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Adhesion mechanism in a DOPA-deficient foot protein from green mussels†

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Abstract

The holdfast or byssus of Asian green mussels, *Perna viridis*, contains a foot protein, pvfp-1, that differs in two respects from all other known adhesive mussel foot proteins (mfp): (1) instead of the hallmark L-3,4-dihydroxyphenylalanine (DOPA) residues in mfp-1, for example, pvfp-1 contains C²-mannosyl-7-hydroxytryptophan (Man7OHTp). (2) In addition, pvfp-1 chains are not monomeric like mfp-1 but trimerized by collagen and coiled-coil domains near the carboxy terminus after a typical domain of tandemly repeated decapeptides. Here, the contribution of these peculiarities to adhesion was examined using a surface forces apparatus (SFA). Unlike previously studied mfp-1s, pvfp-1 showed significant adhesion to mica and, in symmetric pvfp-1 films, substantial cohesive interactions were present at pH 5.5. The role of Man7OHTp in adhesion is not clear, and a DOPA-like role for Man7OHTp in metal complexation (*e.g.*, Cu²⁺, Fe³⁺) was not observed. Instead, cation- π interactions with low desolvation penalty between Man7OHTp and lysyl side chains and conformational changes (raveling and unraveling of collagen helix and coiled-coil domains) are the best explanations for the strong adhesion between pvfp-1 monomolecular films. The strong adhesion mechanism induced by cation- π interactions and conformational changes in pvfp-1 provides new insights for the development of biomimetic underwater adhesives.

Introduction

Marine mussels secrete a variety of mussel foot proteins (mfp) to form a holdfast known as the byssus where they are specifically adapted for functions that include wet adhesion and protective coatings.^{1,2} The modified amino acid L-3,4-dihydroxyphenylalanine (DOPA) features prominently in these proteins and has been shown to play a critical role in both adhesion and cohesion *via* a diverse array of chemical interactions including hydrogen bonding, metal coordination complexes, and covalent cross-links.^{3–7}

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In a recent biochemical analysis of the byssus in the Asian green mussel, *Perna viridis*,^{8,9} a new member of the mussel foot protein-1 family was identified, namely pvfp-1. Pvfp-1 resembles other mfp-1s^{10–12} with its tandemly repeated Pro- and Lys-rich decapeptides; however, DOPA is noticeably deficient (only 2 known residues per pvfp-1), and replaced by a different aromatic functionality C²-mannosyl-7-hydroxytryptophan (Man7OHTrp) (Fig. 1).⁹ Moreover, whereas mfp-1 of the blue mussel (*Mytilus edulis*) consists of DOPA-containing decapeptide repeats, pvfp-1 has four distinct domains: Trp-rich decapeptide repeat (ATPKPWTAWK and APPPAWTAWK) domains confined to the N-terminal two-thirds, a short hinge region, a collagen domain ([Gly-X-Y]₁₈), and a predicted short coiled-coil region before briefly returning to Trp-rich repeats at the C terminus (Fig. 1). Also, pvfp-1 is further decorated with partially characterized glycosyl groups on its protein sequence.⁸

Although it is possible to determine many subtleties of the chemical structure in marine adhesive proteins, correlating this structure to surface forces was not possible until the introduction of the surface forces apparatus (SFA).^{5,6,13} SFA has been extensively used in biologically and non-biologically related research alike and is ideal for measuring the physical forces (both normal and lateral) between surfaces in vapors and liquids, *e.g.*, van der Waals forces, electrostatic forces, adhesion forces, hydrophobic interactions, specific and non-specific biological interactions, and friction and lubrication forces.^{14–16} The SFA has the unique ability to provide a simultaneous direct measurement of the force *F* as a function of the absolute surface separation *D* measured *in situ* and in real time, with a force sensitivity of ~10 nN and distance resolution of less than 0.1 nm determined using multiple beam interferometry.^{14–16} In this study, we have used the SFA to evaluate and compare the contributions of the Man7OHTrp side chains and the collagen domain to adhesion and cohesion of pvfp-1 films on mica surfaces.

Materials and experimental methods

Pvfp-1 purification

Live Asian green mussels (*Perna viridis*) were obtained from seafood vendors in Singapore. After shucking each mussel, the foot was amputated at the base, immediately frozen on dry ice and shipped to UCSB where it was stored at –80 °C until use. Protein extraction from mussel feet was adapted from previous reports^{8,9} as follows: frozen mussel feet were thawed on ice and the brownish pigment on the foot surface was carefully flayed away with a razor blade. The depigmented feet were homogenized in a glass tissue grinder with 50 ml of 5% acetic acid and two protease inhibitors (pepstatin A and leupeptin, both 30 μM). The homogenate was centrifuged (15 000 × *g*, 4 °C) for 30 min, and the recovered supernatant (S1) was chilled in an ice bath. 70% w/v perchloric acid was added drop-wise with stirring to a final perchloric acid concentration of 1.4% w/v. The mixture was further stirred and centrifuged (15 000 × *g*, 4 °C) for 30 min. The supernatant (S2) was collected, dialyzed against 500 volumes of 5% acetic acid for 12 h at 4 °C, freeze-dried at –80 °C, and resuspended in 0.2 ml of 5% acetic acid.

P. viridis foot protein-1 (pvfp-1) was purified in three stages: gel filtration, reversed phase HPLC, and finally, again, gel filtration. Initial isolation was achieved by gel filtration chromatography on a Shodex KW-803 column (5 μm, 8 × 300 mm). The column was equilibrated and eluted with 5% acetic acid at a flow rate of 0.2 ml min^{–1}. A maximum sample volume of 200 μl of S2 was loaded onto the Shodex column per run. The eluted volume was monitored at 280 nm, and the fractions under the pvfp-1 peak were pooled (1–1.5 ml) and further resolved by C8 HPLC (BrownleeAquaporeRP-300, 7 μm, dimensions: 4.6 × 250 mm) at a flow rate of 1 ml min^{–1} using an acetonitrile gradient described in ref. 12. The collected fractions were assayed by redox cycling after acid urea gel

electrophoresis^{8,9} and amino acid analysis, as described below to help identify the pvfp-1 containing fractions. Pvfp-1 fractions were pooled and lyophilized at -80°C . After resuspending in 200 μl of 5% acetic acid, pvfp-1-positive fractions were run once more on Shodex KW-803 (above conditions). Fractions eluting between 33 and 43 min were redox active and had amino acid compositions consistent with previous studies.^{8,9} Purified pvfp-1 fractions were recovered by freeze-drying and stored in evacuated glass vials at -80°C .

Force vs. distance profiles measured by an SFA

The normal force–distance profiles and adhesion forces of the pvfp-1 were determined using an SFA in a configuration reported previously.^{5,6,13,16} The smooth and chemically inert surfaces of mica were used as the substrate surfaces for depositing thin films of the protein used in the experiment. Two thin mica sheets (thickness 1–5 μm) were glued onto two cylindrical silica disks (radius $R = 2\text{ cm}$). The protein film on mica was prepared by the adsorption method. Briefly, a 50 μl droplet of the protein solution ($10\text{ }\mu\text{g ml}^{-1}$, 0.1 M acetic acid, $\text{pH} \approx 3.0$) was placed on a molecularly smooth mica surface glued to a silica disk of radius $R = 2\text{ cm}$, and the proteins were allowed to adsorb/deposit on mica for 20 min in a sealed chamber saturated with water vapour. Then the surface was washed with an appropriate buffer, which was used as the aqueous medium in later specific SFA experiments, before being loaded into the SFA chamber. The adsorption of pvfp-1 to mica was mainly driven by electrostatic attraction between positively charged pvfp-1 molecules (at $\text{pH} \approx 3.0$) and negatively charged mica surfaces. The successful deposition of the pvfp-1 film on mica was confirmed by AFM imaging, as shown in Fig. S3†

After preparing pvfp-1 coated mica surface, the surface was incubated with the testing buffer solution and force–distance profiles were measured. The standard buffer condition for these studies was defined as $\text{pH } 5.5$, 0.25 M potassium nitrate, 0.1 M sodium acetate. The effects of changing pH and ionic strength on the molecular interactions of pvfp-1 were also investigated ($\text{pH} = 3.0$ in 0.1 to 0.6 M potassium nitrate). The two curved and coated mica surfaces (symmetric configuration) or one coated and one uncoated mica surface (asymmetric configuration) were then mounted in the SFA chamber in a crossed-cylinder geometry, which roughly corresponds to a sphere of radius R approaching a flat surface based on the “Derjaguin approximation”.^{15,17} The measured adhesion or “pull-off” force F_{ad} is related to the adhesion energy per unit area W_{ad} by $W_{\text{ad}} = F_{\text{ad}}/2\pi R$ for rigid (undeformable) surfaces with weakly adhesive interactions, and by $W_{\text{ad}} = F_{\text{ad}}/1.5\pi R$ (used in this study) for soft deformable surfaces with strong adhesive contact.^{15,17} The experiments were conducted at room temperature (20°C) unless otherwise indicated. The temperature of the SFA was increased by using two heating rods (100 Watt, 110 V), inserted into the metal walls of the SFA chamber, and the temperature inside was monitored in real time using a thermistor. The chamber temperature was first increased slowly to a desired temperature and then kept at that temperature for a certain waiting time to allow thermal equilibrium, which normally took about 1 hour. During the SFA experiments, the two surfaces were driven together or apart at a constant “driven force rate” of $4.4\text{ }\mu\text{N s}^{-1}$, where the stiffness of the force measuring spring was 880 N m^{-1} which was driven at $\sim 5\text{ nm s}^{-1}$ to make the two surfaces approach or separate. The two surfaces were brought to a maximum compressive load of $\sim 0.7\text{ mN}$ for all measurements, and then kept in contact for different times before separation.

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Results and discussion

Interactions of pvfp-1 under standard buffer conditions

The interaction force between two roughly monomolecular layers of green mussel foot protein (pvfp-1) coated on mica surfaces or between one layer of pvfp-1 and mica was measured using an SFA as described above and ref. 5,6,13 and 16 under the standard buffer conditions (pH 5.5, 0.25 M potassium nitrate, 0.1 M sodium acetate). For the asymmetric configuration (mica supported pvfp-1 vs. uncoated mica), the two surfaces were first brought together to reach a “hard (steric) wall” followed by separation of the surfaces. The “hard wall” distance is defined as the mica–mica separation distance or thickness of confined proteins, which does not appear to change with increase in the normal compressive load or pressure. The hard wall distance measured (~ 8 nm) was slightly less than the hydrodynamic diameter of pvfp-1 (~ 10 nm) in bulk solution by dynamic light scattering (DLS) and the RMS roughness of the pvfp-1 film on mica determined by AFM was ~ 0.9 nm. Thus, the pvfp-1 film on mica used in this study can be roughly considered as a monomolecular layer. A weak adhesion $F_{\text{ad}}/R \approx -0.5 \text{ mN m}^{-1}$ was observed when separation immediately followed approach. When pvfp-1 was kept in contact with mica for 10 and 40 min, the adhesion increased slightly to $F_{\text{ad}}/R \approx -0.6$ and -0.7 mN m^{-1} , respectively (Fig. 2A). Previous studies with mefp-1 films showed that mefp-1 adsorbed on mica did not exhibit any adhesion to the opposing mica (asymmetric configuration) or between two films of mefp-1 (symmetric configuration, at pH 5.5, Table 1).^{6,13}

For the symmetric configuration (a pair of pvfp-1 coated mica surfaces), an adhesion force $F_{\text{ad}}/R \approx -5.5 \text{ mN m}^{-1}$ was measured during an approach–separation cycle when separation occurred within ~ 2 minutes of the approach as shown in Fig. 2B. The adhesion force increased to -8.5 and -11.5 mN m^{-1} when the duration of contact between two surfaces was extended to 10 and 40 min, respectively. The right hand ordinate showed that these forces correspond to surface adhesion energies of $W_{\text{ad}} \approx -1.8$ and -2.4 mJ m^{-2} . Meanwhile, the hard wall distance decreases slightly from 14 nm for contact time $t_c = 2$ min to 9 nm with contact time increased to $t_c = 40$ min. There could be two aspects contributing to the decreased hard wall distances and increased adhesion with increase in the contact time: (i) conformational rearrangement of the protein films under the load/pressure applied during the contact so that more “binding” sites on pvfp-1 chains of each film are exposed at the interface leading to an increased adhesion, (ii) more interpenetration and interdigitation for protein chain ends and loops got across the interface leading to a decreased hard wall distance and an enhanced adhesion. During the separation process, the interdigitated chain ends had to be pulled out from the loops of the opposing film before the two surfaces could be fully detached, similar to the adhesion between two glassy polymer surfaces.^{18–21}

Although it is hard to determine if there was any protein chain transfer or chain rupture associated with the detachment, the probability of such fracture behaviors should be low due to the high energy normally required and relatively higher mobility of protein chains in aqueous solution compared to that in dry glassy polymers. Immediate re-approach and separation with 2 min contact following the 40 min contact measurement showed similar adhesion to the previous 40 min contact measurement, which suggests that once the conformation re-arrangement has happened, it took time for the structure of the pvfp-1 film to recover to the original state. The time- and temperature-dependent recovery is shown in more detail in Fig. 6 and 7. It is also interesting to note that the changes for the hard wall distance and adhesion with contact time for the asymmetric case (protein film vs. bare mica in Fig. 2A) were not as obvious as the symmetric case (Fig. 2B), which further supports the proposed mechanisms of conformational rearrangement of the pvfp-1 proteins and their interpenetration and interdigitation across the interface.

In contrast to mfp-1, pvfp-1 exhibits strong adhesion between two protein films (symmetric configuration) and relatively weaker adhesion between a protein film and uncoated mica (asymmetric case) under standard buffer conditions. Moreover, the adhesion between symmetric pvfp-1 films was 5 times higher than that of mfp-3 which is dominated by DOPA–mica interactions.^{5,6} These results imply that the strong adhesion between pvfp-1 films is mediated by interactions other than those mediated by DOPA.

Effects of pH, ionic strength and oxidation

Previous work has determined that DOPA-containing proteins and polymers generally exhibit stronger adhesion at low pH (pH 3.0–5.5) where DOPA-oxidation to DOPA quinone is limited,^{5,22} whereas at higher pH or with added oxidants, oxidation of DOPA effectively abolished adhesion (>90%).^{5,22} If pvfp-1 behaves like DOPA-containing proteins or polymers in which the side chains of Man7OHTrp mimic DOPA, adhesion would be expected to (1) increase on lowering the pH, and (2) decrease upon treatment with oxidants. To discern any ionic strength and pH effects on the interactions of pvfp-1, the buffer pH was reduced to 3.0.

pH effect—Lowering the solution pH from 5.5 to 3.0 did not significantly alter the adhesion between pvfp-1 and mica for short contact times (<15 minutes) as shown in Fig. 3A (asymmetric configuration). In contrast, at longer contact times (~40 minutes), the adhesion increased by almost 5 times as shown in Fig. 3A. At lower pH, the zeta potential of mica becomes slightly less negative (~10%),^{9,23} but the adsorbed pvfp-1 also becomes more positive due to increased protonation of lysine residues, thereby enhancing the potential for electrostatic interactions with the pvfp-1–mica interface. An alternative interpretation is that the two DOPA residues/pvfp-1 are available for adhesion to mica at pH 3.0 but not at pH 5.5.

It is noteworthy that upon reducing the pH from 5.5 to 3.0, the adhesion force between two pvfp-1 surfaces did not increase as it did in the case of mfp-3 in the symmetric configuration.⁵ As shown in Fig. 3B, the adhesion energy $W_{ad} = F_{ad}/1.5\pi R$ actually decreased slightly from pH 5.5 to 3.0, suggesting that Man7OHTrp might not be the main or only contributor to the strong adhesion observed between two pvfp-1 layers. In previous studies with mfp-3, enhanced adhesion occurred at pH 3.0 by preventing DOPA oxidation. Man7OHTrp clearly does not follow the pH dependent behavior of DOPA.

Effect of ionic strength—The effect of ionic strength on interactions in pvfp-1 was investigated in the SFA at three KNO₃ concentrations, *i.e.*, 0.1 M, 0.25 M or 0.6 M (sodium acetate was fixed at 0.1 M). 0.1 M and 0.6 M salt concentrations were chosen to resemble the ionic strength of physiological fluids (~0.15 M) and seawater (~0.7 M), respectively. The extent to which pvfp-1 adhesion relies on electrostatic interaction before and after secretion should be revealed by measuring adhesion at different salt concentrations. The adhesion forces measured during the separation process under three KNO₃ concentrations at pH 3.0 and the contact time effects are summarized in Fig. 3C and D. The typical force–distance profiles for both asymmetric and symmetric configurations after a brief contact (*e.g.*, separation was followed ~2 minutes after approach) support a salt-dependence for adhesion (shown in Fig. 4). For both asymmetric and symmetric configurations, the adhesion forces decreased with increasing ionic strength. For the interaction between a pvfp-1 film and mica at pH = 3.0, an adhesion force of $F_{ad}/R \approx -6 \text{ mN m}^{-1}$ at 0.1 M KNO₃ declined to $F_{ad}/R \approx -1 \text{ mN m}^{-1}$ at 0.25 M KNO₃, and the interaction appeared to be purely repulsive at high ionic strength (0.6 M KNO₃). The hydrodynamic radius of pvfp-1 measured by dynamic light scattering (DLS) shows a strong dependence on salt concentration (~5 nm at 0.1 M KNO₃, ~50 nm at 0.25 M KNO₃). Similar trends of reduced

adhesion with increasing ionic strength were observed for the symmetric configuration (Fig. 4B).

The reduction of the adhesion forces with increasing $[\text{KNO}_3]$ for asymmetric configurations may be related to the stronger binding of the hydrated K^+ layer onto the bare mica surface at increased KNO_3 concentrations^{23–25} which results in an increase of the zeta potential of mica (less negative). This binding would interfere with the mica-to-pvfp-1 interactions (hydrogen bonding, electrostatics, *etc.*). The reduction of adhesion at higher KNO_3 concentrations may also be related to the cation– π interaction between the side chains of the aromatic amino acids in the pvfp-1 molecules (*e.g.*, tryptophan (Trp) in Man7OHTrp) and positively charged lysine and arginine groups in the pvfp-1 film of the opposing side.²² Increasing concentration of cations such as K^+ would compete with the interaction between Man7-OHTrp and positively charged residues on both pvfp-1 surfaces, thereby interfering with those bridges mediated by the cation– π interaction.

The cation– π interaction is a general, noncovalent binding force comparable in strength to hydrogen bonding and has been established as an important contributor to protein–protein interfaces.^{26–29} The side chains of the aromatic amino acids, *i.e.*, phenylalanine (Phe), tyrosine (Tyr), and tryptophan (Trp) provide a surface of negative electrostatic potential that can bind a wide range of cations including K^+ , Lys and Arg through an interaction that is predominantly electrostatic.²⁶ The Lys or Arg– π interaction may play an important role in the interactions between two pvfp-1 interfaces, and K^+ ions in the buffer are likely to compete with the cation– π interaction between pvfp-1 interfaces. A particular advantage of cation– π interactions in mussel adhesion is their low desolvation penalty.^{26,29} Generally, noncovalent interactions in aqueous media must compete with the substantial solvation energy of ions and it was theoretically demonstrated that the desolvation penalty for cation– π interaction (~60% loss) is much less than that of normal electrostatic interaction (~99% loss).²⁹ Cation– π interactions have not been previously considered to play a role in marine adhesion but definitely should be. A common feature of all the marine adhesive proteins characterized to date is that they contain abundant positively charged (His, Lys, Arg) and aromatic residues (DOPA, Tyr and Trp).¹ In the absence of significant potential charge–charge interactions in pvfp-1 at pH 3–5.5, the present SFA data strongly suggest cation– π interactions. Unfortunately, bond strength variation of cation– π interaction depending on types of medium, ionic strength and types of chemicals which mediate the cation– π interaction is not yet fully understood. Undoubtedly, additional SFA studies of cation– π interactions in well-defined systems will shed critical insights on the extent of their contributions to mussel adhesion.

At all three ionic strengths, the adhesion energy $W_{\text{ad}} = F_{\text{ad}}/1.5\pi R$ increased with longer contact time for both symmetric and asymmetric configurations (Fig. 3C and D). The adhesion energy for the symmetric configuration plateaus after ~10 min contact. The longer contact time enables stronger and more extensive “bonding” at the interface between two pvfp-1 layers, or between one pvfp-1 layer and a mica surface. Fig. 3 also shows that the contact time required to reach the adhesion plateau was less for the symmetric configuration than that for the asymmetric configuration. Such difference was most likely due to the differences in the time associated with the conformational change of the proteins and the time necessary to reach maximal binding of the hydrated K^+ layer to the bare mica surface under the confinement conditions for the two configurations. The jump-in observed for the symmetric configuration with 0.1 M KNO_3 at pH 3.0 could be due to some intermolecular interaction mechanism between the protruded pvfp-1 proteins with certain favored conformation on each surface similar to the so-called “lock-and-key” mechanism. As the two surfaces were close to some critical distance, the adhesion force gradient between these protruded protein chains exceeded the stiffness of the force measuring spring which led to

the jump-in instability. Jump-in was not observed at higher salt concentrations and higher pH (pH 5.5), which was mainly due to the protein conformation changes the high pH and high ionic strength which could increase the steric forces during approach.

Redox effects—The effects of periodate oxidation and ascorbate reduction on pvfp-1 interactions were investigated in both asymmetric and symmetric configurations (Fig. 5). Periodate treatment of DOPA containing proteins or polymers typically results in abolition of adhesion due to oxidation of DOPA.^{3–5,30} Pvfp-1 contains no more than two DOPA residues, thus should not be much affected unless Man7OHTrp is also oxidized by periodate – a reasonable prediction based on hydroxytryptophan chemistry.⁹ The starting point for the oxidation and reduction experiments of Fig. 5A and B was the re-approach and separation immediately after the separation process of a 40 min contact measurement.

Periodate (800 pmol) sufficient to oxidize ~20 mol% Man7-OHTrp only led to a slight reduction of adhesion (~20%), and the adhesion was effectively recovered by reduction with ascorbic acid in the asymmetric configuration (Fig. 5). Higher concentrations of periodate never exceeded ~20% reduction compared with controls. That adhesion was never abolished by periodate treatment implies that chemical functionalities other than DOPA are responsible for adhesion to mica. The sugar groups in pvfp-1, for example, might hydrogen bond to the hydrated mica surface, however, the sensitivity of adhesion to ionic strength makes the electrostatic interaction between lysine side chains and surface Si-O⁻ groups more likely.

For the symmetric configuration, the periodate treatment led to a reduction of adhesion energy by ~20%, but ascorbate addition decreased adhesion by another ~20%. This result further supports that the adhesion between two pvfp-1 surfaces was not mainly due to a DOPA-like activity in Man7OHTrp. Other interactions such as the cation- π interaction²⁶ or conformation-dependent changes in pvfp-1 may also play important roles.

Cation- π interactions are highly orientation dependent, that is, the cation must be directly over one of the π cloud faces of the aromatic ring. To further probe the time dependence of interactions that require proper orientation, two symmetric pvfp-1 surfaces were kept apart after the previous separation and allowed to relax for 9 h (surfaces kept separated in buffer for 9 h); then the force measurement for several approach-separation cycles was resumed (Fig. 6). Relaxation reduced the adhesion to ~20% of the initially observed force. However, increased post-relaxation contact times apparently achieve a full recovery of adhesion. Together with the effects of contact time (Fig. 3C and D), this time-dependent effect points to underlying protein reorganization during the 9 h relaxation period, leading to a reduction of the adhesion force. It should be noted that adhesion values measured with 2, 10 and 40 min contact after 9 h relaxation/incubation (Fig. 6B, C, and D) were ~50% less than the values measured with the same contact times right after the pvfp-1 film deposition (as shown in Fig. 3), which indicates that the conformation of the pvfp-1 film right after protein deposition (adsorption and rinsing with buffer solution) might not have achieved thermal equilibrium. During the long incubation period, certain protein groups, *e.g.*, the hydrophobic indole rings in pvfp-1 could rearrange their conformations, resulting in a reduction in adhesion despite the same contact time. After recovering full adhesion during consecutive force runs (~10), the effect of injecting 0.1 mM of sodium ascorbate into the gap was tested. Adhesion increased ~10% and suggests that antioxidants improve symmetric adhesion of pvfp-1 layers but the effect is a small one.

The time-dependent effects imply that strong adhesion for the symmetric configuration may arise from highly oriented interactions or conformational changes such as collagen or coiled

coil unwinding in pvfp-1. Increasing salt concentration leads to diminished adhesion and agrees well with the proposed cation- π interaction mechanism.²⁶

Effect of temperature

The temperature dependence of pvfp-1 interactions (symmetric configuration) was also investigated. Notably the adhesion force between two pvfp-1 protein surfaces F_{ad}/R decreased from -7 to ~ -0.5 mN m⁻¹ whereas the hard wall distance increased from 20 to ~ 35 nm with a temperature increase from 24 to 40 °C (Fig. 7A). Interestingly, the adhesion force and hard wall distance gradually recovered (although not fully) to their initial levels when the initial temperature was restored (from 40 to 24 °C). The F/R vs. D profiles and the separation rate vs. separation distance profiles shown in Fig. 7 suggest that at small distances the two pvfp-1 surfaces are interconnected by protein tethers and/or interdigitation of pvfp-1 chains which cause them to separate sluggishly to some critical separation D_c beyond which they rapidly accelerate apart (Fig. 7C and D). During the separation process, the surface separation D is a function of time and depends on the previous history, such as the lifetime of the adhesion junction which increases the number of interdigitated protein chains between two surfaces and other system and experimental parameters. An adhesion junction is under a normal stress during the separation, and the lifetime of a stressed junction generally depends also on the temperature T : lifetime $\tau \propto \exp[-F(D)\delta/kT]$, where δ is the effective bond (adhesion junction) length.^{31–33} As the temperature increases, the lifetime of the adhesion junction decreases exponentially. Thus, the maximum pull-off force or adhesion force is both rate and state dependent, similar to that observed in polymer systems.^{31–34} Higher temperature results in a conformation change of the pvfp-1 molecules, *i.e.*, more extended or stretched, as manifested by the higher hard wall thickness. The interdigitation of the pvfp-1 chains at the interface of two protein films is reduced at the higher temperature, leading to a lower adhesion, which is similar to the adhesion of polymers (*e.g.*, polystyrene) reported previously.^{32,33} Curiously, the greatest loss in adhesion (Fig. 7A) occurred in the step from 24 to 30 °C where the latter corresponds to the lower temperature boundary at which collagen domains begin to melt.³⁵

Effect of added metal ions

Since the *ortho*-placement of indolic-N and phenolic OH groups in Man7OHTrp seems well suited to metal ion chelation, efforts have been undertaken to test whether 7OH indole in Man7-OHTrp coordinates transition metals as DOPA does. By SIMS analysis of *P. viridis* byssal threads, we found both Cu and Fe localized to the cuticular layer. Therefore, we speculated that Man7OHTrp residues in pvfp-1 may coordinate Cu²⁺ or Fe³⁺ *via* the indolic N and the phenolic OH. Since indolic-N in Man7-OHTrp is restricted by π -electron density of the indole ring, a soft ligand, we expected Cu²⁺ to form a stable complex with Man7OHTrp. As synthetic Man7OHTrp is not yet available, we used 7-hydroxyindole for the metal coordination study *via* UV spectrometry.

Based on UV-vis spectrophotometry, neither Cu²⁺ nor Fe³⁺ ions formed a stable colored complex with 7-hydroxyindole. Also, when we injected Cu²⁺ and Fe³⁺ at various concentrations (from 100 nM to 100 μ M) between two protein layers in the SFA, there was no observable enhancement of adhesion in the symmetric case (Fig. S1 and S2[†]), in contrast to iron mediated bridging in mefp-1 and mefp-2.^{6,36}

Adhesion mechanism and conclusion

The molecular structure of pvfp-1 is different from the other known adhesive mussel foot proteins in two aspects: (i) pvfp-1 contains Man7OHTrp instead of DOPA residues which

prevail in most mfp proteins, and (ii) pvfp-1 chains form trimers driven by peculiar collagen and coiled-coil domains.

In this work, using an SFA, contributions of the molecular features of pvfp-1 to its mode of adhesion were investigated by exploring the effects of pH, ionic strength, oxidation, temperature and addition of metal ions. Based on the experimental results and discussion above, the adhesion mechanism between a pvfp-1 film and mica surface (asymmetric case) and between two pvfp-1 layers (symmetric case) can be summarized as follows:

1. The adhesion between pvfp-1 and mica is mainly determined by the electrostatic interaction between lysine side chains in pvfp-1 molecules and the negatively charged mica surfaces. Other interactions such as van der Waals forces, hydrogen bonds between the sugar groups in pvfp-1, and hydrated mica surface could also contribute to the adhesion observed.
2. The adhesion between two pvfp-1 films is apparently not dominated by DOPA mediated interactions so typical of other mfps. Although the exact role of Man7OHTrp in adhesion is still not clear, the cation- π interaction between the side chains of the aromatic amino acids in the pvfp-1 molecules (*e.g.*, tryptophan (Trp) in Man7OHTrp) and positively charged lysine and arginine groups in the opposing pvfp-1 film represents the interaction with the greatest potential to stabilize the interface between two pvfp-1 films. In contrast to iron mediated reversible bridging in mfp-1 and mfp-2 reported previously,^{6,36} neither Cu^{2+} nor Fe^{3+} ions contribute to significant bridging adhesion between two pvfp-1 films by shared metal complexation by Man7OHTrp residues. A proposed interaction mechanism is shown in the schematic of Fig. 8 for pvfp-1 trimers interacting through cation- π interactions between lysine and Man7OHTrp in decapeptide repeating units. The cation- π interactions are highly orientation dependent, for example, the amine groups should be perpendicular to the electron-rich plane of the indole rings of Man7OHTrp to initiate effective cation- π interactions, which indicates that the protein conformation changes induced by various solution conditions play a critical role in their intermolecular and surface interactions. The proposed interaction mechanisms are consistent with the effects of pH, ionic strength, oxidation, temperature and addition of metal ions observed in this study.
3. In contrast to iron mediated reversible bridging in mfp-1 and mfp-2 reported previously,^{6,36} neither Cu^{2+} nor Fe^{3+} ions contribute to significant bridging adhesion between two pvfp-1 films through shared metal complexation by Man7OHTrp residues.
4. The adhesion of symmetric pvfp-1 films is highly temperature-dependent and essentially abolished by 30 °C which approximates the melting temperature of short chain collagen domains. Therefore, conformational changes of pvfp-1 protein (*e.g.*, raveling and unraveling of collagen helix and coiled-coil domains) are critical for the protein interactions.

It should be also noted that understanding the adhesion mechanism of mussel proteins in air or dry conditions will also provide important insights into the development of versatile and innovative dry adhesive materials, although only few reports are available.^{1,37} Basically, in air or dry environment, the protein film behaves like a glassy polymer layer, in which dry proteins may partially or totally lose their biological properties (*i.e.*, due to the change of molecular conformation), and van der Waals forces and interdigitation of chain ends or loops of proteins across the contact interface play the most important role in the adhesion, which is similar to the recent reports on engineering polymers.^{21,31,32,38} For adhesion under water, the protein layer is more like a swollen and extended brush close to biological

conditions, where interactions such as electric double layer forces, cation- π interaction, hydration forces, steric interactions, as well as specific ligand-receptor interactions may play more important roles, which is very different from the dry adhesion case. Research on the adhesion mechanisms and contact mechanics of dry pvfp-1 and other mussel foot protein films (*e.g.*, mfp-1, mfp-3 and mfp-5) will be reported in a separate study, which will provide important information for developing biomimetic dry adhesives and a more complete picture of the molecular interaction mechanisms of mussel foot proteins in both wet and dry conditions.

In conclusion, for DOPA-deficient foot protein from green mussels pvfp-1, cation- π interactions with low desolvation penalty between Man7OHTrp and lysyl side chains and the conformational changes of protein molecules (raveling and unraveling of collagen helix and coiled-coil domains) are most likely to induce the adhesion mechanism underwater. The strong adhesion mechanism induced by cation- π interactions and conformational changes in pvfp-1 provides new insights for the development of novel biomimetic underwater adhesive materials. Further research is required to elucidate how the solution environment (pH, ionic strength, metal ions, *etc.*) and experimental conditions (load, contact time, temperature) can impact the exact conformations of protein nano-films.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1.

A comparison of the foot proteins mefp-1 and pvfp-1 from the blue mussel *Mytilus edulis* and green mussel *Perna viridis*, respectively. (A) Domain structures: mefp-1 consists primarily of tandem decapeptide repeats, whereas pvfp-1 is composed of decapeptide repeats as well as collagen and coiled-coil domains. (B) The consensus sequences of the two decapeptide repeats are similar but distinct. Similar amino acids such as Ala, Pro/Hyp, Thr and Lys define the composition, however, the DOPA modification in mefp-1, is substituted by a Trp derivative, C²-mannosyl-7-hydroxyTRP (Man7OHTrp), in pvfp-1. P* P* are hydroxyprolines, Y* is DOPA and W* is Man7OHTrp. *N.B.*: the lower sequence is actually the more abundant one!

Fig. 2. Adhesion forces of pvfp-1 films on mica as a function of contact time at pH 5.5. (A) asymmetric configuration; (B) symmetric configuration. Open circle – approach, closed circle – separation. The measured force, F/R (normalized by the radius of the surfaces, R), is denoted in the ordinate at the left, whereas the corresponding interaction energy per unit area, W , between two flat surfaces, defined by $W = F/1.5\pi R$ is on the right. F_{ad} is the adhesion force. The hard wall corresponds to the thickness of the protein film and is indicated by the arrows (top left corner).

Fig. 3.

The effect of pH (A and B) and ionic strength (C and D) to the energy of adhesion depending on contact time, W_{ad} , for asymmetric and symmetric pvfp-1 films on mica. Lines have been added merely to show the trends.

Fig. 4. Effect of ionic strength on the adhesion force, F_{ad} , of pvfp-1 films on mica at pH 3.0 in asymmetric and symmetric configurations, all for 2 min contact time. Panels (A) correspond to the asymmetric configuration at increasing ionic strength, whereas panels (B) correspond to parallel tests done in the symmetric mode. Open circle – approach, closed circle – separation.

Fig. 5.

Adhesion energy changes in asymmetric and symmetric pvfp-1 films on mica at pH 3.0 before and after periodate treatment (NaIO_4), followed by treatment with ascorbate. Untreated is the control; periodate denotes 800 pmoles of NaIO_4 injected into the gap; ascorbate denotes injecting excess antioxidant ascorbate (~ 0.1 mM) into the gap; all contact times were 2 min in 0.1 M acetate acid, 0.1 M KNO_3 , at pH 3.0. The starting point for the oxidation and reduction experiments of (A) and (B) was the re-approach and separation right after the separation process of a 40 min contact measurement.

Fig. 6.

Time-dependent recovery of adhesion in pvfp-1. Adhesion energy per unit area, W_{ad} , changes between two pvfp-1 coated surfaces in 0.1 M sodium acetate, 0.1 M KNO_3 , pH 3.0 due to contact time and relaxation time. t_{tot} : total time after first force run; t_c = contact time; n = number of force runs at each point. (A) 2 min contact after reaching maximum adhesion energy between two pvfp-1 layers in 0.1 M sodium acetate, 0.1 M KNO_3 , pH 3.0, (B) 2 min contact force run after 9 h separation and relaxation in the same contact point, (C) 10 min contact force run after (B), (D) 40 min contact force run after (C), and (E) 2 min contact force runs after (D).

Fig. 7.

Temperature effects on pvfp-1 symmetric interactions in 0.1 M acetic acid, 0.1 M KNO₃.

(A) Changes in adhesion force between pvfp-1 and pvfp-1 as temperature was increased (24 to 40 °C) and (B) changes in adhesion force between pvfp-1 and pvfp-1 as temperature was decreased (40 to 24.5 °C). (C) Changes in dD/dt between pvfp-1 and pvfp-1 as temperature was increased (24 to 40 °C) and (D) changes in dD/dt between pvfp-1 and pvfp-1 as temperature was decreased (40 to 24.5 °C), where dD/dt is the instantaneous separation rate at D (first derivative of separation distance D to time t).

Fig. 8. Model of pvfp-1 trimers interacting through cation- π interactions between lysine and Man7OHTrp in decapeptide repeating units. *N.B.*: the amino group should be perpendicular to the plane of the ring.

Table 1

Comparison of the work of adhesion (W_{ad}) in homologous proteins mefp-1 and pvfp-1 from two related mytilid species, *Mytilus edulis* and *Perna viridis* after a 40 min contact in 0.1 M sodium acetate buffer, 0.25 M KNO_3 , at pH 5.5

Foot protein	W_{ad} , mJ m ⁻² asymmetric	W_{ad} , mJ m ⁻² symmetric
Mefp-1	~0 (ref. 6 and 13)	~0 (ref. 6 and 13)
Mefp-3	~0.52 (ref. 5 and 18)	~0.56 (ref. 6)
Pvfp-1	0.16 ± 0.1 ($n = 5$)	2.3 ± 0.1 ($n = 5$)