Mechanism of Oligomerisation of Cyclase-Associated Protein from *Dictyostelium discoideum* in Solution

Running Title: CAP oligomerisation in solution

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Cyclase-associated protein (CAP) is a highly conserved modular protein implicated in the regulation of actin filament dynamics and a variety of developmental and morphological processes. The protein exists as a high-molecular weight complex in cell extracts and purified protein possesses a high tendency to aggregate, a major obstacle for crystallisation. Using a mutagenesis approach, we show that two structural features underlie the mechanism of oligomerisation in *Dictyostelium discoideum* CAP. Positively charged clusters on the surface of the N-terminal helix-barrel domain are involved in inter-molecular interactions with the N- or C-terminal domains. Abolishing these interactions mainly renders dimers due to a domain swap feature in the extreme C-terminal region of the protein that was previously described. Based on earlier studies with yeast CAP, we also generated constructs with mutations in the extreme N-terminal region of *Dictyostelium* CAP which did not show significantly altered oligomerisation behaviour. Constructs with mutations in the earlier identified protein-protein interaction interface on the N-terminal domain of CAP could not be expressed as soluble protein. Assessment of the soluble proteins indicates that the mutations did not affect their overall fold. Further studies point to the correlation between stability of full-length CAP with its multimerisation behaviour, where oligomer formation leads to a more stable protein.

Keywords: CAP, Srv2, oligomerisation, protein-protein interaction, structure

Introduction

Cyclase-associated protein (CAP), alias Srv2, is a highly conserved and widely distributed protein required for normal cell growth and development.¹ The protein was first identified in *Saccharomyces cerevisiae*.² Full-length CAP consists of a N-terminal domain (N-CAP), a proline-rich linker region and a C-terminal domain (C-CAP). In *S. cerevisiae*, the N-terminal domain is required for Ras response and it binds to the C-terminal region of adenylyl cyclase by coiled-coil interaction.³ The C-terminal domain of CAP binds monomeric actin with a 1:1 molar stoichiometry and inhibits actin polymerisation.^{4,5} Meanwhile, the proline-rich middle region of *S. cerevisiae* CAP is recognized by the Src homology 3 (SH3) domains of several proteins and is also involved in the correct localisation of CAP in the cell.⁶

Although distinct functions have been allocated to each CAP domain, recent studies revealed that they are not mutually exclusive. Yeast and human CAP have been demonstrated to be involved in recycling actin and cofilin for new rounds of actin depolymerisation and polymerisation.^{7,8} Whereas the C-terminal domain of human CAP1 sequesters monomeric actin as expected, the N-terminal domain is able to bind the actin-cofilin complex.⁷ Thus, it is evident that the functions of the individual CAP domains are intertwined.

Wild-type *Dictyostelium* CAP is a 50 kDa protein that has about 40% identity to its *S. cerevisiae* and human counterparts.⁵ The structure of N-CAP from *D. discoideum* has been determined by both NMR⁹ and X-ray crystallography.¹⁰ The domain consists of six anti-parallel helices arranged into a bundle. In contrast to the N-CAP structures, the C-CAP crystal structures of *S. cerevisiae* and human revealed the fold of a parallel right-handed β -helix where two molecules form a dimer through the domain-swapping of their extreme C-terminal β -hairpins.¹¹

In our attempts to determine the crystal structure of full-length CAP, we have recently solved the structure of the auto-proteolytic N-CAP fragment of the full-length *D. discoideum* protein.¹² To date, structural studies on full-length CAP have been difficult owing to its tendency to precipitate while being concentrated in the presence of membranes, apart from apparently possessing auto-proteolytic activity.¹³ Although crystallisation trials were set up with the full-length protein, only N-CAP crystals were obtained, suggesting the occurrence of auto-proteolytic activity in the crystal drop.

N-CAP has been crystallised in three different space groups (P2₁, P1 and C222₁), allowing structural insights into CAP protein-protein interactions from the crystal packing.^{10,12} While the monoclinic crystal form contained a N-CAP monomer, the two other forms appeared as dimers. The triclinic and orthogonal crystal forms contained similar, but not identical, side-to-side dimers with a common interface involving the same helices. The orthogonal form also allowed characterisation of a head-to-tail dimer. The presence of two different N-CAP oligomer conformations supports the idea that there are various inter-molecular interactions available to the N-terminal domain.

Information obtained from the N- and C-CAP structures support the various functional studies reporting that the full-length protein is able to interact with itself and other CAP molecules to form dimers

and multimeric complexes¹⁴ (Table 1). The size of yeast CAP oligomers was determined to be in the range of tetramers and dodecamers,¹⁷ while *Dictyostelium* CAP was reported to form hexamers in solution.¹⁰ Furthermore, the protein has not been shown to exist as monomers in the cell and its self-association seems to be an important property of CAP. Previous work on cell extracts reported that yeast and mammalian CAP form high molecular weight (HMW) complexes.^{17,18} The complexes are inferred to be the interaction between CAP and actin monomers, adenylyl cyclase, other CAP-binding proteins and other CAP molecules.

Intriguingly to date, there has not been any systematic structural characterisation of full-length CAP in solution since literature reports have mainly concentrated on either the amino or carboxyl domain. Our ongoing efforts on crystallising the full-length protein have been complicated by its multimerisation behaviour. In order to investigate this behaviour in more detail, we have designed four types of mutants of recombinant His-tagged *Dictyostelium* CAP, based on the available crystal structures and information from earlier work, to reduce its oligomerisation tendencies. The oligomerisation behaviour of the mutants able to be expressed in solution have been investigated and compared to the wild-type protein. The results indicate that two structural features of the protein are responsible for the mechanism of CAP oligomerisation .

Results

Protein Expression and Concentration

Out of all the *Dictyostelium* CAP mutants that were generated, apparently not all were expressed in the soluble fraction (Table 2). It is possible that mutants MUT4, 8, 9, 10 and 11 were expressed but are insoluble suggesting that the amino acid changes affected the folding process. Mutants MUT4 and 8 had residue changes on basic clusters on the surface of N-CAP (Figure 1) while MUT9, 10 and 11 were produced to disrupt the formation of N-CAP side-to-side dimers that were discussed earlier. Nevertheless, since our focus was on characterising soluble proteins, we did not further investigate the insoluble constructs.

The first indication of protein oligomerisation behaviour is obtained during the concentration step after purification. For all the proteins analysed in this study, a gel-like phase is rapidly formed in the concentrator device, albeit in varying amounts. Full-length wild-type CAP very easily precipitates and readily forms the gel-like phase as opposed to the C-terminally truncated MUT1-CDEL protein. The latter construct is a truncation of MUT1, where residues at the extreme C-terminal domain were deleted. These deleted residues form the β -hairpin topology, which is responsible for the formation of C-CAP strand-exchanged dimer.¹¹ Attempts to resuspend the gel-like phase were unsuccessful. Various other methods of concentration were tested, including water-absorbing reagents (Aquacide IV, dialysis against sucrose and HMW PEG) and concentration in the presence of detergents (β -octyl glucoside, Tween 20). However, the loss of protein was still very substantial and there was no apparent advantage in using these alternative methods.

Oligomerisation State of *Dictyostelium* CAP

Figure 2 and table 2 summarise the results from SEC-MALLS with wild-type CAP and five mutants. For the wild-type sample, only the supernatant of the gel-like phase after concentration was utilised. Unfortunately, the protein gave only a weak signal for MALLS thereby impairing the accuracy of the molecular weight determination. The supernatant of wild-type CAP after concentration consisted mainly of dimers and, to a lesser extent, monomers.

The MUT1 and MUT2 constructs were produced to emulate the reduced multimerisation behaviour of similar *S. cerevisiae* mutants reported earlier.¹⁶ The elution profile of mutant MUT1 exhibits an entire spectrum of multimers with no dominating species. With MUT2, a dimeric species is evident although the multimers are still present and are of higher order compared to that of MUT1. Comparing MUT1 and MUT2, the cumulative effects of the two additional mutations in the latter construct have evidently reduced the number of multimers that can be formed.

At the molecular level, CAP oligomerisation can occur by interactions between N-CAP/N-CAP, N-CAP/C-CAP or C-CAP/C-CAP molecules. Qualitatively, CAP oligomer formation seems to be based on ionic, as well as hydrophobic, interactions since neither the presence of high ionic strength nor

detergent is sufficient to dissolve the HMW aggregates (Yusof and Hofmann, unpublished data). Another mutational approach, therefore, focuses on general surface properties. Molecular surface potential maps show that C-CAP possesses a predominantly hydrophobic surface with some extended acidic areas. The surface of N-CAP, on the other hand, is a well-balanced mix of hydrophobic, acidic and basic areas (Figure 1). We therefore converted some of the clustered basic residues on N-CAP into acidic residues to interfere with potential ionic/polar interactions. In all these constructs, the mutated amino acids, except L20F, were surface residues in basic patches at the amino domain.

Mutants MUT5 and MUT7 have two and four basic residues respectively on the surface of the amino domain changed to acidic residues. Coincidentally, the elution profiles for both constructs are similar. The predominant species is dimeric although the existence of monomers and multimers could still be detected.

The elution profile of the MUT1-CDEL construct is distinctly different from the other proteins as there are no large multimers observed. However, it is not possible to estimate the fractions of the individual species since the peaks are not separated, which also interferes with an exact determination of molecular weight. It is obvious that the truncation of the β -hairpin at the extreme C-terminal domain has a severe impact on the oligomerisation behaviour of CAP.

Protein Size and Shape

In an attempt to obtain the shape and size information of CAP in solution, we subjected various protein samples to SAXS experiments (Figure 3A). The presence of a number of different oligomeric species makes the data difficult to interpret, as polydisperse systems are not suitable for this technique. In a polydisperse solution, the scattering intensity is the sum of the intensities of the individual scattering particles and the pair distance distribution function is the sum of all pair distance distribution functions. For polydisperse systems, information about the size and shape of the scatterers can only be obtained by comparing the scattering function of a model to the experimental data, a process that usually requires iterative refinement.¹⁹ Therefore, the SAXS results can only be interpreted in a qualitative manner.

Not surprisingly, the main obstacle was to prepare samples with sufficiently high protein concentrations. Since wild-type CAP formed a gel-like phase during concentration, the supernatant of this gel-like phase was used for the SAXS study. Experiments with the wild-type and MUT1 proteins were restrained by the low concentration of the samples. For MUT2, the “standard” SAXS experiments could be carried out as higher concentrations could be achieved. Table 3 shows the results for the three proteins investigated. The R_g values obtained for wild-type CAP are clearly concentration-dependent. Extrapolation yields a R_g of about 33 Å at zero protein concentration (Figure 3D). For MUT1 and MUT2, higher R_g values are observed. While there is only data for MUT1 at one low concentration, a concentration-dependence of R_g is observed for MUT2. The R_g at zero protein concentration is 91 Å for MUT2, which is very close to that of 89 Å for MUT1 (0.12 mg/ml).

In SAXS, the shape of the scattering particle can be inferred from the pattern of the distance distribution function. The three proteins tested display a bell-shaped $P(r)$ function (Figure 3C), which is typically observed with oblate, disc-like particles.²⁰ The estimated maximum diameter of these discs for the three proteins is 210 Å (wild-type), 250 Å (MUT1), and 310 Å (MUT2).

Protein Fold and Stability

Far UV CD spectra of wild-type, MUT1, MUT2, MUT7 and MUT1-CDEL proteins were recorded and yielded nearly identical spectra suggesting a similar fold for these molecules (Figure 4A). The spectra exhibit typical features of a $\alpha + \beta$ protein that has separate mainly α -helix- and mainly β -sheet-rich regions.²¹ The CD spectra of $\alpha + \beta$ proteins usually have a larger intensity in the band around 208 nm than in the band around 222 nm.

To compare the folding stability of the proteins, fluorescence-monitored urea-induced denaturation and thermal denaturation monitored by CD spectroscopy were performed on the wild-type and various mutant proteins (Table 4). Temperature- as well as urea-induced unfolding revealed a simple two-state unfolding process for the wild-type, MUT1, MUT2 and MUT1-CDEL samples (Figure 4B, C). There are no significant differences observed in the thermal folding stability, since the unfolding temperatures are in the narrow range from 43°C to 46°C. Urea-induced unfolding, however, reveals a

destabilisation of MUT1-CDEL and MUT2 by 3.5 kJ/mol and 6.5 kJ/mol, respectively, compared to that of the wild-type molecule. This effect is reflected by the less urea concentration needed for half-maximum unfolding. Notably, MUT2 exhibits a lower m value, which might indicate a higher accessibility of this protein to the denaturing agent. The urea concentrations required to unfold the full-length proteins tested are in the range previously reported by Mattila and coworkers,⁸ although they were only working with the C-terminal domain of yeast CAP.

Discussion

Two structural features underlie the mechanism of CAP oligomerisation

The main difficulty in working with full-length CAP is its tendency to form HMW aggregates that are clearly visible at the concentration stage of protein purification. CAP multimer formation might very well be a physiological property of the protein and a number of structural features are responsible for this tendency. Crystal structures have revealed the capability of CAP to form domain-swapped dimers with their C-terminal domains and to engage in at least two different types of protein-protein interactions with their N-terminal helix-barrel domains.

In the past, only a few N-terminal CAP mutants have been characterised with respect to their functional properties and their ability to (self-) associate. Based on the available structural information, we have decided to characterise the potential role of four types of residues in CAP oligomerisation: (a) residues in the extreme N-terminal region of CAP (MUT1, 2), (b) residues forming positively charged clusters on N-CAP (MUT3, 4, 5, 6, 7, 8), (c) residues forming the N-CAP side-to-side dimer interface (MUT9, 10, 11), and (d) residues at the β -hairpin responsible for the domain-swapping of C-CAP (MUT1-CDEL).

Unlike most of the mutated constructs, none of the cDNA with changes in amino acids identified to be involved in the N-CAP side-to-side dimer interface produced soluble proteins. This suggests that the four residues forming the common interface may be important not only for dimer formation but also in the folding process of full-length CAP. Furthermore, since the protein has always been shown to be part of a HMW complex in the cell, it is possible that the smallest unit of naturally occurring CAP is dimeric.

While all the solubly expressed mutants should be compared to the oligomerisation behaviour of wild-type CAP, this analysis is not straightforward as the wild-type sample very quickly separates into two phases during the concentration process, a gel-like state and a liquid supernatant. Therefore, only the supernatant of the wild-type protein could be utilised in the various methods used in this study. Elucidation of the aggregation state of the gel-like phase is extremely difficult, but it is reasonable to assume that this phase is due to the formation of extensive CAP multimers. In this context, it is not surprising that the supernatant of the wild-type protein sample consists of monomers and dimers, and exhibits a rather small radius of gyration.

Compared to wild-type CAP, mutant MUT1 displayed a less pronounced aggregation behaviour in the concentrator device. Nevertheless, SEC-MALLS revealed that large multimers form the main oligomerisation state of the sample. These findings agree with the large radius of gyration obtained from SAXS experiments. Although there is a distinct dimer peak in the elution profile of the MUT2 sample, the multimer content is still more than 50% indicating that the three mutations at the extreme N-terminus are not sufficient to prevent interactions leading to the formation of multimers. This observation lends further weight to the notion that the main interaction sites for oligomerisation reside at the N-terminal helix-barrel and the C-terminal domain.

Mutants MUT5 and MUT7 were constructed to disable possible ionic interactions between the N-CAP and C-CAP domains that may contribute to the development of multimers. The SEC-MALLS elution profiles of both constructs are very similar, with the major species being dimeric. It is apparent that these mutations have disrupted the multimeric interactions to a greater extent than the changes in MUT2, allowing the formation of mainly dimers.

While an earlier report concluded that the last 27 amino acids of CAP are not responsible for the dimerisation of CAP,¹⁵ we tend to disagree. The C-terminally truncated MUT1-CDEL protein has both monomer and dimer fractions, with the monomer fraction being larger. The smaller fraction of dimers can be explained by the inter-molecular interactions formed by the N-terminal helix-barrel domains.

Formation of CAP oligomers leads to a more stable protein

Similarity in the CD spectra of the proteins emphasises the fact that neither the mutations at the extreme N-terminal domain (MUT1, 2), mutations of four N-CAP surface residues (MUT7) nor the truncation of the extreme C-terminal β -hairpin (MUT1-CDEL) has significantly changed their fold compared to that of the wild-type molecule. However, the stability of mutants MUT2 and MUT1-CDEL were impaired.

The MUT2 construct was about 30% less stable than the wild-type protein and showed a reduced multimerisation behaviour, which concurs with previous results.¹⁶ The stability of mutant MUT1-CDEL was reduced by about 17% although the protein did not exhibit the formation of large multimers. These outcomes strongly suggest that the stability of full-length CAP can be associated with its multimerisation behaviour, where oligomer formation leads to a more stable protein.

CAP dimers are building blocks for further oligomerisation

Taken together, a model of CAP oligomerisation in solution can be envisioned where CAP dimers act as “building blocks” for higher order assemblies involving various N-CAP/N-CAP and N-CAP/C-CAP interactions (Figure 5). As observed in crystal structures, side-to-side and head-to-tail N-CAP interactions can be proposed. No structural details are available for N-CAP/C-CAP interactions, but based on our findings, an involvement of the basic clusters on the N-terminal domain is possible. While the C-CAP/C-CAP interactions are highly specific, the N-CAP/N-CAP and N-CAP/C-CAP interactions are unspecific, a feature which seems to be promoted by the barrel shape of the N-terminal domain. The phenomenon of unspecific interactions enhanced by the cylinder shape of a domain has been seen in green fluorescent protein where the dimer interfaces for different crystal structures are variable.^{22,23} This is further supported by the crystal packing in the monoclinic N-CAP structure,¹⁰ which consists of tightly packed N-CAP barrels arranged in a parallel fashion.

It remains to be clarified how the flexible middle domain contribute to CAP oligomerisation although it has been reported that removing the domain does not impair dimerisation.¹⁵ Nevertheless, the inherent flexibility of this domain will most likely affect the relative orientation of N-CAP and C-CAP moieties of one CAP molecule with other CAP domains. Intra-molecular N-CAP/C-CAP interactions

might also force the protein into a conformation that will allow binding of target proteins such as yeast actin-binding protein, Abp1, to the middle domain. Implications of this conformation for the oligomerisation of full-length CAP remain to be clarified. As discussed previously, since the full-length protein oligomers in solution possess varying stoichiometries and do not seem to carry any symmetric element, we anticipate that these oligomers might disassemble in the presence of target proteins thus allowing specific interactions to occur.

Materials and Methods

Identification of Basic Surface Residues

Surfaces of N-CAP (PDB entry 1TJF) (Figure 1) and C-CAP (PDB entry 1K4Z) crystal structures were inspected with the software GRASP.²⁴ Several basic residues on the surface of N-CAP are clustered and particular surface residues were identified for subsequent mutations inverting the surface charge. The residues chosen were Lys71, Lys72, Lys125, Lys127, Arg131, Lys178, Lys181, Lys203 and Lys206.

Plasmid Construction and Mutagenesis

Full-length wild-type CAP from *D. discoideum* was subcloned as described earlier.¹³ The resulting construct carries a hexa-His-tag at the C-terminal. Table 2 lists the CAP clones prepared in this study.

Mutagenesis for all the constructs except MUT1-CDEL was performed using a method similar to the Quik-Change Site-Directed Mutagenesis approach (Stratagene). A PCR was performed on the wild-type CAP in pRSET_6c vector²⁵ with the appropriate oligonucleotide primers comprising the desired mutation(s). The product was then treated with *DpnI* to digest the parental DNA template before being transformed into *Escherichia coli* XL1-Blue cells (Stratagene).

The template for the MUT2 to MUT11 constructs was the MUT1 DNA. The MUT7 clone was obtained by first performing the K71E and K72E mutagenesis on the MUT1 template, followed by the rest of the changes on the successful L20F, K71E, K72E template. The MUT7

plasmid was then utilised as the template for MUT8. For the MUT1-CDEL construct, the MUT1 DNA was amplified, truncating the sequence at Gln446, thereby losing the previously engineered hexa-His-tag. The amplified product was ligated into the pRSET_C vector using newly engineered *Nhe*I and *Hind*III sites. The resulting sequence has an N-terminally fused hexa-His-tag.

Protein Expression and Purification

Protein expression was carried out in *E. coli* BL21(DE3) cells. A culture of transformed cells was grown overnight at 37°C in LB medium containing 100 µg/ml ampicillin. A 1:20 dilution of the culture was used to inoculate 1 liter cultures in LB (100 mg/l ampicillin), which were grown at 37°C until the absorbance at 600 nm exceeded 1.0. The cells were induced with 0.5 mM isopropyl-1-thio-β-D-galactopyranoside for 16 h at either 30°C, 25°C or 18°C. Fresh ampicillin, 100 mg/l, was added upon induction.

For purification, the cell pellet was resuspended in 20 mM imidazole, 400 mM NaCl, 20 mM TRIS (pH 8.0), before subjected to sonication. The lysate was centrifuged (45 min, 64000 g, 4°C) and applied to a Ni²⁺-NTA column. The protein was eluted with increasing imidazole concentrations in a stepwise method [50 mM, 100 mM, 200 mM, 300 mM imidazole in 20 mM TRIS (pH 8.0), 400 mM NaCl]. Fractions containing the protein were pooled and dialysed against 50 mM NaCl, 20 mM TRIS (pH 9.0), for anion exchange chromatography. This was conducted to separate full-length CAP from the N- and C-terminal individual domains, which are persistent even in the presence of a protease inhibitor cocktail tablet (Roche) when the pellet was first resuspended. The dialysed sample was centrifuged (45 min, 64000 g, 4°C) and applied to a Q-Sepharose column. The protein was eluted with a linear NaCl gradient of 50 mM -1 M in 20 mM HEPES (pH 9.0). Fractions containing CAP were concentrated and buffer-exchanged into 20 mM HEPES (pH 8.0), 100 mM NaCl, using a Vivaspın ultrafiltration device (Vivascience).

Size-exclusion Chromatography Combined with Multiple-angle Laser Light Scattering (SEC-MALLS)

Experiments were performed using a system comprising an online degasser (ERC-3215, ERC Inc., Tokyo, Japan), a HPLC pump (1100 Series, Agilent Technologies, Böblingen, Germany) and a Superose 6 10/300 GL SEC-column (GE Healthcare, Piscataway, NJ, USA). The light scattered by proteins eluting from the SEC-column was detected by a MALLS detector (Dawn DSP, Wyatt, Santa Barbara, CA, USA) equipped with a K5 cell and a He-Ne laser. Protein concentration was measured in an UV/Vis detector (UV-1806, Bio-Rad, Hercules, CA, USA) at 280 nm and a refractive index (RI) detector (Optilab DSP, Wyatt, Santa Barbara, CA, USA). The molar absorption coefficients of the proteins were calculated using the empirical formula of Pace *et al.*²⁶ The refractive index increment (dn/dc) of the proteins was assumed to be the standard value for proteins (0.185 ml/mg). SEC-MALLS data was analysed using the software ASTRA (version 4.90, Wyatt, Santa Barbara, CA, USA).

The column was calibrated with the following proteins of known molecular mass: Rnase A, bovine serum albumin, alcohol dehydrogenase and thyroglobulin. To cover the HMW calibration range, hemocyanins from *Panulirus interruptus*, *Astacus leptodactylus* and *Eurypelma californicum* were used. In all cases, 100 µl of sample with concentrations between 1 mg/ml and 11 mg/ml was injected onto the column and eluted with 50 mM sodium phosphate (pH 7.0), 150 mM NaCl, at a flow rate of 0.5 ml/min.

Small Angle X-ray Scattering (SAXS)

Measurements were performed on the instrument at the University of Aarhus.²⁰ The instrument is a modified version of commercially available small-angle X-ray equipment (NanoSTAR), which is produced by Anton Paar, Graz, and distributed by Bruker AXS. The camera consists of a rotating anode (Cu, 0.3 x 0.3 mm² source point, 6 kW power) and a three-pinhole collimation optimised with respect to flux and background, so that it is ideally suited for solution scattering. The Cu-K_α radiation is monochromatised and made parallel by two Göbel mirrors.

Data collection was performed at 20°C in a reusable thermostated quartz capillary, which is placed in the integrated vacuum chamber of the camera. A home-built capillary holder with good thermal contact to the thermostated surrounding block was used.

The sample-to-detector distance was 66.15 cm, which covered a momentum transfer range of $0.08 < q < 0.35 \text{ \AA}^{-1}$, where $q = (4\pi\sin\theta)/\lambda$ and 2θ is the scattering angle. The two-dimensional data sets were recorded using a two-dimensional position-sensitive gas detector (HiSTAR). The measured data were corrected for variations in detector efficiency and subsequently corrected for spatial distortions. The two-dimensional data were azimuthally averaged (Bruker SAXS software for WindowsTM NT). Background scattering from 20 mM HEPES (pH 8.0), 100 mM NaCl, was subtracted and the scattering intensities were transformed to absolute units using the scattering of water as a standard (software developed in-house). Data were recorded at concentrations ρ^* , $\rho^*/2$ and $\rho^*/4$, where possible. ρ^* was between 0.1 mg/ml and 10 mg/ml.

Measurement times varied from 2 to 12 h. Values for the radii of gyration, R_g , were determined by Guinier analysis on the low s data. The Guinier fit was carried out using the program PRIMUS²⁷ and $q_{\max}$ was chosen so that $R_g \times q_{\max} < 1.3$.²⁸ In cases where no Guinier fit was possible ($R_g \times q_{\max} > 1.3$), the R_g values from the distribution function, $P(r)$, analysis were used. The $P(r)$ was calculated using the program GNOM.²⁹

CD Spectroscopy

Measurements were performed with a JASCO J-810 spectropolarimeter equipped with a JASCO PTC-423S thermostat. Results were analysed using the spectrum analysis program ACDP.³⁰ The concentration of each sample was approximately 0.1 mg/ml in 10 mM HEPES (pH 8.0), 50 mM NaCl (cuvette path length 1 mm). Each spectrum was the average of at least three scans recorded at a speed of 20 nm/min. Far UV spectra were collected at 20°C. For thermal denaturation studies, measurements were done from 20°C to 80°C with a 10 min equilibration before each new temperature scan. All spectra were corrected against the buffer baseline.

Urea-induced Unfolding

Folding stability of various proteins was investigated by urea-induced denaturation. The unfolding process was monitored by intrinsic protein fluorescence. Samples consisted of 2 μ M protein in 20 mM HEPES (pH 8.0), 100 mM NaCl, and a total volume of 300 μ l. Individual samples with urea concentrations ranging from 0 to 8 M were prepared 30 min prior to the measurements. Fluorescence emission spectra were recorded on a Jobin-Yvon FluorMax-3 using two excitation wavelengths, λ_{exc} 280 nm and λ_{exc} 295 nm. Three independent denaturing series were carried out for each protein. All fluorescence spectra were corrected against the buffer baseline and analysed offline with the program AFDP.³⁰ Each excitation set was analysed by an i-c (urea) relation, where $i = I(\lambda_{\text{unfolded}})/I(\lambda_{\text{folded}})$ (emission intensity analysis) and a λ -c(urea) relation (wavelength analysis). Two-state unfolding behaviour allows for a stability analysis according to Pace,³¹ yielding the free stability energy for each protein, $\Delta G(\text{H}_2\text{O})$.

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The abbreviations used are: CAP, cyclase-associated protein; N-CAP, N-terminal domain of CAP; C-CAP, C-terminal domain of CAP; HMW, high molecular weight.

Figure Legends

Figure 1. GRASP surface representation of N-CAP. Red indicates negative surface charge, blue indicates positive surface charge. Surface residues involved in basic clusters are labelled.

Figure 2. SEC-MALLS elution profiles of wild-type CAP and various mutants. For all graphs, the elution volume is shown on the x -axis and the normalised absorption on the y -axis. The labelled peaks correspond to the oligomerisation state of the proteins (see also table 2).

Figure 3. SAXS results for wild-type CAP (closed circles), MUT1 (open triangles) and MUT2 (open boxes). *A* Scattering curves from wild-type CAP (1 mg/ml), MUT1 (0.12 mg/ml) and MUT2 (10 mg/ml). *B* Guinier fit to determine the radius of gyration of the wild-type data set (1 mg/ml). The dotted line represents a linear fit. *C* Normalised pair distance distribution functions using the same data sets as in *A*. Normalisation was carried out using the $I(0)$ forward scattering. *D* Correlation between the radii of gyration and the protein concentrations for the wild-type and MUT2 proteins. The dotted lines represent linear fits to determine the radii of gyration at zero protein concentration.

Figure 4. Protein fold and stability. *A* CD spectra of wild-type CAP (solid line), MUT1 (dashed line), MUT2 (dash-dotted line) and MUT1-CDEL (dotted line). *B* Results from thermal denaturation of wild-type CAP (closed circles), MUT1 (open triangles), MUT2 (open boxes) and MUT1-CDEL (open diamonds) monitored by CD at λ 222 nm. *C* Results from urea-induced unfolding of the same proteins as in *B*. Shown is the bathochromic shift of the intrinsic protein emission wavelength (λ_{exc} 280 nm).

Figure 5. Model of all CAP dimers in solution. N-CAP is depicted as cylinders, C-CAP as trapezoids. *A* Dimer formed by the strand-exchanged β -hairpins of C-CAP. *B*, *C* Dimers formed by the side-to-side and head-to-tail N-CAP interactions. *D*, *E* Dimers from N-CAP/C-CAP interactions.

Table 1. Previously reported CAP mutations and their effects on oligomerisation

Mutation (<i>S. cerevisiae</i>)	Amino acid in <i>D. discoideum</i>	Effects					Reference
$\Delta(499-526)$	438-464	Affects	actin	binding	but	not	15
		oligomerisation					
L16P	L9	Decreased cAMP levels, adenylyl cyclase binding and multimerisation.					16
R19T	R12	Normal cAMP levels and adenylyl cyclase binding. Decreased multimerisation					16
L27F	L20	Normal cAMP levels and adenylyl cyclase binding. Decreased multimerisation					16
D461A/K462A	D399/K400	Affects	actin	binding	but	not	8
		oligomerisation					
K472A/E473A	K410/D411	Affects	actin	binding	but	not	8
		oligomerisation					

Table 2. Mutations of full-length *Dictyostelium* CAP generated in this study

Protein	Mutations	Soluble Expression	SEC-MALLS Results		
			Monomer	Dimer	Multimer
Wild-type CAP	Wild-type	Yes	+ ^a (peak I)	++ ^a (peak II)	- ^a
MUT1	L20F	Yes	+ (peak I)	+ (peak II)	++ (peak III)
MUT2	L9S/R12S/L20F	Yes	-	+ (peak II)	++ (peak III)
MUT3	L20F/K71E/K72E	Yes			
MUT4	L20F/K125E/R127E/R131E	No			
MUT5	L20F/K178E/K181E	Yes	+ (peak I)	++ (peak II)	+ (peak III)
MUT6	L20F/K203E/K206E	Yes			
MUT7	L20F/K71E/K72E/ K203E/K206E	Yes	+ (peak I)	++ (peak II)	+ (peak III)
MUT8	L20F/K71E/K72E/ K125E/R127E/K203E/K206E	No			
MUT9	L20F/H161G/M165G	No			
MUT10	L20F/M165G/S168G/F171G	No			
MUT11	L20F/H161G/M165G/S168G/F171G	No			
MUT1-CDEL	L20F/Δ(446-464)	Yes	++ (peak I)	+ (peak II)	-

^aSupernatant of gel-like phase. +/- indicate the presence/absence of a peak, the frequency of symbols indicates the major species.

Table 3. SAXS results for the CAP proteins studied

Protein	ρ^* (mg/ml)	R_g (Å)	Method	R_g at zero conc. (Å)
Wild-type ^a	0.2	39.3 (1.0)	$P(r)$	
	1.0	71.4 (1.3)	Guinier	
	1.0	65.2 (0.59)	$P(r)$	
	0			33
MUT1	0.12	88.6 (1.0)	$P(r)$	
MUT2	10	99.2 (0.46)	$P(r)$	
	5.0	94.7 (0.45)	Guinier	
	2.5	93.2 (0.68)	Guinier	
	0			91

^aSupernatant of gel-like phase.

The apparent radius of gyration (R_g) was determined either from the Guinier fit or the distribution function. For wild-type CAP and mutant MUT2, the radius of gyration at zero protein concentration was determined by extrapolation.

Table 4. Protein stability parameters

Protein	Thermal		Urea denaturation:		
	denaturation		Pace analysis ^b		
	$T_{1/2}$ (°C)	$c_{1/2}$ (urea) ^a (M)	$\Delta G(H_2O)$ (kJ/mol)	m (kJ* l/mol^2)	$c_{1/2}$ (urea) (M)
Wild-type	43	2.8	20.4	6.0	3.4
MUT1	44	3.1	20.0	5.5	3.6
MUT2	44	2.8	13.9	4.5	3.1
MUT1-CDEL	46	2.8	16.9	5.7	3.0

Fluorescence monitoring of urea-induced protein denaturation was done at an excitation wavelength of 280 nm. ^a $c_{1/2}$ was determined from the observed wavelength shift. ^bFor Pace analysis, fluorescence emission intensity data were used.

Fig. 1

Fig. 2

Fig. 3

Fig. 4

Fig. 5