PROTEIN STRUCTURE REPORT

Solution structure and backbone dynamics of Calsensin, an invertebrate neuronal calcium-binding protein

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(RECEIVED February 15, 2005; FINAL REVISION April 10, 2005; ACCEPTED April 11, 2005)

Abstract

Calsensin is an EF-hand calcium-binding protein expressed by a subset of peripheral sensory neurons that fasciculate into a single tract in the leech central nervous system. Calsensin is a 9-kD protein with two EF-hand calcium-binding motifs. Using multidimensional NMR spectroscopy we have determined the solution structure and backbone dynamics of calcium-bound Calsensin. Calsensin consists of four helices forming a unicornate-type four-helix bundle. The residues in the third helix undergo slow conformational exchange indicating that the motion of this helix is associated with calcium-binding. The backbone dynamics of the protein as measured by ^{15}N relaxation rates and heteronuclear NOEs correlate well with the three-dimensional structure. Furthermore, comparison of the structure of Calsensin with other members of the EF-hand calcium-binding protein family provides insight into plausible mechanisms of calcium and target protein binding.

Keywords: Calsensin; calcium-binding proteins; EF-hand; NMR structure; dynamics; helix-loop-helix; nervous system

Intracellular calcium concentration regulates a variety of cellular processes including neurite extension, cell motility, cell-cycle progression, cell proliferation, and apoptosis (Schafer and Heizmann 1996; Berridge et al. 1998; Donato 2003). Many of these signal transduction events are mediated by members of the EF-hand family of calcium-binding proteins via interaction with target proteins in a calcium-dependent manner (Schafer and Heizmann 1996). The EF-hand family of calcium-binding proteins can be classified as sensor or buffer proteins based on their function (Zimmer et al. 1995; Ikura

1996). The buffer proteins like Calbindin $\text{D}_{9\text{K}}$ maintain calcium homeostasis by regulating the intracellular calcium concentration (Ikura 1996). On the other hand, sensor proteins such as S100B and CaM undergo a conformational change upon calcium binding, thereby altering their affinity for target proteins like caldesmon, tau annexins, and various kinases (Schafer and Heizmann 1996; Donato 2003). EF-hand calcium-binding proteins have been implicated in a variety of pathological diseases including Alzheimer's disease, Down syndrome, and inflammatory disorders (Griffin et al. 1998). Thus, solving the structure of these proteins will be valuable for elucidating their functions.

We have previously cloned and characterized a small 9-kD neuronal EF-hand Ca^{2+} -binding protein, Calsensin (Briggs et al. 1995). Calsensin is expressed in a subset of peripheral sensory neurons fasciculating into a single axon tract in the leech central nervous

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Article published online ahead of print. Article and publication date are at <http://www.proteinscience.org/cgi/doi/10.1110/ps.051412605>.

system (Briggs et al. 1993, 1995). The molecular features of Calsensin and its restricted expression in the nervous system are consistent with the hypothesis that it may participate in protein-complex mediated calcium-dependent signal transduction events in growth cones and axons (Briggs et al. 1995). It has become increasingly clear that changes in intracellular calcium levels can modulate axon fasciculation and growth cone motility (Hong et al. 2000; Zheng 2000) and previous studies have directly implicated calcium-binding proteins in growth cone guidance in *Drosophila* (VanBerkum and Goodman 1995). For these reasons we have undertaken a structural study of Calsensin, determining the structure of calcium-bound Calsensin under reducing condition using multidimensional NMR spectroscopy.

Results and Discussion

3D solution structure of Calsensin

In order to determine the 3D solution structure of Calsensin, sequence-specific resonance assignments were performed according to standard protocols (Wuthrich 1986) using 3D ^{15}N -edited TOCSY and NOESY as well as 3D HNCACB, CBCACONH CCONH, and HCCH-TOCSY (Cavanagh 1995). All experiments were performed at 298 K with purified Calsensin from cell cultures grown in the presence of 1 mM CaCl_2 and resuspended in 50 mM sodium phosphate buffer (pH 6.0) containing 75 mM NaCl, 2 mM DTT and 0.02% NaN_3 . These conditions generated highly reproducible NMR spectra from eight independent purification and sample preparations. The backbone amide resonances were assigned for all but six N-terminal and three C-terminal amino acids. The 513 intra- and 1016 interresidue NOE assignments were obtained by analyzing 2D NOESY as well as 3D ^{15}N -edited and ^{13}C -edited NOESY spectra. The aromatic resonances were assigned based on 2D DQF-COSY and 2D NOESY data. The $^3J_{\text{NH-H}\alpha}$ scalar coupling constants from HNHA data were used to obtain the ϕ angle constraints according to the Karplus equation (Wuthrich 1986). The ϕ angles for the remaining residues and the Ψ angles were obtained using TALOS (Cornilescu et al. 1999). Hydrogen bond restraints were introduced corresponding to slowly exchanging amide protons observed in the deuterium exchange data. A total of 1529 NOE, 44 hydrogen bond (there are two distance restraints for each hydrogen bond), and 78 dihedral angle constraints (Table 1) were used for final calculations with CNS. The 20 lowest energy structures have no distance violations greater than 0.4 Å and angle violations, greater than 5° , and the RMSDs from the experimental constraints and idealized covalent geometry are low. The pairwise RMSD as well as RMSD to the mean structure of these structures

Table 1. Statistics for the NMR solution structure of Calsensin

Experimental restraints used for structure calculation	
Total number of NOEs	1529
Intraresidue NOEs	513
Interresidue NOEs	1016
Hydrogen bonds (two distance restraints each)	22
Dihedral angles (ϕ/Ψ)	78
Refinement statistics ^a	
Overall	333.94 ± 15.45
Bond	21.97 ± 1.48
Angle	115.18 ± 6.56
Improp	19.77 ± 1.79
Vdw	4.92 ± 1.61
Noe	171.56 ± 10.39
Cdih	0.54 ± 0.34
RMSDs from distance constraints and dihedral restraints (Å)	
NOE	0.0380 ± 0.0012
Cdih	0.3272 ± 0.0876
RMSDs from idealized covalent geometry	
Bond (Å)	0.0041 ± 0.00014
Angles (deg)	0.5630 ± 0.0160
Impropers (deg)	0.4560 ± 0.0205
Percent of residues in favorable region of Ramachandran plot ^b	85.10%
Percent of residues in favorable region of Ramachandran plot ^c	100.00%
RMSDs to the mean structure (Å)	
Secondary structure backbone ^c	0.545 ± 0.14
Secondary structure heavy atoms ^c	0.911 ± 0.21
Overall backbone ^b	0.842 ± 0.25
Overall heavy atoms ^b	1.278 ± 0.29
Helix I (E8-L16)	0.26 ± 0.09
Helix II (A26-T35)	0.14 ± 0.05
Helix III (K48-I55)	0.33 ± 0.12
Helix IV (K68-L79)	0.26 ± 0.10

^a Calculated using CNS for the 20 lowest energy structures.

^b Obtained for residues A7–C80 since no long-range NOEs were identified for amino acids 1–6 and 81–83.

^c Includes residues in the helices E8–L16, A26–T35, K48–I55, and K68–L79.

were relatively small (Table 1). Furthermore, the backbone dihedral angles of the majority of residues in the minimum average structure (85.1%) fall inside the most favorable regions of the Ramachandran plot (Table 1).

The ^1H - ^{15}N HSQC spectra of Calsensin were well dispersed suggesting that the protein was in a folded three-dimensional conformation. The tertiary structure was relatively well-ordered except for the unassigned N and C termini and the hinge region connecting the two EF-hands (Fig. 1A). Calsensin was monomeric under the experimental conditions due to the presence of reducing agent and as verified by a lack of concentration effect on the HSQC spectrum. The structure of Calsensin consists of two helix-loop-helix motifs arranged as a unicornate-type four-helix bundle (Fig. 1C). In Calsensin, downfield-shifted $\text{H}\alpha$ protons, slower amide proton exchange rate, and large $^3J_{\text{NH-H}\alpha}$ scalar coupling constants consistent with β -strands

Figure 1. (A) Overlay of the 20 lowest energy structures of Calsensin. The structures were superimposed using all the backbone residues in the secondary structural elements and rendered using MOLMOL. The N-terminal residues M1-K6 and C-terminal Q81-K83 were not assigned due to lack of sequential NOEs. All the helices are well-defined except for H3, which shows chemical exchange and higher RMSD as compared to the mean structure. (B) Surface plot of Calsensin rendered using MOLMOL. The regions colored in blue have positive electrostatic potential, those colored in red have negative electrostatic potential, and the regions in white are nonpolar. The hydrophobic residues in the helices 2, 3, and 4 as well as in the hinge region are exposed to the surface. (C) Stereo view of the energy-minimized average ribbon structure of Calsensin. The labeling of the secondary structural elements follows the standard nomenclature used for EF-hand calcium-binding proteins. (D) Alignment of Calsensin with other members of the EF-hand family of calcium binding proteins. The sequence of Calsensin was aligned with CaM, Plastin-1 and with the members of S100 and polcalcin families. The alignment was generated using AlignX software of the VectorNTI Suite from Invitrogen. The residues in Calsensin that are identical are highlighted in yellow (polcalcin family) or cyan (other EF-hand members), while similar residues are shown in red. The two highly conserved aspartic acid residues are shown in red and highlighted in gray.

were observed for residues Y23-T25 in calcium-binding loop I and K65-S67 in loop II. However, interstrand NOEs characteristic of β -sheet were not observed possibly due to high flexibility of the second EF-hand (as supported by ^{15}N relaxation data). Similar observations have been reported for the regulatory domain of calcium vector protein (Theret

et al. 2001a,b). The characteristic deshielding of the residue at position 8 of both the calcium-binding sites suggested that the EF-hands were likely to be calcium-bound (Biekofsky et al. 1998).

The higher RMSD of the third helix (H3) as compared to the other three helices reflects the lower

number of interhelical long-range NOE restraints in this region and was consistent with the observation that H3 reorients upon calcium-binding in the related S100 family of EF-hand proteins. The extent of this movement varies among the different S100 signaling proteins. For example, the S100B protein shows a large conformational change of the third helix upon calcium-binding as compared to S100A6 (Drohat et al. 1999; Maler et al. 1999). The flexibility of H3 in Calsensin suggests it may be important for promoting conformational exchange between calcium-bound and unbound states. Most of the hydrophobic residues that are in the hinge region as well as in the second and fourth helices are exposed on the surface as would be expected for the calcium-bound form (Fig. 1B). Hydrophobic residues have been implicated in target binding in other calcium-binding proteins (Ikura et al. 1992; Yap et al. 1999).

Comparison of Calsensin with other EF-hand calcium-binding proteins

The highest sequence identity between Calsensin and other members of the EF-hand superfamily is in the calcium-binding loops (Fig. 1D). Sequence alignment further shows that the calcium-binding loops of Calsensin are most similar to those of two members of the polcalcin family of pollen EF-hand calcium-binding proteins (Verdina et al. 2002; Neudecker et al. 2004). Although Calsensin can form dimers via oxidation of cysteine residues (data not shown), it is monomeric in solution under reducing conditions. Furthermore, Calsensin, unlike the members of S100 family (Drohat et al. 1998; Vallyly et al. 2002), does not appear to form noncovalent dimers under experimental conditions. Bet v 4 exists as a monomer whereas another member of the polcalcin family, Phl p 7, revealed a domain-swapped dimer structure (Verdina et al. 2002; Neudecker et al. 2004). In general, the EF-hand calcium-binding proteins are known to exist as monomers, dimers, or oligomers depending on their amino acid composition and function (Inman et al. 2001). Most members of the S100 and polcalcin family are highly acidic (Schafer and Heizmann 1996; Niederberger et al. 1999). In contrast, the isoelectric point (pI) of Calsensin is close to physiological pH and hence could be modulated by small changes in the pH. The anti-parallel packing of the helices in Calsensin is comparable to the open conformation of the N-terminal domain of Ca^{2+} -bound CaM (Nelson and Chazin 1998a) and monomer of S100 proteins (Potts et al. 1996; Vallyly et al. 2002). The packing in polcalcins Bet v 4 and Phl p7 is slightly different due to the extra Z-helix (Verdina et al. 2002; Neudecker et al. 2004).

Backbone dynamics of Calsensin

The global correlation time τ_c of Calsensin was 6.7 ± 0.1 nsec, which is comparable to that observed for proteins of similar size at 298 K (Theret et al. 2001b). The molecule has a statistically significant prolate rotational diffusion tensor ($D_{\parallel}/D_{\perp} = 1.14$), which is consistent with other calcium-binding proteins (Malmendal et al. 1999; Inman et al. 2001). All 74 residues with assigned backbone amide resonances had their corresponding ^{15}N relaxation data fitted to one of five models describing modes of backbone dynamics (Mandel et al. 1995). A majority of the residues (46 out of 74) were satisfied by model 1, 13 by model 2, three by model 3, 11 by model 4, and one by model 5 using the nomenclature of Mandel et al. (1995). The fitted order parameters (S^2) (Fig. 2E) reveal that the regions of high order correlate with the presence of α -helical secondary structural elements. The third helix, suggested to be involved in calcium-induced conformational change in most S100 proteins (Smith et al. 1996; Drohat et al. 1998; Donato 2001) was best fitted by the model having a msec timescale exchange term (R_{ex}) for four out of eight residues (Fig. 2G). This helix also has lower order parameters as compared to the other three helices.

In order to define flexible regions of the molecule the order parameter for each residue constituting secondary structural elements of Calsensin was averaged with standard deviation yielding an estimate for the overall degree of movement of these elements. The order parameters (S^2) averaged over the α -helical secondary structural elements are 0.90 ± 0.03 (H1, E8-L16), 0.91 ± 0.06 (H2, A26-T35), 0.85 ± 0.04 (H3, K48-I55), and 0.90 ± 0.04 (H4, K68-L79), which are well within the range observed for other calcium-binding proteins (Theret et al. 2001b; Henzl et al. 2002). The lowest average order parameters are observed for the hinge region between the two EF-hand motifs (Fig. 2E). Most of the EF-hand calcium-binding proteins exhibit varying degrees of flexibility in the hinge region and in the third helix (Malmendal et al. 1998, 1999; Theret et al. 2001a), which can be attributed to differences among their amino acid sequences (Fig. 1D). The hinge region of Calsensin shows millisecond-timescale conformational exchange, which may indicate concerted motion of the third helix (Biekofsky et al. 1998). This suggests that Calsensin is in an exchange between the open and closed conformations similar to that of the calcium-free C-terminal domain of CaM (Malmendal et al. 1999). The residue D63 in the second EF-hand of Calsensin is not oriented to bind calcium under the experimental conditions and undergoes msec timescale motion. Consequently, the calcium-binding at the second site could be destabilized by the presence of the lysine residue at position 68. Previous studies have found

Figure 2. Backbone dynamics of Calsensin correlate with the observed structural features. (A) The secondary structural elements of Calsensin are shown corresponding to the residue number. The transverse relaxation time (T_1) (B), longitudinal relaxation time (T_2) (C), heteronuclear NOE (D), order parameters (S^2) (E), internal motions (F), and exchange rates (G) are plotted as a function of residue number. All experiments were carried out at 298 K.

that the calcium-binding affinity of Calbindin D_{9K} decreases drastically below pH 7.0 due to protonation of carboxylate side chains (Kesvatera et al. 2001).

Calcium-dependent conformational change

The EF-hand family of calcium-binding proteins that function as buffer proteins has similar structures in both the apo- and calcium-bound form (Nelson and

Chazin 1998b; Yap et al. 1999). In contrast, calcium sensors that mediate signal transduction undergo a significant calcium-dependent conformational change (Nelson and Chazin 1998b; Yap et al. 1999). The mechanism of the calcium-dependent changes for these proteins has been extensively studied using calcium titrations (Aitio et al. 1999) as well as by solving the apo- and calcium-bound structures (Maler et al. 2002). For example, the calcium-induced structural changes for

S100B suggest a large conformational change in the orientation of H3 (Drohat et al. 1998). This reorientation in turn alters the structure of the hinge region and second calcium-binding site. In Calsensin, the relaxation data suggest a high degree of flexibility at the second site on a millisecond timescale (Fig. 2G). Hence, the binding of calcium to the first site might enable a conformational change at the second site allowing calcium-binding at this site. The residues in the hinge region and the C-terminal loop of S100 have been shown to be involved in target binding (Bhattacharya et al. 2003). The binding of calcium leads to a conformational change exposing the hydrophobic residues on the surface (Smith et al. 1996), consequently modulating target binding (Ikura et al. 1992; Malmendal et al. 1999). The hinge region and C-terminal helices of Calsensin consist mostly of hydrophobic residues. This suggests that the calcium-induced structural changes could expose these hydrophobic residues on the molecular surface thereby allowing interaction with target proteins.

Conclusions

Calsensin is a member of the two EF-hand calcium-binding protein family that includes the S100 and pol-calculin families. Molecules like Calsensin that are expressed selectively in certain neurons are candidates to function as signal transducers during axon fasciculation and growth cone guidance (Briggs et al. 1995). We have used multidimensional NMR to solve the structure of calcium-bound Calsensin. The structure of Calsensin reveals an anti-parallel stacking of the two helices of each EF-hand. The relatively higher disorder of H3 in the solution structure as compared to other helices is due to the presence of millisecond-timescale conformational exchange. The observed flexibility of H3 could be attributed to chemical exchange between calcium-loaded and free states and/or closed and open conformations. This indicates that the third helix is important for the calcium-induced conformational changes and that it may be implicated in target protein interactions.

Materials and methods

Protein expression and purification

The full-length Calsensin ORF (Briggs et al. 1995) was PCR amplified and cloned into the pGEX4T3 vector (Amersham Biosciences) to generate the construct pGEX4T3-Cal. For protein purification *Escherichia coli* strain BL21 (DE3) was transformed with pGEX4T3-Cal and the cultures grown in 2XYT medium supplemented with 1 mM CaCl₂ or modified M9 medium supplemented with 1 mM CaCl₂ and 100 mg/mL ampicillin. For unlabeled protein expression in 2XYT medium, the cultures grown at 37°C were induced with 0.1 M

isopropyl β -D-thiogalactopyranoside (IPTG) when O.D.₆₀₀ reached 0.5. For ¹⁵N-single-labeled or ¹⁵N- and ¹³C-double-labeled protein preparations, the modified M9 minimal media contained ¹⁵N-enriched ammonium chloride (1 g/L [Cambridge Isotope Laboratories]) and/or ¹³C-enriched glucose (2 g/L [Cambridge Isotope Laboratories]) as the sole nitrogen and carbon source, respectively. Cells were grown to O.D.₆₀₀ of 0.6 at 37°C, transferred to 30°C, and induced with 1 mM IPTG when O.D.₆₀₀ reached 0.9. The cells were harvested by centrifugation, 6 h and 12 h post-induction for unlabeled and labeled samples, respectively. The cell pellets were resuspended in 50 mL of 50 mM sodium phosphate buffer (75 mM NaCl, 2 mM DTT and 0.02% NaN₃ [pH 6.0]) per liter of culture with lysozyme added to a final concentration of 1 mg/mL. After freezing at -80°C overnight (Brazin et al. 2000) the cells were disrupted upon thawing and protease inhibitor (1 mM PMSF) and DNase I (500 mL of 1 mg/mL stock) were added. The cell extracts were clarified by centrifugation and the supernatant loaded on a glutathione-agarose (Sigma) column. The GST-fusion proteins were eluted with 5 mM reduced glutathione in 50 mM sodium phosphate buffer, concentrated with a Millipore stirred ultrafiltration cell, and separated on a size-exclusion column (Sephacryl S-100 HR, Amersham Pharmacia Biotech) equilibrated with 50 mM sodium phosphate buffer. Fractions containing the fusion proteins were pooled, the NaCl concentration increased to 150 mM, and the GST-tag cleaved off with thrombin by incubation at room temperature for 12–16 h. The GST-tag was subsequently removed from the recombinant Calsensin protein using a glutathione-agarose column. The Calsensin protein was further purified by gel-filtration (Sephacryl S-100 HR). The collected fractions were analyzed by SDS-PAGE for purity, pooled, and concentrated to 1–2 mM for NMR experiments. The final NMR samples contained 10% ²H₂O.

NMR spectroscopy

The NMR samples were prepared in 50 mM sodium phosphate buffer (pH 6.0) containing 75 mM NaCl, 2 mM DTT and 0.02% NaN₃. All NMR data were acquired at 298 K on a Bruker DRX500 spectrometer operating at ¹H frequency of 499.867 MHz. A 5-mm triple-resonance (¹H/¹⁵N/¹³C) probe with XYZ field gradients was used for all experiments. A gradient-enhanced HSQC experiment with minimal water saturation (Mori et al. 1995) was used for all ¹H-¹⁵N correlation experiments. 3D ¹⁵N-edited TOCSY, ¹⁵N-edited NOESY (Talluri and Wagner 1996) and ¹⁵N-HMQC-NOESY-HMQC (Andersson et al. 1998) spectra were collected for ¹⁵N-labeled Calsensin sample using mixing times of 80 msec for TOCSY and 125 msec for NOESY experiments. For ¹⁵N/¹³C double-labeled samples 3D CBCA(CO)NH, HNCACB (Muhandiram and Kay 1994), CCONH (Muhandiram and Kay 1994), HCCH-TOCSY (Kay et al. 1993), and ¹³C-edited NOESY (Muhandiram et al. 1993) spectra were acquired using standard experimental procedures. The backbone coupling constants (³J_{NH-H α}) were measured by a HNHA (Kuboniwa et al. 1994) experiment. Additionally, 2D homonuclear ¹H-¹H TOCSY (Fulton et al. 1996), NOESY (Lippens et al. 1995), as well as DQF-COSY (Piantini et al. 1982) data were obtained. Deuterium exchange experiments were performed as described by Roberts (1993). The proton chemical shifts were referenced to DSS (Markley et al. 1998) and ¹⁵N and ¹³C chemical shifts were referenced indirectly. The data were processed on a Linux workstation using NMRPIPE software

package (Delaglio et al. 1995) and assignments were carried out using NMRView (Johnson and Blevins 1994).

Structure calculation

The NOE and distance restraints were generated using resonance assignments from 2D and 3D data sets analyzed with NMRView. Additionally, the deuterium exchange as well as the backbone dynamics data were interpreted using NMRView. The peak volumes obtained from NOEs were classified as strong, medium, weak, and very weak restraints, corresponding to upper bound interproton distances of 2.8, 3.4, 4.3, and 5.0–6.0 Å. Pseudo-atom corrections were added for methylene and methyl protons (Wuthrich 1986). The interproton distances and backbone torsion angle constraints served as input for structure calculations using distance geometry and simulated annealing with CNS version 1.1 (Brünger et al. 1998). Hydrogen bond constraints of $r_{\text{NH-O}}$ 1.5–2.8 Å and $r_{\text{N-O}}$ = 2.4–3.5 Å were introduced during structure calculations based on $^2\text{H}_2\text{O}$ exchange data, and in the regions of secondary structure having characteristic NOEs. The refinement of 200 structures yielded several structures (>150) with no distance violations greater than 0.4 Å and no dihedral angle violations greater than 5° . The final 20 structures were selected on the basis of lowest total energies and having minimal restraint violations. The statistics of the 20 lowest energy structures are represented in Table 1 and the coordinates have been deposited in the Protein Data Bank (accession number 6519). All the structures were visualized and rendered using MOLMOL (Koradi et al. 1996). The complete resonance assignments have been deposited into BioMagRes Bank as accession codes 1YX7 and 1YX8.

Relaxation data analysis

Measurement of ^{15}N longitudinal relaxation rates R_1 , transverse relaxation rates R_2 and $\{^1\text{H}\}$ - ^{15}N NOE were obtained at 11.7 T and 298 K as previously described (Farrow et al. 1994 [refer to Fig. 10 for recycle delays]). The auto-relaxation rate constants R_1 and R_2 were calculated by nonlinear optimization using the rate analysis tool in NMRView. The heteronuclear NOEs were obtained as a ratio of HSQC cross-peak intensities measured with and without steady-state saturation of proton magnetization (Farrow et al. 1994). The global correlation time τ_c was calculated using 53 residues after excluding residues for which the resonance frequencies overlap, or those that undergo large-scale internal motions and/or conformational exchange (Tjandra et al. 1995). The rotational diffusion tensor was determined for the energy minimized average structure and the ^{15}N relaxation parameters using TENSOR2 (Dosset et al. 2000). The data were analyzed using extended model-free formalism with the statistical model selection of Mandel et al. (1995) as implemented in TENSOR2.

Acknowledgments

This work was supported by NIH grant NS28857. We thank Dr. Monica Sundd for valuable discussions during the preparation of this manuscript. We also appreciate the technical help provided by Jayandran Palaniappan for structure calculations.

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