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Measuring protein self-association using pulsed-field-gradient NMR spectroscopy: Application to myosin light chain 2

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Summary

At the millimolar concentrations required for structural studies, NMR spectra of the calcium-binding protein myosin light chain 2 (MLC2) showed resonance line widths indicative of extensive self-association. Pulsed-field-gradient (PFG) NMR spectroscopy was used to examine whether MLC2 aggregation could be prevented by the zwitterionic bile salt derivative 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS). PFG NMR measurements indicated that CHAPS was capable of preventing MLC2 self-association, but only at concentrations well above the critical micelle concentration of ~7.5 mM. CHAPS was most effective at a concentration of 22.5 mM, where the apparent molecular mass of MLC2 corresponded to a protein monomer plus seven molecules of bound detergent. The resolution and sensitivity of 2D ^{15}N-^{1}H HSQC spectra of MLC2 were markedly improved by the addition of 25 mM CHAPS, consistent with a reduction in aggregation following addition of the detergent. The average amide nitrogen T_2 value for MLC2 increased from ~30 ms in the absence of CHAPS to ~56 ms in the presence of 25 mM CHAPS. The results of this study lead us to propose that PFG NMR spectroscopy can be used as a facile alternative to conventional techniques such as analytical ultracentrifugation for examining the self-association of biological macromolecules.

Introduction

A prerequisite for protein structure determination using NMR spectroscopy is that the protein does not self-associate to give complexes larger than ~35 kDa (King and Mackay, 1995). The rapid T_2 relaxation associated with such large complexes makes coherence transfer, even via large heteronuclear couplings, very inefficient. Hence, the first step in the determination of protein structures using NMR spectroscopy is generally the elucidation of solution conditions that obviate protein self-association at the millimolar concentrations necessary for NMR analysis.

Myosin light chain 2 (MLC2) is an 18.7-kDa calcium-binding regulatory protein (Kretsinger and Nockolds, 1973), which is similar in structure to calmodulin (Ikura et al., 1992), troponin C (Herzberg and James, 1985), and calcineurin B (Anglister et al., 1993). It is located near the head/rod junction of the myosin molecule and appears to be involved in modulating the calcium sensitivity of cross-bridge cycling and stabilising the head/rod junction in skeletal muscle (Lowey et al., 1993; Sweeney et al., 1993). Although crystal structures of MLC2 bound to the myosin heavy chain have recently become available (Rayment et al., 1993; Xie et al., 1994), we are interested in determining the solution structure of MLC2 in order to investigate the conformational changes induced by calcium binding and phosphorylation.

Recombinant MLC2 self-associates extensively at millimolar concentrations, and is therefore unsuitable for NMR structural studies. It was recently shown (Anglister et al., 1993) that the related protein calcineurin B also aggregates at these concentrations, but that self-association could be prevented by addition of a 10- to 20-fold molar excess of the zwitterionic bile salt derivative 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS). From an NMR perspective, CHAPS is an ideal detergent for disaggregating proteins, as it is soluble over a wide pH range, it is not prone to precipitation by

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divalent cations, and it is nondenaturing (Hjelmeland et al., 1983; Partearroyo et al., 1988). Furthermore, protein dissociation is accompanied by the binding of only a small number of detergent molecules to the protein; for example, the self-association of cytochrome b_5 is prevented by the binding of ~12 CHAPS molecules to each protein monomer (Hori et al., 1988). Amide proton T_2 measurements on calcineurin B were consistent with binding of ~10 detergent molecules to the protein in the presence of 25 mM CHAPS (Anglister et al., 1993).

Thus, we decided to determine the optimal conditions for use of CHAPS as a protein disaggregating agent and to investigate whether it is capable of preventing self-association of MLC2. Since its introduction by Stejskal and Tanner (1965), pulsed-field-gradient (PFG) spin-echo NMR spectroscopy has been widely used for studying the translational diffusion of small and large molecules in chemical and biological systems (e.g., Haner and Schleich, 1989; Kuchel and Chapman, 1991,1993). We show that PFG NMR is a convenient method for measuring the self-association of detergents and biological macromolecules and that the measured translational diffusion coefficients can be used, albeit with some caveats, to estimate the molecular weights of complexes in solution.

Materials and Methods

Materials

$(^{15}NH_4)_2SO_4$ was purchased from Isotec (Miamisburg, OH). Isopropyl-β-D-thiogalactopyranoside (IPTG) was obtained from Sigma Chemical Co. (St. Louis, MO). The pET-3c expression vector and *Escherichia coli* BL21 cells were a gift from Dr. N.E. Dixon (Research School of Chemistry, Australian National University). All other materials were analytical grade.

Expression and purification of MLC2

A mutant version of the chicken skeletal *mlc2* gene ($Cys^{125} \rightarrow Arg$; Boey et al., 1994) was cloned into the pET-3c expression vector (Studier et al., 1990) to produce the plasmid pMLCII (N.E. Dixon, A.J. Dingley and G.F. King, unpublished results). *E. coli* BL21 cells, which also contain the plasmid pLysS, served as host for pMLCII. ^{15}N-labelled MLC2 was grown in a defined minimal medium, as previously described (Kralicek et al., 1993). After induction with 0.1 mM IPTG for cells grown in rich medium, or 0.4 mM IPTG for cells grown in minimal medium, incubation was continued for a further 4 h before harvesting. MLC2 was purified as previously described (Boey et al., 1994).

Pulsed-field-gradient NMR experiments

PFG NMR spectra were acquired at 298 K on a Bruker AMX-400 (9.4 T) wide-bore spectrometer. The magnetic field gradients were generated by an actively shielded coil assembly, mounted in fiberglass/epoxy resin around the radiofrequency (rf) coils of a multinuclear (15–162 MHz) probe designed for 10-mm sample tubes. Gradients were applied in the same direction as the field from the spectrometer magnet. ^1H NMR spectra were acquired via the ^1H decoupler coil. The magnitude of the magnetic field gradient (G) was calibrated as previously described (Kuchel and Chapman, 1991) to be 105.5 and 211 mT m^{-1} for experiments on CHAPS and MLC2, respectively.

Samples for PFG NMR experiments were placed in a cylindrical semimicro bulb (Cat. 529-E-10; Wilmad, Buena, NJ), which was subsequently inserted into a 10-mm NMR tube containing 2.5 ml of CCl_4 (in order to match the magnetic susceptibility on either side of the bulb). The PFG longitudinal eddy-current delay (LED) pulse sequence (Gibbs and Johnson, 1991; see Fig. 1) was used to measure the translational diffusion coefficients of CHAPS and MLC2. The PFGLED sequence minimises the effects of gradient-induced eddy currents and is well suited to studying the diffusion of macromolecules with $T_2 \ll T_1$. The pulse sequence was modified by the incorporation of homospoil gradient pulses after the second and fourth rf pulses, in order to eliminate any transverse magnetization that may have otherwise arisen during the longitudinal storage periods, T and T_e (see Fig. 1; Waldeck et al., 1993). Each diffusion measurement was obtained from a series of 12 PFGLED spectra in which the delay periods ($\tau = 20$ ms, $\Delta = 50$ ms, $T = 30$ ms, and $T_e = 14$ ms) and the magnitude of G were held constant, but the length of the field-gradient pulse (δ) was incremented in 1.5-ms steps from 0 ms in the first spectrum to 16.5 ms in the final spectrum.

All spectra consisted of 8096 data points with a spectral width of 4.4 kHz. Spectra of CHAPS were derived from 32 summed transients, whereas 256–1024 transients were used to collect spectra of MLC2 with and without CHAPS. Free induction decays were multiplied by a decaying exponential function with a line-broadening factor of 3 Hz prior to Fourier transformation. A third-order baseline correction was applied to the spectra of MLC2 with and without CHAPS. For each series of experiments, a 'dummy' PFGLED spectrum ($G = 0$ T m^{-1}) was acquired before recording the PFG NMR experiment, to ensure that the sample had reached thermal equilibrium.

Theory and analysis of PFG NMR experiments

For unbounded diffusion of a molecule in an isotropic medium, the signal intensity recorded in a PFG NMR experiment, normalized to the signal obtained in the absence of the magnetic field-gradient pulses, is given by (Stejskal and Tanner, 1965):

$$R = \exp(-\gamma^2 G^2 D \delta^2(\Delta - \delta/3)) \quad (1)$$

where G and δ are the magnitude and duration of the magnetic field-gradient pulses, respectively, Δ is the time

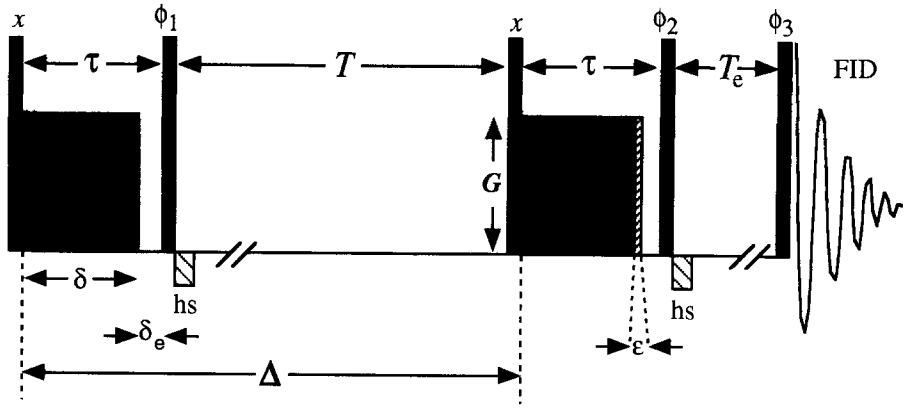

Fig. 1. Modified PFGLED pulse sequence, used for measuring translational diffusion coefficients. Solid bars represent 90° pulses. Any imbalance between the time integrals of the two magnetic field-gradient pulses (shaded bars) was compensated for by an experimentally determined correction factor, ε. Negative homospoil (hs) gradient pulses were employed to eliminate any transverse magnetisation that may have otherwise arisen during the longitudinal storage periods (T and T_e). Phase cycling was as follows: $\phi_1 = x,-x$; $\phi_2 = 2(x),2(-x)$; $\phi_3 = 4(x),4(y),4(-x),4(-y)$; receiver = x,2(−x),x,y,2(−y),y,−x,2(x),−x,−y,2(y),−y.

between field-gradient pulses, and γ is the gyromagnetic ratio of the observed nucleus. Thus, nonlinear least-squares regression of an exponential onto the data, using $x = \gamma^2 G^2 \delta^2 (\Delta - \delta/3)$ as the independent variable, yields the translational diffusion coefficient, D, and an estimate of its standard deviation (σ). A three-parameter fit of the form $R = R_\infty + R_0 \exp(-Dx)$ was used to fit the data, where R_0 and R_∞ are the normalised resonance intensities at zero and infinite time, respectively.

The D of CHAPS was calculated using the singlet resonance arising from the magnetically equivalent $-N^+(CH_3)_2-$ protons (see Fig. 2). For both CHAPS and MLC2, the weighted mean and grouped standard deviation ($D_m \pm \sigma_m$) were calculated, using $1/\sigma^2$ as the weights, when more than one PFG experiment was performed under identical conditions (Spiegel, 1972).

Calculation of the effective molecular weights of CHAPS and MLC2

The translational diffusion coefficient is related to the molecular weight, M, of the diffusing species by the following equation (Cantor and Schimmel, 1980):

$$M = \left(\frac{kT}{6\pi\eta FD}\right)^3 \left(\frac{4\pi N_A}{3[\bar{v}_2 + \delta_1 \bar{v}_1]}\right) \qquad (2)$$

where k is the Boltzmann constant ($J\,K^{-1}$), T is the temperature (K), η is the viscosity of the solution ($kg\,m^{-1}\,s^{-1}$), D is the translational diffusion coefficient ($m^2\,s^{-1}$), N_A is Avogadro's number (mol^{-1}), $\bar{v}_2$ is the partial specific volume of the molecule ($m^3\,kg^{-1}$), δ_1 is the fractional amount of water bound to the molecule (g H_2O per g diffusant), and $\bar{v}_1$ is the partial specific volume of solvent water ($m^3\,kg^{-1}$). The dimensionless Perrin factor, F, which relates to the shape of the molecule, is equal to the ratio of the frictional coefficient of the diffusant (f) to that of a hard sphere of equivalent mass (f_{sphere}). F can be readily calculated for ellipsoids of revolution in which two of the semiaxes are identical; for an oblate ellipsoid, in which the two long semiaxes are identical, F is defined by the following equation (Cantor and Schimmel, 1980):

$$F = \frac{f}{f_{sphere}} = \frac{(p^2-1)^{1/2}}{p^{2/3}\arctan\left[(p^2-1)^{1/2}\right]} \qquad (3)$$

where p = a/b, and a and b are the long and short semiaxes, respectively.

Molecular weights of CHAPS complexes were calculated using Eq. 2, assuming that F = 1 (i.e., a hard sphere), and that negligible water is bound to CHAPS (i.e., $\delta_1 \bar{v}_1 = 0$). The partial specific volume of CHAPS is $0.81 \times 10^{-6}\,m^3\,kg^{-1}$ (Hjelmeland et al., 1983). The microviscosity of water is relatively unaffected by small volume fractions of protein (Endre and Kuchel, 1986); hence, since the largest concentration of MLC2 used in this study (2.8 mM) corresponds to a volume fraction of only ~0.04, the solution viscosity was taken to be that of water at 298 K ($8.904 \times 10^{-4}\,kg\,m^{-1}\,s^{-1}$; Weast, 1984).

The molecular weight of MLC2 was calculated using a partial specific volume of $0.73 \times 10^{-6}\,m^3\,kg^{-1}$, calculated on the basis of amino acid composition (Perkins, 1986). An F value for MLC2 was calculated from measurements of the semiaxis lengths in the solution structure of the related calcium-binding protein calmodulin, bound to a fragment of myosin light chain kinase (Brookhaven accession number 2bbm; Ikura et al., 1992). The solution structure of calmodulin approximates an oblate ellipsoid with semiaxis lengths of 1 : 1.82 : 1.84; this gives an F value of 1.03 when p = 1.83 is substituted into Eq. 3. Molecular weights were calculated using a range of δ_1 values considered typical of most proteins (0.30–0.40 g H_2O per g protein; Cantor and Schimmel, 1980); a value of 0.34 corresponds to a protein hydration shell approximately one water molecule thick.

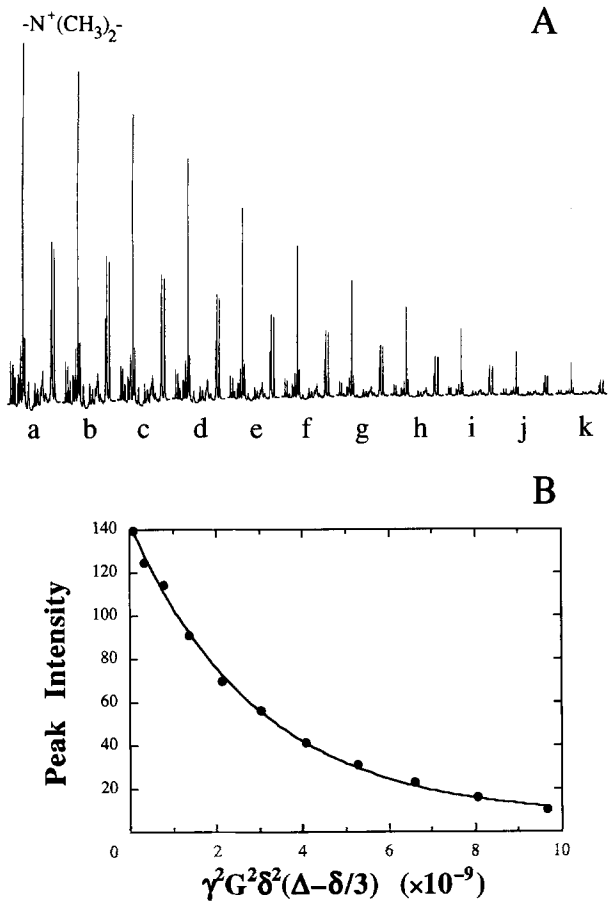

Fig. 2. (A) PFGLED NMR spectra of CHAPS. Each panel (labelled a–k) represents a 1600 Hz region of the ^{1}H NMR spectrum encompassing the -$N^+(CH_3)_2$- resonance. The magnetic field-gradient pulses were incremented by 1.5 ms between sequential spectra, starting at 1.5 ms in spectrum a. (B) Peak intensity of the CHAPS -$N^+(CH_3)_2$- resonance plotted as a function of $\gamma^2 G^2 \delta^2(\Delta - \delta/3)$. Nonlinear regression of a decaying exponential onto the data (solid line) yielded the translational diffusion coefficient (D).

Heteronuclear NMR experiments

Two-dimensional (2D) NMR spectra of MLC2 were acquired at 310 K on a Bruker AMX-600 (14.1 T) narrow-bore NMR spectrometer. Samples contained 1.5 mM uniformly ^{15}N-labelled MLC2 in 50 mM KCl, 15 mM DTT, 10 mM NaH_2PO_4 / Na_2HPO_4, pH 6.8 (95% H_2O/5% D_2O). 2D ^{1}H-^{15}N heteronuclear single-quantum coherence (HSQC) spectra were acquired without solvent presaturation, as previously reported (Kralicek et al., 1993). Spectral widths were 6.6 and 1.5 kHz for F_2 and F_1, respectively, and time-proportional phase incrementation was employed to achieve quadrature detection in F_1 (Marion and Wüthrich, 1983). Spectra consisted of 256 t_1 increments, each collected as 1024 complex data points. The digital resolution was improved by forward linear prediction to 384 data points in t_1, followed by zero-filling to 2048×512 ($F_2 \times F_1$) real data points. After phase correction, base lines were corrected by subtracting a third-order polynomial fitted to the base line.

An approximate average T_2 (strictly $T_{1\rho}$) for the backbone amide nitrogens of MLC2 was obtained using a 1D version of the sensitivity-enhanced 2D experiment described previously by Kördel et al. (1992), except that a single low-power ^{15}N spin-lock pulse was used instead of a CPMG sequence to retain ^{15}N magnetisation in the transverse plane during the spin–spin relaxation period (τ). Eleven separate experiments were recorded, each with a different value of τ. The τ values ranged from 1 to 90 ms for the sample of MLC2 alone, and from 5 to 300 ms for the sample containing CHAPS; an intertransient delay of 4 s was used for both experiments. The strength of the spin-lock field was 2.9 kHz, and all other parameters were as described above for the 2D HSQC experiments. Plots of signal intensity versus τ were made at three chemical shift values within the amide proton envelope and an exponential function of the form $I = I_\infty + I_0 \exp(-\tau/T_2)$, where I_0 and I_∞ are the resonance intensities at zero and infinite time, respectively, was fitted to the data using a nonlinear least-squares protocol. The 'average' ^{15}N T_2 value was calculated as the mean of these three values.

Results

Self-association of CHAPS

PFGLED NMR spectra were recorded at various CHAPS concentrations. The resonance intensities arising from the -$N^+(CH_3)_2$- protons of CHAPS were measured as a function of the duration of the magnetic field-gradient pulses (see Fig. 2A). Note that J modulation of multiplets and the effects of T_2 relaxation can be disregarded as the spin-echo delay time (τ) and the total length of the pulse sequence ($2\tau + T + T_e$ + Acq.) are the same for all PFGLED experiments. Thus, the diminution in signal intensity with increasing δ is solely a manifestation of diffusion during the field-gradient pulses and the interval between them; hence, values of the translational diffusion coefficient were estimated by nonlinear regression of Eq. 1 onto the data (see Fig. 2B and Materials and Methods). Figure 3A illustrates that, as the detergent concentration was increased, the translational diffusion coefficient decreased, reflecting an increase in the apparent molecular mass of the detergent (see Fig. 3B).

The calculated apparent molecular mass of CHAPS at 1 mM is ~1200 Da (see Fig. 3B), which corresponds to a dimer. The abrupt decrease in the translational diffusion coefficient of CHAPS at a concentration of ~7.5 mM corresponds to the critical micelle concentration (CMC). However, this is not a sharp (infinitely cooperative) transition and, as illustrated in Fig. 3B, there is a monotonic increase in the apparent (population average) mass of CHAPS as the detergent concentration is increased beyond the CMC. We interpret this as representing the thermodynamic equilibrium between CHAPS dimers and

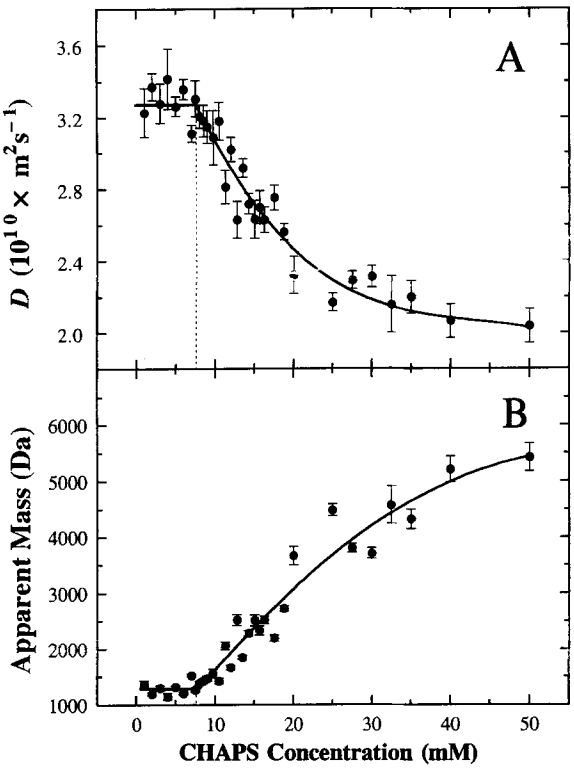

Fig. 3. (A) Plot of the translational diffusion coefficient of CHAPS as a function of its concentration. Error bars denote ± 1 SD. The solid line is simply intended to guide the eye. The dotted line represents the approximate CMC of the detergent. (B) Plot of the apparent molecular mass of CHAPS as a function of the detergent concentration, calculated from the measured diffusion coefficients using Eq. 2. The solid line is simply intended to guide the eye. CHAPS is initially dimeric, even at very low concentrations, but forms larger aggregates as the detergent concentration is increased.

micelles, which shifts towards micelles as the detergent concentration is increased. At 50 mM CHAPS, the calculated mass (see Fig. 3B) corresponds to a nonamer.

Self-association of MLC2

PFGLED experiments were used to measure the translational diffusion coefficient of MLC2 as a function of the CHAPS concentration (Fig. 4A). Equation 2 was used to calculate the apparent molecular mass of MLC2 from the measured diffusion coefficients over a range of protein hydration values (see Fig. 4B). In the absence of CHAPS, MLC2 self-associated to give aggregates with an apparent molecular mass of ~60 kDa, corresponding to trimers of MLC2 molecules. The average backbone amide proton T_2 of ~8 ms is consistent with this high level of aggregation.

Upon titration with CHAPS, the apparent molecular mass of MLC2 did not change significantly until well beyond the CMC of the detergent (see Fig. 4B). However, the apparent mass decreased dramatically from ~50 kDa at 15 mM CHAPS to ~23 kDa at 22.5 mM CHAPS. The latter mass was equivalent to a monomer of MLC2 (18.7 kDa) plus approximately seven molecules (~4.3 kDa) of bound CHAPS. Increasing the CHAPS concentration beyond 22.5 mM caused the molecular mass of the protein–detergent complex to increase, consistent with binding of additional detergent molecules to monomeric MLC2.

2D ^1H-^{15}N HSQC spectra of MLC2

The resolution and sensitivity observed in the HSQC spectrum of MLC2 were markedly improved by the addition of 25 mM CHAPS (compare Figs. 5A and B). As observed for calcineurin B (Anglister et al., 1993), the resonances that were resolved in both spectra had similar chemical shifts, suggesting that the improvement in spectral quality resulted from a marked reduction in MLC2 self-association, without a large detergent-induced alteration in conformation. The average backbone amide nitrogen T_2 value increased from ~30 ms in the absence of CHAPS to ~56 ms in the presence of 25 mM CHAPS, consistent with the PFGLED and HSQC results.

Discussion and Conclusions

We have used PFG NMR spectroscopy to probe the apparent molecular mass of MLC2 in the presence and absence of the zwitterionic detergent CHAPS. PFGLED measurements on CHAPS alone indicated that the deter-

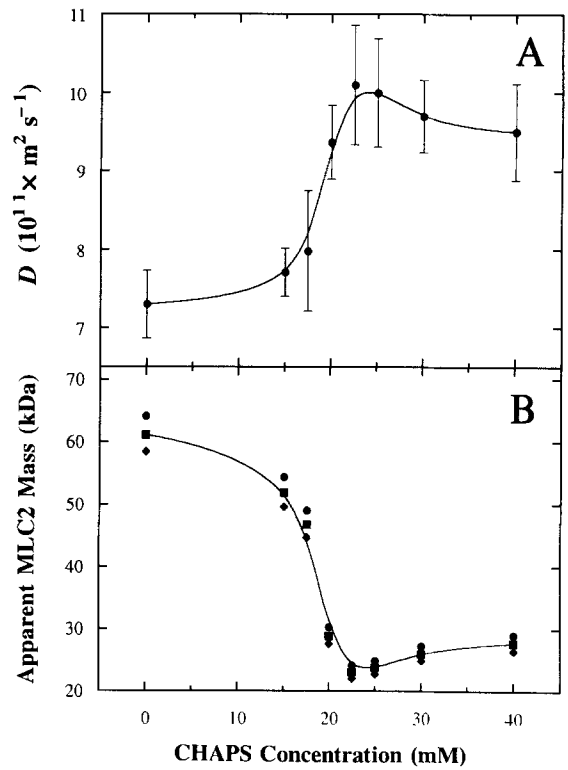

Fig. 4. (A) Plot of the translational diffusion coefficient of MLC2 as a function of the CHAPS concentration. Error bars denote ± 1 SD. (B) Plot of the apparent molecular mass of MLC2 as a function of CHAPS concentration, calculated from the measured diffusion coefficients using Eq. 2. The circles, squares, and diamonds represent calculations using hydration values of 0.30, 0.35, and 0.40 g H_2O per g of protein, respectively.

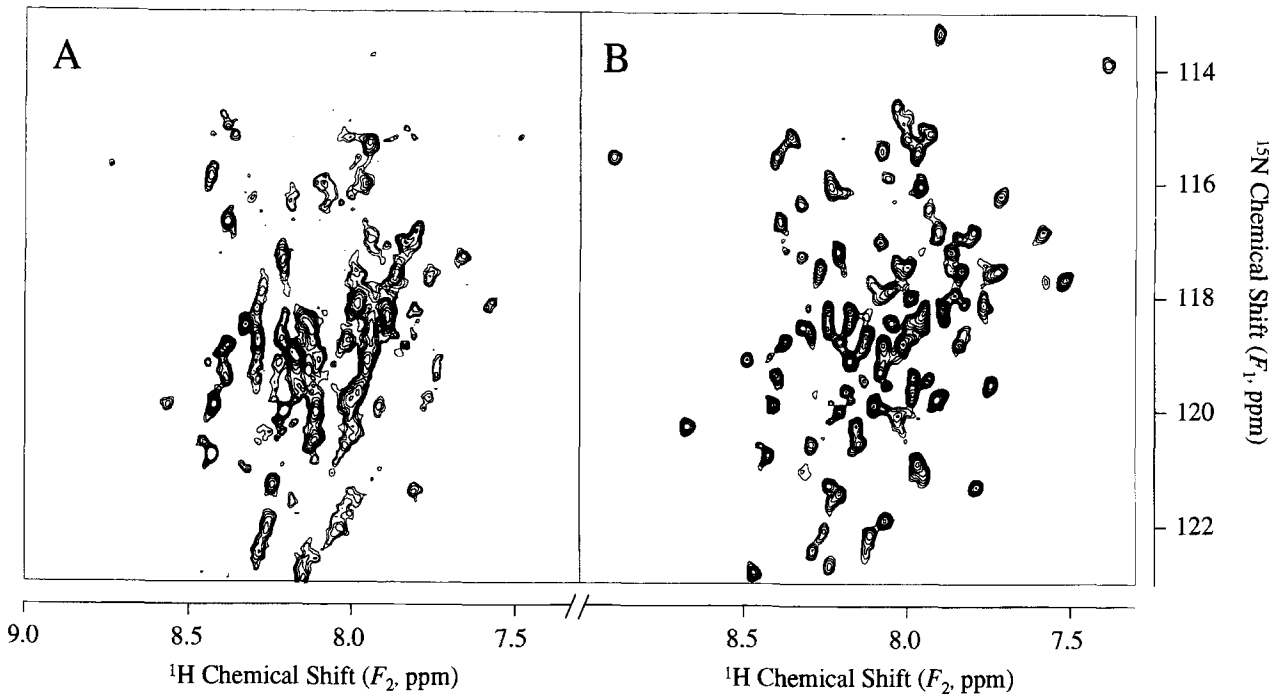

Fig. 5. The most crowded region of the 2D ^{1}H-^{15}N HSQC spectrum of MLC2 acquired in the absence (A) or presence (B) of 25 mM CHAPS. The spectra were acquired and processed using identical parameters, and are plotted at the same contour levels. The spectrum acquired in the presence of CHAPS displays markedly enhanced resolution and sensitivity.

gent has a critical micelle concentration of ~7.5 mM; this is consistent with previous estimates that ranged from 4 to 11 mM, depending on the measurement technique employed (Hjelmeland et al., 1983; Chattopadhyay and London, 1984; Stark et al., 1984; Partearroyo et al., 1988). The CMC of CHAPS was unaltered by the presence of 50 mM KCl (data not shown), as expected for a zwitterionic detergent for which micelle formation does not involve a 'common ion' effect (Roda et al., 1983).

The measured translational diffusion coefficients were used to calculate apparent molecular masses. At low concentrations, CHAPS formed a dimer. The apparent mass increased monotonically as the detergent concentration was increased, with the calculated average mass at 50 mM CHAPS corresponding to a nonamer, consistent with a thermodynamic equilibrium between a small fraction of dimers and a larger proportion of micelles with an aggregation number of 10, as reported previously (Hjelmeland et al., 1983). Thus, the NMR data are consistent with CHAPS forming small micelles of ~10 molecules in size, in contrast with detergents such as sodium dodecylsulfate and Triton X-100, which form very large aggregates of 60–140 molecules (Helenius and Simons, 1975).

Analytical ultracentrifugation studies suggested that, even at rather low concentrations (≥0.2 mM), MLC2 self-associates (data not shown). The translational diffusion coefficients and T_2 values measured for MLC2 in the absence of CHAPS support this result. However, PFGLED NMR measurements indicated that MLC2 aggregation is prevented at CHAPS concentrations above 22 mM, even for relatively high concentrations of protein (≥2 mM). In the presence of 22.5 mM CHAPS, MLC2 was monomeric with approximately seven molecules of bound detergent. Note that the number of bound detergent molecules corresponds approximately to the CHAPS aggregate number, suggesting that dissociation of the protein may correspond to the binding of one CHAPS micelle. Adding additional detergent beyond the optimal concentration range (22–25 mM) caused a gradual increase in the apparent mass of the protein, presumably due to the binding of additional detergent molecules to MLC2. Hence, when attempting to use detergents for preventing protein self-association during NMR structural studies, PFG NMR provides a simple means of determining the detergent concentration that gives maximal alleviation of protein self-association and a minimal number of detergent molecules bound to the protein.

CHAPS was largely ineffective at dissociating MLC2 aggregates at concentrations close to the CMC. Significant disaggregation of MLC2 did not occur until the detergent concentration was at least twice the CMC value, and optimal prevention of MLC2 self-association occurred at a CHAPS concentration of 22.5 mM. These observations are consistent with previous studies, which showed that CHAPS is most effective at preventing protein self-association at concentrations greater than 20 mM (Hori et al., 1988; Anglister et al., 1993). This contrasts with *solubilization* of proteins by CHAPS, which simply requires detergent concentrations close to or higher than the CMC (Hjelmeland, 1980; Simonds et al., 1980).

As evidenced by the data in Fig. 4B, protein disaggregation does not suddenly occur at some critical value of the [CHAPS]:[protein] ratio. Rather, if we assume that it is the micellar form of CHAPS which preferentially binds to the protein, then the extent to which protein self-association is prevented will depend upon both the equilibrium constant describing micelle formation ($K_{micelle}$, which determines the CMC) and the equilibrium constant describing binding of the micelle to the protein ($K_{protein}$). Detergents with higher values of $K_{micelle}$ (i.e., lower CMCs) are therefore likely to prevent protein self-association at lower detergent concentrations as long as the affinity of the micelles for the protein is not dramatically altered. It would therefore be interesting to investigate the dissociative properties of bile salt derivatives with CMCs lower than that of CHAPS – subtle covalent modifications can give bile salt derivatives with CMCs as low as 1.8 mM (Roda et al., 1983).

Studies of the interaction between calcineurin B and CHAPS (Anglister et al., 1993) showed that it is the hydrophobic portion of the detergent which interacts directly with the protein, and the same is probably true for MLC2. Recent crystallographic studies (Rayment et al., 1993; Xie et al., 1994) showed that MLC2 interacts with the myosin heavy chain through a large number of hydrophobic residues, in a manner similar to that observed for the binding of calmodulin to myosin light chain kinase (Ikura et al., 1992). These hydrophobic residues lie on the surface of MLC2 and are likely to cause aggregation of this and other related proteins. Thus, the reduction of protein aggregation observed following the addition of CHAPS is probably the result of the interaction of these surface-exposed hydrophobic residues with the cholate moiety of the detergent. Circular dichroism spectra revealed a small conformational change in MLC2, corresponding to a 4% increase in the α-helical content, upon addition of 25 mM CHAPS (data not shown). This suggests that CHAPS stabilises a nascent helical region of MLC2, perhaps the region involved in the interaction with the myosin heavy chain, which is largely helical in the crystal structure of the myosin light chain–heavy chain complex (Rayment et al., 1993). Elimination of MLC2 self-association by CHAPS resulted in a drastic improvement in 2D ^{1}H-^{15}N HSQC spectra of the protein (see Fig. 5), which has enabled us to commence NMR structural analysis.

PFG NMR spectroscopy represents a facile alternative to conventional methods such as sedimentation analysis (Morris and Ralston, 1994) and quasi-elastic light scattering (Mikol et al., 1990) for studying the self-association of proteins and other biological macromolecules. The technique is likely to find wide application, given the recent advent of commercial PFG probes for NMR structural analysis. However, molecular weights need to be extracted from translational diffusion coefficients with some caution. The shape factor (F) and water solvation of the protein (δ_1), which appear in Eq. 2, are generally not known a priori. Wherever possible, experimentally determined values of these parameters should be used. However, as shown in Fig. 4B, δ_1 can be varied widely without significantly altering the calculated molecular weight. While the Perrin factor only increases to ~1.2 for proteins modelled as prolate or oblate ellipsoids with axial ratios as large as 5:1, this parameter has a more dramatic effect on the calculated molecular weight because of the inverse cubic dependence of M on this term. However, for most proteins, F values of 1.00–1.18 (covering shapes from spheres to prolate and oblate ellipsoids with axial ratios of 4:1) should suffice for most molecular weight calculations.

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References

Anglister, J., Grzesiek, S., Ren, H., Klee, C.B. and Bax, A. (1993) *J. Biomol. NMR*, **3**, 121–126.

Boey, W., Huang, W., Bennetts, B., Sparrow, J., dos Remedios, C. and Hambly, B. (1994) *Eur. J. Biochem.*, **219**, 603–610.

Cantor, C.R. and Schimmel, P.R. (1980) *Biophysical Chemistry, Part II: Techniques for the Study of Biological Structure and Function*, W.H. Freeman, New York, NY, pp. 539–590.

Chattopadhyay, A. and London, E. (1984) *Anal. Biochem.*, **139**, 408–412.

Endre, Z.H. and Kuchel, P.W. (1986) *Biophys. Chem.*, **24**, 337–356.

Gibbs, S.J. and Johnson Jr., C.S. (1991) *J. Magn. Reson.*, **93**, 395–402.

Haner, R.L. and Schleich, T. (1989) *Methods Enzymol.*, **176**, 418–447.

Helenius, A. and Simons, K. (1975) *Biochim. Biophys. Acta*, **415**, 29–79.

Herzberg, O. and James, M.N.G. (1985) *Nature*, **313**, 653–659.

Hjelmeland, L.M. (1980) *Proc. Natl. Acad. Sci. USA*, **77**, 6368–6370.

Hjelmeland, L.M., Nebert, D.W. and Osborne Jr., J.C. (1983) *Anal. Biochem.*, **130**, 72–82.

Hori, A., Hayashi, F., Kyogoku, Y. and Akutsu, H. (1988) *Eur. J. Biochem.*, **174**, 503–508.

Ikura, M., Clore, G.M., Gronenborn, A.M., Zhu, G., Klee, C.B. and Bax, A. (1992) *Science*, **256**, 632–638.

King, G.F. and Mackay, J.P. (1995) In *NMR in Drug Design* (Ed., Craik, D.J.), Chapter 4, CRC Press, Boca Raton, FL, pp. 101–200.

Kördel, J., Skelton, N.J., Akke, M., Palmer III, A.G. and Chazin, W.J. (1992) *Biochemistry*, **31**, 4856–4866.

Kralicek, A.V., Vesper, N.A., Ralston, G.B., Wake, R.G. and King, G.F. (1993) *Biochemistry*, **32**, 10216–10223.

Kretsinger, R.H. and Nockolds, C.E. (1973) *J. Biol. Chem.*, **248**, 3313–3326.

Kuchel, P.W. and Chapman, B.E. (1991) *J. Magn. Reson.*, **94**, 574–580.

Kuchel, P.W. and Chapman, B.E. (1993) *J. Magn. Reson. Ser. A*, **101**, 53–59.

Lowey, S., Waller, G.S. and Trybus, K.M. (1993) *Nature*, **365**, 454–456.

Marion, D. and Wüthrich, K. (1983) *Biochem. Biophys. Res. Commun.*, **113**, 967–974.

Mikol, V., Hirsch, E. and Giege, R. (1990) *J. Mol. Biol.*, **213**, 187–195.

Morris, M.B. and Ralston, G.B. (1994) In *Physicochemical Methods in the Study of Biomembranes* (Eds., Hilderson, H.J. and Ralston, G.B.), Subcellular Biochemistry, Vol. 23, Plenum Press, New York, NY, pp. 25–82.

Partearroyo, M.A., Goñi, F.M., Katime, I. and Alonso, A. (1988) *Biochem. Int.*, **16**, 259–265.

Perkins, S.J. (1986) *Eur. J. Biochem.*, **157**, 169–180.

Rayment, I., Rypniewski, W.R., Schmidt-Bäse, K., Smith, R., Tomchick, D.R., Benning, M.M., Winkelmann, D.A., Wesenberg, G. and Holden, H.M. (1993) *Science*, **261**, 50–65.

Roda, A., Hofmann, A.F. and Mysels, K.J. (1983) *J. Biol. Chem.*, **258**, 6362–6370.

Simonds, W.F., Koski, G., Streaty, R.A., Hjelmeland, L.M. and Klee, W.A. (1980) *Proc. Natl. Acad. Sci. USA*, **77**, 4623–4627.

Spiegel, M.R. (1972) *Theory and Problems of Statistics*, McGraw-Hill, New York, NY.

Stark, R.E., Leff, P.D., Milheim, S.G. and Kropf, A. (1984) *J. Phys. Chem.*, **88**, 6063–6067.

Stejskal, E.O. and Tanner, J.E. (1965) *J. Chem. Phys.*, **42**, 288–292.

Studier, F.W., Rosenberg, A.H., Dunn, J.J. and Dubendorff, J.W. (1990) *Methods Enzymol.*, **185**, 60–89.

Sweeney, H.L., Bowman, B.F. and Stull, J.T. (1993) *Am. J. Physiol.*, **264**, C1085–C1095.

Waldeck, A.R., Lennon, A.J., Chapman, B.E. and Kuchel, P.W. (1993) *J. Chem. Soc., Faraday Trans.*, **89**, 2807–2814.

Weast, R.C. (Ed.) (1984) *CRC Handbook of Chemistry and Physics*, 64th ed., CRC Press, Boca Raton, FL.

Xie, X., Harrison, D.H., Schlichting, I., Sweet, R.M., Kalabokis, V.N., Szent-Györgyi, A.G. and Cohen, C. (1994) *Nature*, **368**, 306–312.