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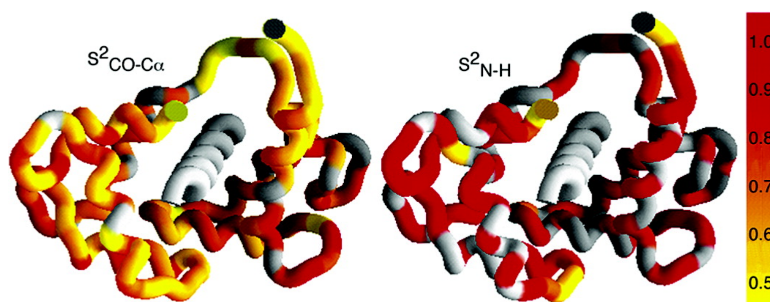

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Changes in Calmodulin Main-Chain Dynamics upon Ligand Binding Revealed by Cross-Correlated NMR Relaxation Measurements

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It has long been recognized that proteins are dynamic systems and that this dynamic quality is important to protein function.[1] NMR spectroscopy has evolved to become a powerful method for experimental quantification of conformational dynamics of proteins.[2] Historically, the sampling of ^{15}N relaxation due to dipole−dipole interactions with its attached hydrogen has been the principal measurement of subnanosecond motion of the polypeptide backbone in proteins.[3] In general, these experiments have suggested that the protein backbone is highly rigid on the picosecond−nanosecond time scale.[2,4] In an effort to more fully characterize the motion of the polypeptide chain of proteins, we have developed a complementary approach based on the transverse cross-correlated relaxation rates between ^{13}CO chemical shift anisotropy and the ^{13}CO−^{13}Cα dipolar interactions which are sensitive to the motion the ^{13}CO−^{13}Cα bond vector.[5]

Here, we investigate the changes in subnanosecond time scale backbone dynamics of calcium-saturated calmodulin (CaM) upon binding of a peptide (smMLCKp) corresponding to the calmodulin binding domain of the smooth muscle myosin light chain kinase.[6,7] CaM contains two globular domains each containing two calcium-binding EF-hand motifs, connected by a central helix that is dynamically disordered at its center.[8] In the presence of ligand, the two domains form a clam-shell around the bound peptide.[9] Only small structural changes occur within the N- and C-terminal domains.[9] The ligand-induced conformational changes are accompanied by the diminishment in the amplitude of subnanosecond time scale dynamics of the methyl-bearing side chains, as detected by deuterium relaxation in methyl groups.[10] In contrast, the amplitude of subnanosecond time scale dynamics of the protein backbone as monitored by ^{15}N relaxation in amide N−H groups is not affected, except for the region bridging the two globular domains.[10] These findings implied that peptide binding causes a major loss of conformational entropy of the side chains, but seemed to indicate that the entropy of the backbone was not affected by the binding process.[10] Here we show that the ^{13}CO−^{13}Cα vectors report a significant loss in dynamics upon ligand binding, suggesting that the entropy of the backbone contributes to the binding free energy after all.

^{13}CO−^{13}Cα cross-correlated relaxation rates were measured with a 3D HNCO experiment[5a,e] without Cα decoupling in the constant-time carbonyl evolution period. For consistency, the S^2_{N-H} order parameters for CaM in the two states were redetermined.[11] As we previously reported,[10a] the ^{15}N relaxation data suggest that the protein backbone, in both states, is homogeneously rigid in regions of defined secondary structure with some flexibility in the loops, linker, and terminal regions (Figure 1A). The average change in S^2_{N-H} ($\langle \Delta S^2_{N-H} \rangle$) upon ligand binding is a very small +0.01 ± 0.003 units (bound minus free, not including the data for the linker),[12] also in agreement with the previous report.[10a]

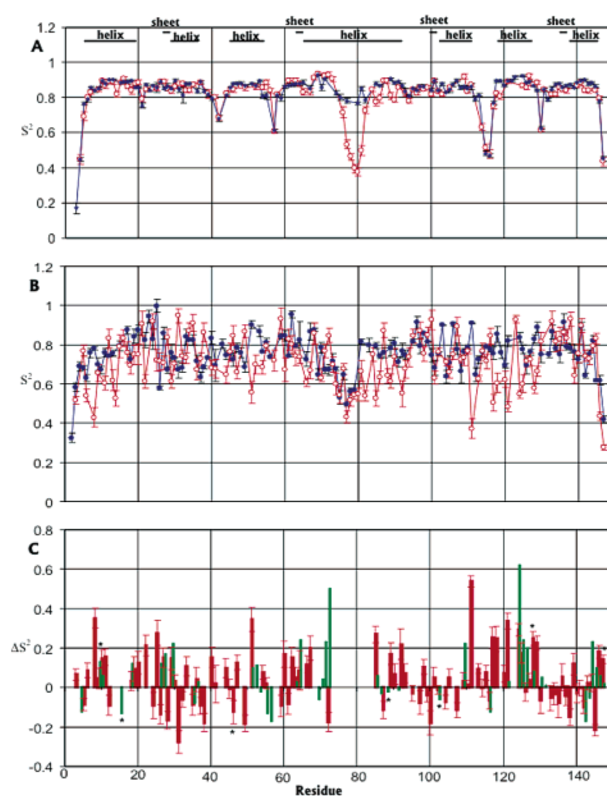

Figure 1. Generalized order parameters and their differences for smMLCKp-complexed CaM (blue) and free CaM (red). All data werr recorded at 11 T and 308 K using a single sample of uncomplexed CaM and a single sample of CaM in complex with the smMLCKp peptide. Both forms were calcium-saturated. Details are provided in the Supporting Information. (Panel A) for the NH vectors. The data and error bars were computed with the program Modelfree.[11d] (Panel B) for the ^{13}CO−^{13}Cα vectors, as derived from the (^{13}CO−CSA) − (^{13}CO−^{13}Cα) dipole R_2 cross-correlated relaxation. The error bars represent the spread obtained from five (CaM-free) and three (CaM-complexed) CT-HNCO data sets. (Panel C) Red: differences $\Delta S^2_{CO-C\alpha}$ (smMLCKp-complexed minus CaM free). Green: $\Delta S^2_{CH_3}$ for the side-chain methyl symmetry axes, derived from ^{2}H quadrupolar relaxation, obtained and replotted from ref 10a for comparison. The asterisks indicate alanine residues.

The ^{13}CO−^{13}Cα cross-correlated relaxation rates depend on the global tumbling time, the $S^2_{CO-C\alpha}$ order parameters, and the site-to-site differences in the ^{13}CO chemical shift anisotropy[5e] (CSA), see Supporting Information. The effective isotropic tumbling time

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for each state was obtained from the ^{15}N relaxation data (anisotropy in rotational diffusion is insignificant for CaM-free[8c] and CaM-complexed[10a]). Figure 1b shows significant differences between the cross-correlated relaxation rates of the backbone of calmodulin in its free and complexed states. This is especially true in the C-terminal globular domain. To minimize the influence of the variability of the ^{13}CO CSA on our interpretation, we discuss here only the differences in the order parameters, $\Delta S^2_{CO-C\alpha}$, between the ligand-bound and ligand-free state for those residues that showed a change in isotropic ^{13}CO chemical shift of less than 0.5 ppm. The $\Delta S^2_{CO-C\alpha}$ parameters shown in Figure 1C suggest that the backbone dynamics of CaM is strongly perturbed upon the formation of the CaM−smMLCKp complex, and that it overall becomes more rigid, with an average increase $\langle \Delta S^2_{CO-C\alpha} \rangle$ of 0.048 ± 0.005.[12]

In contrast to the view afforded by the ^{15}N relaxation data, the ^{13}CO−^{13}Cα cross-correlated relaxation data presented here reveal that the backbone of unliganded CaM contains residual motion, which is affected and partially quenched upon binding of the target domain. Qualitatively, this finding corresponds to the increase in order parameter of the methyl group symmetry axes ($\langle \Delta S^2_{CH_3} \rangle = +0.07$) upon peptide binding as reported previously.[10a] Figure 1c reveals a few interesting correlations between $\Delta S^2_{CH_3}$ and the corresponding $S^2_{CO-C\alpha}$ parameters. For example, in alanine residues, changes in $S^2_{CO-C\alpha}$ are correlated with changes in $S^2_{CH_3}$. In addition, a significant rigidification of the C-terminal domain is reported by both dynamic measures. Otherwise the correlation is weak.

Using a harmonic motional model to interpret the changes of the subnanosecond reorientational dynamics of methyl-bearing side chains upon binding of the smMLCKp domain, in terms of a change in configurational protein entropy (ΔS^{Conf}), a value on the order of −35 kcal/mol at room temperature was estimated.[10a] Using the same model,[10a,13b] the average change in ^{13}CO−^{13}Cα order parameters corresponds to a ΔS^{Conf} of −24 kcal/mol. As pointed out before,[2c,10a,c] simple addition of the entropy changes of side chains and the main chain can only be expected to provide a crude estimate of changes in total residual protein entropy since some correlation of motion must occur (see also above). Nevertheless, adding the values for ΔS^{Conf} estimated for side chains and main chain yields a value (−59 kcal/mol) in reasonable agreement with the range for $\Delta S^{Conf} = -70$ to −140 kcal/mol as was estimated from calorimetric data and theoretical considerations by Wintrode and Privalov.[14]

Significantly, the change in backbone dynamics as sensed by the ^{13}CO environment is not sensed at all by the NH vector, despite the fact that both are part of the same peptide plane. This is reminiscent of our recent findings[5e] that the ^{13}CO−^{13}Cα order parameters decrease faster than the corresponding NH order parameters upon a temperature increase. The apparent lack of full correlation of the NH and ^{13}CO−^{13}Cα detectors of protein backbone motion may be explained by anisotropic local motion of the peptide planes[5] and/or dynamic pyrimidalization[15] of the nitrogen atom which partially decouples the motion of the NH vector from that of the peptide plane.[16]Our findings indicate that the investigation of protein backbone dynamics by NMR spectroscopy should be expanded to routinely include dynamical information derived from ^{13}CO−^{13}Cα cross-correlated relaxation experiments; this holds especially true if subtle changes in dynamical properties, summed over many residues, are to be evaluated in terms of change of conformational entropy.

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Supporting Information Available: Three tables with relaxation rates, methodology used to extract order parameters from the relaxation rates. This material is available free of charge via the Internet at http://pubs.acs.org.

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(12) $\langle S^2_{CO-C\alpha} \rangle$ and the average experimental uncertainties are: calmodulin + peptide: 0.7646, 0.02514; calmodulin-free: 0.7171, 0.0385. A *t*-test on these data (95 points each) yields a two-tailed *P* value of less than 0.0001, demonstrating that the difference between the average order parameters in the two states is extremely statistically significant. Press, W. H.; Flannery, B. P.; Teukolsky, S. A.; Vetterling, W. T. *Numerical Recipes-Fortran Version*; Cambridge University Press: Cambridge, 1988; p 467. An equivalent formulation is that the uncertainties in the two averages are given by the average uncertainties/$\sqrt{95}$ (0.0026 and 0.004, respectively) and that the uncertainty in the difference of the averages ($\langle \Delta S^2_{CO-C\alpha} \rangle = 0.048$) is $\sqrt{(0.0026^2 + 0.004^2)} = 0.005$.

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