Exploring the hydrophobic binding sites of calmodulin with fluorescent probes: Dynamic formation of the excimer†

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Molecular probes consisting of hydrophobic and hydrophilic functional groups separated by a flexible linker have been investigated for their binding with calmodulin, a ubiquitous calcium binding protein. For example, 9-anthryl)methylamine HCl (AMAC), 3-(9-anthryl)propylamine HCl (APAC), N-ethyl-(9-anthryl)methylamine HCl, (N-Et-AMAC), and 4-(1-pyrenyl)butylamine HCl (PBAC) bind to calmodulin. The cationic and hydrophobic moieties of these probes separated by a linker provide multiple sites for interaction with the protein. Length and flexibility of the linker play a key role in the binding. For example, the isomeric probes N-Et-AMAC and APAC show major differences in their binding interactions. Probes with long linkers (APAC and PBAC) bind better than the probes with short linkers (AMAC and N-Et-AMAC). The electronic absorption spectra of the anthryl probes AMAC, APAC and N-Et-AMAC undergo only minor changes upon binding to calmodulin. In contrast, the absorption spectrum of the pyrenyl analog, PBAC, undergoes a large red shift (~6 nm) and ~20% decrease in the extinction coefficients. Such a red shift is consistent with the binding of the pyrenyl moiety to hydrophobic regions of calmodulin. The decrease in the absorption is indicative of stacking interactions between the pyrenyl chromophore and the aromatic residues of the protein. The fluorescence spectra are red shifted, accompanied by quenching of the overall intensity in the cases of AMAC, N-Et-AMAC or APAC. Binding of PBAC to calmodulin results in a new emission band at 475 nm assigned to the pyrene excimer, consistent with the literature reports. Fluorescence quenching experiments with potassium iodide coupled with lifetime measurements indicate that the calmodulin-bound probes are significantly protected from the aqueous phase. The decreases in the quenching constants are 42, 22 and 69% for APAC, N-Et-AMAC and PBAC when compared to those of the corresponding free probes, respectively. The extent of protection from iodide depends upon the length of the linker and hydrophobicity. The observed order of protection, PBAC > APAC > AMAC > N-Et-AMAC, parallels the hydrophobicity of the aromatic moiety as well as the length of the linker. Binding of PBAC to calmodulin results in induced circular dichroism bands, establishing the asymmetric environment of the bound probe. Time-resolved fluorescence studies indicate that the protein-bound probes exhibit multi-component decays with lifetimes, 7.7 and 11.6 ns for APAC; 2.0 and 11 ns for N-Et-AMAC; 13, 80 and 198 ns for PBAC. Time-resolved emission spectra of PBAC bound to calmodulin clearly show the growth and decay of the excimer emission with a maximum around 475 nm. The excimer grows with a time constant of 12 ns and the growth rate is independent of the protein concentration. No such excimer emission is observed with the anthryl probes. The excimer formation is found to depend upon temperature, with an activation barrier of 53 kJ/mol. The magnitude of the barrier and the excimer growth rate constant suggest that the excimer formation is perhaps controlled by the local segmental motion or bending of the protein. If so, the present suggest a dynamic flexible structure for calmodulin, in aqueous solutions. In general, the binding is enhanced with the increase in hydrophobicity of the probe as well as length of the linker separating the hydrophobic and hydrophilic moieties.

Introduction

Calmodulin is a calcium binding protein consisting of 148 amino acid residues. It is present in all eukaryotic cells and regulates the activity of more than 20 different enzymes. A number of drugs, peptides and proteins have been shown to interact with calmodulin. The interaction was found to be quite sensitive to the concentration of Ca²⁺. In fact, calmodulin was shown to be a key player in calcium dependent signal-transduction in biological systems. The three dimensional structure of calmodulin in solid state was shown to consist of two homologous lobes joined by a central α-helix of nearly 8 turns. The solution structure of calmodulin was thought to be

†Dedicated to Professor M.V. George on the occasion of his 70th birthday.
flexible and differ significantly from the solid state structure\(^6\). NMR and light scattering experiments with calmodulin suggest that the central helix is perhaps bent, and the two lobes of the protein are \(\sim 10\text{\AA}\) apart. Binding of calcium to calmodulin was shown to induce major structural changes, and results in the exposure of hydrophobic side chains for interaction with proteins, peptides and drugs. Drugs such as trifluoperazine and chlopromazine were suggested to bind at such hydrophobic sites, in the presence of calcium ions. The positively charged regions of these drugs were suggested to interact with the negatively charged side chains of the protein at the binding site\(^6\). Calmodulin is therefore chosen as a model protein to investigate the binding properties of selected ligands.

Understanding the molecular details of the binding of small molecules to biopolymers is important in the rational design of new and more efficient therapeutic agents that can recognize and interact with specific biomolecular sites. In this context, a systematic investigation of the binding of a new class of molecules containing hydrophobic and cationic functions separated by a flexible linker (Chart 1) was carried out. The biopolymers selected for these studies were DNA\(^7\), serum albumin\(^8\), lactoglobulins, calmodulin and low density lipoprotein\(^9\). Use of these probes provided a facile method to investigate the role of hydrophobic, hydrogen bonding, and electrostatic interactions in controlling the binding affinity and specificity of small molecules.

The fluorescent probes used in the present studies, represented as \(\text{Ar-(CH}_2\text{)}_n\text{-NH}_2\text{R}^+\), are shown in Chart 1. Our design includes a cationic function attached to a hydrophobic fluorophore through a flexible linker. Anthryl and the pyrenyl fluorophores were chosen as the hydrophobic moieties (Ar) such that they would promote binding to hydrophobic regions of the protein. These fluorophores also provide a convenient spectroscopic handle to investigate the tell-tale events that occur at the binding site. For example, the photophysical properties of these fluorophores are sensitive to the local environment\(^10\). Therefore, they can serve as convenient spectroscopic reporters. The fluorescence lifetime of the pyrenyl chromophore is much longer (\(\sim 100\text{ ns, in air-saturated aqueous solutions}\)) than that of the anthryl (\(\sim 10\text{ ns}\)). Therefore, the pyrenyl moiety can be used to investigate events that occur on longer time-scale when compared to that of the anthryl. For example, the long-lived fluorescence of the pyrenyl chromophore may lead to excimer formation at the binding site. Excimers are produced when a complex is formed between the ground and excited states of the same molecule and these complexes emit at longer wavelengths than the corresponding parent excited state. Therefore, excimer formation can be a bimolecular event and it may be related to the relative diffusional rates of the excited and the ground state molecules. In addition, the pyrenyl excited state may result in exciplex formation with amino acid side chains surrounding the binding site. Therefore, exciplex emission can be useful in characterizing the residues at the binding site.

In our design, ammonium is selected as the cationic, hydrophilic group such that it may hydrogen bond with polar side chains, or the peptide backbone,
and/or favorably interact with negatively charged residues at the binding site. The ionic function also helps in improving the solubility of the hydrophobic chromophores in aqueous media. The ammonium group, in combination with the aromatic residue, is expected to promote binding of the probe to anionic sites at the protein-water interface. Therefore, the bifunctional nature of the probe is expected to result in preferential binding of these probes to hydrophobic/hydrophilic interfaces.

The flexible linker separating the hydrophobic and hydrophilic groups of the probe is expected to play an important role in the binding. Long linkers are expected to allow the cationic function to be close to the aqueous phase while keeping the hydrophobic moiety in the less polar interior of the protein, away from the aqueous phase. The linker may also be important for selectivity. For example, binding is expected at sites that provide a hydrophobic path arranged at an appropriate orientation and distance from the anionic and/or hydrogen bonding groups. Binding sites that do not feature such an arrangement are expected to be the poor affinity sites. Multiple interactions at the binding site can result in greater stabilization of the bound probe, and binding to such sites should be thermodynamically favored other sites where only one of the probe components can be accommodated. This discrimination between binding sites can result in selectivity, and provide a basis for site recognition. The effective distance of separation between the charge center and the hydrophobic fluorophore is determined by the number of intervening atoms in the linker, and the linker conformation. Length of the linker increases in the order (AMAC = N-Et-AMAC)<APAC<PBAC. A comparison of the binding properties of AMAC, APAC and N-Et-AMAC should reveal the role of the side chain in their interaction with the protein. On the other hand, lengths of the linkers in APAC and PBAC are comparable, whereas the latter is much more hydrophobic than the former. Flexibility of the linker should allow the probe to explore different binding geometries until it is trapped in a favorable conformation at the binding site. Therefore, these probes are expected to bind to proteins, and show selectivity for protein-water interfaces decorated with anionic and/or hydrogen bonding functions.

Protein binding properties of (9-anthryl)methylamine hydrochloride (AMAC), 3-(9-anthryl)propylamine hydrochloride (APAC), N-ethyl (9-anthryl)methylamine hydrochloride (N-Et-AMAC), and 4-(1-pyrenyl)butylamine hydrochloride (PBAC) with calmodulin are presented here. The linker plays a significant role in determining the binding properties and the affinity seems to increase with hydrophobicity of the aromatic moiety. High affinity, coupled with selectivity, can be used in the development of reagents for site-specific photocleavage of proteins\textsuperscript{11,12}. Site-specific probes may also be useful in investigating protein local structure, local polarity, protein denaturation, and folding. These bifunctional probes also bind to double helical DNA\textsuperscript{7}, polyelectrolytes\textsuperscript{13}, and layered zirconium phosphate\textsuperscript{14}, with a high affinity. Owing to the hydrophobicity of the probes, equilibrium dialysis experiments were difficult and hence the binding interactions of these probes were investigated in spectroscopic studies. Spectral changes and the dynamics observed for these probes with calmodulin as a model protein are described here.

**Experimental Section**

Calmodulin (from bovine brain, MW \approx 16,700) was purchased from Sigma Chemical Co., and used without further purification. Calmodulin solutions were prepared by dissolving an appropriate amount of the protein in 100 mM Tris-HCl buffer, pH 7.2, containing 0.1 mM CaCl\textsubscript{2}. When calcium-free conditions were required 2 mM EDTA was added to the buffer, instead of calcium chloride. The protein solutions were prepared fresh and used within 24 hr. When necessary, calmodulin concentrations\textsuperscript{15} were estimated by using an extraction coefficient of 3,300 M\textsuperscript{-1} cm\textsuperscript{1} at 277 nm. All the absorption spectra were recorded on a Perkin-Elmer Lambda 3B spectrophotometer. The fluorescence spectra were recorded on a Perkin-Elmer LSS spectrometer. Both were interfaced with an Apple Macintosh computer and the data were collected/processed with software developed in our laboratory.

The absorption and fluorescence titrations with calmodulin were performed by keeping the concentration of the probe constant while varying the protein concentration. This was done by dissolving an appropriate amount of the probe in calmodulin stock solution. Various proportions of the probe solutions were then mixed with the calmodulin stock solution while keeping the total volume constant (1 mL). This procedure resulted in a series of solutions with varying concentrations of calmodulin but with a constant concentration of the probe. For fluorescence
measurements, the solutions containing the anthryl probes were excited at 385 nm and the fluorescence intensity was monitored at 415 nm. The corresponding wavelengths for PBAC were 340 and 376 nm, respectively.

In the fluorescence quenching experiments with iodide, the data were analyzed according to the Stern-Volmer equation\(^{17}\):

\[
I_0/I = 1 + K_{sv} [Q] \quad (1)
\]

Fluorescence lifetimes and time-resolved fluorescence spectra were measured using a home-built single photon counting spectrometer. The excitation source was a deuterium filled nanosecond flash lamp (F-195) from Edinburgh Instruments. The spark-gap between the electrodes was adjusted to 0.5 mm and the full width at half maximum of the excitation pulse was \(1.6 \text{ ns}\). The fluorescence signal from the sample was collected with appropriate optics, passed through a monochromator (H-10, ISA Instruments, 2 mm slits) and focused onto a Hamamatsu R-955 photomultiplier. The single photon counting data were collected with a EG&G multichannel analyzer card installed in an IBM PS/2 model 30, personal computer. The time-resolved emission spectra at various delay times were constructed from the decay traces recorded at various wavelengths. The software necessary for the collection and manipulation of the fluorescence decay data was developed in our laboratory. The fluorescence decay profiles were deconvoluted with software from PRA Inc., and the goodness of the fits was tested using various parameters. Typically, the residuals, \(\chi^2\), Durbin-Watson parameter, and the correlation function were used in the data analysis\(^{18}\). The data were fitted by choosing different initial values for the lifetimes and amplitudes. Only the parameters that resulted in good, reproducible fits to the experimental decay curves have been considered. Each sample was run several times, and each curve was deconvoluted with several trials to ensure that the values obtained were reproducible best descriptions of the observed data.

The circular dichroism spectra (CD) were recorded on a JASCO model 710 spectropolarimeter, interfaced with a NECA 486 microprocessor. All the software needed to control the spectrometer were supplied by JASCO. The sample compartment was thermostatted with a computer-controlled circulating bath. The temperature was maintained at 20 °C unless mentioned otherwise. Baseline, with the buffer alone, was first recorded for each measurement and the sample spectra were then recorded. Under our experimental conditions, the baseline was flat and the data have been reported as obtained without smoothing or baseline subtractions. Several spectra were averaged when necessary to improve the signal to noise ratio.

The probes AMAC, APAC, \(N\)-Et-AMAC and PBAC were synthesized as described previously\(^4\). The probe concentrations were maintained at 5 \(\mu\text{M}\) or 2\(\mu\text{M}\) in Tris-HCl buffer described above. All solutions were prepared fresh prior to each experiment, and were used within 24 hr. All studies were conducted without degassing and at room temperature, unless stated explicitly. Protein solutions were gently stirred for uniformity and no precipitation or turbidity was detected under the experimental conditions.

**Results and Discussion**

The binding of the probe (Chart 1) with calmodulin was investigated using absorption fluorescence, and circular dichroism (CD) spectral methods. The aromatic chromophore present in these probes is sensitive to the binding site and the spectral changes that occur when this family of probes bind to calmodulin can be compared among the individual members. Length of the linker and hydrophobicity of the fluorophore make significant contributions to the binding affinity.

**Absorption studies.** Absorption spectra of AMAC, APAC, and \(N\)-Et-AMAC undergo small changes when calmodulin was added to the probe solution (Figure 1). In these experiments, the probe concentration was kept constant by adding an appropriate amount of the probe to the protein stock solution. The absorption spectra of APAC (5 \(\mu\text{M}\)) in the presence and absence of calmodulin (7 \(\mu\text{M}\)) are shown in Figure 1A. Small red-shift at the foot of the absorption band and a decrease in the intensity are apparent in the spectra. Further increase in calmodulin concentration did not make any substantial change in the absorption spectra.
and these small changes made it difficult to construct binding plots. Similar, but small changes were observed with AMAC, and N-Et-AMAC (data not shown). Perhaps, these probes bind close to the protein-water interface due to their short linkers, and are well exposed to the solvent. Such binding is not expected to result in dramatic changes in the probe environment or in their absorption spectra. In contrast, binding of AMAC, APAC or N-Et-AMAC to calf thymus DNA induces extensive broadening of the vibronic bands accompanied by strong hypochromism, due to electronic interactions between DNA bases and the anthryl chromophore. Additional experiments described below support the view that these chromophores bind at the protein-water interface and their exposure to the solvent depends upon the length of the linker. These changes in the absorption spectra are also in contrast to the dramatic changes observed when the probes bind to the layered inorganic phosphates.

The absorption spectrum of PBAC (5μM) undergoes a large red shift of ~6 nm upon binding to calmodulin (60μM) (Figure 1B). When the spectra were recorded at increasing concentrations of calmodulin, an isosbestic point was observed at 342 nm. Only two spectra are shown for the sake of clarity. The spectral changes suggest that PBAC environment is substantially hydrophobic when bound to calmodulin. Similar red shift of the PBAC absorption spectrum was observed in non-polar solvents such as benzene. The molar extinction coefficient of PBAC at the 0-0 band \( (ε_{00} = 2.8 \times 10^{4} \text{ M}^{-1} \text{cm}^{-1}) \) in the presence of calmodulin was considerably lower than that of the free probe \( (ε_{00} = 3.4 \times 10^{4} \text{ M}^{-1} \text{cm}^{-1}) \). The decrease in the molar extinction coefficient cannot be explained by a change in polarity, because a decrease in solvent polarity was found to increase the extinction coefficient. The extinction coefficient of PBAC was found to decrease upon binding to the double helical DNA, due to π-π stacking interactions with DNA bases. Similar π-π stacking interactions can be expected in the present case, if PBAC binds to hydrophobic sites of calmodulin lined with aromatic side chains. Thus, the observed red shift and hypochromism could be due to stacking interactions with aromatic residues at the binding site. In the absence of calcium ions, no such changes were observed and hence calcium ions are essential for the binding of PBAC. No such calcium dependency was observed with non-calcium binding proteins such as bovine serum albumin or lactoglobulin. These interpretations are consistent with the binding of PBAC to hydrophobic sites of calmodulin exposed by calcium binding. The binding of these probes was further examined in fluorescence experiments to determine their access to the aqueous environment.

Fluorescence studies. The fluorescence spectrum of APAC (5μM) undergoes red shift and decreases in intensity when calmodulin (7μM, Fig. 2A) was added. To avoid cluttering, only two emission curves are shown in Figure 2A. No excimer emission could be detected under these conditions. In these experiments, the probe-protein solutions were excited at the corresponding isosbestic points to keep the absorbance of the sample constant at the excitation wavelength, throughout the fluorescence titration. The decrease in intensity as a function of the protein concentration was quite small, and the corresponding Stern-Volmer quenching constants estimated from this data were found to be in the range 1-2 ×10^4 M\(^{-1}\). These estimates are considerably larger than the corresponding \( K_{sv} \) values observed with bovine serum albumin (~2×10^4 M\(^{-1}\)). Quenching of probe...
fluorescence by calmodulin can be due to the interaction of the anthryl chromophore with protein side chains at the binding site. The red shift in emission maxima can be due to changes in local polarity of the probe upon binding to the protein. Fluorescence maxima of APAC undergo red shifts with a decrease in solvent polarity. For example, the 413 nm band observed in water shifts of 414 nm in acetonitrile, 418 nm in dimethyl sulfoxide, and 416 nm, in benzene. The relative quantum yield of APAC fluorescence also decreases with the solvent polarity\(^6\). Even though the changes observed with APAC were small, they are significant when compared to little or no changes observed with AMAC, and N-Et-AMAC. This is interesting as APAC and N-Et-AMAC are isomeric and they differ only with respect to the distance of separation between anthryl and the charge center. The large separation in APAC can position the anthryl group deep inside the protein when the charge center is located at the protein-water interface. These suggestions were further probed in fluorescence quenching experiments described later.

In contrast to the above results, the fluorescence spectra of PBAC undergo dramatic changes in the presence of calmodulin, with a new band appearing around 475 nm (Figure 2B). The Stern-Volmer quenching constant estimated from these data was \(-2 \times 10^5\) \(M^{-1}\). The intensity of the new band at 475 nm increases steadily with protein concentration, and reaches a plateau. Subtraction of the monomer emission from the composite spectrum resulted in a broad band (Figure 3). This new emission was attributed to PBAC excimer, based on literature reports\(^{14}\). The ratio of the intensity of the excimer to the monomer \(I_e/I_{mono}\) increases with the protein concentration, reaches a maximum at a ratio of \(\sim 1:3\) (Figure 3 inset). These data suggest that the excimer formation is promoted by calmodulin. At high concentrations of protein, the probe is diluted among protein molecules such that the excimer formation is reduced.

The excimer emission is centered around 475 nm and the time resolved measurements show a slow growth at this wavelength (presented below) indicating the dynamic formation of the excimer. Emission from static excimers is known to occur at shorter wavelengths\(^{10,14}\). No excimer band was observed when the experiments were carried out in the absence of calcium ions. Therefore, binding of calcium to calmodulin is a prerequisite for the excimer formation. No excimer emission was observed when PBAC binds to bovine serum albumin\(^8\) or DNA\(^7\).

**Fluorescence quenching studies.** Fluorescence quenching experiments with iodide can provide a simple method to distinguish between the bound and free probes. If the probe binds at the protein-water interface with its hydrophobic chromophore far away from the aqueous phase, then its access to the solvent is restricted by the protein matrix. Extent of protection depends upon how deep the chromophore is located from the surface, and permeability of the protein matrix to iodide. No protection is expected if the probe is close to the surface. Iodide ion quenches fluorescence from APAC, N-Et-AMAC, and PBAC at nearly diffusion controlled rates\(^8\). This behavior was exploited to determine the relative accessibilities of the probes bound to bovine serum albumin\(^8\), DNA\(^7\), and layered zirconium phosphate\(^{14}\).

Fluorescence intensity of the probe-calmodulin mixture was monitored at various iodide concentrations, while keeping the probe and protein concentrations constant. Stern-Volmer quenching plots were constructed from these data using equation...
(1). The quenching plots for APAC and PBAC, in the presence and absence of calmodulin are shown in Figure 4. To avoid cluttering, data for N-Et-AMAC are not shown (supplementary material #1) but the corresponding $K_{sv}$ values are listed in Table 1. The quenching plots were linear for all the probes, with or without calmodulin. The quenching constants obtained for the bound parobes are substantially lower than the corresponding value for free probes. For example, $K_{sv}$ for APAC decreases from 72 M$^{-1}$ in the absence of the protein to 42 M$^{-1}$ in the presence of the protein. Even more dramatic decrease in the quenching was observed with PBAC, 98 M$^{-1}$ for free PBAC to 30 M$^{-1}$ for the bound form. The % decrease in the quenching constants, upon binding to calmodulin, were 22, 42 and 69 for N-Et-AMAC, APAC and PBAC, respectively. Therefore, the degree of protection offered by the protein matrix varies with the probe.

Given the small size of iodide and its ability to penetrate the interior of proteins\textsuperscript{21}, large reductions in the quenching slopes are very strong indications for the tight binding of the probe to sites that are relatively inaccessible to iodide. This conclusion is also based on the observation that the fluorescence lifetimes of the probes are increased upon binding to the protein. Hence, the decrease in the quenching constants are not due to decreases in the fluorescence lifetimes of the bound probe. The observed reductions in $K_{sv}$ values are to be compared with the moderate protection obtained when AMAC or APAC binds to calf thymus DNA despite the fact that DNA is negatively charged and it should repel iodide\textsuperscript{7}. In the presence of calmodulin, APAC was protected to a greater extent than its isomer, N-Et-AMAC. Therefore, the latter is exposed to solvent whereas the former is not. Thus, the longer side chain of APAC can position the hydrophobic chromophore farther away from the surface while keeping the charged ammonium group closer to the aqueous phase. Therefore, the linker plays an important role in determining the probe accessibility to the aqueous phase. The largest reduction in the quenching slope was observed with PBAC (from 98/M to 30/M), indicating that the more hydrophobic probe with a longer linker was the least accessible to the quencher. The longer side chain of PBAC can position the hydrophobic chromophore farther away from the aqueous phase and lead to greater protection from iodide.
Induced circular dichroism studies. The probe binding environment was investigated by monitoring the circular dichroism (CD) spectra in the presence of calmodulin. The probes (Chart I) have no asymmetric centers and hence, do not give rise to CD spectra. However, if the bound probe environment is asymmetric, then one may expect to observe induced CD spectra. No induced CD spectra were observed for AMAC or N-Et-AMAC in the presence of calmodulin. Only weak bands were observed for APAC while strong induced CD bands were evident with PBAC (10 μM) in the presence of calmodulin (10 μM) (Figure 5). The positions of these CD bands correspond to the absorption bands of the bound probe. Such induced CD spectra were not observed when PBAC binds to bovine serum albumin. Therefore, PBAC binds to asymmetric sites in the protein and the environment sensed by PBAC is different from that of the anthryl probes.

Fluorescence lifetime measurements. The fluorescence lifetimes of these probes were measured in the presence and in the absence of calmodulin. The free probes exhibit single exponential decays with lifetimes 8.8, 10.4 and 96.3 ns for APAC, N-Et-AMAC and PBAC, respectively. Fluorescence lifetimes change upon binding to calmodulin as shown in Table II. In the presence of calmodulin, fluorescence decay curves arising from APAC and N-Et-AMAC were fitted satisfactorily to two characteristic lifetimes, while that of PBAC required three exponentials (Figure 6). For APAC, the lifetimes were 11.6 (70%) and 7.7 ns (30%), while for N-Et-AMAC lifetimes of 11 (93%) and 2 ns (7%) were observed. With PBAC, the longer-lived component had a lifetime of 198 ns (25%), nearly twice the lifetime observed for the free probe. The shorter-lived components had lifetimes of 13 (10%) and 80 ns (65%) at 395 nm. The long-lived emission component clearly indicates extensive protection of the probe fluorescence by the protein matrix. The 13 ns component corresponds to the growth of the excimer emission at 475 nm. The 2 ns component in case of N-Et-AMAC, could be due to quenching of fluorescence by amino acid residues present at the probe binding site.

When PBAC emission (10 μM) was monitored at 500 nm, away from the monomer emission, in the presence of 20 μM of calmodulin, a slow growth was observed (Figure 7). The data could be fitted satisfactorily to a growth component of 12 ns. The growth rate is independent of the protein concentration (55, 30, and 20 μM) or PBAC concentration and matches with the 13 ns component (10%) discussed above. No such growth was observed at the monomer emission wavelengths and the growth is due to dynamic formation of the excimer. Since the growth is slow when compared to the lamp pulse-width (1.6 ns), and independent of the protein concentration, it can be assigned to the excimer formation between two probes bound to the same protein molecule. If it involves two protein molecules, the growth rate would depend on protein concentration. Perhaps, the protein segments carrying these probes come together to form excimer or the probe molecules move from one site to the other on these time scales. The time-resolved emission spectra confirm that the growth corresponds to the excimer formation.
Time-resolved fluorescence spectra. The fluorescence decay profiles were collected at various wavelengths and the time-resolved emission spectra at varying delay times were constructed from this data. The fluorescence spectra of PBAC (10 μM) in the presence of calmodulin (55 μM) showed a long wavelength emission with a maximum at 475 nm (Figures 8A and 8B) which grows on nanosecond time scale. No such long wavelength emission was observed with the anthryl probes or when PBAC binds to albumin or lactoglobulin. The red shifted emission of PBAC observed in these measurements corresponds to the excimer emission detected in steady-state experiments. The intensity of the peak at 475 nm increases with time, up to 10 ns delay and then starts to decay, as shown in Figure 8A. At longer time delays both components decay significantly. Therefore, the growth corresponds to the formation of the excimer. The growth of the excimer was further investigated by varying the temperature.

Effect of temperature. Since the excimer growth rate was independent of protein concentration, it is clear that the excimer formation takes place between probes that were bound to the same protein molecule. Temperature dependence studies indicate that the ratio of the intensity of the excimer to monomer increases as a function of temperature up to 30 °C and then reaches a plateau (Figure 9). The activation energy calculated from the linear part of the temperature dependence data was 53 ± 3 kJ/mol. This value is much larger than the activation energies measured for intramolecular excimer formation between pyrene chromophores (14-20 kJ/mol)

<table>
<thead>
<tr>
<th>Probe</th>
<th>( \tau_0 ) (No Calmodulin)</th>
<th>( \tau_1 )</th>
<th>( \tau_2 )</th>
<th>( \tau_3 )</th>
</tr>
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<tbody>
<tr>
<td>N-Et</td>
<td>10.4</td>
<td>11.0</td>
<td>2.0</td>
<td>---</td>
</tr>
<tr>
<td>AMAC</td>
<td>8.8</td>
<td>11.6</td>
<td>7.7</td>
<td>---</td>
</tr>
<tr>
<td>APAC</td>
<td>96.3</td>
<td>198.2</td>
<td>80.2</td>
<td>13.3</td>
</tr>
<tr>
<td>PBAC</td>
<td></td>
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Figure 5 — The induced circular dichroism of PBAC (10 μM, solid line) in the presence of calmodulin (14 μM). The dashed line indicates the baseline observed for PBAC, in the absence of calmodulin.
formation may provide insights into the protein segmental motion.

The saturation of $I_{ex}/I_{mono}$ value at higher temperatures is interesting. Similar saturation behavior was also observed for intramolecular excimer formation, however, at much higher temperature. This was attributed to the kinetic condition $k_d = 1/\tau_e$, where $k_d$ is the rate constant for the dissociation of excimer to monomer, and $\tau_e$ is the excimer lifetime. Calmodulin may be serving as a flexible spacer between the bound pyrenyl chromophores, and modulates the excimer dynamics. The large activation energy estimated for the excimer formation is in support of such a model. It is also consistent with the fact that the excimer growth rates are independent of calmodulin concentration and compare well with the correlation times measured in NMR studies. Due to the high thermal stability of calmodulin, loss of excimer formation above 30 °C could not be due to protein denaturation. Attempts to
increase the solvent viscosity by the addition of ethylene glycol or glycerol or sucrose inhibited the binding of the probe and hence, direct evidence for the segmental motion being responsible for excimer formation could not be obtained. The excimer accounts for only about 10% of the total emission.

Intensities of the induced CD bands of PBAC were found to diminish rapidly with increase in temperature from 5°C to 40°C (~45% decrease). No changes were detected in the corresponding absorption spectra in this temperature range. Therefore, the decrease in the intensity of the CD bands with temperature is not due to a decrease in the binding affinity. Since calmodulin is stable to temperatures as high as 60°C,2 minor perturbations in the probe environment is sufficient to reduce the local asymmetry and decrease the intensity of the CD bands. Thus, the PBAC environment would permit local motion that can rapidly destroy the asymmetry with temperature and enhance the excimer formation.

Conclusions

The cationic hydrophobic bifunctional probes described here bind to calmodulin. A strong relation between the probe structure and the binding properties was observed. For example, pyrenyl (PBAC) shows greater changes in all the spectral properties described above, when compared to the anthryl analogs. Clearly, increased hydrophobicity of the pyrenyl group promotes binding to the protein. Similar results were also observed with bovine serum albumin, lactoglobulin, and low density lipoprotein. These conclusions are in contrast to the high affinity offered by DNA helices for the anthryl probes. Not only the hydrophobicity of the fluorophore is important, but also the linker plays a major role. For example, the
degree of protection provided by calmodulin depends upon the number of atoms separating the charge center from the hydrophobic groups. The per cent decrease in the Stern-Volmer constant for APAC is much larger than for N-Et-AMAC. These probes differ in terms of the distance of separation between the anthryl and the ammonium groups. Longer linkers facilitate burial of the chromophore into the interior of the protein matrix. Such linker dependence was not observed in DNA binding studies, consistent with the relatively shallow binding sites of DNA. The small size of iodide and its ability to penetrate the protein matrix as well as strong induced CD signals observed here. Binding of the anthryl probes to calmodulin shows weak or no induced CD bands, even though these probes show strong induced CD bands upon binding to DNA. While dynamic formation of the excimer between probes bound to the protein was observed with PBAC, no excimer emission can be seen with the anthryl probes. No excimer emission was observed with any of these probes bound to DNA. In the present case, the excimer was formed with a rate constant of 0.08 ns\(^{-1}\) and an activation barrier of 53 kJ/mol. Understandably, this barrier is much larger than what was observed for the intramolecular excimer formation with 1,3-dipyrenyl-propane. Quenching studies suggest that a large fraction of the chromophores is buried in the protein matrix and only a small fraction of them is exposed. Therefore, the observed barrier is perhaps due to the bending and flexing of the protein matrix needed to bring these chromophores together or the barrier for the reorganization of the probes to form excimers at the binding site. In solution, the two lobes of calmodulin were thought to come together due to the bending of the central helix. Such a bent structure for calmodulin was supported by NMR and light scattering studies\(^{24}\). The motional correlation times of the two halves of calmodulin containing the hydrophobic binding sites were estimated to be ~7 ns\(^{-1}\), a value that corresponds to the excimer growth rate observed in our experiments.

These spectral data when coupled with the fact that the side chain has been systematically altered, suggest that probes with long side chains and large hydrophobic moieties prefer binding to proteins\(^{8,9}\). The long side chains seem to have minimal effect on their DNA binding affinities\(^{7}\). Therefore, DNA binding drugs with large hydrophobic groups and long cationic side chains are susceptible to binding to proteins such as serum albumin, lactoglobulin and calmodulin. This inference, although limited, will be important in the design of anticancer drugs targeted for DNA.

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18 Extensive hyperchromism and broadening of the pyrenyl transitions were observed when PBAC binds to calf thymus DNA. In addition, marginal red-shift of few nm was also observed.

19 The relative fluorescence quantum yields of APAC (when measured at the 0-0 band) were benzene (1.0) and water (1.76). The Emission maxima also shift with solvent polarity: ethanol (412 nm), water (413 nm), acetonitrile (414 nm), benzene (416 nm), and dimethyl sulfoxide (418 nm).


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