft with the four drugs, chloroquine, chlorpromazine, quinacrine and trifluoperazine is shown in panels (*a–d*), respectively. Drug binding in the carboxy terminal cleft with the four drugs, chloroquine, chlorpromazine, quinacrine and trifuoperazine is shown in panels (*e–h*), respectively. The spheres represent 70% of van der Waal radii. The drugs are in pink and the hydrophobic pockets of calmodulin are in blue.

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Table 4. *Summary of the inhibition studies on P. falciparum* by Ca^{2+} and calmodulin antagonists

	assay		
antagonists	invasion I-IC_{50}/μM	maturation I-IC_{50}/μM	red cell pretreatment I-IC_{50}/μM
W12	125	175	350
W13	15	30.5	51
chlorpromazine	9.2	12.5	55
trifluoperazine	5.4	6.3	45
verapamil	19	19	ND

to see whether these proteins are a suitable new target for novel antimalarials.

(*d*) *Inhibitor studies with Ca^{2+} calmodulin inhibitors*

We have assessed the effects of two naphthalene sulphonamides (W12 and W13), two phenothiazines (CPZ and TFP), and verapamil on parasite invasion and maturation. The results are summarized in table 4. As expected from these and other data (Scheibel *et al.* 1987; Matsumoto *et al.* 1987; Scheibel *et al.* 1989; Tanabe *et al.* 1989) using another pair of naphthalene sulphonamides (W5 and W7) the parasite is more sensitive to the chlorinated homologues W7 and W13. Both the phenothiazines and the naphthalene sulphonamides were more effective at inhibiting parasite invasion than schizont maturation, whereas verapamil, a Ca^{2+}-channel blocker, was not selective. Higher concentrations of both W12 and W13 and the two phenothiazines were required to perturb parasite invasion when the drugs were used to pretreat the red cells. This suggests that host calmodulin is involved in parasite invasion but has a lower sensitivity to the drugs. These concentrations are in agreement with those described for the regulation of red cell shape (Nelson *et al.* 1983). These and other data (Kristiansen & Jepsen 1985; Geary *et al.* 1986; Scheibel *et al.* 1987; Matsumoto *et al.* 1987; Scheibel *et al.* 1989; Tanabe *et al.* 1989) suggest that the naphthalene sulphonamides and the phenothiazines interact with parasite rather than host calmodulin. However, it must be noted that some of the effects of chlorpromazine, trifluoperazine and verapamil may be independent of their action directly or indirectly on parasite calmodulin as at high concentrations these compounds cause hemolysis. Further experiments examining the speed at which these agents perturb schizogony suggest that this occurs within 30 min of adding these compounds to schizont infected red cells (Data not shown).

These results encouraged us to pursue our hypothesis that the increased sensitivity of *P. falciparum* to known calmodulin antagonists might be a consequence of sequence changes in the hydrophobic clefts.

(*e*) *Model building*

Before we could examine the nature of the interaction of various calmodulin antagonists with the hydrophobic pockets of Ca^{2+}-activated calmodulin, it was necessary to build complete atomic models for these proteins from *P. falciparum*, *T. brucei* and *S. cerevisiae*, using the known mammalian structure (Babu *et al.* 1988). A stereo picture of the *P. falciparum* calmodulin molecule is shown in figure 2. The overall length of the molecule is approximately 63Å. In general, the molecule is helical and consists of two similar lobes connected by a long, flexible central helix, a conformation which is unique and found only in a closely related protein troponin C (Herzberg & James, 1985). It is evident from the crystal structure of mammalian calmodulin (Babu *et al.* 1988) that the central helix is involved in interacting with target enzymes. The significant differences in the three model structures are highlighted below:

(i) *Overall conformation*

There are four amino acid changes in the central helix of *P. falciparum*, and *T. brucei* and twelve in *S. cerevisiae*. Nevertheless, a cluster of charged residues is maintained in this helix (table 1).

(ii) *Calcium coordination*

The four Ca^{2+}-binding loops in the calmodulin molecule (figure 3), two in each lobe, exhibit a typical EF-hand (helix-loop-helix) conformation and are located on the surface of the molecule. All these binding sites follow sevenfold coordination, a general Ca^{2+} coordination number as observed in several Ca^{2+} complexes. There is a significant difference in the *S. cerevisiae* fourth Ca^{2+}-binding site, where there is a deletion of an amino acid residue at position 130 (table 1).

(iii) *Hydrophobic surfaces*

Ca^{2+}-activated calmodulin, in general, contains a large hydrophobic cleft in each lobe (figure 2). These clefts face towards each other and are located on opposite sides of the central helix. Sequence changes in the two hydrophobic clefts are summarized in table 3. More sequence variation is found in the carboxy terminal cleft. The deletion of residue 130 affects the size of the carboxy terminal cleft in *S. cerevisiae*.

(iv) *Active lysine*

It is known that lysine 115 is trimethylated in most calmodulins by an irreversible enzymatic reaction. This residue is conserved in all calmodulins except *S. pombe* where lysine has been replaced by an arginine (table 1). Of all the lysines, lysine 115 is the most solvent accessible.

(v) Drug binding

We have modelled the nature of the interaction of calmodulin antagonists with the hydrophobic pockets of Ca^{2+}-activated calmodulin from *P. falciparum*. The drugs chosen included two known antimalarials, quinacrine (Courseille *et al.* 1973*a*) and chloroquine (Courseille *et al.* 1973*b*), as well as two known calmodulin antagonists, chlorpromazine (McDowell 1969) and trifluoperazine (McDowell 1980) (figure 4).

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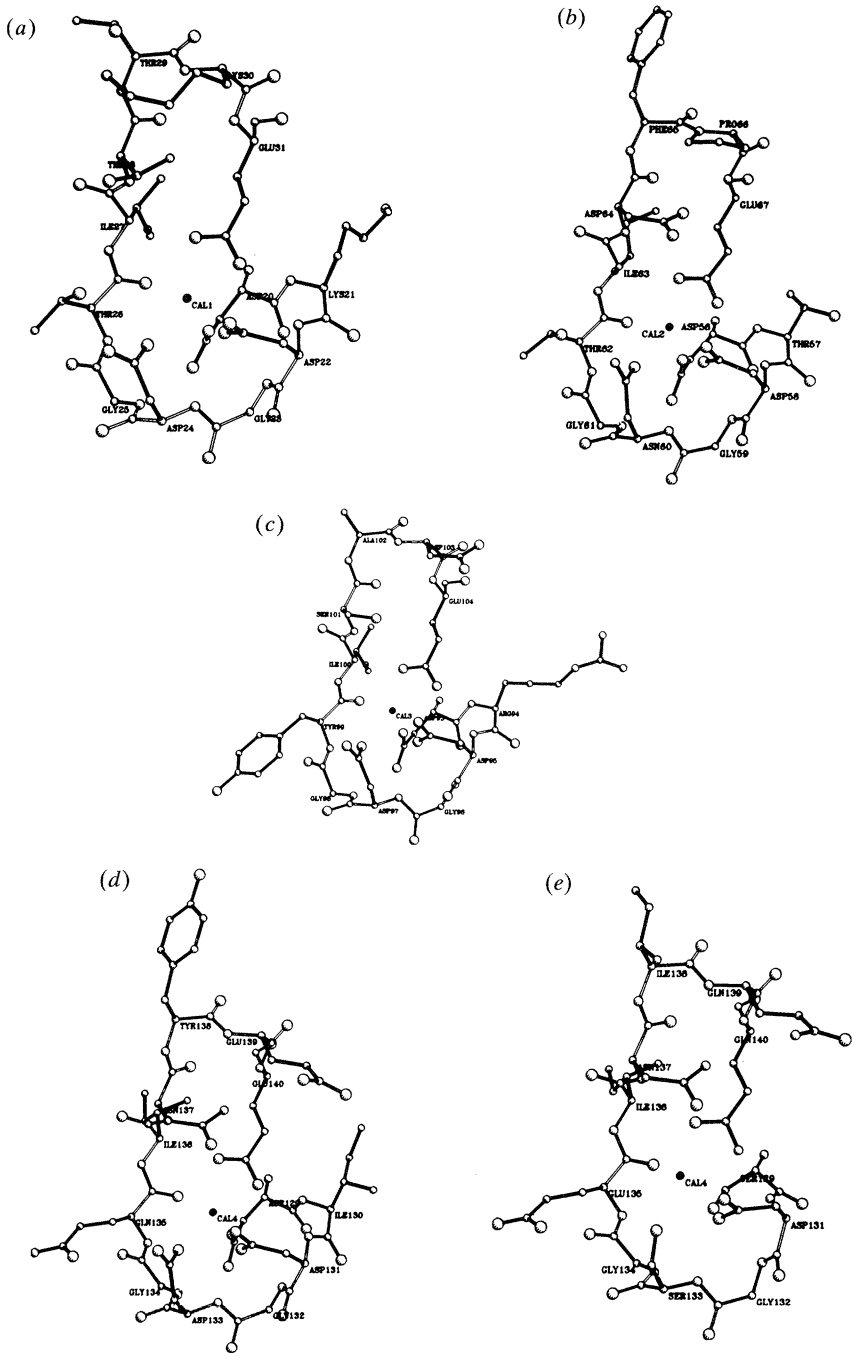

Figure 3. Stereo drawings of the four Ca^{2+}-binding loops in calmodulin from *P. falciparum* (*a–d*) and the fourth Ca^{2+}-binding loop of calmodulin from *S. cerevisiae* (*e*).

That the hydrophobic clefts in either half of the calmodulin molecule are the sites of interaction with these drugs is suggested from preliminary crystallographic results (Babu *et al.* 1988) who showed that the aromatic portion of the drug molecule, trifluoperazine, could fit into the hydrophobic pocket with the positively charged end of the molecule extending towards the central helix (Babu *et al.* 1988). There are a number of chemical and spectroscopic studies available in the literature to support this model (Brzeska *et al.* 1983; Vogel *et al.* 1984; Giedroc *et al.* 1985). Our model building studies suggested that it is feasible to place all four of these drugs into both hydrophobic pockets (figure 5*a–h*). The chlorine and fluorine atoms of the drug compounds are buried deep within the pockets and the hydrophobic rings are stacked against the residues of the hydrophobic cleft. This supports the observations that drug compounds with halide atoms are more active (table 4), probably having increased affinities with calmodulin. Our model building studies using the four drug molecules and calmodulin from *P. falciparum* has shown that trifluoperazine fitted most tightly (-94 kcal mol^{-1}) in the hydrophobic pockets in different orientations with respect to the central helix. Chloroquine fitted least well (-10 kcal mol^{-1}).

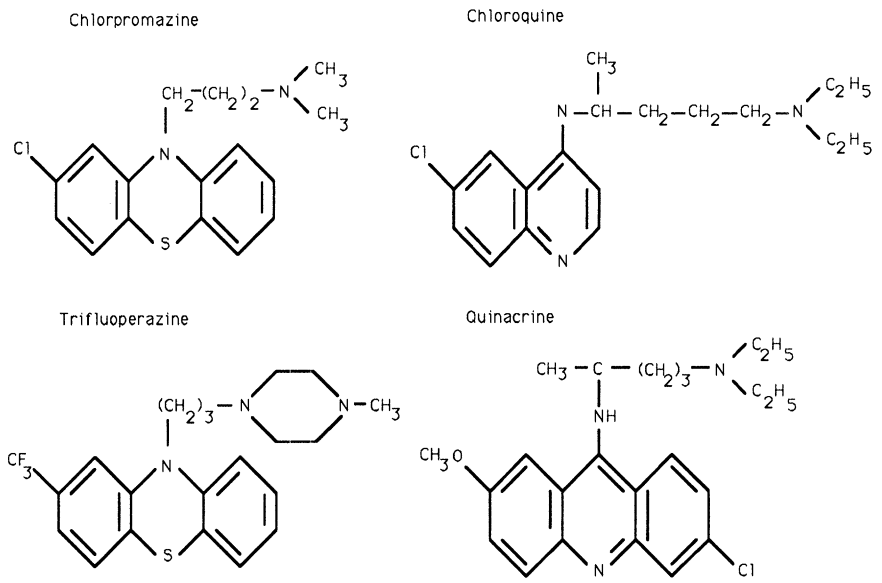

Figure 4. The chemical structures of the drug compounds chlorpromazine, trifluoperazine, chloroquine and quinacrine.

4. DISCUSSION

The structures of homologous proteins may be predicted with some confidence when the structure of one or more members of the family is known. The advent of DNA technology has meant that many sequences are becoming available for families of proteins. Many proteins belong to relatively few families of protein structures. Thus it may be possible to derive the three-dimensional structures of all the members of a family provided there are one or more experimental structures available. This could be a more rational way of comparative model building than predictions based on primary sequence alone and is true for calmodulin.

The high degree of sequence identity between calmodulins of different origins has allowed us to perform molecular modelling studies following this principle. Our interests have centered on two parasite proteins, from *P. falciparum* and *T. brucei*, as well as that from *S. cerevisiae*. We have investigated the suitability of calmodulin from *P. falciparum* as a target for novel antimalarials. In general, the nature of sequence changes that occur in calmodulin from *T. brucei* are such that our model building observations for *P. falciparum* hold true for *T. brucei*.

It is known that the functional properties of calmodulin are conserved throughout evolution. However, the amino acid sequence of the *S. cerevisiae* protein is the least conserved and has only 60% identity with vertebrates. There are significant differences in the ability of calmodulins from *S. cerevisiae* and vertebrates to activate target enzymes such as myosin light chain kinase and cyclic nucleotide phosphodiesterase using *in vitro* assays (Luan *et al.* 1987). However, using an *in vivo* assay where vertebrate calmodulin replaced yeast calmodulin the two proteins were shown to be functionally interchangeable (Davis & Thorner 1989). From the model building studies it is clear that significant changes have occurred in the central helix, in the hydrophobic clefts and in particular EF-hand 4. The deletion of the amino acid at position 130 has some interesting consequences.

Matsuura *et al.* (1991) have shown that this domain is unlikely to bind Ca^{2+} despite the observation that compensations in the amino acid sequence further on would make it possible to maintain the seven-fold coordination without altering the overall structure (see figure 3). Recent experimental data (Geiser *et al.* 1991) in which yeast strains containing mutant calmodulins in which the Ca^{2+}-binding loops have been altered, such that the side chains of the first and last amino acids in the EF-hands can no longer coordinate Ca^{2+}, are still viable. Furthermore, none of these mutant proteins changed conformation even in the presence of high levels of Ca^{2+}.

The two parasite proteins (*T. brucei* and *P. falciparum*) show a high level of sequence identity with vertebrate calmodulin. Some of the sequence changes occur in the central helix of the molecule which is important in maintaining function, other changes have occurred in the hydrophobic clefts. Significant sequence changes in the hydrophobic clefts also occur in *S. cerevisiae*. Other unicellular fungi also show considerable amino acid differences, particularly in the C-terminal cleft.

From our modelling studies examining the interactions of the four drug molecules with calmodulin from *P. falciparum* we can conclude that chloroquine fitted least well, suggesting that calmodulin is not a major target for this drug. The high degree of amino acid sequence identity shared by protozoan and mammalian calmodulins together with structural similarity does not provide a basis for differential drug binding. However, the number of amino acid sequence changes in the hydrophobic clefts of calmodulins from the unicellular fungi suggest that these may be a suitable

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target for novel calmodulin antagonists and its seems likely that these changes might have a role in the interaction with hydrophobic molecules.

The invasion data presented here do not differ significantly from those of other workers who used related drugs W-5 and W-7 (Scheibel *et al.* 1987; Matsumoto *et al.* 1987; Scheibel *et al.* 1989; Tanabe *et al.* 1989). W-12 and W-13 have been thought to be more potent than these other two naphthalene sulphonamides. This class of drugs was believed to be specific for calmodulin but there is now accumulating evidence (Schatzman *et al.* 1983) that these drugs interact with a phospholipid-sensitive calcium-dependent protein kinase. It is therefore likely that it is the hydrophobic nature of these compounds together with their three-dimensional structure that gives these compounds their ability to interact with the hydrophobic clefts in calmodulin. This is in agreement with the general principle that most quaternary structures exhibit symmetrical properties and involve associations through complementary surfaces primarily due to hydrophobic interactions (Wodak *et al.* 1987). Recently Meador *et al.* (1992) have determined the crystal structure of Ca^{2+} bound calmodulin to a peptide analogue of the calmodulin-binding region of chicken smooth muscle myosin light chain kinase at 2.4Å resolution. Their results explain that due to the peptide-protein interactions, the closely associated domains of the calmodulin molecules form a pseudo-twofold symmetry and the hydrophobic surfaces on either side of the central helix come closer and form a tunnel covering the hydrophobic region of the helical peptide. The tight association of the peptide in the tunnel thus makes the complex structure rather compact in nature. However, we do not anticipate such a conformational change with small hydrophobic molecules as discussed above, which has been supported by preliminary X-ray results (Babu *et al.* 1988).

Comparison of protein sequences allows construction of a phylogenetic tree which includes *P. falciparum* and differs from that constructed from the nucleic acid sequences of small subunit ribosomal RNA. Our phenogram for calmodulin (figure 1) includes 37 amino acid sequences and agrees with the analysis of Moncrief *et al.* (1990) which included the 15 calmodulin sequences available at that time. These types of comparison are difficult to interpret, nevertheless, it is of interest that in all of the phenograms generated the protozoan calmodulins seem to be distantly related to those of the plant kingdom. Recent evidence, of mitochondrial RNA editing (Simpson & Shaw, 1989) and extrachromosomal elements related to chloroplasts (Wilson *et al.* 1991), have also suggested a similar relationship. This could mean that new drugs based on an understanding of the modes of action of herbicides may prove rewarding.

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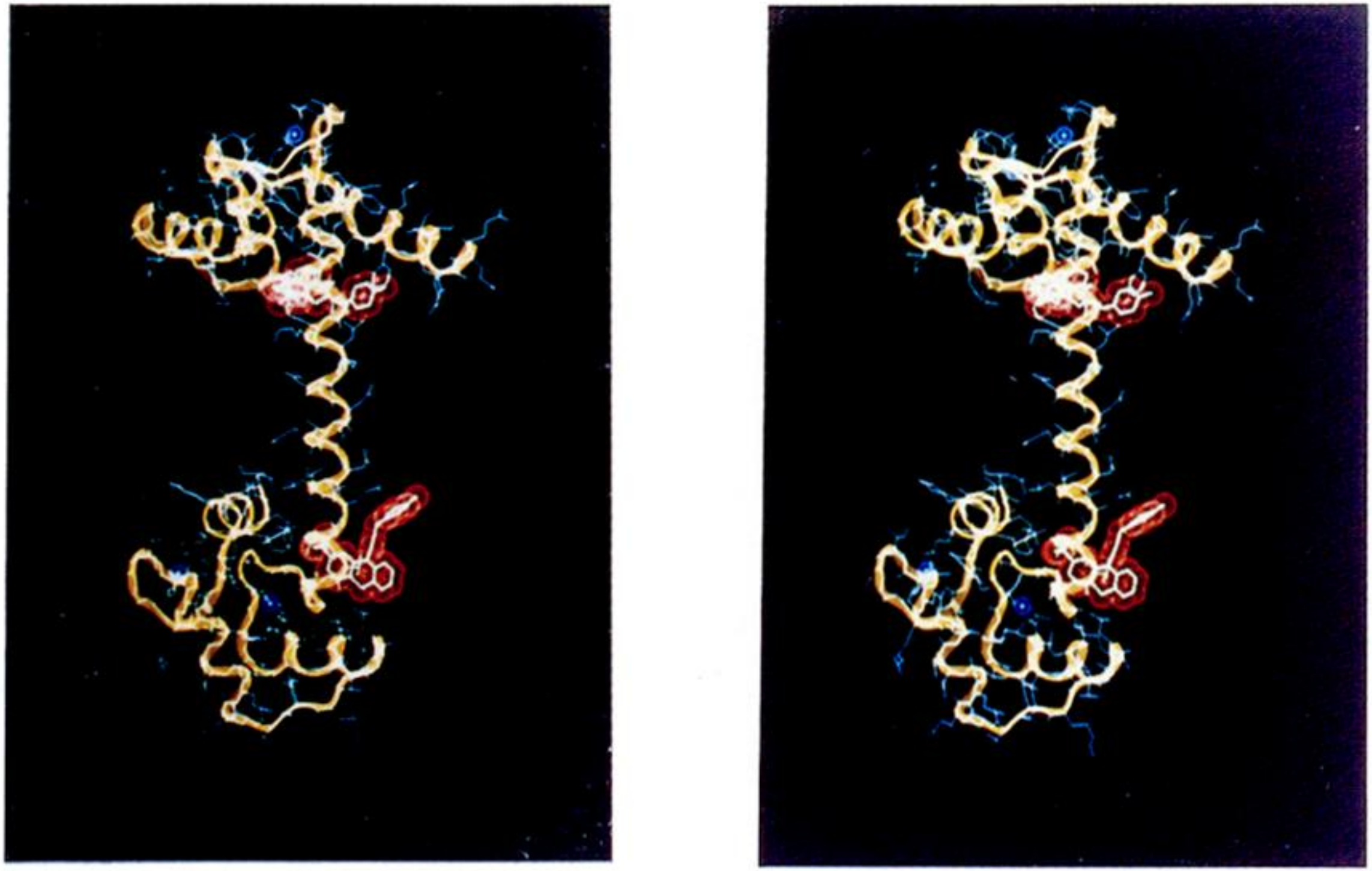
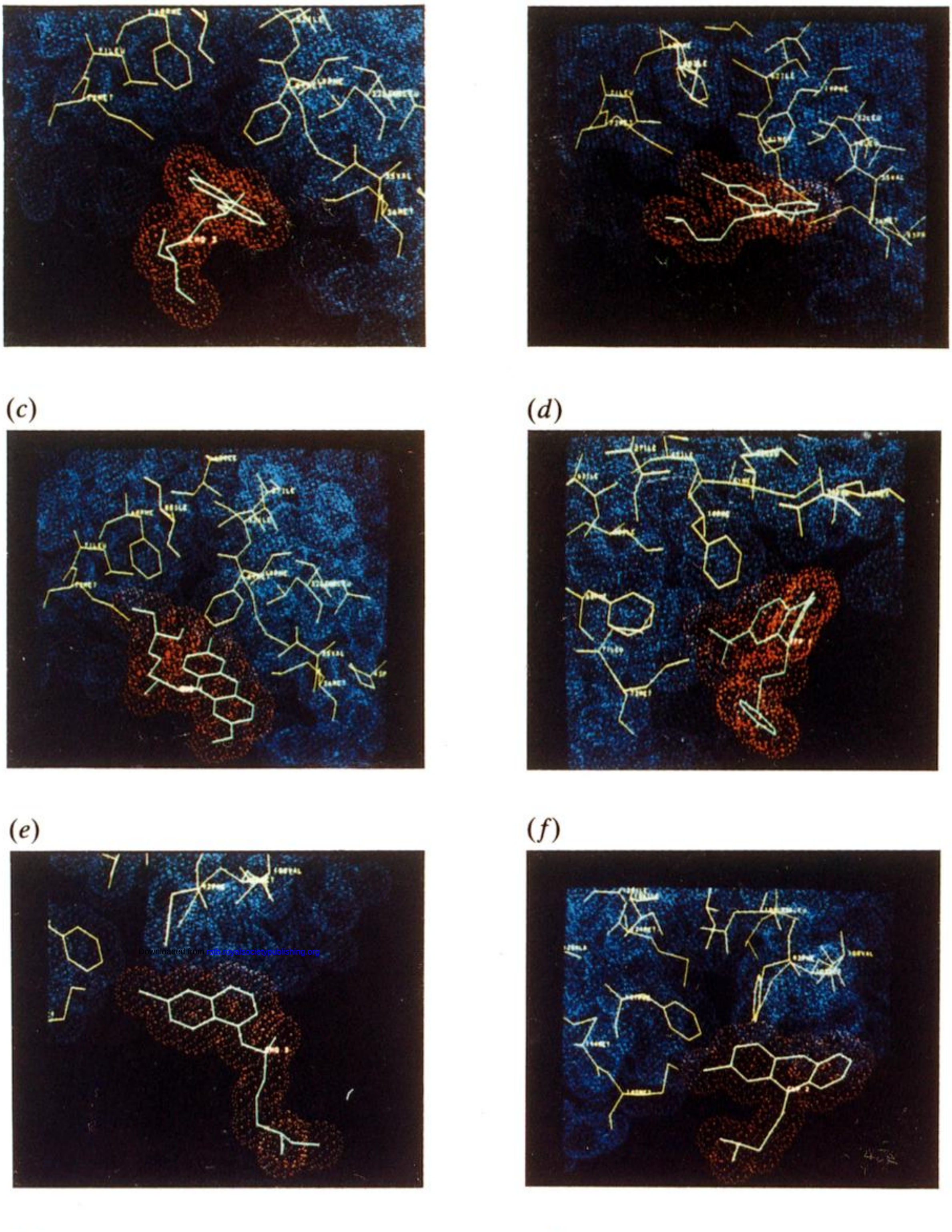


gure 2. Stereo picture of *P. falciparum* calmodulin. Residues 5–147 are included. The ckbone structure is highlighted in orange, the side chains are in pale blue with the lcium ions in the four binding sites being in dark blue. TFP(red) is located in the two ausible drug-binding pockets.



igure 5. Drug binding in the amino-terminal cleft with the four drugs, chloroquine, hlorpromazine, quinacrine and trifluoperazine is shown in panels (*a–d*), respectively. rug binding in the carboxy terminal cleft with the four drugs, chloroquine, hlorpromazine, quinacrine and trifuoperazine is shown in panels (*e–h*), respectively. he spheres represent 70% of van der Waal radii. The drugs are in pink and the ydrophobic pockets of calmodulin are in blue.