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Thermal perturbation correlation of calcium binding Human centrin 3 and its structural changes ☆

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HIGHLIGHTS

- PCMW2D correlation spectroscopy has proven useful in defining T_m of protein motifs.
- The aspartates and glutamates vibrational modes were analyzed.
- The N-terminal domain has high affinity for calcium and therefore high T_m.
- The sequential orders of events were validated.

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ABSTRACT

Perturbation-correlation moving-window two-dimensional (PCMW2D) correlation spectroscopy was applied for the determination of the individual transition temperatures of different vibrational modes located within structural components of a calcium binding protein known as Human centrin 3. This crucial information served to understand the contribution individual calcium binding sites made towards the stability of the EF-hand and therefore the protein without the use of probes. We are convinced that the general application of PCMW2D correlation spectroscopy can be applied to the study of proteins in general to ascertain the differences in the stability of structural motifs within proteins and its relationship to the actual transition temperature of unfolding.

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1. Introduction

Human centrin 3 shares common structural elements to other EF-hand proteins, and respond to cellular calcium influx by selectively binding Ca^{2+} ions in highly conserved helix-loop-helix motifs [1–6]. EF-hand proteins have a dumbbell shape where two EF-hand motifs make up a globular domain linked by a tethered helix. The actual site for binding calcium lies within the loop region, known as the calcium binding site (CaBS). The geometry adopted for calcium binding is that of a pentagonal bipyramid, which can be described as X, Y, Z, -X, -Y, and –Z. The X, Y, Z and –Z positions are usually comprised of Asp^- or Glu^- residues that coordinate Ca^{2+}, while in the –Y position, the coordination is *via* a backbone carbonyl group and in the –X position, the residue coordinates indirectly through a water molecule [1–4]. In canonical EF-hand proteins, like *Chlamydomonas reinhardtii* centrin, there are two high affinity sites and two low affinity sites for calcium [1–5]. These domains behave independently from one another [4]. The dissociation constants range from 1 nM to 0.1 mM. Recently, we performed a comparative molecular biophysical study of related Human centrin isoforms, specifically centrin 1, centrin 2, and centrin 3 to assess their relative stability and established qualitatively the differences in molecular flexibility and dynamics as a function of temperature [6]. Herein, for the first time, we have determined quantitatively the actual transition temperature range at which each calcium binding site within Human centrin 3 occurs. The unique method Perturbation-correlation Moving-window Two-dimensional (PCMW2D) correlation spectroscopy used was defined by Morita for the determination of transition for each type of vibrational mode analyzed [7–10]. The spectroscopic results provide the relationship between the molecular behavior of the calcium binding sites, through the aspartate and glutamate carboxylate stretching modes and the secondary structure carbonyl stretching of the peptide bonds located in the side chain and amide I′ bands, respectively; as a function of temperature [5,6,11–13]. This analysis allowed for the assignment of the

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relative stability of the calcium binding sites, and relates these differences to their lower and higher calcium affinity sites. Consequently, these findings have effectively increased our understanding of the role of calcium binding in the stability of the EF-hand motif and its domain.

2. Experimental methods

Purified recombinant *Homo sapiens* centrin 3 in 50 mM HEPES, 150 mM NaCl, 4 mM $CaCl_2$, and 4 mM $MgCl_2$ at pD 6.6 was fully H → D exchanged and 35 μL of 60 mg/mL protein was placed on a 49 mm × 4 mm CaF_2 window with a fixed path length of 40 μm [5–6]. Reference was prepared in a similar manner as described for the protein containing sample. The loaded CaF_2 windows were set in a custom dual cell holder for a thermal dependence analysis within the temperature range of 5–95 °C with 5 °C intervals, once the desired temperature was reached 10 min equilibrium times were provided followed by data acquisition using a Jasco model 6200 FT-IR spectrophotometer equipped with a narrowband MCT detector, and a sample shuttle. Spectral acquisition was performed by co-adding 512 scans which were apodized with a triangular function and Fourier transformed to provide a 4 cm^{-1} resolution with data encoded every 2 cm^{-1}.

2.1. PCMW2D correlation spectroscopy

PCMW2D correlation synchronous and asynchronous plots were generated using the 2Dshige Software with a spectral intensity data matrix comprised of rows along the spectral variable (i. e., wavenumber) and columns along the perturbation variable (temperature). The correlation intensities are calculated in a sub-matrix named "moving-window" along the perturbation. A window size set to 2 m + 1 = 3 °C was used. A linear function was applied and therefore the synchronous PCMW2D correlation is proportional to the gradient of the spectral variation as a function of the thermal perturbation [7,8] such that:

$$\Pi_{\phi(\upsilon,p)} \sim \left(\frac{\partial y(\upsilon,\ p)}{\partial p}\right)_{\upsilon} \tag{1}$$

where y, υ, and p are: the spectral intensity, the spectral variable (wavenumber) and the perturbation (temperature) [7].

For the asynchronous PCMW2D correlation represents the relationship between the spectral variation and the Hilbert–Noda transformation [9] where a linear function is applied and the asynchronous PCMW2D is proportional to the curvature of the spectral variation as a function of the thermal perturbation:

$$\Pi_{\psi(\upsilon,p)} \sim -\left(\frac{\partial^2 y(\upsilon,p)}{\partial p^2}\right)_{\upsilon} \tag{2}$$

Therefore, the resulting contour map increases the resolution of overlapped peaks as a function of the temperature perturbation [7,10].

3. Results and discussion

Human centrin 3 was fully H → D exchanged as shown in Fig. 1, the intensity ratios of the amide I′ (1700–1600 cm^{-1}) and amide II′ (1500–1380 cm^{-1}) bands at 20 °C upon heating and cooling is consistent with a fully exchanged protein. The FT-IR spectra of the amide I′ and side chain bands are shown in Fig. 2 (panels A–C) for the temperature ranges of 5–40 °C, 40–60 °C and 60–95 °C as in the previously published manuscript [6]. We then generated the PCMW2D correlation spectroscopy synchronous and asynchronous plots, to experimentally discern the actual temperature at which each CaBS (Supplementary Fig. 4) was perturbed. The PCMW2D correlation plots are shown in Fig. 2. The white and gray peaks within the contour plots represent the positive and negative correlation intensities, respectively. In addition, the band assignments and the sequential order of event plots are shown in Fig. 3. These band assignments have been validated by our group [6,14]. The side chain assignments have also been validated by Chirdgadze [11] and the calcium coordination peak shifts have been observed for the Asp^- and Glu^- vibrational modes in other calcium binding proteins as well [12,13].

3.1. PCMW2D synchronous plots

The pre-transition temperature range (5–40 °C) Fig. 2D, clearly shows the negative correlation peak assigned to the Glu^- vibrational mode as having the a large intensity change suggesting the loss of coordination to calcium in its lowest affinity site (CaBS III) at about 30 °C also slight π-helix flexibility is observed as well. In the transition range (40–60 °C) the PCMW2D synchronous plot (Fig. 2F) is less informative except that at 40 and 55 °C the side chain modes as well as the backbone are equally perturbed, this is not so when observing the PCMW2D asynchronous relationship. Our interpretation is that the C-terminal domain comprised of CaBS III and IV whereby the third CaBS has the lowest affinity for calcium and whose aspartates and glutamates were perturbed within the pre-transition temperature range has induced the flexibility observed at 46 °C. As the temperature is increased CaBS IV becomes perturbed 55–58 °C. Finally, in the temperature range of 60–95 °C (Fig. 2H) a gradient intensity change is observed at around 82–85 °C and to a much lower extent about 68–72 °C these two transitions may be due to the high affinity CaBS located within the N-terminal domain where both CaBS (I and II) are bound to calcium providing the stability to this domain.

PCMW2D asynchronous plot was more informative (Fig. 2 panels E,G,I), since it provided greater resolution of the spectral region of interest, 1710–1500 cm^{-1}. We focused on the determination of the temperature perturbations within the glutamate and aspartate side chain modes since these residues are located mostly within or near the CaBS. In the temperature range of 5–40 °C (Fig. 2E) the aspartate peaks located at 1557.8 cm^{-1} ($\upsilon_{a\text{-}s}(COO^-)$) were perturbed at two different temperatures 10 and 24 °C. The arginine peaks associated with the antisymmetric N-D stretching mode at 1604 cm^{-1} was perturbed at ~15 and 28 °C. Also, a strong negative correlation was observed for a peak at 1546.9 cm^{-1} for the glutamates ($\upsilon_{a\text{-}s}(COO^-)$) at 31 °C; positive peaks are observed at 1625 cm^{-1} for the β-sheet (υ(C═O)) at 15 and 30 °C, suggesting the short β-sheet segments within this low affinity calcium binding site were perturbed prior to the glutamates within the (CaBS III). Finally, the 1650 cm^{-1} assigned to the π-helix (υ(C═O)) was also perturbed around 30 °C. Furthermore, the π-helix is found exclusively within the C-terminal end of the protein, thus acting as an internal probe. Yet, the perturbation of the π-helix located exclusively at the C-terminal end of the protein provided a degree of confidence in the aspartate and glutamate assignments within the CaBS III (5–40 °C) and IV (40–60 °C) which were the least stable EF-hand motifs. In the temperature range of 40–60°C (Fig. 2G) presumably the calcium binding site being perturbed is neighbor of the low affinity site, also located at the C-terminal domain (CaBS IV); three strong negative correlation peaks were observed for the aspartate peak located at 1557.8 cm^{-1} ($\upsilon_{a\text{-}s}(COO^-)$) had a perturbation temperature of about 52 °C, the β-sheet peak at 1625 cm^{-1} (υ(C═O)) was perturbed at 50 and 60 °C, were also negatively correlated. For the positive correlation we observed the glutamates ($\upsilon_{a\text{-}s}(COO^-)$) at 57 °C and the arginine peaks at 1600 cm^{-1} were perturbed at 47 and 57 °C, this second perturbation temperature was accompanied by a large intensity change suggesting partial unfolding. Also, the loop hinge region at 1668.1 cm^{-1} (υ(C═O))