J. Mol. Biol. (1994) 239, 545–554

Two-dimensional NMR Studies of Selenomethionyl Calmodulin

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Calmodulin (CaM) is a ubiquitous calcium regulatory protein that can interact with almost 30 different target proteins. The majority of the CaM-binding domains of the target proteins are believed to interact with two hydrophobic surfaces on Ca^{2+}-CaM; these two regions are very rich in Met residues. To obtain more information about the role of these residues, we have biosynthetically incorporated selenomethionine (SeMet) in place of the nine Met residues of CaM. Amino acid analysis shows that the SeMet-CaM contains 15% Met and 85% SeMet. SeMet-CaM retains many of the properties of the wild-type protein; it activates the enzyme cyclic nucleotide phosphodiesterase, it binds to phenyl-Sepharose and myosin light chain kinase (MLCK) in a calcium-dependent manner, and it experiences a calcium-dependent band shift during SDS-gel electrophoresis. Moreover, by comparing the natural abundance (^{1}H,^{13}C)-heteronuclear multiple quantum coherence (HMQC) spectra of the calcium, apo and target peptide-bound forms of wild-type CaM and SeMet-CaM, we have found that the two proteins have very similar, if not identical, structures. Thus, the substitution of SeMet for Met does not cause a change in the conformation and function of CaM, in agreement with the results obtained for other proteins. The apo, calcium and target peptide-bound forms of SeMet-CaM were subsequently studied by natural abundance (^{1}H,^{77}Se)-heteronuclear multiple bond correlation (HMBC) and (^{1}H,^{13}C)-HMQC NMR. Nine well-resolved ^{77}Se resonances could be observed. Substitution of SeMet for Met gave rise to the same ^{1}H and ^{13}C chemical shift changes for each individual Met residue, this facilitated making the assignments from known ^{1}H, ^{13}C assignments of the Met residues. Some of these assignments were confirmed by studying Met→Leu mutants of CaM. With the exception of Met76, which always remains solvent exposed, all resonances experienced large ^{77}Se chemical shift changes upon the addition of Ca^{2+} and the MLCK peptide. The large shift changes indicate that the electron distribution in the SeMet side-chain can be adjusted for the different states of CaM, suggesting that the polarizability of sulfur or selenium may be important for the proper functioning of CaM. This study also shows that the natural abundance (^{1}H,^{77}Se)-HMBC experiment provides a sensitive approach for the study of SeMet proteins.

Keywords: calmodulin; selenomethionine; two-dimensional (^{1}H,^{77}Se)-NMR; two-dimensional (^{1}H,^{13}C)-NMR; methionine-rich regions

1. Introduction

Calmodulin (CaM‡) is a major intracellular Ca^{2+} receptor in all eukaryotic cells. It can regulate various biological events by binding and activating almost 30 different target enzymes (for reviews, see Hiraoki & Vogel, 1987; Means *et al.*, 1991). There are four Ca^{2+}-binding sites in CaM, and the binding of Ca^{2+} to CaM induces a major conformational change, which converts CaM from an "inactive" to an "active" state. The three-dimensional structure of Ca^{2+}-CaM shows that the protein contains two homologous globular domains that are connected by a long solvent-exposed α-helix (Babu *et al.*, 1988; Chattopadhyaya *et al.*, 1992; Rao *et al.*, 1993). Each globular domain of CaM consists of two related helix-loop-helix Ca^{2+}-binding motifs. In addition, each domain contains one hydrophobic surface patch that is involved in the binding of target

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‡ Abbreviations used: CaM, calmodulin; SeMet, selenomethionine; 1D, 2D; one and two-dimensional, respectively; PDE, cyclic nucleotide phosphodiesterase; MLCK, myosin light chain kinase; HMQC, heteronuclear multiple quantum coherence; wt-CaM: wild-type CaM; HMBC, heteronuclear multiple bond correlation; p.p.m., parts per million; TSP, (trimethylsilyl)-propionic-d4 acid; M51L-CaM, CaM mutant with Met51 mutated to Leu.

0022–2836/94/240545–10 $08.00/0

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proteins (Vogel *et al.*, 1983; Ikura *et al.*, 1992; Meador *et al.*, 1992, 1993).

Mammalian CaM contains nine methionine residues out of a total of 148 amino acid residues. The Met residues account for 6·2% of the total number of residues of CaM, this is approximately three times higher than the statistical average for the occurrence of Met residues in other proteins. Of these nine Met residues, Met36, 51, 71 and 72 are located in the N-terminal hydrophobic patch; Met109, 124, 144 and 145 are part of the C-terminal hydrophobic patch; and Met76 is located in the central helix of the protein (Babu *et al.*, 1988; Chattopadhyaya *et al.*, 1992; Rao *et al.*, 1993). The eight Met residues in the two globular domains contribute as much as 46% of the accessible surface area of the hydrophobic patches of the protein (O'Neil & DeGrado, 1990). The binding of CaM to its target enzymes proceeds mainly *via* interactions between the hydrophobic patches on CaM and the hydrophobic regions provided by the CaM-binding domains of target enzymes (Ikura *et al.*, 1992; Meador *et al.*, 1992, 1993). Previous studies have shown that nearly all of the Met residues in the two hydrophobic patches are involved when CaM interacts with its binding domains from myosin light chain kinases (Ikura *et al.*, 1992; Meador *et al.*, 1992), CaM-dependent protein kinase IIα (Meador *et al.*, 1993), caldesmon and nitric oxide synthase (Zhang and Vogel, 1994*a*,*b*). Thus, it is of interest to ask why CaM has such a high abundance of Met residues. The unique properties of the Met side-chain that may help us understand its role in binding so many target proteins in a sequence-independent fashion have been discussed by Gellman (1991). The author points out that the thioether sulfur atom in the Met side-chain is not simply an equivalent of methylene. Instead, it is characterized by a unique flexibility and by polarizability of the sulfur atoms, features that can contribute to the generation of an adaptable binding site that is tailored for strong interactions with nonpolar surfaces of different shapes (Gellman, 1991). Indeed, we have recently shown that Met→Leu mutations in CaM can weaken its interactions with target enzymes (Zhang *et al.*, 1994*a*).

One approach to the study of Met residues in proteins is the use of the Met analog seleno-Met (SeMet). The structural and functional effects that result from the simultaneous replacement of all Met residues with SeMet can be investigated in the purified proteins (Huber & Criddle, 1967; Coch & Greene, 1971; Frank *et al.*, 1985; Boles *et al.*, 1991, 1992). The strong anomalous diffraction of the Se atom provides a very valuable avenue for solving the phase problem in X-ray crystallography. Using the multiwavelength anomalous diffraction (MAD) technique, Hendrickson and co-workers (1990) have been able to determine the three-dimensional structures of several SeMet-containing proteins without a need for phase information from other sources. Selenium-77 NMR spectroscopy potentially offers another approach for the study of Met residues in SeMet-labeled proteins. ^{77}Se has a spin of 1/2, and a natural abundance of 7·6%. Earlier work on ^{77}Se-labeled proteins has already shown the potential of the technique in probing the active site of enzymes (House *et al.*, 1992; Gettins & Crews, 1991). The two major limiting factors for the application of ^{77}Se NMR in the study of proteins are: (1) the low sensitivity of 1D ^{77}Se NMR at natural abundance; and (2) the unfavorable relaxation characteristics (Luthra *et al.*, 1982; Gettins & Wardlaw, 1991). The first problem can sometimes be overcome by using isotopically enriched material (Gettins & Crews, 1991; House *et al.*, 1992, and references therein); however, the generality of this method is limited. To date, the detection of ^{77}Se NMR signals at natural abundance arising from biosynthetically substituted SeMet residues in a protein has only been reported once (Boles *et al.*, 1992).

In this paper, we describe the incorporation of SeMet residues into the Met-rich protein, calmodulin. SeMet-CaM and wild-type CaM were compared in terms of their ability to activate the target enzyme PDE. Moreover, their binding to phenyl-Sepharose and to the CaM-binding domain of MLCK were compared. The structural effects of the replacement of Met with SeMet were assessed by comparing the natural abundance (^{1}H,^{13}C)-heteronuclear multiple quantum coherence (HMQC) NMR spectra of SeMet-CaM and wt-CaM in the apo and Ca^{2+} forms of the protein. Two-dimensional (^{1}H,^{77}Se)-heteronuclear multiple bond correlation (HMBC) NMR spectra were used to detect the SeMet residues in CaM. Three different forms of SeMet-CaM were studied; namely, apo-CaM, Ca^{2+}-CaM and a complex of CaM with the CaM-binding domain of MLCK.

2. Materials and Methods

(a) *Materials*

SeMet was purchased from Sigma; this compound is not isotopically labeled and hence should contain 7·6% of the NMR-active ^{77}Se isotope. Bovine brain CaM-dependent PDE was a generous gift from Dr J. Wang (University of Calgary). Unless otherwise stated, all other reagents were purchased from Sigma Chemical Company. Wild-type CaM was expressed, purified, decalcified and quantitated as described earlier (Zhang & Vogel, 1993). The synthetic peptide encompassing the CaM-binding domain of skeletal muscle MLCK was obtained and used exactly as described elsewhere (Zhang & Vogel, 1993; Zhang *et al.*, 1993).

(b) *SeMet incorporation in CaM*

The CaM expression vector, which encodes the synthetic bovine CaM gene, was a gift from Dr T. Grundström (University of Umeå, Sweden). It has been described in detail elsewhere (Waltersson *et al.*, 1993; Zhang & Vogel, 1993). The *Escherichia coli* strain DL41 (ΔMet) was obtained from the *E. coli* genetic stock center at Yale University; the properties of this strain have been discussed by Hendrickson *et al.* (1990). The CaM-containing plasmid was transformed into *E. coli* DL41 using the standard $CaCl_2$ method (Sambrook *et al.*,

1989). A chemically defined, enriched Mops medium (Neidhardt *et al.*, 1974) was used to incorporate SeMet into CaM. For expression of the SeMet protein, we followed the same method used for [*methyl*-^{13}C]Met-labeled CaM (Zhang & Vogel, 1994*b*). SeMet-CaM could be purified using phenyl-Sepharose chromatography as described earlier (Vogel *et al.*, 1983; Putkey *et al.*, 1985), and the purity of the protein was greater than 95% as judged by SDS/polyacrylamide gel electrophoresis. Amino acid analysis was used to determine the efficiency of SeMet incorporation. SeMet-CaM was hydrolyzed in 6 M HCl, 0·1% β-mercaptoethanol under vacuum at 150°C for 1 h, and the amino acid composition was determined on a Beckman 6300 amino acid analyzer. For the NMR studies, the required amount of CaM or SeMet-CaM was dissolved in 99·9% ^2H_2O (MSD Isotopes), containing 0·1 M KCl, pH 7·5 (0·45 ml final volume). The assay of the CaM-dependent PDE activity has been described in detail elsewhere (Zhang *et al.*, 1994*b*). The binding studies with the CaM-binding domain of MLCK in the presence of 4 M urea were carried out following the procedure reported by Erickson-Vittanen & DeGrado (1987).

(c) *NMR spectroscopy*

All NMR spectra were obtained on a Bruker AMX500 spectrometer using a 5 mm broad band inverse detection probe. The ^2H_2O solvent was used to provide a spectrometer lock. Natural abundance (^{1}H,^{13}C)-HMQC spectra of the CaM samples were recorded in the phase-sensitive mode (time proportional phase increment) using a pulse scheme described by Bax *et al.* (1983). In each 2D spectrum, 360 experiments (128 scans each) were collected, and the total experimental time was approx. 18 h. The carrier for ^{13}C was centered at ~40 p.p.m. and the ^{1}H frequency was centered at the residual H^2HO resonance. The sweep widths for ^{13}C and ^{1}H were around 80 p.p.m. and 10 p.p.m., respectively. All the (^{1}H,^{13}C)-HMQC spectra were processed on a Bruker X32 computer using UXNMR software. The data in the F1 dimension was zero filled once, and a sine square window function with a 72° phase shift was applied in both dimensions prior to the Fourier transformation. The chemical shifts are reported using TSP as the reference (0 p.p.m. for ^{1}H and ^{13}C). Natural abundance 2D (^{1}H,^{77}Se)-HMBC spectra of the amino acid SeMet and the various SeMet-CaM samples were acquired in the magnitude mode using a published HMBC pulse sequence (Bax & Summers, 1986). This sequence is essentially similar to that of an HMQC experiment optimized for a small coupling constant. A delay time of 50 ms, which equals $1/(2 \times {}^3J_{H\text{-}Se})$, was found to be optimal to observe the long-range correlation between ^{77}Se and ^{1}H using SeMet as a model compound. A typical (^{1}H,^{77}Se)-HMBC spectrum, for a sample containing approx. 1·5 mM SeMet-CaM, was recorded from 256 experiments (96 scans each), resulting in a total experimental time of 12 h. The carrier for ^{77}Se was centered at 95·39 MHz with a sweep width of approx. 150 p.p.m. and the carrier for ^{1}H was set at approx. 3 p.p.m. with a sweep width of 5 p.p.m.. The ^{77}Se chemical shifts are referenced to external neat $(CH_3)_2Se$ at 0 p.p.m.

3. Results

(a) *Incorporation of SeMet into CaM*

There are in principle two methods of obtaining a SeMet-containing protein from bacteria. First of all, one can grow bacteria on a medium containing selenate rather than sulfate (Huber & Criddle, 1967). Secondly, it is possible to feed SeMet instead of Met to a Met auxotrophic strain (Hendrickson *et al.*, 1990). The former approach has provided proteins in which about 55% of the Met residues were replaced with SeMet (Huber & Criddle, 1967). Greater incorporation of SeMet has been obtained using the Met auxotroph approach (Hendrickson *et al.*, 1990; Boles *et al.*, 1992; Graber *et al.*, 1993). Our objective in this study was to obtain a high level of SeMet incorporation, and hence we chose the latter approach. The best results were obtained when we pregrew the cells on rich L-broth medium and, following collection by centrifugation, they were resuspended in the defined Mops-based production medium. Under these conditions we obtained approximately 35 mg of CaM per liter of culture; this is about 50% of what is obtained for wt-CaM. The level of substitution of Met by SeMet was determined by amino acid analysis. The amino acid SeMet eluted at the same position as Ile, and we have indeed observed a significant increase in the amount of Ile in the protein sample. However, SeMet is known not to be stable under these conditions even in the presence of β-mercaptoethanol (Shepherd & Huber, 1969), thus we have used the number of residual Met residues to quantitate the incorporation of SeMet. About 15% of the Met residues were recovered during the amino acid analysis, hence we conclude that approximately 85% of the Met residues in CaM are replaced by SeMet. The quantitation of the incorporation of SeMet was further confirmed by an analysis of the intensity of (^{1}H,^{13}C)-HMQC spectra of CaM samples as discussed below.

(b) *Functional effects of substituting SeMet for Met in CaM*

Since CaM has such a high abundance of Met residues, it was important to study the functional effects of replacing Met with SeMet. We have assayed the ability of SeMet-CaM to activate the CaM-dependent enzyme PDE, and the results show that there is no noticeable difference in the maximal activity or in the level of protein required for activation, between wt-CaM and SeMet-CaM (Figure 1). SeMet-CaM could be purified in exactly the same manner as wt-CaM by Ca^{2+}-dependent hydrophobic interaction chromatography on phenyl-Sepharose (Vogel *et al.*, 1983; Putkey *et al.* 1985). This suggests that SeMet-CaM also undergoes the same Ca^{2+}-dependent exposure of hydrophobic surfaces as wt-CaM. A characteristic, but poorly understood, feature of CaM is its Ca^{2+}-dependent band shift that occurs during SDS-gel electrophoresis (Klee *et al.*, 1979). SeMet-CaM also displays a Ca^{2+}-dependent band shift on SDS-gel electrophoresis, although the effect was less pronounced than for wt-CaM (see Figure 2(a)). In fact, a slightly slower migration rate was observed for SeMet-CaM in both the Ca^{2+} and EDTA forms of the protein

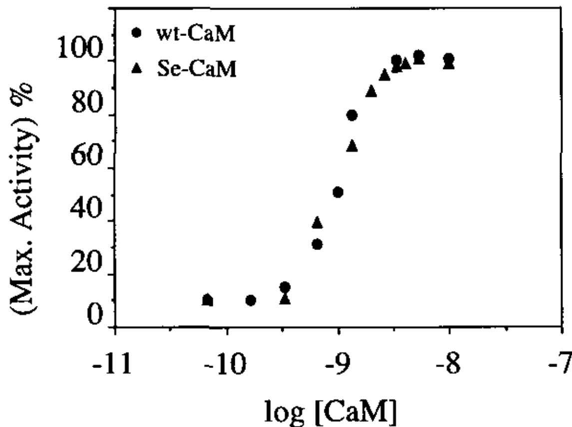

Figure 1. Activation of CaM-dependent cyclic nucleotide phosphodiesterase by wild-type CaM (●) and SeMet-CaM (▲). The activation is expressed as a percentage of the maximal activation (100%) obtained with wt-CaM.

(Figure 2(a)). We have also studied the binding of SeMet-CaM to a 23-residue peptide encompassing the CaM-binding domain of MLCK. It has been demonstrated that the specific binding of peptides to CaM can be conveniently studied by gel electrophoresis in the presence of 4 M urea (Erickson-Vittanen & DeGrado, 1987). By using different ratios of peptide to protein, it is often possible to obtain an estimate of the stoichiometry of binding (see, for example, Zhang & Vogel, 1994*a*). The results obtained with SeMet-CaM show the same band shift pattern (Figure 2(b)) as wt-CaM, which binds to the MLCK peptide with a 1 : 1 stoichiometry. The above results indicate that CaM can tolerate the substitution of Met with SeMet although the Se atom is significantly larger than the S atom (atomic radii 1·17 Å *versus* 1·04 Å).

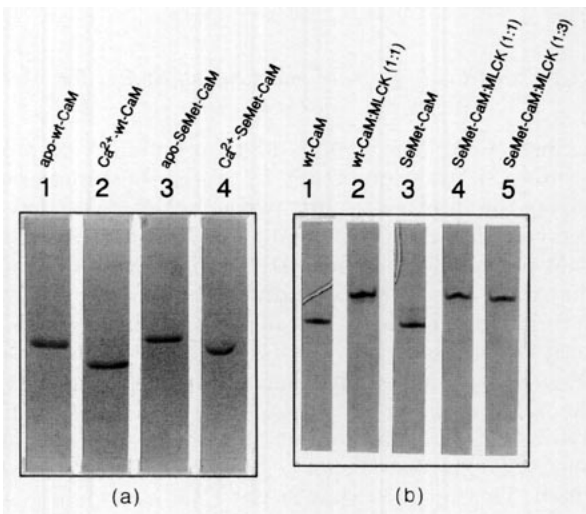

Figure 2. (a) A 15% SDS-polyacryamide gel electrophoresis of wt-CaM and SeMet-CaM in the presence and absence of Ca^{2+}. For the calcium proteins, 5 mM Ca^{2+} was added in the sample buffer; for the apoproteins, 5 mM EDTA was added instead. (b) Non-denaturing polyacrylamide (15%) gel electrophoresis of CaM and complexes of CaM with varying amounts of the MLCK peptides in the presence of 4 M urea, 0·1 mM Ca^{2+}. The data show that wt-CaM and SeMet-CaM both bind with 1 : 1 stoichiometry to the MLCK peptide.

(a) *Spectroscopic characterization of Ca^{2+}-saturated SeMet-CaM*

The UV, CD, FTIR and 1D ^1H NMR spectra of Ca^{2+}-saturated SeMet-CaM were identical to that of wt-CaM (spectra not shown), suggesting that the protein keeps the same fold after the substitution of Met by SeMet. Further structural information about SeMet-CaM was obtained by comparing the natural abundance (^1H,^{13}C)-HMQC spectrum of SeMet-CaM with that of wt-CaM. The complete assignment of the resonances for Ca^{2+}-CaM has been reported earlier (Ikura *et al.*, 1991) and these could be directly identified in our spectrum of wt-CaM. Figure 3 shows the methyl region of the (^1H,^{13}C)-HMQC spectrum of Ca^{2+}-saturated SeMet-CaM, and this region of the spectrum is nearly identical to that of wt-Ca^{2+}-CaM, with the exception that the intensity of the resonances for the methyl groups of the remaining 15% Met residues is significantly reduced. Furthermore, a set of new intense resonances appears in the top left corner of the spectrum, which arise from the methyl groups of the SeMet residues. However, the chemical shifts of the methyl groups of the other aliphatic amino acid side-chains appear to be virtually identical in wt-CaM and SeMet-CaM. The above results clearly demonstrate that the structural perturbations resulting from the substitution of Met by SeMet are minimal.

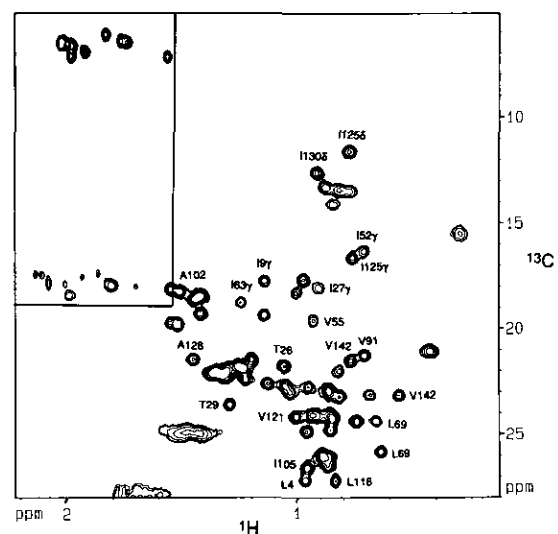

Figure 3. The methyl region of the natural abundance (^1H,^{13}C)-HMQC spectrum of Ca^{2+}-SeMet-CaM (30°C). The spectrum is identical to that of wt-CaM except for the boxed region, and some of the resonance assignments are indicated in the Figure. They were taken from published assignments for Ca^{2+}-CaM (Ikura *et al.*, 1991).

The intensity of the Met methyl resonances in Figure 3 is significantly weaker than the other resonances in the spectrum; the new SeMet methyl resonances that appear upfield in the ^{13}C dimension are comparable in intensity to the other methyl resonances. Figure 4a shows an expanded plot of the region of the (^{1}H,^{13}C)-HMQC spectrum showing the methyl groups of Met and SeMet in Ca^{2+}-saturated SeMet-CaM. Volume integration of these peaks also shows that approximately 85% of the Met residues in the protein were replaced by SeMet. The ratio of Met/SeMet was virtually the same for each resonance, suggesting that the incorporation of SeMet is random throughout the protein.

We have earlier assigned the methyl groups of the nine Met residues in Ca^{2+}-CaM, apo-CaM and target peptide-bound CaM by mutating all Met residues individually to Leu residues; the assignments were made by identifying the "missing peak" in 2D (^{1}H,^{13}C)-HMQC spectra of selectively [*methyl*-^{13}C]Met-labeled CaM mutant proteins (M. Zhang, K. Siivari, A. Palmer & H. J. Vogel, unpublished results). The complete assignment derived in this fashion for Ca^{2+}-CaM is indicated in Figure 4a. The substitution of Se for S in a Met residue causes an approximately 11 p.p.m. and an approximately 0·1 p.p.m. upfield shift for the ^{13}C and ^{1}H chemical shifts, respectively. This upfield shift is probably due to the slightly lower electron-negativity of Se compared to S. The upfield shifts of the carbon resonances of the methyl groups in the SeMet residues leaves them in a distinct spectral window, which does not overlap with any other resonances of the protein. This is generally not the case for methyl groups of Met residues in a protein, which overlap with resonances from other amino acids. One interesting feature of Figure 4a is that the pattern of the methyl resonances from Met and SeMet appears to be virtually identical. This suggests that the substitution of Se for S produces almost identical ^{1}H and ^{13}C chemical shift changes for all of the methyl groups. On this basis, one can obtain the assignment for the methyl groups of the SeMet residues directly from the known assignment of the Met residues. We have used four SeMet-CaM mutants in which we have replaced the individual Met residues with Leu (M51L, M71L, M109L, M124L, respectively) to confirm the assignments derived in this manner. Figure 4b shows one example of the Met and SeMet methyl region of the (^{1}H,^{13}C)-HMQC spectrum of Ca^{2+}-saturated M51L-SeMet-CaM. Indeed in each mutant, the two corresponding peaks are missing in the methyl regions of the Met and SeMet residues (see Figure 4b). In this fashion, we have found that SeMet methyl resonances can indeed be assigned from their corresponding Met resonances by applying the same Δ p.p.m. chemical shift values (Table 1).

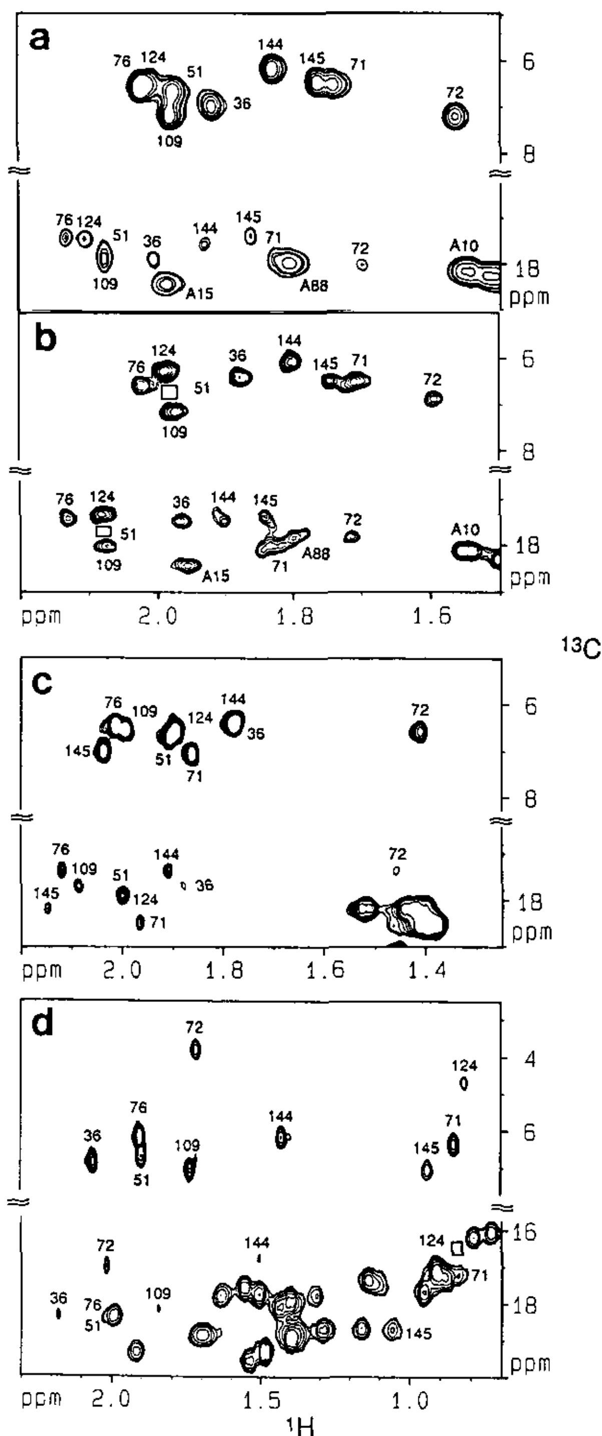

Figure 4. The Met and SeMet region of the natural abundance (^{1}H,^{13}C)-HMQC spectrum of: a, Ca^{2+}-SeMet-CaM; b, the SeMet-M51L-CaM mutant; c, apo-SeMet-CaM; and d, the SeMet-CaM/MLCK peptide complex. In b, the boxes represent the missing methyl resonances from Met51 and SeMet51, respectively. Note the different scale for panels c and d.

We next attempted to detect the natural abundance ^{77}Se NMR spectrum of SeMet-CaM. The excellent sensitivity of the inverse detected natural abundance (^{1}H,^{13}C)-HMQC spectra of CaM and other proteins, combined with the fact that ^{77}Se has a two times higher receptivity and a seven times higher natural abundance compared to ^{13}C, prompted us to consider similar inverse detection

Table 1
Proton and carbon-13 chemical shifts determined for the Met and SeMet residues of Ca^{2+}-CaM

	^{1}H shifts			^{13}C shifts		
	Met-CaM	SeMet-CaM	Δ (p.p.m.)	Met-CaM	SeMet-CaM	Δ (p.p.m.)
M36	2·00	1·91	0·09	17·9	6·9	11·0
M51	2·07	1·97	0·10	17·7	6·7	11·0
M71	1·83	1·74	0·09	17·9	6·6	11·3
M72	1·80	1·56	0·24	18·1	7·2	10·9
M76	2·13	2·03	0·10	17·4	6·6	10·8
M109	2·07	1·98	0·09	18·0	7·2	10·8
M124	2·10	2·01	0·09	17·5	6·6	10·9
M144	1·93	1·83	0·10	17·6	6·3	11·3
M145	1·86	1·76	0·10	17·4	6·5	10·9

The assignments for the SeMet resonances of Met51, Met71, Met109 and Met124 were obtained directly by studying Met→Leu mutants as depicted in Figure 5. The other SeMet peaks are assigned by assuming analogous carbon-13 chemical shifts upon substitution of Se for S.

experiments. However, there are no protons directly attached to the Se atom in SeMet. From the natural abundance 1D ^{77}Se NMR spectrum of the amino acid SeMet (data not shown), which served as a model compound, we have measured the two bond coupling constant between ^{1}H and ^{77}Se as $^2J_{H\text{-}Se}$ = 9·4 Hz, which is in agreement with a previously reported value (Odom *et al.*, 1979). A 9·4 Hz coupling constant should be sufficient to record a 2D inverse detected (^{1}H,^{77}Se)-HMBC spectrum for SeMet. Figure 5a shows the 2D (^{1}H,^{77}Se)-HMBC spectrum of Ca^{2+}-saturated SeMet-CaM, and it is clear that all nine SeMet residues can be resolved. The initial assignment of the ^{77}Se resonances was obtained from the ^{1}H chemical shifts, and is indicated in the Figure. We have also recorded (^{1}H,^{77}Se)-HMBC spectra of several SeMet-CaM mutants to confirm these assignments further. Figure 5b gives an example of an (^{1}H,^{77}Se)-HMBC spectrum of M51L-SeMet-CaM, and it is directly

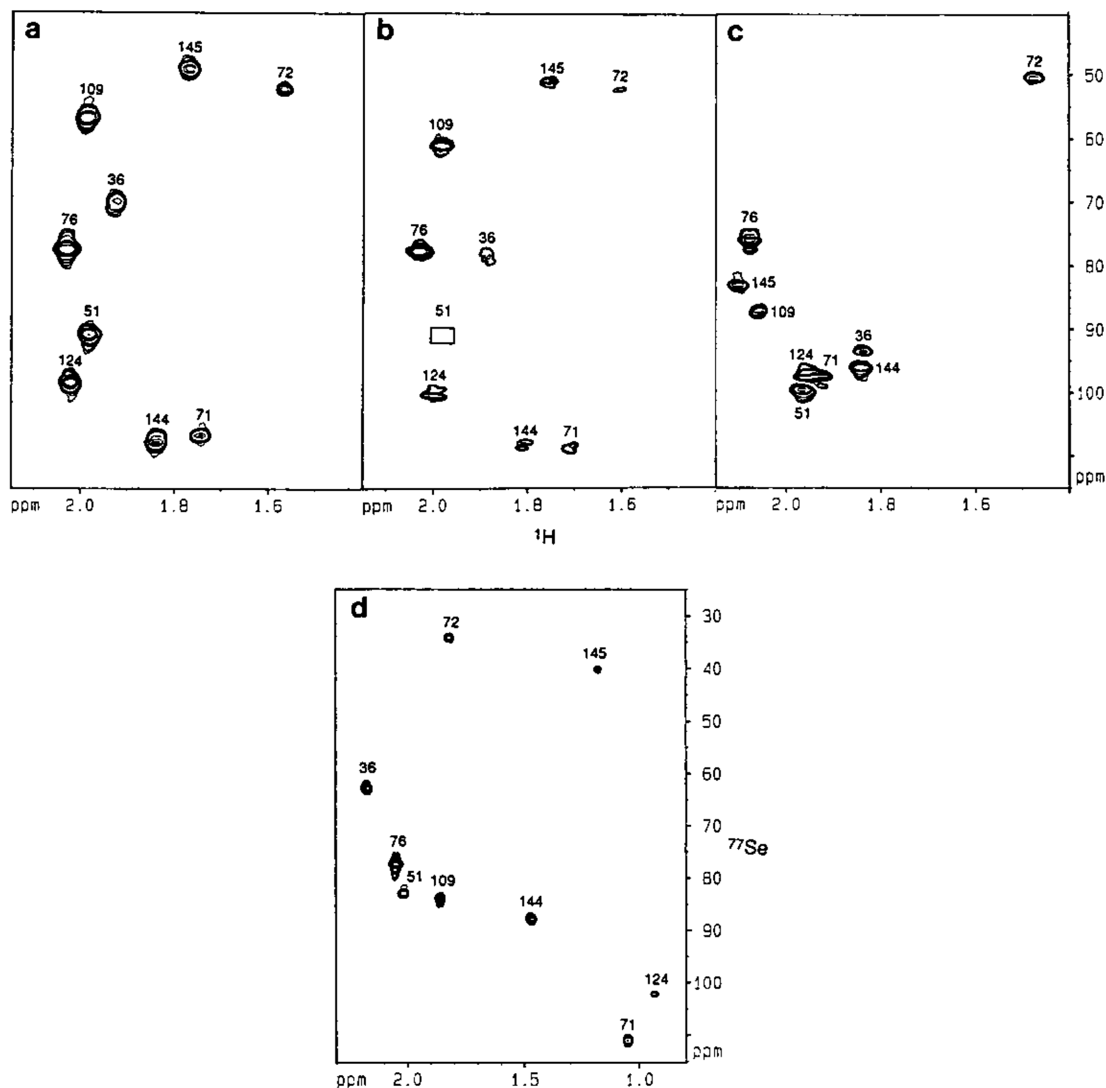

Figure 5. (^{1}H,^{77}Se)-HMBC spectrum of: a, Ca^{2+}-SeMet-CaM; b, the SeMet-M51L-CaM mutant; c, apo-SeMet-CaM; and d, the SeMet-CaM/MLCK-peptide complex (at 55°C). In b, the box represents the missing Met51 resonance. The chemical shift change of Met36 that results from the mutation of Met51 is seen in panel b. Note the different scale in d.

obvious from the Figure that the corresponding resonance of SeMet51 is missing. In this spectrum, we have also noted that a small chemical shift change occurs for SeMet36 upon mutating Met51 to Leu. This small change can be explained by the proximity of the Met36 and Met51 residues; their sulfur atoms are within 5 Å in the crystal structure (Babu *et al.*, 1988). We also tried to observe the γ and β-CH_2 protons of the SeMet, since these would give a direct assignment from the known αCH Met assignments. Unlike what is seen for the amino acid SeMet, we could not detect the crosspeaks for the γ and β-CH_2 protons of the protein SeMet residues. The latter are extensively spin coupled and hence their crosspeaks are of much lower intensity than the one for the εCH_3 group (data not shown). In addition, because of the anticipated relaxation rate difference between the Met methyl group and its methylene groups in a protein, these would probably be of even lower intensity than observed for the amino acid SeMet.

The chemical shift of ^{77}Se is known to be very sensitive to the local electronic environment (Luthra & Odom, 1986). We have observed an approximately 60 p.p.m. chemical shift dispersion for ^{77}Se resonances of Ca^{2+}-saturated SeMet-CaM (Figure 5a), while the chemical shift dispersion of the methyl -^{13}C and ^{1}H resonances is less than ~1 p.p.m. This large chemical shift dispersion illustrates that ^{77}Se NMR could have some advantages for studying the properties of Met residues in proteins. The different intensities observed could arise from the fact that some of the resonances (e.g. Met71, Met72) have broader lines; moreover, the HMBC delay time of 50 milliseconds may not represent the optimal value for every SeMet residue in the protein.

(d) *Apo-form of SeMet-CaM*

Apo-CaM represents a conformational state that is distinct from Ca^{2+}-CaM. Thus, it is of interest to determine whether apo-CaM will tolerate the substitution of SeMet for Met as well as Ca^{2+}-CaM. We have compared the (^{1}H,^{13}C)-HMQC spectrum of wt-apo-CaM with that of SeMet-CaM, and found that there is virtually no difference between the two spectra except for the Met resonances (data not shown). The above results indicate that the substitution of Met with SeMet also causes no structural perturbations in apo-CaM. Figure 4c shows an expansion of the region of the (^{1}H,^{13}C)-HMQC spectrum containing the methyl resonances from SeMet and the remaining Met residues in apo-SeMet-CaM. The chemical shifts for the methyl groups of the remaining Met residues in apo-SeMet-CaM again have the same chemical shifts as those in wt-CaM. We also note that the substitution of Se for S gives rise to virtually the same chemical shift change for every methyl group, as we have observed earlier for Ca^{2+}-SeMet-CaM (Table 1). Thus, on this basis we have obtained the complete assignment for the methyl groups of the SeMet residues in apo-SeMet-CaM; this assignment is indicated in Figure 4c. This assignment was further confirmed using M51L, M109L-CaM mutants in the same fashion as described for Ca^{2+}-SeMet-CaM (spectra not shown). We have also recorded a (^{1}H,^{77}Se)-HMBC spectrum of apo-SeMet-CaM (Figure 5c). As in the case of Ca^{2+}-SeMet-CaM, we can observe nine resonances in the HMBC spectrum, which correspond to the nine SeMet residues in the apo-protein. The assignment of the SeMet resonances was obtained from Figure 4c by matching the ^{1}H chemical shift of each residue, and they are indicated in Figure 5c. The two SeMet-labeled CaM mutants (M51L, M109L) were again used to partially confirm the assignment in Figure 5c. The resonances of Met145, Met109, Met51 and Met36 shift significantly downfield upon the removal of Ca^{2+}, indicating that they move to a relatively more hydrophobic environment in apo-CaM than in Ca^{2+}-CaM.

(e) *Complex of SeMet-CaM with the MLCK peptide*

In order to complete the characterization of the SeMet residues in the three functionally important states of CaM, we have also studied a complex of SeMet-CaM with the CaM-binding domain of MLCK. Figure 4d shows an expansion of the (^{1}H,^{13}C)-HMQC spectrum of the complex of SeMet-CaM with the MLCK peptide. Similar to what we observed for the Ca^{2+} and apo-SeMet-CaM, the pattern of the methyl groups of the remaining Met in the complex is the same after the majority of the Met residues have been substituted by SeMet. The pattern of the methyl groups for the SeMet residues closely resembles that for the remaining Met methyl groups; hence, we can again obtain the assignment for the methyl groups of the SeMet residues in the complex. Upon complexation with the peptide, all the Met residues, except Met76, undergo significant chemical shift changes. The resonances for the Met residues in the complex have an even larger chemical shift dispersion compared to that observed in the spectrum of Ca^{2+}-CaM (Figure 4a and d). Furthermore, many of the resonances (in particular, Met124, Met145) in the complex have broader lines compared to Ca^{2+}-CaM. The above results suggest, but do not prove, that the Met residues in CaM are directly involved in binding to its target proteins. Figure 5d shows the (^{1}H, ^{77}Se)-HMBC spectrum of the complex of SeMet-CaM with the MLCK peptide acquired at 55°C. Nine well-resolved resonances that represent the nine SeMet residues from SeMet-CaM in the complex were detected. The ^{77}Se assignment for the resonances in the spectrum was obtained from the ^{1}H chemical shifts derived in the corresponding (^{1}H,^{13}C)-HMQC spectrum (Figure 4d). Except for Met76, all residues experience dramatic chemical shift changes upon complex formation.

4. Discussion

To date, SeMet has been incorporated as a substitute for Met in a variety of enzymes. In most cases

it has been shown that this substitution does not perturb the enzymatic activity (Huber & Criddle, 1967; Coch & Greene, 1971; Boles *et al.*, 1991, 1992; Graber *et al.*, 1993). However, this outcome may have been fortuitous, since the Met residues in many of these enzymes do not participate directly in their activity. As discussed in the Introduction, the Met residues of CaM are directly involved in target protein binding. This study of SeMet-CaM therefore represents an example of a SeMet-substituted protein in which a clear functional role for the Met residues has been established (O'Neil & DeGrado, 1990; Ikura *et al.*, 1992; Meador *et al.*, 1992, 1993; Zhang *et al.*, 1994*a*). Our (^{1}H,^{13}C)-HMQC NMR data show that substitution of SeMet in the Met-rich regions of CaM does not perturb the overall structure of the protein. Moreover, the various functional assays suggest that SeMet-CaM binds calcium and exposes its hydrophobic interaction surfaces in an identical manner to wt-CaM, allowing it to activate and bind to typical target enzymes such as cyclic nucleotide phosphodiesterase and myosin light chain kinase. Thus, our data provide compelling support for the notion that the introduction of SeMet is a nonperturbing substitution.

Although a number of NMR studies of selenium-containing proteins have been reported to date, all of these have utilized 1D ^{77}Se NMR. Because ^{77}Se relaxation in macromolecules is dominated by chemical shift anisotropy, the line width of the resonances increases with the square of the field strength (Luthra *et al.*, 1982; Gettins & Wardlaw, 1991). Hence, one can generally not take advantage of the better sensitivity of high-field spectrometers, because the extensive broadening of the resonances makes them difficult (if not impossible) to detect. As a result, most ^{77}Se studies of proteins reported to date have used extremely high protein concentrations, lower field spectrometers, and relied at the same time on extensive signal averaging. In this paper, we show that the (^{1}H,^{77}Se)-HMBC experiment provides a superior method for detecting protein SeMet residues. Because there is no proton directly attached to Se, the magnetization was transferred to the three protons on the methyl groups instead, thus allowing the inverse detection. The sensitivity of this experiment is very good because the three protons on the methyl group are degenerate, thus giving rise to an easily detectable peak. An additional bonus is that a 2D spectrum with improved resolution is obtained. The acquisition of a natural abundance (^{1}H,^{77}Se)-HMBC spectrum required much less time, and used only 10% of the amount of protein used in the earlier 1D natural abundance ^{77}Se experiments (Boles *et al.*, 1992). Hence under these conditions there is no need to utilize isotopically enriched ^{77}SeMet, even though the natural abundance of the ^{77}Se isotope is only 7·6%.

Our results also show that the (^{1}H,^{13}C)-HMQC experiment is very useful for the analysis of SeMet residues in proteins. As we have shown this experiment allows us to study the remaining 15% Met, as well as the 85% SeMet, simultaneously. The substitution of Se for S gives rise to an approximately 11 p.p.m. upfield shift for the carbon of the methyl groups, while its protons are shifted upfield by approximately 0·1 p.p.m. We note with interest that these shifts are almost identical for each of the nine Met residues; this has allowed us to suggest the assignment of the SeMet resonances, directly from the known ^{1}H and ^{13}C assignment for the Met residues. The results obtained with the Met→Leu mutants further corroborated the validity of this approach. Since the sensitivity of the natural abundance (^{1}H,^{13}C)-HMQC experiment of the methyl region of the spectrum is comparable to that of the (^{1}H,^{77}Se)-HMBC, it is obvious that this experiment will also prove very useful for future studies of other SeMet-containing proteins. As stated above, the natural abundance (^{1}H,^{13}C)-HMQC spectrum also provides a useful finger-print spectrum showing the correct folding of a protein, which can be readily used to compare the wild-type and SeMet substituted protein.

Our primary objective in incorporating SeMet for Met into CaM was to obtain information about the side-chains of this residue. The NMR properties of ^{77}Se are far superior to those of the only NMR active isotope of sulfur, the quadrupolar ($I = 3/2$) ^{33}S (Barbarella, 1993). Selenium and sulfur exhibit similar polarizabilities (Fersht, 1985), and the only difference is that Se is somewhat larger than S. The chemical shift of ^{77}Se is known to be extremely sensitive to the bonding pattern of the nucleus, as well as to the solvent. For example, selenide compounds with two linear alkyl substitutions, such as the side-chain of SeMet, can be found in a chemical shift window that spans almost 200 p.p.m. Likewise, the polarity of the solvent has a major influence, and it has been established that the ^{77}Se NMR shift of $(CH_3)_2Se$ can vary up to 50 p.p.m. depending on the choice of solvent (a more polar solvent gives a more upfield shift; Luthra & Odom, 1986). These factors all suggest that the electron distribution around the ^{77}Se atom in dialkyl-substituted selenides is readily adjustable. In our studies of SeMet-CaM, we noted that the ^{77}Se chemical shift of eight Met residues can change substantially (up to approx. 43 p.p.m. for Met145, for example) in response to the conformational state of the protein (Table 2). Since there is no obvious correlation between the solvent accessibility of the Met side-chains and their ^{77}Se chemical shift in Ca^{2+}-CaM, it would appear that other factors have an overriding effect on the shift. In all likelihood they reflect primarily the polarizability of the Se atoms in the side-chains of the eight Met residues that are part of the two hydrophobic interaction surface of CaM. The only residue that does not change significantly is Met76, which is in the central helix of CaM; its chemical shift remains nearly identical to that of the amino acid SeMet in water, and hence this residue is in a solvent exposed state in apo, Ca^{2+}- and target protein bound CaM. To our knowledge, no theoretical calculations of ^{77}Se

Table 2
The solvent accessibility of the Met residues in Ca^{2+}-CaM and the chemical shift values of their ^{77}Se resonances in different states of CaM

Residue no.	Solvent accessibility (Å²)	^{77}Se chemical shifts (p.p.m.)		
		apo-CaM†	Ca^{2+}-CaM‡	CaM : MLCK§
36	33	93·9	70·0	62·7
51	60	100·0	91·1	82·9
71	52	97·6	106·8	111·1
72	43	50·6	51·9	34·1
76	91	76·6	77·6	77·4
109	41	87·2	57·0	83·9
124	80	98·0	97·7	102·3
144	81	96·1	108·0	87·8
145	105	83·7	48·7	40·1

Data were obtained from X-ray structure of bovine Ca^{2+}-CaM at 2·2 Å (Babu *et al.*, 1988).

† Chemical shift values are reported as measured at 30°C.

‡ The line widths of most resonances for Ca^{2+}-SeMet-CaM decreased when the temperature of the sample was raised to 55°C. Most ^{77}Se resonances only experienced a minor (<2 p.p.m.) chemical shift change when the temperature was varied from 30°C to 55°C; the only exception being the resonances of Met72, Met109 and Met145, which moved approx. 5 p.p.m. towards the random coil shift of SeMet.

§ Chemical shift values measured at 55°C. At 30°C, we were only able to observe 7 out of a total of 9 SeMet resonances in the MLCK peptide SeMet-CaM complex; those for Met124 and Met145 were missing. It should be noted that the resonances for these 2 residues also have the broadest lines in the (^{1}H,^{13}C)-HMQC spectrum (Figure 4d); this is probably caused by an exchange process.

chemical shifts have been reported to date, and hence it is difficult at this time to attach a more detailed interpretation to the chemical shift changes. Be that as it may, the large effects are in keeping with the notion that the Met side-chains of CaM play a major role in the function of this important calcium regulatory protein.

This project was supported by an operating grant of the Medical Research Council (MRC) of Canada. The AMX500 NMR spectrometer used in this work was funded by the MRC and the Alberta Heritage Foundation for Medical Research (AHFMR). H.J.V. is a Scholar of the AHFMR. The authors also thank Mrs Kirsi Siivari for providing some of the mutants used in this work.

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Edited by P. E. Wright

(Received 15 December 1993; accepted 21 March 1994)