Dynamics of the Transition between Open and Closed Conformations in a Calmodulin C-Terminal Domain Mutant

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Summary

Background: Calmodulin is a ubiquitous Ca$^{2+}$-activated regulator of cellular processes in eukaryotes. The structures of the Ca$^{2+}$-free (apo) and Ca$^{2+}$-loaded states of calmodulin have revealed that Ca$^{2+}$ binding is associated with a transition in each of the two domains from a closed to an open conformation that is central to target recognition. However, little is known about the dynamics of this conformational switch.

Results: The dynamics of the transition between closed and open conformations in the Ca$^{2+}$-loaded state of the E140Q mutant of the calmodulin C-terminal domain were characterized under equilibrium conditions. The exchange time constants ($\tau_{ex}$) measured for 42 residues range from 13 to 46 $\mu$s, with a mean of 21 $\pm$ 3 $\mu$s. The results suggest that $\tau_{ex}$ varies significantly between different groups of residues and that residues with similar values exhibit spatial proximity in the structures of apo and/or Ca$^{2+}$-saturated wild-type calmodulin. Using data for one of these groups, we obtained an open population of $p_o = 0.50 \pm 0.17$ and a closed $\rightarrow$ open rate constant of $k_o = (2.7 \pm 1.0) \times 10^4$ s$^{-1}$.

Conclusions: The conformational exchange dynamics appear to involve locally collective processes that depend on the structural topology. Comparisons with previous results indicate that similar processes occur in the wild-type protein. The measured rates match the estimated Ca$^{2+}$ off rate, suggesting that Ca$^{2+}$ release may be gated by the conformational dynamics. Structural interpretation of estimated chemical shifts suggests a mechanism for ion release.

Introduction

The conformational fluctuations and dynamical properties of a protein are intimately coupled to its function [1–6]. Conformational transitions on microsecond to millisecond timescales are believed to be particularly important for processes such as molecular recognition by "induced fit" and enzyme activity [7–10]. Nuclear magnetic resonance (NMR) spectroscopy provides a powerful means to study the dynamics of proteins and other biomolecules at atomic resolution over a wide range of timescales [11–14]. Heteronuclear spin relaxation measurements commonly identify regions of proteins that experience conformational fluctuations on a timescale of microseconds to milliseconds, but detailed analyses of the microscopic time constants and the structural nature of the exchange process have so far been rare [15–18]. Recent developments in NMR enable characterization of conformational exchange with correlation times as short as ~10 $\mu$s in solution, using off-resonance rotating-frame $^1$H NMR relaxation [19–22]. In the present study, we have utilized this method to characterize the dynamics of a large-scale transition between folded conformations in the C-terminal domain of calmodulin (CaM).

CaM is a ubiquitous eukaryotic protein that couples transient increases in intracellular Ca$^{2+}$ concentration with numerous regulatory processes by undergoing a conformational transition that triggers target recognition [23]. CaM consists of two structurally homologous domains joined by a flexible linker. Each domain contains two Ca$^{2+}$ binding helix-loop-helix motifs called EF-hands [24, 25] that are packed in a head-to-head orientation with a short $\beta$-type interaction connecting the two loops. The $\alpha$ helices and Ca$^{2+}$ binding loops of CaM are denoted A–H and I–IV, respectively. Each CaM domain binds two calcium ions with positive cooperativity and dissociation constants in the micromolar range [26]. Upon Ca$^{2+}$ binding to CaM, the helices in each EF-hand change their relative orientations from approximately antiparallel to orthogonal, corresponding to a "closed-to-open" structural transition of the domain [27–31], which also involves significant repacking of the hydrophobic core. In the open conformation, the domain exposes a large hydrophobic patch that forms the binding sites for various targets [23, 32–35]. This conformational switch is thus central to CaM-mediated signal transduction in the cell. The time-averaged structures of the open and closed states have been characterized by X-ray crystallography [27, 28] and NMR spectroscopy [29–31]. Recent X-ray crystallographic studies of calcium-loaded CaM at 1.0 Å resolution reveal correlated disorder of the backbone and side chains, suggesting a hierarchy of conformational substates [36]. $^1$H NMR spin relaxation experiments have been used to characterize the intramolecular dynamics of apo [37] and Ca$^{2+}$-loaded CaM [38], as well as of a complex between Ca$^{2+}$–CaM and a target peptide [39]. In contrast, the dynamics of the structural transition have not been addressed in detail, although semiquantitative results have been obtained for the C-terminal domain, indicating that the apo state transiently samples open conformations on

Key words: NMR; dynamics; off-resonance rotating-frame $^1$H spin relaxation; conformational exchange
a timescale of microseconds to milliseconds [40]. In addition, molecular dynamics simulations have been used to study motions on timescales of nanoseconds that appear to sample the initial stages of the transition process [41]. Recent studies have indicated that conformational transitions in CaM occur with rates similar to due to rapid amide proton exchange with the solvent [42]. Recent studies have indicated that conformations were not observed in the NMR spectra, presumably due to rapid amide proton exchange with the solvent. Measurements could not be obtained for N97 and Q140 due to severe spectral overlap. For the remaining 69 residues, Rs values were obtained at nominal tilt angles ranging from 19° to 64° (measured at the midpoint of the 15N spectrum). The rapidly decaying intensities of I100, Y138, and E139 precluded reliable Rs measurements for these residues at the largest nominal tilt angle, i = 64°, where the Rs contribution is largest. Except for the cases mentioned above, fits of single-exponential decays to the experimental data were accepted on the 95% confidence level for all residues at all tilt angles. Representative relaxation curves at a nominal tilt angle of φ = 54° are shown in Figure 1a. The eight data pairs (i, Rs) form a relaxation dispersion curve [c.f. equation (1)], as exemplified in Figure 1b. An alternative representation of these data are given by (R1 - R0)/sinφ plotted as a function of ω2r, as shown in Figure 1c.

**Exchange Model and Optimization of Dynamic Parameters**

Based on our previous observations indicating two major conformations [43, 45], a minimal two-state exchange model was applied here. Two parameters, τex and φ, were included to describe the exchange for each residue. The exchange time constant (τex) is the effective mean lifetime of the two conformations and is related to the microscopic opening and closing rates k+ and k− [see equation (3)]. The parameter φ contains information on the populations and 15N chemical shifts of the exchanging conformations [see equation (4)]. The optimized model included also as free parameters the longitudinal and transverse relaxation rate constants R1opt and R2opt. For each 15N spin, these four parameters (τex, φ, R1opt, and R2opt) were fit to the experimental relaxation data (R1, R2, R0, and the eight values of Rs), as outlined in equations (9)–(12). The significance of any resulting relaxation dispersion was assessed by F-statistical testing [48], comparing the four-parameter fit with a two-parameter fit that included only the optimized parameters R1opt and R2opt (i.e., conformational exchange was excluded in the latter fit). For 57 residues out of the 69, the quality of the fit was significantly improved when exchange was included.

**Results and Discussion**

**R1 Relaxation Dispersion Measurements**

We have measured off-resonance rotating-frame 15N spin relaxation rate constants (R1) at eight different effective fields and a temperature of 301 K. E140Q Tr1C (corresponding to residues M76–K148 of CaM and including the mutation E140Q) contains 73 backbone amide groups. The resonances of the two N-terminal residues were not observed in the NMR spectra, presumably due to rapid amide proton exchange with the solvent. Measurements could not be obtained for N97 and Q140 due to severe spectral overlap. For the remaining 69 residues, Rs values were obtained at nominal tilt angles ranging from 19° to 64° (measured at the midpoint of the 15N spectrum). The rapidly decaying intensities of I100, Y138, and E139 precluded reliable Rs measurements for these residues at the largest nominal tilt angle, i = 64°, where the Rs contribution is largest. Except for the cases mentioned above, fits of single-exponential decays to the experimental data were accepted on the 95% confidence level for all residues at all tilt angles. Representative relaxation curves at a nominal tilt angle of φ = 54° are shown in Figure 1a. The eight data pairs (i, Rs) form a relaxation dispersion curve [c.f. equation (1)], as exemplified in Figure 1b. An alternative representation of these data are given by (R1 - R0)/sinφ plotted as a function of ω2r, as shown in Figure 1c.

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Figures 1b and 1c show representative Rs relaxation dispersion curves. Precise measurements of φ and τex were obtained for 42 residues. The remaining 15 residues exhibit small degrees of relaxation dispersion, yielding either fits of poor quality or substantial uncertainties in the optimized parameters (with few exceptions, τex falls in the range of 10–100 μs). Limited relaxation dispersion is expected for these residues because the 15N chemical shift changes observed upon Ca2+ binding to wt-Tr1C are small, with a mean value of <Δδ(wt)> = 0.91 ± 0.65 ppm and a maximal shift difference of 2.8 ppm (observed for G98). The following discussion involves only the set of 42 residues for which well-determined exchange parameters were obtained.
The optimized values of $R_{1opt}$ and $R_{0opt}$ agree with the experimentally determined values $R_1$ and $R_0$ [43] to within the experimental errors, yielding mean deviations of $<R_{1opt} - R_1> = -0.01 \pm 0.02$ s$^{-1}$ and $<R_{0opt} - R_0> = -0.02 \pm 0.08$ s$^{-1}$. This observation indicates that the different sets of relaxation rates, obtained from experimental data acquired several months apart and on different samples, are mutually consistent.

Equation (12) is derived for the fast exchange limit $\tau_{ex} C_0 \ll 1$. The optimized parameters obtained here (see below) yield $\tau_{ex} C_0 < 0.1$ in all cases, suggesting that the fast exchange limit is fulfilled. We investigated further the extent of agreement between actual and optimized parameters by simulating off-resonance $R_1$, relaxation data using the homogeneous Bloch-McConnell equations [49-52] using MATLAB (The MathWorks, Inc.), and subsequently fitting equation (12) against the simulated data (J. E. and M. A., unpublished data). The simulations indicate that for the ranges of $\tau_{ex}$ and $\phi$ values reported here [13-46 $\mu$s and $(0.02 - 0) \times 10^5$ s$^{-1}$, respectively] the results should be accurate to within 15% ($\tau_{ex}$) and 12% ($\phi$). These maximal errors are obtained for heavily skewed populations, $p_c = 0.9$, and large chemical shift differences, $|\Delta R| = 10$ ppm, while for more equal populations and smaller chemical shift differences the errors are substantially smaller. Thus, for the large majority of residues (see below) the systematic error is within 5% for both $\tau_{ex}$ and $\phi$, which is less than the estimated random errors of $\sim 6\%-60\%$. Faster exchange processes ($\tau_{ex} < 5 \mu$s) are not subject to misinterpretation, because they do not yield appreciable dispersion, given the effective fields used in the present study. In summary, the simulations validate the exchange parameters extracted using equation (12).

Residue-Specific Exchange Time Constants

The resulting values of $\tau_{ex}$ are shown as a function of amino acid sequence in Figure 2a. The $\tau_{ex}$ values range between 13 and 46 $\mu$s, with a weighted mean of $<\tau_{ex}> = 21$ and weighted uncertainty of $<\sigma_{\tau_{ex}} > = 3 \mu$s.

The well-defined mean exchange time constant for the opening-closing process corroborates earlier estimates of $\tau_{ex} \sim 17-20 \mu$s [43]. Although the majority of residues exhibit values of $\tau_{ex} \sim 20 \mu$s, the results suggest that there is a significant variation in $\tau_{ex}$ between different residues. The heterogeneity among the $\tau_{ex}$ values is further revealed by the shape of the sum of normal distributions derived from the $\tau_{ex}$ values and their standard deviations (Figure 2b). The current experiment does not detect conformational exchange processes with time constants much less than 10 $\mu$s, implying that the skewed distribution could potentially be due to conformational limitations. Geary’s test of normality [48] indicates that the distribution of $\tau_{ex}$ values deviates only weakly from a gaussian. The site-to-site variability in $\tau_{ex}$ was estimated as follows. The standardized variable $Z = (\tau_{ex} - <\tau_{ex}>)/\sigma_{\tau_{ex}}$, in which $\sigma_{\tau_{ex}}$ is the estimated standard deviation of $\tau_{ex}$ for the individual residue, was evaluated for the entire set of 42 residues. The standard deviation of $Z$, denoted $\sigma_Z$, is related to the standard deviation in site-to-site variability in $\tau_{ex}$, denoted $\Lambda$, through the relationship $\sigma_Z^2 = 1 + (\Lambda <\sigma_{\tau_{ex}} >)^2$ [53]. Here, $\sigma_Z = 1.50$, corresponding to $\Lambda = 3.2 \mu$s. We analyzed the variation in $\tau_{ex}$ in more detail by partitioning objectively the 42 residues into three different groups using cluster analysis based on hill-climbing algorithms [54], modified to weight each datum by its estimated uncertainty. The resulting three groups indicated in Figure 2 have the following weighted mean values of $\tau_{ex}$: 18.5 $\pm$ 2.6 $\mu$s.

Figure 1. Off-Resonance Rotating-Frame $^1$H Relaxation Data

Representative data are shown for R86 (filled circles), S101 (filled triangles), and D131 (open squares). (a) $R_{1p}$ decay curves obtained at a nominal tilt angle of $\theta = 54^\circ$. The lines show the single-exponential fits of the equation $I(t) = I(0)\exp(-R_1 t)$ to the experimental data for each residue. The bars represent the estimated uncertainties (one standard deviation) in the measured intensities. The tilt angles and optimized values of $R_1$ are 57.5, 8.0 $\pm$ 0.2 s$^{-1}$ (R86); 57.5, 15.8 $\pm$ 0.5 s$^{-1}$ (S101); and 52.1, 10.4 $\pm$ 0.2 s$^{-1}$ (D131). The rotating-frame $^1$H relaxation rate constants ($R_0$) are shown as a function of (b) tilt angle, $\theta$, and (c) the effective field squared, $\omega_p^2$ [c.f. equations (1) and (2)]. $R_0$ is included at $\theta = 0^\circ$, and $R_0$ (inverted symbols) and $R_{1p}$ at $\theta = 90^\circ$ [43]. The solid lines represent the nonlinear fits, as described in detail in the text [c.f. equations (9)–(12)]. Error bars represent one standard deviation. The dashed lines show the expected intensity in absence of conformational exchange, $I_{ex} = 0$ [c.f. equation (1)]. The optimized values of $\tau_{ex}$ and $\phi$ are 20.3 $\pm$ 2.1 $\mu$s, (269 $\pm$ 27) $\times 10^3$ s$^{-1}$ (R86); 16.9 $\pm$ 1.7 $\mu$s, (980 $\pm$ 104) $\times 10^3$ s$^{-1}$ (S101); and 23.3 $\pm$ 1.5 $\mu$s, (484 $\pm$ 38) $\times 10^3$ s$^{-1}$ (D131).
As stated above, the results of the cluster analysis do not necessarily imply that there are only three distinct exchange processes active. An alternative view is that there exists a continuous distribution of effective exchange time constants that can be rationalized as follows. The open and closed conformations are probably best viewed as two different basins of attraction in the energy landscape [5, 55, 56]. Within each of the open and closed basins, the molecule is interconverting between lower level conformational substates on a timescale faster than that studied here. It is thus likely that the conformational transition can be initiated from a large number of substates, such that there exists a multitude of possible paths across the energy landscape connecting the two basins. (Note, however, that the transition process does not involve a significant population of unfolded molecules, as gauged from the fast timescale [ps-ns] order parameters [43]). Because the $^{15}$N chemical shift is sensitive primarily to local interactions [57], the chemical shift modulation that is studied in the present experiment reflects mainly local conformational changes. Based on these considerations, we propose that the distribution of residue-specific $\tau_{\text{ex}}$ values may reflect ensemble-averaged hierarchical formation of local structure characteristic of the open and closed basins. Further experiments conducted over a range of temperatures and static magnetic fields will be needed to address this issue.

**Relative Populations and Chemical Shift Differences**

The quadratic dependence of $\phi$ on the chemical shift difference between the exchanging conformations results in a wide range of values, $\phi = (0.02-2) \times 10^6$ s$^{-2}$, c.f. Equation (4). The $\phi$ parameter encapsulates both the populations and the chemical shift difference, and these two terms are not directly separable in the present analysis. However, we compared $\phi^{/15}$ and the absolute $^{15}$N chemical shift differences, $|\Delta \delta_{\text{wt}}|$, between the apo and (Ca$^{2+}$) states of wt-Tr2C in order to investigate the extent of agreement between the current data and the assumed exchange process between open and closed conformations (Figure 3a). The largest $\phi$ values are obtained for residues I100 and E139, which also exhibit the largest chemical shift changes upon Ca$^{2+}$ binding to wt-Tr2C (13.6 and 7.3 ppm, respectively). Weighted least-squares minimization of $|\phi^{/15} \gamma_{\text{wt}}|_{\text{r}}$ versus $|\Delta \delta_{\text{wt}}|$ using all 42 residues yields a slope corresponding to an open population of $p_0 = 0.88 \pm 0.03$, as indicated by the black line in Figure 3a; the sample correlation coefficient of the data is $r_c = 0.66$. However, a variation in $\tau_{\text{ex}}$ between different groups of residues likely implies a variation also in the effective populations. A substantially better correlation is obtained if we consider only the red group (22 residues, excluding I100 and I130, as explained below), which contains residues with well-determined exchange parameters and the largest variation in $\phi$. These data yield $p_0 = 0.65 \pm 0.15$, which was based on the intensities of the two mutually exclusive sets of NOEs [45]. Thus, the results obtained for the red group strongly support the notion that the observed exchange involves open and closed conformations similar to apo and Ca$^{2+}$-saturated wild-type CaM. In contrast, the green and blue groups exhibit more limited ranges of $\phi$ and poorer correlations with $|\Delta \delta_{\text{wt}}|$ that correspond to more skewed populations. The slopes of the linear fits are dominated by only a few points for these two groups, and the results can hardly be taken as significant. It is possible that the exchange
and blue groups. The presence of two calcium ions in exchange contributions to the laboratory frame trans-

The local geometry and hence the chemical shifts of terms, indicating that they exhibit similar fluctuations.

$Dd$ between residues from the respective

text for details. of $E140Q$ and the two apo states are observed for residues

Figure 3. Populations and Chemical Shift Differences

Comparison of the $\phi$ values of (Ca$^{2+}$)$_2$-E140Q and the Ca$^{2+}$-induced $^{15}N$ chemical shift changes in wt-Tr$_2$C at 301 K. The data are color coded (red triangles, green circles, and blue diamonds) according to clusters, based on the values of $\tau_r$, see Figures (2)–(4) and the text for details.

(a) $[\phi^{\text{closed}}(\gamma_N B_N)]$ plotted versus $[\Delta \delta(\text{wt})]$. Black line: linear regression using all data, yielding $p_\phi = 0.88 \pm 0.03$ ($r_r = 0.66$). Red line: linear regression using the red group only, yielding $p_\phi = 0.50 \pm 0.17$ ($r_r = 0.86$). Open red triangles indicate residues that have not been included in the linear regression of the red group (see the text for details).

(b) The $^{15}N$ chemical shift differences $[\Delta \delta(\text{wt})]$ (open circles) and $[\Delta \delta(\text{E140Q})]$ (colored symbols) are shown as a function of the amino acid sequence. $[\Delta \delta(\text{E140Q})]$ was calculated from the $\phi$ values, assuming an open population of $p_\phi = 0.50$.

observed for the green and blue groups does not corre-

tion between open and closed conformations but that additional processes may be active for these residues (see also discussion above).

$^{15}N$ chemical shift differences between the exchanging conformations, $[\Delta \delta(\text{E140Q})]$, were calculated for each residue from the respective $\phi$ values and $p_\phi = 0.50$ (as obtained for the red group), yielding values ranging between 0.8 and 7.5 ppm. Figure 3b shows a comparison between $[\Delta \delta(\text{E140Q})]$ and $[\Delta \delta(\text{wt})]$, plotted against the amino acid sequence. As expected, the two sets of values are highly similar for the residues in the red group, whereas larger deviations are observed for the green and blue groups. The presence of two calcium ions in the closed conformation of E140Q most likely distorts the local geometry and hence the chemical shifts of residues located in and around the Ca$^{2+}$ binding sites, as compared to the apo state of wt-Tr$_2$C. The removal of one side chain oxygen not only impairs the bidentate coordination of Ca$^{2+}$ but also affects the hydrogen bond network in the Ca$^{2+}$ binding loop [45, 58], which can result in significant chemical shift differences for certain residues between the open conformation of the mutant and wild-type proteins. For example, the shift deviation estimated for I130 may be caused by such mutation-specific effects, because the mutated side chain cannot simultaneously form the hydrogen bonds that are observed in the wild-type protein to this amide group and to those of D131 and N137 [27, 28, 45]. Clearly, discrepancies between the $[\Delta \delta(\text{E140Q})]$ and $[\Delta \delta(\text{wt})]$ values could also result from other structural differences between the two conformations of (Ca$^{2+}$)$_2$-E140Q and the apo and Ca$^{2+}$-saturated states of wt-Tr$_2$C. Another possible source of chemical shift discrepancies might be that the dynamic chemical shift averaging between substates within each of the open and closed conformations of (Ca$^{2+}$)$_2$-E140Q may be different from that occurring in the wild-type protein. We emphasize that the detailed structures of the open and closed conformations of (Ca$^{2+}$)$_2$-E140Q are unknown, although the present data together with the observed NOEs as well as the $^1H$ and $^{15}N$ chemical shifts indicate that the conformations resemble the apo and Ca$^{2+}$-saturated states of wild-type CaM [43, 45].

Rate Constants of the Opening and Closing Processes

In the case of the red group, the determination of exchange time constant as well as populations enables extraction of the forward and backward rate constants. Using equation (3) with a mean exchange time constant of $<\tau_r>$ = 18.5 $\pm$ 2.6 $\mu$s and an open population of $p_\phi = 0.50 \pm 0.17$, we estimate the rate constants for the opening (closed→open) and closing (open→closed) processes to be $k_0 = k_1 = (2.7 \pm 1.0) \times 10^4$ s$^{-1}$. In a previous study we noted that the estimated conformational exchange rate was of the same magnitude as the estimated Ca$^{2+}$-off rate from loop IV and speculated that Ca$^{2+}$ dissociation from loop IV may occur predominantly from the closed conformation so that it is effectively gated by the conformational dynamics [43]. Given a Ca$^{2+}$ binding constant of $K = 1.4 \times 10^4$ M$^{-1}$ [45] and assuming an on-rate of $k_{\text{on}} \sim 10^7$ M$^{-1}$ s$^{-1}$ [59, 60], the off-rate is estimated to $k_{\text{off}} \sim 7 \times 10^4$ s$^{-1}$. The present measurement of the actual rate constant for the closing process is in general agreement with the estimated Ca$^{2+}$-off rate.

Relevance for the Ca$^{2+}$-Induced Structural Activation of Calmodulin

The present results on (Ca$^{2+}$)$_2$-E140Q are likely to be relevant for understanding the conformational transition also in the wild-type protein. NMR studies of apo CaM [29, 30, 37] and apo wt-Tr$_2$C [40] have revealed pronounced conformational exchange for many residues in the C-terminal domain. Importantly, the patterns of exchange contributions to the laboratory frame transverse relaxation rates $R_{\text{NCPMR}}$ are similar in all three systems, indicating that they exhibit similar fluctuations. Again, the largest differences in $R_{\text{NCPMR}}$ between (Ca$^{2+}$)$_2$-E140Q and the two apo states are observed for residues
located in the Ca\textsuperscript{2+} binding loops (c.f. discussion above). Disregarding six significant outliers, least-squares minimization of $\phi$ (determined here for E140Q) against $R_{\text{ex,CPMG}}$ (determined previously for apo wt-Tr\textsubscript{2C} at a temperature of 291 K [40]) yields a slope of $\phi/R_{\text{ex,CPMG}} = 51.044 \pm 2697 \text{s}^{-1}$, with a medium degree of correlation of $r_e = 0.62$. The slope yields an approximate value of the rate constants in apo wt-Tr\textsubscript{2C} as follows. Assuming that the second term of equation (8) can be neglected and that the exchange samples identical conformations in E140Q and apo wt-Tr\textsubscript{2C} [i.e., $\Delta \phi(E140Q) = \Delta \phi(apo)$], the slope $\phi/R_{\text{ex,CPMG}}$ corresponds to $k_{\text{ex}}(apo)p_i(E140Q) p_{i'=o}(E140Q)/[p_{i=apo}(apo)]$. Taking $p_{i=apo}$ $\sim$5\%–10\% as a reasonable estimate of the populations in apo wt-Tr\textsubscript{2C} [40], we obtain $k_{\text{ex}}(apo) \sim \{(1.0–1.8) \times 10^4 \text{s}^{-1}\}$. Although the temperature dependence of the rate is not known, the inference is that the rate constants of E140Q and apo wt-Tr\textsubscript{2C} agree well, in accord with previous conclusions [40]. This may suggest that the E140Q mutation affects the rates mainly by altering the free energy of the open conformation and to a lesser extent the energies of the closed conformation and the effective barrier between the conformations; in this case, the exchange rate of E140Q cannot exceed that of wt-Tr\textsubscript{2C} by more than a factor of two. The conformational exchange observed here most likely involves repacking of interior side chains and significant displacement of backbone atoms, implying excursions across the energy landscape over a large number of local barriers connecting distinct substates. The E140Q mutation is likely to cause small effects on the barriers related to the repacking of interior side chains, even though it significantly affects the difference in free energy between the two major conformations. Because the populations are nearly equal in E140Q, the effects of conformational exchange on the transverse spin relaxation are significantly enhanced (approximately by a factor of three to ten) relative to the apo states of intact CaM and wt-Tr\textsubscript{2C}. To this extent, E140Q may serve as a model system for probing in detail the dynamics and energy landscape of the structural transition in wt-Tr\textsubscript{2C} and CaM.

**Detailed Structural Information from Chemical Shift Differences**

Valuable information on the structural details of the exchanging species is further provided by the chemical shift differences of I100 [$\Delta \delta(wt) = 13.6 \text{ ppm}$] and V136 [$\Delta \delta(wt) = 5.5 \text{ ppm}$], located in the middle of the $\beta$ strand (corresponding to loop position 8) of loops III and IV, respectively. The $^{15}$N chemical shifts of these two residues have been shown to be reliable indicators of Ca\textsuperscript{2+} coordination [61]. Ca\textsuperscript{2+} coordination in loop III involves the carbonyl group of Y99 in loop III, causing appreciable deshielding of the adjacent $^{15}$N nucleus of I100 due to polarization of the amide group, with a resulting increase of the chemical shift by 4–8 ppm [61]. The remaining chemical shift change of $\sim$7 ppm of I100 is attributed to a change in the side chain $\chi_1$ torsion angle from gauche to trans, which is likely to be associated with the open-closed transition [61]. In the present study, the estimated value for I100 is $\Delta \delta(E140Q) = 7.0 \text{ ppm}$. This finding is consistent with persistent coordination of Ca\textsuperscript{2+} in loop III by the backbone oxygen of Y99 and further suggests that the exchange between closed and open conformations is associated with conformational changes of the I100 side chain. In the case of V136, the Ca\textsuperscript{2+}-induced chemical shift change in the wild-type protein is caused only by polarization effects due to the Ca\textsuperscript{2+} coordination by the backbone oxygen of Q135 in loop IV [61]. Previous characterization of Ca\textsuperscript{2+} binding to E140Q has established that the (Ca\textsuperscript{2+}) state of E140Q is populated to 98\% under the present experimental conditions. Given the chemical shifts and Ca\textsuperscript{2+} exchange rates estimated, any relaxation contributions due to Ca\textsuperscript{2+} exchange between fully saturated and half-saturated states are at least one order of magnitude
smaller than those observed here and can thus be safely neglected [43, 45]. The present estimate of $\Delta \delta_{E140Q} = 4.5$ ppm for V136 therefore suggests that the backbone oxygen of Q135 coordinates Ca$^{2+}$ only in the open conformation. This is in agreement with the structural difference of the Ca$^{2+}$-binding site between the closed and open states of the wild-type protein [28–30], which reveals a hinge-like motion around residue 6 of the loop [29, 42]. The hinge positions the carbonyl oxygen of residue 7 (Q135) close to the Ca$^{2+}$ site in the open conformation but significantly further away from the site in the closed conformation; the distance is 2.2 Å in the crystal structure of the Ca$^{2+}$-loaded state (Protein Data Bank [PDB] entry 1CCL [28]) and 8.5 ± 0.2 Å in the NMR structure ensemble of the apo state (PDB entry 1CFC [29]), as measured by superimposing the first five loop residues of the apo and Ca$^{2+}$-loaded structures. The proposed hypothesis that Ca$^{2+}$ dissociation from loop IV may be gated by the conformational dynamics is supported by the present interpretation of the V136 shift differences in terms of a reduced number of Ca$^{2+}$ ligands contributed by the protein in the closed conformation. For comparison, in loop III the distances are 2.2 Å and 2.4 ± 1.9 Å, respectively, suggesting that Ca$^{2+}$ coordination by the carbonyl oxygen in position 7 is maintained in the closed conformation. This difference between the two loops is mirrored by the N-terminal domain, where the corresponding distances are 2.3 Å and 1.9 ± 0.2 Å for loop I and 2.3 Å and 3.2 ± 0.4 Å for loop II. Interestingly, loop II of Mg$^{2+}$-saturated wt-Tr1C also exchanges between two conformations, only one of which appears to coordinate the ion with the backbone oxygen in position 7 [42]. Also in this case, the exchange rate is similar to the rate of ion release [42]. The similarities between the exchange processes in (Ca$^{2+}$)$_2$-E140Q and (Mg$^{2+}$)$_2$-wt-Tr1C suggest that the phenomenon is not specific to the mutant but rather is general for the second loop of each domain. However, the exchange in (Mg$^{2+}$)$_2$-wt-Tr1C appears to be restricted to the loop region and does not involve the hydrophobic core. In this context, it is instructive to consider the differences in ion coordination by the residue in position 12: in (Mg$^{2+}$)$_2$-wt-Tr1C, the E in position 12 coordinates the Ca$^{2+}$ with a single oxygen and approximately equal populations of open and closed conformations are observed; in (Ca$^{2+}$)$_2$-wt-Tr1C, the E in position 12 coordinates Ca$^{2+}$ with two oxygens, and the conformation is predominantly open. The emerging picture shows that interactions between the ion and the oxygen(s) in position 12 are required for stabilizing the open conformation [42, 45, 46, 62] and that loss of these interactions promotes fluctuations in the second loop of the EF-hand pair, such that the carbonyl oxygen in position 7 moves away from the ion, possibly triggering ion release. The comparative analysis of exchange in (Mg$^{2+}$)$_2$-wt-Tr1C and (Ca$^{2+}$)$_2$-E140Q suggests that these fluctuations occur also in the wild-type protein. However, in the Ca$^{2+}$-saturated state the population of the open conformation totally dominates, which makes the exchange contributions to the spin relaxation rates undetectably small.

Residues with Similar Exchange Time Constants Cluster in the Structure

In Figure 4, the residues are color coded by $\tau_{ex}$ groups in the apo and Ca$^{2+}$-loaded structures of wt-Tr1C. Certain
patterns can be identified from the spatial distribution of residues belonging to the different groups. Residues belonging to the red group are primarily located on the surface of the protein (Figure 4). In addition, the red group includes the central residues of the β sheet, I100 and V136, as well as a cluster of residues, I85, M109, L116, and M145, located at the “bottom” (in the view of Figure 4) of the hydrophobic core in the apo structure (Figure 4a). The latter cluster is not present in the Ca$^{2+}$-loaded structure, although the interactions are maintained between residues I85 and M145 and between M109 and L116 (Figure 4b). Similar observations are made for the green group: F89 packs against V108, forming a hydrophobic cluster together with L112 in the apo state (Figure 4a) but not in the Ca$^{2+}$-loaded state (Figure 4b). The green group includes several residues located in helix F. The different sides of helix F and to that (Figure 4a). The latter cluster is not present in the Ca$^{2+}$apo state (Figure 4a) but not in the Ca$^{2+}$-saturated wild-type CaM. These observations suggest that the conformational exchange involves locally collective processes, which depend on the structural topology. Transition rates were estimated for a subset of residues, yielding $k_e = (2.7 \pm 1.0) \times 10^{-4}$ s$^{-1}$. Comparisons with previous results indicate that similar processes occur also in the wild-type protein. The measured rates match the estimated Ca$^{2+}$ off-rate, suggesting that Ca$^{2+}$ release may be gated by the conformational dynamics. Structural interpretation of estimated chemical shifts suggests a mechanism for ion release.

Experimental Procedures

Theory

The off-resonance rotating-frame relaxation rate constant $R_o$ is given by [19, 63, 64]

$$R_o = R_t \cos^2 \theta + R_s \sin^2 \theta + R_{ns} \sin^2 \phi$$

where $R_t$ and $R_s$ are the longitudinal and exchange-free transverse autorelaxation rate constants, respectively; $R_{ns}$ is the conformational exchange contribution to transverse relaxation; $\theta$ is the tilt angle between the reduced static magnetic field and the effective field $\omega_0 = (\omega_0 + \omega_0)^2$ in the rotating frame; $\omega_0$ is the spin-lock frequency; $\omega_0$ is the population-averaged Larmor frequency; and $\omega_0$ is the precession frequency around the spin-lock field. The conformational exchange contribution is dependent on $\omega_0$ and $\omega_0$, such that an excess dispersion in the relaxation rate reveals the existence of conformational exchange (c.f. Figures 1b and 1c).

For a two-state exchange process between closed (c) and open (o) conformations [63, 64], the exchange contribution in the fast-exchange limit ($\tau_ex < \tau_{ns}$) is given by

$$R_{ex} = \phi \tau_{ex} (1 + \tau_{ex} \omega_0)$$

in which

$$\tau_ex = 1/k_{ex} = p_c/k_{ex} = p_o/k_{ex} = 1/(k_{ex} + k_c)$$

and

$$\phi = p_o \delta_{i o} \omega_0^2$$

where $\tau_{ex}$ is the time constant for the exchange process; $k_{ex}$ is the exchange rate constant; $k_c$ and $k_o$ are rate constants for the opening and closing reactions, respectively; $p_0$ is the population of spins in state i; $\delta_{i o} = \gamma_i B_i \Delta H$; $\gamma_i$ is the gyromagnetic ratio of $^1$H; $B_i$ is the strength of the static magnetic field; and $\Delta H$ is the chemical shift difference (in ppm) between the two states.

The exchange-free transverse autorelaxation rate constant can be obtained as [65]

Biological Implications

The conformational fluctuations and dynamics of a protein are intimately coupled to its function, as evidenced by a large number of atomic resolution structures of proteins revealing significant structural rearrangements upon binding of low molecular weight ligands or macromolecular receptors. While there is a rich database available on the amplitudes of structural change obtained from the static pictures of the end states, very little is known about the dynamics of these transitions. The ultimate goal of understanding protein function from a molecular perspective requires that the dynamic processes and energy landscape of structural changes be investigated.

CaM is a ubiquitous Ca$^{2+}$-activated regulator of numerous cellular processes in eukaryotic organisms. Ca$^{2+}$ binding to CaM triggers a structural transition in each of its two domains from a closed (inactive) to an open (active) conformation that is central to target recognition. Here, we have used NMR relaxation experiments to characterize the dynamics of this transition in a model system—the E140Q mutant of the C-terminal domain of CaM.

The conformational transition occurs with a mean time constant of $\tau_{ex} = 21 \pm 3$ µs. The results suggest that $\tau_{ex}$ varies significantly between different groups of residues and that residues with similar values exhibit spatial proximity in the structures of apo and/or Ca$^{2+}$-saturated wild-type CaM. These observations suggest that the conformational exchange involves locally collective processes, which depend on the structural topology. Transition rates were estimated for a subset of residues, yielding $k_e = (2.7 \pm 1.0) \times 10^{-4}$ s$^{-1}$. Comparisons with previous results indicate that similar processes occur also in the wild-type protein. The measured rates match the estimated Ca$^{2+}$ off-rate, suggesting that Ca$^{2+}$ release may be gated by the conformational dynamics. Structural interpretation of estimated chemical shifts suggests a mechanism for ion release.
$$R_\parallel = \gamma_{H}/\gamma_{N} (R_0 - 1.249\tau_{\text{mho}}) + 1.079\tau_{h}$$

where $\gamma_H$ and $\gamma_N$ are the gyromagnetic ratios of $^1H$ and $^{15}N$, respectively. Equation (5) is based on the fact that $\tau_{h}$ and $\tau_{mho}$ depend on the same interaction strengths and that $\tau_{mho}$ does not contain contributions from exchange [65, 67]. Furthermore, it is assumed that $\tau_{h}$ is negligibly affected by $^1H$–$^1H$ cross-relaxation. Equation (5) is derived using reduced spectral density mapping [68–70], based on the assumption that spectral densities at frequencies in the interval $(\omega_{r} + \omega_{c} - \omega_{r} - \omega_{c})$ may be written as $\mathcal{J}((\omega_{r} - \omega_{c})) = (0.87/\tau_{l})(0.87/\tau_{mho})$ [65, 68].

The transverse auto-relaxation measured in a CPMG experiment [71, 72] can be represented by

$$R_\perp = R_0 + R_{\text{CPMG}}$$

(7)

The exchange contributions $R_{\text{ex,CPMG}}$ can be interpreted in terms of the microscopic exchange time constants using the following approximate expression for two-site exchange [73]

$$R_{\text{ex,CPMG}} = \phi_{\text{ex}} [1 - 2\tau_{\text{spin}}/\tau_{\text{rel}} \tanh(\tau_{\text{rel}}/2\tau_{\text{spin}})]$$

(8)

in which $\tau_{\text{ex}}$ is the delay between the $^{15}N$ 180° pulses in the CPMG sequence. Numerical calculations indicate that equation (8) is accurate to within 6% for parameter values expected here: $\tau_{\text{ex}} < 100 \mu s$, $\tau_{\text{rel}} = 1.2 \text{ ms}$, $0.5 < \phi_{\text{ex}} < 0.9$, and $\delta h = 10 \text{ ppm}$. NMR Spectroscopy

Expression and purification of $^{15}$N-labeled E140Q were carried out as reported previously [45]. The NMR sample contained 600 $\mu$l of 2.3 mM protein, 0.2 mM NaN$_3$, 0.1 mM DSS, and 41 mM CaCl$_2$, dissolved in 90% H$_2$O/10% D$_2$O at pH 6.0. At this Ca$^{2+}$ level, the (Ca$^{2+}$), state of E140Q is populated to 98% and the (Ca$^{2+}$), state to 2%, as calculated from the binding constants determined previously [45]. $^1H$ and $^{15}N$ assignments of (Ca$^{2+}$), E140Q at 301 K have been reported previously [45]. All NMR experiments were run at 301 K on a 600 MHz Varian Inova spectrometer operating at a $^1H$ Larmor frequency of 599.89 MHz and using a Varian single-axis pulsed field gradient inverse broadband probe. The pulse sequence used to record the $^{15}N$ $R_1$ spectra is shown in Figure 5. At point b, where longitudinal $^{15}N$ magnetization is established, the pulsed field gradient (g1) is applied to dephase any remaining transverse magnetization, and the $^{15}N$ carrier frequency is switched to a value determined by the desired effective field. The magnetization is aligned along the effective field using a 5 ms hyperbolic secant-shaped amplitude-modulated adiabatic pulse [74] applied along the z axis. Calculations indicate that the adiabatic pulse achieves an alignment better than 99.9% for the largest tilt angle, where the performance of the pulse is poorest. During the relaxation delay (T) the magnetization is spin locked in the tilted frame using continuous-wave irradiation. In order to eliminate effects of cross-correlation between $^1H$–$^{15}N$ dipolar and $^{15}N$ CSA relaxation mechanisms in the $R_1$ experiments, $^{1H}$ 180° pulses are applied during the relaxation delay [75–77]. After the relaxation delay, the remaining magnetization is returned to the z axis using a reverse adiabatic pulse, and the $^{15}N$ carrier frequency is switched back to the value corresponding to the midpoint of the $^{15}N$ spectrum. In other respects, this $^{15}N$ off-resonance $R_1$ experiment is virtually identical to the $^{15}N$ $R_1$ experiment described previously [78]. The recycle delay between transients was 2.3 s, including the acquisition period. $^{15}N$ decoupling during acquisition was achieved using the GARP-1 decoupling sequence [79].

The spin lock may cause substantial heating of the sample. Differential sample heating during the $R_1$ series was avoided by applying continuous-wave irradiation far off-resonance ($\Delta = \pm 300$ kHz) during the recycle delay for a period $T = T_{\text{mho}} - T$, with $T_{\text{mho}}$ held constant at 500 ms for all tilt angles. The sample temperature was calibrated by measuring the frequency difference between the temperature-independent resonance signal of DSS [80] and the carrier frequency, which follows the temperature-dependent HDO resonance used for the field/frequency lock [81]. The set point temperature of the VT unit was adjusted so that the sample temperature was stable at the target value of 301 K after a preparation period of “dummy scans.”

$R_\parallel$ values were measured at eight different effective fields, achieved by varying the radio frequency of the spin lock (w) while keeping the B field strength constant at a value corresponding to $\omega_r = 2164 \pm 43$ Hz. The B field strength was measured by monitoring residual scalar couplings as a function of off-set during continuous-wave irradiation. Measurements were performed with the following nominal tilt angles (corresponding to the midpoint of the spectrum), with the range between the smallest and largest actual tilt angles (corresponding to residues G132 and A147, respectively) given within parentheses: 19° (17–20°), 22° (20–25°), 27° (24–30°), 34° (30–39°), 39° (34–46°), 46° (39–54°), 54° (45–64°), and 64° (53–76°). At each tilt angle, NMR spectra were obtained for eight different relaxation delays, and duplicate spectra were recorded for two of these. The longest relaxation delay used for each tilt angle was chosen such that the magnetization of each spin had decayed to less than 30% of its initial value. All spectra were recorded with spectral widths of 8000 Hz over 2048 complex points in $\omega_0$ ($^1H$) and 1300 Hz over 128 complex points in $\omega_0$ ($^{15}N$). The $^1H$ carrier frequency was set on the H$_2$O signal.

NMR Data Processing and Analysis

Processing and analysis of the NMR spectra were performed using Felix97 (MSI). All spectra were processed using two protocols to optimize either signal-to-noise or resolution of the crosspeaks. The former protocol involved exponential and cosine bell apodization functions in $\omega_0$ and $\omega_1$, respectively, while the latter involved Lorentzian-to-Gaussian transformation in $\omega_0$ and extension of the interferogram by linear prediction followed by a cosine bell in $\omega_1$. The final size of the matrices was 1024 × 1024 real points after zero filling in $\omega_0$ and Fourier transformation. Peak intensities were measured as peak heights. Uncertainties of the intensities were estimated from duplicate spectra and from standard deviations of the base-plane noise [92]. $R_1$ values were obtained at each tilt angle by nonlinear optimization of single-exponential decays to the experimental data [83]. The rate constants at 301 K for the $^{15}N$ longitudinal and transverse auto-relaxation ($R_1$ and $R_2$), respectively, the longitudinal and transverse cross-relaxation ($R_0$ and $R_\perp$), respectively due to interference between $^1H$–$^{15}N$ dipolar and $^{15}N$ CSA relaxation mechanisms, and the steady-state $^{1H}$–$^{15}N$ NOE values have been reported previously [43].

### Determination of Dynamic Parameters

The four parameters $R_\text{tot}$, $R_\text{off}$, $R_\text{on}$, and $\phi_\text{on}$ were optimized simultaneously for each $^{15}N$ spin against the experimental values of $R_1$, $R_2$, and the eight values of $R_\perp$, as a function of $\phi_\text{on}$, using the following relations [c.f. equations (1), (2), (7), and (8)]:

$$R_\text{tot} = R_\text{off}$$

(9)

$$R_\parallel = R_\text{off}$$

(10)

$$R_\text{tot} = R_\text{tot} + \phi_{\text{on}} [1 - 2\tau_{\text{spin}}/\tau_{\text{rel}} \tanh(\tau_{\text{rel}}/2\tau_{\text{spin}})]$$

(11)

$$R_\text{off} = R_\text{off} \cos^2 \phi + R_\text{on} \sin^2 \phi + [\phi_{\text{on}}/(1 + \tau_{\text{mho}}/2\tau_{\text{spin}})] \sin \phi$$

(12)

Nonlinear least-squares optimization using the Levenberg–Marquardt algorithm and Monte Carlo error analyses were performed following standard procedures [83]. In order to assess the statistical significance of any obtained relaxation dispersion, an additional fit was performed that excluded the conformational exchange parameters $\phi$ and $\tau_{\text{spin}}$ (i.e., only two optimized parameters, $R_\text{off}$ and $R_\text{on}$, were used in this fit). F-statistical testing was used to select between the two- and four-parameter models [48].

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