# Rotational dynamics of calcium-free calmodulin studied by ${ }^{15} \mathrm{~N}$-NMR relaxation measurements 

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#### Abstract

The backbone motions of calcium-free Xenopus calmodulin have been characterized by measurements of the ${ }^{15} \mathrm{~N}$ longitudinal relaxation times $\left(T_{1}\right)$ at 51 and 61 MHz , and by conducting transverse relaxation $\left(T_{2}\right)$, spin-locked transverse relaxation $\left(T_{1_{\rho}}\right)$, and ${ }^{15} \mathrm{~N}-\left\{{ }^{1} \mathrm{H}\right\}$ heteronuclear NOE measurements at 61 MHz ${ }^{15} \mathrm{~N}$ frequency. Although backbone amide hydrogen exchange experiments indicate that the N -terminal domain is more stable than calmodulin's C-terminal half, slowly exchanging backbone amide protons are found in all eight $\alpha$-helices and in three of the four short $\beta$-strands. This confirms that the calcium-free form consists of stable secondary structure and does not adopt a 'molten globule' type of structure. However, the C-terminal domain of calmodulin is subject to conformational exchange on a time scale of about $350 \mu \mathrm{~s}$, which affects many of the C -terminal domain residues. This results in significant shortening of the ${ }^{15} \mathrm{~N} T_{2}$ values relative to $T_{1 \rho}$, whereas the $T_{1 \rho}$ and $T_{2}$ values are of similar magnitude in the N terminal half of the protein. A model in which the motion of the protein is assumed to be isotropic suggests a rotational correlation time for the protein of about 8 ns but quantitatively does not agree with the magnetic field dependence of the $T_{1}$ values and does not explain the different $T_{2}$ values found for different $\alpha$-helices in the N -terminal domain. These latter parameters are compatible with a flexible dumbbell model in which each of calmodulin's two domains freely diffuse in a cone with a semi-angle of about $30^{\circ}$ and a time constant of about 3 ns , whereas the overall rotation of the protein occurs on a much slower time scale of about 12 ns . The difference in the transverse relaxation rates observed between the amides in helices C and D suggests that the change in interhelical angle upon calcium binding is less than predicted by Herzberg et al. Strynadka and James [Strynadka, N. C. J. \& James, M. N. G. (1988) Proteins Struct. Funct. Genet. 3, 1-17].


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Calmodulin (CaM) is a ubiquitous $16.7-\mathrm{kDa}$ intracellular protein of 148 residues that plays a key role in coupling $\mathrm{Ca}^{2+}$ transients, caused by a stimulus at the cell surface, to events in the cytosol (Cohen and Klee, 1988). It performs this role by binding to a host of intracellular enzymes in a calcium-dependent manner. In the crystalline state, $\mathrm{Ca}^{2+}$-ligated calmodulin resembles a dumbbell, in which the small globular amino- and carboxy-terminal domains are linked by a 26 -residue $\alpha$-helix, frequently referred to as the 'central helix' (Babu et al., 1985, 1988; Taylor et al., 1991). However, ${ }^{15} \mathrm{~N}$-NMR relaxation experiments (Barbato et al., 1992) and small-angle X-ray scattering studies (Heidorn and Trewhella, 1988) both indicated that the helical linker, found in the crystalline state, is highly flexible in solution. Moreover, the NMR study unequivocally identified residues $\mathrm{K} 77-\mathrm{S} 81$, located near the middle of this 'central helix' as non-helical and highly flexible. The functional significance of the flexibility of the linker was shown in subsequent NMR and X-ray studies of $\mathrm{Ca}^{2+}-\mathrm{CaM}$ structures complexed with

[^0]peptide fragments of the binding sites of CaM-target enzymes: each of these complexes adopts a globular shape, in which the target peptide is clamped between the N - and C-terminal halves of CaM (Ikura et al., 1992; Meador et al., 1992, 1993).

No structure has been reported yet for $\mathrm{Ca}^{2+}$-free CaM , although a model for its structure has been proposed (Strynadka and James, 1988) by analogy with a crystal structure of the homologous protein troponin C , in which the C -terminal domain is $\mathrm{Ca}^{2+}$-ligated, whereas the N -terminal domain is not (Sundaralingam et al., 1985; Herzberg and James, 1988). Attempts to crystallize the $\mathrm{Ca}^{2+}$-free form of CaM have been unsuccessful, but a recent NMR study of the C-terminal domain suggests a conformational change upon $\mathrm{Ca}^{2+}$ ligation which qualitatively confirms the Strynadka and James model (Finn et al., 1994). The spectral dispersion of the ${ }^{1} \mathrm{H}-{ }^{15} \mathrm{~N}$ two-dimensional NMR spectrum is considerably worse compared to the $\mathrm{Ca}^{2+}$-ligated form and exhibits severe line broadening for many of the backbone amide resonances. These observations have led to speculations that the $\mathrm{Ca}^{2+}$-free state might not adopt a stable and welldefined structure, but instead might exist as a 'molten globule'. The present study provides evidence that $\mathrm{Ca}^{2+}$-free CaM adopts a well-defined structure with a large fraction of its backbone amide protons significantly protected from exchange with the solvent. The backbone dynamics are also similar in nature to those observed previously for $\mathrm{Ca}^{2+}$-ligated CaM (Barbato et al.,
1992), indicating that $\mathrm{Ca}^{2+}$-free CaM also consists of two small globular domains, connected by a flexible linker.
${ }^{15} \mathrm{~N}$ relaxation measurements of isotopically enriched proteins, using sensitive ${ }^{1} \mathrm{H}$-detected two-dimensional experiments, have become an accepted procedure for characterizing overall protein motion, and for determining the degree of local backbone flexibility (Kay et al., 1989; Clore et al., 1990b; Szyperski et al., 1993; Schneider et al., 1992; Redfield et al., 1992; Akke et al., 1993; Torchia et al., 1993; Wagner et al., 1993; Palmer, 1993; van Mierlo et al., 1993; Peng and Wagner, 1992, 1994 ; Orekhov, 1994; Alexandrescu and Shortle, 1994; Fushman et al., 1994). Most commonly, a set of three different relaxation measurements, corresponding to the ${ }^{15} \mathrm{~N}$ longitudinal relaxation time, $T_{1}$, its transverse relaxation time, $T_{2}$, and the heteronuclear ${ }^{15} \mathrm{~N}-\left\{{ }^{1} \mathrm{H}\right\}$ NOE are interpreted in a model-free approach to yield an overall rotational correlation time, $\tau_{c}$, and an order parameter, $S^{2}$, which is a measure for the local rigidity (Lipari and Szabo, 1982a,b).

In most previous ${ }^{15} \mathrm{~N}$ relaxation studies of protein dynamics the protein has been assumed to tumble isotropically. However, Barbato et al. (1992) demonstrated that this approximation is not entirely valid for $\mathrm{Ca}^{2+}$-ligated CaM , although the observed degree of anisotropy was far smaller than the anisotropy predicted for a rigid dumbbell model. In the present study, the relaxation data for $\mathrm{Ca}^{2+}$-free CaM are interpreted using several different models and indicate that $\mathrm{Ca}^{2+}$-free CaM behaves qualitatively similar to the $\mathrm{Ca}^{2+}$-ligated form of the protein, i.e. its rotational dynamics show evidence for a limited degree of anisotropy.

## MATERIALS AND METHODS

Sample preparation. Recombinant Xenopus calmodulin was overexpressed in Escherichia coli (strain AR58) containing the expression vector pTNco12. Uniform ${ }^{15} \mathrm{~N}$ labeling at a level of more than $95 \%$ was obtained by growing the bacteria in M9 minimal media with ${ }^{15} \mathrm{NH}_{4} \mathrm{Cl}$ (Isotec Inc.) as the sole nitrogen source. Protein was purified as described previously (Ikura et al., 1990). The final concentration used for this study was 1.8 mM $\left[{ }^{15} \mathrm{~N}\right] \mathrm{CaM}$ in $100 \mathrm{mM} \mathrm{KCl}, 1.5 \mathrm{mM}$ EDTA, $100 \mu \mathrm{M}$ sodium azide, $5 \% \mathrm{D}_{2} \mathrm{O}, 95 \% \mathrm{H}_{2} \mathrm{O}, \mathrm{pH} 6.3$ in $250 \mu$ l, using a Shigemi (Shigemi Inc. Allison Park PA, USA) microcell. Addition of 1.5 mM EDTA was found to be sufficient for ligating all divalent cations; addition of more than 1.5 mM did not generate any further changes in the ${ }^{15} \mathrm{~N}-{ }^{1} \mathrm{H}$ two-dimensional shift correlation NMR spectrum.

NMR spectroscopy. All experiments were performed at $23^{\circ} \mathrm{C}$, using Bruker AMX-500 and AMX-600 spectrometers, each equipped with a triple-resonance $5-\mathrm{mm}$ probehead containing a self-shielded $z$-gradient coil. The pulse sequences used for measurement of the ${ }^{15} \mathrm{~N} T_{1}$ and $T_{2}$ values have been adapted from those reported by Barbato et al. (1992) by including pulsed field gradients to remove unwanted coherences (Bax and Pochapsky, 1992), and by the addition of the Watergate scheme (Piotto et al., 1992) for suppressing the $\mathrm{H}_{2} \mathrm{O}$ signal. Also, a semiconstant time evolution period (Grzesiek and Bax, 1993b) was used to optimize the acquired signal in the $t_{1}$ dimension. All experiments used States-TPPI $t_{1}$ quadrature detection in the $t_{1}$ dimension (Marion et al., 1989a).

In order to obtain independent information regarding the reproducibility of the relaxation time measurements (vide infra), ${ }^{15} \mathrm{~N} T_{1}$ measurements at 600 MHz were performed twice. A $T_{1}$ measurement was also conducted at $500-\mathrm{MHz}{ }^{1} \mathrm{H}$ frequency. Measurement of the ${ }^{15} \mathrm{~N} T_{2}$ values was conducted at 60.8 MHz ( $600 \mathrm{MHz}{ }^{1} \mathrm{H}$ frequency), both by using a CPMG-type sequence (Kay et al., 1992; Palmer et al., 1992) and by using continuous
${ }^{15} \mathrm{~N}$ spin-locking with a $2.5-\mathrm{kHz}$ radiofrequency field (Peng et al., 1991), i.e. effectively measuring $T_{1 p}$. For the CPMG-type $T_{2}$ measurement, the radiofrequency field strength of the $180^{\circ}$ pulses was 5.6 kHz , and $180^{\circ}$ pulses were spaced 1 ms apart.

Each $T_{1}, T_{2}$, and $T_{1 \rho}$ measurement required approximately 32 h measuring time. The ${ }^{15} \mathrm{~N} T_{1}$ data were acquired using ${ }^{15} \mathrm{~N}$ relaxation delays of $8,96,200,336,488,688,968$, and 1296 ms . Similarly, ${ }^{15} \mathrm{~N} T_{2}$ and $T_{1 \rho}$ measurements were performed using relaxation delays of $8,16,32,48,64,80,104$, and 136 ms . Each data matrix consisted of $150^{*} \times 768^{*}$ data points ( $n^{*}$ denotes $n$ complex data points), and acquisition times of 91 ms in $t_{1}$ and 83 ms in $t_{2}$ were used for each $T_{1}$ and $T_{2}$ experiment.

For measurement of the ${ }^{15} \mathrm{~N}-\left\{{ }^{1} \mathrm{H}\right\}$ NOE, a pulse sequence which returns the $\mathrm{H}_{2} \mathrm{O}$ magnetization to the $+z$ axis at the end of each scan was used, thereby minimizing the effect of the slowly relaxing water magnetization on the NOE measured for amides with rapidly exchanging protons (Grzesiek and Bax, 1993a). The ${ }^{15} \mathrm{~N}-\left\{{ }^{1} \mathrm{H}\right\}$ NOE values results from the difference between two two-dimensional spectra, acquired in an interleaved manner, each consisting of a $180^{*} \times 768^{*}$ data matrix with acquisition times of 109 ms and 83 ms in the $t_{1}$ and $t_{2}$ dimensions, respectively. For all experiments, the ${ }^{1} \mathrm{H}$ carrier was positioned on the $\mathrm{H}_{2} \mathrm{O}$ frequency and the ${ }^{15} \mathrm{~N}$ carrier at 116.5 ppm . The spectral widths used were 15.4 ppm for ${ }^{1} \mathrm{H}$ and 27.0 ppm for ${ }^{15} \mathrm{~N}$. A repetition delay of 3 s was used for the NOE measurements. Incomplete magnetization recovery during the relaxation delay in the NOE experiment to first order can be compensated by a correction factor (Grzesiek and Bax, 1993a):

$$
\begin{equation*}
N O E=(1-a) N O E_{\mathrm{m}} /\left(1-a \times N O E_{\mathrm{m}}\right), \tag{1}
\end{equation*}
$$

where $a=\exp \left(-T / T_{1}\right), N O E_{\mathrm{m}}$ is the measured ratio of intensities with and without ${ }^{1} \mathrm{H}$ irradiation, $T$ is the recovery delay between scans, and $T_{1}$ is the non-selective ${ }^{1} \mathrm{H}$ longitudinal relaxation time of the protein ( $\approx 1.4 \mathrm{~s}$ ). This correction factor, which lowers the NOE by a small percentage relative to the measured intensity ratio, was used in determining NOE values throughout this study.

All data sets were processed using $60^{\circ}$-shifted squared-sinebell filtering in both dimensions, and zero filling to $512 \times 2048$ data points. Data were processed using the program nmrPipe (F. Delaglio, unpublished) and spectra were analyzed using the software package PIPP (Garrett et al., 1991). Resonance intensities were used rather than integrated peak volumes in calculating the relaxation rates and NOE values. Note that the peak shapes for a given amide in the interleaved two-dimensional spectra are identical and the use of volume integration or peak heights in principle yields identical results except that the fractional error in volume integrals tends to be larger than for peak heights. Moreover, many of the ${ }^{1} \mathrm{H}-{ }^{15} \mathrm{~N}$ correlations are insufficiently resolved to permit accurate peak integration. Errors in the derived $T_{1}$ values were estimated both by Monte-Carlo type procedures (Kamath and Shriver, 1989), which involves randomly adding or subtracting a number corresponding to the root-mean-square ( rms ) spectral noise from the measured intensities, and by comparing the reproducibility of $T_{1}$ values measured at 600 MHz on two separate occasions, several weeks apart. Errors in the derived $T_{2}$ values were estimated also by the Monte-Carlo procedure, and by comparing the $T_{2}$ and $T_{1 \mathrm{p}}$ values for the N terminal domain of CaM . The two measurements indicated the absence of any systematic difference. The error in the NOE is estimated from assuming that the uncertainty in the peak heights in the two interleaved two-dimensional spectra, recorded with and without ${ }^{1} \mathrm{H}$ saturation, equals the rms noise in each of these two spectra.

Amide exchange measurements. Slowly exchanging backbone amide hydrogens were identified by dissolving a freshly
lyophilized NMR sample into $99.9 \% \mathrm{D}_{2} \mathrm{O}$ at $4^{\circ} \mathrm{C}$ and transferring this solution into a regular $5-\mathrm{mm}$ NMR sample tube. A twoscan two-dimensional heteronuclear single quantum correlation (HSQC) experiment was started 2 min after inserting the sample into the NMR probe, thermostatted at $23^{\circ} \mathrm{C}$. The time between adding $\mathrm{D}_{2} \mathrm{O}$ to the lyophilized sample and inserting the sample into the NMR magnet was 5 min . The total duration of the HSQC measurement was also 5 min . The HSQC experiment was repeated with 8 scans $/ t_{1}$ increment immediately after the first experiment was finished, i.e. 7 min after the sample temperature was raised to $23^{\circ} \mathrm{C}$, and again at 63,100 and 246 min after the sample was inserted into the magnet. After completion of the NMR experiment, its pH was measured to be 6.36 , uncorrected for the deuterium isotope effect.

## MODELS FOR DATA ANALYSIS

Measurement of NMR relaxation parameters such as ${ }^{15} \mathrm{~N} T_{1}$, $T_{2}$, and ${ }^{15} \mathrm{~N}-\left\{{ }^{1} \mathrm{H}\right\}$ NOE are related in a direct and simple manner to spectral densities, $J(\omega)$ :

$$
\begin{gather*}
1 / T_{1}=d^{2}\left[J\left(\omega_{\mathrm{H}}-\omega_{\mathrm{N}}\right)+3 J\left(\omega_{\mathrm{N}}\right)\right.  \tag{2a}\\
\left.+6 J\left(\omega_{\mathrm{H}}+\omega_{\mathrm{N}}\right)\right]+c^{2} J\left(\omega_{\mathrm{N}}\right) \\
1 / T_{2}=0.5 d^{2}\left[4 J(0)+J\left(\omega_{\mathrm{H}}-\omega_{\mathrm{N}}\right)+3 J\left(\omega_{\mathrm{N}}\right)\right.  \tag{2b}\\
 \tag{2c}\\
\left.+6 J\left(\omega_{\mathrm{H}}\right)+6 J\left(\omega_{\mathrm{H}}+\omega_{\mathrm{N}}\right)\right]+1 / 6 c^{2}\left[3 J\left(\omega_{\mathrm{N}}\right)+4 J(0)\right]
\end{gather*}
$$

$N O E=1+\left(\gamma_{\mathrm{H}} / \gamma_{\mathrm{N}}\right) d^{2}\left[6 J\left(\omega_{\mathrm{H}}+\omega_{\mathrm{N}}\right)-J\left(\omega_{\mathrm{H}}-\omega_{\mathrm{N}}\right)\right] T_{1}$
where $d^{2}=0.1\left[\left(\gamma_{\mathrm{H}} \gamma_{\mathrm{N}} h\right) /\left(2 \pi\left\langle r_{\mathrm{HN}}^{3}\right\rangle\right)\right]^{2}$ and $c^{2}=2 / 15\left[\omega_{\mathrm{N}}^{2}\left(\sigma_{\|}-\right.\right.$ $\left.\left.\sigma_{\perp}\right)^{2}\right], J(\omega)$ is the spectral density function, $\gamma_{i}$ is the gyromagnetic ratio of spin $i, h$ is Planck's constant $r_{\mathrm{HN}}$ is the internuclear ${ }^{1} \mathrm{H}-{ }^{15} \mathrm{~N}$ distance ( 102 pm ), and $\sigma_{\|}-\sigma_{\perp}$ is the difference between the parallel and perpendicular components of the axially symmetric ${ }^{15} \mathrm{~N}$ chemical shift anisotropy tensor, estimated at 160 ppm (Hiyama et al., 1988). Although these spectral densities are sometimes considered as the final result of a protein ${ }^{15} \mathrm{~N}$ relaxation study, the intrinsic value of ${ }^{15} \mathrm{~N}$ relaxation studies is to evaluate to what degree plausible physical models are either supported or contradicted by the relaxation data. Three possible models for motion of the protein are considered below: (I) isotropic tumbling of the entire protein with fast internal dynamics of limited amplitude for the individual amide $\mathrm{N}-\mathrm{H}$ bond vectors, (II) axially symmetric anisotropic tumbling of the entire protein with fast internal dynamics, and (III) isotropic tumbling of the protein, with internal motions of the entire amino- and carboxyterminal domains on an intermediate time scale, in addition to fast internal dynamics. The relation between spectral density and these models is briefly summarized below.

Isotropic model. For a protein tumbling isotropically with rotational correlation time, $\tau_{c}$, and with fast internal dynamics on a time scale, $\tau_{\mathrm{f}}$, the spectral density function has the form (Lipari and Szabo, 1982a):

$$
\begin{equation*}
J(\omega)=S^{2} \tau_{c} /\left[1+\left(\omega \tau_{c}\right)^{2}\right]+\left(1-S^{2}\right)\left\{\tau^{\prime} /\left[1+\left(\omega \tau^{\prime}\right)^{2}\right]\right\} \tag{3a}
\end{equation*}
$$

with

$$
\begin{equation*}
1 / \tau^{\prime}=1 / \tau_{\mathrm{c}}+1 / \tau_{\mathrm{f}} . \tag{3b}
\end{equation*}
$$

$S^{2}$ is the generalized order parameter which describes the amplitude of the fast internal motion and assumes a value of 1 when the amplitude of the internal motion approaches zero. It is derived from the NMR data in a model-free manner but, for any given model, $S^{2}$ can be readily calculated. For example, in the commonly used model where the $\mathrm{N}-\mathrm{H}$ bond vector freely diffuses in a cone of semi-angle $\alpha, S^{2}$ is given by:

$$
\begin{equation*}
S^{2}=[\cos \alpha(1+\cos \alpha) / 2]^{2} \tag{4}
\end{equation*}
$$

Axially symmetric model. In the Lipari-Szabo formalism
(Lipari and Szabo, 1982a,b), the spectral density function for a molecule with an axially symmetric rotational diffusion tensor (Woessner, 1962a; Huntress, 1968; Hubbard, 1970) is extended to account for fast internal motions:

$$
\begin{align*}
J(\omega) & =S^{2}\left\{A_{1} \omega \tau_{1} /\left[1+\left(\omega \tau_{1}\right)^{2}\right]\right. \\
& +A_{2} \omega \tau_{2} /\left[1+\left(\omega \tau_{2}\right)^{2}\right]  \tag{5a}\\
& \left.+A_{3} \omega \tau_{3} /\left[1+\left(\omega \tau_{3}\right)^{2}\right]\right\}+\left(1-S^{2}\right)\left\{\tau^{\prime} /\left[1+\left(\omega \tau^{\prime}\right)^{2}\right]\right\}
\end{align*}
$$

with

$$
\begin{align*}
& A_{1}=0.75 \sin ^{4} \alpha  \tag{5b}\\
& A_{2}=3 \sin ^{2} \alpha \cos ^{2} \alpha  \tag{5c}\\
& A_{3}=\left(1.5 \cos ^{2} \alpha-0.5\right)^{2} \tag{5~d}
\end{align*}
$$

where $\alpha$ is the angle between the $\mathrm{N}-\mathrm{H}$ bond vector and the cylinder axis. The correlation times $\tau_{1}, \tau_{2}$, and $\tau_{3}$ depend on the rotational diffusion coefficients:

$$
\begin{align*}
& \tau_{1}=\left(4 D_{\|}+5 D_{\perp}\right)^{-1}  \tag{6a}\\
& \tau_{2}=\left(D_{\perp}+5 D_{\|}\right)^{-1}  \tag{6b}\\
& \tau_{3}=\left(6 D_{\perp}\right)^{-1} .
\end{align*}
$$

The time constant $\tau^{\prime}$ is dominated by the time constant of the fast internal motion, $\tau_{\mathrm{f}}$. When the internal motion is at least tenfold faster than $\tau_{1}, \tau_{2}$, and $\tau_{3}$, the value for $\tau^{\prime}$ may be approximated by (Barbato et al., 1992):

$$
\begin{equation*}
1 / \tau^{\prime}=1 / \tau_{\mathrm{f}}+1 / \tau_{\mathrm{c}, \text { eff }} \tag{7a}
\end{equation*}
$$

with

$$
\begin{equation*}
\tau_{\text {c.eff }}=\left(2 D_{\|}+4 D_{\perp}\right)^{-1} \tag{7b}
\end{equation*}
$$

Both for axially symmetric and general anisotropic motion, Lipari and Szabo (1982b) have shown that $J(\omega)$, to a good approximation, can be approximated by a simpler function of the form

$$
\begin{gather*}
J(\omega)=S^{2}\left\{A \tau_{1} /\left[1+\left(\omega \tau_{1}\right)^{2}\right]+(1-A) \tau_{2} /\left[1+\left(\omega \tau_{2}\right)^{2}\right]\right\} \\
+A\left(1-S^{2}\right) \tau^{\prime \prime} /\left[1+\left(\omega \tau^{\prime}\right)^{2}\right]+(1-A)\left(1-S^{2}\right) \tau^{\prime \prime} /\left[1+\left(\omega \tau^{\prime \prime}\right)^{2}\right] \tag{8a}
\end{gather*}
$$

with

$$
\begin{align*}
1 / \tau^{\prime} & =1 / \tau_{\mathrm{f}}+1 / \tau_{1}  \tag{8b}\\
1 / \tau^{\prime \prime} & =1 / \tau_{\mathrm{f}}+1 / \tau_{2} . \tag{8c}
\end{align*}
$$

In the case of axially symmetric motion, $J(\omega)$ is, for all practical purposes, described equally well by Eqn (8), using fewer parameters, as by Eqn (5). Similarly, the rather lengthy spectral density equation derived for the general case of anisotropic motion (Woessner, 1962b) can also be approximated by Eqn (8). However, the relation between $\tau_{1}, \tau_{2}$, and the molecular diffusion tensor can no longer be described by an equation with the simplicity of Eqn (7).

In Eqn (8), $\tau_{1}$ and $\tau_{2}$ are time constants that apply to the entire protein. Their magnitudes are related to the rate of tumbling and to the degree of anisotropy. for the isotropic case one has $\tau_{1}=\tau_{2}$, and the value of $A$ is meaningless; for axially symmetric anisotropic motion with $L_{11}>D_{1}, \tau_{1}$ will be slightly shorter than $\tau_{3}$ of Eqn (6), and $\tau_{2}$ will be slightly longer than $\tau_{1}$ of Eqn (6). When applying Eqn (8) to the study of amide ${ }^{15} \mathrm{~N}$ relaxation, it is important to note that, in contrast to its use by others, the value of $A$ is not a constant for the entire protein; its value depends on the orientation of the amide N-H bond vector with respect to the molecular diffusion tensor.

Global internal motion model. The spectral density function for isotropic motion has been adapted by Clore et al.
(1990a,b) to account for cases where internal protein motions of significant amplitude occur on an intermediate time scale, $\tau_{s}$ $\left(\tau_{\mathrm{c}}>\tau_{\mathrm{s}} \gg \tau_{\mathrm{f}}\right)$. The spectral density function than takes on a form which is rather similar to Eqn (8):

$$
\begin{align*}
& J(\omega)=S^{2} \tau_{c} /\left[1+\left(\omega \tau_{\mathrm{c}}\right)^{2}\right]+\left(S_{\mathrm{f}}^{2}-S^{2}\right) \tau_{\mathrm{s}}^{\prime} \\
& /\left[1+\left(\omega \tau_{\mathrm{s}}^{\prime}\right)^{2}\right]+\left(1-S_{\mathrm{f}}^{2}\right) \tau_{\mathrm{f}}^{\prime} /\left[1+\left(\omega \tau_{\mathrm{f}}^{\prime}\right)^{2}\right] \tag{9}
\end{align*}
$$

with $\tau_{\mathrm{s}}^{\prime}=\tau_{\mathrm{s}} \tau_{\mathrm{c}} /\left(\tau_{\mathrm{s}}+\tau_{\mathrm{c}}\right)$ and $\tau_{\mathrm{f}}^{\prime}=\tau_{\mathrm{f}} \tau_{\mathrm{c}} /\left(\tau_{\mathrm{f}}+\tau_{\mathrm{c}}\right)$. To find the simplest description consistent with the available data, $\tau_{\mathrm{f}}$ is commonly assumed to be so short that it provides a negligible contribution to $J(\omega)$, reducing Eqn (9) to two terms. For internal motions of an entire protein domain that occur on an intermediate time scale, $\tau_{\mathrm{s}}$, it is expected that the amplitude of such motions is homogeneous over the entire domain, and that all occur on the same time scale. The same approximation was made by Pastor et al. (1988) for analyzing the relaxation of methylene carbons in lipids. Thus, in contrast to the more common use of this model, we will calculate a best fit of all relaxation data to Eqn (9) using only a single value of $\tau_{\mathrm{s}}$ and $S_{\mathrm{f}}^{2}-S^{2}$. Moreover, as numerous previous protein relaxation studies have shown that the order parameter ( $S_{\mathrm{f}}^{2}$ ) for residues with little ${ }^{15} \mathrm{~N}-\left\{{ }^{1} \mathrm{H}\right\}$ NOE effect has a rather uniform value of about 0.85 , we will fix $S_{\mathrm{f}}^{2}$ at this value when searching for the values of $\tau_{\mathrm{c}}, \tau_{\mathrm{s}}$, and $S^{2}$. Assuming that the very fast motions are axially symmetric, an order parameter $S_{\mathrm{s}}^{2}=S^{2} / S_{\mathrm{f}}^{2}$ may be defined which is related directly to the amplitude of the slow internal motions (Clore et al., 1990a).

## RESULTS AND DISCUSSION

Complete resonance assignments of the backbone amides were obtained using triple-resonance $\mathrm{CBCA}(\mathrm{CO}) \mathrm{NH}$ and CBCANH (Grzesiek et al., 1993b) experiments on a sample of uniformly ${ }^{13} \mathrm{C} /{ }^{15} \mathrm{~N}$-enriched CaM under conditions that are otherwise identical to those used in the present study of the internal dynamics, supplemented by information from a three-dimensional ${ }^{15} \mathrm{~N}$-separated NOE spectrum (Zuiderweg and Fesik, 1989; Marion et al., 1989b). These assignments are summarized in Table 1. The two-dimensional ${ }^{1} \mathrm{H}-{ }^{15} \mathrm{~N}$ HSQC shift correlation map exhibits significant resonance overlap (Fig. 1), and reliable relaxation rates could only be established for 117 out of 144 observed backbone amide resonances.
$T_{1}, T_{2}$ and NOE data. $T_{1}, T_{1 \rho}$, and $T_{2}$ decay data were fit to single exponentials. These data, together with the ${ }^{1} \mathrm{H}-\left\{{ }^{15} \mathrm{~N}\right\}$ NOEs are presented in Table 1. The $T_{1}$ measurement at 600 MHz was repeated twice, several weeks apart, and the pairwise rms difference was $2.6 \%$ of their averaged value, indicating an rms error of $1.3 \%$ in the averaged $T_{1}$ value. Similarly, the pairwise difference between $T_{2}$ and $T_{1 \mathrm{p}}$ data for residues in the N -terminal half of CaM was $3 \%$ of their averaged value, suggesting an error of about $2 \%$ in the individual $T_{2}$ and $T_{1 \rho}$ measurements. These errors are very similar in magnitude to the maximum deviations obtained by fitting the intensity decay multiple times, randomly adding to or subtracting from the measured peak heights a number that corresponds to the rms noise measured from a sig-nal-free region of the corresponding spectrum. The error for the $T_{2}$ data measured at 500 MHz , obtained in this latter manner, is estimated at $2 \%$. Analogously, an error of about $3 \%$ in the ${ }^{1} \mathrm{H}$ $\left\{{ }^{15} \mathrm{~N}\right\}$ NOE is based on the rms noise present in the two spectra obtained with and without saturation of the ${ }^{1} \mathrm{H}$ spectrum. The relaxation data are presented graphically in Fig. 2 together with the secondary structure derived from NOE and chemical shift data (H. Kuboniwa, unpublished results). This secondary structure is very similar to that observed in $\mathrm{Ca}^{2+}$-ligated CaM .

Slow conformational exchange. Comparison of ${ }^{15} \mathrm{~N} T_{2}$ data and $T_{1 \rho}$ data, obtained with a $2.5-\mathrm{kHz}$ spin lock field, show very good agreement for residues in the N -terminal half of the protein but very large differences in the C-terminal domain of the protein. Resonances with the largest difference between $T_{2}$ and $T_{1 \rho}$ also exhibit, as expected, weak resonance intensity in the twodimensional ${ }^{15} \mathrm{~N}-{ }^{-1} \mathrm{H}$ HSQC spectrum (Fig. 1). Assuming that the conformational exchange process involves two states, A and B , with populations $P_{\mathrm{A}}$ and $P_{\mathrm{B}}$, and rate constants $k_{\mathrm{A} \rightarrow \mathrm{B}}$ and $k_{\mathrm{B} \rightarrow \mathrm{A}}$, the exchange contribution to the ${ }^{15} \mathrm{~N}$ transverse relaxation time is given by (Szyperski et al., 1993)

$$
\begin{gather*}
1 / T_{2}=P_{\mathrm{A}} P_{\mathrm{B}}(\Delta \omega)^{2} \tau_{\text {exch }} /\left[1+\left(\omega_{1} \tau_{\text {exch }}\right)^{2}\right]+1 / T_{2, \text { intr }}  \tag{10a}\\
\tau_{\text {exch }}=\left(1-P_{\mathrm{A}}\right) / k_{\mathrm{A} \rightarrow \mathrm{~B}} \tag{10b}
\end{gather*}
$$

where $\Delta \omega$ is the difference in angular ${ }^{15} \mathrm{~N}$ chemical shift frequency between states A and B , and $T_{2, \text { intr }}$ is the intrinsic ${ }^{15} \mathrm{~N}$ $T_{2}$, in the absence of conformational exchange. As the $180^{\circ}$ pulses used during the ${ }^{15} \mathrm{~N} T_{2}$ relaxation delay are spaced 1 ms apart, this pulse train has an effective spin lock field strength of 500 Hz . Fitting of the observed ${ }^{15} \mathrm{~N} T_{2}$ rates derived from the ${ }^{15} \mathrm{~N}$ line widths in the HSQC experiment and the $T_{2}$ and $T_{1 e}$ data to Eqn (10a) yields a value for $\tau_{\text {exch }}$ of $350 \pm 100 \mu \mathrm{~s}$. Although $\Delta \omega$ is not known, it may be estimated to be at least a few ppm as even minor conformational changes frequently result in chemical shift changes of such magnitude. With this assumption, Eqn (10) yields a $P_{\mathrm{A}} / P_{\mathrm{B}}$ ratio of about $25: 1$, i.e. a low population for one of the two conformers involved in the two-site exchange.

Hydrogen exchange. The rates of hydrogen exchange for slowly exchanging backbone amide protons were obtained by monitoring the HSQC intensity as a function of time after dissolving the protein in $\mathrm{D}_{2} \mathrm{O}$, and relating these intensities to those observed under the same conditions for a sample dissolved in $\mathrm{H}_{2} \mathrm{O}$. Considering the strong dependence of the hydrogen exchange rates on temperature, hydrogen exchange taking place during the initial sample preparation time of $300 \mathrm{~s}\left(\right.$ at $\left.4^{\circ} \mathrm{C}\right)$ was not accounted for when calculating the exchange rates, but the 120 s time during which the sample temperature was raised from $4^{\circ}$ to $23^{\circ} \mathrm{C}$ (in the NMR probe) was included in full as being part of the hydrogen exchange time. The exchange time was counted until the mid-point of the data collection time. In the first HSQC spectrum, initiated 2 min after raising the sample temperature to $23^{\circ} \mathrm{C}, 45$ amides that are part of the N -terminal domain and 23 amides of the C -terminal domain could be observed, indicating exchange times longer than about 200 s , i.e. protection factors (Jeng and Englander, 1991; Bai et al., 1993) greater than $\approx 100$. The approximate exchange rates for these amides are presented graphically in Fig. 2D. Although the slowest amide exchange is found for the first and last helix of the N -terminal domain, a substantial number of slowly exchanging amides is also present in the carboxy-terminal domain, confirming that both domains contain numerous stable hydrogen bonds.

Determination of the rotational correlation time for the isotropic motion model. For an isotropically tumbling protein, very fast internal motions on a time scale $\tau_{\mathrm{f}}\left(\tau_{\mathrm{f}} \ll \omega_{\mathrm{H}}^{-1}\right)$ do not affect the $T_{1} / T_{2}$ ratio (cf. Eqn 2a,b), and this ratio then uniquely characterizes the rotational correlation time, $\tau_{c}$, of the protein. Residues with an NOE lower than 0.6 are not used during this procedure as for such residues the condition $\tau_{\mathrm{f}} \leftrightarrow \omega_{\mathrm{H}}^{-1}$ is clearly not valid. Although the $\tau_{c}$ value may be obtained from the average of the $\tau_{c}$ values derived individually for each residue from its $T_{1} / T_{2}$ ratio (Kay et al., 1989), it is better to search for

Table 1. Chemical shifts, ${ }^{15} \mathrm{~N}$ relaxation times, order parameters ( $S^{2}$ ), internal correlation times ( $\tau_{\mathrm{e}}$ ), and hydrogen exchange rates ( $\tau_{\text {exch }}$ ) for the backbone amide protons in $\mathrm{Ca}^{2+}$-free calmodulin. Order parameters ( $S^{2}$ ) and internal correlation times ( $\tau_{e}$ ), were calculated using the isotropic diffusion model (cf. Eqn 3), with $\tau_{c}=8.0 \mathrm{~ns}$ for residues Q3-D80, and $\tau_{\mathrm{c}}=7.5 \mathrm{~ns}$ for residues $\mathrm{S} 81-\mathrm{K} 148$. ${ }^{1} \mathrm{H}$ chemical shifts are relative to sodium 3 -trimethylsilyl- $\left(2,2,3,3-{ }^{2} \mathrm{H}_{4}\right)$ propionate TSP and ${ }^{15} \mathrm{~N}$ chemical shifts are relative to liquid ammonia at $25^{\circ} \mathrm{C}$. Sample conditions: 1.8 mM , $\mathrm{pH} 6.3,100 \mathrm{mM} \mathrm{KCl}, 1.5 \mathrm{mM}$ EDTA, 0.1 mM sodium azide, $23^{\circ} \mathrm{C}$.


|  | ppm |  | ms |  |  |  |  |  | ps | S |
| :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: |
| Q3 | 8.21 | 118.4 | 759 | 654 | 195 | 193 | -0.004 | 0.629 | 142 | $<200$ |
| L4 | 8.31 | 121.8 | 729 | 608 | 149 | 149 | 0.369 | 0.733 | 103 | $<200$ |
| T5 | 8.78 | 112.9 | 665 | 557 | 109 | 108 | 0.649 | 0.858 | 76 | 300 |
| E6 | 9.03 | 120.1 | 734 | 609 | 90 | 90 | 0.693 | 0.784 | 32 | $<200$ |
| E7 | 8.73 | 119.2 | 727 | 642 | 82 | 80 | 0.717 | 0.777 | 25 | $<200$ |
| I9 | 8.24 | 118.3 | 700 | 615 | 84 | 88 | 0.723 | 0.811 | 29 | 4000 |
| E11 | 7.80 | 120.1 | 729 | 612 | 79 | 79 | 0.690 | 0.792 | 34 | 1000 |
| F12 | 8.84 | 120.3 | 678 | 569 | 88 | 86 | 0.744 | 0.863 | 32 | 4000 |
| K13 | 9.24 | 121.4 | 690 | 580 | 85 | 82 | 0.760 | 0.854 | 24 | $<200$ |
| A15 | 7.64 | 120.7 | 741 | 643 | 80 | 78 | 0.731 | 0.768 | 20 | 5000 |
| F16 | 8.54 | 117.8 | 687 | 582 | 85 | 86 | 0.754 | 0.847 | 25 | 5000 |
| S17 | 8.44 | 110.9 | 732 | 585 | 81 | 80 | 0.722 | 0.818 | 30 | 1000 |
| F19 | 7.40 | 114.6 | 736 | 705 | 85 | 86 | 0.701 | 0.733 | 23 | 3000 |
| D20 | 7.31 | 122.4 | 716 | 587 | 109 | 104 | 0.612 | 0.795 | 58 | 200 |
| K21 | 8.09 | 123.6 | 756 | 616 | 126 | 127 | 0.435 | 0.727 | 80 | $<200$ |
| D22 | 8.74 | 117.0 | 767 | 636 | 107 | 110 | 0.436 | 0.711 | 73 | $<200$ |
| G23 | 8.02 | 110.3 | 734 | 626 | 110 | 106 | 0.569 | 0.751 | 55 | $<200$ |
| D24 | 8.80 | 120.7 | 698 | 591 | 121 | 120 | 0.616 | 0.804 | 61 | $<200$ |
| G25 | 10.20 | 111.9 | 649 | 557 | 104 | 106 | 0.674 | 0.868 | 69 | $<200$ |
| T26 | 7.60 | 109.7 | 722 | 619 | 85 | 84 | 0.702 | 0.781 | 29 | 800 |
| I27 | 8.27 | 110.3 | 669 | 535 | 75 | 82 | 0.756 | 0.891 | 34 | 3000 |
| T28 | 8.35 | 110.6 | 639 | 535 | 99 | 102 | 0.727 | 0.909 | 65 | 600 |
| T29 | 8.30 | 112.4 | 688 | 605 | 88 | 87 | 0.706 | 0.821 | 36 | $<200$ |
| K30 | 7.64 | 118.8 | 713 | 599 | 80 | 80 | 0.736 | 0.814 | 25 | $<200$ |
| E31 | 7.52 | 117.4 | 657 | 537 | 98 | 98 | 0.712 | 0.894 | 64 | 500 |
| G33 | 8.82 | 105.4 | 696 | 579 | 91 | 97 | 0.763 | 0.840 | 20 | $<200$ |
| T34 | 7.53 | 118.3 | 710 | 614 | 93 | 90 | 0.757 | 0.810 | 18 | $<200$ |
| V35 | 7.99 | 122.7 | 624 | 537 | 95 | 94 | 0.733 | 0.919 | 70 | 6000 |
| R37 | 8.54 | 119.4 | 687 | 577 | 85 | 86 | 0.787 | 0.855 | 13 | 3000 |
| S38 | 8.12 | 119.0 | 642 | 574 | 90 | 88 | 0.688 | 0.876 | 68 | 600 |
| G40 | 7.92 | 107.1 | 769 | 647 | 87 | 86 | 0.712 | 0.749 | 22 | $<200$ |
| Q41 | 7.78 | 117.7 | 735 | 632 | 77 | 79 | 0.740 | 0.784 | 20 | 500 |
| N42 | 8.69 | 116.5 | 873 | 746 | 106 | 109 | 0.450 | 0.619 | 45 | $<200$ |
| T44 | 8.80 | 113.2 | 711 | 599 | 98 | 96 | 0.715 | 0.810 | 31 | 200 |
| E45 | 8.87 | 120.4 | 657 | 561 | 100 | 103 | 0.718 | 0.866 | 45 | $<200$ |
| A46 | 8.34 | 121.0 | 660 | 560 | 95 | 94 | 0.689 | 0.865 | 59 | 2000 |
| L48 | 8.35 | 120.0 | 636 | 539 | 98 | 97 | 0.712 | 0.901 | 70 | 5000 |
| Q49 | 8.07 | 117.5 | 642 | 558 | 94 | 89 | 0.730 | 0.897 | 55 | 5000 |
| D50 | 7.85 | 118.9 | 630 | 532 | 95 | 93 | 0.764 | 0.922 | 45 | 800 |
| M51 | 7.98 | 119.1 | 623 | 540 | 100 | 101 | 0.749 | 0.930 | 67 | 3000 |
| I52 | 8.35 | 119.0 | 649 | 518 | 92 | 93 | 0.736 | 0.912 | 60 | 6000 |
| N53 | 8.28 | 117.3 | 642 | 576 | 90 | 91 | 0.756 | 0.882 | 33 | 200 |
| E54 | 7.59 | 117.3 | 658 | 557 | 98 | 100 | 0.674 | 0.867 | 69 | < 200 |
| V55 | 7.64 | 112.8 | 649 | 542 | 40 | 126 | 0.191 | 0.749 | 300 | 200 |
| D56 | 8.48 | 121.9 | 679 | 577 | 96 | 96 | 0.677 | 0.836 | 51 | $<200$ |
| A57 | 8.17 | 124.9 | 715 | 632 | 121 | 114 | 0.562 | 0.762 | 61 | $<200$ |
| D58 | 8.45 | 114.8 | 719 | 622 | 117 | 117 | 0.534 | 0.761 | 68 | $<200$ |
| G59 | 7.91 | 108.9 | 738 | 610 | 104 | 103 | 0.590 | 0.767 | 55 | $<200$ |
| N60 | 9.29 | 119.8 | 734 | 627 | 105 | 106 | 0.606 | 0.768 | 51 | $<200$ |
| T62 | 7.57 | 110.7 | 622 | 544 | 106 | 102 | 0.702 | 0.910 | 88 | $<200$ |
| I63 | 8.92 | 118.8 | 683 | 591 | 94 | 96 | 0.689 | 0.826 | 43 | 2000 |
| D64 | 8.50 | 124.3 | 660 | 572 | 94 | 89 | 0.776 | 0.867 | 19 | $<200$ |
| F65 | 8.66 | 118.6 | 760 | 625 | 74 | 77 | 0.711 | 0.759 | 23 | 500 |
| E67 | 8.10 | 117.5 | 761 | 662 | 72 | 74 | 0.752 | 0.749 | 14 | 2000 |
| F68 | 8.49 | 122.4 | 744 | 626 | 80 | 82 | 0.763 | 0.783 | 14 | 10000 |
| T70 | 7.73 | 115.4 | 781 | 675 | 74 | 76 | 0.717 | 0.724 | 18 | 5000 |
| M71 | 7.74 | 121.3 | 727 | 605 | 72 | 68 | 0.758 | 0.814 | 18 | 8000 |
| A73 | 8.30 | 121.0 | 721 | 583 | 75 | 75 | 0.736 | 0.822 | 26 | 4000 |
| R74 | 7.47 | 116.8 | 756 | 699 | 75 | 72 | 0.679 | 0.720 | 26 | 600 |
| K75 | 7.73 | 118.2 | 734 | 617 | 82 | 80 | 0.689 | 0.787 | 33 | $<200$ |

Table 1. Continued.

| Residue | Shift of |  | $T_{1,600}$ | $T_{1,500}$ | $T_{2,600}$ | $T_{1 p, 600}$ | $N O E_{600}$ | $S^{2}$ | $\tau_{\text {e }}$ | $\tau_{\text {exch }}$ |
| :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: |
|  | ${ }^{1} \mathrm{H}^{\mathrm{N}}$ | ${ }^{15} \mathrm{~N}$ |  |  |  |  |  |  |  |  |
|  | ppm |  | ms |  |  |  |  |  | ps | $s$ |
| M76 | 7.96 | 117.8 | 714 | 604 | 92 | 93 | 0.614 | 0.777 | 52 | $<200$ |
| D78 | 8.34 | 122.0 | 716 | 618 | 118 | 120 | 0.325 | 0.711 | 105 | $<200$ |
| T79 | 8.17 | 114.9 | 737 | 661 | 121 | 124 | 0.347 | 0.682 | 84 | $<200$ |
| D80 | 8.47 | 123.1 | 707 | 637 | 127 | 123 | 0.394 | 0.721 | 91 | $<200$ |
| S81 | 8.48 | 117.2 | 702 | 608 | 100 | 110 | 0.569 | 0.770 | 63 | $<200$ |
| E82 | 8.43 | 121.8 | 719 | 593 | 92 | 93 | 0.575 | 0.747 | 55 | $<200$ |
| E84 | 8.02 | 119.1 | 696 | 568 | 69 | 84 | 0.698 | 0.798 | 34 | 1000 |
| E87 | 8.42 | 116.9 | 719 | 579 | 43 | 68 | 0.733 | 0.785 | 22 | $<200$ |
| A88 | 7.51 | 121.3 | 640 | 530 | 73 | 87 | 0.769 | 0.869 | 22 | 600 |
| F89 | 7.49 | 114.2 | 710 | 599 | 55 | 69 | 0.731 | 0.761 | 19 | 1000 |
| R90 | 8.25 | 117.9 | 701 | 602 | 45 | 71 | 0.752 | 0.787 | 17 | < 200 |
| V91 | 7.17 | 117.0 | 606 | 503 | 73 | 85 | 0.675 | 0.906 | 113 | 300 |
| D93 | 7.84 | 121.3 | 750 | 650 | 77 | 80 | 0.701 | 0.720 | 22 | $<200$ |
| K94 | 8.44 | 124.4 | 719 | 607 | 73 | 99 | 0.444 | 0.723 | 80 | 1000 |
| D95 | 8.63 | 116.2 | 645 | 584 | 76 | 109 | 0.538 | 0.791 | 86 | $<200$ |
| G96 | 8.06 | 110.1 | 648 | 557 | 99 | 109 | 0.675 | 0.836 | 54 | $<200$ |
| N97 | 9.02 | 118.7 | 656 | 575 | 107 | 113 | 0.644 | 0.823 | 62 | $<200$ |
| Y99 | 7.79 | 118.2 | 631 | 518 | 68 | 88 | 0.721 | 0.897 | 62 | 300 |
| S101 | 8.95 | 117.3 | 640 | 566 | 79 | 91 | 0.710 | 0.839 | 41 | $<200$ |
| A102 | 8.81 | 124.5 | 669 | 575 | 80 | 85 | 0.713 | 0.814 | 33 | $<200$ |
| A103 | 8.28 | 119.0 | 662 | 594 | 78 | 88 | 0.738 | 0.809 | 24 | $<200$ |
| E104 | 7.75 | 120.2 | 670 | 527 | 81 | 92 | 0.683 | 0.846 | 54 | 700 |
| R106 | 8.06 | 117.2 | 707 | 577 | 72 | 80 | 0.725 | 0.782 | 23 | $<200$ |
| V108 | 8.14 | 119.5 | 671 | 574 | 79 | 80 | 0.664 | 0.808 | 48 | 300 |
| T110 | 7.95 | 110.8 | 669 | 591 | 54 | 70 | 0.721 | 0.802 | 28 | $<200$ |
| N111 | 7.65 | 119.8 | 628 | 568 | 95 | 98 | 0.627 | 0.824 | 69 | $<200$ |
| L112 | 7.74 | 119.8 | 725 | 635 | 86 | 98 | 0.614 | 0.727 | 41 | $<200$ |
| G113 | 8.19 | 108.0 | 664 | 567 | 135 | 136 | 0.527 | 0.787 | 87 | $<200$ |
| E114 | 8.19 | 120.2 | 638 | 547 | 149 | 147 | 0.465 | 0.800 | 127 | $<200$ |
| K115 | 8.27 | 120.1 | 620 | 574 | 123 | 136 | 0.454 | 0.798 | 132 | $<200$ |
| L116 | 7.79 | 121.1 | 687 | 615 | 89 | 110 | 0.419 | 0.731 | 92 | $<200$ |
| T117 | 8.99 | 113.5 | 652 | 546 | 108 | 112 | 0.586 | 0.831 | 92 | $<200$ |
| D118 | 8.84 | 120.9 | 641 | 559 | 107 | 109 | 0.692 | 0.842 | 50 | $<200$ |
| E119 | 8.59 | 117.9 | 647 | 523 | 87 | 99 | 0.667 | 0.853 | 65 | $<200$ |
| E123 | 7.88 | 119.2 | 645 | 516 | 88 | 94 | 0.684 | 0.862 | 62 | 500 |
| M124 | 7.91 | 118.7 | 638 | 530 | 92 | 97 | 0.647 | 0.852 | 76 | 500 |
| I125 | 8.34 | 118.3 | 615 | 518 | 72 | 92 | 0.709 | 0.886 | 62 | 2000 |
| R126 | 7.96 | 119.6 | 627 | 521 | 51 | 75 | 0.693 | 0.871 | 63 | 900 |
| E127 | 7.85 | 116.8 | 640 | 577 | 50 | 75 | 0.668 | 0.816 | 50 | $<200$ |
| A128 | 7.60 | 121.5 | 608 | 520 | 89 | 97 | 0.664 | 0.893 | 104 | 300 |
| D129 | 8.37 | 119.6 | 716 | 595 | 88 | 94 | 0.591 | 0.752 | 52 | $<200$ |
| I130 | 7.85 | 121.0 | 691 | 586 | 98 | 99 | 0.562 | 0.766 | 65 | $<200$ |
| D131 | 8.63 | 124.2 | 653 | 564 | 64 | 72 | 0.574 | 0.803 | 79 | $<200$ |
| G132 | 8.38 | 107.9 | 653 | 578 | 96 | 102 | 0.545 | 0.798 | 88 | $<200$ |
| D133 | 8.30 | 119.3 | 676 | 585 | 113 | 116 | 0.581 | 0.778 | 64 | $<200$ |
| G134 | 8.54 | 109.5 | 622 | 541 | 114 | 114 | 0.599 | 0.856 | 109 | $<200$ |
| Q135 | 8.33 | 119.4 | 688 | 577 | 108 | 107 | 0.595 | 0.784 | 62 | $<200$ |
| V136 | 9.43 | 119.4 | 678 | 508 | 80 | 101 | 0.645 | 0.864 | 85 | $<200$ |
| N137 | 8.81 | 125.1 | 673 | 553 | 80 | 94 | 0.684 | 0.819 | 44 | $<200$ |
| Y138 | 7.53 | 122.4 | 586 | 527 | 87 | 85 | 0.677 | 0.894 | 94 | $<200$ |
| F141 | 7.56 | 119.0 | 611 | 528 | 75 | 86 | 0.663 | 0.873 | 83 | 600 |
| V142 | 8.15 | 120.0 | 647 | 543 | 92 | 94 | 0.714 | 0.861 | 47 | 600 |
| M144 | 7.77 | 118.0 | 667 | 566 | 75 | 90 | 0.673 | 0.817 | 48 | 200 |
| M145 | 7.99 | 116.0 | 614 | 533 | 59 | 86 | 0.649 | 0.873 | 92 | $<200$ |
| T146 | 7.71 | 110.4 | 668 | 566 | 66 | 90 | 0.600 | 0.798 | 66 | $<200$ |
| A147 | 7.61 | 125.5 | 649 | 620 | 118 | 116 | 0.463 | 0.750 | 89 | $<200$ |
| K148 | 7.71 | 125.5 | 736 | 662 | 283 | 274 | 0.109 | 0.570 | 85 | 100 |

the $\tau_{\mathrm{c}}$ value which minimizes the total error function, $E$ (Dellwo and Wand, 1989):

$$
\begin{aligned}
E & =(1 / k N) \Sigma_{N}\left(T_{1,500}^{\text {calc }}-T_{1,500}^{\text {meas }}\right)^{2} /\left(\Delta T_{1,500}\right)^{2} \\
& +\left(T_{1,600}^{\text {calc }}-T_{1,600}^{\text {meas }}\right)^{2} /\left(\Delta T_{1,600}\right)^{2}+\left(T_{2}^{\text {calc }}-T_{2}^{\text {meas }}\right)^{2} /\left(\Delta T_{2}\right)^{2}
\end{aligned}
$$

$$
\begin{equation*}
+\left(N O E^{\text {calc }}-N O E^{\text {meas }}\right)^{2} /(A N O E)^{2} \tag{12}
\end{equation*}
$$

where the summation extends over all $N$ residues with $N O E>0.6$, and $k$ denotes the number of measured variables


Fig. 1. A small region of the $600-\mathrm{MHz}{ }^{1} \mathbf{H}-{ }^{15} \mathrm{~N}$ correlation spectrum of $\mathrm{Ca}^{2+}$-free $\mathbf{C a M}$. Horizontal dashed lines connect sidechain $\mathrm{NH}_{2}$ resonances of Gln and Asn residues. For each of these, the second amide proton resonates outside the region shown.


Fig. 2. Graphical representation of the relaxation data measured for $\mathbf{C a}^{2+}$-free $\mathbf{C a M}$ at $\mathbf{2 3}{ }^{\circ} \mathbf{C}, \mathbf{p H} \mathbf{6 . 3}, \mathbf{1 0 0} \mathbf{~ m M ~ K C l}$. (A) ( ${ }^{(\boldsymbol{*})}{ }^{15} \mathrm{~N}$ relaxation data measured at $60.8 \mathrm{MHz}\left(600 \mathrm{MHz}^{1} \mathrm{H}\right.$ frequency) and (x) at 50.7 MHz . (B) $T_{2}$ ( - ) and $T_{1 \rho}$ (x) data, both measured at 60.8 MHz . (C) Heteronuclear ${ }^{15} \mathrm{~N}-\left\{{ }^{1} \mathrm{H}\right\}$ NOE, measured at $600 \mathrm{MHz}{ }^{1} \mathrm{H}$ frequency. (D) Hydrogen exchange times ( $\tau_{\mathrm{HX}}$ ) and secondary structure. Open bars correspond to $\alpha$-helices, open arrows correspond to the short $\beta$-strands.
used in the fit ( $k=4$, for the case where $T_{1,500}, T_{1,600}, T_{2}$, and $N O E$ data were fitted). $\Delta T_{1}, \Delta T_{2}$ and $\triangle N O E$ are the standard errors in the $T_{1}, T_{2}$ and NOE measurements, the subscripts 500 and 600 (e.g. $T_{1, \text { soo }}$ ) refer to the ${ }^{1} \mathrm{H}$ resonance frequency at which experiments were conducted, the superscript 'meas' refers to measured data, and 'calc' refers to the value calculated on the basis of Eqn (2), using the spectral density function of Eqn (3). Standard errors of $2 \%$ were used for the $T_{1,500}, T_{1,600}$, and $T_{2}$ data, and $3 \%$ for the NOE. In the search for the optimal $\tau_{c}$ value,
$\tau_{\mathrm{f}}$ is assumed to be zero and $S^{2}$ is allowed to vary by residue between zero and one. The last term in Eqn (12) is not used when searching for the optimal $\tau_{c}$ value as all reasonable values for $\tau_{c}$ yield $N O E^{\text {calc }}$ values of 0.8 (using the $\tau_{\mathrm{f}}=0$ approximation). The model describes the measured data accurately if the error function approaches one. Once the value of $\tau_{c}$ has been determined, the search is repeated without the $\tau_{\mathrm{f}}=0$ assumption and taking the NOE term of Eqn (12) into account as well. This then yields order parameters, $S^{2}$, and $\tau_{\mathrm{f}}$ values for each residue.


Fig. 3. Magnetic field dependence of ${ }^{15} \mathbf{N}$ relaxation times. (A) Ratio of the ${ }^{15} \mathrm{~N} T_{1 \rho}$ and $T_{2}$ values measured at 60.8 MHz (corresponding to $600 \mathrm{MHz}{ }^{1} \mathrm{H}$ frequency). (B) Ratio of the ${ }^{15} \mathrm{~N} T_{1}$ values measured at 50.7 and 60.8 MHz .
$N$-terminal domain. For the N -terminal domain the $T_{1 \mathrm{p}} / T_{2}$ ratio is close to 1 (Fig. 3A), indicating the absence of conformational exchange processes on a time scale slower than 2.5 kHz . Moreover, the $T_{2}$ values are relatively uniform within the individual $\alpha$-helices (which have their N-H bond vectors in nearly parallel orientations), suggesting that conformational exchange does not have any large effect on the measured $T_{2}$ or $T_{1 \varrho}$ data. The fact that different helices show different $T_{2}$ values is related to motional anisotropy (vide infra), but will be ignored when attempting to derive an isotropic $\tau_{c}$ value. Using this approach, optimizing $E$ for the 47 N -terminal residues which have an $N O E$ larger than 0.6 yields a $\tau_{\mathrm{c}}$ value of $8.4 \pm 0.2 \mathrm{~ns}$. The error is estimated from the largest and smallest $\tau_{c}$ value obtained when repeating the search 200 times, randomly omitting $15 \%$ of the measured residues for each search.

As $T_{2}$ values in the C-terminal domain are significantly shortened by a slow conformational averaging process, only $T_{1}$ and NOE data can be used for determining the apparent rotational correlation time in this domain. To demonstrate the validity of such an approach we first apply it to the N-terminal domain and demonstrate that it gives a $\tau_{c}$ value which is close to the value obtained with the regular procedure, used above. Previous ${ }^{15} \mathrm{~N}$ relaxation studies on isotropically tumbling proteins indicate that for residues with $N O E>0.6$, the generalized order parameter, $S^{2}$, adopts a rather uniform value of $0.85 \pm 0.05$. Using this assumption of a fixed $S^{2}=0.85$ value for all residues, $E$ may be minimized using only the $T_{1}$ and NOE data. This results in a $\tau_{\mathrm{c}}$ value of $7.9 \pm 0.1 \mathrm{~ns}$ (Table 2). The fact that the error function ( $E$ ) is somewhat lower compared to the case where $T_{1}$ and $T_{2}$ data are fitted does not indicate that this $\tau_{c}$ value is a better estimate for the rotational correlation time; it merely reflects the fact that the $T_{1}$ values and NOE data are more homogeneous than the $T_{2}$ values.

The $\tau_{\mathrm{c}}$ value obtained from $T_{1}$ and NOE data is slightly shorter than the value obtained from $T_{1}$ and $T_{2}$ data. This is caused in part by the low measured NOE values, which fall in the $0.6-0.8$ range, and which result in a lowering of $\tau_{c}$ when constraining $\tau_{\mathrm{f}}$ to be zero. NOE values in this range are observed for stable regions in all previously studied proteins and reflect the fact that the $\tau_{f}=0$ approximation is not entirely valid. Therefore, we also have tried to fit the $T_{1}$ data alone, again assuming $S^{2}=0.85$. This results in a $\tau_{c}$ value of $8.0 \pm 0.1 \mathrm{~ns}$ (Table 2).

C-terminal domain. As mentioned above, comparison of $T_{2}$ and $T_{1 \mathrm{p}}$ data (Fig. 3A) suggests that $T_{2}$ values for this domain are unreliable as they are significantly affected by conformational exchange. A spin lock field considerably stronger than the
2.5 kHz used in the present study would be needed to ensure that the exchange contribution to $T_{1 \rho}$ is negligible. Such strong radiofrequency fields of long durations result in unacceptable sample heating and can damage the probehead and therefore could not be used. However, as shown above, use of $T_{1}$ data alone, or of both $T_{1}$ and NOE data, yields $\tau_{\mathrm{c}}$ values that are very similar to what is obtained with the more conventional approach, using $T_{1}$ and $T_{2}$ data. Using $T$, and NOE data, for all 31 residues that have $N O E>0.6$, a $\tau_{c}$ value of $7.4 \pm 0.2 \mathrm{~ns}$ is obtained. Using exclusively $T_{1}$ data results in a slightly longer $\tau_{c}$ value of $7.5 \pm 0.1$ ns (Table 2). For $\mathrm{Ca}^{2+}$-ligated CaM , Barbato et al. (1992) also observed a slightly shorter $\tau_{\mathrm{c}}$ value for the C-terminal domain relative to that found for the $N$-terminal domain, which was attributed to its number of residues.

Anisotropic model. Considering the large value of the error function $E$ (Table 2), the isotropic model is in poor agreement with the experimental data. Also, as mentioned above, in the N terminal domain the $T_{2}$ values are very similar within each of the four individual $\alpha$-helices, but the average $T_{2}$ values for the four helices differ significantly from one another. Considering that in an $\alpha$-helix, the $\mathrm{N}-\mathrm{H}$ bond vectors are parallel to the helix axis, with a rms deviation of about $15^{\circ}$, anisotropic tumbling will cause amides within a given helix to relax rather uniformly, but amides in different helices will generally relax at different rates, depending on the orientation of the helix axis relative to the anisotropic molecular diffusion tensor. Because the structure of $\mathrm{Ca}^{2+}$-free CaM has not yet been completed, it is not possible to characterize the degree of anisotropy at a detailed level. However, a lower limit for the degree of anisotropy may be estimated by considering that the $T_{2}$ values for amides in helix C are about $25 \%$ longer than for helix D. If the protein were tumbling with an axially symmetric diffusion tensor, and helices $C$ and $D$ were oriented perpendicular and parallel to this symmetry axis, Eqn (5) and (6) indicate that this $T_{2}$ ratio, which is dominated by $J(0)$ spectral density, corresponds to a $D_{\mathrm{i} 1} / D_{\perp}$ ratio of 1.6 . This degree of anisotropy is larger than that previously observed for $\mathrm{Ca}^{2+}$-ligated CaM at $35^{\circ} \mathrm{C}$, but considerably less than the ratio of 2.5 , expected from hydrodynamic calculations for a rigid dumbbell model (Barbato et al., 1992).

The $\alpha$-helical backbone N-H bond vectors are not perfectly colinear with the helix axis and, on average, they make angles of about $15^{\circ}$ with this axis. Assuming axially symmetric anisotropic diffusion, the amide in helix D which has the longest $T_{2}$ value ( $\mathrm{F} 68,81 \mathrm{~ms}$ ) is expected to make an angle with the unique axis of the diffusion tensor that is $\approx 15^{\circ}$ larger than the angle between the unique axis and helix D. Similarly, the amide of

Table 2. Correlation times and values for the error function, $E$, derived for the N - and C -terminal domains of $\mathrm{Ca}^{\mathbf{2 +}-f r e e ~} \mathbf{C a M}$ at $\mathbf{2 3}^{\circ} \mathrm{C}$, assuming isotropic motion. The error function, $E$, extends only over the input parameters used. Uncertainties correspond to the maximum deviation when repeating the search 200 times, omitting randomly $15 \%$ of the residues. $N$ and $p$ are the number of residues and the number of variables used in the fitting procedure.

| Input parameters | Domain | $\tau_{c}$ | $E$ | $N$ | $p$ |
| :--- | :--- | :--- | :--- | :--- | :--- |
|  |  | ns |  |  |  |
| $T_{1,600} ; T_{1,500} ; T_{2,600} ; T_{\mathrm{c} \rho, 600}$ | N | $8.4 \pm 0.2$ | $19 \pm 3$ | 47 | 48 |
| $T_{1,600} ; T_{1,500} ; N O E$ | N | $7.9 \pm 0.1$ | $15 \pm 2$ | 47 | 1 |
| $T_{1,600} ; T_{1,500}$ | N | $8.0 \pm 0.1$ | $16 \pm 2$ | 47 | 1 |
| $T_{1,600} ; T_{1,500} ; N O E$ | C | $7.4 \pm 0.2$ | $16 \pm 2$ | 31 | 1 |
| $T_{1,600} ; T_{1,500}$ | C | $7.5 \pm 0.1$ | $18 \pm 2$ | 31 | 1 |

Q49 in helix D which has the shortest transverse relaxation rate ( 91 ms ) is expected to make an angle with the unique axis which is $\approx 15^{\circ}$ smaller than the angle between this axis and helix C . Considering that the fastest relaxing amide in helix C relaxes considerably slower than the slowest relaxing amide in helix D , the angle between helix D and the unique axis must be smaller by significantly more than $30^{\circ}$ compared to the angle between the unique axis and helix $C$. The angle between helices $C$ and D is therefore also significantly larger than $30^{\circ}$. Helices A and B, which exhibit ${ }^{15} \mathrm{~N} T_{2}$ values intermediate between those of helices $C$ and $D$, must make angles with the unique axis of the diffusion tensor that fall between those of helices C and D . Note that small non-uniformity of the order parameter, $S^{2}$, and/or small random errors in the $T_{2}$ measurements statistically bring the shortest $T_{2}$ value in helix C and the longest $T_{2}$ value in helix D closer to one another, and the above analysis of the minimum angle between helices C and D is therefore very conservative.

In the $\mathrm{Ca}^{2+}$-free N -terminal domain of troponin C , helices C and $D$ have an interhelix angle of $146^{\circ}$, whereas helix $D$ makes angles of $115^{\circ}$ and $53^{\circ}$ with helices A and B , respectively (Strynadka and James, 1989). In $\mathrm{Ca}^{2+}$-ligated CaM these angles are $84^{\circ}(\mathrm{C} / \mathrm{D}), 115^{\circ}(\mathrm{A} / \mathrm{D})$ and $34^{\circ}(\mathrm{B} / \mathrm{D})$. Herzberg et al. (1986) proposed that binding of calcium is responsible for the large difference in helical angles between the $\mathrm{Ca}^{2+}$-free N -terminal domain of troponin C and its $\mathrm{Ca}^{2+}$-ligated C -terminal domain. Strynadka and James (1988) suggest that $\mathrm{Ca}^{2+}$-free CaM adopts a similar helical arrangement as observed in the N -terminal domain of troponin C, with a C/D angle of $151^{\circ}$. However, as argued above, an angle of $151^{\circ}$ (equivalent to $180^{\circ}-151^{\circ}=$ $29^{\circ}$, from an NMR relaxation viewpoint) appears incompatible with the rather large difference in transverse relaxation rates observed for amides in helices C and D , suggesting that at a quantitative level the Strynadka and James model may not be quite accurate.

Global internal motion model. Both for the isotropic and anisotropic tumbling models, it is readily seen that in the motional correlation time range applicable for $\mathrm{CaM}(>5 \mathrm{~ns}), T_{1}$ values at $600 \mathrm{MHz}{ }^{1} \mathrm{H}$ frequency are expected to be longer than $T_{1}$ values at 500 MHz by approximately the ratio of the corresponding spectral densities, $J\left(2 \pi \times 51 \times 10^{6}\right) / J\left(2 \pi \times 61 \times 10^{6}\right)$. Taking into account the increase in chemical shift anisotropy at the higher field (expressed in Hertz), a $T_{1,600} / T_{1.500}$ ratio of about 1.28 is expected. Indeed, this is very close to the average ratio previously found for staphylococcal nuclease (Kay et al., 1989) ( $\tau_{\mathrm{c}} \approx 9 \mathrm{~ns}$ ). However, the $T_{1,600} / T_{1,500}$ ratios observed for $\mathrm{Ca}^{2+}$ free CaM are considerably smaller than 1.28 (Fig. 3B) and have an average ratio of only 1.18 . This smaller than anticipated $T_{1,600} / T_{1,500}$ ratio cannot be explained by motional anisotropy because the low value is observed throughout the protein. Note
that in a model of a protein tumbling with an axially symmetric diffusion tensor the amides oriented along the cylinder axis relax at the same rate as in a protein isotropically tumbling with a correlation time, $\tau_{\mathrm{c}}=1 /\left(6 D_{\perp}\right)$. So, regardless of $D_{\mathrm{f}}$, at least for helix $D$ one would expect a $T_{1,600} / T_{1,500}$ ratio close to 1.28 , in contrast to what is observed. A second indication that the smaller than expected $T_{1,000} / T_{1,500}$ ratio is not caused by motional anisotropy is the fact that the degree of anisotropy in the $T_{1}$ values is considerably less than what is observed for the $T_{2}$ values.

These observations can be explained by assuming that the entire N -terminal domain undergoes large amplitude motions on a time scale that significantly shortens the ${ }^{15} \mathrm{~N} T_{1}$ values relative to that of a slowly tumbling rigid dumbbell. Previously, Clore et al. (1990a, b) proposed one should use the extended spectral density function of Eqn (9) to account for such motions. When using this equation, $\tau_{c}$ is typically fixed at a constant value which is determined from residues with relaxation times that can be fit with the simple Lipari-Szabo spectral density function of Eqn (3), and $S_{\mathrm{f}}^{2}, S^{2}$ and the correlation time for slow internal motion, $\tau_{s}$, are optimized for each residue individually. In the present case, where an entire protein domain is subject to motion on an intermediate time scale, $\tau_{\mathrm{s}}$ is expected to be the same for all residues and the order parameter for this motional process, $S_{\mathrm{s}}^{2}$, is also expected to be uniform. In addition, as argued above, a $S_{\mathrm{f}}^{2}$ value of 0.85 is commonly observed in proteins for residues with a ${ }^{15} \mathrm{~N}-\left\{{ }^{1} \mathrm{H}\right\}$ NOE larger than 0.6 . Therefore, we have searched for the $\tau_{\mathrm{c}}, \tau_{\mathrm{s}}$, and $S^{2}$ values that optimize the fit between Eqn (9) and the observed $T_{1,600}, T_{1,500}, T_{2}, T_{1 \rho}$ and NOE data. The results of these searches are summarized in Table 3 where it can be seen that this extended model provides a considerably better fit to the experimental data than does the simple isotropic tumbling model (Table 2). The global internal motion model suggests an overall correlation time, $\tau_{c}$, in the $10-13-\mathrm{ns}$ range, with internal motions of the two individual domains on a time scale of $\approx 3 \mathrm{~ns}$. An internal correlation time of 3 ns is reasonable considering that small proteins the size of a single CaM domain are expected to tumble on this time scale (Peng and Wagner, 1992; Akke et al., 1993; Barchi et al., 1994).

The global internal motion as used in the present study is clearly oversimplified as it does not include the effects of anisotropy of the overall protein motion. The slow overall tumbling contributes relatively little to $T_{1}$ relaxation, but dominates $T_{2}$ relaxation and causes a pronounced dependence of the $T_{2}$, observed for a given amide, on the orientation of the $\mathrm{N}-\mathrm{H}$ bond vector relative to the principal axes of the overall diffusion tensor. Therefore, this model cannot account for the noticible anisotropy in the ${ }^{15} \mathrm{~N} T_{2}$ values, mentioned above, and the error function remains large when the $T_{2}$ data is included in the fitting procedure (Table 3). A model which includes the effect of

Table 3. Global rotational correlation time ( $\tau_{c}$ ), and correlation time ( $\tau_{\mathrm{s}}$ ) for the rigid body domain motions of the $\mathbf{N}$ - and $\mathbf{C}$-terminal domains of $\mathrm{Ca}^{2+}-$ free $\mathbf{C a M}$ at $23^{\circ} \mathrm{C}$, obtained using the extended spectral density function of $\mathbf{E q n}(9)$, and assuming $\tau_{\mathrm{f}}=0$. The generalized order parameter ( $S^{2}$ ) includes the effects of motion on the $\tau_{\mathrm{s}}$ time scale and of very fast ( $\tau_{\mathrm{f}} \approx 0$ ) internal motions, described by a uniform order parameter, $S_{\mathrm{f}}^{2}=0.85$. The error function, $E$, extends only over the input parameters used. Uncertainties correspond to the maximum deviations when repeating the search 200 times, omitting randomly $15 \%$ of the residues. $N$ and $p$ are the number of residues and the number of variables used in the fitting procedure.

| Input parameters | Domain | $\tau_{\text {c }}$ | $\tau_{\text {s }}$ | $S^{2}$ | $E$ | $N$ | $p$ |
| :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: |
|  |  | ns |  |  |  |  |  |
| $T_{1,600} ; T_{1,500} ; T_{2,600} ; T_{19,600}$ | N | $12.0 \pm 0.4$ | $2.8 \pm 0.2$ | $0.56 \pm 0.03$ | $15 \pm 4$ | 47 | 3 |
| $T_{1,600} ; T_{1,500} ;$ NOE | N | $12.8 \pm 0.5$ | $2.8 \pm 0.2$ | $0.56 \pm 0.02$ | $8 \pm 2$ | 47 | 3 |
| $T_{1,600} ; T_{1,500}$ | N | $13.6 \pm 1$ | $3.4 \pm 0.9$ | $0.53 \pm 0.04$ | $10 \pm 4$ | 47 | 3 |
| $T_{1,600} ; T_{1,500} ;$ NOE | C | $9.6 \pm 0.5$ | $2.2 \pm 0.2$ | $0.60 \pm 0.02$ | $7 \pm 2$ | 31 | 3 |
| $T_{1,600} ; T_{1.500}$ | C | $10.6 \pm 0.5$ | $2.8 \pm 0.4$ | $0.57 \pm 0.3$ | $9 \pm 3$ | 31 | 3 |

anisotropy of the overall motion requires knowledge of the orientation of each of the $\mathrm{N}-\mathrm{H}$ bond vectors within each of calmodulin's domains and therefore cannot be used in the present case.

The value for $S^{2} \approx 0.56$ corresponds to a value of $S_{\mathrm{s}}^{2}=$ $S^{2} / S_{\mathrm{f}}^{2}=0.66$. The assumption of restricted diffusion of the individual domains in a cone of semi-angle $\alpha$ may be used to obtain an approximate idea regarding the magnitude of these motions. Use of Eqn (4) indicates a value for $\alpha$ of $\approx 30^{\circ}$. Considering that both domains would freely diffuse in their own cones, the maximum angular excursion of one domain relative to the other one would be approximately $60^{\circ}$ in such a model. Indeed, the linker between helices D and E, extending over residues M76S81 is found to exhibit considerable flexibility, with $T_{2}$ values that are nearly $50 \%$ longer compared to helices $D$ and $E$. The ${ }^{15} \mathrm{~N}-\left\{{ }^{1} \mathrm{H}\right\}$ NOE values for these residues also drop to low values which is characteristic of high flexibility, indicating that these residues serve as the flexible tether between the two domains, completely analogous to that which was observed previously for $\mathrm{Ca}^{2+}$-ligated CaM (Barbato et al., 1992), except that the residual degree of anisotropy is somewhat larger in the present case.

## CONCLUSIONS

In the absence of calcium, CaM adopts a well-folded structure with numerous slowly exchanging backbone amide protons. Its dynamics cannot adequately be described by a model of an isotropically tumbling protein with fast, small, amplitude internal motions. In particular, the ${ }^{15} \mathrm{~N} T_{1}$ data measured at 51 and $61 \mathrm{MHz}{ }^{15} \mathrm{~N}$ frequency are incompatible with a model of a rigid protein tumbling anisotropically. Significantly better agreement with the measured relaxation data is obtained in a model where the individual domains have restricted mobility on a time scale of about 3 ns , superimposed on the overall tumbling of the molecule which occurs on a time scale which is about 4 times slower. Residues M76-S81 are located between helix D of the N-terminal domain and helix $E$, the first helix of the C -terminal domain. These residues show relaxation behavior characteristic of a flexible linker. In the simplest of models, where each entire domain is subject to restricted diffusion in a cone of semi-angle $\alpha$, the ${ }^{15} \mathrm{~N}$ relaxation data are compatible with $\alpha$ values of $\approx 30^{\circ}$, corresponding to a maximum fluctuation of $\pm 60^{\circ}$ in the relative orientations of helices D and E.

Hydrogen exchange data indicate that, on average, the N terminal domain is more stable than the C-terminal domain, confirming an earlier report on the melting behavior of the individual domains (Brzeska et al., 1983). However, slowly exchang-
ing amide protons are observed in all eight $\alpha$-helices and in three of the four short $\beta$-strands, indicating that these all form welldefined hydrogen-bonded structures. Substantial line broadening of many of the ${ }^{15} \mathrm{~N}$ resonances in the C -terminal domain is caused by conformational exchange, on a time scale of a few hundred microseconds. At the temperature where the present dynamics study was conducted $\left(23^{\circ} \mathrm{C}\right)$, one of the two conformers has a low population $(<10 \%)$. There is no correlation between the extent to which the ${ }^{15} \mathrm{~N}$ relaxation time of the individual C terminal residues is affected by conformational exchange and the rates at which the backbone amides exchange with solvent. It is therefore unlikely that the conformational exchange process is the cause for the faster hydrogen exchange in the C-terminal domain relative to that observed in the N -terminal domain. Considering that the conformational exchange occurs on the same time scale for all C-terminal residues affected by this process, it is also unlikely that the ${ }^{15} \mathrm{~N}$ broadening is the result of several independent conformational changes.

The overall tumbling of $\mathrm{Ca}^{2+}$-free CaM is somewhat slower and noticeably more anisotropic than for $\mathrm{Ca}^{2+}$-ligated CaM (Barbato et al., 1992). However, this difference is caused in part by the difference in temperature at which the two forms of the protein were studied. In fact, when studying the temperature dependence of the dynamics of $\mathrm{Ca}^{2+}$-ligated CaM , we found that, at the same temperature, the $\mathrm{Ca}^{2+}$-ligated form of the protein actually tumbles slower and with a similar degree of anisotropy as the $\mathrm{Ca}^{2+}$-free form (Do, R. et al., unpublished results). This is in agreement with small-angle X-ray scattering results which indicate a smaller maximum length vector for the $\mathrm{Ca}^{2+}$-free form (Yoshino et al., 1989; Heidorn and Trewhella, 1988).

The large difference in transverse ${ }^{15} \mathrm{~N}$ relaxation rates observed for amides in helix $C$ versus those in helix $D$ appears incompatible with the nearly antiparallel arrangement of these helices in the model for $\mathrm{Ca}^{2+}$-free CaM proposed by Strynadka and James (1988). This finding suggests that their troponin-Cbased model of the calcium-dependent conformational switch may need refinement. A definitive answer to this question is expected from the determination of the $\mathrm{Ca}^{2+}$-free CaM structure, presently in progress.

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## REFERENCES

Akke, M., Skelton, N. J., Kordel, J., Palmer, A. G. \& Chazin, W. J. (1993) Biochemistry 32, 9832-9844.

Alexandrescu, A. T. \& Shortle, D. (1994) J. Mol. Biol. 242, 527-546.
Babu, Y. S., Sack, J. S., Greenhough, T. J., Bugg, C. E., Means, A. R. \& Cook, W. J. (1985) Nature 315, 37-40.
Babu, Y. S., Bugg, C. E. \& Cook, W. J. (1988) J. Mol. Biol. 204, 191204.

Bai, Y., Milne, J. S., Mayne, L. \& Englander, S. W. (1993) Proteins, Struct. Funct. Genet. 17, 75-86.
Barbato, G., Ikura, M., Kay, L. E., Pastor, R. W. \& Bax, A. (1992) Biochemistry 31, 5269-5278.
Barchi, J. J., Grasberger, B., Gronenborn, A. M. \& Clore, G. M. (1994) Protein Sci. 3, 15-21.
Bax, A. \& Pochapsky, S. (1992) J. Magn. Reson. 99, 638-643.
Brzeska, H., Venyaminov, S. V., Grabarek, Z. \& Drabikowski, W. (1983) FEBS Lett. 153, 169-173.
Clore, G. M., Szabo, A., Bax, A., Kay, L. E., Driscoll, P. C. \& Gronenborn, A. M. (1990a) J. Am. Chem. Soc. 112, 4989-4991.
Clore, G. M., Driscoll, P. C., Wingfield, P. T. \& Gronenborn, A. M. (1990b) Biochemistry 29, 7387-7401.
Cohen, P. \& Klee, C. B. (1988) Calmodulin, Elsevier, New York.
Dellwo, M. J. \& Wand, A. J. (1989) J. Am. Chem. Soc. 111, 4571-4578.
Finn, B. E., Drakenberg, T. \& Forsen, S. (1994) FEBS Lett. 336, 368 374.

Fushman, D., Ohlenschlager, O. \& Rüterjans, H. (1994) J. Biomol. Struct. Dyn. 11, 1377-1402.
Garrett, D. S., Powers, R., Gronenborn, A. M. \& Clore, G. M. (1991) J. Magn. Reson. 94, 214-220.
Grzesiek, S. \& Bax, A. (1993a) J. Am. Chem. Soc. 115, 12593-12594.
Grzesiek, S. \& Bax, A. (1993b) J. Biomol. NMR 3, 185-204.
Heidorn, D. B. \& Trewhella, J. (1988) Biochemistry 27, 909-915.
Herzberg, O. \& James, M. N. G. (1988) J. Mol. Biol. 203, 761-779.
Herzberg, O., Moult, J. \& James, M. N. G. (1986) J. Biol. Chem. 261, 2638-2644.
Hiyama, Y., Niu, C., Silverton, J. V., Bavoso, A. \& Torchia, D. A. (1988) J. Am. Chem. Soc. 110, 2378-2383.

Hubbard, P. S. (1970) J. Chem. Phys. 52, 563-568.
Huntress, W. T. (1968) J. Chem. Phys. 48, 3524-3533.
Ikura, M., Marion, D., Kay, L. E., Shih, H., Krinks, M., Klee, C. B. \& Bax, A. (1990) Biochem. Pharmacol. 40, 153-160.
Ikura, M., Clore, G. M., Gronenborn, A. M., Zhu, G., Klee, C. B. \& Bax, A. (1992) Science 256, 632-638.
Jeng, M.-F. \& Englander, S. W. (1991) J. Mol. Biol. 221, 1045-1061.
Kamath, U. \& Shriver, J. W. (1989) J. Biol. Chem. 264, 5586-5592.
Kay, L. E., Nicholson, L. K., Bax, A. \& Torchia, D. A. (1992) J. Magn. Reson. 97, 359-375.

Note added in proof: Preliminary NMR structure calculations for $\mathrm{Ca}^{2+}$ free calmodulin indicate a CDD angle of $125^{\circ}$.

Kay, L. E., Torchia, D. A. \& Bax, A. (1989) Biochemistry 28, 89728979.

Lipari, G. \& Szabo, A. (1982a) J. Am. Chem. Soc. 104, 4546-4558.
Lipari, G. \& Szabo, A. (1982b) J. Am. Chem. Soc. 104, 4559-4570.
Marion, D., Ikura, M., Tschudin, R. \& Bax, A. (1989a) J. Magn. Reson. 85, 393-399.
Marion, D., Kay, L. E., Sparks, S. W., Torchia, D. A. \& Bax, A. (1989b) J. Am. Chem. Soc. 111, 1515-1517.

Meador, W. E., Means, A. R. \& Quiocho, F. A. (1992) Science 257, 1251-1255.
Meador, W. E., Means, A. R. \& Quiocho, F. A. (1993) Science 262, 1718-1721.
Orekhov, V. Y., Pervushin, K. V. \& Arseniev, A. S. (1994) Eur. J. Biochem. 219, 887-896.
Palmer, A. G. (1993) Curr. Opin. Biotechn. 4, 385-391.
Palmer, A. G., Skelton, N. J., Chazin, W. J., Wright, P. E. \& Rance, M. (1992) Mol. Phys. 75, 699-711.

Pastor, R. W., Venable, R. M., Karplus, M. \& Szabo, A. (1988) J. Chem. Phys. 88, 1128-1140.
Peng, J. W. \& Wagner, G. (1992) Biochemistry 31, 8571-8586.
Peng, J. W. \& Wagner, G. (1994) Methods Enzymol. 239, 563-596.
Peng, J. W., Thanabal, V. \& Wagner, G. (1991) J. Magn. Reson. 94, 82100.

Piotto, M., Saudek, V. \& Sklenar, V. (1992) J. Biomol. NMR 2, $661-$ 665.

Redfield, C., Boyd, J., Smith, L. J., Smith, R. A. G. \& Dobson, C. M. (1992) Biochemistry 31, 10431-10437.

Schneider, D. M., Dellwo, M. J. \& Wand, A. J. (1992) Biochemistry 31, 3645-3652.
Strynadka, N. C. \& James, M. N. G (1988) Proteins Struct. Funct. Genet. 3, 1-17.
Strynadka, N. C. J. \& James, M. N. G. (1989) Annu. Rev. Biochem. 58, 951-998.
Sundaralingam, M., Bergstrom, R., Strasburg, S., Rao, S. T., Roychowdhury, M., Greaser, M. \& Wang, B. C. (1985) Science 227, 945948.

Szyperski, T., Luginbühl, P., Otting, G. \& Wüthrich, K. (1993) J. Biomol. NMR 3, 151-164.
Taylor, D. A., Sack, J. S., Maune, J. F., Beckingham, K. \& Quiocho, F. A. (1991) J. Biol. Chem. 266, 21375-21380.

Torchia, D. A., Nicholson, L. K., Cole, H. B. R. \& Kay, L. E. (1993) NMR of proteins (Clore, G. M. \& Gronenborn, A. M., eds) pp. 190219, MacMillan, London.
van Mierlo, C. P., Darby, N. J., Keeler, J., Neuhaus, D. \& Creighton, T. E. (1993) J. Mol. Biol. 229, 1125-1146.

Wagner, G., Hyberts, S. \& Peng, J. W. (1993) NMR of proteins (Clore, G. M. \& Gronenborn, A. M., eds) pp. 220-257, MacMillan, London.

Woessner, D. T. (1962a) J. Chem. Phys. 37, 647-654.
Woessner, D. T. (1962b) J. Chem. Phys. 36, 1-4.
Yoshino, H., Minari, O., Matsushima, N., Ueki, T., Miyake, Y., Matsuo, T. \& Izumi, Y. (1989) J. Biol. Chem. 264, 19706-19709.

Zuiderweg, E. R. P. \& Fesik, S. W. (1989) Biochemistry 28, 2387-2391.


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    Abbreviations. CaM, calmodulin; HSQC, heteronuclear singlequantum correlation; $T_{1}$, longitudinal relaxation time; $T_{2}$, transverse relaxation time; $T_{1 e}$, spin-locked transverse relaxation time; rms, root-mean-square.

