Diffusion of Intrinsically Disordered Coral Acid-Rich Proteins

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Introduction

Coral acid-rich proteins (CARP) of skeletal organic matrix (SOM) are responsible for biomineralization. It is anticipated that their interactions with calcium cations could determine the crystal form of the skeleton, *i.e.* that these proteins can control early stages of crystallisation by driving the amorphous calcium carbonite to calcite or aragonite crystal nuclei. Little is known about such proteins at the molecular level. Only four CARP SOM have been cloned and partially characterised. Here we show expression, purification and hydrodynamic characterisation of two new CARPs SOM, an N-terminal part of secreted acidic protein 1A and aspartic and glutamic acid rich protein, discovered recently in *Acropora millepora* [1], a model organism to study environmental changes in the oceans due to global climate-warming. At issue is how to analyse diffusion of such biopolymers to observe changes in the Stokes radius upon interactions with calcium ions. The classical polymer formalism is based on the number of units in the polymer [2], which is applicable for denatured proteins in the random coil form [3] and proteins with well-defined 3D structures [4]. However, intrinsically disordered proteins (IDPs) have more intricate characteristics, hence a sequence–dependent model for IDPs was also proposed [4]. The goal of our study is to analyze hydrodynamic properties of the CARPs in the absence and presence of Ca^{2+} by means of fluorescence correlation spectroscopy.

Coral Acid-Rich Proteins (CARPs)

N-terminal secreted acidic protein 1A (SAP 1A) | **Aspartic and glutamic acid-rich protein (AGARP)**

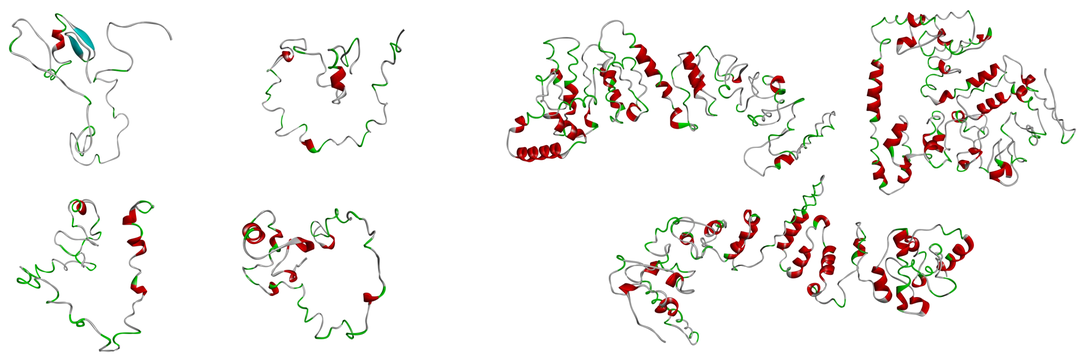

Figure 1. Best example models of possible 3D structures of the *A. millepora* N-terminal secreted acidic protein 1A (SAP 1A) and Aspartic and glutamic acid-rich protein (AGARP) predicted *ab initio* with QUARK [5] and LOMETS3 [6], respectively. Both of these proteins are intrinsically disordered.

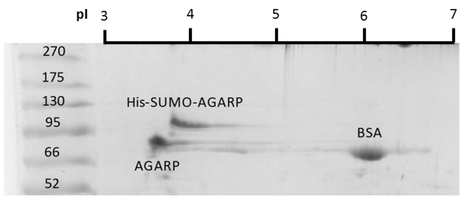

Figure 2. 2D IEF SDS PAGE of CARPs showing their low isoelectric point in the range of 3 to 4.

Table 1. Parameters of CARPs used in this study.

Protein	Molar mass (g/mol)	Molar extinction coefficient (cm^{-1} M^{-1})	Isoelectric point	Net charge at pH 8
N-terminal SAP 1A	15 856	-*	3.4	-63
His-SUMO-N-terminal SAP 1A	29 263	1 490	4.0	-58
AGARP	58 339	20 400	3.9	-152
His-SUMO-AGARP	71 746	21 890	4.1	-147

*N-terminal SAP 1A protein sequence does not contain any aromatic residue

Secreted acidic protein 1A

N-terminal SAP 1A

GLPLPLKNENAIVDGDGTSVVTTKEDASTIFERDPNPANQVSAMVTGVILDENGDPGESDESVENVDNDGEGGDKDDDKNGEDNDLDNKEHEEEKGD
DDRGDDEEEDDAEGDNDSNDNEGDDDDDDDSGDDDDVDESGADEDDDDDSGD

His-SUMO-N-terminal SAP 1A

MGSSHHHHHHSSGLVPRGSHMSDSEVNQEAKPEVKPEVKPETHINLKVSDGSSEIFFKIKKTTPLRRLMEAFAKRQGKEMDSLRFLYDGIRIQADQT
PEDLDMEDNDIIEAHREQIGGGLPLPLKNENAIVDGDGTSVVTTKEDASTIFERDPNPANQVSAMVTGVILDENGDPGESDESVENVDNDGEGGDKD
DDKNGEDNDLDNKEHEEEKGDDDRGDDEEEDDAEGDNDSNDNEGDDDDDDDSGDDDDVDESGADEDDDDDSGD

Aspartic and glutamic acid-rich protein

AGARP

SPLRNRFNEDHDEFSKDDMARESFDTEEMYNAFLNRRDSSESQLEDHLLSHAKPLYDDFFPKDTSPDDDEDSYWLESRNDDGYDLAKRKRGYDDEEAYDDFDEVDDRADDEGARDVDESDFEEDDKLPAEEESKNDMDEETFEDE
PEEDKEEAREEFAEDERADERDEDDDADFDFNDEEDEDEVDNKAESDIFTPEDFAGVSDEAMDNFRDDNEEEYADESDDEAEEDSEETADDFEDDPEDESDETFRDEVEDESEENYQDDTEEGSEIKQNDETEEQPEKKFDADKEH
EDAPEPLKELSDESKARAEDESDKSEDAAKEIKEPEDAVEDFEDGAKVSEDEAELLDDEAELSDDEAELSKDEAESQSDEAEKSEDKAEKSEDEAELSEDEAKQSEDEAEKAEDAAGKESNDEGKKREDEAVKSKGIARDESEF
AKAKKSNLALKRDENRPLAKGLRESAAHLRDFPSEKKSKDAAQGNIENELDYFKRNAFADSKDAEPYEFDK

His-SUMO-AGARP

MGSSHHHHHHSSGLVPRGSHMSDSEVNQEAKPEVKPEVKPETHINLKVSDGSSEIFFKIKKTTPLRRLMEAFAKRQGKEMDSLRFLYDGIRIQADQTPEDLDMEDNDIIEAHREQIGGSPLRNRFNEDHDEFSKDDMARESFDTE
EMYNAFLNRRDSSESQLEDHLLSHAKPLYDDFFPKDTSPDDDEDSYWLESRNDDGYDLAKRKRGYDDEEAYDDFDEVDDRADDEGARDVDESDFEEDDKLPAEEESKNDMDEETFEDEPEEDKEEAREEFAEDERADERDEDDDAD
FDFNDEEDEDEVDNKAESDIFTPEDFAGVSDEAMDNFRDDNEEEYADESDDEAEEDSEETADDFEDDPEDESDETFRDEVEDESEENYQDDTEEGSEIKQNDETEEQPEKKFDADKEHEDAPEPLKELSDESKARAEDESDKSE
DAAKEIKEPEDAVEDFEDGAKVSEDEAELLDDEAELSDDEAELSKDEAESQSDEAEKAEDAAGKESNDEGKKREDEAVKSKGIARDESEFAKAKKSNLALKRDENRPLAKGLRESAAHLRDFPSEKKSKDAAQGNIENELDYFKRNAFADSKDAEPYEFDK

Figure 3. Sequences of proteins used in this study [1].

ED – acidic amino acid residues
H – His-tag
XXX – SUMO-tag
XXX – folded part of the SUMO-tag

Cloning and Purification of N-terminal SAP 1A and AGARP

N-terminal SAP 1A and AGARP were cloned for the first time. The proteins in fusion with the SUMO-tag providing higher expression were purified by histidine – nickel affinity chromatography. Process of removing His-SUMO fragment is described in **Figure 4** caption.

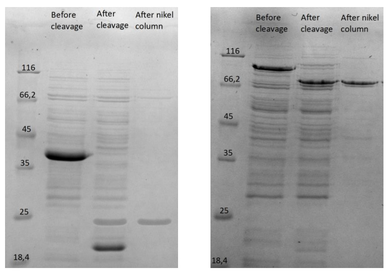

Figure 4. Enzymatic digestion of His-SUMO-N-terminal SAP 1A (left panel) and His-SUMO-AGARP (right panel). SUMO-fused proteins (before cleavage) were enzymatically digested by specific SUMO-protease (after cleavage) and purified by Ni-chromatography to remove His-SUMO (after nickel column).

Fluorescence Correlation Spectroscopy

Hydrodynamic properties of CARPs and reference proteins (lysozyme, BSA and apoferritin) labelled with Alexa Fluor 488 NHS ester (**Figure 5**) has been studied with Fluorescence Correlation Spectroscopy. Measurements were performed on Axio Observer LSM 780 Zeiss inverted confocal microscope (40x/1.2 water immersion lens) with a ConfoCor3 kit for FCS measurements with MBS 488 beam splitter and a BP 495-555 filter. Each drop had the volume of 25 μL and the temperature of $T \sim 23$ °C. The diffusion times, τ_D, and then the diffusion coefficients D for each protein were determined based on 150 measurements. The hydrodynamic radii R_H for the CARPs were calculated from the Stokes-Einstein relation, assuming the T/η value was constant and equal for all sample drops, and can be estimated from the measurements done for standard proteins of known R_H values [7]:

$$D = \frac{k_B T}{6\pi\eta R_H}$$

η - viscosity

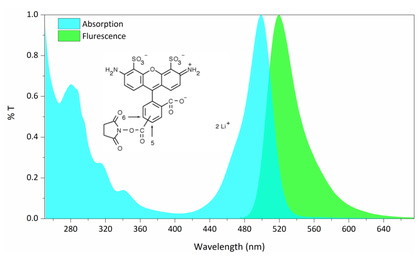

Figure 5. The NHS ester (succinimidyl ester) of Alexa Fluor 488 is a tool for conjugating the dye to the primary amines (R-NH_2) of proteins, *i.e.,* the side chains of lysine and arginine residues and the N-terminus. The absorption and emission spectra are shown.

Diffusion Coefficients and Hydrodynamic Radii of CARPs

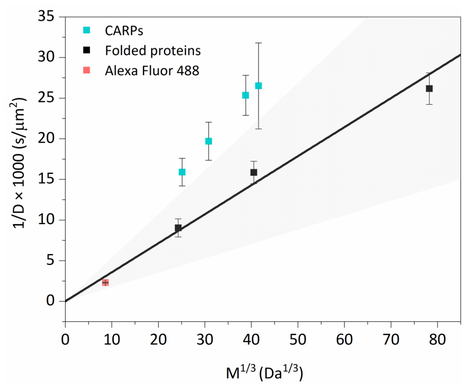

Figure 6. The FCS results. Linear dependence of $1/D$ vs. $M^{1/3}$. CARPs outlie the 99% CI (grey area) of the standard line. CARPs are characterized by smaller diffusion coeffcients as compared to folded proteins of the same molar mass.

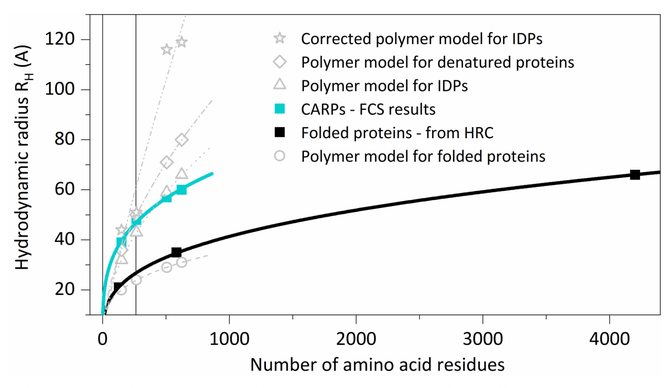

Figure 7. Estimated experimental hydrodynamic radii of CARPs (cyan squares) and standard proteins (black squares) represented as a function of the biopolymer length. Both classes follow the power function [2] $R_H = R_0 N^{\upsilon}$ but with different coefficients R_0 and υ. Previously described models [4] (grey hollow figures) could not be applied to describe CARPs due to limited range of these models (indicated by two vertical lines).

Effect of Ca^{2+} on CARP Diffusion

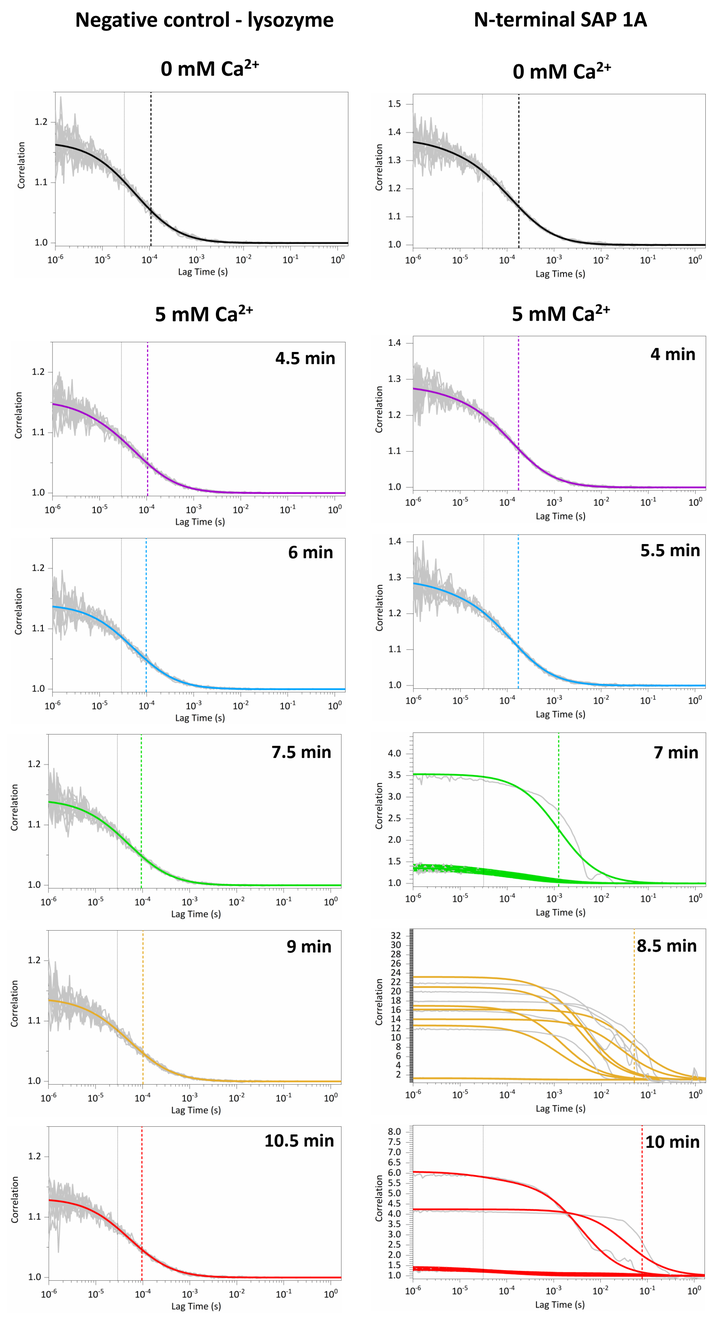

Figure 8. Autocorrelation curves (ACs) of lysozyme (left panel) and N-terminal SAP 1A (right panel) obtained in the FCS experiment before (0 mM Ca^{2+}) and after (5 mM Ca^{2+}) calcium addition. ACs were measured after a given time of incubation (shown in the upper left corner of each graph). Thick vertical lines indicate diffusion times of proteins in case of lysozyme and N-terminal SAP 1A till 5.5 min of incubation and apparent diffusion times for N-terminal SAP 1A after 7 and more minutes of incubation. Thin vertical line denotes the diffusion time of Alexa Fluor 488.

We investigated how the diffusion times of N-terminal SAP 1A change in time upon addition of calcium ions. Our negative control in this case was lysozyme which diffusion time did not change under the influence of calcium ions (**Figure 8**, left panel). Whereas, starting from 7 minutes of incubation of N-terminal SAP 1A with Ca^{2+}, the diffusion process slows down by three orders of magnitude on the lag time scale after 10 minutes of incubation (**Figure 8**, right panel).

FCS measurements revealed that N-terminal SAP 1A at a nanomolar concentration in the presence of calcium ions at 5 mM appears in the form of different individuals with apparent diffusion times – 10^3 times greater than that for the monomeric protein. Since this interaction occur at nanomolar concentrations they must be specific.

This indicates that N-terminal SAP 1A aggregation is mediated by the calcium ions and happens during the diffusion of particles through the confocal volume, which we observe in the form of distortions of the autocorrelation curve in millisecond time scale (**Figure 8**, right panel, 8.5 min).

We were able to observe individual processes of CARP aggregation upon interaction with calcium ions shown in **Figure 9** as changing apparent diffusion times for every single measurement in the confocal volume. This CARP-Ca^{2+} interactions may be involved in the early stages of biomineralization.

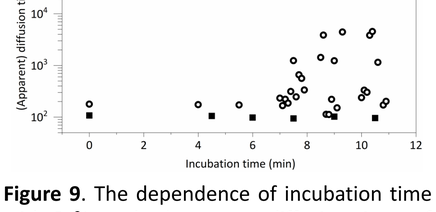

Figure 9. The dependence of incubation time with Ca^{2+} on the (apparent) diffusion times of lysozyme (black squares) and N-terminal SAP 1A (hollow circles).

Conclusions

1. We **cloned and characterized for the first time** two new coral acid-rich proteins (CARPs) from skeletal organic matrix of *Acropora millepora.*
2. These proteins have **smaller diffusion coefficients** and **greater hydrodynamic radii** as compared to folded proteins of the same molar mass. Our FCS results discern unambiguously the intrinsically disordered from folded proteins in a broad range of molecular masses.
3. By means of FCS, we can directly observe **the specific CARP interactions with calcium ions** resulting in formation of aggregates that can lead to early stages of protein-driven biomineralization.

Literature

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Acknowledgements

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