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Cation- and peptide-binding properties of human centrin 2

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Abstract Centrin and calmodulin (CaM) are closely related four-EF-hand Ca^{2+}-binding proteins. While CaM is monomeric, centrin 2 is dimeric and binds only two Ca^{2+} per dimer, likely to site IV in each monomer. Ca^{2+} binding to centrin 2 displays pronounced negative cooperativity and a $[Ca^{2+}]_{0.5}$ of 30 μM. As in CaM, Ca^{2+} binding leads to the exposure of a hydrophobic probe-accessible patch on the surface of centrin 2. Provided Ca^{2+} is present, centrin 2 forms a 1:1 peptide:monomer complex with melittin with an affinity of 100 nM. The complex binds four instead of two Ca^{2+}. Our data point to surprising differences in the mode of activation of these homologous proteins.

Key words: Centrosome; Ca^{2+}-binding protein; EF-hand motif; Conformational change; Protein–peptide interaction

1. Introduction

In cells of higher eukaryotes, the number, direction and polarity of microtubules is organized by the centrosome. This structure, composed of two cylindrical centrioles surrounded by pericentriolar material, duplicates prior to mitosis and forms the two poles of the mitotic spindle [1]. The centrioles themselves are composed of α- and β-tubulin, whereas the ordered lattice of the pericentriolar material merely consists of pericentrin and γ-tubulin. About 100 proteins are permanently or temporarily part of this matrix, several of them being essential for duplication or chromosome movement. Among them are several regulatory proteins such as centrin, also named caltractin. Centrins are members of the EF-hand protein family, closely related to the CaM (about 30% sequence identity). Middendorp et al. [2] pointed to an intriguing divergence of two subfamilies within the centrin family: human centrin 1 and 2 and *Chlamydomonas* centrin are probably involved in centrosome localization and segregation, whereas human centrin 3 and yeast Cdc31p would be involved in centrosome duplication [3]. This functional diversity is also reflected in the amino acid sequence. The Ca^{2+}-binding properties of *Chlamydomonas* centrin have been reported [4]: it binds two Ca^{2+} with high affinity and two Ca^{2+} with low affinity and undergoes Ca^{2+}-dependent conformational changes. For the other centrins no direct Ca^{2+}-binding studies have been reported, but from sequence comparison it can be inferred that the stoichiometries are different. In yeast Cdc31p the EF-hands 1 and 4 are active and Ca^{2+} binding affects the conformation [5].

Cdc31p interacts with Kar1p protein, which is a component of the half bridge of the spindle pole body, the homologue of the centrosome in yeast [6]. The binding site was identified as a 19-residue-long segment in the center of Kar1p and is composed of a partly amphiphilic α-helix with an excess of positive charges [5]. This segment binds CaM, but not efficiently, and differs from the much-studied CaM-binding motif [7] by the fact that the hydrophobic side is interspersed with three negatively charged side chains. Mutation of these side chains to Ala strongly increased its affinity for CaM without affecting that for Cdc31p. The binding of Cdc31p, of *Scherffelia* centrin and of human centrins 1 and 2 to the isolated peptide is Ca^{2+} dependent and displays dissociation constants ranging from 50 (Cdc31p) to 250 nM (human centrin 2). The binding of Ca^{2+} to the fourth EF-hand of Cdc31p is the trigger for the interaction with Kar1p [5].

In this paper we report the Ca^{2+}-binding characteristics, i.e. affinity, selectivity, cooperativity of human centrin type 2, as well as the effect of Ca^{2+} on the conformation of this protein. We report also the interaction of centrin 2 with melittin (ME), a peptide that was used as a model in the interaction of CaM with its target proteins [8].

2. Materials and methods

2.1. Materials

ME was purified as previously described [9]. Its concentration was assessed spectrophotometrically using the extinction coefficient ε_{280nm} of 5500 M^{-1} cm^{-1}.

2.2. Protein expression and purification

Expression of human centrin 2 was based on the plasmid construction and protein induction procedure described by Wiech et al. [10]. The bacterial pellet was diluted 15-fold in 40 mM Tris–HCl buffer, pH 7.4 containing 5 mM EDTA and antiproteases (Complete-Boehringer, PMSF, 1 mM). The cells were lysed by ultrasonication in ice, centrifuged and the supernatant was heated for 10 min at 60°C. The soluble fraction was incubated overnight at 4°C, in $(NH_4)_2SO_4$ at 50% saturation. The supernatant was fractionated on a Phenyl-TSK (Toso-Haas) column, equilibrated in 40 mM sodium glycerophosphate buffer, pH 7.2, 5 mM EDTA, 1.8 M $(NH_4)_2SO_4$ and 3 mM β-mercaptoethanol. The elution was done by gradually decreasing the $(NH_4)_2SO_4$ concentration to zero. Two additional chromatography steps were necessary for obtaining the suitable homogeneous samples: one on DEAE TSK 650S (Tris–HCl buffer, pH 8.0, elution by NaCl gradient)

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Abbreviations: CaM, calmodulin; ME, melittin; $[Ca^{2+}]_{0.5}$, calcium concentration at half-maximal change; K_{Ca}, stoichiometric Ca^{2+}-binding constant; TNS, 2-*p*-toluidinylnaphthalene-6-sulfonate; ANS, 8-anilino-1-naphthalenesulfonate

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and a second on HW 40S-TSK (NH_4CO_3 buffer). The purity of the intermediate and final samples was assessed by SDS–PAGE and reverse-phase HPLC (column Poros R1, PerSeptive Biosystems).

2.3. Metal removal

Centrin were precipitated with 3% trichloroacetic acid and then passed through a 40×1 cm Sephadex G-25 column equilibrated in 50 mM Tris–HCl, pH 7.5, 150 mM KCl (buffer A). Typically the contaminating Ca^{2+} represents less than 3% of the total binding capacity. Total Ca^{2+} and Mg^{2+} concentrations were determined with a Perkin-Elmer 2380 atomic absorption spectrophotometer. The protein concentration was determined from the ultraviolet absorption spectrum using the molar extinction coefficient $\varepsilon_{259nm} = 2450\ M^{-1}\ cm^{-1}$, based on the Tyr and Phe content.

2.4. Cation binding

Flow dialysis in the absence or presence of 2 mM Mg^{2+} was carried out at 25°C in buffer A with protein concentrations of 20–30 μM according to the modified method of Colowick and Womack [11]. In the flow dialysis experiments on an equimolar mixture of 50 μM ME and centrin 2, 2 μM ME was present in the perfusion buffer. At the end of the experiment the concentration of ME was estimated spectrophotometrically to evaluate the loss of ME from the upper compartment. Treatment of the raw data and evaluation of the metal-binding parameters were done as described previously [12]. Since the Ca^{2+}-binding isotherms display negative cooperativity, the data were analyzed with the Adair equation for two (centrin alone) or four (equimolar complex) binding sites. Equilibrium gel filtration was performed and analyzed as described [12].

2.5. Interaction with hydrophobic probes

The Ca^{2+}-dependent changes in the hydrophobic core of centrin were followed by monitoring the fluorescence properties of 2-*p*-toluidinylnaphthalene-6-sulfonate (TNS) and 8-anilino-1-naphthalenesulfonate (ANS) as described previously [13]. A 400 μl solution of 40 μM indicator and 2 μM metal-free protein was excited at 322 (TNS) or 380 nm (ANS) with slits set at 10 nm. EGTA (20 μM), Ca^{2+} (1 mM) or Mg^{2+} (2 mM) were added to obtain the different forms.

2.6. Interaction with melittin

The interaction of centrin with ME was monitored by Trp fluorimetry at 25°C on mixtures of 1–2.5 μM centrin and ME in buffer A. Both slits were at 10 nm. Ca^{2+} or EGTA to final concentrations of 1 mM were added to obtain the respective apo and Ca^{2+}-saturated forms. The spectra of the Ca^{2+} and apo form show an isosbestic point at 375 nm. In the titration experiment the ratio of fluorescence at 324 nm and of the isosbestic points was plotted as function of the concentration of centrin 2 monomers. The fluorescence values were corrected for the contribution of centrin 2, which was obtained from a blank titration of buffer A by this protein. The interactions were also monitored by non-denaturing gel electrophoresis as previously reported [8,9].

3. Results

3.1. Secondary and tertiary structure

The far UV CD spectra of the apoprotein, Mg^{2+} and Ca^{2+} forms, measured with a Jasco J-715 spectropolarimeter either at low or moderate ionic strength, are nearly identical (data not shown). The absence of any change in the secondary structure upon binding of Ca^{2+} was also reported for *Chlamydomonas* centrin [4]. Analysis of the spectra using a program for secondary structure prediction [14] indicates that there is 48% of α-helix, 9% 3_{10}-helix, 3% β-pleated sheet, 15% turn, 25% others. The Tyr fluorescence spectrum of human centrin 2 shows a maximum at 305 nm, but the intensity is not much influenced by binding or dissociation of Ca^{2+} (dashed and dotted lines in Fig. 3). This was confirmed by UV difference spectrophotometry (not shown): the 'Ca^{2+}–apo' spectrum shows rather small positive peaks at 280 and 288 nm, i.e. in the area of Tyr absorption. Since the single Tyr is placed at the real C-terminal position, it is likely that its environment is not much influenced by Ca^{2+} binding. In contrast, this difference spectrum shows strong positive bands at 253, 259, 265 and 269 nm, suggesting that Ca^{2+} binding changes the microenvironment of some of the nine Phe residues.

Upon gel filtration of 2–4 mg of centrin 2 on a 1×120 cm

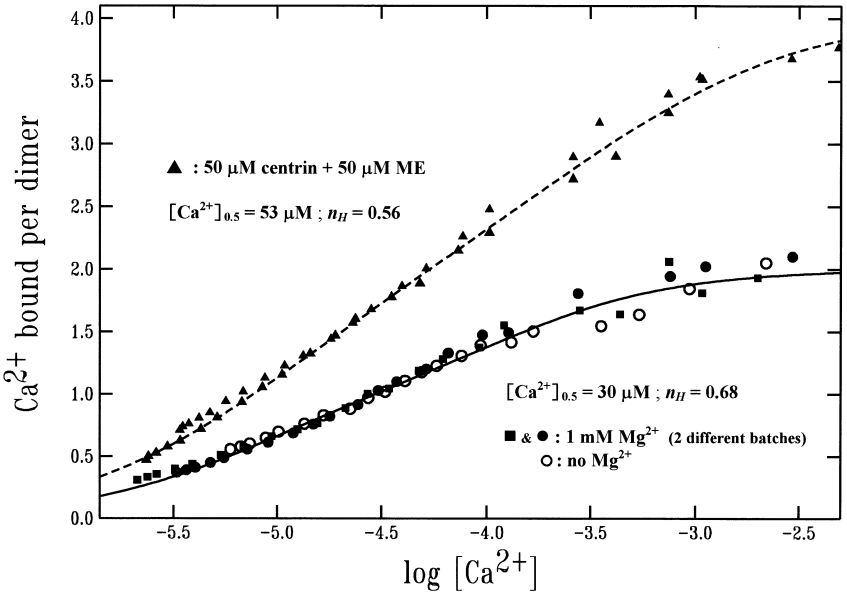

Fig. 1. Calcium binding to human centrin 2 in the absence (open symbols) and presence of 2 mM Mg^{2+} (closed symbols; the rectangles and circles represent experiments on different batches and periods) and in the presence of an equimolar concentration (50 μM) of ME (triangles). Calcium binding was measured by flow dialysis at 25°C in 50 mM Tris–HCl, pH 7.5, 150 mM KCl. The solid and dashed lines represent the theoretical isotherms generated with the following stoichiometric binding constants (K_n): 1.5×10^5 and $7.5\times10^3\ M^{-1}$ (solid) and 3.0×10^5, 5.0×10^4, 7.0×10^3 and $1.0\times10^3\ M^{-1}$ (dashed).

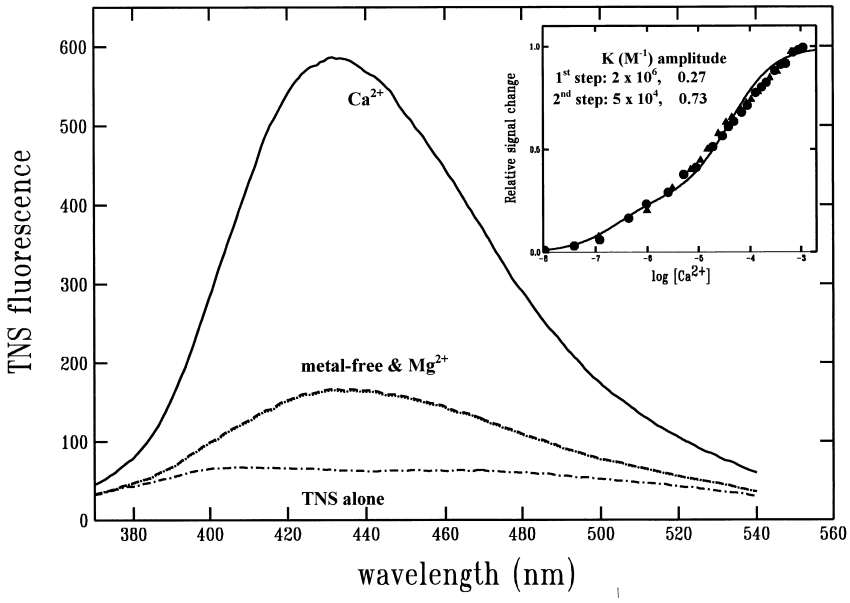

Fig. 2. Hydrophobic exposure as monitored by fluorescence enhancement of TNS. Spectra of 40 μM bis-ANS in the presence of 3.3 μM human centrin 2 in the absence of divalent cations (dotted lines) or in the presence of 1 mM μM Ca^{2+} (solid line) or 2 mM Mg^{2+} (dashed line). The dashed and dotted lines represent the fluorescence of 40 μM TNS alone. Inset: Ca^{2+} titration of the fluorescence enhancement of TNS by centrin 2. Triangles and circles represent two independent experiments. The line is calculated with the Adair equation for two sites assuming K_1 and K_2 values of 2×10^6 and 5×10^4 M^{-1}, respectively, and signal contributions of 0.27 and 0.73 for the first and second binding steps, respectively.

Sephacryl S200 column equilibrated in buffer A containing either 100 μM Ca^{2+} or 1 mM EGTA the protein elutes as a *single symmetrical* peak with apparent molecular weights of 45 and 52 kDa (compared to the Bio-Rad standards) in the presence and absence of Ca^{2+}, respectively. No traces of lower molecular weight are visible. Since under very similar conditions the monomeric, asymmetric CaM displays an apparent molecular weight of 33 kDa [15], our data suggest that essentially all centrin 2 is in a dimeric state. In this equilibrium gel filtration system the dimer binds 1.5 ± 0.15 ($n=3$) moles of Ca^{2+}, in agreement with the results of flow dialysis presented below.

3.2. Direct cation-binding studies

In flow dialysis nearly identical isotherms are obtained in the presence and absence of 2 mM Mg^{2+} (Fig. 1): per dimer two Ca^{2+}-binding sites were titrated with $[Ca^{2+}]_{0.5}$ of 30 μM and n_H of 0.68. This points to a negative allosteric effect and this is only conceivable in the dimeric state. Since Mg^{2+} does not influence the isotherm (closed versus open symbols in Fig. 1), the site is of the so-called Ca^{2+}-specific type, as are the four sites of CaM. Two flow dialyses were also performed on an equimolar mixture of apo centrin 2 and ME. The loss of ME (mass 2935 Da) in the flow cell during the dialysis is reasonably low (8–12%). Interestingly, the complex binds four Ca^{2+} ions with $[Ca^{2+}]_{0.5}$ of 53 μM and n_H of 0.56. ME binding thus activates two Ca^{2+}-binding sites which are silent in centrin 2 alone. The stoichiometric binding constants listed in the legend of Fig. 1 are related via the appropriate statistical factors to the intrinsic constants, with values of 7.5×10^4, 3.3×10^4, 1.1×10^4 and 4.0×10^3 M^{-1}, for K'_{Ca1} to K'_{Ca4}, respectively. From these values we can deduce that four, and not two constants can yield a satisfactory fit for this isotherm, implying that the complex is a heterotetramer, not a heterodimer.

3.3. Interaction with hydrophobic probes

The fluorescence of TNS is enhanced slightly (1.2–2-fold) by the apo and Mg^{2+} form of centrin, but strongly (7-fold) by the Ca^{2+} form (Fig. 2). The same is true for ANS, although the enhancement is less pronounced (data not shown). The instantaneous fluorescence changes upon binding or dissociation of Ca^{2+} suggest that the apo protein does not adopt a molten globule conformation, and that Ca^{2+}-loaded centrin exposes a hydrophobic surface, as in CaM [16]. The Ca^{2+} dependence of the fluorescence increase occurs in at least two steps (Fig. 2, inset). In an (over)simplified model the first increase to 27% of the total signal change occurs with a $[Ca^{2+}]_{0.5}$ of 0.5 μM, whereas the major change occurs with a $[Ca^{2+}]_{0.5}$ of 20 μM. These values suggest that the affinities of Ca^{2+} for the TNS–centrin 2 complex are about one order higher than in centrin 2 alone. This qualitatively fits with the rules of linked functions [12].

3.4. Interaction with melittin

Although the Ca^{2+}-binding parameters and the self-association propensity of centrin are very different from those of CaM (CaM shows only a minor dimerization, see [17]), the two proteins share significant sequence identity and exhibit a Ca^{2+}-dependent exposure of hydrophobic patches on their surface. Therefore we examined if centrin 2 can interact with ME, which was considered as a model for target binding to CaM [8]. ME contains one Trp, whose fluorescence strongly increases upon binding of CaM. Addition of Ca^{2+} to an equimolar mixture of centrin 2 (monomer) and ME leads to a 1.6-fold increase of the fluorescence intensity and a blue shift from 343 to 333 nm (Fig. 3). The apocentrin *plus* ME spectrum is different from that of ME alone, but close to the calculated sum of the individual apocentrin and ME, suggesting that no complex is formed in the absence of Ca^{2+}. All the spectral changes are perfectly and instan-

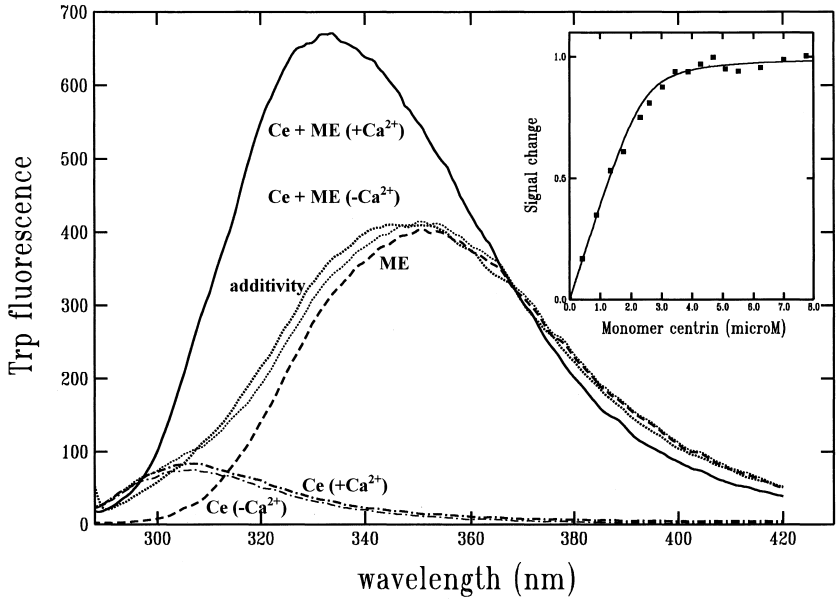

Fig. 3. Centrin–ME interaction measured by the Trp fluorescence of ME in 50 mM Tris–HCl, pH 7.5, 150 mM KCl. Ce stands for centrin 2. Slits are 5 and 5 nm. Excitation is at 278 nm. Samples contained 6.6 μM of the individual compounds or of the equimolar mixtures. ME+Ca^{2+}-saturated centrin (solid line); ME+metal-free centrin (experimental: dotted line; calculated sum: thin dotted line); ME (dashed line); Ca^{2+}-saturated and metal-free centrin (thick and thin dashed and dotted lines). Inset: titration of 2.4 μM of ME with centrin 2 as monitored by the change in Trp fluorescence. The *X* axis shows the added concentration for monomers. The lines represent the theoretical curves calculated with a K_D of 100 nM.

taneously reversible when either Ca^{2+} or EGTA are neutralized.

The stoichiometry of the centrin–ME complex could be evaluated from the fluorescence titration of 2.4 μM ME with centrin in the presence of 2 mM Ca^{2+} (Fig. 3, inset). The Trp fluorescence change yielded the stoichiometry of 0.99 moles of centrin monomer per mole of ME. The curvature around the inflection point allows the estimation of the K_D which amounts to 100 nM. Thus each monomer of centrin can bind one mole of this model peptide with an affinity which is about 30 times lower than the affinity of ME for CaM. This complex was not stable when a mixture of 3 mg centrin and a 2-fold molar excess of ME was chromatographed on a 1×100 cm Sephacryl S200 equilibrated in buffer A plus 100 μM Ca^{2+}. The 1 ME/1 monomer stoichiometry was confirmed by native PAGE on mixtures of 5 μg centrin and increasing amounts of ME (data not shown).

4. Discussion

In this study we showed that human centrin 2 possesses only one functional Ca^{2+}-binding site (likely site IV, see below) per monomer, i.e. two sites in the dimeric protein. Gel filtration strongly suggests that all centrin 2 is in a homodimeric form. Thus, homodimerization allows allosteric interactions between subunits and may be the reason for the pronounced negative cooperativity in the binding curve. The $[Ca^{2+}]_{0.5}$ value of 30 μM is rather low for the protein to detect efficiently the Ca^{2+} signal in stimulated cells, especially since the centrosome is not surrounded by organelles that can generate steep Ca^{2+} gradients. Nevertheless, several intracellular Ca^{2+}-binding proteins have similar binding properties and an affinity increase due to binding to a target protein is often invoked to explain the low affinity. *Chlamydomonas* centrin is reported to bind two Ca^{2+} with high and two Ca^{2+} with low affinity [4] and sites I and II have been reported to be the high affinity sites. It can thus be deduced that there is poor evolutionary conservation of the EF-hand motifs in the centrin subfamily.

From the sequence analysis it can be predicted that only site IV is canonical, i.e. oxygen-bearing carboxyl side chains are present in the critical positions for Ca^{2+} binding and segments with a propensity to form α-helices surround the Ca^{2+}-binding loop. All the other EF-hands bear a Ser or Thr in position +Z, which is unusual [18]. EF-hand III has an Asn in position −Z, which normally is occupied by a bidentate carboxyl group. We therefore suppose that EF-hand IV constitutes the unique functional Ca^{2+}-binding site in centrin 2. The fact that the NMR spectrum of the C-terminal half of centrin is highly sensitive to Ca^{2+} (C.T. Craescu, unpublished data) is in agreement with this assignment.

The absence of changes in the secondary structure does not preclude that Ca^{2+} binding leads to important conformational changes. Indeed, upon the binding of Ca^{2+} hydrophobic patches are exposed on the surface of centrin 2, which is reminiscent of the behavior of CaM [16,19]. Binding of the fluorescent probes TNS and ANS was highly sensitive to these conformational changes. Contrary to the case of CaM, the Ca^{2+} dependence of fluorescence enhancement of these probes is complex with two or even more steps. No changes occur in the fluorescence of the single Tyr, but this can easily be explained by its position at the real C-terminus of the protein. Termini are often highly flexible and this seems to be the case for centrin 2, as its fluorescence spectrum is that of a free Tyr.

The study of the interaction of CaM with model peptides has been very helpful for understanding how CaM activates its target proteins [20]. The sequence homology of centrin with CaM and the fact that centrin also exposes hydrophobic sur-

face patches, incited us to examine the interaction of ME with centrin. Interestingly, the influence of centrin on the Trp fluorescence of ME is very similar to that of CaM, with a similar intensity increase and blue shift. Seminalplasmin, another high-affinity CaM-binding model peptide [21], also forms a complex with centrin 2 and again the Trp fluorescence characteristics are similar to those described here for ME (J.A.C., data not shown). Based on this similarity and on the fact that Trp is a good conformational probe, we suppose that centrin wraps up around ME (or seminalplasmin) in a similar way as CaM [20]. However, the affinity of CaM for ME is 30-fold higher than that of centrin 2 for ME. It should be noted that the putative centrin target Kar1p, which also possesses one Trp and shows sequence homology to ME (and with seminalplasmin), displays the same affinity for centrin as ME and shows the same spectral enhancement upon binding of centrin as the model studied here [5]. Surprisingly, Ca^{2+} binding to the centrin–Me complex revealed that two more sites per centrin dimer are titrated as compared to centrin 2 alone. Fitting of the data to the Adair equation is distinctly better when using four affinity constants instead of two, suggesting that ME binding is not accompanied by centrin dissociation and that a $(centrin)_2(ME)_2(Ca^{2+})_4$ complex is formed. The Ca^{2+}-dependent centrin–ME interaction revealed a very new aspect of the principle of free energy coupling: in the complex the affinity for Ca^{2+} is not increased as is usual for CaM [12], but the number of Ca^{2+}-binding sites has doubled. It is likely that one of the 'dead' EF-hands in the N-terminal half of centrin is activated to bind Ca^{2+} upon complex formation. Our data suggest that the mode of target activation by centrin 2 is very different from the well-established model for CaM.

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