Three intrinsically unstructured mussel adhesive proteins, mfp-1, mfp-2, and mfp-3: Analysis by circular dichroism

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Abstract: Mussel foot proteins (mfps) mediate fouling by the byssal holdfast and have been extensively investigated as models for versatile polymer-mediated underwater adhesion and coatings. However, insights into the structural properties of mfps have lagged far behind the nanomechanical advances, owing in part to the inability of these proteins to crystallize as well as their limited solubility. Here, solution secondary structures of mfp-1, mfp-2, and mfp-3, localized in the mussel byssal cuticle, adhesive plaque, and plaque–substratum interface, respectively, were investigated using circular dichroism. All three have significant extended coil solution structure, but two, mfp-1 and mfp-2, appear to have punctuated regions of structure separated by unstructured domains. Apart from its punctuated distribution, the structure in mfp-1 resembles other structural proteins such as collagen and plant cell-wall proteins with prominent polyproline II helical structure. As in collagen, PP II structure of mfp-1 is incrementally disrupted by increasing the temperature and by raising pH. However, no recognizable change in mfp-1's PP II structure was evident with the addition with Ca^{2+} and Fe^{3+} . In contrast, mfp-2 exhibits Ca^{2+} - and disulfide-stabilized epidermal growth factor-like domains separated by unstructured sequence. Mfp-2 showed calcium-binding ability. Bound calcium in mfp-2 was not removed by chelation at pH 5.5, but it was released upon reduction of disulfide bonds. Mfp-3, in contrast, appears to consist largely of unstructured extended coils.

Keywords: mussel adhesive protein; mfp-1; mfp-2; mfp-3; secondary structure; circular dichroism

Abbreviations: diHyp, *trans*-2,3-*cis*-3,4-dihydroxyproline; Dopa, 3,4-dihydroxyphenylalanine; EGF, epidermal growth factor; Hyp, hydroxyproline.

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Introduction

The marine mussel (*Mytilus* sp.) adheres to a variety of wet surfaces by producing a fibrous protein holdfast structure called byssus that resists cyclic mechanical stresses related to tides, wave turbulence, and abrasion.¹ Mussel adhesive proteins are thus important molecular factors in marine fouling as well as useful paradigms of the robust, yet-to-be-invented underwater adhesive and coating polymers that are needed for wide-ranging medical and industrial applications.^{2,3} At the end of each byssal, thread is an adhesive plaque where interfacial adhesion between the byssus and a foreign substratum occurs. The byssus is mainly made of various

Figure 1. Solution conformation of three mussel byssal proteins. CD of (A) mfp-1, (B) mfp-2, and (C) mfp-3 in 50 mM sodium acetate buffer, pH 5.0.

proteins secreted from a phenol gland of mussel feet and the proteins that comprise byssus are called mussel foot proteins (mfps). To date, eight different types of mfps have been isolated and interestingly, most display extensive post-translational modifications of their primary sequences.² At present, attempts to mimic mussel-inspired adhesives and coatings have been conducted largely on the basis of primary sequence information^{4,5}; however, an understanding of biomechanical and structural properties of mfps is also prerequisite for mimicking mussel adhesion and coating to invent novel adhesives and coatings.

The transformative power of biomimetics applied to engineering and inspired by biomacromolecular materials such as mussel byssus depends in large part on the depth of understanding of structure–property relationships. Recruitment of the atomic force microscope, nanoindentation, and the surface force apparatus (SFA) has contributed much to understanding the physical and mechanical properties of mussel byssus and byssal proteins particularly the mfps.^{5–9} However, structural studies at different length scales have been hampered by considerable challenges, including a menagerie of peculiar post-translational modifications, mfp sequence polymorphism,^{2,10} the inability to crystallize any of the mfps, difficulty of purifying mg quantities, and the instability of mfps in solution.¹¹ This has led to a variety of experimental approximations, which may or may not be biologically relevant. Mfp-1 is an excellent case in point. Extensive circular dichroism (CD), proton nuclear magnetic resonance (NMR), and molecular modeling, for example, have been applied to synthetic and recombinant analogs of the protein's consensus decapeptide sequence repeated 1–20 times, with no or partial post-translational modifications.^{11–14}

In this study, we investigate the secondary structures in aqueous solution of three dominant mfps, mfp-1, mfp-2, and mfp-3, as detected by CD. Mfp-1 (from *Mytilus californianus*) is the only protein known to be present in the byssal cuticle remarkable for its combination of high stiffness and

high extensibility.⁶ Mfp-1 has a mass between 88 and 92 kDa, a basic isoelectric point (8–10), and 60 or more tandem repeats of a decapeptide sequence (PKISYO*OTY*K in which Y* is 3,4-dihydroxyphenyl-L-alanine (Dopa), O is *trans*-4-hydroxyproline, and O* denotes *trans*-2,3-*cis*-3,4-dihydroxyproline (diHyp)).¹⁵ Mfp-2 (from *M. edulis*) is an abundant protein in the plaques (~25% of adhesive plaque by dry weight) with a mass of 45 kDa and consists of 11 tandem repeats of a 40-res long epidermal growth factor (EGF) motif.^{16,17} Mfp-3 (from *M. edulis*) is present at the interface with the substratum as a protein family where it is presumed to function as an adhesive primer. Mfp-3 is the most polymorphic of all known plaque proteins with more than 30 different known sequence variants ranging in mass from 5 to 7 kDa.¹⁰ Mfp-3 has 10–20 mol % of Dopa and ~4 mol % of 4-hydroxyarginine. Our main purpose here is to compare and contrast the secondary structure of three dominant mfps, mfp-1, mfp-2, and mfp-3, under identical solution conditions.

Results

Comparing secondary structures of mfp-1, mfp-2, and mfp-3

Secondary solution structures of mfp-1, mfp-2, and mfp-3 were investigated by CD spectroscopy. To maximize solubility of mfps and minimize the pH-dependent oxidation of Dopa residues, 50 mM sodium acetate, pH 5.0, at 20°C was chosen as the assay buffer. Under these conditions, the CD spectrum of Mfp-1 features a prominent maximum with molar ellipticity of about 1100 de cm²/dmol at 230 nm and a molar ellipticity of about –13,500 deg cm²/dmol at the minimum of 200 nm [Fig. 1(A)]. The CD spectrum of mfp-1 is fairly typical of a polyproline II (PP II) helix with a deep minimum around 198–200 nm (π – π^* transition), a slight maximum around 229 nm (n – π^* transition).^{18–20} PP II helix is a left-handed helix with *trans* peptide bonds and three residues per turn. Backbone dihedral angles (ϕ , ψ) are about –75 and 150°, resulting in a more extended helix than the α -helix. Peptide and protein sequences

Figure 2. Thermal stability of mfp-1 conformation. (A) CD spectra in 50 mM sodium acetate buffer, pH 5.0, with increasing of temperature. (B) Thermal titration of mfp-1 by continuous measurement of CD value at 200 and 230 nm from 4 to 28°C.

which form polyproline II (PPII) have slightly positive maxima at around 215–230 nm and strong negative minima around 195–200 nm in solution CD.^{19,20}

The CD spectrum of mfp-2 in the same buffer reveals a minimum at about 193 nm, a shoulder at ~200 nm (~−23,000 deg cm²/dmol), and slight maximum around 230 nm (~2000 deg cm²/dmol, Fig. 1(B)). The CD spectra of mfp-1 and mfp-2 exhibit similar weak maxima around at 230 nm. In contrast to the sequence of mfp-1, mfp-2 consists of 11 tandem repeats of an EGF-like motif and the CD spectrum of mfp-2 is consistent with that of other proteins in EGF family.^{21,22} A weakly positive maximum at 230 nm and higher has been attributed to tryptophan and tyrosine residues in recombinant EGF.²³

In contrast, the measured CD spectrum of mfp-3 exhibits predominantly an extended chain conformation with minimal helical content in aqueous solutions at 20°C [Fig. 1(C)]. Positive ellipticities between 230 and 250 nm probably correspond to π – π^* transitions among the abundant aromatic Dopa and tryptophan side chains.²⁴ Temperature-

dependent effects on mfp-3 structure were investigated by CD but mfp-3 spectra showed little change at 200–215 nm, suggesting overall random coil characteristics (Supporting Information Fig. 1). In the range of 220–235 nm, temperature-dependent effects are consistent with solvent-dependent transitions in tyrosine and tryptophan residues that may reflect localized helix to coil structure changes.²⁴

Temperature, pH, and iron (III) effect on secondary structures of mfp-1

The thermal stability of structure in mfp-1 was studied by monitoring ellipticity in 50 mM sodium acetate buffer, pH 5.5, between 4 and 28°C [Fig. 2(A)]. A gradual loss of the maximum and minimum at 230 and 200 nm, respectively, was observed but the overall polyproline II tendency of mfp-1 remained (Fig. 2). The PPII content in mfp-1 decreased with increasing temperature. Using a PP II prediction model based on short polyproline peptides,²⁵ PP II structure reduction in mfp-1 with increasing temperature was observed but it is slight at best (from ~54% at 4°C to 51% at 28°C). pH effects on the CD spectrum of mfp-1 were also investigated between

Figure 3. pH and the conformational stability of mfp-1. (A) CD spectra of mfp-1 in 50 mM sodium acetate buffer, with variation of pH at 20°C. (B) Estimated polyproline II helical content in mfp-1 depending on pH.

Figure 4. Effect of metals and the conformational stability of mfp-1. CD spectra of mfp-1 in 50 mM sodium acetate, 1 mM Bis-tris buffer (black), with addition of 0.1 mM FeCl_3 at 20°C (gray and black dash).

pH 3.0 and 8.2 (Fig. 3). The overall pattern was not changed but a gradual decrease in the 230 nm maximum was observed as the buffer pH was raised. As protonation of few if any amino acid side chains occurs in the pH range of 3.0–8.2, a pH-independent structure according to CD is not surprising. PP II content in mfp-1 decreased from ~60 to 46% upon increasing the pH from 3.0 to 8.2 [Fig. 4(B)]. A small reduction in the maximum at 230 nm and % proline content is caused by oxidative losses of DOPA, leading to some crosslinking and precipitation of mfp-1 as the pH is raised.⁸

We speculated that iron binding by mfp-1 would initiate conformational changes of mfp-1 and hence the effect of Fe (III) addition on the CD spectra of mfp-1 was measured. Bis-tris (1 mM), a weak Fe (III) chelator, was also added to prevent Fe (III) oxidation and precipitation at neutral and high pH.^{9,26,27} Slight perturbation of the CD spectrum was observed but overall ellipticity remained as before (Fig. 4). As the bonding between Dopa in mfp-1 and iron (III) will be stronger and the probability of forming *tris*-Dopa-Fe (III) complexes in mfp-1 greater at basic pH, we raised the pH of Fe (III)

containing mfp-1 solution from pH 5.0 to 7.0. The pink color characteristic of tri-Dopa-Fe (III) complexes in mfp-1 became more intense at pH 7.0,²⁶ but the CD spectrum remained essentially unchanged except at very short wavelengths (<195 nm). These results suggest that Fe (III)-Dopa coordination in solution is not accompanied by recognizable secondary structure changes in mfp-1. In the byssal cuticle where mfp-1 is localized, a high concentration of Ca^{2+} ions was also reported by secondary ion mass spectroscopy and the presence of various fatty acids (myristic acids, lauric acids, and palmitic acids) was proposed.²⁸ Therefore, Ca^{2+} and the above-mentioned fatty acids were added separately or together to mfp-1 containing solutions. However, no detectable secondary structure changes in CD were evident (data not shown).

Effect of temperature, reductants, and calcium binding on secondary structures of mfp-2

The conformational stability of mfp-2 was also investigated by CD. The fact that the spectra of mfp-2 were not significantly changed by heating is consistent with the previous CD measurements of EGF family [Fig. 5(A)].²³ When the disulfides remain intact, mfp-2, like other EGF modular domains, is largely stable to heating.²⁹ The shoulder at 197 nm, however, which is particularly indicative of Ca^{2+} -binding EGF modules, disappears reversibly with temperature [Figs. 1(B) and 5(B)]. The disulfides in EGF modules are generally assumed to lock the protein into an EGF-distinctive conformation. Reducing the disulfides leads to the formation of α -helices in some EGF modules and β -sheets in others.²² Disulfide bonds in mfp-2 were reduced by the addition of 2 mM *tris*(2-carboxyethyl) phosphine or dithiothreitol (DTT). In stark contrast to other experiments using recombinant EGF, there was no apparent induction of secondary structure in mfp-2 with a twofold molar excess of reducing agents; however, disulfide reduction did abolish the 197-nm peak associated with calcium binding [Fig. 5(B)]. To investigate

Figure 5. Conformational stability of mfp-2 depending on (A) temperature, (B) redox, and (C) calcium binding. (A) CD spectra of mfp-2 in 50 mM sodium acetate, pH 5.5, buffer, with increasing temperature. (B) CD spectra of mfp-2 in 20 mM sodium acetate, pH 5.5, at 20°C (black) and with 2 mM DTT (gray). (C) CD spectra of mfp-2 in 20 mM sodium acetate, pH 5.5, at 20°C buffer (black), with 0.1 mM CaCl_2 (gray), with 0.1 mM CaCl_2 and 1 mM EDTA (black dash), and with 0.1 mM CaCl_2 , 1 mM EDTA, and 1 mM DTT (gray dash).

calcium-binding effect of mfp-2 in greater detail, we added 0.1 mM CaCl_2 (excess concentration sufficient to occupy all calcium-binding domains in dissolved mfp-2) to mfp-2 and monitored CD spectrum (Fig. 5(C), gray line). Interestingly, peak around 197 nm was strongly enhanced with calcium addition, the peak persisted despite treatment with 1 mM ethylenediaminetetraacetic acid (EDTA) (Fig. 5(C), black dash line) but subsequent addition of 1 mM DTT eliminated the strong peak around 197 nm (Fig. 5(C), gray dashline).

Discussion

Mfp-1 is localized to a protective outer cuticle in byssal threads. The cuticle is both hard and reversibly extensible up to 100% strain.^{6,28} How these peculiar mechanical properties of the cuticle are related to mfp-1's unusual structure continues to intrigue. A variety of techniques have been performed to elucidate the structure of mfp-1. For various reasons, few, if any, of these were applied to native full-length protein. Instead, nearly all investigations have used synthetic or recombinant analogs, none of which contained all post-translational modifications known to be present.^{11–14,30} CD analysis of a shortened recombinant mfp-1 analog suggested a random-coil shape,¹¹ whereas 2D-NMR and dynamics simulation of a mfp-1 decapeptide consensus sequence suggested a kinked left-handed polyproline II helix.^{12,14} Collagen-like peptides such as $[\text{Gly-Pro-Pro}]_n$ generally exhibit polyproline II helix-like CD spectra when properly folded in a collagen triple helix.³¹ The maxima and minima in molar ellipticity that we observed are roughly six times less intense than those reported for collagens.

Present ellipticity data contrast with a previous CD analysis of recombinant mfp-1 analog that suggested a random coil conformation unaffected by temperature or addition of 6M guanidine hydrochloride.¹¹ A left-handed polyproline II helix CD spectrum of mfp-1 is more consistent with ^1H and ^{13}C 2D-NMR conformation analysis of the consensus decapeptide sequence of Mfp-1 (*M. edulis*).¹² Indeed, mfp-1 has ~6 mol % diHyp and 18.2 mol % Hyp, which provide strong inductive effects to favor the *trans*-proline required in the polyproline II helix.³² The polyproline II helix also occurs in Hyp-rich extensins of the plant cell wall³³ with typical molar ellipticities of –16,100 and 3900 at 197 and 225 nm, respectively. These helices are monomeric and depend on extensive arabinosylation of Hyp residues to further stabilize the polyproline II helices.

Rather inextensible in tension (strain, <10%), neither collagen nor Hyp-rich cell-wall proteins provide particularly compelling molecular prototypes for extensible tissues. Given its high extensibility and Pro content, the PEVK domain of the muscle protein titin, however, is an intriguing model for byssal cuticle and mfp-1. The PEVK domain consists of tandem

repeats of a 28-residue long proline (P), glutamate (E), valine (V), and lysine (K)-rich module. CD and nuclear overhauser effect–NMR analysis of a single module found PPII structures between four and six residues long that are interspersed within disordered flexible regions.¹⁸ Given the similar distribution of Pro/Hyp clusters in the repeat sequence of mfp-1, that is P/O-S-Y-O*-O, the model of interspersed order and disorder has considerable appeal. In the context of the measured hydrodynamic radius of 11 nm for mfp-1, the CD data are consistent with a structure in which short, localized regions of order (PPII) are separated by unstructured sequences, and thus resulting in a flexible molecular “necklace” with small structured domains. In contrast, uninterrupted PPII structures such as tropocollagen and plant cell-wall proteins have much larger hydrodynamic radii, namely 200 nm³⁴ and 89 nm,³⁵ respectively.

Based on homologies in primary sequence between EGF family and mfp-2,¹⁷ mfp-2 was deduced to be a distinctive knot-like EGF structure stabilized by three disulfide bonds.^{23,36} Indeed, CD of mfp-2 in the far UV was almost identical to that of oxidized recombinant human EGF,²² but there is a further insight. The 197-nm shoulder of the minimum at 193 nm [Figs. 1(B) and 5(A)] is particularly indicative of the Ca^{2+} -binding EGF modules of fibrillin^{29,37} and can be disrupted by temperature or reduction of disulfides. SFA studies have independently shown that mfp-2 is Ca^{2+} binding⁷ and a partially Ca^{2+} -bound form of mfp-2 was apparently used in the CD studies. Bound calcium on mfp-2 was not easily removed by chelation with excess EDTA at pH 5.5 but released with reduction of disulfide bonding [Fig. 5(B,C)]. The results suggest that strong Ca^{2+} binding in the Ca^{2+} -binding EGF modules of mfp-2 may depend on a tertiary structure that is maintained by the disulfide bonds. Recombinant EGFs often acquire significant new secondary structures upon reduction of disulfides,²² but no such changes were observed in mfp-2. Previous studies allow the estimation of the hydrodynamic radius of mfp-2 at 3–4 nm.⁷ In contrast to mfp-1, mfp-2, although also a flexible molecular necklace, has larger (40–45-res long) structured EGF domains.

Mfp-3 together with mfp-5 is an adhesive primer for adhesion by the byssal plaque. A recent study based on Sum Frequency Generation vibrational spectroscopy³⁸ reported that mfp-3 adopts different conformations at different interfaces. Our analyses by CD are limited to detecting a random coil at solution conditions consistent to those present during secretion.³⁹ Perhaps, a random coil conformation of mfp-3 in solution is beneficial for anticipating a variety of conformations adopted on different surface chemistries.

In summary, the secondary solution structures of the mussel adhesive proteins mfp-1, mfp-2, and mfp-3 were investigated by CD and exhibit distinctly

different tendencies. Mfp-1 had a CD reminiscent of the polyproline II helix in collagen, plant cell-wall protein, and the PEVK domains in titin. In contrast, mfp-2 showed EGF-like structure with a knot-like structure stabilized by three disulfide bonds and calcium, and mfp-3 exhibits an unstructured extended coil structure that is well adapted to conform to a variety of surfaces. Interestingly, although the global protein backbone in all three remains flexible, repeated regions of localized structure increase from five to six residues in length (PPII) in mfp-1 to about 40 residues (EGF) in mfp-2.

Materials and Methods

mfp preparation

The purification of each mfps is described in detail elsewhere.^{10,15,16} Mfp-1 used for this study was prepared from *M. californianus* feet, whereas mfp-2 and mfp-3 were prepared from *M. edulis* feet. Live *M. californianus* were collected from Goleta Pier on Sandspit Road (Goleta, CA); flash-frozen *M. edulis* feet were purchased from Northeast transport (Waldoboro, ME). Mfp-1 and mfp-2 were extracted from dissected phenol glands containing mussel feet with cold 5% (v/v) acetic acid with 0.1M EDTA, 0.1 mM leupeptin, and 0.1 mM pepstatin. Mussel feet were initially crushed using a garlic press and then homogenized with 50 mL tissue grinders from Kontes (Vineland, NJ), and homogenates were spun in a refrigerated (4°C) centrifuge 20,000g for 40 min to produce a supernatant S1 and pellet P1. Mfp-1 and mfp-2 were further purified from S1 by the addition of 1.4% (v/v) perchloric acid and resulting insolubles were removed by centrifugation as before. Supernatant containing Mfp-1 and mfp-2 was dialyzed with 5% acetic acids to remove small impurities and perchloric acid at 4°C and concentrated by freeze-drying. The freeze-dried sample was resuspended with small volume of 5% acetic acid (~200 µL) and injected to gel filtration column (Shodex KW 803) and eluted with 5% acetic acid at 0.25 mL/min at room temperature and monitored at 280 nm.⁴⁰ Mfp-1 (88–92 kDa) typically elutes just after the void volume, whereas mfp-2 (45 kDa), depending on the sample volume injected, elutes as a peak about 10 min after mfp-1. Fractions under the peaks were examined for their mfp content using acid–urea gel electrophoresis.⁴⁰ Those fractions of mfp-1 without mfp-2, or mfp-2 without mfp-1, respectively, were pooled, freeze-dried, reconstituted in a small volume of 5% acetic acid (~1 mL), and polished by C8 reversed-phase high-performance liquid chromatography (HPLC) using an acetonitrile gradient in water with 0.1% trifluoroacetic acid. Both proteins elute at about 22% acetonitrile.⁷ Following HPLC, the proteins were freeze-dried and stored at –80°C.

Mfp-3 was obtained by further extraction of P1. Mfp-3 was extracted by homogenization (tissue grinder) of pellets with 8M urea in 5% acetic acid (50 mL/5g) at 4°C. Centrifugation (20,000g with 40 min)

of this resulted in a pellet P2 and supernatant S2. S2 was collected and 30% (w/v) ammonium sulfate was added and stirred for 1 h at room temperature. Insolubles were removed by centrifugation (as above) and the supernatant was dialyzed against Q-water at 5°C with dialysis tubing having a nominal molecular weight cutoff of 1000 (Spectrum Industries). Mfp-3 forms a floc during dialysis and settles. This was harvested by a 5-min spin at 15,000g on a microfuge, redissolved in a small volume of 5% acetic acid, and polished by C8 reversed-phase HPLC using an acetonitrile gradient in water with 0.1% trifluoroacetic acid. Purity and homogeneity of mfp-1, mfp-2, and mfp-3 were determined by measuring the Dopa content of hydrolyzed proteins by amino acid analysis following a 158°C hydrolysis *in vacuo* for 1 h and by acid–urea gel electrophoresis, respectively.⁴¹

Circular dichroism

CD spectra of mfps were acquired using an Olis RSM spectrophotometer (Olis, Bogart, GA) in the far UV region (190–250 nm) with quartz cuvettes of 0.5 mm path length. Baseline spectra were subtracted from sample spectra and smoothed using the software supplied by the manufacturer. We used 50 mM sodium acetate buffers with pH adjusted from 3.0 to 8.2 depending on the experiment. Mean residual ellipticity, θ , as a function of wavelength, was estimated from spectra obtained using a scan rate of 12 nm/min. Temperature scans were performed from 4 to 37°C to monitor the melting of secondary structure. All spectra shown here are averages of more than five individual scans. Estimates of PPII helix content in mfp-1 were obtained by using the following equation.^{25,42,43}

$$\%PPII = \frac{[\theta]_{\max} + 6700}{13,700} \times 100$$

where $[\theta]_{\max}$ is the molar ellipticity at the characteristic maximum, –6100 deg dmol^{–1} cm² is taken as the lower limit (~0% PPII), and 7600 deg dmol^{–1} cm² is taken as the upper limit (PPII, ~100%).

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