Structure–Function Studies of the Lustrin A Polyelectrolyte Domains, RKSY and D4

Brandon A. Wustman, 1 James C. Weaver, 2 Daniel E. Morse, 2 and John Spencer Evans 1

1 Laboratory for Chemical Physics, New York University, New York, USA
2 Department of Molecular, Cellular, and Developmental Biology and Materials Research Laboratory, University of California, Santa Barbara, California, USA

The lustrin superfamily represents a unique group of biomineralization proteins localized between layered aragonite mineral plates (i.e., nacre layers) in mollusk shell. These proteins not only exhibit elastomeric behavior within the mineralized matrix, but also adhesion to the aragonite-containing composite layer. One member of the lustrin superfamily, Lustrin A, has been sequenced; the protein is organized into defined, modular sequence domains that are hypothesized to perform separate functions (i.e., force unfolding, mineral adhesion, intermolecular binding) within the Lustrin A protein. Using nuclear magnetic resonance (NMR) and in vitro mineralization assays, we investigated structure-function relationships for two Lustrin A putative mineral binding domains, the 30 AA Arg, Lys, Tyr, Ser-rich (RKSY) and the 24 AA Asp-rich (D4) sequence regions domain of the Lustrin A protein. The results indicate that both sequences adopt open, unfolded structures that represent either extended or random coil states. Using geologic calcite overgrowth assays and scanning electron microscopic analyses, we observe that the RKSY polypeptide does not significantly perturb calcium carbonate growth. However, the D4 domain does influence crystal growth in a concentration-dependent manner. Collectively, our data indicate that D4, and not the RKSY domain, exhibits structure-function activity consistent with a mineral binding region.

Keywords Biomineralization, Lustrin A, Polyelectrolyte, Secondary Structure.

INTRODUCTION

Molecular elasticity is associated with a select number of polypeptides and proteins [1–10]. As an example, the lustrins, a protein superfamily localized within layered aragonite mineral plates (i.e., nacre layers) in mollusk shell, have been postulated to play a role in the enhancement of nacre layer fracture toughness [1, 2]. Recent atomic force microscopic (AFM) pulling studies have demonstrated that the organic layer of the nacre of the red abalone, Haliotis rufescens, exhibits a typical sawtooth force-extension curve with hysteretic recovery [2]. The AFM pulling experiments also demonstrated that this lustrin-containing organic matrix layer possessed adhesive interactions, either with the underlying aragonite mineral phase or with other organic matrix components [2]. One particular lustrin protein, Lustrin A (Pacific red abalone H. rufescens, 116 kDa) is a component of the “insoluble” intercrystalline organic matrix lying between the layers of aragonite tablets [1]. This intercrystalline organic layer also contains β-chitin and β-silk fibroin protein [1, 3]. Using scanning electron microscopic (SEM) and immunolocalization techniques, the Lustrin A protein has been localized to the region between parallel aragonite mineral layers within the nacre; effectively, the protein resides in an organic “strand” that resembles a supramolecular “bungee cord” stretching between adjacent aragonite crystal tablet layers (D. Morse, UCSB, unpublished observations).

Consequently, there is considerable interest in defining the structure-function relationships within the lustrin superfamily and establishing their participation in the shell fracture resistance. We recently investigated the solution-state structure of a 12-residue consensus sequence found near the N-terminal end of the C1-C8 domains within the Lustrin A protein [4]. This consensus sequence, believed to be one of several putative elastic motifs within Lustrin A, was found to adopt a loop structure at pH 7.4 [4]. Not surprisingly, loop regions are believed to behave as entropic springs and are capable of reversible motion or flexibility within proteins [5, 6]. In this report, we continue the use of the model peptide strategy to probe the sequence-structure relationships of modular motifs within Lustrin A. Our present focus is on two domains: the first, a 24- AA Asp-containing domain (–GKGASYDTDADSG-SCNRSPGYLPG- sequence positions...
1251–1274), termed “D4”; and the second, an Arg, Lys, Ser, Tyr-rich 30- AA domain (-YRGPIAPRSSRYLAKYLQGRSGKRLKQPK-, sequence positions 1354–1383), termed “RSKY” [1]. Our rationale for studying these two domains is that each possesses either putative Ca (II) (D4: Asp) or CO3
\[^{-2}\] (RKSY: Arg, Lys, Tyr, Ser; D4: Arg) binding residues that could permit Lustrin A to “adhere” to mineral surfaces. Using “capped” versions of both peptides, we determined secondary structure preferences for both peptides and explored putative interactions of each peptide with nucleating calcium carbonates in vitro. We find that both peptides adopt an extended and/or “random coil” conformation and that the D4 domain, and not the RSKY domain, exhibits positive interactions with calcium carbonates in vitro.

**EXPERIMENTAL PROCEDURES**

**Peptide Synthesis and Sample Preparation**

Purified, N-acetyl, C-amide capped RSKY, and D4 polypeptides were synthesized by Dr. Janet Crawford, Yale University HHMI Biopolymer/Keck Biotechnology Resource Laboratory, using an Applied Biosystems 431A Peptide Synthesizer and N\[^{\beta}\]-L-FMOC-amino acids as described elsewhere [4]. The crude peptides were separately dissolved in deionized distilled water, extracted three times with diethyl ether, and then concentrated and lyophilized. Peptide purification involved C18 reverse phase high performance liquid chromatography (HPLC) column, using 0.1% TFA/water mobile phase and eluting with an 80% acetonitrile/0.1% TFA/water linear gradient. Peptide elution was monitored at 230 nm. Individual HPLC fractions were analyzed using a custom made matrix-assisted laser desorption/ionization-time of-flight (MALDI/TOF) delayed extraction mass spectrometer. The experimental molecular weights for the capped RSKY and D4 polypeptides were determined to be 3575.6 Da and 2499.96 Da, in agreement with the theoretical molecular weights of 3572.1 Da and 2499.55 Da, respectively. For NMR studies each peptide was dissolved in 1 mM Na\[^{2+}\]HPO\[^4\] in deionized distilled water, pH 7.4. NMR samples contained 10% v/v deuterium oxide (99.9% atom D, Cambridge Isotope Labs) and 10 \(\mu\)M d4 TSP. Final peptide concentrations were 2 mM for NMR. For in vitro calcium carbonate assays, the peptides were dissolved in deionized distilled water and the pH was adjusted to 7.0 using NaOH.

**NMR Experiments**

NMR experiments were performed on a Varian UNITY 500 spectrometer equipped with variable temperature controller and a three-channel (\(^{13}\)C/\(^{15}\)N/\(^2\)H) z-axis PFG 5-mm solution probehead. With the exception of the proton amide temperature shift experiments, all reported NMR experiments were conducted at 278 K to slow down conformational exchange within the peptide. Proton scalar coupling assignments, \(^3\)J\(_{\text{NH-CH}}\) coupling constants, and short, medium, and long-range nOes were obtained using “excitation sculpting” 2-D PFG homonuclear experiments [7–12]. Using PFG TOCSY or NOE experiments at 278, 283, 288, 293, and 298 K, amide proton temperature coefficients were determined from the slope of the temperature versus amide proton chemical shift curves for each residue [4, 13–15].

**In Vitro Calcium Carbonate Overgrowth Assays**

We employed the geologic calcite fragment overgrowth assay [16, 17] with the following modifications. Briefly, Iceland spar calcite (Ward Scientific) was cleaved into 9–16-mm\(^2\) fragments that were used immediately. Assays were conducted in borosilicate scintillation vials and were initiated by mixing equal volumes of 12.5 mM CaCl\(_2\) and 12.5 NH\(_4\)HCO\(_3\) solutions (3 mL total volume). Microliter volumes of RSKY, D4, and bovine serum albumin (Pierce Scientific) were added and mixed with reaction media in individual vials; typically a concentration series (6.7 \times 10^{-9}, 1.3 \times 10^{-8}, 3.3 \times 10^{-8}, 1.7 \times 10^{-7}, and 3.3 \times 10^{-7} \text{ M polypeptide}) was run in triplicate. Negative controls consisted of reaction media alone. The calcite fragment was introduced into each vial, the vial was sealed and incubated at 15\(^{\circ}\)C for 3 hr. Upon conclusion of the incubation period, calcite fragments were washed 3 time with 3 mL calcium carbonate-saturated methanol, then dried overnight at 37\(^{\circ}\)C to evaporate remaining solvent. Calcite fragments were then mounted on carbon conductive tabs and sputter coated with gold under vacuum/Ar gas to generate a 3–5 nm layer of gold on the mineral surfaces. Imaging of overgrowth films was performed using a JEOL JSM 6300F cold cathode field emission scanning electron microscope, operating at 5 kV.

**RESULTS AND DISCUSSION**

**NMR Secondary Structure Analyses of D4 and RSKY**

Figures 1 and 2 list the sequential intra- and interresidue nOes, nOe ratios, \(^3\)J\(_{\text{NH-CH}}\) coupling constants, and amide temperature shift coefficients (ATC) for RSKY and D4, respectively. Using PFG-NOESY and ROESY experiments (50 ms to 200 ms mixing times) we were unable to detect sequential distance between residues (nOe) (i, i+1), medium or long-range backbone nOes, and for some residues for either peptide; in addition, some intraresidue distance (nOe) (i, i) nOes were not observed. The absence of intra- and interresidue nOes also have been noted in conformationally labile random coil states and in extended peptide structures [19–21]. The presence of extended or random coil structure is also confirmed by calculation of nOe intensity ratios (\(\alpha\text{N}(i,i+1)/\alpha\text{N}(i,i))\) (Figures 1 and 2). A value of 2.3 is predicted for the population-weighted random coil model, whereas values >4 are predicted for \(\beta\)-strand [19, 22]. For RSKY, with the exception of the terminal V1-R2 region, the obtained nearest-neighbor residue pairs possess nOe ratios >2.3, consistent with the presence of extended structure in these regions [22]. By comparison, for D4, the obtained nOe ratios are smaller, indicating a stronger preference for random coil structure in this sequence.

It has been shown that random coil or extended peptides exhibit amide temperature shift coefficients in the range of \(-6.6\) to \(-9.0 \text{ ppb/K in aqueous solution}\) [13–15]. For both D4 and RSKY, we found that the temperature coefficients varied from \(-6.0\) to \(-9.4 \text{ ppb/K (Figures 1 and 2)}, indicating both peptides exist...
Figure 1. Summary of NMR parameters for Lustrin A RKSY peptide in 1 mM Na₂HPO₄, 90% water/10% D₂O, 278 K pH 7.4. The summary includes interresidue sequential CH/residue “i” to NH/residue “i+1” nOes [denoted as αN(i, i+1), presented as contiguous bars for corresponding interresidue proton pairs] and intraresidue CH/residue “i” to NH/residue “i” nOes [denoted as αN(i, i)]. The histogram bar heights reflect the cross-peak intensities of corresponding nOes for each residue or residue pair, determined via integration of the NOESY or ROESY spectra. The αN ratio denotes the ratio of interresidue αN(i, i+1) nOe cross-peak intensities to intraresidue αN(i, i) cross-peak intensities, determined via cross-peak integration from the spectra. “x” denotes observed nOe but due to cross-peak overlap, relative intensities could not be integrated. Figure 1 also presents 3J couplings (Hz) and amide temperature shift coefficients (ATC, in ppb/K). N/A = not available.

Figure 2. Summary of NMR parameters for Lustrin A D4 peptide in 1 mM Na₂HPO₄, 90% water/10% D₂O, 278 K pH 7.4. Refer to Figure 1 legend for explanation of terms.
in an extended conformation with no significant shielding of backbone amide (NH) protons from solvent exchange [13–15]. Thus, we conclude that both Lustrin A domains do not adopt α-helix or β-sheet structure; rather, they appear to exist largely in a conformationally labile random coil state or, in the case of RKSY, in a random coil state that is in conformational exchange with an extended state.

**SEM Analysis of In Vitro Calcite Overgrowth Assays**

Direct investigations of polypeptide interactions with aragonite are difficult to pursue, owing to the fact that aragonite is thermodynamically unstable in solution (i.e., transforms to calcite). Thus, we conclude that both Lustrin A domains do not adopt a conformationally labile random coil state or, in the case of RKSY, a conformational exchange with an extended state.

The in vitro thermodynamically unstable in solution (i.e., transforms to calcite) are difficult to pursue, owing to the fact that aragonite is thermodynamically unstable in solution (i.e., transforms to calcite). Thus, we conclude that both Lustrin A domains do not adopt an extended state. Obviously, further experimental studies are required to resolve these issues.
Figure 3. Scanning electron micrographs of calcium carbonate overgrowth on the [104] cleavage plane of geological calcite. (A) Negative control (no peptide added). Arrows indicate characteristic rhombohedral growth steps. In B–E the overgrowth nucleation was observed in the presence of (B) $3.3 \times 10^{-8}$ M bovine serum albumin; (C) $1.7 \times 10^{-7}$ M RKSY polypeptide; (D) $6.7 \times 10^{-9}$ M D4 polypeptide; and (E) $1.7 \times 10^{-7}$ M D4 polypeptide. Scalebars in A–E 1 µm.
ACKNOWLEDGMENTS

Support for this study has been made possible by grants from the National Science Foundation (DMR 99-01356, MCB 98-16703 to NYU) and the Army Research Office (MURI DAAH04-96-1-0443 to UCSB).

REFERENCES


[18] Michenfelder, M., Thompson, J., Shimizu, K., Le, K., Smith, B.L., Weaver, J., Lawrence, C., Stucky, G., Hansma, P., and Morse, D. Characterization of two crystal-modulating proteins isolated from the gastropod mollusk, Biopolymers, in press.


