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Ca²⁺ ATPase conformational transitions in lipid bilayers mapped by site-directed ethylation and solid-state NMR

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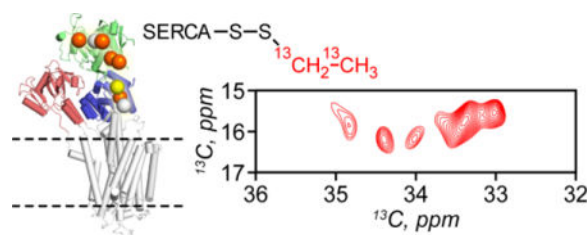
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Abstract

To transmit signals across cellular compartments, many membrane-embedded enzymes undergo extensive conformational rearrangements. Monitoring these events in lipid bilayers by NMR at atomic resolution has been challenging due to the large size of these systems. It is further exacerbated for large mammalian proteins that are difficult to express and label with NMR-active isotopes. Here, we synthesized and engineered ¹³C ethyl groups on native cysteines to map the structural transitions of the sarcoplasmic reticulum Ca²⁺-ATPase, a 110 kDa transmembrane enzyme that transports Ca²⁺ into the sarcoplasmic reticulum. Using magic angle spinning NMR, we monitored the chemical shifts of the methylene and methyl groups of the derivatized cysteine residues along the major steps of the enzymatic cycle. The methylene chemical shifts are sensitive to the ATPase conformational changes induced upon nucleotide and Ca²⁺ ion binding and are ideal probes for active and inactive states of the enzyme. This new approach is extendable to large mammalian enzymes and signaling proteins with native or engineered cysteine residues in their amino acid sequence.

Graphical abstract



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Supporting Information.

Identification of the ¹³C-EMTS labeled sites. Synthesis of the NMR and spin labels. This material is available free of charge via the Internet at <http://pubs.acs.org>.

Author Contributions

The manuscript was written through contributions of all authors.

Keywords

Site-directed ethylation; Solid-state NMR; conformational transitions; membrane proteins; Ca²⁺-ATPase

Introduction

Cell signaling events are mediated by membrane-bound enzymes and proteins that transmit their messages across lipid membranes through chemical, structural and/or dynamical changes. Since membrane proteins are difficult to crystallize in lipid membranes, solid-state NMR (ssNMR) spectroscopy is the ideal high-resolution spectroscopic technique to monitor structural changes in liquid crystalline lipid membranes^{1–3}. When the proteins or protein complexes exceed 100 kDa, the analysis of structural transitions becomes cumbersome as the number of structural probes (resonances) crowds the NMR spectra. For solution NMR spectroscopy, Kay and co-workers introduced recombinant protein expression protocols that enable amino acid specific ¹³C methyl labeling^{4, 5}. The higher gyromagnetic ratio of ¹³C with respect to ¹⁵N in concert with longer relaxation times of the methyl groups makes it possible to image the methyl fingerprint region of larger proteins^{4, 6}. For example, such labeling technique was used to monitor the conformational transitions of large enzymes by solution NMR⁷. For larger globular proteins containing cysteine residues, it is also possible to covalently link ¹³C-labeled methyl groups by site-directed labeling, incubating the protein with ¹³C methyl methanethiosulfonate (MMTS)⁸. Similar approaches have been utilized to probe the conformational landscape of membrane proteins by modification of Lys side chains^{9–11}, or by introducing ¹⁹F probes^{12–15}. To date, these studies have been limited to detergent micelles that can affect protein structure, topology and function compared to native lipids^{16–19}.

Larger eukaryotic membrane proteins are very challenging to express recombinantly in amounts required for NMR spectroscopy²⁰. Nonetheless, their structural and dynamic transitions could be still monitored by ssNMR using cysteine reductive methylation⁸. However, this method presents severe spectroscopic challenges as the ¹H signals of non-deuterated proteins are typically broad and unresolved²¹. Moreover, the ¹³C background signals arising from natural abundance ¹³C from proteins and lipids complicate the appearance of the spectra. To overcome these problems, we synthesized ¹³C-ethyl methanethiosulfonate (¹³C EMTS) and used it to alkylate the native Cys side chains (Figure 1). This approach has three major merits: i) it enables one to filter out the natural abundance ¹³C signals using dipolar assisted rotational resonance (DARR) experiments²², making it possible to study membrane proteins in native lipid bilayers; ii) it provides better resolution of the engineered aliphatic resonances with respect to methyl groups, iii) the ¹³C-EMTS probe is small and relatively non-perturbing, and labeling can be carried out in aqueous milieu, retaining enzymatic activity.

As a proof-of-principle, we used site-directed ethyl labeling to monitor the conformational transitions of the sarcoplasmic reticulum Ca²⁺-ATPase (SERCA), EC: 3.6.3.8, a 110 kDa integral membrane protein that plays a key role in muscle contractility^{23, 24}. To monitor

SERCA's conformational transitions, we labeled the most reactive cysteine residues with ^{13}C -EMTS and followed the chemical shift changes of both CH_2 and CH_3 signals by ssNMR DARR experiments. To assign the resonances, we employed a combination of paramagnetic relaxation enhancements (PRE) experiments. We were able to obtain domain specific assignments for all of the resonances in the P- and N-domains of SERCA, including two unambiguous assignments for ethyl groups linked to C674 and C636 of the P-domain, defining the NMR signatures for the different conformational states of the enzyme.

Materials and Methods

The synthesis of ^{13}C -EMTS was accomplished by refluxing equimolar amounts of ^{13}C bromoethane and sodium methanethiosulfonate for 5 h in ethanol (Figures 1 and S1). The resulting product was filtered and the solvent was removed by evaporation. The final product was identified by solution NMR and used without further purification. To label the ATPase with EMTS, we incubated $9\ \mu\text{M}$ C_{12}E_8 -solubilized SERCA purified from rabbit skeletal muscle²⁵ in reconstitution buffer (20 mM HEPES, 100 mM KCl, 1 mM Mg^{2+} , 5% glycerol, 0.02% NaN_3) with $45\ \mu\text{M}$ (5-fold molar excess) of EMTS for 3 h. Enzymatic activity of labeled SERCA was measured by ATP hydrolysis assay²⁶. The incubation time and the EMTS concentrations were varied to minimize activity loss of SERCA. Under conditions of 5-fold excess of EMTS relative to and 3 h incubation, the ATPase retains full activity (Figure 1). Additionally, we have tested SERCA labeling with 10-fold and 20-fold excess of EMTS to assess whether all the cysteine residues in the enzyme are accessible to the labeling reagent. The enzymatic assay shows only in the presence of large excess of EMTS, suggesting that the least accessible cysteines are essential for the SERCA function (Figure S2).

For SERCA reconstitution in lipid membranes, 9 mg of DMPC-*d*₅₄ were solubilized in 18 mg of C_{12}E_8 and added to the ATPase. Detergent was removed by incubation with a 30-fold weight excess of biobeads for 3 h. The sample was centrifuged at $100,000\times g$, and the pellet was re-suspended in reconstitution buffer, dialyzed twice to remove any unreacted EMTS, and pelleted down at $100,000\times g$. The proteoliposome pellet was re-suspended in 1 ml reconstitution buffer with appropriate ligands, sedimented by ultracentrifugation at $350,000\times g$ for 20 h, and the resulting hydrated pellet was transferred to a 3.2 mm MAS rotor for NMR experiments. The ligands used to induce the enzyme different SERCA conformations were obtained using the following conditions: 5 mM CaCl_2 (E1- Ca^{2+}), 2.5 mM AMPPCP (E2-ATP), 5 mM CaCl_2 and 2.5 mM AMPPCP (E1- Ca^{2+} -ATP), or 5 mM CaCl_2 , 2.0 mM AlCl_3 , 2.5 mM ADP and 20 mM NaF (E1- Ca^{2+} -P~ADP). To increase the resolution, a constant-time version of the DARR experiment, [^{13}C , ^{13}C]-CT-DARR, was used^{22, 27}. The spectrum was acquired at 4°C with a spinning rate of 10 kHz on a 600 MHz Varian spectrometer. Acquisition parameters were 4000 points with a spectral width of 100 kHz (direct dimension). The corresponding values in the indirect dimension were 36 points and 5 kHz. 1024 scans were acquired with 800 μs CP transfer, 100 ms DARR mixing time and $\omega_{\text{RF}}/(2\pi) = 100\ \text{kHz}$ TPPM decoupling.

Results and Discussion

The spectrum of labeled SERCA in DMPC bilayers obtained with a 5-fold excess of EMTS is shown in Figure 1. The SERCA spectrum with non-hydrolyzable ATP analog AMPPCP without Ca^{2+} shows well-resolved cross peaks between CH_2 and CH_3 moieties of the probe. Importantly, these are the only off-diagonal signals in the spectrum, as DARR transfer occurs only between adjacent ^{13}C nuclei, eliminating the natural abundance ^{13}C resonances of lipids and SERCA, which dominate one-dimensional ^{13}C spectra²⁸. Thus, the EMTS labeling strategy enables spectral editing in 2D, enhancing the resolution. Since the transfer of magnetization in the DARR experiment is mediated by dipolar couplings, it is sensitive toward the mostly rigid groups. The differential mobility of the NMR probes can lead to distinct efficiencies of magnetization transfer, manifested as the variations in the peak intensity. The dynamics of the NMR probes can be further evaluated and quantified with the appropriate techniques^{29,30}. The ethyl cross peaks are clearly visible in the spectrum at ~17 ppm (CH_3 -) and ~35 ppm ($-\text{CH}_2$ -) with much greater resolution in the $-\text{CH}_2$ - resonances. To identify the ethylated Cys residues of SERCA, we utilized trypsin digestion in combination with electrospray ionization tandem mass spectrometry (LC-MS/MS). We detected small peptides corresponding to 16 out of 23 Cys residues of SERCA, covering all Cys in the cytoplasmic P-, N- and A-domains. High yield of Cys ethylation was achieved at six sites: 364, 471, 498, 561, 636 and 674 (Figures S3–S5). To estimate the labeling efficiency, a large excess of iodoacetamide was added to SERCA immediately after the ethylation reaction and prior to trypsin cleavage. For two of the modified Cys (614 and 636), we detected carbamidomethylated (CAM) peptides with higher relative abundance than of the ethylated peptides (Figure S5). Thus, we conclude that these residues have much lower percentage of ethylation and the peaks in the [^{13}C - ^{13}C]-CT-DARR spectrum correspond to Cys 364, 471, 498, 561, 674 and 636 (Figure 2).

While the DARR experiment enables one to measure short-range distances between the probes^{22,31}, the distance separation greater than 10 Å between the labeled cysteines precludes its use for distance measurement in case of SERCA. To obtain domain specific assignments of the ethyl group fingerprint, we incubated SERCA in the E2-ATP state with various spin labels to selectively quench the NMR signals based on their proximity to the unpaired electron using paramagnetic relaxation enhancement (PRE) (Figure 1). Specifically, we engineered the TEMPO spin-label coupled to the lipid head group or to the 3'/5' hydroxyl groups of the nucleotide (Figure S1). Additionally, we selectively attached iodoacetamide TEMPO to C674, as described previously³², and performed the experiments with the oxidized and reduced spin label. The NMR spectra of the ethyl group fingerprint and EPR spectra corresponding to the spin-labels are shown in Figure 1. Both DOPE-TEMPO and C674-MTSL are close to the lipid-water interface and quench the ethyl groups of the Cys in the P-domain. The spin labeled nucleotide, ADP-TEMPO, bound in the N-domain away from the membrane, partially quenched residues both in the P- and N-domains. Based on the quenching pattern, we assigned the resonances between 33.5 and 34.5 ppm to the ethyl groups cross-linked to the cysteines in the N-domain, whereas the resonances in the range between 34.5 and 35 ppm and 32.5 and 33.5 to those of the P-domain. Furthermore, we employed 10 mM ascorbic acid to reduce free radical of C674-

MTSL to hydroxylamine. The quenched spin label does not exert any effect on NMR relaxation of the adjacent ^{13}C nuclei, and the only missing signal can be assigned to the ethyl group on C674. Indeed, the 34.8 ppm peak is absent in the NMR spectrum, and therefore it is assigned to C674. Therefore, the remaining P-domain resonance at 33.1 ppm consequently is assigned to C636.

The labeled Cys in the P and N domains of SERCA are ideally positioned to probe the allosteric communication between Ca^{2+} binding in the TM domain and nucleotide binding in the cytoplasmic domain (Figures S6–S7). It has been proposed that the enzymatic cycle of the ATPase comprises several different conformational states^{24, 33}. For example, the structures of SERCA in the Ca^{2+} -free nucleotide-bound (E2-ATP)³⁴, Ca^{2+} - and nucleotide-bound (E1- Ca^{2+} -ATP)³⁵ and Ca^{2+} -bound nucleotide-free (E1- Ca^{2+})³⁶ states show distinct conformations in the cytoplasmic domains^{37–39}. To test whether the ^{13}C -EMTS labeled sites are sensitive to the conformational transitions of SERCA, we mimicked these major states of the enzymatic cycle⁴⁰, saturating the enzyme with AMPPCP, ADP/AlF₄, or Ca^{2+} . The ethyl group signatures of the enzyme's states display pronounced differences. In the Ca^{2+} -free form, SERCA populates the E2 conformational state, while addition of Ca^{2+} shifts the equilibrium to the E1 state^{33, 40}. Upon Ca^{2+} addition, the P domain peaks of the E2 state spectrum change substantially (Figure 3). These peaks are sensitive probes for tracing the enzyme's interconversion between the E1/E2 states.

The center of mass of the cytoplasmic domains has the largest separation in the E1- Ca^{2+} structure (Figure 3), which has been predicted to be highly dynamic^{37, 38}. It is notable that the resonance pattern and signal intensity of SERCA in this state differ significantly from the rest of the spectra. We speculate this may be attributed to the distinct motion time scale of this state. During the E1/E2 transition, the N-domain undergoes rotation and translation, with the ATP molecule bridging the N- and P-domains, stabilizing the enzyme. Accordingly, in the E2-ATP state we observed all of the Cys ethyl groups, suggesting reduced conformational dynamics. The crystal structures of the E1- Ca^{2+} -ATP and E1- Ca^{2+} -P~ADP states are nearly identical (1.2 Å RMSD), and one can expect the ethyl fingerprints to be similar. Nevertheless, the pattern differs in both N-domain signals as well as C636, indicating structural and/or dynamic changes of SERCA going from ATP-bound to the transition mimic ADP-P-bound state.

The activity of SERCA is regulated by several factors: lipids⁴¹, other proteins^{42, 43}, and post-translational modifications⁴⁴. The associated structural changes have been monitored using site-directed spin labeling of SERCA's Cys residues^{32, 45–53}. Although most of the chemical modifications of SERCA's Cys residues did not completely inactivate this enzyme, their size and mobility represent a problem for the accurate distance measurement, or monitoring allosteric coupling between ATP hydrolysis and Ca^{2+} transport^{47–49, 51, 54, 55}. These problems are even more apparent when small molecules inhibitors or activators are screened for drug discovery⁵⁶. In such cases, the combination of ethylation and ssNMR can provide a powerful approach to monitor ligand-induced conformational changes with SERCA function in a native membrane environment.

In summary, we demonstrated that site-directed ethyl labeling in combination with paramagnetic spin-labeling and solid-state NMR is a powerful method to study SERCA in lipid bilayers under fully functional conditions. Paramagnetic relaxation enhancement strategy enabled domain-specific assignments of the spectra, with two resonances unambiguously assigned to the individual cysteine residues. Importantly, the ethyl chemical shifts are sensitive to conformational changes of the enzyme, giving distinct signatures for the major structural states and will constitute specific reporters for active and inhibited states of the ATPase. This method will be widely applicable to monitor the conformational states of large proteins or protein complexes, purified from natural sources. It can be easily extended to smaller proteins and receptors expressed recombinantly, including GPCRs, where site-directed mutagenesis can make the assignments unambiguous. Moreover, it can be utilized to monitor dynamic changes via nuclear spin relaxation experiments. Finally, spectral overlap can be resolved by introducing new probes in the ^{13}C -EMTS groups, such as ^{19}F , enabling multidimensional NMR spectroscopy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

DARR	Dipolar assisted rotational resonance
EMTS	ethyl methanethiosulfonate
SERCA	sarco(endoplasmic reticulum Ca^{2+} -ATPase
ssNMR	solid state NMR
TM	transmembrane

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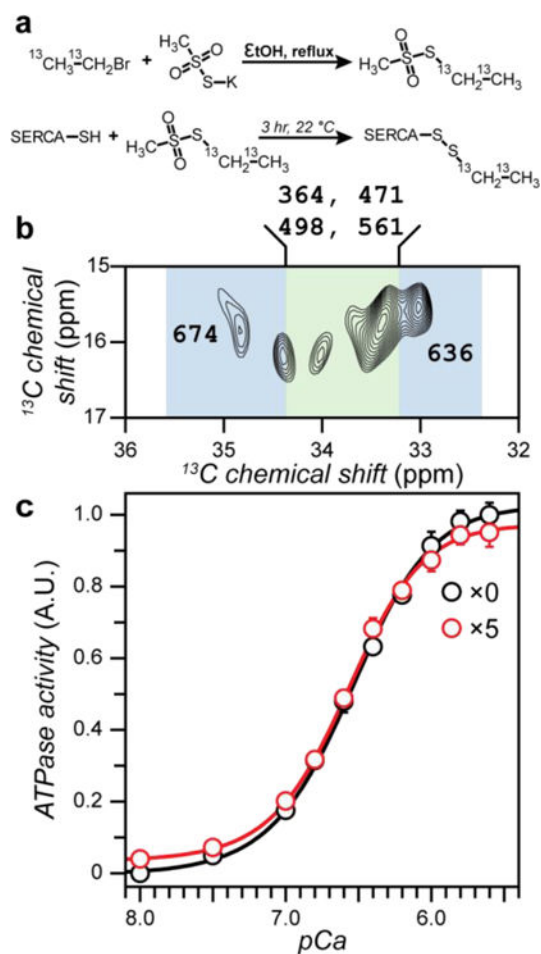
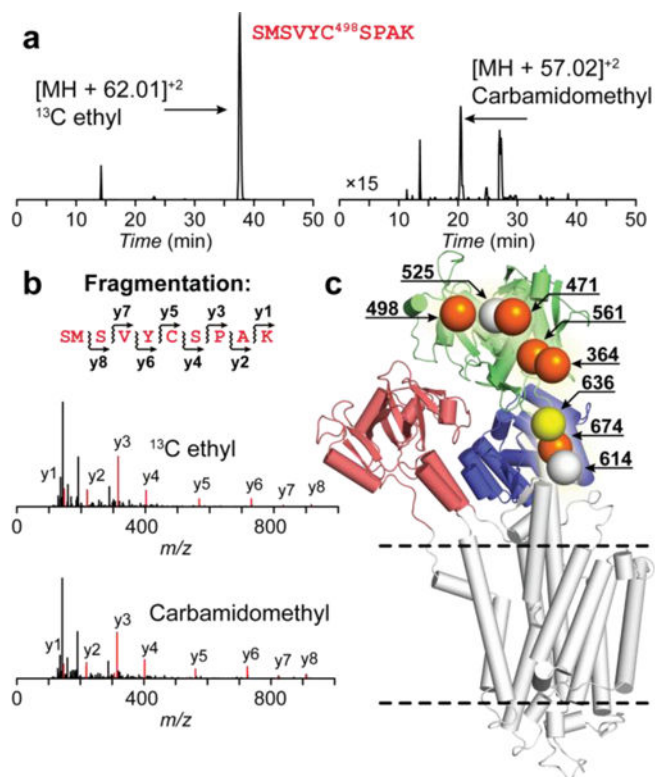


Figure 1.
a: Synthesis of ^{13}C EMTS and its usage for the labeling of cysteines of SERCA. **b:** [^{13}C , ^{13}C]-CT-DARR spectrum of ^{13}C -EMTS labeled SERCA. **c:** Normalized ATPase activity of the ethylated SERCA: reaction with 5-fold excess of EMTS did not modify the activity of the enzyme.

**Figure 2.**

Mass spectrometry of ¹³C-EMTS labeled SERCA. **a:** representative LC-MS chromatograms of a tryptic peptide with EMTS (+62) or CAM (+57) modifications. **b:** MS2 fragmentation of the indicated peaks was used to confirm the peptide identity. **c:** Cys in SERCA that are preferentially labeled (orange), partially labeled (yellow) and not labeled (white) with EMTS.

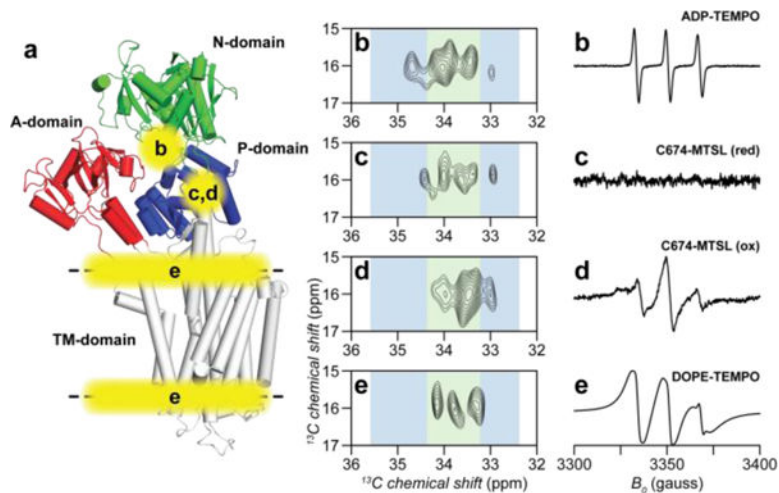


Figure 3.
a: Crystal structure of SERCA with the spin label positions indicated. **b–e:** NMR and EPR spectra of the ethyl labeled enzyme with the spin labels indicated in panel a. Residues in the proximity of the spin label are absent in NMR spectra due to paramagnetic relaxation enhancement. EPR acquisition conditions were identical to the ones described previously²⁸.

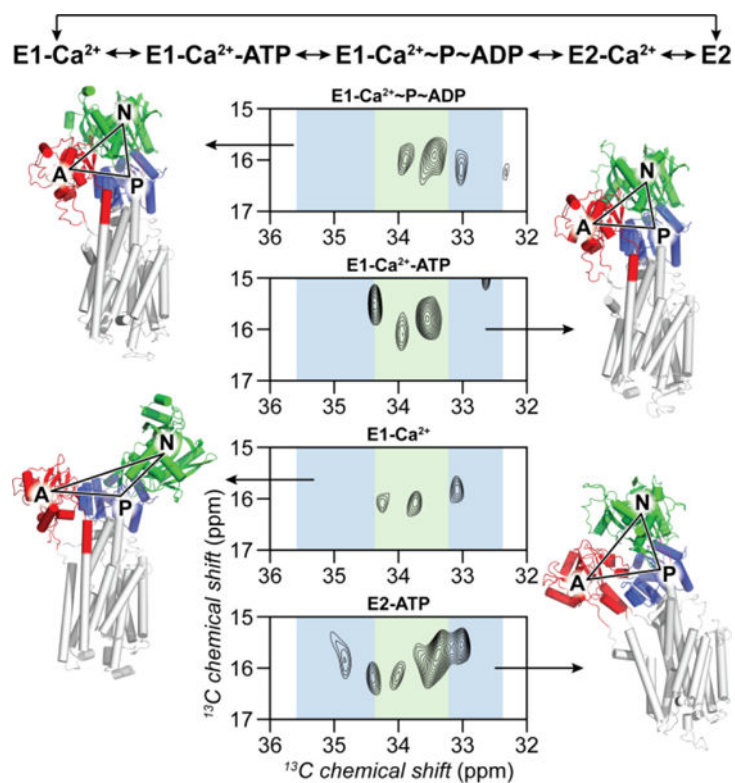


Figure 4. Enzymatic cycle⁵⁷ and conformations of SERCA by ethyl fingerprints. The crystal structures are Ca^{2+} -free E2 state (PDB ID: 1IWO), E1- Ca^{2+} (1SU4), E1- Ca^{2+} -ATP (1T5S) and E1- $Ca^{2+}\sim P\sim ADP$ (2ZBD). Triangle vertices correspond to the centers of mass of the three cytoplasmic domains.