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Isotope-edited multidimensional NMR of calcineurin B in the presence of the non-deuterated detergent CHAPS

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SUMMARY

At the concentration needed for NMR, the calcium-saturated form of calcineurin B dissolved in water shows resonance line widths that indicate aggregation of this protein. Although the line width or aggregation state can be influenced to some degree by temperature, pH, and salt concentrations, in the absence of detergent no conditions could be found where the protein behaved as a monomeric unit. In the presence of a 10- to 20-fold molar excess of the zwitterionic detergent 3-[(3-cholamidopropyl)-dimethyl-ammonio]-1-propanesulfonate (CHAPS), resonance line widths were considerably narrower and were compatible with a protein of ~25 kDa. The presence of the NMR signals of the non-deuterated CHAPS does not interfere with modern isotope-directed NMR studies as the signals from protons not attached to ^{15}N or ^{13}C are removed by isotope filtering and purge pulses.

A prerequisite for determining the solution structure of a protein by NMR is that it must be soluble and non-aggregating at a concentration of at least ~1 mM. Aggregation of proteins causes an increase in the rotational correlation time, which increases the resonance line width of the protons and heteronuclei. As a result, for such aggregating proteins the sensitivity of experiments measuring through-bond correlations drops dramatically and in many cases is beyond detection. Also, the resolution of multidimensional spectra rapidly decreases with increasing line widths, which usually makes it impossible to study aggregating proteins. Aggregation can be the result of interprotein electrostatic interactions or of the presence of hydrophobic patches on the protein surface. The degree of protein aggregation may depend on pH, temperature, and the type and concentration of salts added. The first step in every protein NMR study therefore involves optimization of the measurement conditions, which are usually geared towards obtaining the narrowest proton line widths without affecting the structure of the protein.

Detergents that form micelles can be used to study the structures of peptides and proteins that

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are insoluble in H_2O or to induce a conformational change in those peptides that adopt a random coil conformation in aqueous solution. To date, the most commonly used detergents for detailed protein ^1H NMR studies are sodium dodecyl sulfate (SDS) (O'Neil and Sykes, 1989) and dodecyl phosphocholine (DPC) (Wider et al., 1982). The popularity of these detergents is due in part to the fact that both can be obtained commercially in a fully deuterated form, making it possible to observe the protein ^1H NMR spectra in the presence of high detergent concentrations. However, as was recently demonstrated in detail for bacteriorhodopsin, the rotational correlation time of the solubilized protein and its temperature stability depend strongly on the type(s) of detergent used (Seigneuret et al., 1991a,b). The use of non-deuterated detergents in their work did not interfere with the NMR measurements as only ^{13}C nuclei were detected, with no detergent ^{13}C resonances being observed in the regions of interest. Here we demonstrate the use of the zwitterionic detergent 3-[(3-cholamidopropyl)-dimethyl-ammonio]-1-propanesulfonate (CHAPS, Hjelmeland et al., 1983) to minimize the degree of aggregation observed for the protein calcineurin B. As will be demonstrated, the use of a protonated detergent does not interfere with many of the recently introduced proton-detected multinuclear protein NMR experiments when applied to uniformly $^{13}C/^{15}N$-enriched proteins.

Calcineurin is a calmodulin and calcium-stimulated protein phosphatase present in all eukaryotic cells, but primarily in nerve cells (Guerini and Klee, 1991). Recently, it has been found that calcineurin is a target for the complexes of the immunosuppressive drugs cyclosporin and FK-506 with their respective receptor proteins, cyclophilin and FKBP (Liu et al., 1991). Calcineurin consists of two non-covalently tightly bound subunits, calcineurin A and calcineurin B, with molecular weight of 59 and 19 kDa, respectively. The primary sequence of calcineurin B indicates that it contains four calcium binding sites of the EF-hand type (Guerini and Klee, 1991). Natural calcineurin B is myristoylated at its amino-terminal glycine residue (Aitken et al., 1982), but the present NMR work deals with genetically engineered calcineurin B, which is overexpressed and isotopically enriched in *Escherichia coli*, and which lacks the post-translational myristoylation.

An approximate value for the rotational correlation time, τ_c, of a protein can be derived from the average transverse relaxation time, T_2, of the amide protons. The spectral dispersion of these protons also can be used as a qualitative indication of structural integrity; upon denaturation, these amides tend to cluster at ~8 ppm. The 1–1 echo sequence (Sklenar and Bax, 1987) provides a convenient means for measuring the approximate T_2 of the amide protons because of the absence of homonuclear H_N–H_α J modulation and the high degree of suppression of the H_2O solvent resonance in this experiment. Although a substantial degree of variation in T_2 is often observed for the various backbone amide protons, most resonances tend to cluster in a relatively narrow range, and it is the average T_2 value of this 'majority' of resonances that is used to estimate τ_c. As was found by comparing the average H_N line width with the τ_c values derived from ^{15}N relaxation studies for the proteins staphylococcal nuclease and calmodulin (Kay et al., 1989; Barbato et al., 1992), τ_c is approximately equal to $1/(5T_2)$ ns, where T_2 is the numerical value of T_2 expressed in seconds.

NMR spectra of calcineurin B were recorded with and without Ca^{2+} at 35 °C and at pH 6.0, using a protein concentration of 0.8 mM in 5% D_2O/95% H_2O, in the presence of 20 mM dithiothreitol to prevent disulfide bond formation involving the two cysteine residues of the protein. In the absence of Ca^{2+}, calcineurin B showed a spectrum with very broad resonances, and the approximate T_2 value for the amide protons was 1.6 ms. After saturation of the protein with

Ca^{2+} (14 mM), the amide proton T_2 increased to 8.3 ms. Although much longer than that measured without calcium, this T_2 is still a factor of 3–4 shorter than what is observed for other proteins in the 16–20 kDa range.

The effect of various factors on the T_2 relaxation time has been investigated. Raising the temperature by 8 °C increases the T_2 to ~11 ms. At 43 °C, increasing the pH to 7.8 results in a decrease in the T_2 by about 10%. Lowering the pH to 4.7 increases the T_2 by about 10% but this lower pH also reduces the protein solubility. Clearly, temperature has the most pronounced effect on T_2, caused in part by the temperature dependence of aggregation and solvent viscosity. However, an increase in temperature also increases internal mobility, resulting in poorer definition of the protein structure, and we therefore prefer to carry out our experiments at or below 37 °C.

Because the degree of aggregation depends only weakly on the ionic strength of the solution, the interprotein contact is presumably hydrophobic in nature. Addition of non-denaturing detergent is expected to disrupt these hydrophobic intermolecular interactions, alleviating the aggregation problem. For this purpose we chose the detergent CHAPS, which is commonly used to delipidate and solubilize membrane proteins as it does not change their secondary and tertiary structure (Hjelmeland et al., 1983; Hori et al., 1988; Kline et al., 1989). Its non-denaturing character is also responsible for its most widespread commercial use, baby shampoo. The aggregation number of CHAPS is only 10 (Hjelmeland et al., 1983), much lower than for other commonly used detergents. A correlation between the number of detergent molecules bound to the protein and the aggregation number of the detergent has been suggested (Robinson and Tanford, 1975; Hori et al., 1988), and this number is therefore expected to be small for CHAPS. Indeed, Hori et al. (1988) found that, on average, 12 CHAPS molecules were attached to a

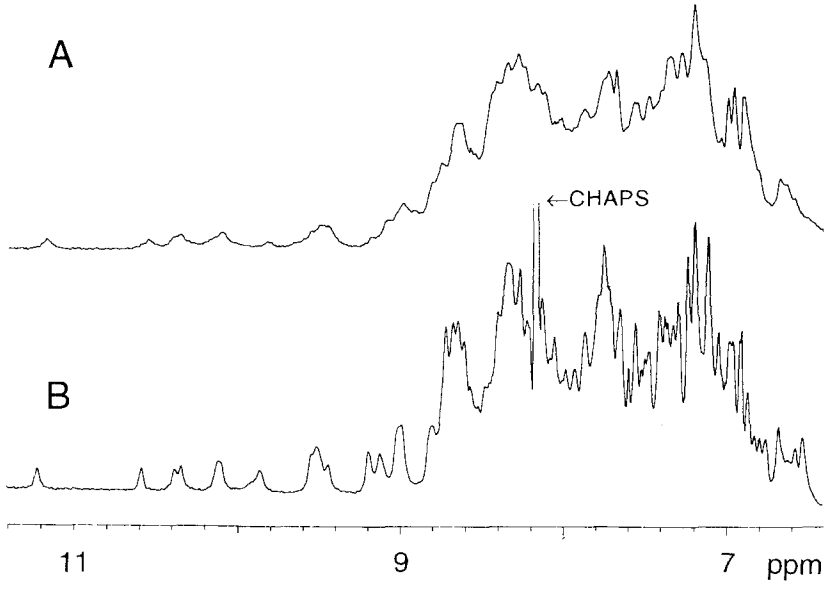

Fig. 1. Amide region of 0.8 mM calcineurin B at 35 °C, pH 4.9, in the (A) absence and (B) presence of 18 mM CHAPS. The position of the very intense amide proton resonance of CHAPS (8.13 ppm) is marked.

17-kDa fragment of cytochrome b_5. This number is expected to vary strongly, however, depending on the protein studied.

Figure 1 compares the spectrum of the amide region of calcineurin B in the absence and presence of CHAPS. Upon addition of 10 mM CHAPS at 35 °C, the average amide T_2 increased from ~8.3 to ~17.5 ms. In the presence of 20 mM CHAPS, only a small further increase was observed (T_2 ~19 ms). Further addition of CHAPS actually decreased T_2 (~16 ms in the presence of 50 mM CHAPS). Addition of 50 mM KCl to the calcineurin B solution containing 20 mM CHAPS, or lowering the pH from 6.0 to 4.9, did not cause any observable change in T_2.

To gain more insight into the nature of the interaction between the detergent and the protein, the proton $T_{1\rho}$ relaxation times of the CHAPS resonances were measured in the absence and presence of calcineurin B, using 25 mM CHAPS and 2.3 mM calcineurin B in D_2O at 35 °C, pH 7. In the absence of protein, the $T_{1\rho}$ of the CHAPS protons was relatively uniform at ~100 ms. In the presence of protein, the CHAPS protons of the cholic acid moiety, including its methyl groups, showed much shorter $T_{1\rho}$ values (~25ms). In contrast, the protons of the sulfobetaine side chain of CHAPS did not show a significant decrease in $T_{1\rho}$. These results indicate that the CHAPS molecules interact with calcineurin B. However, while the hydrophobic moiety is strongly immobilized by the interaction with the protein, the polar side chain retains a very high degree of flexibility.

The ^{15}N–$^{1}H_N$ correlation spectra of calcineurin B in the absence and presence of CHAPS are shown in Figs. 2a and 2b, respectively. The HSQC pulse scheme used included a scrambling pulse (Messerle et al., 1989) which removes the need for H_2O presaturation and simultaneously strongly

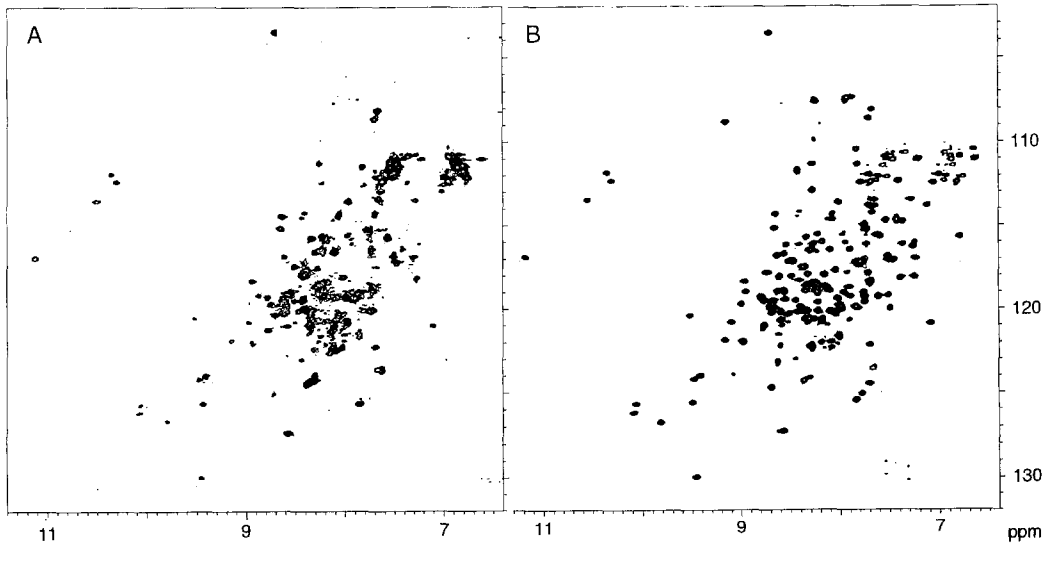

Fig. 2. ^{1}H–^{15}N correlation spectra of 5% D_2O/95% H_2O solution of 0.8 mM ^{15}N-labeled calcineurin B at 35 °C, pH 4.9, in (A) the absence and (B) the presence of 18 mM CHAPS. Spectra were recorded at 600-MHz ^{1}H frequency, on a Bruker AMX-600 spectrometer. Acquisition times in the t_1 (102.8 ms) and t_2 (120.8 ms) dimensions and the total number of scans and measuring time (2 h per spectrum) were identical for both spectra. Both data sets were processed with identical digital filtering (squared sine shifted by 45° in the t_1 dimension and by 60° in the t_2 dimension), and both data sets were zero filled to yield a 512 x 1024 matrix size for the absorptive part of the 2D spectrum.

attenuates the CHAPS signals. The spectra illustrate the dramatic increase in sensitivity and resolution obtained by the addition of CHAPS. For resonances that were resolved in both spectra, the differences in chemical shifts were all very small. However, there were also a large number of resonances observed in the presence of CHAPS that were extremely weak or completely absent from the spectrum without CHAPS, suggesting that in the absence of CHAPS the protein may also undergo conformational averaging on a time scale that broadens the ^{1}H and ^{15}N resonances. Considering that the resonances that were resolvable in both spectra had approximately identical chemical shifts, it seems likely that the regions of calcineurin B that are not affected by conformational averaging retain their conformation upon addition of CHAPS. This is in agreement with the high activity of Ca^{2+}-saturated calcineurin in the presence of 10 mM and 20 mM CHAPS; 55% and 40%, respectively.

The NMR signals from the protonated detergent do not interfere with the signals of interest in the ^{15}N- or ^{13}C-edited 3D spectra, which are needed for resonance assignment and structure determination. The intense ^{1}H detergent signals were eliminted by the use of scrambling pulses and by the isotope filtration used in these experiments, enabling us to make virtually complete ^{1}H, ^{15}N and ^{13}C assignments for calcineurin B and to determine its secondary structure. As an example of the spectral quality obtainable with such experiments, Fig. 3 presents two 2D 'strips' taken from the 4D $^{15}N/^{13}C$-separated NOESY spectrum (Kay et al., 1990) showing the ^{1}H and ^{13}C resonances for protons that interact with the amide protons of Gln^{128} and Lys^{135}.

The present study indicates that detailed structural studies of isotope-enriched proteins do not require the use of deuterated detergents. Although the synthesis of deuterated detergents does not pose any fundamental problems, only SDS and DPC are currently commercially available in

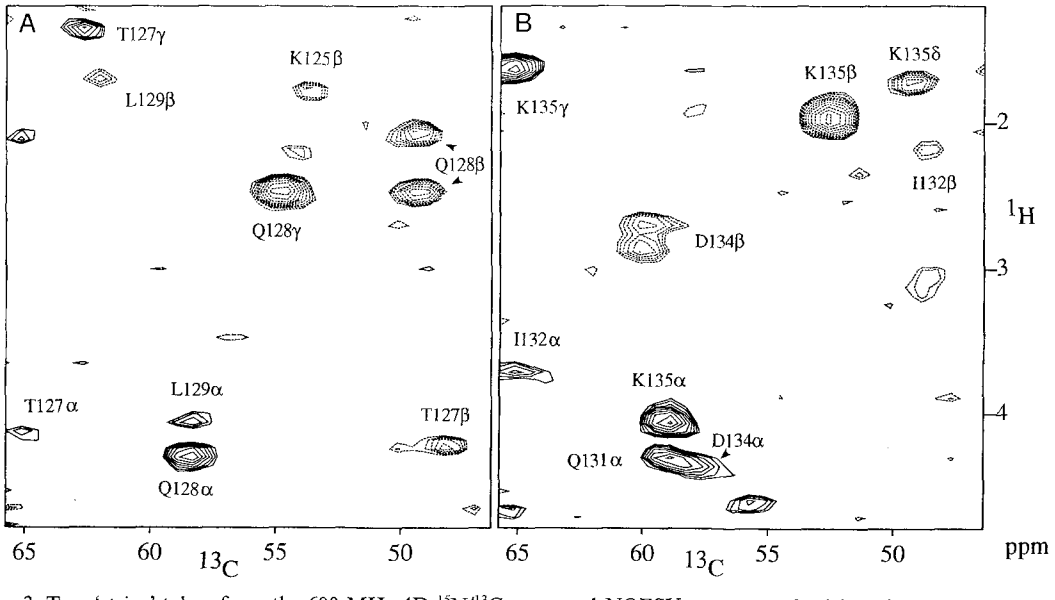

Fig. 3. Two 'strips' taken from the 600-MHz 4D $^{15}N/^{13}C$-separated NOESY spectrum of calcineurin B, 2.3 mM in the presence of 25 mM CHAPS, dissolved in 5% D_2O/95% H_2O, pH 4.9, displaying the interaction between aliphatic resonances and (A) the amide of Gln^{128} and (B) the amide of Lys^{135}. Dashed contours correspond to resonances with negative intensity, caused by aliasing an odd number of times in the ^{13}C dimension.

deuterated form. Hence, the possibility of using protonated detergents dramatically increases the range of detergents applicable to NMR studies. No general recipe can be given for which detergent is best for any given protein, but the fact that all detergents are now potential candidates for use in NMR structural studies increases the power of NMR for the study of poorly soluble and aggregating proteins, including protein domains.

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