Notes

Use of Attenuated Total Internal Reflection Fourier Transform Infrared Spectroscopy To Investigate Interactions between *Mytilus edulis* Foot Proteins at a Surface

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The sea mussel *Mytilus edulis* has become a model organism for investigations of adhesion. Academic interest stems from curiosity about the molecular interactions involved in a highly evolved and complex biological adhesion process. These investigations are facilitated by a relatively advanced biochemical characterization of the various components that condense to form the adhesive plaque. From an engineering perspective the sea mussel offers design possibilities for a biomimetic glue. Both academic and more practical interests are inspired by the sea mussel's ability to form a tenacious attachment to surfaces with a wide range of chemistries within minutes in a seawater environment.

The protein components of the adhesive plaque are numbered roughly in the order in which they were successfully purified to homogeneity. *M. edulis* foot protein 1 (Mefp-1) has become a model protein of this model organism. Mefp-1 has an intriguing primary structure that is crucial to the adhesion process as suggested by the relatively few sequence variations that are found within the species and by its appearance as a common motif across different species. Mefp-1 consists primarily of tandem repeats of a decapeptide sequence which incorporates two unusual amino acids, L-3,4-dihydroxyphenylalanine (L-dopa) and *trans*-2,3-*cis*-3,4-dihydroxyproline, two lysine residues and a proline residue.[1,2] These primary structural characteristics translate into a conformation that is open,[3] but not random.[4] Mefp-1 is a major component of the protective varnish that covers the plaque and is also present in the interior of the plaque, concentrated near the substratum interface.[5] Mefp-1 is thought to have an exceptional affinity for surfaces.[6]

About 90−95% of the plaque is proteinaceous.[7] Thus far a family of four Mefp proteins, each of which incorporates L-dopa, have been isolated from the phenol gland of *M. edulis*. Mefp-2, the second protein in the family to be fully characterized, contributes about 25% to the protein content.[7] Mefp-2 is approximately a third the size of Mefp-1 (42-47 kDa compared to 125 kDa). It is highly cross-linked via cystine disulfide bonds (15 mol %). It has been proposed that Mefp-2 is a structural component and that Mefp-1 mediates surface bonding.[7]

In addition to the Mefp series (1−4) there is at least one catechol oxidase that makes a significant contribution to the plaque.[8] The proposed function of this enzyme is to convert the relatively unreactive catechol functionalities of the L-dopa residues to the highly reactive quinone form which then induces formation of, as yet uncharacterized, cross-links. It was at first proposed that these cross-links were formed from nucleophilic addition of lysine into the quinone ring; however, recent results weaken this hypothesis.[9−11]

The Mefp family as well as catechol oxidase(s) and at least one collagen[12] are all extruded into the cavity of the foot and condense rapidly to form the adhesive plaque. The (between-protein) interactions have not been characterized. One approach for studying these interactions is to use methodology based on attenuated total reflection Fourier transform infrared (ATR-FTIR) spectroscopy.

Details pertaining to spectroscopy have been described previously.[9,13] ATR-FTIR spectroscopy was used to follow the kinetics of appearance of proteins at the surface. A flow cell designed to accommodate a germanium (Ge) trapezoidal prism[14] was used to deliver protein solutions to the interfacial region. The Ge substratum was exposed to a solution of Mefp-1 for 60 min. This was followed by

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a 30 min rinse period. At this dilute bulk concentration, the signal originating from the solution phase protein is negligible. This is confirmed by the lack of any detectable decrease in signal upon rinsing (data not shown). Following the rinse period, Mefp-2 was introduced into the flow cell for 60 min followed by a 30 min rinse period. Difference spectra were used to follow the interaction of solution phase Mefp-2 with the preadsorbed Mefp-1 adlayer. In one experiment the Mefp-1 adlayer was exposed to 3 mM sodium periodate for 30 min to oxidize the catechols of the L-dopa residues to the quinone form.[15] This was followed by a 10 min rinse period. Surface coverage of protein was estimated on the basis of baseline subtracted areas of the amide II band. Values reported in Table 1 and Figure 2 were obtained by a published method for converting amide II areas into surface coverage using bovine serum albumin as the standard in a transmission cell.[13] A conversion factor was also obtained by measuring the signal from solutions of Mefp-1 and -2 that had been dried onto the surface of the prism.[16] Estimates of surface coverage using this latter conversion factor are approximately 1.5 times greater than those reported, both for Mefp-1 and for Mefp-2. Curve fits to spectra were generated by GRAMS/386 software (Galactic Industries Corp.) using mixed Guassian−Lorentzian component bands. Kinetic curves were fit using TableCurve 2D (Jandel Scientific Corp.).

Mefp-1 was purchased from Bioscience Laboratory (Floda, Sweden). Mefp-2 was a gift from J. Herbert Waite (Marine Science Institute, University of California, Santa Barbara). Purity of Mefp-1 and -2 was confirmed by acetic acid−urea polyacrylamide gel electrophoresis.[17] Protein solutions (Mefp-1 or -2) were in 10 mM sodium phosphate (pH 7.2). Concentration for adsorption was 0.05 mg/mL except for one experiment. The Mefp-1 adlayer with 0.03 μg/cm^2 surface coverage (Table 1, row 2) was obtained by exposing the Ge surface to a 0.02 mg/mL solution.

Figure 1 shows spectra of Mefp-1 and Mefp-2 adlayers adsorbed directly onto Ge. It was previously proposed that the sharp band indicated in the figure (D) originated from the catechol functionality of the L-dopa residues. Mefp-2 incorporates fewer L-dopa and tyrosine residues than Mefp-1. This is consistent with the relative size of the putative L-dopa band for the spectra of Mefp-1 and -2. Areas of the amide II band were computed for the ranges indicated in Figure 1. Areas were corrected for appearance of the L-dopa band in the amide II region by subtracting its contribution on the basis of a curve fit to this region. On the basis of this fit the ratio of the area of the putative L-dopa band to the amide II band was 0.136 for Mefp-1 and 0.012 for Mefp-2.

Figure 2 shows the kinetics of appearance of Mefp-2 in the interfacial region of the Ge surface for a clean Ge surface and for surfaces coated with adlayers of Mefp-1. The signal originates essentially only from protein that accumulates by adsorption either to the Ge surface or to the Mefp-1 adlayer. The kinetic curves are fit well ($r^2 >$ 0.98) with an equation describing the sum of two first order formation rates

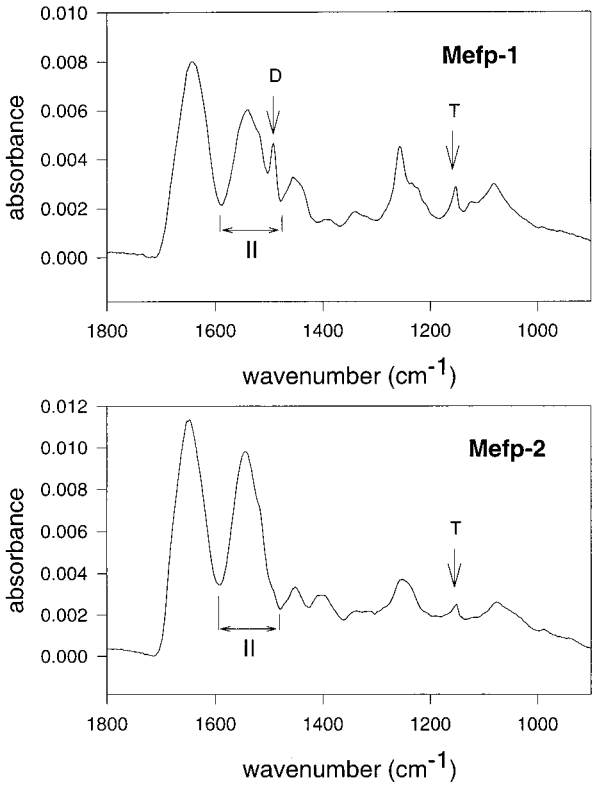

Figure 1. Spectra of Mefp-1 (top) and Mefp-2 (bottom) adlayers adsorbed directly onto Ge. Limits for computing baseline-subtracted areas of the amide II band (II), putative L-dopa band (D), and residual features that likely originate from Teflon spacers of the flow cell (T) are indicated.

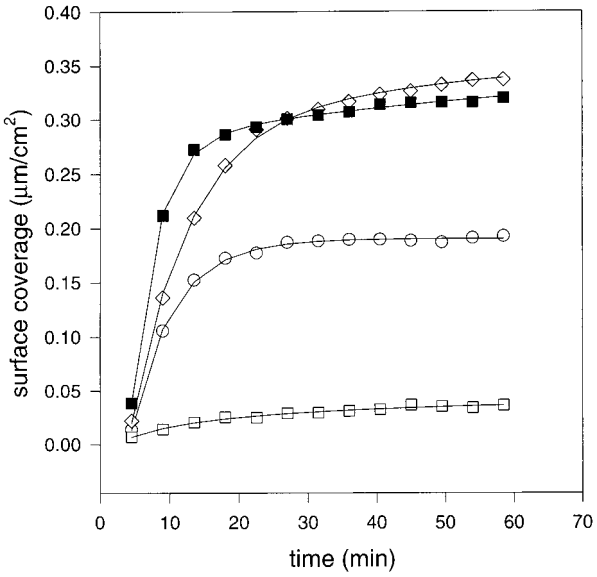

Figure 2. Kinetics of appearance of Mefp-2 in the interfacial region measured by ATR-FTIR. Symbols are data points: solid squares, Mefp-2 onto Ge; open circles, Mefp-2 onto Mefp-1 adlayer (low coverage, see Table 1, row 2); open squares, Mefp-2 onto Mefp-1 adlayer (high coverage, see Table 1, row 3); open diamonds, Mefp-2 onto oxidized Mefp-1 adlayer. Solid lines: fit of model (eq 1).

$$K + C_1 (1 - \exp(k_1 t)) + C_2(1 - \exp(k_2 t)) \qquad (1)$$

where t is time. Estimates of K, C_1, C_2, k_1, and k_2 were found by a least-squares fit criterion. In all cases curves were fit better by the double exponential than the analogous single exponential. A similar model describes

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Table 1. Adsorption Parameters for Mefp-2 onto Ge and Mefp-1 Adlayers

$C_s(1)$[a]	$C_s(2)$[b]	Γ[c]	k_1[d]	k_2[e]
0	0.28 (0.30)[f]	0.29 (0.35)	0.272 (0.174)	0.016 (0.11)
0.03[g]	0.20	0.20	0.184	0.126
0.19 (0.11)	0.04 (0.04)	0.04 (0.08)	0.162 (0.174)	0.032 (0.11)
0.16[h] (0.12)	0.38 (0.22)	0.50 (0.22)	0.117 (0.170)	0.005 (0.071)

[a] Surface coverage (μg/cm^2) of Mefp-1 at end of first rinse period. [b] Surface coverage (μg/cm^2) of Mefp-2 at end of second rinse period. [c] Surface coverage (μg/cm^2) at infinite adsorption time predicted by fit to eq 1 ($C_1 + C_2 + K$). [d] Rate constant (min^{-1}) for adsorption (eq 1). [e] Rate constant (min^{-1}) for adsorption (eq 1). [f] Numbers in parentheses are values for a replicate experiment. [g] Adsorption was from a 0.02 mg/mL solution. [h] Mefp-1 adlayer reacted with sodium periodate.

kinetics of adsorption of cationic surfactants and was used to determine thermodynamic parameters of the two-step first-order adsorption reaction.[18] Use of eq 1 is semiempirical here. The floating parameter, K, releases the constraint that the predicted curve pass through the origin, and thus effects of the entrance kinetics on delivery of protein to the interfacial region, that are expected to be appreciable during the first 5 min, are ignored. The kinetics are regulated by both diffusion into the interfacial region and the rate of adsorption. On the basis of the flow cell geometry, flow regime, adsorption protocol, and an estimate of the diffusion coefficient for Mefp-2, it requires a maximum of 8 min for the solution concentration adjacent to the surface to reach 90% of the bulk concentration by diffusion from the edge of the estimated parabolic-shaped plug of protein.

Table 1 lists best estimates of parameters (eq 1) describing adsorption of Mefp-2. Fits were to data presented in Figure 2 or to data for a replicate experiment (in parentheses). In row 1 are parameters for Mefp-2 adsorption onto clean Ge. In rows 2 through 4 are parameters for Mefp-2 adsorption onto Ge with preadsorbed Mefp-1 adlayers. In the last row are parameter estimates for adsorption of Mefp-2 onto an adlayer of Mefp-1 that was oxidized with sodium periodate. Both the surface coverage measured at the end of the rinse period ($C_s(2)$) and the surface coverage predicted for infinite adsorption time by the fit to eq 1 ($\Gamma = C_1 + C_2 + K$) indicate that the presence of a preadsorbed (nonoxidized) adlayer of Mefp-1 excludes Mefp-2 from the interfacial region. Exposure of the Mefp-1 adlayer to the oxidant, sodium periodate, increases $C_s(2)$ to levels comparable to those for Mefp-2 adsorption onto a clean Ge substratum.

Survival of the sea mussel depends on rapidly securing an anchor to a surface in the intertidal zone. An essential step is formation of an adhesive plaque at the distal end of each byssus. Thus it is critical that components that are extruded into the cavity of the foot interact with both each other (cohesion) and the intended substratum (adhesion) with a high probability of success. The patterns of order exhibited by the primary structures of the Mefp family suggest a self-assembly process in which the different proteins have specialized roles. For example, one possible model would be a cascade of reactions (perhaps resembling in some respects those involved in blood clotting). Regardless of the extent of specialization, one or more proteins in the foot cavity must rapidly form bonds to the intended substratum, while leaving functionalities free to form bonds with solution phase matrix components. The catalyst in the adhesive mixture, one or more catechol oxidase(s), may function in part to regulate the rate of cross-linking of the solution phase components so that adhesion to the substratum can be ensured.

Results here introduce a methodology for investigating interactions of the Mefp family at a surface. The hypothesis that can be posed is that Mefp species have different specialized roles. A corollary is that one or more biomolecules in the mixture is endowed with a bifunctionality that enables it to bond with the intended substratum and solution-phase components. ATR-FTIR is ideal for testing this corollary since kinetics of adsorption of hydrated biomolecules can be measured in real time and the technique is surface sensitive. The results presented here show that Mefp-1 excludes Mefp-2 from the surface until the L-dopa residues are converted to the quinone form. This suggests that Mefp-1 serves as a bifunctional primer that requires activation by the catechol oxidase. This is speculative based on the evidence presented, especially since the results suggest that Mefp-2 adsorbs to the Ge substratum at least as strongly as Mefp-1.

The methodology used to obtain these results can be exploited to investigate interactions between other proteins in the Mefp family. Surfaces are not restricted to Ge. We have previously modified the Ge surface with thin polymer films and organosilanes for studies of adsorption of biomolecules.[19,20] The laminar flow cell design provides controlled hydrodynamics allowing investigation of the effect of diffusion on kinetics of adsorption by regulating the flow rate. The influence of different catalysts on the interfacial interactions can also be investigated.

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