Molecular Recognition by Calmodulin: Pressure-Induced Reorganization of a Novel Calmodulin−Peptide Complex†

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ABSTRACT: The interaction of apocalmodulin (apoCaM) with a peptide ($Neuro_p$) based on the primary sequence of the calmodulin-binding domain of neuromodulin has been studied by fluorescence spectroscopy. The 1:1 complex (12 μM) formed between apoCaM and the $Neuro_p$ peptide is extremely sensitive to salt and is half dissociated in less than 0.1 M KCl, suggesting that electrostatic interactions contribute strongly to complex formation. Ion pair interactions are frequently sensitive to high hydrostatic pressure due to electrostriction effects in the solvated ion state. Application of high pressure to the apoCaM·$Neuro_p$ complex causes a red shift of the $Neuro_p$ tryptophan emission center of mass and a reduced residual anisotropy but with insignificant reduction in quantum yield. The transition is smooth, reversible, and apparently two-state with a midpoint pressure of approximately 0.8 kbar. The residual anisotropy, quantum yield, and center of mass of the emission spectrum are consistent with the movement of the tryptophan side chain to a more solvated, slightly less restricted environment upon the pressure-induced transition. The pressure effect is independent of the concentration of the complex. These and other data are consistent with the pressure-induced reorganization being a unimolecular event not requiring dissociation of the complex. A volume change of approximately 66 mL mol^{-1} and a free energy change of approximately 1.7 kcal mol^{-1} are associated with the reorganization. The residual interactions maintaining the complex under high pressure are characterized by low standard molar volume and/or high standard free energy changes upon disruption but are destroyed by 200 mM KCl. It is postulated that the main effect of salt on the complex at high pressure is to drive the collapse of the hydrophobic pocket to which the peptide is binding.

Calmodulin is a small (148 residue) acidic protein that is present in all eucaryotic cells and often serves as the primary transducer for calcium-mediated signal transduction. The mediation of cellular responses by calmodulin as a function of calcium concentration is an example of the coupling of extracellular messengers to fundamental biological responses. Calmodulin binds four calcium ions with dissociation constants in the micromolar range. The crystal structure reveals a dumbbell-shaped molecule in which there are two helix−loop−helix (EF-hand) Ca^{2+}-binding motifs in each globular domain (Babu et al., 1988). The calcium-binding loops of each pair of EF-hands participate in a short antiparallel β-sheet. The calcium-binding sites of calmodulin show a varying but relatively limited range of binding affinities (Minowa & Yagi, 1984; Pedigo & Shea, 1995). Most regulation involving calmodulin results from its binding to target proteins in response to an increased concentration of intracellular calcium. Possible exceptions to this general mode of complexation are a pair of neural specific proteins, neuromodulin and neurogranin, which will bind to calmodulin only in the absence of Ca^{2+} (Apel et al., 1990; Baudier et al., 1991). Neuromodulin is exclusive to neuronal tissue and has been found in membrane bound and cytosolic fractions. It has been proposed that it functions by sequestering calmodulin at specific regions of the neuronal membrane, releasing it in the presence of elevated cytosolic calcium to produce localized high concentrations of calmodulin (Alexander et al., 1987). This phenomenon appears in rapidly growing regions of the neuron, linking this process to nerve growth.

A 17 amino acid sequence corresponding to the calmodulin-binding domain of neuromodulin has been defined as QASFRGHITRKKLKGEK. The subsequence RGHITRKK is essential for binding (Alexander et al., 1988). The full sequence binds to calmodulin in both the presence and the absence of Ca^{2+} whereas neuromodulin will bind only to apocalmodulin (apoCaM).[1] Though the peptide does not entirely mimic the calcium-dependent binding of intact

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[1] Abbreviations: AEDANS-apoCaM, calcium free calmodulin AEDANS adduct; apoCaM, calcium free calmodulin; apoCaM·$Neuro_p$, the 1:1 complex of calcium free calmodulin and $Neuro_p$; Bis-Tris, [bis-(2-hydroxyethyl)imino]tris(hydroxymethyl)methane; CaM, calcium-saturated calmodulin; *E*, fluorescence acceptor enhancement efficiency; CaM·$smMLCK_p$, the 1:1 complex of calcium saturated calmodulin and $smMLCK_p$; EDTA, ethylenediaminetetraacetate; I-AEDANS, 5-[[[(iodoacetyl)amino]ethyl]amino]-1-naphthalenesulfonic acid; *J*, spectral overlap integral; MLCK, myosin light chain kinase; $Neuro_p$, peptide based on the calmodulin-binding domain of neuromodulin as defined in the text; NMR, nuclear magnetic resonance; NOE, nuclear Overhauser effect; R_0, Forster resonance energy transfer distance; *R*, resonance energy transfer distance; $smMLCK_p$, peptide based on the calmodulin-binding domain of the smooth muscle MLCK as defined by Roth et al. (1991); Tris, tris(hydroxymethyl)aminomethane.

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neuromodulin to calmodulin, it does provide a unique opportunity to probe the origins of the thermodynamic and structural linkage between binding of calmodulin-binding domains and calcium to calmodulin by offering the ability to investigate the interactions of the same domain with calmodulin in the presence and absence of calcium.

The amino acid sequence of the calmodulin-binding domain of neuromodulin does not possess the pronounced potential for amphiphilic helix formation common to other CaM-binding domains. Nevertheless, transfered NOE studies of the bound peptide indicate that a peptide corresponding to the calmodulin-binding domain of neuromodulin adopts a helical conformation while bound to apoCaM (Zhang et al., 1994). Details of the structural consequences of the binding of this domain to apocalmodulin have only recently begun to emerge (Urbauer et al., 1995). The NMR spectra of both apocalmodulin and its 1:1 complex with the $Neuro_p$ peptide have recently been assigned by triple resonance and NOE-based strategies (Urbauer et al., 1995). The NMR studies indicate that the apoCaM·$Neuro_p$ complex is in fast exchange with its dissociated components at millimolar concentrations of the complex with an apparent off-rate greater than 10^2 s^{-1}. ApoCaM displays many of the same basic structural features as calcium-saturated calmodulin (Babu et al., 1988; Ikura et al., 1991). Under low salt conditions, the two globular domains of calcium-saturated calmodulin persist in apoCaM though there is a clear separation of the loops of the two EF-hands from each other in the C-terminal domain, creating a disordered, solvent-exposed hydrophobic pocket (Urbauer et al., 1995).

The apoCaM·$Neuro_p$ complex is particularly amenable to binding studies by fluorescence as the neuromodulin peptide ($Neuro_p$) contains a single tryptophan while calmodulin contains no tryptophan. In this paper, we demonstrate that high hydrostatic pressure does not dissociate the apoCaM·-$Neuro_p$ complex but produces an intermediate structure. In contrast to temperature variation, the use of pressure as a thermodynamic variable allows one to vary the volume of a system without signficant changes in the thermal energy (Weber & Drickamer, 1983). Different types of interactions (e.g., ion pairs, van der Waals) often have quite different volume changes upon their disruption and therefore display quite different sensitivities to pressure. An apparent conformational change induced in the apoCaM·$Neuro_p$ complex is indicated by a change in the local environment of the fluorescent reporting tryptophan residue of the bound peptide. Relatively large molar volume and small free energy changes are associated with this pressure-induced transition. These data provide a novel view of the relative importance of ionic and hydrophobic interactions to the stability of complexes formed between calmodulin and its target proteins.

MATERIALS AND METHODS

Materials. The chicken calmodulin gene (Putkey et al., 1985) was expressed in *Escherichia coli* and purified by phenyl-Sepharose affinity chromatography (Gopalakrishna & Anderson, 1982; Seeholzer & Wand, 1989) as described previously (Roth et al., 1992). An N-terminal acetylated and C-terminal amidated peptide corresponding to the neuromodulin calmodulin-binding domain (Apel et al., 1990) was synthesized in a manner described elsewhere (Roth et al., 1991). The sequence of the $Neuro_p$ peptide used in the studies reported here is as follows: Acetyl-Gln-Ala-Ser-Trp-Arg-Gly-His-Ile-Thr-Arg-Lys-Lys-Leu-Lys-Gly-Glu-Lys-NH_2. 5-[[[(Iodoacetyl)amino]ethyl]amino]-1-naphthalenesulfonic acid (I-AEDANS) was obtained from Sigma and used without further purification.

Preparation of AEDANS-Calmodulin. The I-AEDANS derivative of calmodulin (AEDANS-apoCaM) was prepared with conditions similar to those employed by Walsh and Stevens (1977) for modification of the methionines of calmodulin with iodoacetate. Calmodulin contains no cysteine. The reaction mixture (LaPorte et al., 1981) contained 10 mg of calmodulin, 200 mM sodium acetate, pH 5.0, 1 mM $CaCl_2$, and 10 mM I-AEDANS (Hudson & Weber, 1973) in a final volume of 6 mL. After incubation in the dark for 6 h, the reaction mixture was dialyzed three times against 200 mL of 10 mM sodium acetate, pH 5.0, 300 mM NaCl, 8 M urea, and 3 mM EDTA to remove unreacted I-AEDANS. This was followed by dialysis three times against 50 mM Tris, pH 6 (first) and 7.5 (subsequent 2), and 1 mM $CaCl_2$. The dialysate was then subjected to purification by phenyl-Sepharose affinity chromatography. A portion of the purified product was dialyzed into 25 mM ammonium acetate buffer, pH 5.5, and diluted 10-fold into 0.1% formic acid for analysis on a Fisons VG Quattro quadrapole mass spectrometer.

Fluorescence Emission and Anisotropy Measurements. Samples of the apoCaM·$Neuro_p$ complex were prepared using a 10−20% molar excess of calmodulin to ensure binding of the synthetic neuromodulin peptide at 12 μM. The buffer was composed of 5 mM EDTA, 5−60 mM KCl as noted, and 10 mM Bis-Tris, pH 6.50. High pressure measurements were performed using a high pressure bomb with quartz windows (Paladini & Weber, 1981a). Ambient pressure data were collected on an ISS PC1 spectrofluorometer. High pressure data were collected on an ISS GREG PC spectrofluorometer or on a homebuilt spectrofluorometer. For these experiments an excitation frequency of 295 nm was used. The emission was collected between 253 and 518 nm with a diode array detector (Princeton Instruments IRY-700). The following equation was used to calculate the center of mass of the emission spectrum (305−450 nm):

$$\langle \nu_p \rangle = \frac{\sum \nu_i F_i}{\sum F_i} \qquad (1)$$

where F_i is the intensity collected at the diode corresponding to frequency ν_i (cm^{-1}).

High pressure anisotropy data were corrected for photoelastic birefringence bias using a sample of *N*-acetyl-L-tryptophanamide in glycerol and the equations of Paladini and Weber (1981a). Fluorescence polarization measurements were made in the L-format using a photon-counting polarization instrument of the type described by Jameson et al. (1978). The polarized emission was collected through a 340 nm cutoff filter. The data were fit to a reorganization factor analogous to the one used for emission data using the appropriate anisotropy values.

Fluorescence Resonance Energy Transfer. Atmospheric pressure fluorescence resonance energy transfer data were collected on an ISS PC1 spectrofluorometer using 1 mm slits (8 nm bandpass). The AEDANS−apoCaM·$Neuro_p$ complex (1.3:1) was prepared in buffer consisting of 1 mM EDTA, 5 mM KCl, and 10 mM Bis-Tris, pH 6.5. The complex was formed by titrating the peptide with AEDANS−apoCaM and

following the tryptophanyl steady state anisotropy. A Schott WG 345 high pass filter and a Corning 9863 bandpass filter were used together to achieve a transmission maximum of 70% at 366 nm and a transmission of <1% at 329 nm and at 420 nm. The anisotropy at each titration point was the average of five measurements. This filter pair was placed immediately in front of the emission photon counter to eliminate scattering and reduce the contribution of AEDANS anisotropy. A buffer blank was simultaneously titrated to further reduce the contribution of AEDANS. Energy transfer from the tryptophan of $Neuro_p$ to an AEDANS acceptor on apoCaM was measured using the technique of acceptor enhancement. The enhancement was calculated using the following relationship:

$$E = \frac{F_{295}^{450}(\text{AEDANS-apoCaM}\cdot\text{Neuro}_p)/F_{340}^{450}(\text{AEDANS-apoCaM}\cdot\text{Neuro}_p)}{F_{295}^{450}(\text{AEDANS-apoCaM})/F_{340}^{450}(\text{AEDANS-apoCaM})} - 1 \quad (2)$$

corresponding to the ratio of fluorescence emission intensities (450 nm) upon excitation at the absorption lines of tryptophan (295 nm) and AEDANS (340 nm) in the presence and absence of bound $Neuro_p$. The ratio in the in the numerator of eq 2 represents the ratio of the AEDANS emission measured at 450 nm when the AEDANS−apoCaM·$Neuro_p$ complex is excited at 295 nm or at 340 nm. The denominator represents the same experimental relationship but performed on a sample lacking the $Neuro_p$ peptide. This term serves to normalize the numerator, i.e., the emission intensities obtained from excitation at 340 nm serve to normalize the data between the two samples as these intensities are independent of energy transfer. Acceptor enhancement and tryptophan anisotropy were measured as a function of KCl concentration to allow for interpretation of high pressure data collected in the high pressure bomb at 1 bar and 2400 bar in the same manner.

RESULTS

It has been shown by NMR spectroscopy that the $Neuro_p$ peptide will form a unique 1:1 complex with apoCaM under low salt conditions (Zhang et al., 1994; Urbauer et al., 1995). The bound peptide is apparently helical (Zhang et al., 1994), and binding mostly affects the C-terminal domain of calmodulin (Urbauer et al., 1995). The apoCaM·$Neuro_p$ complex contains only one tryptophan located at the fourth position from the amino terminus of the peptide. When apoCaM is added to a solution of the $Neuro_p$ peptide, the emission center of mass of the tryptophan fluorescence shifts 12−13 nm to the blue and exhibits a marked increase (>70%) in quantum yield. The tryptophan also becomes protected from iodide quenching upon complexation, suggesting that the indole side chain is inserted into a hydrophobic pocket in apoCaM (Alexander et al., 1988; Chapman et al., 1991). At atmospheric pressure and low ionic strength conditions, the center of mass of the emission of this tryptophan occurs at 347 nm (Figure 1) and the fluourescence shows considerable residual anisotropy (Figure 2). Both are indicative of a relatively immobilized tryptophan side chain in a comparatively apolar environment. When the complex is subjected to high hydrostatic pressure, there is a shift of the emission distribution to longer wavelengths (352 nm at 2.2 kbar) which is accompanied by only a minimal decrease in quantum yield. The residual anisotropy of the fluorescence after the pressure-induced transition is decreased as well but is significantly greater than that of the free peptide ($r \sim 0.05$) (Figure 2). All measured spectral properties of the pressure-induced intermediate are unaffected by changing the concentration of the complex between 2.5 and 40 μM.

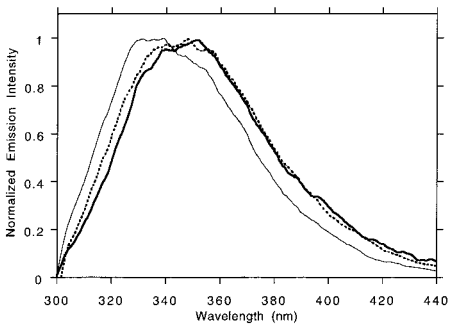

FIGURE 1: Normalized emission spectra of the tryptophan fluorescence of $Neuro_p$ in the 1:1 apoCaM·$Neuro_p$ complex in 10 mM KCl and 1 bar (thin solid line), 10 mM KCl and 2.2 kbar (dotted line), and 60 mM KCl and 2.2 kbar (thick solid line). The complex was approximately 12 μM in 10 mM Bis-Tris buffer at pH 6.5 and 20 °C.

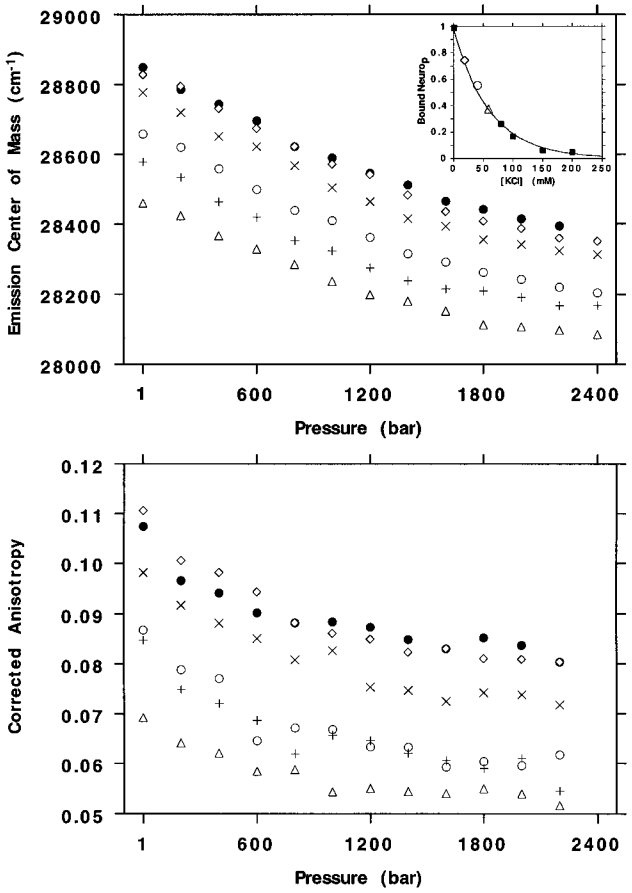

FIGURE 2: The pressure and salt sensitivity of the center of mass of the apoCaM·$Neuro_p$ fluorescence emission spectrum. Data were collected in 200 bar increments and at KCl concentrations of 10 (●), 20 (◊), 30 (×), 40 (○), 50 (+), and 60 (△) mM (top panel). The center of mass of the fluorescence emission spectrum of free $Neuro_p$ is found near 28 100 cm^{-1}. The inset shows the fraction of $Neuro_p$ which is bound to apoCaM at a given concentration of KCl at atmospheric pressure. Matching symbols are used for values determined for 20, 40, and 60 mM KCl. The titration began with 10 μM apoCaM·$Neuro_p$ (see Results). The bottom panel shows the corrected fluorescence anisotropy of apoCaM·$Neuro_p$ versus pressure at KCl concentrations of 5 (◊), 10 (●), 20 (×), 30 (○), 40 (+), and 60 (△) mM. The tryptophan fluorescence anisotropy of free $Neuro_p$ in solution is 0.055 ± 0.005.

Table 1: Effect of Ionic Strength and Hydrostatic Pressure on Acceptor Enhancement Fluorescence Energy Transfer Experiments on 10 μM AEDANS−ApoCaM·Neuro$_p$

[KCl] (mM)	enhancement (%)	[KCl] (mM)	enhancement (%)
5	45	109	17
35	35	284	7
63	25		

hydrostatic pressure (bar)	enhancement (%)
1 (start)	37
2400	25
1 (return)	32

Increasing ionic strength causes the complex to dissociate (inset of Figure 2). These data are in good agreement with the results of Chapman et al. (1991) who also demonstrated dissociation of the apoCaM·Neuro$_p$ complex with increasing [KCl] using steady state fluorescence anisotropy. To further explore the effects of pressure on the complex, both emission center of mass and steady state anisotropy data were collected at a series of salt concentrations and hydrostatic pressures (Figure 2).

As the salt concentration is increased, the shapes of both the emission center of mass and anisotropy curves as a function of pressure are virtually unaffected with the exception of a vertical shifting of the curves toward the respective values for the peptide free in solution. Minimal hysteresis was observed during decompression, and the 1 bar values collected at the start and finish of each experiment were within experimental error (Table 1).

The concentration independence and size of the residual anisotropy of fluorescence remaining after the pressure-induced transition suggest that the structural reorganization that is occurring does not result in dissociation of the peptide from apoCaM. To confirm that the peptide is not being dissociated from apoCaM during the pressure-induced transition, the resonance energy transfer between the Neuro$_p$ peptide Trp and an AEDANS moeity attached to apoCaM was monitored.

AEDANS-apoCaM was prepared by chemical modification with I-AEDANS. The modified calmodulin was analyzed by electrospray mass spectrometry and was found to be 58% unlabeled (*m*/*z* 16 706 amu) (see Figure 3). Of the labeled material, 73% was singly labeled (*m*/*z* 17 012 amu) and 27% doubly labeled (*m*/*z* 17 318 amu) with AEDANS (MW 306 amu) (Figure 3). The calculated molecular weight of calmodulin with all neutral residues is 16 662, for an expected M + 1 ion at 16 663. Given the mass accuracy of the instrument of ±0.01%, this leaves a discrepancy of 43 ± 2 amu. This likely corresponds to an associated acetate. It has been demonstrated by proteolytic cleavage and subsequent optical analysis of AEDANS−apoCaM that 70−80% of the labeling occurs on Met-144 and Met-145 of calmodulin (Olwin et al., 1983).

The presence of high KCl concentration reduces the acceptor enhancement resonance energy transfer from 45% to 7% at 284 mM KCl (Table 1). When the fluorescence resonance energy transfer data are fitted to a single exponential, the fitted value for the enhancement observed at 284 mM KCl is near 3%. This residual enhancement likely represents a small amount (∼5%) of complexed material at this ionic strength and is in qualitative agreement with the measurements made using tryptophan anisotropy shown in the inset of Figure 2. Application of hydrostatic pressure of 2.4 kbar, well above the midpoint of the pressure-induced transition, causes only a 30% loss in acceptor enhancement. This corresponds to a movement of approximately 1 Å of the donor−acceptor pair away from each other. This is further strong evidence that high pressure is not producing dissociation of the complex.

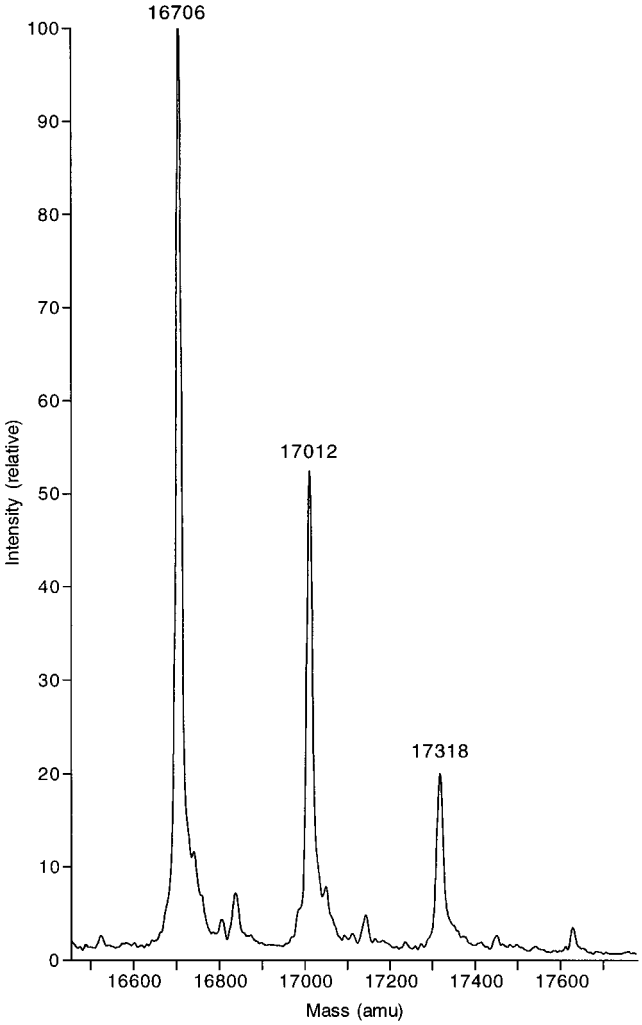

FIGURE 3: Electrospray mass spectrum of AEDANS−apoCaM.

Orientation of the Bound Peptide. Though not the main purpose of the study of the fluorescent enhancement of the apoCaM·Neuro$_p$ complex, the approximate distance between the tryptophan of Neuro$_p$ and an AEDANS moiety can also be estimated and provides an indication of the orientation of the bound peptide on apoCaM. The Förster distance was calculated using eq 3, with the refractive index

$$R_0 = 9790(\kappa^2 n^{-4} \phi_d J)^{1/6} \quad (3)$$

(n) taken to be 1.4 and the orientation factor (κ^2) taken to correspond to free rotation of the transition dipoles of the donor and/or of the acceptor (i.e., $^2/_3$). If the anisotropy of the donor or acceptor is less than 0.22, as is the case here, then the distance error associated with this assumption is likely to be less than 10% (Haas et al., 1978). The quantum yield of the donor Trp (ϕ_d) was found to be 0.099. The overlap integral, J, was calculated using eq 4 using absorbance measurements on 100 μM AEDANS−apoCaM to

$$J = \frac{\sum F_d(\lambda)\, \epsilon_a(\lambda) \lambda^4\, \Delta\lambda}{\sum F_d(\lambda)\, \Delta\lambda} \quad (4)$$

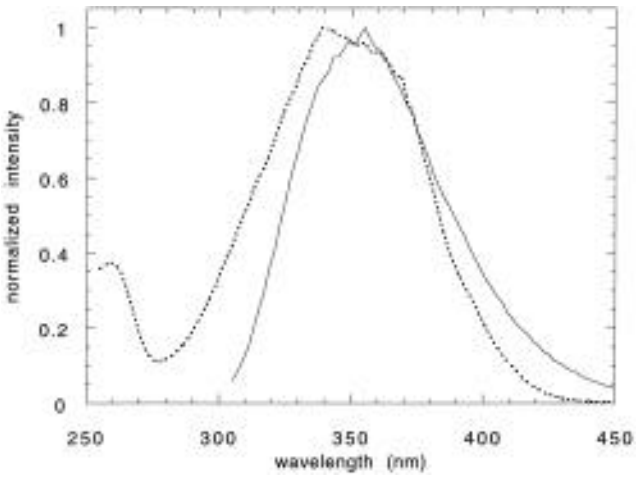

FIGURE 4: The normalized plots of the emission spectrum of the tryptophan fluorescence of $Neuro_p$ (solid line) and the excitation spectrum of the AEDANS of AEDANS−apoCaM (dashed line) show a large overlap, in part accounting for the efficient 45% acceptor enhancement observed at atmospheric pressure and 5 mM KCl.

calculate its molar extinction coefficient (ϵ_a) in 2 nm steps ($\Delta\lambda$) and using the normalized fluorescence emission intensity of $Neuro_p$, each evaluated between 300 and 450 nm (Figure 4). An R_0 of about 21 Å was determined using values of 0.667, 0.26, 0.099, and 5.6×10^{-15} M^{-1} cm^3 for κ^2, n^{-4}, ϕ_d, and J, respectively. Using eq 5 and a value of

$$R = R_0 \times (E^{-1} - 1)^{1/6} \tag{5}$$

21 Å for R_0, the observed acceptor enhancement efficiency (0.45) corresponds to a resonance energy transfer distance of about 22 Å. The obtained distance of 22 Å is most consistent with a C-terminal domain of calmodulin to the N-terminal end of the $Neuro_p$ peptide orientation. This result must be considered qualitative as it assumes that the AEDANS label is in one site and that the second site in the 27% of doubly labeled material is more distant and contributes little to the enhancement given the R^6 dependence.

The orientation of the bound $Neuro_p$ peptide appears then to be analagous to that of the smMLCK calmodulin-binding domain peptide when bound to calmodulin (Roth et al., 1992) and opposite to that of the bee venom peptide melittin (Seeholzer et al., 1987). NMR studies of the complex intended to confirm this are ongoing. It should also be noted that the distance between Met-144, the dominant site of labeling by AEDANS, and Trp-5 of the bound peptide in the CaM-smMLCKp complex is much closer, on the order of 4 Å (Meador et al., 1992).

Thermodynamics of the Pressure-Induced Transition. All data presented are consistent with the pressure-induced transition occurring with a midpoint of about 0.8 kbar being a structural change resulting in a reorganized biomolecular complex. In that case, the degree of reorganization (α_P) at pressure P is given by:

$$\alpha_P = \left[1 + \frac{Q_F}{Q_I}\left(\frac{\langle\nu_P\rangle - \langle\nu_F\rangle}{\langle\nu_I\rangle - \langle\nu_P\rangle}\right)\right]^{-1} \tag{6}$$

where ν_I, ν_F, and ν_P represent the emission center of mass of the initial state, final state, and state at pressure P for each salt concentration. Q_F and Q_I represent the quantum yields of the final and initial states (Paladini & Weber, 1981b).

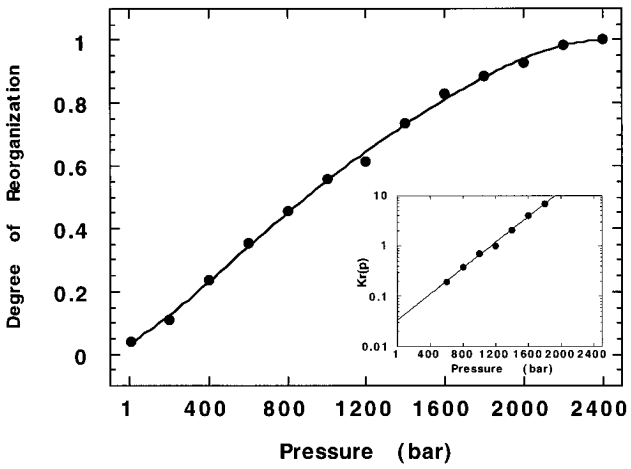

FIGURE 5: Thermodynamic analysis of the pressure-induced reorganization of the apoCaM·$Neuro_p$ complex. Shown is the the degree of reorganization of apoCaM·$Neuro_p$ calculated for the 20 mM KCl fluorescence emission center of mass data shown in Figure 2. The 1 bar value for ν_I and the 2.4 kbar value for ν_F were used in eq 6 (see Results). In the inset, the determined reorganization equilibrium constants Kr(P) are plotted against hydrostatic pressure and fitted to the exponential function of eq 7 (see Results). This procedure was used at each KCl concentration to produce the thermodynamic results given in Table 2.

Table 2: Thermodynamics Governing the Pressure-Induced Reorganization of the ApoCaM·$Neuro_p$ Complex

[KCl]	Kr_0	ΔG (kcal/mol)	ΔV (mL/mol)
10	0.04	1.9	68
20	0.03	2.0	70
30	0.04	1.9	72
40	0.06	1.7	61
50	0.11	1.3	58
60	0.06	1.7	66
av	0.06 ± 0.02	1.7 ± 0.1	66 ± 5

Figure 5 shows the degree of reorganization calculated from the change in emission center of mass using the end points of the 20 mM KCl titration. The end point of the transition is most clearly seen in the anisotropy data. The reorganization data were used to calculate a standard unimolecular equilibrium constant for the pressure transition using the following equation:

$$\mathrm{Kr}(P) = \frac{\alpha_P}{1 - \alpha_P} = \mathrm{Kr}_0 \exp\left(\frac{P\,\mathrm{d}V^\circ}{RT}\right) \tag{7}$$

where dV° is the standard molar volume change and Kr_0 is the reorganization equilibrium constant at 1 bar (Silva et al., 1992). The observed reorganization equilibrium constants are plotted against pressure in Figure 5, and the thermodynamic parameters resulting from fitting the data are tabulated in Table 2 using the following equation to determine ΔG:

$$\mathrm{d}G(P) = -RT \ln \mathrm{Kr}_0 - P\mathrm{d}V^\circ \tag{8}$$

The underlying thermodynamic parameters of the transition are insensitive to salt with a standard molar volume change of about -66 ± 5 mL mol^{-1} and a free energy change of about 1.7 ± 0.1 kcal mol^{-1}.

DISCUSSION

Pressure and ionic strength have been used to probe the origin of the high affinity interaction between apocalmodulin and a peptide based on the calmodulin-binding domain of

neuromodulin. Pressure is a particularly interesting potential perturbant of interactions whose formation involves large changes in volume. Application of high pressure to the apoCaM·$Neuro_p$ complex results in a red shift in the emission spectrum and a reduced but significant residual anisotropy of the bound peptide's Trp. The distance between the Trp and an AEDANS label primarily located at Met-144 or Met-145 of apoCaM does not change appreciably through the pressure-induced structure transition. These results suggest that the application of pressure causes a reorganization of the complex that results in greater solvation of the Trp ring of the bound peptide with an increase in its mobility.

On the basis of the fluorescence resonance energy transfer experiments, the peptide appears to be oriented in a manner analagous to that of the complex between a peptide (sm-$MLCK_p$) corresponding to the smMLCK calmodulin-binding domain (Roth et al., 1992; Meador et al., 1992) with its N-terminus located in the C-terminal domain of calmodulin. Given that the general orientation of the peptide is similar to that of the $smMLCK_p$·CaM complex, then the Trp side chain will interact with the C-terminal globular domain of apoCaM. It has recently been shown that, under low salt conditions, there is a solvent exposed, disordered hydrophobic pocket in the C-terminal domain of apoCaM (Urbauer et al., 1995). This region of the protein becomes more ordered upon binding of the $Neuro_p$ peptide.

The minimal sequence required for binding to apoCaM includes five basic residues—two lysines, two arginines, and one histidine. The C-terminal flanking sequence contains two additional lysines. All seven of these basic residues are potentially capable of forming salt links with glutamates of apoCaM in a fashion analagous to that seen in the $smMLCK_p$·-CaM complex (Meador et al., 1992). The gross sensitivity of the stability of the complex with respect to the presence of salt is consistent with ionic interactions being necessary for the binding of the peptide to apocalmodulin.

Origin of the Pressure Sensitivity. The sensitivity of ionic interactions to high pressure has generally been ascribed to the phenomenon of electrostriction where the negative molar volume change seen upon dissociation of an ion pair arises from the ordering and contraction of solvent around the separated charges. The result is a lower molar volume of the separated charges than for the ion pair (Weber, 1992). The volume changes are often on the order of 15 mL mol^{-1} but in principle can be much lower if the ion pair itself is well packed. Similarly, the free energy change associated with the disruption of specific ion pairs can, in the context of proteins, show a tremendous range and be highly context dependent. For example, an aspartate−arginine ion pair located in an α-helix on the surface of a barnase mutant contributes 0.33 kcal of free energy to the stabilization of the protein (Serrano et al., 1991) while a buried aspartate−histidine ion pair contributes 3−5 kcal of stability to T_4 lysozyme (Anderson et al., 1990).

The measured free energy change for the pressure-induced reorganization described here is on the order of 1.7 kcal mol^{-1}. The molar volume change is about 66 mL mol^{-1}. It is tempting to ascribe this transition to the disruption of salt linkages as, using a standard molar volume change of 15 mL mol^{-1} per salt link, one would estimate that about four salt links are being disrupted by the reorganization. This in turn would imply an average free energy change of about 0.4 kcal mol^{-1} per salt link. As there are five basic residues involved in the minimal consensus sequence required for high affinity binding, this viewpoint would suggest that most if not all of the ionic interactions being disrupted by pressure are central to binding of the peptide. However, it is also possible that the low volume and free energy changes are the result of a simple repacking of the van der Waals contacts of the complex. Nevertheless, the data presented here clearly show a plasticity of complexation that has not been previously detected in complexes involving calmodulin; i.e., there are at least two stable conformations of this complex which are separated by less than 2 kcal mol^{-1} in free energy.

There appear to be two possible reasons as to why the pressure-induced transition does not lead to dissociation of the peptide from apoCaM while high salt does. On the one hand, one could postulate that there is a class of ionic interactions which are distinct from those revealed here. Disruption of this class of interactions would need to be characterized by very low standard volume change and/or relatively high standard free energy change. Another, more likely possibility, is the presence of a van der Waals type of interaction which is sensitive to salt. The recent examination of this complex by NMR spectroscopy suggests a structural feature consistent with the latter notion (Urbauer et al., 1995).

While the N-terminal domain of apoCaM in complex with the $Neuro_p$ peptide displays all of the main structural features of the calcium-saturated calmodulin, the C-terminal domain does not (Urbauer et al., 1995). Specifically, under low salt conditions, the EF-hands of the C-terminal domain are separated such that the short antiparallel sheet that is usually formed between them is disrupted. The resulting reorganization leads to the disruption of close packing involving several aromatic residues of the core and their potential exposure to solvent (Urbauer et al., 1995). As it seems clear that the aromatic ring of Trp-4 of the bound $Neuro_p$ peptide is buried in the C-terminal domain, this region seems a likely point of contact. This burial is reduced upon reorganization by pressure and exposes the Trp ring and presumably this portion of the core of the C-terminal domain to solvent. This would provide a mechanism for high salt to disrupt the basically hydrophobic interaction. By increasing the dielectric constant of the solvent, the exposure of hydrophobic residues becomes increasingly disfavored. The end result is the explusion of the hydrophobic Trp side chain and the closure of the core of the domain under high salt conditions. The insensitivity of these proposed hydrophobic interactions to pressure is not unexpected as the molar volume changes associated with van der Waals interactions are generally very small. This model can only be tested by examining a high resolution model for the structure of the apoCaM·$Neuro_p$ complex under low and high pressure conditions. That work is currently in progress. It is also interesting to note that addition of cations such as K^+ has been shown to have some of the same effects as calcium binding to calmodulin (see Haiech et al., 1981; Linse et al., 1991). Here, however, potassium (and chloride) destabilizes the apoCaM·$Neuro_p$ complex while the binding of calcium has a stabilizing effect (Urbauer et al., 1995).

Implications for Molecular Recognition by Calmodulin. The binding of calcium to calmodulin has been proposed to promote a conformational change that exposes a hydrophobic surface to initiate binding of the target protein. Complexation of calcium-loaded calmodulin with peptides corresponding to the calmodulin-binding domains of regulated proteins has been studied in great detail (Crivici & Ikura, 1995). Peptides which comprise the calmodulin-binding domains of target proteins typically have the ability to form basic amphiphilic α-helices and usually bind calmodulin only

in the presence of Ca^{2+} (O'Neil & De Grado, 1990). The amphiphilic helix model for the structure of a peptide corresponding to a calmodulin-binding domain bound to CaM was first directly confirmed by NMR-based studies on the complex of CaM with a peptide analog of the myosin light chain kinase CaM-binding domain (Roth et al., 1991) and has now been demonstrated for two additional cases (Meador et al., 1993; Zhang et al., 1994). For the two complexes studied in comprehensive detail, the interaction between the bound peptide and CaM in the compact complex involves both extensively buried salt linkages and general hydrophobic interactions (Ikura et al., 1992; Meador et al., 1992, 1993). An emerging view of how the binding of peptides to CaM leads to the final structure is that the initial stages of molecular recognition are driven by relatively nonspecific hydrophobic interactions while the later stages require helix formation and creation of specific pairwise ionic interactions (Fisher et al., 1994; Ehrhardt et al., 1995). These pairwise interactions guide the collapse of the initial encounter complex to the unusual compact structure where the helical peptide is essentially buried in between the two globular domains. It is then interesting to note that an increase in salt concentration leaves the basic thermodynamic parameters governing the pressure-induced transition unchanged and merely results in a shift in the equilibrium between bound and dissociated states of the peptide. This would seem to suggest that the formation of salt linkages between the bound peptide and calmodulin is highly cooperative and that complexes with less than the final number of ion pairs are difficult to populate.

The presence of interactions between apocalmodulin and the $Neuro_p$ peptide in the absence of calcium which are sufficient to cause binding of significant affinity (K_d ~0.4 μM) is unusual and appears to find its origin in the salt-dependent conformation of the C-terminal domain of calmodulin. We have shown elsewhere (Urbauer et al., 1995) that apoCaM exists as a mixture of conformations at physiological salt concentrations. The fact that the $Neuro_p$ peptide binds only to the conformation favored at low ionic strength presents the possibility that neuromodulin and neurogranin actually bind to the minor species of apoCaM under physiological conditions.

Summary. We have shown that pressure can cause a reorganization of the apoCaM·$Neuro_p$ complex. A volume change of approximately 66 mL mol^{-1} and a free energy change of approximately 1.7 kcal mol^{-1} are associated with the reorganization. This transition does not lead to significant dissociation of the peptide from apoCaM, indicating that residual interactions whose disruption is characterized by low volume and/or high free energy changes must be present. These residual interactions are destroyed by salt, and it is postulated that this sensitivity arises from the collapse of the hydrophobic pocket to which the peptide is binding in response to the increase in dielectric constant. These observations represent an interesting separation of the hydrophobic and electrostatic interactions responsible for the binding of amphiphilic peptides to calmodulin.

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