High-Resolution Triple-Resonance NMR Spectroscopy of a Novel Calmodulin·Peptide Complex at Kilobar Pressures

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Protein−protein complexes are a dominant feature of signal transduction pathways in biology.[1,2] There is therefore a keen interest in identifying which interactions control the affinity and specificity of such complexes.[2,3] Hydrostatic pressure apparently offers one of the few routes to the explicit separation of the contributions of hydrophobic and ionic interactions to the formation of protein complexes.[3−5] For example, protein interfaces are frequently composed of both intermolecular salt links as well as extensive hydrophobic surface contacts. Ionic interactions often have significant negative ΔV of dissociation and are therefore usually exquisitely sensitive to pressure, having midpoint dissociation pressures of a few hundred bar.[3−5] In contrast, van der Waals interactions are usually characterized by small ΔV of dissociation and are relatively pressure insensitive often having midpoint dissociation pressures of a few thousand bar.[3−6] This difference offers perhaps the only means to isolate and define the relative contributions of each type of interaction to the affinity and specificity of protein complexes. Such interactions are, of course, central to our understanding of the fundamental chemical principles underlying basic biological phenomena such as signal transduction.[2] Here, we demonstrate the first instance of high-pressure triple-resonance NMR spectroscopy and use this capability to trace the origin of the high-pressure reorganization of a novel complex involving calmodulin, a major element of calcium-mediated signal transduction.

Many aspects of the structure−function relationships of calmodulin have been illuminated by the structures of apocalmodulin,[7,8] calcium-saturated calmodulin,[9−11] and calcium-saturated calmodulin in complex with the calmodulin-binding domains of a few target proteins.[12−16] Most target proteins are bound by calcium-saturated calmodulin only. In contrast, neurogranin and neuromodulin bind to calmodulin in the absence of calcium and not in the presence of bound calcium.[17−20] We have recently reported fluorescence[21] and NMR[22] studies of the interaction between a peptide corresponding to the calmodulin-binding domain of neuromodulin (neuro-p) and calcium-free apocalmodulin. The acidity of calmodulin and the seven basic amino acids of this domain suggest that electrostatic interactions contribute strongly to complex formation. The apocalmodulin·neuro-p complex is 50% dissociated in 40 mM KCl. Application of high hydrostatic pressure red shifts the neuro-p tryptophan fluorescence emission spectrum indicating a regional change in polarity.[21] Importantly, this event is *unimolecular* and does not involve dissociation of the complex.[21]

Since the pioneering work of Benedek and Purcell,[23] two types of approaches to achieve high-pressure NMR capability have evolved. The high-pressure *probe* technique takes the design principle that the entire rf (radiofrequency) coil and sample are to be pressurized. This original design strategy has been adopted and extended by Jonas and co-workers.[6,24,25] The second general approach is the high-pressure *cell* technique which employs thick-walled glass,[26] Vespel,[27] or sapphire[28] tubes or reinforced quartz capillaries.[29] We have developed a high-pressure cell that allows state-of-the-art NMR spectroscopy to be safely performed at kilobar pressures without modification of the spectrometer. We have employed a sapphire tube fitted to a BeCu charging valve and have used liquid pressurization to increase the reliability and safety of the device. A detailed description of the design and operation of the pressure cell will be presented elsewhere.

The NMR spectra both of free apocalmodulin and in the 1:1 complex with neuro-p have recently been assigned using triple-resonance spectroscopy.[22] Upon binding to apocalmodulin, the ^{15}N-HSQC spectrum of the peptide changes significantly (compare panels A & B, Figure 1). Especially noteworthy are the two resonances associated with the Arg side chain guanidino moieties of the peptide. In the free peptide, hydrogen exchange with solvent is efficient enough to cause total loss of crosspeaks from these groups. Upon binding to apocalmodulin, strong crosspeaks arising from these groups appear in the ^{15}N-HSQC indicating their participation in hydrogen-bonding interactions with apocalmodulin. Application of high pressure results in significant changes in the spectrum of the bound peptide (Figure 1). At 1.0 kbar there is a marked reduction in the intensity of the two guanidino crosspeaks indicating increased hydrogen exchange with solvent. Under the conditions used here, the effect of a 1 kbar change in pressure on the underlying chemistry of hydrogen exchange is expected to increase the intrinsic exchange rate by only ∼3-fold (see Carter et al.[30] and references therein). This cannot explain the observed changes in crosspeak intensities even if significant hydrogen exchange can occur from the salt-linked (hydrogen-bonded) state, which, as Figure 1

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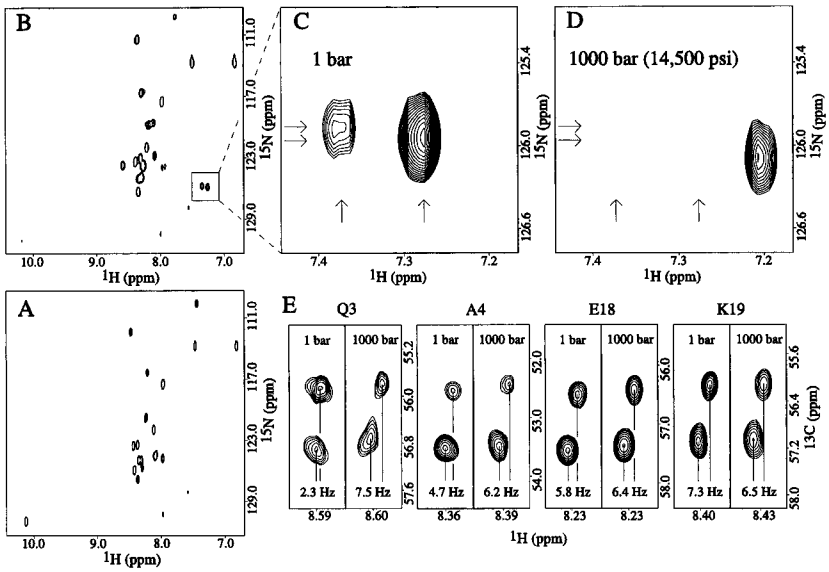

Figure 1. Characterization of the pressure-induced reorganization of the apocalmodulin·neuro-p complex by high-resolution NMR. The ^{15}N-labeled peptide used has the sequence GSQASWRGHITRKKLKGEKG. Panel A shows the ^{15}N HSQC spectrum[34] of the free neuro-p peptide in a 5 mm (o.d.) NMR tube. Panel B shows the ^{1}H−^{15}N HSQC spectrum of the 1:1 apocalmodulin·neuro-p complex (4 mM) in the sapphire high-pressure tube [5 mm (o.d.)/1.5 mm (i.d.), ∼80 μL active volume] at ambient pressure (∼1 bar). Samples were prepared in 2.5 mM imidazole-d_4, 2.5 mM KCl, 0.025 mM EDTA, and 0.02% NaN_3 at pH 6.5 (uncorrected for the isotope effect). Spectra were acquired at 25 °C. HSQC spectra were acquired as 1024 (^{1}H) by 64 (^{15}N) complex point data sets (64 scans/FID) using a sensitivity-enhanced pulsed field gradient technique.[35] Expansions showing the ϵ ^{1}H−^{15}N correlations of R7 and R12 of the bound peptide (aliased from ∼85 ppm) at ambient pressure (∼1 bar) and 1 kbar are shown in panels C and D, respectively. Arrows indicate peak centers at ambient pressure. Main chain backbone assignments of the neuro-p peptide bound to apocalmodulin were obtained using triple-resonance HNCA and HN(CO)CA spectra[36,37] acquired in the sapphire high-pressure tube using sensitivity-enhanced pulsed field gradient techniques[38] (see Supporting Information). The conformation of the main chain of the bound neuro-p peptide was characterized by HNCA-J spectra[39] obtained at ambient pressure and at 1 kbar (panel E). All spectra were acquired with a Varian Unity Inova 600 MHz spectrometer using a standard *z*-gradient triple-resonance probe.

indicates, is not the case. The only remaining interpretation is also the simplest: pressure disrupts the ionic interactions between the peptide and protein resulting in exposure of the Arg guanidino hydrogens of the peptide to solvent. As noted above, the nonsalt-linked state has intrinsic hydrogen exchange rates resulting in total loss of crosspeak intensity. Thus, under the conditions used here, the residual intensities of the guanindo resonances are a direct measure of the salt-linked state of the Arg side chain where hydrogen exchange is significantly retarded. Crosspeak intensities therefore provide a direct measure of the equilibrium between the salt-linked and separated ion states and indicate, through the usual expression $K(p) = K^{\circ}$ exp($p\Delta V/RT$), an apparent free energy change of ∼2 kcal mol^{-1} and a standard volume change of ∼−60 mL mol^{-1}. These values are consistent with those determined from the pressure sensitivity of the fluorescence of the Trp side chain of the peptide.[21] Comparison of triple-resonance HNCA-J spectra of the bound peptide obtained at 1 bar and at 1.0 kbar provide access to changes in the main chain torsion angle φ via $^{3}J_{HNH\alpha}$. These data show that the main chain of the bound peptide is only slightly perturbed during this reorganization (Figure 1). The absence of significant perturbations of the chemical shifts of backbone amide NH resonances of the bound peptide also reinforces the conclusion that the secondary structure of the peptide remains largely unchanged through the pressure-induced transition.

Although the central elements embodied in the basic amphiphilic helix were identified some time ago as critical to the maintenance of a high-affinity complex with calmodulin,[31] almost nothing is known about the relative importance of ionic and hydrophobic interactions in the generation of a high-affinity complex and in the selection of a unique structure. The contrast between specificity and affinity is of critical importance for calmodulin which must bind a wide array of proteins. Here, we have demonstrated that the disruption of two ionic interactions between calmodulin and the neuro-p domain is the origin of the pressure sensitivity of the complex, as suspected previously,[21] and that these interactions contribute ∼2 kcal mol^{-1} toward the total binding free energy. It is has also been shown that the main ionic interactions between the peptide and calmodulin are not required for binding nor are they required for the maintenance of helical peptide structure. Taken together, these results also support a recently proposed model for the molecular recognition events leading from the initial encounter complex to the final low-energy complex structure, where general hydrophobic interactions dominate affinity while ionic interactions dominate in maintaining the uniqueness of the complex.[32,33]

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Supporting Information Available: One figure illustrating triple-resonance assignments (2 pages). See any current masthead page for ordering and Internet access instructions.

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