Calcium-dependent Self-assembly of Human Centrin 2*†§

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Human centrin 2 (HsCen2) is a member of the EF-hand superfAMILY of calcium-binding proteins, often associated with the centrosomes and basal bodies. These organelles exhibit different morphological aspects, including a variety of centrin-containing fibers that connect the two centrioles or other structural elements of the pericentriolar space. The molecular basis of the Ca\(^{2+}\)-sensitive fibers and their precise role in centrosome duplication are not known. To explore the possible structural role of HsCen2, we initiated a physicochemical study of the self-assembly properties of the purified protein in vitro. Using light scattering experiments, we investigated the temporal evolution of the assembly process and characterized the dependence on various chemical and physical factors, including temperature, dication concentration, ionic strength, protein concentration, and pH. The reversible self-assembly revealed many features of a large-size protein polymerization, with nucleation and elongation steps. Kinetic and equilibrium experiments show that a hydrophobic fluorescent probe (ANS) inhibits the polymerization by interfering with the nucleation step, probably through interactions with the apolar exposed sites on the protein surface. A truncated form of HsCen2, lacking the first 25 residues (Δ25HsCen2), shows no detectable self-assembly, pointing to the critical role played by the N-terminal domain in the supermolecular organization of HsCen2. As revealed by isothermal titration experiments, the isolated N-terminal domains bind with a significant affinity (2 × 10\(^{5}\) m\(^{-1}\)) to preformed oligomers of Δ25HsCen2 through an entropy-driven mechanism.

Centrin is a small acid proteins (about 170 residues and a molecular mass of 20 kDa) from the highly conserved Ca\(^{2+}\)-binding EF-hand superfamily, which were identified in a variety of species from the eukaryote kingdom of protists, fungi, plants, and animals. Spatial and time distribution within the mitotic organizer centers and the critical role of these proteins in cell cycle control have been the subject of intensive investigation in cellular biology for the last decade (1–4). Although lower eukaryote organisms (yeast, algae) have generally only one centrin gene, higher vertebrates have four distinct centrin isoforms (5) with variable sequence and different tissue and cell distribution. HsCen1 and HsCen2 are highly similar to each other (sequence identity, 81%) and to the algae centrin (68 and 71%, respectively), whereas HsCen3, discovered lately (6), has a more distant sequence; it shows only 58 and 57% identity with HsCen1 and HsCen2, respectively, and is slightly closer to Cdc31, the centrin equivalent in yeasts. Saccharomyces cerevisiae (59% sequence identity). Mammalian centrin 4 is philogenetically related to HsCen1 and HsCen2 with 72 and 74% sequence identity, respectively, and a restricted expression profile resembling that of HsCen1 (5). A large part of the sequence diversity among the centrins occurs within the first 20 residues of the amino-terminal domain, which have no counterpart in the “standard” Ca\(^{2+}\)-binding protein calmodulin (CaM).

Historically, centrins were first identified as the major component (63% of the soluble proteins) of the striated flagellar roots from the Prasinophycean green alga Tetraselmis striata (7). This seems to be a common feature of unicellular green algae where the centrin-containing fibers connect the basal bodies to one another, to the cytoplasm of the cell body, to the plasma membrane, or to the nucleus (8). It was observed that all of these fibers could contract upon increase in intracellular Ca\(^{2+}\) concentration (7, 9), and in some instances this may induce microtubule severing and a consequent excision of the flagellum (7). The way in which the flagellar roots contract in response to elevated calcium levels suggested that centrin is largely responsible for the motile behavior of the system by forming contractile centrin fibers. Centrin is also one of the major components of the infraciliary lattice spanning the whole cell surface in Paramecium (10). Despite the presence of other polypeptide components, the sensitivity to Ca\(^{2+}\) and EGTA of this cytoskeletal network suggests a central regulating and structural role for the centrin component.

Similar structural and motility roles for centrin were also suggested in higher eukaryotic cells. Thus, in cultures of Ptk2 epithelial cells, it was observed that at high Ca\(^{2+}\) concentrations, centrin can form detectable large spots near the cell center or pericentriolar well-formed satellites (11). The authors proposed that the pericentriolar satellites and the centrosomal matrix may be interconvertible forms of the same fibrillar material, essentially composed of centrin. HsCen1 and/or HsCen2 were also suggested to be involved in ciliary beating of human nasal epithelial cells in culture (12). Within the

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1 The abbreviations used are: HsCen, human centrin; CaM, calmodulin; ANS, 8-anilino-naphthalene-1-sulfonic acid; C-HsCen2, the C-terminal domain of HsCen2 (T\(_{94}-Y_{172}\)); N-HsCen2, the N-terminal domain of HsCen2 (M\(_{1}-S_{98}\)).
plex structure of the centrosomes, centrin evolve among many other proteins (about 100 in the pericentriolar material) of larger size, exposing significant hydrophobic surface areas (13). The intermolecular interaction of these components may explain the high density and viscous character of the pericentriolar environment.

In an earlier comparative study focused on the physicochemical diversity of centrins, Wiech et al. (14) showed that centrins from yeast (Cdc31p), algae (Scherfelia dubia) centrin (SdCen), or humans (HsCen1, HsCen2) may form multimers in the presence of Ca$^{2+}$, in clear contrast with the yeast calmodulin. Among the proteins studied, the algir calcin exhibits a considerably higher propensity to associate into high-density material that can be sedimented by low-speed centrifugation. These results raise at least three important questions related to 1) the biological significance of the observations, 2) the molecular mechanism of the self-association, and 3) the relevance of the weaker interactions observed with the human isoforms. The aim of the present work is mainly related to the last two points, focusing on the physicochemical and molecular basis of HsCen2 self-assembly. To address these objectives, we used the high-purity recombinant protein and various constructs, derived from its sequence, overexpressed in Escherichia coli. On the basis of spectroscopic and thermodynamic observations (light scattering, fluorescence, and calorimetry), we characterized the self-assembly of purified HsCen2 and its dependence on various physicochemical parameters (temperature, pH, Ca$^{2+}$, ionic strength, and protein concentration). Experiments using isolated domains or a truncated form of the protein lacking the first 25 residues suggest that interactions between N- and C-terminal domains from different subunits play a crucial role in the observed process.

MATERIALS AND METHODS

Protein Expression and Purification—Recombinant proteins or domains (HsCen2 and C-HsCen2) were overexpressed in E. coli and purified as described previously (15, 16). The cDNA of the domain N-HsCen2 (residues 1–98) and of the construct Δ25HsCen2 (which lacks the first 25 residues) were obtained by polymerase chain reaction from the cDNA of HsCen2 and subcloned into the expression vector pET Blue-1 (Novagen). The vector was transferred into E. coli Tuner (DE3) pLacI, which were incubated at 37 °C. At an optical density of 1.0 (at 600 nm), protein synthesis was induced using isopropyl-β-D-thiogalactopyranoside (1 mM) for 2 h. After purification, the proteins were lyophilized and kept at −80 °C. The stock protein solutions in the appropriate buffer were conserved in liquid nitrogen and thawed before the experiments. The usual buffer was 50 mM Bis-Tris, 20 mM KCl, pH 7.1 (buffer A), which may contain various concentrations of divalent cations (Ca$^{2+}$, Mg$^{2+}$).

Light Scattering Measurements—Solution turbidity changes induced by rapid temperature increase were monitored by the absorbance at various wavelengths (>300 nm) of the protein samples in quartz cells of 1-mm or 1-cm optical path. By analogy with the extinction coefficient, the turbidity parameter ($I$) of a solution of concentration $c$ in a 1-cm cell is defined by the reduction in intensity of an incident light of intensity $I_0$.

$$I = I_0 \exp(-\tau \times c \times l)$$

For particles much smaller than the wavelength, this parameter may be simply related to the Rayleigh ratio at 90° (17).

$$\tau = (16 \pi^3) \times (8 \pi^3 \alpha^3) / \lambda^4 = (16 \pi^3) \times R_0$$

A spectrophotometer Hitachi U-2001, equipped with an external temperature control unit (Lauda RMI), was used for turbidimetric experiments. In the absence of specific absorption, the optical density, at a given wavelength, of a macromolecular solution allows estimation of the elastic light diffusion that in turn is related to the average threedimensional size of the species present in solution. Some of the light scattering observations at 90° were also performed with a fluorescence spectrometer Jasco FP777, using the same excitation and emission wavelengths.

ANS Binding—ANS (ammonium salt) was purchased from Sigma. Fluorescence emission of the fluorescent hydrophobic probe was recorded at a constant excitation (375 nm) in a 1-cm path length cuvette. The time dependence of the emission fluorescence was recorded on a Jasco FP777 fluorimeter, equipped with a temperature control device.

Isothermal Titration Calorimetry—Thermodynamic parameters of intermolecular interactions at constant temperature were investigated by isothermal titration calorimetry using a MicroCal MCS instrument (MicroCal Inc., Northampton, MA). The samples were equilibrated in the same buffer and degassed before their use. In a standard experiment, one sample (usually 250 μM) in a 250-μl syringe is diluted into the 1.337-ml calorimeter cell using 20–30 automatic injections of 8–15 μl each. The reaction triggered by each injection generally produces heat absorption or heat emission, recorded as positive or negative peaks, respectively, on a thermogram. Integration of the peaks corresponding to each injection and correction for the baseline, performed using Origin-based software, give the enthalpy variation along the mixing path, which represents the reaction isotherm. Fitting of the data to various interaction models results in the stoichiometry (n), equilibrium binding constant (K$^\circ$), and enthalpy of the interaction (ΔH).

RESULTS

The Thermal-induced HsCen2 Self-assembly Is Reversible—When a solution of HsCen2, buffered at physiological pH, is transferred from ice to a higher temperature in the spectrophotometer, the turbidity changes according to a reproducible pattern including a delay time, a fast increasing phase, and a final plateau. The kinetic and equilibrium parameters depend on the final temperature (Fig. 1). Generally, this pattern is associated with a reversible, nucleation-controlled polymerization starting with the nucleation process (the delay time), during which several monomers associate to form nuclei or seeds serving as initiators of the polymer formation. The slope of the elongation step is associated with the rate of polymer elongation, whereas the final plateau is determined by the size of the scattering objects. In cases where this size is much lower than the incident light wavelength, the turbidity is proportional to 1/A$^4$. In our experiments, we used relatively low KCl concen-
tractions in the buffer (usually 20 mM) to keep the working temperature range under the physiological values. The three phases are better distinguished at lower temperatures (Fig. 1A).

In all of our experiments, the observed process is reversible and highly reproducible. When the temperature is switched back to 0 °C, the sample is again optically transparent, and repetitive experiments with the same sample give superimposable curves.

At higher temperatures, the lag phase decreases considerably, and the larger final amplitude imposes use of 1-mm cells (Fig. 1B). Because of a relatively slow temperature equilibration of the sample, estimation of the initial lag phase or of the elongation rate is less reliable, and only the equilibrium final turbidity could be confidently considered. We noted that this last parameter increases with temperature and reaches a plateau between 30° and 40 °C. The back extrapolation of the temperature dependence curve permits a rough estimation of the minimal self-assembly temperature (T min) between 30° and 40 °C. The back extrapolation of the turbidity could be confidently considered. We noted that this elongation rate is less reliable, and only the equilibrium final concentration of the sample, estimation of the initial lag phase or of the elongation rate is less reliable, and only the equilibrium final concentration of the sample, estimation of the initial lag phase or of the equilibrium final turbidity could be confidently considered. We noted that this last parameter increases with temperature and reaches a plateau between 30° and 40 °C. The back extrapolation of the temperature dependence curve permits a rough estimation of the minimal self-assembly temperature (T min) in given experimental conditions (Fig. 1B, inset). The order of the final temperature in a series of experiments using the same sample does not influence the pattern of the curve or the extrapolated T min value.

The general properties of the temperature-induced self-assembly of HsCen2, observed in these experiments, are similar to those of the polymerization process observed for other biomolecules, such as hemoglobin S (18), actin (19–21), or tubulin (22–24).

**The Self-assembly Is a Heterogeneous and Multi-step Process**—A series of light scattering experiments were conducted at different wavelengths, between 330 and 800 nm. The equilibrium final intensity of the scattered light increases continuously with the decreasing wavelength of observation, but the curves show a similar global trend (Fig. 2). A more detailed analysis revealed several specific characteristics of the self-association process, which may be related to the size of the assemblies, the heterogeneity, and the interconversion rate between the different components.

The expected 1/λ 4 dependence of the light scattering is not fulfilled, indicating the presence of large particles, with sizes comparable to the observation wavelength (hundreds of nm). Small but reproducible differences may be observed among the patterns of various curves. Thus, at 330 nm the rate of polymer elongation is higher than at other wavelengths, and the initial lag phase is not detectable. In addition, the final plateau exhibits a continuous slow decrease, in contrast with the increasing tendency observed at 800 nm. An intermediate behavior was noted between these two wavelengths. Taken together, the above observations suggest that the fast self-assembly phase produces scattering species of heterogeneous size distribution, which may rearrange slowly in a subsequent phase. In the simplest case, we can assume the presence of two main classes of turbid objects, with smaller (<<λ) and larger (∼λ) sizes. The observed pattern at 330 nm, where the scattering is more sensitive to the smaller size population (1/λ 4 dependence), suggests that formation of the smaller particles has faster kinetics (Fig. 2). The decreasing tendency of the plateau may be explained by a slow conversion of these species into larger objects. On the other hand, at 800 nm where the scattering is less sensitive to smaller sizes, the plateau shows a positive slope, in agreement with the hypothesis of a progressive increase in particle average size.

**Ca 2+ Dependence**—In the absence of Ca 2+, it was not possible to detect any increase in the light scattering at temperatures up to 40 °C, protein concentrations up to 170 μM, and a Mg 2+/protein ratio up to 12. A systematic search for di-cation dependence of HsCen2 self-assembly revealed that a minimum of one Ca 2+ ion per protein and an excess of divalent cations (Ca 2+, Mg 2+, Mn 2+) are necessary to trigger the molecular association process. We have demonstrated recently that HsCen2 has only a strong binding site for Ca 2+, which discriminates against Mg 2+ ions (15). Because Mg 2+ alone is not able to initiate the self-assembly, we conclude that highly scattering species can form only with the holo form of the protein. Fig. 3A shows that the maximum turbidity increases with the divalent cation concentration and shows a plateau at high concentrations. Mn 2+ and Ca 2+ appear to be more efficient than Mg 2+ in inducing the self-association, as reflected in the tighter cation dependence of DO max. These experiments demonstrate that HsCen2 self-assembly requires one Ca 2+ ion, specifically bound to the C-terminal domain, and a moderate molar excess of Ca 2+ or other divalent cations. Tight binding of the first Ca 2+ ion stabilizes an open conformation of the C-terminal half, with the exposure of a large hydrophobic surface that may constitute a strong interaction site with other molecules (16, 25). Additional divalent cations, required for the self-assembly, may bind to the low-affinity Ca 2+–binding motifs. Indeed, EF-hand III exhibits a binding constant on the order of 10 4 M –1 (16), whereas the N-terminal motifs have an even lower affinity (on the order of 10 5 M –1).

It may be noted that G-actin polymerization is also modulated by divalent cations (Ca 2+ and Mg 2+), but in this case, Mg 2+ is more efficient in decreasing the lag phase and increasing the elongation rate (26). Similarly to HsCen2, G-actin exhibits two classes of metal-binding sites, a strong one (∼10 9 M –1), in which the metal is linked to ATP, and several weaker binding sites (∼10 7 to 10 8 M –1) (27).

**Dependence on Protein Concentration**—Increasing the protein concentration, while keeping constant the other physicochemical parameters (buffer A, 1.2 mM Ca 2+ at 38 °C), results in a faster self-assembly process and a higher plateau (Fig. 3B). In these conditions, a concentration of 50 μM (1 mg/ml) appeared as the minimum requirement for the experimental observations and was mainly used throughout this work.

**Other Physicochemical Factors (pH, Ionic Strength)**—A clear pH dependence, in the range of common physiological values, was pointed out by our experiments. The acid pH favors centrin polymerization, whereas basic pH is an unfavorable factor. For instance, a solution of 250 μM HsCen2 in buffer A, 2 mM CaCl 2...
shows a $T_{\text{min}}$ of 13 °C, 20 °C, and 24 °C at pH 6.5, 7.1, and 7.6, respectively. Similar to many members of the CaM superfamily, human centrins are acidic proteins, with a pI around 4.8. However, HsCen1 and HsCen2 exhibit a common distinctive feature consisting of an asymmetric charge distribution among the two EF-hand domains; although the C-terminal half has an acidic character, the N-terminal half is distinctly basic (pI, 7.9), essentially because of the Arg and Lys content of the first 25-residue segment. Therefore, this last domain is much more sensitive to the proton concentration in the working pH domain, becoming less positive from pH 6 to pH 8. One may conclude that the positive charge of the N-terminal EF-hand is favorable to the self-assembly, probably by interacting with negative charges of the C-terminal domain of another subunit.

In contrast to the divalent cations, higher (>/=50 μM) monovalent salt concentrations (Na/K) have the tendency to inhibit centrin polymerization. The monovalent ion inhibitory effect should be related to a possible reduction in Ca$^{2+}$ affinity (as it was demonstrated for CaM (28)) and/or to a screening of electrostatic attractive interactions that contribute to the self-assembly.

A different behavior was noted for actin polymerization (29, 30), for which the minimal temperature of polymerization is lower at higher salt concentration (KCl).

**ANS Binding**—ANS is a hydrophobic fluorescence probe generally used to detect exposed apolar surfaces in protein structures (31). Interaction of the probe with a hydrophobic site results in an increased fluorescence intensity, usually accompanied by a blue-shift of the maximum. We showed recently that binding of a similar molecular probe (2-p-toluidinylnaphthalene-6-sulfonate) to the C-terminal domain of HsCen2 is considerably increased upon Ca$^{2+}$ binding, suggesting that the main binding site is situated in the hydrophobic pocket, which is also the target interaction site (16). In agreement with this hypothesis, use of a slightly longer C-terminal construct, in which an intra-protein interaction blocks the main hydrophobic pocket, showed a dramatic decrease in 2-p-toluidinylnaphthalene-6-sulfonate binding.

To investigate the role of the Ca$^{2+}$-induced hydrophobic surface in the centrin self-association process, we assessed whether binding of a fluorescent probe interferes with the protein/protein interaction. As showed in Fig. 4A, increasing concentrations of ANS result in a lower final intensity of the scattered light (the height of the plateau), whereas the kinetics characteristics (nucleation and elongation steps) seem to be less affected. In addition, experiments conducted at different temperatures showed that the minimum polymerization temperature increases linearly with the fluorescent probe concentration up to 800 μM (Fig. 4B). The inhibitory effect of the hydrophobic probes strongly suggests that the primary binding
sites of ANS are identical or close to the intermolecular interaction sites inducing polymer elongation. In contrast with the temperature-dependent curves (Fig. 1), where the decrease in plateau is coupled to a slower nucleation (producing more nuclei) and elongation phases, the ANS effect concerns only the final light scattering intensity, most probably attributable to a reduced number of active molecules.

The ANS inhibition effect was further explored using the fluorescence spectroscopy. In buffer A containing 2 mM Ca\(^{2+}\), the fluorescence emission of the probe has a maximum at 520 nm and a low quantum yield. When HsCen\(_2\) (50 \(\mu\)M) is added, ANS shows a 9-fold enhancement of the fluorescence emission and a blue-shifted maximum at 466 nm, reflecting its binding to a hydrophobic protein region. The light scattering at 450 nm (\(\lambda_{ex}, 450\) nm) as well as the fluorescence emission (\(\lambda_{ex}, 375\) nm; \(\lambda_{em}, 466\) nm) of the mixture HsCen\(_2\)/ANS (50 \(\mu\)M/250 \(\mu\)M) was monitored as functions of time, at different temperatures. Signal recording started immediately after the sample transfer from ice to the thermostated cell holder of the spectrofluorimeter. As may be seen in Fig. 5, the self-association process, revealed by the light scattering measurement (Fig. 5B), is accompanied by a considerable decrease in ANS fluorescence emission (Fig. 5A) and a slight red-shift of the \(\lambda_{max}\) (Fig. 5D). Control experiments showed that the same thermal variations induced only a modest (~5\%) increase of the fluorescence emission of ANS alone, as was also observed for the ANS dimeric conjugate (bis-ANS) (32). In light of the previous results presented here, the decrease in ANS fluorescence, after the temperature switch, may be explained by a release of protein-bound fluorescent probes into the more polar buffer environment. An additional contribution may come from the thermal inactivation of the probe excited state, but we expect it to be much smaller. For instance, only a moderate fluorescence decrease (8\%) for bis-ANS bound to \(\alpha\)-crystallin, observed between 20 °C and 38 °C, was related to this mechanism (33).

The temperature profiles of the two observed parameters are clearly different (Fig. 5C), with fluorescence changes taking place in the absence of (or before) the light scattering enhancement. This suggests that ANS binding is in competition with the protein/protein interactions taking place during the nucleation period. At 20 °C, the assembly size is too small to affect markedly the light scattering, whereas the ANS fluorescence is sensitive enough to detect nuclei formation. This interpretation also requires that the binding affinity of ANS at higher temperatures be smaller than those driving the intermolecular associations, in agreement with the data in Fig. 4. A similar behavior was observed in the study of the 2-p-toluidinylnaphthalene-6-sulfonate binding to short and long variants of the C-terminal domain of HsCen\(_2\) (16), showing that the binding of the probe to the longer C-terminal domain, where the hydrophobic pocket is occupied by the extra-helix (D-helix), is largely inhibited.

The N-terminal Fragment of 25 Amino Acids Is Necessary for Polymerization—At millimolar concentrations, the C-terminal domain (94–172) of HsCen\(_2\), including the strongest Ca\(^{2+}\) binding site (loop IV), has the tendency to form dimers (16) but exhibits no temperature-induced light scattering. Also, the N-terminal half (1–98), having a much lower affinity for divalent cations, does not show any self-assembly tendency. These observations suggest strongly that polymerization requires the integral protein and is determined by heterologous interactions between domains belonging to two different subunits.

The members of the centrin family are characterized by an additional N-terminal segment of about 20 residues with a basic character and high sequence variability. To assay the
possible role of this fragment in the polymerization process of HsCen2, we overexpressed and purified a truncated centrin 2 variant lacking the first 25 residues (Δ25HsCen2: 26–172). CD experiments showed that the removal of this N-terminal fragment does not affect significantly either the global secondary structure or the structural stability (data not shown). At concentrations as high as 100 μM and 4 mM Ca2+, Δ25HsCen2 shows no turbidity changes up to 42 °C. At 5 °C, the ANS fluorescence is enhanced in the presence of the variant and saturating Ca2+ concentrations. However, for the same probe/protein ratio, the fluorescence enhancement is about three times lower for Δ25HsCen2 than for the wild-type protein. A similar variation of the probe fluorescence in the presence of the wild-type protein was noted when the ionic strength was increased from 20 to 100 mM KCl at similar protein concentrations. More significantly, transfer of the Δ25HsCen2/ANS solution from 0 °C to higher temperatures failed to produce any change in light scattering or ANS fluorescence (data not shown). Taken together, these data suggest that the N-terminal fragment, with its +5 net charge, is essential for centrin assemblies, emphasizing the electrostatic component of the underlying interactions. The difference in ANS fluorescence enhancement at low temperatures between the wild-type protein and the truncated form (Δ25HsCen2) should be attributable to additional charge-dependent probe binding by the wild-type protein.

Calorimetric Investigation of Intermolecular Interactions—Absence of polymerization of isolated HsCen2 domains, disymmetric charge distribution between the two halves, and the properties of the truncated construct suggest that self-assembly of centrin molecules involves Ca2+-modulated interactions between the C-terminal and N-terminal domains from different subunits. To explore this hypothesis, we performed calorimetric experiments designed to characterize homo- and heteromolecular interactions between the integral protein and its isolated domains.

Dilution of C-HsCen2 solutions (250 μM) by the injection of small aliquots into the calorimeter cells containing only buffer results in series of positive heat pulses that reflect an endothermic dissociation process of dimers or higher oligomers (Fig. 6A). Successive injections give progressively smaller peaks as the domain concentration builds up in the calorimeter cell. Fit of the absolute values of heat exchange per injection along the titration pathway to a model for dimer-monomer dissociation (suggested by our previous experiments (16)) enabled us to estimate a dissociation constant of 74 mM and an enthalpy of dissociation of 16 kcal/mol. However, analysis of these isothermal titration data alone does not allow a non-ambiguous identification of the dissociating species as dimmers or higher oligomers (34). Comparison of the lineshape in the 1H NMR spectra of the centrin domains (see the supplementary material) with that of a purely monomeric (11-kDa) protein (apo-neocarzinostatin (35)) strongly suggests that the majority of C-HsCen2 oligomer species in solution should not be larger than tetramers.

If C-HsCen2 is now titrated into a solution containing 10 or 18 μM HsCen2 (Fig. 6, B and C), the endothermic exchange heat is progressively cancelled, strongly suggesting that the newly dissociated C-terminal domains may interact with C-terminal domains of integral protein molecules. This hypothesis is supported by titration experiments conducted with the truncated form of HsCen2, which is a non-polymerizing species. Indeed, dilution of a 250 μM solution of Δ25HsCen2 into the buffer shows a similar endothermic dissociation as C-HsCen2 (Fig. 7A). Oligomerization of the truncated centrin form was also apparent from the line broadening of the proton NMR spectra.
(see the supplementary material). In contrast, injection of the wild-type protein at 250 μM (a turbid solution) into the buffer exhibits a highly reduced heat exchange (Fig. 7B), suggesting more complex energetics of the polymer dilution.

As we showed earlier in this work, an increased monovalent ion concentration (50 mM) inhibits the polymerization of HsCen2. At 200 mM NaCl, both the wild-type (which is no more turbid) and the truncated form are still oligomeric and dissociate with a comparable dissociation constant (Fig. 7, C and D). This demonstrates the oligomerization capacity of HsCen2 and its weak sensitivity to high ionic strengths. Although the oligomer formation seems to be mainly driven by interactions between C-terminal domains, it is likely that higher-order polymers build up on these intermediate complexes and require the participation of the basic N-terminal 25 residues.

To investigate this last hypothesis further, we performed an additional titration experiment using the N-terminal domain. As shown in Fig. 6D, dilution of 250 μM C-HsCen2 into 13 μM N-HsCen2 results in a dissociation isotherm, similar to that observed earlier in the absence of the N-terminal domain (Fig. 6A). This means that, at weak concentrations where the molecules are mainly monomers, the two isolated centrin halves show no significant interaction. A similar conclusion may be drawn from the titration of the diluted integral protein (13 μM) by the N-terminal domain (Fig. 6E). We therefore designed a titration experiment where centrin oligomers (but not higher polymer species) are already formed in the calorimeter cell, taking advantage of the particular properties of the truncated centrin. When N-HsCen2 (380 μM) is injected into a 120 μM solution of Δ25HsCen2 (Fig. 6F), a clear binding isotherm could be recorded, which can be fitted to a simple one-site interaction model. This results in a moderate binding constant $K_a = 2.1 \times 10^9$ M$^{-1}$ and a weak exothermic binding enthalpy $\Delta H = -0.56 \pm 0.04$ kcal/mol. From the free energy $\Delta G = -7.4$ kcal/mol and the enthalpy of interaction, one can calculate a positive entropy contribution, $T\Delta S = +6.9$ kcal/mol, which constitutes the driven force of the interaction. Interestingly, the optimized value for the reaction stoichiometry is 0.29, confirming the initial hypothesis that three or four associated molecules of Δ25HsCen2 are necessary to bind one molecule of N-HsCen2.

The two main components of the entropy term, generally identified in the protein/protein interactions, correspond to a decrease in translational and rotational degrees of liberty of the reacting molecules (having a negative value) and to the protein dehydration, resulting in a global positive entropy change of the solvent. The large positive entropy, observed in our experiment, suggests that the heterologous interaction of the EF-hand domains is largely accounted for by the dehydration of the monomers and the accompanying increase in water disorder.

**Fig. 7.** Isothermal titration calorimetry dilution experiments using the wild-type and the truncated form of HsCen2. Δ25HsCen2 (A and C) and HsCen2 (B and D) at 250 μM were titrated at 35 °C into the buffer (50 mM 4-morpholinepropanesulfonic acid, pH 6.8, 1 mM CaCl$_2$) containing 20 mM NaCl (A and B) or 200 mM NaCl (C and D). X, experimental artifacts.
DISCUSSION

Energetic and Structural Nature of the Self-assembly—On the basis of a number of physicochemical techniques, we characterized the reversible self-assembly properties of the highly purified human centrin HsCen2. At least one Ca\(^{2+}\) ion, an excess of divalent cations, a minimal protein concentration, and a floor temperature are necessary for the initiation of the assembly process. The ensemble of experimental observations suggests that molecular interactions are mediated by both ionic and hydrophobic forces. Thus, the pH effect, the inhibition by higher salt concentrations, and the role of the positively charged N-terminal fragment support the importance of the electrostatic component. On the other hand, molecular associations triggered by higher temperatures generally have a hydrophobic character. Ca\(^{2+}\) binding by regulatory EF-hand domains induces an active conformation characterized by a large exposed hydrophobic surface, which is usually involved in the intermolecular interactions with target molecules or apolar fluorescent probes. Hence, the ANS inhibition and the Ca\(^{2+}\) requirement fully support the hydrophobic contribution to the self-assembly.

Together with the spectroscopic experiments, the calorimetric titrations of various protein constructs provided some key elements for a structural and energetic understanding of the centrin reversible self-assembly. The Ca\(^{2+}\)-saturated HsCen2 molecules have the propensity to form small oligomers (through an enthalpy-driven mechanism), with a dissociation constant in the 50–100 \(\mu\)M range, most likely attributable to intermolecular interactions between the C-terminal domains. Evolution of these complexes toward larger polymers requires the participation of a highly basic N-terminal segment including the first 25 residues. Indeed, isothermal titration calorimetry experiments showed that the N-terminal domain binds with a significant affinity to the preformed oligomers in a process that is entropically driven. NMR structural studies on the N-HsCen2, in progress in our laboratory indicate that the first 25 residues, 6 of which are positively charged, are very flexible in solution and show no persistent secondary or tertiary structure elements. This segment may recognize and bind to negatively charged areas surrounding the hydrophobic pocket of C-terminal domains in a process that is pH- and ionic strength-sensitive. The present data suggest that formation of oligomers is a prerequisite of these interactions, but an alternative model in which the N-terminal domains activate the fusion of three or more monomers may also be conceived. Finding of appropriate conditions, necessary for obtaining high resolution images (14), either by negative staining electron microscopy, cryomicroscopy, or atomic-force microscopy, will allow us to build a more precise structural model of the centrin complexes.

Biological Implications—A number of cell biology studies, mainly on lower eukaryotic cells, such as paramecium or green algae, demonstrated that centrin plays an important structural role by contributing to the formation of Ca\(^{2+}\)-sensitive contractile filaments (36). A fraction of HsCen2 is constantly found associated with the highly regular structure of centrioles, as well as with other proteins within the viscous pericentriolar material (1, 37), suggesting that human centrioles also may contribute to some supramolecular assemblies. The results presented here, obtained with highly purified preparations of HsCen2, support this hypothesis and constitute a useful physicochemical basis for understanding the cellular observations. The self-assembly of HsCen2 is a reversible process, Ca\(^{2+}\)-dependent, and shows many common properties with other polymerizing proteins such as actin, tubulin, or hemoglobin S.

The biological significance of the present results is further supported by the similarity of the experimental conditions used here and the cellular context. The pH, Mg\(^{2+}\) concentrations, and temperature values used in the in vitro assays are clearly within the range of cell parameters. As concerns the protein concentration in a given subcellular compartment, it may be tissue-dependent but also can fluctuate in time as a function of the metabolic or pathologic state of the cell. Actually, the average experimental estimations, similar to those for CaM concentration (8–40 \(\mu\)M) (38), are fully compatible with our conditions. Ca\(^{2+}\) concentrations necessary for in vitro self-assembly correspond to those necessary for saturating the high-affinity site. These values are larger than the average intracellular concentration, but fluctuations attributable to various types of signaling may locally reach significantly higher values. Because of the non-ideal conditions of the intracellular environment (macromolecular crowding), the chemical activity of the compounds should be much larger than the theoretical concentration, favoring the intermolecular associations (39, 40).

Nevertheless, experiments conducted with highly purified proteins, albeit easier to analyze, constitute a simplification of the biological system. Thus, it is highly probable that heterologous interactions, involving one (or more) centrosome protein, and kinase-controlled phosphorylation (41) may take place in the cell, resulting in new structural and physicochemical properties. In addition, the same centrin may participate in structures of diverse morphology, and centrin from various organisms exhibit distinct self-association propensity (14).

The recent discovery of a novel centrin target (Sfi1p) in the yeast spindle pole body and the human centrosome (42) constitutes a stimulating key element for the exploration of these kinds of interactions. In the analysis of the functional role of Sfi1p-HsCen2 complex or of any other potential centrosome interaction, the self-association properties of HsCen2, characterized in the present work, should be taken into account. In the cell context, the capacity of nucleation and polymerization of centrin is most probably in competition with Sfi1p interactions, and the relative affinities of the corresponding molecular interactions, as well as their Ca\(^{2+}\) modulation, are critical parameters.

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REFERENCES

1. Salisbury, J. L. (1995)Curr. Opin. Cell Biol. 7, 39–45
2. Salisbury, J. L., Suino, K. M., Busby, R., and Springett, M. (2002)Curr. Biol. 12, 1287–1292
3. Schiebel, E., and Bornens, M. (1995) Trends Cell Biol. 5, 197–201
4. Wolfrum, U., Giesel, A., and Pulvermüller, A. (2002) in Photoceptors and Calcium (Baehr, W., and Palczewski, K., eds) pp. 155–178, Kluwer Academic, New York
5. Gavet, O., Alvarez, C., Gaspar, P., and Bornens, M. (2003) Mol. Biol. Cell 14, 1818–1834
6. Middendorf, S., Paolotti, A., Schiebel, E., and Bornens, M. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 9141–9146
7. Salisbury, J. L., Baron, A., Surek, B., and Melkonian, M. (1984) J. Cell Biol. 99, 962–970
8. McFadden, G. I., Schulze, D., Surek, B., Salisbury, J. L., and Melkonian, M. (1987) J. Cell Biol. 105, 963–972
9. Salisbury, J. L., and Floyd, G. L. (1978) Science 202, 975–977
10. Beisson, J., Clérot, J.-C., Fleury-Aubasson, A., Garreau de Loubresse, N., Ruiz, F., and Klotz, C. (2001) Protist 152, 339–354
11. Baron, A. T., Suman, V. J., Nemeth, E., and Salisbury, J. L. (1994) J. Cell Sci. 107, 2993–3003
12. Laoukili, J., Perret, E., Middendorf, S., Houchine, O., Guennou, C., Marano, F., Bornens, M., and Tournier, F. (2000) J. Cell Biol. 119, 1355–1364
13. Wigge, P. A., Jensen, O. N., Holmes, S., Souèes, S., Mann, M., and Kilmartin, J. V. (1988) J. Cell Biol. 114, 967–977
14. Wiech, H., Geier, B. M., Paschke, T., Spang, A., Grein, K., Steinköetter, J., Melkonian, M., and Schiebel, E. (1996) J. Biol. Chem. 271, 22453–22461
15. Durussel, I., Blouquit, Y., Middendorf, S., Craescu, C. T., and Cox, J. A. (2000) FEBS Lett. 472, 208–212
16. Matei, E., Miron, S., Blouquit, Y., Duchambon, P., Durussel, I., Cox, J. A., and Craescu, C. T. (2003) Biochemistry 42, 1439–1450
17. Campbell, I. D., and Dwek, R. A. (1984) Biological spectroscopy, The Benjamin/ Cummings Publishing Company, Inc., Menlo Park
18. Eaton, W. A., and Hofrichter, J. (1990) Adv. Prot. Chem. 40, 63–279
19. Buzan, J. M., and Frieden, C. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 91–95
20. Belmont, L. D., Orlova, A., Drubin, D. G., and Egelman, E. H. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 29–34
21. Chen, X., and Rubenstein, P. A. (1996) J. Biol. Chem. 270, 11406–11414
22. Carlier, M., and Pantaloni, D. (1978) Biochemistry 17, 1908–1915
23. Na, G. C., and Timasheff, S. N. (1982) Methods Enzymol. 85, 393–408
24. Liliom, K., Wagner, G., Kovacs, J., Drubin, D. G., and Goode, J. (1999) Biochem. Biophys. Res. Commun. 264, 605–610
25. Popescu, A., Miron, S., Blouquit, Y., Duchambon, P., and Craescu, C. T. (2003) J. Biol. Chem. 278, 40252–40261
26. Steinmetz, M. O., Goldie, K. N., and Aebi, U. (1997) J. Cell Biol. 138, 559–574
27. Carlier, M. (1991) J. Biol. Chem. 266, 1–4
28. Linse, S., Helmersson, A., and Forsen, S. (1991) J. Biol. Chem. 266, 8050–8054
29. Cooper, J. A., and Pollard, T. D. (1982) Methods Enzymol. 85, 182–210
30. Greer, S. C. (2002) Annu. Rev. Phys. Chem. 53, 173–200
31. Lakowicz, J. R. (1999) Principles of Fluorescence Spectroscopy, 2nd Ed., Kluwer Academic/Plenum Press, New York
32. Tang, D., and Borchman, D. (1998) Exp. Eye Res. 67, 113–118
33. Das, K. P., and Surewicz, W. K. (1995) FEBS Lett. 360, 321–325
34. McPhail, D., and Cooper, A. (1997) J. Chem. Soc. Faraday Trans. 93, 2283–2289
35. Mispelter, J., Izadi-Pruneyre, N., Quiniou, E., and Adjadj, E. (2000) J. Magn. Reson. 145, 229–232
36. Salisbury, J. L. (1998) J. Eukaryot. Microbiol. 45, 28–32
37. Paoletti, A., Moudjou, M., Paintrand, M., Salisbury, J. L., and Bornens, M. (1996) J. Cell Biol. 139, 3089–3102
38. Black, D. J., Tran, Q.-K., and Persechini, A. (2004) Cell Calcium 35, 415–425
39. Ellis, R. J. (2001) Trends Biochem. Sci. 26, 597–604
40. Medalia, O., Weber, I., Frangakis, A., Daniela, N., Gerisch, G., and Baumeister, W. (2002) Science 298, 1209–1213
41. Lutz, W. H., Lingle, W. L., McCormick, D., Greenwood, T. M., and Salisbury, J. L. (2001) J. Biol. Chem. 276, 20774–20780
42. Kilmartin, J. V. (2003) J. Cell Biol. 162, 1211–1221
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