NMR Studies of Caldesmon−Calmodulin Interactions†

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Received October 14, 1996; Revised Manuscript Received January 10, 1997⊗

ABSTRACT: The binding of the calcium-regulatory protein calmodulin (CaM) to caldesmon (CaD) contributes to the regulation of smooth muscle contraction. Two regions of caldesmon have been identified as putative calmodulin-binding domains. We have earlier reported on the binding of one of these domains to calmodulin (Zhang & Vogel (1994) *Biochemistry 33*, 1163−1171). Here we have studied the binding of CaM to synthetic peptides of CaD which contain: (1) both the first and second CaM-binding domains; (2) the second CaM-binding domain; and (3) the sequence between the first and second CaM-binding domains. Two-dimensional transferred nuclear Overhauser enhancement proton NMR measurements as well as circular dichroism studies of a 22-residue peptide NKETAGLKVGVSSRINEWLTK, which contains the second CaM-binding domain, show that only the C-terminal half of the peptide becomes α-helical upon binding to CaM. Somewhat surprisingly, the shorter 9-residue peptide SRINEWLTK was sufficient to form a 1:1 complex with CaM; this peptide appears to bind as a 3_{10}-helix. Proton−carbon-13 correlation NMR titration studies with specifically labeled [*methyl*-^{13}C]methionine CaM were used to study the participation of the hydrophobic regions in both domains of the dumbbell shaped CaM in peptide binding. Binding of a 54-residue CaD peptide containing both CaM-binding domains affects all the 8 Met residues in the two hydrophobic domains of CaM (only Met 76 in the linker region of CaM is not involved), while binding of the second CaM-binding domain of CaD influences principally Met 51, 71, and Met 124, 144. Simultaneous binding to CaM of two peptides comprising the first and the second CaM-binding domains also caused changes to all Met residues except Met 76. Taken together, these data demonstrate that both CaM-binding domains of CaD can bind simultaneously to the two hydrophobic regions of CaM.

Caldesmon (CaD)[1] is found in all smooth muscle cells and in many nonmuscle tissues. It binds to the contractile proteins actin, tropomyosin, and myosin, and hence it has the capacity to cross-link the thick and thin filaments in these tissues. Numerous *in vitro* studies have demonstrated that CaD acts as an inhibitor of the actomyosin ATPase (for reviews, see: Marston & Redwood, 1991; Sobue & Sellers, 1991; Allen & Walsh, 1994). CaD can be phosphorylated by a wide range of protein kinases which modulate its interaction with the other contractile proteins. For example, phosphorylation of Ser 73 in the N-terminal domain of smooth muscle CaD by casein kinase or CaM kinase II weakens the interaction of CaD with myosin (Bogatcheva et al., 1993; Hemric et al., 1993; Sutherland et al., 1994). In addition, phosphorylation of Ser 667, Thr 673, Thr 696, and Ser 702 in the C-terminal domain by proline-directed kinases, such as the cell-cycle regulatory p34^{cdc2} kinase and MAP-kinase, reduces the binding of CaD to actin (Mak et al., 1991a,b; Yamashiro et al., 1991; Childs et al., 1992).

The interaction of CaD with actin and tropomyosin is also regulated by the binding of Ca^{2+}-calmodulin (CaM); again an activation of the actomyosin ATPase is observed upon binding CaM. Originally it was envisaged that competition between actin and CaM for the same binding site on CaD could provide the rationale for the presence of this calcium regulatory mechanism (Sobue et al., 1981; Kakiuchi & Sobue, 1983). However, subsequent studies have shown that the situation is somewhat more complex. For example, the binding of Ca^{2+}-CaM to CaD is relatively weak, suggesting that this calmodulin-controlled mode of release of the actomyosin ATPase inhibition would not be very effective *in vivo*. On the other hand, kinetic studies (Kasturi et al., 1993) and *in vivo* cross-linking studies (Mangels & Gnegy, 1992) have provided experimental support for such a role. Furthermore, addition of CaM-binding peptides of CaD to permeabilized smooth muscle tissue generates effects consistent with a regulatory role for the CaM/CaD interaction (Katsuyama et al., 1992).

Smooth muscle CaD is a fairly large (87 kDa) protein, with a highly irregular amino acid sequence and an elongated structure (Wang et al., 1991a). The protein is generally described as containing three domains, the N- and C-terminal domains which are believed to be globular, and an extended α-helical central domain which is very rich in Lys, Glu, and Ala and contains numerous repeated sequences (Marston & Redwood, 1991). Using proteolytic fragments and bacterially expressed and mutated domains of CaD, the binding sites for the contractile proteins on CaD have been deter-

† Supported by the Alberta Heart and Stroke Foundation (to H.J.V.) and the Medical Research Council of Canada (to A.S.M.). H.J.V. is supported by a Scientist Award from the Alberta Heritage Foundation for Medical Research.

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⊗ Abstract published in *Advance ACS Abstracts,* February 15, 1997.

[1] Abbreviations: CaD, caldesmon; CaM, calmodulin; CD, circular dichroism; cNOS, constitutive nitric oxide synthase; MLCK, myosin light chain kinase; HMQC, heteronuclear multiple quantum coherence; NMR, nuclear magnetic resonance; NOE, nuclear Overhauser enhancement; PDE, phosphodiesterase; SIV, simian immunodeficiency virus; SP, Ser-Pro; TP, Thr-Pro; TFE, trifluoroethanol; TOCSY, total correlation spectroscopy; 1D/2D, one/two-dimensional.

S0006-2960(96)02571-8 CCC: $14.00

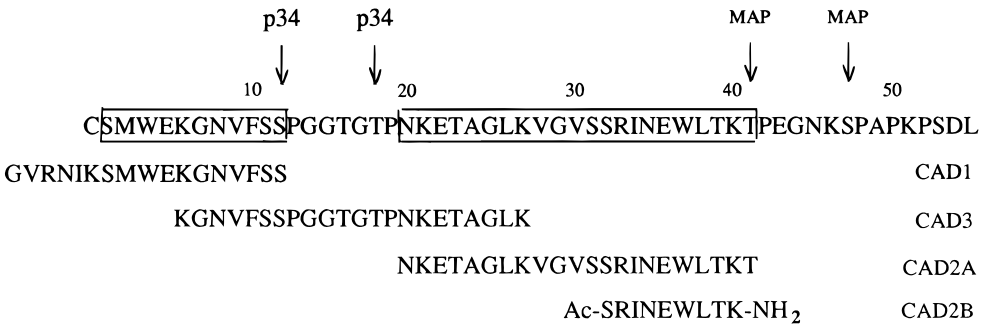

FIGURE 1: Diagram showing the amino acid sequences for the caldesmon synthetic peptides used in this and a previous study (Zhang & Vogel, 1994a). The 54-residue peptide represents amino acids 657−710 in the sequence of chicken gizzard caldesmon (with the exception of the N-terminal Cys residue, which was added to allow attachment of labels). The boxed regions indicate the two potential CaM-binding domains in the C-terminal domain of caldesmon (for discussion, see: Wang et al., 1991b; Zhan et al., 1991; Marston et al., 1994; Mezgueldi et al., 1994; Wang et al., 1996). The shortest CAD2B peptide was acetylated and amidated to remove end charges on the peptide, which could influence its binding to CaM. The preferred positions for phosphorylation by the proline-directed kinases p34 ^{cdc2} kinase and MAP kinase are also indicated. For NMR measurements, we used the 54-residue peptide (without the N-terminal Cys, which caused dimerization), the CAD1 peptide containing the first CaM-binding domain, the CAD2A and CAD2B peptides containig the second CaM-binding domain, and the CAD3 peptide containing part of two CaM-binding domains as well as their intervening sequence which also encompasses the p34 kinase sites.

mined. Myosin interacts with the N-terminal domain, and actin, tropomyosin, and calmodulin bind primarily to the C-terminal domain of CaD; these results are in agreement with the effects of the protein phosphorylation studies (Bartegi et al., 1990; Wang et al., 1991b; Chalovich et al., 1992; Wang et al., 1996).

The binding site for CaM on chicken gizzard CaD was originally determined to be in the region around Trp 659 (Wang et al., 1991b). This was consistent with observations that binding of CaM to CaD gave rise to a considerable change in the Trp fluorescence wavelength and intensity (Shirinsky et al., 1988). From studies with various fragments of CaD, it appeared that the heptapeptide W^{659}EKGNVF might be sufficient for CaM binding (Wang et al., 1991b). A 17-residue peptide with the amino acid sequence GVRNIKSMW^{659}EKGNVFSS (CAD1; see Figure 1) was later shown to bind to CaM with an affinity comparable to that of intact CaD ($K_D \sim 10^{-6}$M). In addition, it also displayed inhibitory properties similar to intact CaD as well (Zhan et al., 1991). NMR and CD spectroscopic studies have confirmed that this peptide binds with a short α-helix to the two Met-rich hydrophobic surface regions of Ca^{2+}-CaM (Zhang & Vogel, 1994a). Subsequent studies, however, have identified a second CaM-binding domain slightly C-terminal to the original region near Trp 659 (Marston et al., 1994; Mezgueldi et al., 1994). Both these studies identified the sequence SRINEW^{692}LTK as part of the second CaM-binding domain. Note that again a Trp residue is present which is consistent with the enhanced fluorescence that was observed upon CaM binding in studies of intact CaD (Shirinsky et al., 1988). Mezgueldi et al. (1994) suggested a longer 22-residue binding region (NKETAGLKVGVSSRINEW^{692}-LTKT). The latter two studies also demonstrate that the CaM induced release of the actomyosin inhibition by CaD was primarily associated with this second CaM-binding domain (Marston et al., 1994; Mezgueldi et al., 1994). A model in which a single CaM molecule could bind to both CaM-binding domains of CaD simultaneously was proposed (Marston et al., 1994; Huber et al., 1996; see also Wang et al., 1996). It should be noted, however, that others have argued that this site may be indirectly involved through a conformational change caused by binding of CaM to the site around Trp 659 (Zhuang et al., 1995).

In this work, we have investigated the CaM-binding properties of synthetic peptides encompassing the second CaM-binding domain of CaD by NMR and CD spectroscopy. Two synthetic peptides, which were identical to the amino acid sequences for the second CaM-binding domain identified by Mezgueldi et al. (CAD2A) and Marston et al. (CAD2B), were studied (see Figure 1). Morever, a 54-residue synthetic peptide containing the two CaM-binding regions and their intervening sequence was studied. As a control, a peptide containing mostly the intervening sequence (CAD3; see Figure 1) was studied as well; this peptide is of importance because it is a good substrate for Pro-directed protein kinases. Finally, the binding of various mixtures of CAD1 and CAD2A or CAD2B peptides was studied. Our data suggest that both CaM-binding domains of CaD can bind simultaneously to CaM.

MATERIALS AND METHODS

All peptides used were obtained from the Peptide Synthesis Facility at Queens University (Kingston, ON, Canada). Peptides were synthesized using standard FMOC chemistry on an Applied Biosystems Synthesizer. All the shorter peptides (<25 amino acid residues) were purified by preperative reversed-phase HPLC and were >98% pure as judged by proton NMR, amino acid analysis, and mass spectrometry. The longer 54-residue peptide was not further purified following synthesis: it was >90% pure as determined by analytical reversed-phase HPLC. Calmodulin was purified using repeated chromatography on phenyl-Sepharose from an *Escherichia coli* strain harboring an expression plasmid containing a synthetic gene for CaM (Zhang & Vogel, 1993; Waltersson et al., 1993). [*methyl*-^{13}C]Met labeled CaM was prepared as described elsewhere (Zhang et al., 1994a; Siivari et al., 1995). Trifluoroethanol was obtained from Sigma and trifluoroethanol-d_3 was purchased from MSD Isotopes (Montreal, Canada).

All NMR experiments were performed on a Bruker AMX-500 as described earlier (Zhang & Vogel, 1994a; Siivari et al., 1995). Transferred NOE experiments were performed at a peptide to protein ratio of 25:1; the peptide concentration in these experiments was 2.0 mM. The buffer used was 90% H_2O−10% D_2O, 100 mM KCl, 10 mM $CaCl_2$, pH 7.0. All

transferred NOE spectra were collected at 20 °C; data were processed with standard Buker UXNMR software. Mixing times from 100 to 250 ms were used, and no evidence for spin diffusion was obtained.

One-dimensional and two-dimensional $^{1}H,^{13}C$ HMQC spectra were obtained using the pulse sequences of Bax et al. (1983). Titration experiments were performed by adding aliquots of concentrated peptide solutions to [*methyl*-^{13}C]-Met labeled CaM samples (0.5 mM in 100% D_2O, 100 mM KCl, 10 mM $CaCl_2$, pH 7.0). The final stoichiometry of the CAD2A, CAD2B peptide−CaM interaction was determined by measuring the UV absorption at 280 nm of the peptides and CaM stock solutions.

CD spectra were acquired on a Jasco J-500C CD spectropolarimeter as described before (Yuan et al., 1995). The concentration of the peptide or CaM used was 10 μM. CD spectra of the peptides, CaM, and the complex of CaM and peptide were measured at ambient temperature in 10 mM Tris buffer, pH 7.2, with 0.5 mM $CaCl_2$. Nondenaturing urea polyacrylamide gel electrophoresis was performed using reported methods (Erickson-Viitanen & DeGrado, 1987; Yuan et al., 1995).

RESULTS

Synthetic peptides encompassing the CaM-binding domains of target proteins of CaM generally are devoid of regular secondary structure in aqueous solution (Vogel & Zhang, 1995; Ikura, 1996). They acquire an α-helical structure upon binding to CaM, or following addition of the helix-inducing solvent trifluoroethanol (Roth et al., 1991; Ikura & Bax, 1992; Zhang et al., 1993; Zhang & Vogel, 1994a; Yuan et al., 1995). One- and two-dimensional proton NMR spectra of the CAD2A, CAD2B and the 54-residue peptide in aqueous solution demonstrated that these were also devoid of such structures (data not shown, see also CD spectra below). Therefore, our first objective was to determine whether CaM was capable of inducing α-helical structures in these peptides. Information about the structure of peptides bound to proteins can be obtained from the transferred NOE effect, which can be observed if the peptide exchanges fast on the NMR time scale between free and protein-bound states (Ni & Scheraga, 1994). Transferred NOESY 2D experiments were performed with the CAD2A and CAD2B peptides in the presence of a small amount of CaM (peptide to protein ratio at 25:1). Several peptide peaks shifted and broadened slightly upon the addition of CaM, suggesting fast exchange between free and protein-bound peptides. In addition, the number of NOESY cross peaks and their peak intensities increased dramatically upon addition of CaM to the peptide samples. Complete sequential assignments for CAD2A and CAD2B were obtained by analysis of TOCSY and NOESY spectra, using standard two-dimensional NMR analysis techniques (Wüthrich, 1986). The NOE summary data derived from these spectra for the CAD2A and CAD2B peptides are given in Figure 2. Continuous medium range NOE's ($d_{\alpha\beta}(i,i+3)$, $d_{\alpha N}(i,i+3)$) are found for the 22-residue CAD2A peptide from Val 11 to Thr 22, indicating that this region of the peptide becomes α-helical (Wüthrich, 1986) upon binding to CaM. Such cross peaks could not be found for the N-terminal 11 residues of the peptide, suggesting that this region retains a nonhelical structure upon binding to CaM. The NOE summary for the

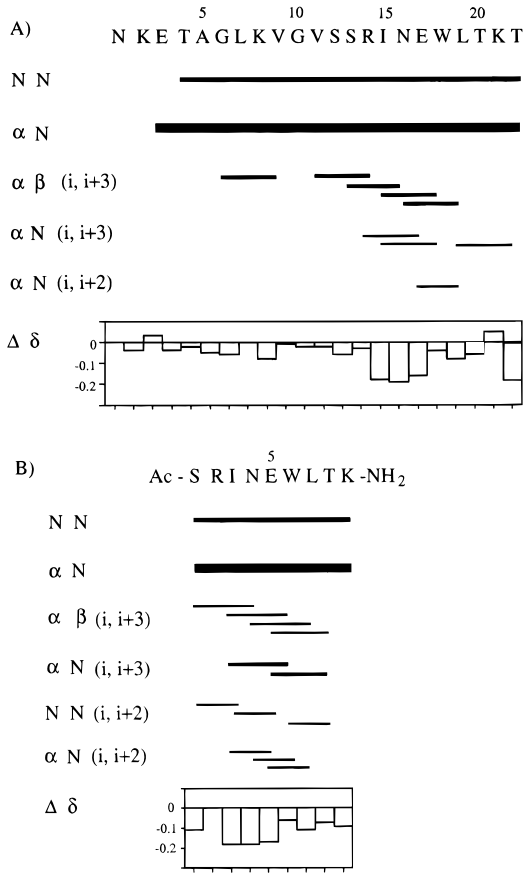

FIGURE 2: (A) Summary of the NOE's observed in a transferred NOESY experiment of the CAD2A peptide in the presence of calmodulin. The thickness of the lines corresponds to the observed NOE intensities. The lower panel indicates the difference between the observed α CH proton chemical shifts and its random coil shift (the latter were taken from Wüthrich, 1986). (B) NOE-summary and chemical shift difference plot for the CAD2B peptide.

CAD2B peptide is shown in Figure 2B. In this case many medium range NOE's were found for the entire 9-residue peptide, which suggests the formation of helical turns in this peptide upon binding to CaM. However, we note that six $d_{NN}(i,i+2)$ and $d_{\alpha N}(i,i+2)$ NOE cross peaks can be found for this shorter peptide; in addition, the $d_{\alpha\beta}(i,i+3)$ cross peaks are relatively weak; these NOE data are therefore possibly more characteristic for 3_{10}-helix rather than α-helical turn formation (Wüthrich, 1986). However, it should be stressed that it is difficult to distinguish between α-helix and 3_{10}-helix on the basis of such peptide NMR data alone. Our NMR data for CAD2B may thus indicate the presence of interconverting structures of α-helical and 3_{10}-helical turns, as well as extended structures. We also calculated the difference between the observed αCH proton chemical shifts and their random coil chemical shifts for the two peptides (see Figure 2). In both peptides we find the expected upfield shift for the αCH proton chemical shift, indicative of the formation of helical turns (Wishart et al., 1991). However, these shifts are quite small, because the transferred NOE experiments are typically run at a large excess of peptide; hence these data alone do not provide conclusive evidence for the absence or presence of helical turns. However, they are consistent with the NOE data, as they suggest α-helix formation in the C-terminal residues of CAD2A and throughout CAD2B. Finally, it should be mentioned that serious overlap problems in the proton NMR spectra of the 54-

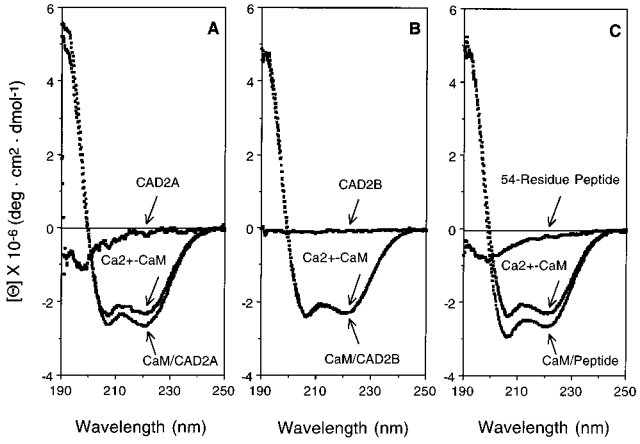

FIGURE 3: Circular dichroism spectra for the binding of the (A) CAD2A, (B) CAD2B, and (C) the 54-residue peptide to calmodulin. All three panels contain traces for the peptide, calcium-saturated CaM, and the CaM−peptide complex. The concentration of either CaM or peptide was 10 μM, dissolved in 10 mM Tris buffer, pH 7.2 with 0.5 mM $CaCl_2$.

residue peptide precluded us from analyzing its spectra in detail. In addition, several minor peaks appeared and some of these gave rise to substantial NOE's which further complicated matters. Hence at this stage no structural information about its binding to CaM could be obtained from the NMR data.

Further evidence for the formation of α-helices in the two CaM-binding domains of CaD was obtained by CD spectroscopy. The spectra in Figure 3 clearly show the increase in α-helicity upon binding of the CAD2A and the 54-residue peptide to CaM. None of these peptides appears to have any α-helical structure in the absence of CaM. CaM is an extremely α-helical protein, consisting of two domains, which do not change much in structure upon the binding of target proteins (Ikura et al., 1992; Roth at al., 1992). The major change that occurs upon binding of typical target peptides is for the helical linker region which unravels somewhat to allow for a different orientation of the two domains of CaM (Ikura et al., 1992; Meador et al., 1992, 1993). Thus the increase in α-helicity observed in the CD spectra of CaM peptide complexes does not arise from an increase in the α-helicity of CaM, because the protein does in fact experience a small decrease in helix content upon peptide binding. Rather it indicates α-helix formation of the target peptide upon binding to CaM. If the CaM conformational change is neglected, the CD spectral changes of both CAD2A and the 54-residue peptide upon CaM binding can be interpreted as caused by formation of approximately a 9-residue long α-helix (Scholtz et al., 1991). This interpretation of the CD spectra has also been supported by isotope-edited Fourier transform infrared spectroscopy studies of CaM-binding domain peptides (Zhang et al., 1994b). Consequently, the data in Figure 3 indicate formation of α-helix in the CAD2A and 54-residue peptide upon binding to CaM, as an enhancement in the characteristic ellipticities for α-helix at 208 and 222 nm is observed. The data for the 9-residue CAD2B peptide show no increased α-helicity; this may be related to its lower affinity for CaM. However, it can also be due to its helix being too short, or too irregular to give rise to the appearance of the characteristic helical ellipticities; such observations are not uncommon in CD studies of relatively short peptides (for discussion, see Manning, 1989; Johnson, 1990).

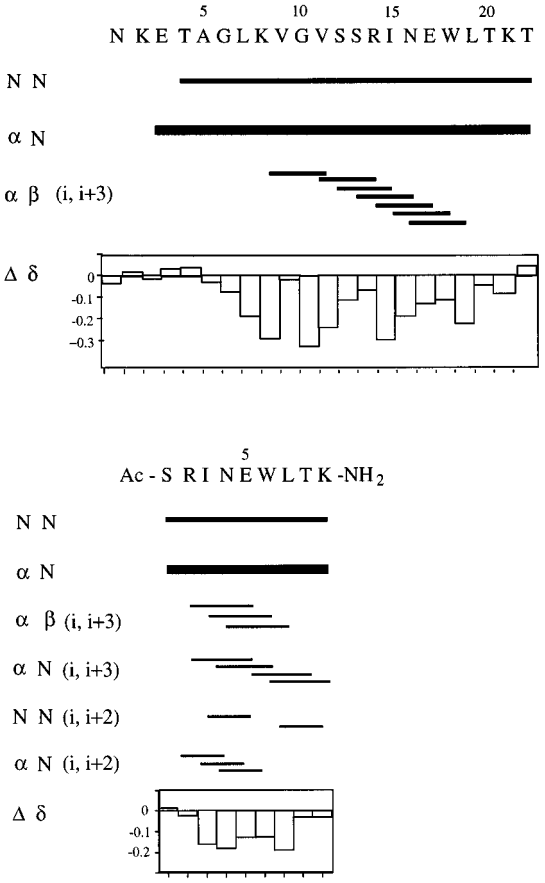

FIGURE 4: A summary of the NOE and chemical shift change data obtained for the CAD2A peptide in 40% TFE aqueous solution (top) and for the CAD2B peptide in 45% TFE aqueous solution (bottom).

The NMR and CD data obtained for the CAD2A and CAD2B peptides in the presence of CaM in aqueous solution suggest that these two peptides may have a preference for forming distinct secondary structures, despite their similarity in amino acid sequence. We have therefore also investigated their structures in 40−45% aqueous trifluoroethanol. The cosolvent TFE is generally capable of inducing α-helical structures in peptides that form α-helical regions in intact proteins (see, e.g., Lehrman et al., 1990, and Segawa et al., 1991) and in many CaM-binding domain peptides (Vogel & Zhang, 1995). The NMR data recorded for these two peptides under these conditions are summarized in Figure 4. Based on the medium range NOE and chemical shift change data, the CAD2A now forms a slightly longer α-helical structure than that observed upon binding to CaM (see above), starting at its Leu 7 residue and probably extending to the penultimate Lys residue. For the CAD2B peptides in aqueous TFE solution, we observe a substantial number of *i*,*i*+3 as well as *i*,*i*+2 medium range NOE interactions (see Figure 4). The latter are again indicative of the formation of 3_{10}-helical turn structures in this peptide (Wüthrich, 1986). The 54-residue peptide was also studied in aqueous TFE solution. Many additional cross peaks appeared in the NOESY spectra, which were consistent with peptidal α-helix formation; however, as before, the spectral resolution did not allow a complete assignment for this peptide (data not shown). Circular dichroism studies of the

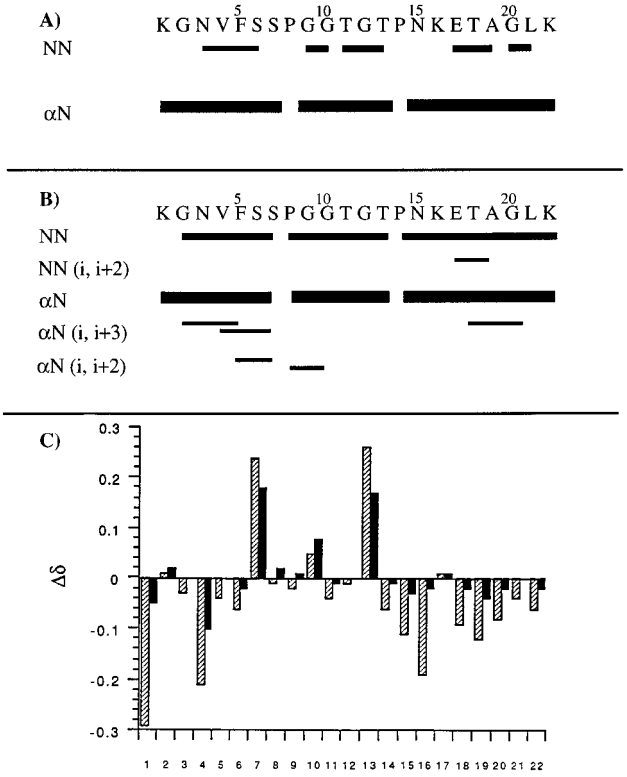

FIGURE 5: NOE summary obtained for the CAD3 peptide, in (A) the absence and (B) the presence of 40% TFE. The thickness of the lines corresponds to the observed NOE intensities. (C) The chemical shift difference between the observed and random coil α CH proton chemical shifts, as observed in the absence (solid bars) or presence (hatched bars) of trifluoroethanol.

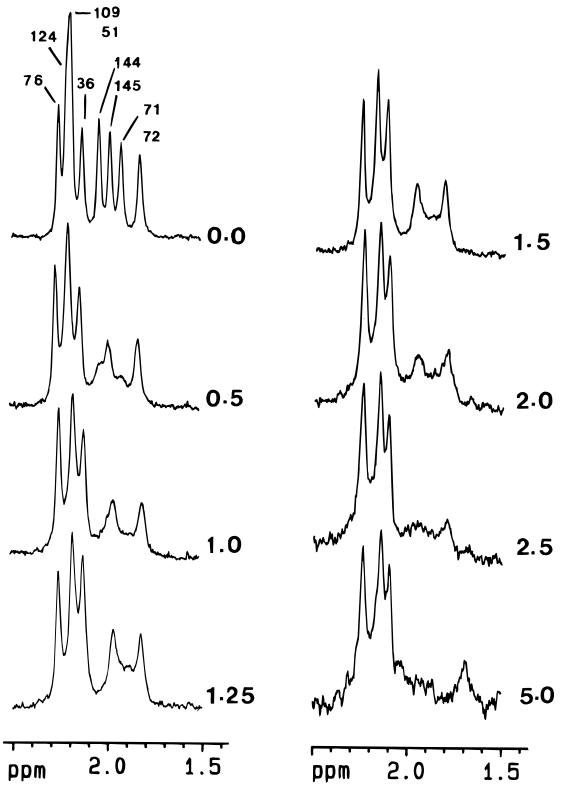

FIGURE 6: One-dimensional $^1H,^{13}C$ HMQC spectra of [*methyl*-^{13}C]-Met calmodulin during a titration with the CAD2A peptide. The ratio of peptide to calmodulin is indicated in the figure. The assignments for the Met residues were taken from Siivari et al. (1995). At the end of the titration (peptide to CaM ratio 5 :1), only the peaks for Met 36, 72, 76, 109, and 145 are still detected reliably.

CAD2A, CAD2B, and the 54-residue peptide in 40% TFE show increased α-helical content in the two longer peptides (data not shown).

We have also characterized a synthetic CAD3 peptide, which contains parts of both CaM-binding domains as well as the Pro- and Gly-rich region between them. In addition, it encompasses the two primary phosphorylation sites for the cell cycle regulatory $p34^{cdc2}$ kinase (see Figure 1). This peptide can be efficiently phosphorylated by $p34^{cdc2}$ kinase, suggesting that it contains the required determinants for recognition by this protein kinase (Mak et al., 1991a). Secondary structure prediction programs assign a very high probability of turn formation to the SP and TP sequences of this peptide (data not shown); however, NMR data acquired for the CAD3 peptide in aqueous solution only show a few short range d_{NN} and $d_{\alpha N}$ cross peaks, indicating the absence of a substantial amount of turns under these conditions (see Figure 5A). The cosolvent trifluoroethanol, which is well-known for its α-helix stabilizing ability (Lehrman et al., 1990; Segawa et al., 1991), has also been shown to be able to stabilize β-turn structures in some peptides (Blanco et al., 1994). However, addition of TFE to the CAD3 peptide (Figure 5B) did not generate stable turn formation for the SP or TP regions either (for summary of characteristic NOE patterns for turn formation, see Wüthrich, 1986). In addition, all the $^3J_{NH\alpha}$ coupling constants all remained around 7 Hz, indicating a predominance of extended structures. The appearance of some medium range NOE's for the N- and C-terminal end of this peptide upon TFE addition suggests that some helical turns may form in these regions; this is consistent with the chemical shift difference data for this peptide (Figure 5C), which show slightly upfield shifts for the majority of the α CH protons in these two regions. In this diagram, it can be noted that the α CH resonances of the Ser and Thr preceding the proline residues experience a characteristic downfield shift, which has been observed for nearly all amino acid residues immediately preceding Pro in peptides and proteins (Wishart et al., 1991).

Our next objective was to study whether these peptides bound to the hydrophobic regions in the two domains of CaM, which have been shown previously to be involved in the binding of the CAD1 peptide (Zhang & Vogel, 1994a), the MLCK and CaM kinase II peptides (Ikura et al., 1992; Meador et al., 1992, 1993) as well as the PDE and cNOS CaM binding domains (Zhang et al., 1994a, 1995). Our earlier NMR studies with peptides comprising various CaM-binding domains relied primarily on the use of [*methyl*-^{13}C]-Met which was biosynthetically incorporated into CaM. The two hydrophobic regions of CaM are extremely rich in Met−46% of their surface area is contributed by Met side chains (O'Neil & DeGrado, 1990)−making these residues a good marker for studying peptide binding to these regions. Figure 6 shows a titration experiment with the CAD2A peptide followed by 1D proton-detected $^1H,^{13}C$ correlated spectra. The assignments for the initial spectrum were determined by comparing proteins containing Met → Leu mutations of all individual Met residues in CaM (Siivari et al., 1995). Upon binding the first equivalent of CAD2A, the resonances for Met 71 and 144 broaden drastically. Some changes also occur for the partially overlapping peaks for Met 36, 51, 109, and 124, which could be followed more easily by recording 2D $^1H,^{13}C$ HMQC spectra (see

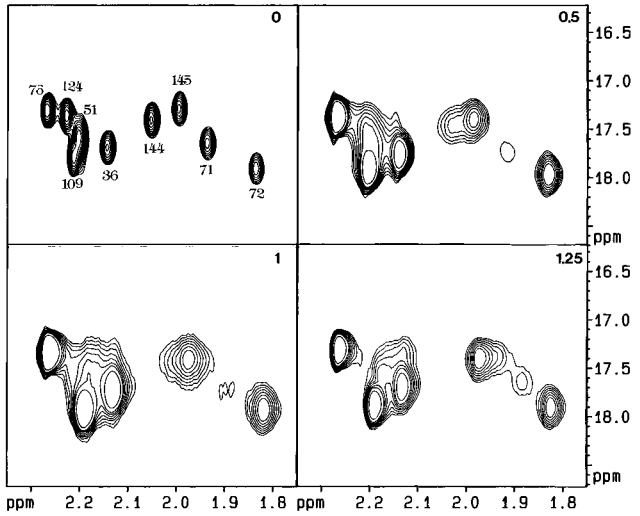

FIGURE 7: Two-dimensional $^{1}H,^{13}C$ HMQC spectra of the same titration experiment of CAD2A with [*methyl*-^{13}C]Met calmodulin as shown in Figure 6 (the ratio of peptide to calmodulin is indicated in each panel). Broadening of the peaks for Met 51, 71, 124, and 144 is observed here by changes in the contour pattern and the contour levels.

Figure 7). Apart from Met 71 and 144, also the Met 51 and 124 resonances broaden somewhat. However, the resonances for the other five Met residues of CaM—including Met 76 which is not in the hydrophobic regions, but is in the central linker region of CaM—are not much affected by the addition of 1 equiv of CaD2A. Addition of more than 1 equiv of peptide gave rise to broadening and shifting of the peaks for Met 72 and 145 (see Figure 6). This indicates the formation of multimeric (CAD2A) peptide structures bound to CaM at higher peptide concentrations.

In order to further investigate the nature of the process that leads to the broadening of the Met residues, the temperature dependence of the spectra was recorded for a sample containing equal amounts of CaM and CAD2A peptide. As shown in Figure 8, all the broadened peaks reappear at higher temperatures, as was also demonstrated in 2D $^{1}H,^{13}C$ HMQC spectra of the same sample (data not shown). In addition, shifting peaks, which give rise sometimes to peak overlap and hence changes in peak intensities, are observed. This behavior appears to be consistent with an exchange process between peptide-bound and unsaturated CaM, where the resonances of some of the Met residues in the bound state must be very broad and hence undetectable in our current analysis. The rationale for this broadening effect upon CAD2A binding is unclear at present, as saturation of CaM with other tightly binding peptides derived from PDE, cNOS, and MLCK, for example, does give rise to substantially shifted, but not to broad Met residues in the peptide-bound state (Zhang et al., 1994b, 1995; Siivari et al., 1995). In all likelihood, it indicates the presence of conformational heterogeneity and exchange between these conformers in the peptide-bound state, similar to what we have reported for the complex of CaM with an SIV glycoprotein peptide (Yuan et al., 1995). Since peptide binding to the hydrophobic regions generally leads to rather large chemical shift changes for the Met residues, this could explain the broad bound resonances observed here with the CAD2A peptide binding.

Titration of [*methyl*-^{13}C]Met CaM with the CAD2B peptide gave rise to similar results as obtained with the

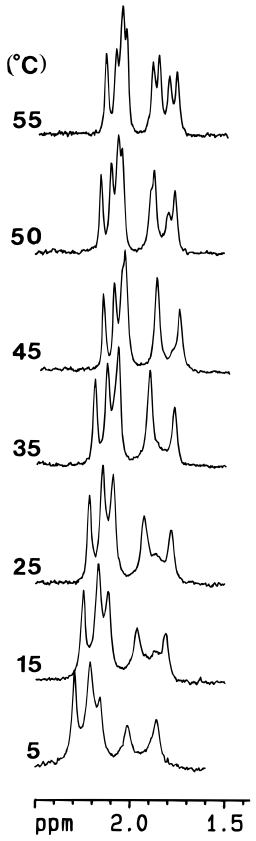

FIGURE 8: Temperature dependence of the 1D $^{1}H,^{13}C$ HMQC spectra for the [*methyl*-^{13}C]Met calmodulin sample with CAD2A (1:1 ratio). The temperatures are indicated in the figure.

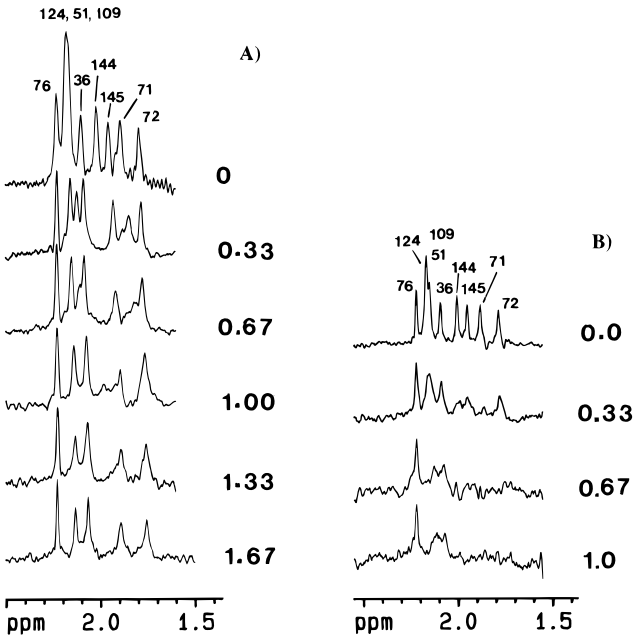

FIGURE 9: (A) 1D $^{1}H,^{13}C$ HMQC spectra of [*methyl*-^{13}C]Met-CaM with the CAD2B peptide and (B) with the 54-residue peptide. The ratio of peptide to calmodulin is indicated in the figures (experimental conditions identical to Figure 5).

CAD2A peptide (see Figure 9A). Again, Met 71 and 144 are dramatically broadened, followed by Met 51 and 124. This is also seen in the 2D HMQC spectra (data not shown). It should be noted that no further changes occur in the spectra upon further peptide additions, unlike the results obtained with peptide CAD2A (compare Figures 6 and 9A).

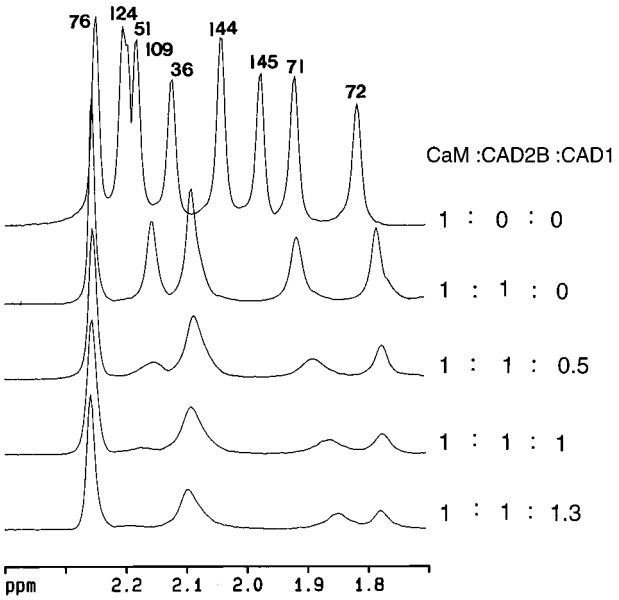

FIGURE 10: Titration experiment of [*methyl*-^{13}C]Met-CaM with CAD2B and CAD1 peptides followed by one-dimensional ^{1}H,^{13}C HMQC spectra. The addition of 1 equiv of CAD2B (or CAD2A) peptide causes similar changes as observed in Figure 6 or Figure 9A. Subsequent addition of 0.5, 1.0, or 1.5 equiv of peptide CAD1 causes further changes in the Met resonances, so that the final spectrum resembles that obtained with the 54-residue peptide. If CAD1 and CAD2A (or CAD2B) are added in the reversed order, the final spectra are identical to the bottom panel in this figure.

We also performed titrations of [*methyl*-^{13}C]Met CaM with the 54-residue peptide and the CAD3 peptide. The latter introduced very few changes in the spectra, suggesting that it does not bind with high affinity to this region of CaM (data not shown). However, addition of the 54-residue peptide showed that all Met in the two hydrophobic regions of CaM are now broadened beyond detectability after addition of 1 equiv of peptide; the peak which is still relatively sharp and visible is that of Met 76 (see Figure 9B). This pattern is distinct from that obtained upon titration of CaM with the peptides CAD2A and -2B, or with the peptide CAD1 (see Figure 8 in Zhang & Vogel, 1994a). Finally, we have studied titration of [*methyl*-^{13}C]Met-CaM with 1:1 mixtures of CAD1 and CAD2A (or CAD2B) added in different order. All these mixtures give identical final spectra which are similar to that observed for the titration of the 54-residue peptide to CaM. An example of such a titration is shown for the addition of the CAD1 peptide to a complex of CaM/CaD2B in Figure 10. These data clearly demonstrate that both CAD1 and CAD2A/B peptides can bind simultaneously to the two hydrophobic regions of CaM.

We have also studied the binding of the CAD1, CAD2A, CAD2B, CAD3, and the 54-residue peptide to CaM by nondenaturing urea polyacrylamide gel electrophoresis. Tight complex formation of peptide with CaM generally gives rise to a characteristic band shift (Erickson-Viitanen & Degrado, 1987; Vogel & Zhang, 1995). However, no band shift was observed for any of the CAD peptide under conditions where the MCLK peptide gave rise to an obvious band shift (data not shown). This is consistent with the notion that peptides that give rise to a band shift generally bind with dissociation constants in the nanomolar range, while those that do not give a band shift bind with lower affinity (micromolar range). Thus all CaD peptides bind relatively weak to CaM, in agreement with the reported K_D for binding of CaM to the intact CaD protein ($K_D \sim 10^{-6}$ M, see Marston & Redwood, 1991).

DISCUSSION

The binding of target peptides that are derived from CaM-modulated proteins and enzymes to calmodulin has been studied extensively by NMR in recent years (Vogel, 1994; James et al., 1995). As such, considerable knowledge has been gained about the binding of MLCK (Roth et al., 1991, 1992; Ikura & Bax, 1992; Ikura et al., 1992; Meador et al., 1992), nitric oxide synthase (Zhang & Vogel, 1994b; Zhang et al., 1995), CaM kinase II (Meador et al., 1993), and the HIV/SIV (Yuan et al., 1995) CaM-binding domain peptides. In addition, various studies have probed the atypical binding of the B50/neuromodulin CaM-binding peptide to CaM (Zhang et al., 1994c; Gerendasy et al., 1995; Urbauer et al., 1995). In all cases, the CaM-binding domain is believed to be contained in a single contiguous region in the amino acid sequence, and this mode of interaction occurs in the vast majority of CaM-binding proteins. Some calcium−CaM activated proteins display multiple CaM-binding domains, but many of these bind more than 1 equiv of CaM (e.g., Goold & Baines, 1994; Menegazzi et al., 1994). However, there are limited examples of proteins that bind only 1 mole equiv of CaM where two distinct CaM-binding domains have been found, that are separated in the amino acid sequence. This has been most clearly demonstrated in the case of phosphorylase kinase (Dasgupta et al., 1989; Juminaga et al., 1994). Recent studies have suggested that CaD also contains two distinct CaM-binding domains, and it has been proposed that these bind simultaneously to CaM (Marston et al., 1994; Mezgueldi et al., 1994; Wang et al., 1996). In a previous NMR study of the CAD1 domain which has the sequence GVRNIKSMW^{659}EKGNVFSS and was originally studied by Zhan et al. (1991), we found that a short 10-residue α-helix was formed in this peptide upon binding to CaM, and that it bound with 1:1 stoichiometry to the two Met-rich regions of CaM (Zhang & Vogel, 1994a). Our current transferred NOE NMR data on peptides encompassing the second CaM-binding domain of CaD show that binding to CaM is again capable of inducing helical structures in the peptides. Our CD data clearly show α-helix formation in the 22-residue CAD2A peptide and in the 54-residue peptide. However, no significant increase in α-helicity is observed for the shorter 9-residue peptide (CAD2B), probably because this helix is too short or too irregular to give a typical helical CD spectrum. Nevertheless, its numerous medium range NOE peaks clearly suggest the formation of a mixture of 3_{10}-helix and α-helix in the CAD2B peptide upon binding to CaM. The increase in α-helicity in the 54-residue peptide upon binding to CaM must be caused primarily by induction of α-helical structure in the CAD2A region; our earlier CD studies with the CAD1 CaM-binding domain showed a very small increase in α-helicity in this region upon binding to CaM, and since the 54-residue contains only the C-terminal part of the 17-residue CAD1 sequence, it is unlikely that this part contributes very much to the drastic increase of helicity observed in Figure 3C. No structural changes were detected by transferred NOE or CD measurements for the CAD3 peptide upon adding CaM.

We note that CaM only induces an increase in α-helical structure in the C-terminal 11 residues of the 22-residue CAD2A (see Figure 2A) containing the 9-residue CAD2B

peptide sequence. The remaining residues of CAD2A do not appear to have any particular secondary structure when bound to CaM, and it seems possible that these do not bind to CaM. In fact, given that this N-terminal part of the CAD2A sequence does not contain any bulky hydrophobic groups, it is unlikely that this region binds to the hydrophobic regions of CaM. However, fluorescence competition experiments show that the CAD2A peptide binds CaM with higher affinity than CAD2B (Weljie and Vogel, unpublished data), suggesting that the N-terminal region of CAD2A may contribute to the binding possibly by making some salt linkages to the acidic CaM.

The ability of the CAD2A and CAD2B peptides to form secondary structure appears to be remarkably different. This is somewhat surprising in view of the similarity in amino acid sequence. When binding to CaM or dissolved in TFE, CAD2A prefers to form α-helical structures in its C-terminal end. However, evidence for the existence of 3_{10}-helical turns was found in our studies of the shorter CAD2B peptide under these conditions. The evidence for the presence of interconverting 3_{10} and α-helical structures in peptides has recently been reviewd in detail (Millhauser, 1995; Fiori & Millhauser, 1995). It was concluded that mixtures of these two types of helical structures may be difficult to analyze by CD and NMR spectroscopy alone. Based on other spectroscopic studies, however, the authors propose that the detection of (*i*,*i*+2) medium range NOE interactions is indicative of the presence of at least some 3_{10}-helical structures. Therefore, we interpret our data in this fasion; furthermore, the absence of any *i*,*i*+4 NOE's in our NMR spectra argues against stable α-helix formation in CAD2B. The rationale for CAD2B having different structural properties than CAD2A may simply relate to the different length of these two peptides: shorter peptides generally display a greater propensity to form 3_{10}-helical structures (Millhauser, 1995; Fiori & Millhauser, 1995). In addition, we cannot exclude the possibility that the N-terminal acetylation and C-terminal amidation of the CAD2B peptide may influence its structural properties; we are not aware of any studies in which the effects of these substitutions on secondary structure formation in peptides have been investigated systematically.

Titration experiments of [*methyl*-^{13}C]Met CaM were performed with CAD2A, CAD2B, CAD3, the 54-residue peptide, and (in our earlier study) with CAD1. The CAD1 peptide binds in fast exchange, and chemical shift changes for all the Met peaks in the two hydrophobic domains could be followed throughout the titration (Zhang & Vogel, 1994a). CAD3 did not cause significant changes in the spectra; this suggests that the numerous Pro and Gly residues in between the two CaM-binding sites are preventing cooperative helix formation between these and in doing so, prevent the binding of CAD3 to CaM. Titration with CAD2A and CAD2B resulted in the selective disappearance of the resonances for Met 51 and 71 in the N-terminal domain of CaM, as well as the disappearance of Met 124 and 144 in homologous positions in the C-terminal domain. This suggests that CAD2A and CAD2B bind in a very similar manner to both domains of CaM. It should be noted that this behavior is unique; with all other CaM-binding peptides tested to date (PDE, cNOS, MLCK, SIV, melittin), all 8 Met residues in the two domains experience large shifts. Since the binding of CAD1 also affected all 8 Met in both domains, our data shows that the binding of CAD1 and CAD2 peptides to the two Met-rich regions is distinct. Hence it might be possible that both peptides can be accommodated simultaneously by CaM. In order to test this, we have performed a titration of CaM with the 54-residue peptide as well as with all possible mixtures of the CAD1 and CAD2A/B peptides added in different order. We noted that this led to a complete broadening of all Met residues (except Met 76) in the two hydrophobic domains of CaM. This behavior is indicative of a binding mode where both the CAD2A and CAD1 segments bind simultaneously to CaM. In order to determine how these two peptides are arranged with respect to each other and to CaM, other kinds of studies, for example, involving nitroxide spin-labeled peptides, will have to be performed (see, for example: Zhang et al., 1995; Millhauser, 1995). Such studies have been initiated in our laboratories, with the aim of shedding further light on this unique two-domain CaM-binding motif in CaD. In this respect, it is noteworthy that a peptide containing the two CaM-binding domains of phosphorylase kinase forms a complex with CaM, in which CaM retains an extended structure (Juminaga et al., 1994), contrary to the compact structures observed with MLCK and CaM kinase II peptides (Ikura et al., 1992; Meador et al., 1992, 1993).

ACKNOWLEDGMENT

We are indebted to Ms. Sook Shin (Queens University) for peptide synthesis and purification, to Mr. Wei Guo for initial NMR measurements on the CAD3 peptide, to Dr. Deane McIntyre for upkeep of the NMR spectrometers, and to Dr. J. H. van de Sande and Mr. B. Kalisch (University of Calgary) for allowing the use of their CD spectrometer.

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BI9625713