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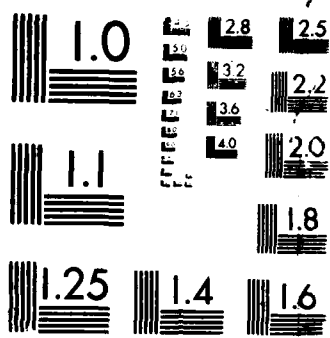
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Our long-term objective is an understanding of the molecular basis of calmodulin activation of target enzymes. We have chosen the interaction of calmodulin with peptides as an initial paradigm to simplify the study of the molecular details of the peptide-protein interaction. Our initial focus has been the development of methodology for the experimental study of the molecular dynamics of calmodulin binding-peptides, and of calmodulin-peptide complexes, in particular an evaluation of the conjoint use of fluorescence and NMR spectroscopy in such studies.

#### DETAILED PROGRESS REPORT

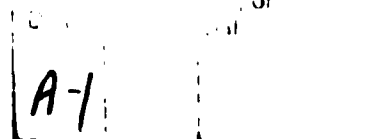
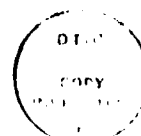
Most molecular dynamics simulations of protein structure have so far focused on events occurring in the low picosecond regime -  $\leq 200$  picosecond (1). The results of such simulations have been interesting and provocative, but they need corroboration from experimental data to be validated. However, quantitative measurement of picosecond motions is difficult. Both NMR and fluorescence spectroscopy are, in principle, able to gain access to this time domain and to provide quantitative data, but to date it has been difficult to use these two experimental methods to study motions in precisely the same system. Conjoint use of both methods would allow the data from one experimental technique to corroborate (or refute) the inferences drawn from the data of the other.

Our basic experimental approach has thus been to develop an appropriate paradigm. To achieve this goal we have synthesized a tryptophan molecule with  $^{13}\text{C}$  enrichment in the  $\text{C}_6$  position of the indole ring (2).

Subsequently, we have incorporated this tryptophan into two sets of peptides - first a set of small hydrophobic peptides, and second into melittin and a series of melittin analogs. These peptides have then been examined by the use of  $^{13}\text{C}$ -NMR and fluorescence spectroscopy. The results have shown convincingly the value of this approach.

(I) Details of the initial study with the small hydrophobic peptides are provided in the manuscript attached to this report and so won't be considered here. A simple summary suffices.

1. The measurements of  $T_1$ ,  $T_2$ , and NOE for the  $^{13}\text{C}$ -H vector of the  $^{13}\text{C}$ -enriched tryptophan appears to provide realistic values of the rotational correlation times of the tryptophan side chain. This inference is based on the similarity of the times calculated from the NMR and fluorescence data.
2. The rotational correlation times of the indole side chain of the peptides in solution are generally in excess of 100 ps. These values are 1 to 2 orders of magnitude different from those predicted from molecular dynamics simulations of peptides (*in vacuo*) and suggest that meaningful time constants from such simulations are likely to be obtained only if these simulations are done with the peptides (or for that matter proteins) in a solvent environment.
3. Detailed mathematical analysis of the NMR data have thus far relied exclusively on the use of the Lipari and Szabo model (3). The results



have shown the inherent complexity of the analysis in that the local rotational (or librational) rate of a molecule (e.g., tryptophan side chain) calculated by use of the Lipari and Szabo formalism ( $\tau_e$ ) is very sensitive to the order parameter(S) and to the overall rotational correlation time (i.e.,  $\tau_m$ ) of the substrate. Consequently, the accuracy of the NMR measurement is constrained. Fortunately, the error is unlikely to be even as great as an order of magnitude, and with fluorescence anisotropy data, especially time-dependent fluorescence anisotropy to buttress the NMR data, reasonable correlation times will be realizable. However, a different formalism seems needed particularly for the analysis of the dynamics of flexible chains. We are currently examining the possibility that an appropriate theory might appear from treatment of the peptide as a flexible polymer whereupon Flory-type polymer dynamics may be applied to assess dynamics. As we noted above, the details of the initial study are in the manuscript attached.

(II) To continue the assessment of  $^{13}\text{C}$ -NMR as a method for studying peptide and protein dynamics, we have synthesized and purified the following peptides.

i. Melittin (MLT)	G <sub>1</sub> GAVLKVLTTGLPALISWIKRKRQQCONH <sub>2</sub> * * * *
ii. Melittin <sub>17</sub>	G <sub>1</sub> GAVLKVLTTGLPALWSILKRKRQQCONH <sub>2</sub> **
iii. Melittin <sub>11</sub>	G <sub>1</sub> GAVLKVLTWGLPALISLIKRKRQQCONH <sub>2</sub> * *
iv. Melliting	G <sub>1</sub> GAVLKVWTTGLPALISLIKRKRQQCONH <sub>2</sub>

In each of these peptides two  $^{13}\text{C}$ -enrichment sites were included - one on the  $\alpha$ -carbon of glycine at position 13 in the sequence and the other at the  $\text{C}_6$  position of the aromatic ring (as described above). All of the analogs of melittin showed properties akin to that of the parent peptide. Most notably, (i) they all showed a strong tendency to form  $\alpha$ -helices in heliogenic solvents such as methanol or trifluoroethanol; (ii) they all lyse red blood cells; (iii) they all bind to calmodulin. However, there were also some notable differences from melittin. The most significant among them being (i) the marked variation in cytolytic potency (rank potency order  $\text{MLT} > \text{MLT}_{17} > \text{MLT}_{11} > \text{MLT}_9$ ); and (ii) the failure of *any* of the analogs to form a tetramer. By use of attenuated total reflectance FT-IR procedures we have shown, moreover, that the pattern of interaction of the analog peptides with lipids varies. The apparent lipid bilayer penetrability shows the same rank order among the peptides as for cytolysis. The greatest difference is seen for  $\text{MLT}_9$  which appears to reside primarily on the lipid surface and displays a partially helical/partially  $\beta$  sheet configuration - c.f., melittin whose amino terminal dodecapeptide appears to penetrate into the lipid bilayer as a helix whose long axis lies normal to the plane of the bilayer (4).

The  $^{13}\text{C}$ -enriched melittin and melittin analogs therefore provide us with an excellent model for studying basic features of the  $^{13}\text{C}$ -NMR approach to quantitation of molecular motion. The  $^{13}\text{C}$ - $\alpha$  carbon group of glycine, kept in a common location in all of the peptides, provides direct information on the dynamics of the peptide main chain and, therefore, allows assessment of

the overall dynamics of the peptide. The  $^{13}\text{C}$ -enriched indole ring provides data almost exclusively on the mobility of the aromatic side chain. We have now studied in detail the solution dynamics of all four peptides under conditions where we expect the peptides to be (i) in random (coil) configuration; (ii) existing as monomeric helices (e.g., in 70% methanol/water mixtures, V/V); and (iii) tetrameric - i.e., in solutions with high salt concentration. The measurements have been very productive. So far, data have been analyzed, as described above, by the use of the Lipari and Szabo "model-free" approach as implemented by Weaver et al. (see manuscript attached). The results and inferences are briefly summarized below.

(i) For the random coil peptides, the apparent rotational correlation times calculated from  $T_1$ , and NOE measurements show an expected, distinct difference between the motions of the  $^{13}\text{C}$ -glycine site and the  $^{13}\text{C}$ -tryptophan (detailed values are provided in Table 1). The motion of the indole side chain showed rotational correlation times of *ca* 200 ps. for the peptides dissolved in  $\text{DMSO-d}_6\text{:D}_2\text{O}$  (90:10 V/V) (see table I), whereas the glycine  $\alpha$ -carbon showed correlation times  $\geq 5$  nsec even for the peptides in free solution (as monomers?)

(ii) An increase in salt concentration is known to make MLT helical and to provoke formation of a melittin tetramer. The  $^{13}\text{C}$ -NMR results showed clearly the evolution of this tetrameric structure as the ionic strength was increased in a solution of the  $^{13}\text{C}$ -enriched melittin. The process was reflected in a progressive up-field shift in the position

of the  $^{13}\text{C}$ -resonance of *both* the tryptophan side chain and the glycine  $\alpha$ -carbon.

Interestingly, the NMR data also suggested the presence of oligomeric species other than tetramer (most likely melittin dimers). The formation of the tetramer was also reflected in a tenfold increase in the apparent rotational correlation time of tryptophan side chain motion and a similar change in correlation time for the glycine  $\alpha$ -carbon. However, increased ionic strength failed to significantly affect the  $^{13}\text{C}$ -NMR spectra,  $T_1$ , or NOE of the  $^{13}\text{C}$ -resonances in any of the melittin analogs supporting the view that these analogs do not form oligomers.

The changes in chemical shift for both the tryptophan and glycine  $^{13}\text{C}$ -resonances support the inference that both carbon moieties move into less polar environments as melittin becomes a tetramer. This inference is supported by examination of the x-ray crystal structure of the tetramer. The increases in rotational correlation time stem from the effective increase in molecular volume attending oligomer formation, and from the increased restriction on motion, as both the tryptophan side chain and the glycine residues are part of the peptide-peptide interaction surfaces in the tetramer. The  $^{13}\text{C}$ -NMR results on tryptophan side chain behaviour are strongly corroborated by the results of fluorescence measurements. A manuscript describing this work is currently being prepared for publication.

The data we have gathered during the past year have thus established the usefulness of the combined  $^{13}\text{C}$ -NMR and fluorescence approach to study of peptide dynamics. The process needs now to be applied to more complex systems (see plans for grant year 1987-1988).

### (III) Molecular Dynamics Simulations

As stated earlier, one of the principal long-term objectives of this research is the study of the influence of protein dynamics on the function of calmodulin as a  $\text{Ca}^{++}$ -dependent regulator of enzyme function. Work on this aspect of the project has only just now begun because of the lack of a crystal structure for calmodulin i.e., lack of the x-ray crystal coordinates. (The crystal structure of calmodulin is known, but the coordinates have yet to be deposited in the Brookhaven data bank.) During the past two months we have attempted to surmount this problem in the following way. The  $\alpha$ -carbon backbone (main chain) coordinates are available. Since the gross secondary form of the protein is known, it should be possible to use the molecular dynamics algorithm with energy minimization procedures to reconstruct the side chain orientations. The problems inherent in such reconstructions are, of course, well known to those who have tried to fold a protein of known amino acid sequence into a secondary/tertiary structure. In our case the difficulty of the latter procedure is lessened considerably since the secondary structure is essentially already provided by the configuration of the  $\alpha$ -carbon chain. However, to give the greatest credence to the results of any calculations we might do on calmodulin, we have first

secured the complete coordinate set for the structurally homologous troponin-C (turkey). These have been kindly supplied by Dr. M. N. James of the University of Alberta. We have reduced the data on troponin-C to the  $\alpha$ -carbon set and are currently using energy minimization and molecular dynamics simulations with CHARMM to "build" the troponin-C. The calculated structure will then be compared with that from the x-ray coordinates (see "experimental plans below).

#### EXPERIMENTAL PLANS FOR 1987-1988

##### A. NMR and Fluorescence Measurements

1. Now that the studies on peptides in solution have been completed, we will begin analysis of the calmodulin-peptide complexes. We know that melittin and the three analogs described above all bind to calmodulin. Also, we know from other studies that these peptides assume a (presumably amphiphilic) helical form when bound. Our objectives with the  $^{13}\text{C}$ -NMR measurements will be to determine the dynamics of the  $\alpha$ -carbon of the  $^{13}\text{C}$ -glycine and of the indole ring in the complexes, under conditions in which (i) the tryptophan forms part of the interacting surface (i.e., lies on the apolar face of the amphiphilic helix) and should, therefore, be tightly "packed" in the overall structure of the protein, peptide complex, or (ii) the tryptophan is relatively free to rotate i.e., when the indole side chain lies on the hydrophilic surface of the CAM-bound

peptide (e.g., MLT<sub>11</sub>). In all cases, the <sup>13</sup>C-glycine resonance should show highly restricted motion in the complex i.e., should display an effective order parameter of 1.

2. It will be necessary, concomitantly with the NMR measurements, to re-examine the reliability of the Lipari and Szabo model for calculation of dynamic parameters. Our studies to date have shown clearly the limitation of the model under conditions where the overall rotational correlation time ( $\tau_m$  in our notation) cannot be specified i.e., has not been corroborated by data from some other independent measurement. The problem is that  $\tau_m$  is not easily measured. However, in a folded protein whose molecular dimensions are known, a reasonable estimate of  $\tau_m$  can be calculated by use of the Stokes-Einstein equation. Moreover, time-dependent anisotropy data should provide another estimate of  $\tau_m$  which one hopes would be similar to that calculated from the molecular dimensions. Thus, all NMR measurements will continue to be complemented with determinations of time-dependent fluorescence anisotropy decays. For the latter our Nd:YAG-pumped, picosecond resolving multifrequency phase fluorometer will be used. These measurements will be employed for the assessment of the proteins' rotational correlation times. Unfortunately, studies of peptide dynamics do not enjoy such an advantage. A detailed study of polymer dynamics will be undertaken during the coming year to determine possible applicability to the analysis of the NMR data.

## B. Molecular Dynamics Calculations

In work supported by other grants, we have completed molecular dynamics studies of cyclosporine, scorpion neurotoxin variant 3 and ribonuclease T<sub>1</sub>. The program CHARMM was used. The experience gained has considerably facilitated our initial attempts to "build" TnC from  $\alpha$ -carbon coordinates. We expect to have solved the problems with this construction within a few weeks of the new grant year. The next stage is to build calmodulin from its  $\alpha$ -carbon coordinates and to proceed with molecular dynamics calculations. The latter will not be straightforward for several reasons. First, the x-ray structure was done on the molecule at acidic pH. All available evidence suggests that under such conditions, CAM has a significantly different configuration from that existing at neutral pH. Moreover, the protein's Ca<sup>++</sup> binding properties perforce are different at acidic pH. The second problem is strongly related to the first, namely that the high anionic charge complicates our modeling because it requires that we account carefully for a high electrostatic charge. In our view, this negates a simple "vacuum" simulation and necessitates inclusion of water. Even with the latter, the electrostatic problem is ameliorated but, we realize, certainly not solved. Thus, we know from CD measurements that the conformational response of CAM to the binding of Ca<sup>++</sup> ions is different if the protein is dissolved in media of low or "physiological" ionic strengths. We cannot currently correct the simulations for ionic

effects but would argue that simulations in an aqueous environment should provide at least a good, if preliminary, assessment. We will most likely employ a periodic boundary calculation in order to include water.

Once we are satisfied with the results of our construction of the CAM structure we will proceed with i) a calculation of a likely structure for CAM in its fully  $\text{Ca}^{++}$ -replete form; ii) a molecular dynamics simulation of the CAM; iii) a molecular graphics/molecular dynamics assessment of the likely peptide binding site on the  $\text{Ca}^{++}$  replete molecule. Clearly item number ii will present the most formidable challenge and the fate of item iii is utterly dependent on the success of the initial dynamics studies. CAM is large enough that a simulation of the entire molecule for a sufficiently long time and inclusive of water is probably impracticable. An initial approach will be to simulate each major domain separately and then try to reconstruct the entire protein.

The cost in computer and personnel time will be high. Dr. Chris Haydock, who has become expert in the use of molecular dynamics calculations will be fully employed on the project. Simulations will continue to be performed on CRAY computers.

Overall this is a very ambitious program considering the limited personnel -- approximately 1.5 FTE total with 1 FTE fully utilized for molecular dynamics simulations.

END

JAN.

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