BBA - Proteins and Proteomics xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

BBA - Proteins and Proteomics

journal homepage: www.elsevier.com/locate/bbapap

Utilizing tagged paramagnetic shift reagents to monitor protein dynamics by NMR☆

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ARTICLE INFO

Keywords:
^{19}F NMR
Calmodulin
Conformational dynamics
Paramagnetic shifts
CPMG
DEER
Pulsed EPR

ABSTRACT

Calmodulin is a ubiquitous calcium sensor protein, known to serve as a critical interaction hub with a wide range of signaling partners. While the holo form of calmodulin (CaM-4Ca^{2+}) has a well-defined ground state structure, it has been shown to undergo exchange, on a millisecond timescale, to a conformation resembling that of the peptide bound state. Tagged paramagnetic relaxation agents have been previously used to identify long-range dipolar interactions through relaxation effects on nuclear spins of interest. In the case of calmodulin, this lead to the determination of the relative orientation of the N- and C-terminal domains and the presence of a weakly populated peptide bound like state. Here, we make use of pseudocontact shifts from a tagged paramagnetic *shift reagent* which allows us to define minor states both in ^{13}C and ^{15}N NMR spectra and through ^{13}C- and ^{15}N-edited ^{1}H-CPMG relaxation dispersion measurements. This is validated by pulsed EPR (DEER) spectroscopy which reveals an ensemble consisting of a compact peptide-bound like conformer, an intermediate peptide-bound like conformer, and a (dumbbell-like) extended ground state conformer of CaM-4Ca^{2+}, where addition of the MLCK peptide increases the population of the peptide-bound conformers. This article is part of a Special Issue entitled: Biophysics in Canada, edited by Lewis Kay, John Baenziger, Albert Berghuis and Peter Tieleman.

1. Introduction

Proteins constitute roughly half of the cell's dry weight fraction and carry out a myriad of functions including that of structural scaffolds, transporters, receptors and facilitators of signaling, catalysis, and DNA replication. While the protein database provides an exquisite high resolution perspective of representative protein structures, the majority of proteins are inherently dynamic and are better represented by a complex conformational landscape, where distinct functional conformers often exchange on microsecond and millisecond timescales. The soluble eukaryotic calcium-sensing protein, calmodulin, is no exception. Consisting of distinct N- and C-terminal domains which are connected by a flexible helical linker, this 150-residue soluble protein is remarkably flexible in solution. This flexibility is believed to be key to its property of serving as a signaling hub with literally hundreds of binding partners. Upon binding of calcium, the holo form of the protein (CaM-4Ca^{2+}) populates an extended "dumbbell-shaped" conformer in which the N- and C-terminal domains reorient semi-independently. Protein recognition is initiated when the N- and C-terminal domains 'clamp' onto peptides derived from protein binding partners in a so-called closed form of the protein. Through such calcium-dependent interactions, calmodulin is well-known to regulate the action of a variety of kinases, potassium channels, and G-protein-coupled receptors (GPCRs) [1–3]. Given its significant interaction promiscuity and its prevalence as a calcium-based regulator in eukaryotes, it is of great interest to determine the representative states or conformers and the associated dynamics that relate to function. Here we investigate the utility of paramagnetic (shift reagent) tags in combination with relaxation dispersion measurements to better distinguish excited state conformers from the ground state. This approach is applied to the study of functional states of CaM-4Ca^{2+} and their characteristic exchange rates.

1.1. Calmodulin structure and NMR methods for structure determination

The X-ray crystal structure of CaM-4Ca^{2+} reveals a dumbbell-shaped structure, connected by an alpha helix, as shown in Fig. 1 [4].

Abbreviations: 3-FPhe, 3-fluorophenylalanine; CaM, calmodulin; CPMG, Carr-Purcell-Meiboom-Gill; HSQC, heteronuclear single quantum coherence; DEER, double electron-electron resonance; CEST, chemical exchange saturation transfer; PRE, paramagnetic relaxation effect; PCS, pseudocontact shift

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http://dx.doi.org/10.1016/j.bbapap.2017.09.011
Received 28 June 2017; Received in revised form 11 September 2017; Accepted 18 September 2017

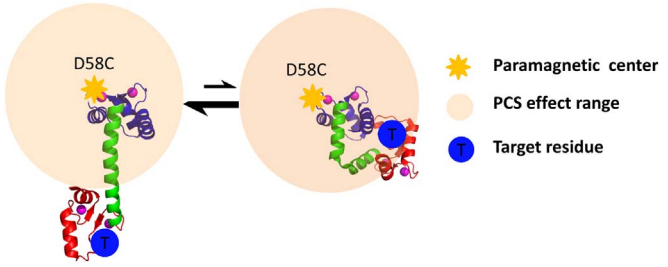

Fig. 1. Proposed equilibrium between the ground state "open" form of CaM-4Ca^{2+} (PDB: 1CLL) [4] and the peptide-bound like state wherein the N- and C-termini are in close contact. For simplicity we make use of the published peptide-bound state where CaM-4Ca^{2+} is in complex with the MLCK peptide (PDB: 1CDL) [8]. However, in the absence of a binding partner, the peptide-bound like state is thought to be more "open" than the crystal structure to allow for diffusional access and entry by the binding peptide. The figure illustrates the idea that a paramagnetic shift reagent in the N-terminal domain should give rise to increased pseudocontact shifts on target residues (T) in the C-terminal domain, particularly when the protein adopts closed conformations.

The N- and C-termini each consist of two EF-hand motifs responsible for calcium binding, share 46% sequence homology, and possess a similar overall fold. However, the C-terminal domain binds Ca^{2+} more strongly than the N-terminal domain [5]. This difference in Ca^{2+}-affinity between the two domains renders the protein highly responsive to calcium concentrations between sub-micromolar and high micromolar concentrations. The N-terminal domain has also been noted to be more dynamic both on a sub-microsecond timescale and in terms of sampling the peptide-bound like state (i.e. a compact form of the protein resembling the protein bound state in which the N- and C-terminal domains are in closer proximity) [5,6]. This added plasticity has been proposed to provide for greater freedom to interact with a multitude of binding partners. While the 27-residue helix bridging the N- and C-terminal domains was shown to be extended in the X-ray crystal structure [4], solution state NMR studies reveal a flexible hinge in the middle of the helix. This hinge allows the N- and C-terminal domains to reorient semi-independently, undergoing restricted diffusion within a cone of semi-angle 50–80° [5,7].

Traditionally, backbone triple-resonance NMR provides high-resolution structures of proteins in solution via detailed measurements of ^{13}C and ^{15}N chemical shifts, various homo- and hetero-nuclear scalar couplings, which relate to dihedral angles and secondary structure, and NOE-based interproton distance measurements reaching ~10 Å [9]. Together, these experimental measures are used as pseudo-potentials to computationally derive a structure or ensemble of structures that satisfy the NMR data. Such datasets can be further refined, as was done with calmodulin, by weakly aligning the protein in a medium such that orientational measurements can be performed (usually in the form of chemical shift perturbations and residual dipolar couplings) [5,10]. Importantly, residual dipolar couplings provide valued long-range constraints on relative orientations of distinct domains, which is clearly needed in multi-domain proteins such as calmodulin.

1.2. Delineating minor conformers and excited states of proteins

While the above approach provides a high resolution dynamic structure of the ground state of a multidomain protein such as calmodulin, protein function is often best understood in terms of a conformational ensemble. For example, in the case of CaM-4Ca^{2+}, we might expect to observe a peptide-free extended state in equilibrium with a peptide-bound like state, in addition to potential intermediate states. Moreover, these functional states may also exhibit some degree of conformational heterogeneity of their own, reflecting local conformational sampling and possible sub-states relating to the complex interaction proteome associated with calmodulin. In principle, NMR methods are ideal for the identification of major and minor protein conformers (states), whose exchange invariably appears on an NMR timescale [11–13]. In situations where the ground state has been first determined, the presence of an excited state may be manifested by an entirely distinct, albeit weaker set of peaks. Even under conditions where the minor state population represents less than 1% of the ensemble, the excited state spectrum can be to some extent recapitulated by a so-called CEST experiment. Here, specific saturation of minor peaks, gives rise to a decrease in the intensities of the corresponding major peaks, assuming that the ground and excited states exchange on a timescale comparable to the spin-lattice relaxation time (i.e. tens of milliseconds to seconds). Remarkably, by determining the ^{13}C and ^{15}N chemical shift signatures of the excited state, it is in principle possible to reconstruct a high-resolution structure of the excited state [12,13]. A protein may also adopt a fast equilibrium (i.e. high microseconds to tens of milliseconds) between ground and excited states where a shift or perturbation in the ground state chemical shift spectrum is observed, at least for resonances associated with the part of the protein undergoing conformational exchange. Via T_2-based CPMG relaxation dispersion experiments, the ^{13}C and ^{15}N chemical shift signatures of the excited state (in addition to the excited state population and lifetime) can be ascertained. A CPMG relaxation dispersion experiment measures the decay rate associated with single or multiple quantum coherence, where a train of refocusing pulses with an interpulse delay, $\tau_{CP,}$ is repeatedly applied during a constant relaxation period [14]. In situations where the equilibrium between states A and B can be approximated as a simple two-state exchange defined by

$$A \underset{k_{BA}}{\overset{k_{AB}}{\rightleftharpoons}} B \qquad \text{and} \qquad k_{ex} = k_{AB} + k_{BA}, \tag{1}$$

the CPMG dispersion rate may be approximated by [15,16]

$$R_2(\tau_{CP}) = R_2(0) + p_A p_B \Delta\omega^2 k_{ex} / [k_{ex}^2 + (p_A^2 \Delta\omega^4 + 144/\tau_{CP}^4)^{1/2}], \tag{2}$$

where $\Delta\omega$ represents the separation in radial frequency units between the coherences designating the states A and B, and the ground state population, p_A, is assumed to be significantly greater than that of the excited state population, p_B [14]. In general CPMG dispersions may be used to ascertain information on the equilibrium (p_A, p_B, k_{ex}) in addition to the frequencies characterizing the excited state. Again, the chemical shift dataset can be used to determine a structure of the excited state, particularly in the case of (^1H,^{13}C,^{15}N) NMR where there are a sufficient number of reporters to define a state.

Fluorine (^{19}F) NMR is often a convenient tool in the NMR repertoire, given the sensitivity of the fluorine nucleus to local electrostatic and van der Waals environments [17–19]. By strategically ^{19}F-labeling a protein at sites where functional states can be most easily distinguished (through known differences in topology), ^{19}F NMR can readily delineate functional states or intermediates which more conventional NMR nuclei fail to resolve [20]. This is quite simply a consequence of the fact that ^{19}F NMR chemical shift dispersions typically exceed those of other nuclei and have been known to range by as much as 10 ppm in studies of proteins, due purely to environmental effects [19]. Labeling by ^{19}F probes in proteins is also straightforward and is generally accomplished by either biosynthetic means and induced auxotrophy or by chemical tagging of thiol-specific fluorinated probes [17]. Large chemical shift dispersions also provide an advantage in the delineation of states which are in fast exchange and are only visible through CPMG relaxation dispersion experiments. This is because the magnitude of such relaxation dispersions typically depends on the square of the difference of the chemical shifts between ground and excited states (i.e. $\Delta\omega^2$, as shown in Eq. 2). Recently, ^{19}F NMR CPMG relaxation dispersions were employed in a study of conformational exchange of calcium-bound calmodulin, using the chemical shift signature from all eight fluorophenylalanines [6]. Whereas ^{15}N or ^{13}C relaxation dispersions did not reveal any substantive conformational exchange processes for CaM-4Ca^{2+}, ^{19}F NMR CPMG relaxation dispersions revealed a low-millisecond exchange between a native extended state and a near-native