Journal of Colloid and Interface Science **230,** 340–348 (2000)
doi:10.1006/jcis.2000.7120, available online at http://www.idealibrary.com on IDEAL®

Influence of Sodium Periodate and Tyrosinase on Binding of Alginate to Adlayers of *Mytilus edulis* Foot Protein 1

Peter A. Suci[1] and Gill G. Geesey

Center for Biofilm Engineering and Department of Microbiology, Montana State University, Bozeman, Montana 59717-3980

Received February 7, 2000; accepted July 31, 2000

***Mytilus edulis* foot protein 1 (Mefp-1) is the most well-characterized component of this sea mussel's adhesive plaque. The plaque is a condensed, heterogeneous mixture consisting of a large proportion of cross-linked biopolymers that bonds the mussel to a chosen mooring. Mefp-1 is densely populated with lysine and L-3,4-dihyroxyphenylalanine (L-dopa) residues incorporated into a repeating amino acid sequence motif. It has been proposed that one plaque cross-linking reaction is the nucleophilic addition of the ε-amino groups of the lysine residues into the oxidized catechol (o-diphenol) functionality (quinone) of the L-dopa residues. In order to determine if this reaction occurs in adlayers of Mefp-1, a previously developed assay for ε-amino groups was applied. Adlayers of Mefp-1 were exposed to an oxidant, either the enzyme, mushroom tyrosinase, or sodium periodate. Binding of alginate to adlayers was used to probe for accessibility of ε-amino groups. It was found that lysine residues lose the ability to bind alginate after exposure to sodium periodate, but that this loss is not clearly due to a reaction with L-dopa residues. There is a slight decrease of binding of alginate to adlayers of Mefp-1 exposed to either active or thermally deactivated mushroom tyrosinase, probably due to the obstruction of binding sites by bound enzyme. Adsorption kinetics of mushroom tyrosinase onto adlayers of Mefp-1 for active and thermally inactivated enzyme were nearly identical. Attenuated total reflection Fourier transform infrared spectroscopy was used to characterize these interactions at a germanium (Ge) interface.** © 2000 Academic Press

Key Words: **mussel adhesive protein; Mefp-1; alginate; tyrosinase; periodate; ATR-FTIR.**

INTRODUCTION

Sea mussels, such as *Mytilus edulis*, tether themselves to natural or engineered materials in marine intertidal zones by fabricating a set of byssal threads, each of which is anchored to the surface via an adhesive plaque. The attachment is completed within minutes under conditions that would seriously challenge bond formation by commercially available adhesives. Biochemical characterization of the plaque components has been fairly extensive. For this reason the sea mussel, and especially *M. edulis*, has become a model organism for studies aimed at understanding biological adhesion at the molecular level.

The plaque is composed of a protein matrix that is extruded as a foam (1) or gel (2) into a cavity formed by a retractable appendage known as the foot. The components harden to form the plaque by participating in a set of, as yet, uncharacterized cross-linking reactions. The finished plaque is covered by a varnish that also extends as a protective skin around the attached thread. A major component of the varnish is a protein known as *M. edulis* foot protein 1 (Mefp-1). Mefp-1 is also present in the interior of the plaque, concentrated near the substratum interface (3).

Mefp-1 is composed of tandem repeats of a hexa- and decapeptide sequence (4). The most abundant repeated motif of Mefp-1 is the decapeptide sequence. The sequence includes two lysine residues and the unusual amino acid L-3,4-dihydroxyphenylalanine (L-dopa). When the catechol functionality present on the L-dopa is oxidized it becomes a quinone, prone to participate in numerous side reactions (5, 6). One of the protein components of the adhesive plaque is a catechol oxidase (7), suggesting strongly that one or more possible reaction pathways involving quinones are essential for cross-linking.

There exist a number of possible reactions between quinones and other moieties present in the plaque reaction mixture that would result in covalent cross-links. Among these is nucleophilic addition (Michael addition) of the ε-amino group of the lysine into the quinone (1). The reaction has been demonstrated to occur in aqueous solution between small organic molecules having appropriate functional groups and is fairly well characterized (8–11). In these studies either the conversion of the catechol to the quinone is catalyzed by a catechol oxidase (typically mushroom tyrosinase from *Agaricus sporus*) or the oxidation is induced chemically by sodium periodate. Previously, it was shown that alginate adsorption to adlayers of Mefp-1 at a Ge interface can be used as a quantitative probe for ε-amino groups of lysine residues using attenuated total reflection Fourier transform infrared spectroscopy (ATR-FTIR) (12). In this paper, this assay is used to characterize the influence of exposure of adlayers of Mefp-1 to mushroom tyrosinase or sodium periodate on the density of ε-amino groups.

[1] To whom correspondence should be addressed.

0021-9797/00 $35.00
Copyright © 2000 by Academic Press
All rights of reproduction in any form reserved.

MATERIALS AND METHODS

Materials

Mefp-1 was purchased from Bioscience Laboratory (Floda, Sweden) and stored desiccated at −40°C. Purity was checked by acetic acid–urea polyacrylamide gel electrophoresis (13) indicating no trace of other L-dopa containing proteins. Stock solutions of Mefp-1 were made at 1 mg/ml in dilute HCl (pH 2.5) which had been deaerated by bubbling with nitrogen and stored at 5°C. The buffer used for adsorption experiments was 10 mM sodium phosphate (pH 7.2). Mushroom tyrosinase (EC 1.14.18.1), poly-L-lysine (P-1274), sodium alginate from *Macrocystis pyrifera* (A-2158), L-proline (P-0380), and sodium (m)-periodate (S-1878) were from Sigma Chemical Co. (St. Louis, MO). Water was ultra pure (Barnstead water purification system, Dubuque, IA). Ethyl alcohol and chloroform were HPLC grade. Isopropyl alcohol was analytical grade.

Surface Preparation

Single crystal, cylindrical germanium (Ge) internal reflection elements (IRE) (Spectra Tech, Stamford, CT) were cleaned by ultrasonication in a base bath (saturated KOH in isopropyl alcohol) for 10 min followed by a series of rinses which all consisted of ultrasonication in various liquids for 10 min. Following the base bath were two rinses in ultrapure water followed by a gentle scrubbing with undiluted Micro™ cleaning solution using cotton swaps. The cleaning solution was flushed off in a hard stream of ultrapure water. The IRE was then subjected to the following rinses: ultrapure water (2×), ethyl alcohol, and chloroform.

Flow Chamber

The cylindrical IRE was positioned within a stainless steel flow chamber (Circle Cell™, Spectra Tech), held in place by two Teflon O-rings. The interior cavity is cylindrical (diameter, 0.476 cm; length, 2.7 cm; fluid volume with IRE in place, 0.289 cm^3). Fluid was drawn through the flow chamber at 0.5 ml/min using a peristaltic pump (Cole-Parmer Instrument Co., Niles, IL) coupled to the silicone effluent tubing. All influent tubing and fittings were Teflon (0.08 cm I.D.) which were cleaned by sonicating in base bath. A Teflon valve (Cole-Parmer Instrument Co.) channeled influent feed from among two vessels. All glassware were cleaned in base bath.

FTIR Spectroscopy

During the course of each experiment infrared (IR) spectra were acquired during 5 min intervals. A Nicolet 740 Fourier transform infrared (FTIR) spectrophotometer equipped with a liquid N_2 cooled, medium range mercury–cadmium–telluride detector (5000–580 cm^{-1}) was used to collect the FTIR spectra. Interferograms were double sided, acquired with nominal resolution of 4 cm^{-1}, and apodized with a Happ–Genzel function. At least 200 interferograms were averaged per spectrum; when necessary, water vapor bands were removed by subtraction of a pure water vapor spectrum. Fluctuations in intensity of the strong water band at 1640 cm^{-1} resulted in the appearance of this band in the difference spectra. This residual water absorption band was removed by subtracting out a pure buffer spectrum using the ratio of areas of the absorption water band centered at 2120 cm^{-1} as a normalization factor (14). Variation in absorbance values resulting from slight differences in alignment of the flow chamber were normalized by using the area of the water absorption band at 1640 cm^{-1} (area: 1540 to 1740 cm^{-1}) as an internal standard (15). Areas of spectral features were computed for the region bounded by the data curve and a linear baseline drawn between the two end points of the integration. Theoretical descriptions of the evanescent field which penetrates from the surface of the IRE in the attenuated total reflection (ATR) geometry are available (16). Briefly, for a 45° incident angle, the evanescent field intensity decreases exponentially with distance from the IRE surface such that 95% of the energy is contained within 0.477 μm of a Ge–water interface at 2000 cm^{-1} and within 1.05 μm at 900 cm^{-1}. Protein surface coverage was estimated based on a previously published expression using bovine serum albumin as a standard (17).

Adsorption Protocol

For adsorption of proteins a vial containing 1 ml of the appropriate concentration in a phosphate buffer was inserted into the flow system and the protein solution was immediately pumped through a short (∼20 cm) section of leader tubing and through the flow chamber for 120 s. Flow was stopped during the adsorption period. Adsorption of protein was done under these static conditions to conserve material. Exposure to other solutions was done under flowing conditions.

The general methodology used here was developed previously (12) and is illustrated in Fig. 1. A biopolymer, in this case Mefp-1 or polylysine, is adsorbed to the Ge surface from a bulk solution. A rinse period follows. Adsorption of Mefp-1 or polylysine

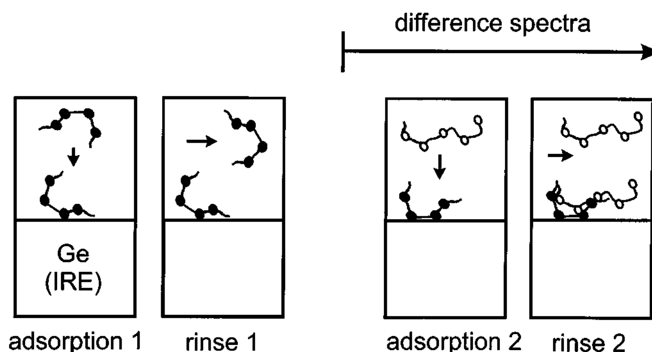


FIG. 1. Schematic of experimental design. An adlayer is deposited on the surface of a Ge substratum from the bulk liquid (adsorption 1). The bulk solution is replaced with buffer (rinse 1). The adlayer is exposed to a solution of a second substance (adsorption 2), followed by a rinse (rinse 2). The spectrum acquired just prior to the introduction of the second substance is used as background for difference spectra used to characterize interactions between the adlayer and the second substance.

results in the formation of a stable film, i.e., an adsorbed adlayer for which desorption during subsequent rinsing is negligible. At the end of this rinse period a second biopolymer is introduced, in this case alginate or mushroom tyrosinase. Difference spectra acquired during adsorption of the second biopolymer and the subsequent rinse period are analyzed to obtain information about interaction of the two biopolymers at the interface.

Assay for ε-amino Groups

Results obtained previously suggested that alginate could be used to probe for ε-amino groups of lysine side chains in adlayers of Mefp-1 (12). Alginate is a linear copolymer composed of the subunits mannuronic and guluronic acid. Difference spectra of alginate adsorbed to surfaces presenting primary amines exhibit distinctive features. This result provides the basis for an assay based on ATR-FTIR.

For experiments here, adlayers of Mefp-1 (or polylysine) were exposed to either mushroom tyrosinase or sodium periodate between the end of rinse 1 and the beginning of adsorption 2 (Fig. 1). For polylysine and Mefp-1, adsorption 1 was from a 0.05 mg/ml bulk solution. Based on previous results (12) this bulk concentration was found to result in a surface coverage of approximately 60% of the saturation coverage. The strategy was to optimize the surface coverage, while conserving Mefp-1. Choice of the buffer and pH was aimed at approximating conditions in which Michael addition has been characterized (8–11). Cross-linking of adlayers of Mefp-1 on graphite, catalyzed by mushroom tyrosinase, was reported under similar buffer conditions (18).

Figure 2 shows a spectrum of bulk alginate and representative difference spectra of alginate adsorbed onto adlayers of either Mefp-1 or polylysine. Difference spectra were acquired at the end of rinse 2. Most features of difference spectra of alginate adsorbed on Mefp-1 and polylysine are either altered or distinct from features of bulk alginate. In some cases features of bulk alginate appear in the difference spectra (e.g., Fig. 2c), probably due to nonspecific adsorption to some interfacial component. However, in these cases spectral features that have no component in the bulk spectrum can be used to follow the interaction with the ε-amino groups. The spectral feature that can be used most reliably for this purpose is the band indicated on Fig. 2b. This band is referred to as alg II, for consistency with the previous study (12). The surface coverage obtained in adsorption 1 is somewhat variable. In order to normalize for these differences in surface coverage between experiments the area of alg II (indicating relative density of ε-amino groups in the adlayer) is normalized to the area of the amide II band (proportional to protein surface coverage (15)). This normalized alg II area is referred to as alg II/II.

Sodium Periodate Oxidation

A 1-ml stock solution was made in phosphate buffer at 100 mM less than 5 min prior to use. The appropriate dilution was made into a 20 ml phosphate buffer, stirred vigorously, and pumped through the flow chamber. For experiments with L-proline, the 20 ml of phosphate buffer contained 20 mM L-proline.

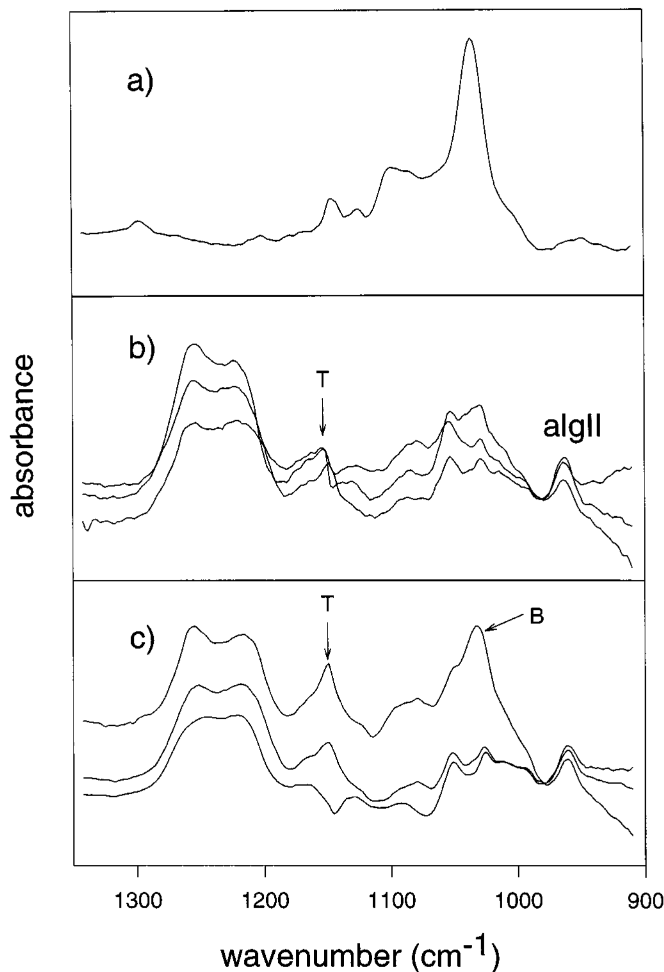


FIG. 2. Spectra of alginate: (a) 10 mg/ml in solution in phosphate buffer, (b) adsorbed onto a Mefp-1 adlayer, (c) adsorbed onto a polylysine adlayer. Spectra for (b) and (c) were acquired as difference spectra as in Fig. 1. Band (alg II) used to quantify binding to lysine residues is indicated in (b). Feature of bulk alginate originating from nonspecific adsorption (B) is indicated in (c). Feature originating from Teflon O-rings (T), not completely subtracted out, is indicated in (b) and (c).

RESULTS

Figure 3 shows alg II/II at the end of rinse 2 for exposure of adlayers of Mefp-1 to various concentrations of sodium periodate for 30 min followed by a 30-min rinse period. Data points are the mean of three experiments and error bars are standard deviations. A reaction time of 30-min is sufficient to allow reactions in solution to come to completion (first order time constants about 3–5 min) (10). The concentrations of sodium periodate used are considerably lower than those used for the solution studies (e.g., 1.44 mM (10)). At these concentrations there is no detectable

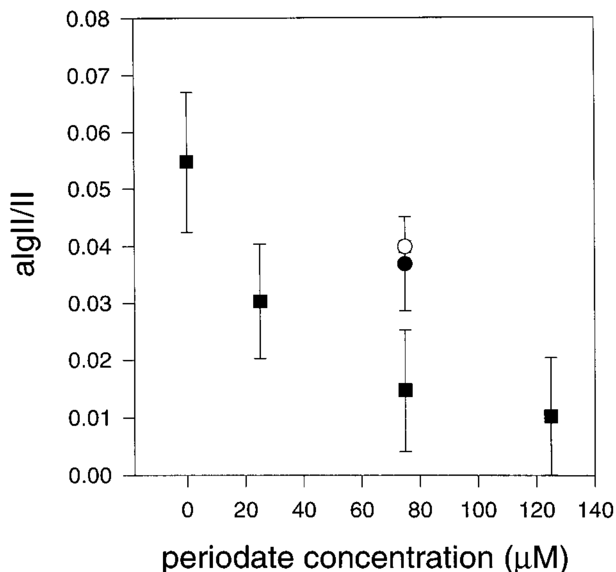


FIG. 3. Mefp-1 adlayers were exposed for 30 min to various concentrations of sodium periodate. Extent of binding of alginate to amines of lysine residues in the adlayer is indicated by alg II/II. Error bars are standard deviations for three experiments except for data at 0 μM sodium periodate (five experiments). Circles are experiments in which 100 mM proline was included with sodium periodate. In one set of three experiments exposure to the sodium periodate–proline mixture was followed by an additional exposure to sodium periodate for 30 min (open circles).

IR signal from bulk sodium periodate. Thus difference spectra acquired during the exposure period can be used to search for small spectral changes associated with chemical reactions in the adlayer. Difference spectra, as well as first and second derivatives of these spectra, were scrutinized for such changes. The only reproducible change observed was in the relative area of the small band indicated in Fig. 4a. A plot of the area of this band normalized to the amide II area versus time is shown in Fig. 4b for adsorption 1, rinse 1, exposure to sodium periodate, and the 30-min rinse period. Normalization to amide II was to remove variations in band area that may have arisen from small changes in signal intensity and/or surface coverage. The curve indicates that reaction with sodium periodate increases the area in a manner consistent with possible first order kinetics of the reaction (10). The position of this band is in the range expected for vibrational modes associated with substituted aromatics and may be an enhanced ring mode originating from the aromatic ring of either the L-dopa or the tyrosine residues. Michael addition into this ring might be expected to cause a change in relative band area. In this case there should be a (negative) correlation between the change in this band area and the loss of ε-amino groups. Figure 5 shows the relation between the change in this band area during exposure to periodate (the quantity X in Fig. 4b) versus alg II/II, for all the experiments used to accumulate data for Fig. 3. The correlation for the linear regression is quite poor ($R^2 = 0.23$), suggesting that the processes responsible for the loss of ε-amino groups and increase in the band presented in Fig. 4b are independent. However, the change in area of this band is on the edge of our detection limit and this may account for the poor correlation.

Proline has been shown to add into the quinone ring through Michael addition (8, 10) and, in fact, the reaction is the basis for one assay for conversion of catechol to quinone (19). It is expected that proline in solution would add into the quinone form of the L-dopa residues under appropriate conditions. Once addition of proline into the quinone ring occurred this would prevent reaction with ε-amino groups of the Mefp-1 lysine residues. Therefore, proline in solution would be expected to compete with lysine residues for addition into the quinone and to moderate the loss of ε-amino groups of Mefp-1 (Fig. 3) if the reaction responsible for this loss is Michael addition of ε-amino groups into the oxidized L-dopa residue. The results of this experiment are presented in Fig. 3. After rinse 1, Mefp-1 adlayers were exposed to a mixture of 20 mM L-proline and 75 μM sodium periodate for 30 min followed by a 30-min rinse (three experiments). In another three experiments the 30-min rinse was followed by exposure to 75 μM sodium periodate for 30 min followed by another 30-min rinse. This last set of experiments was performed

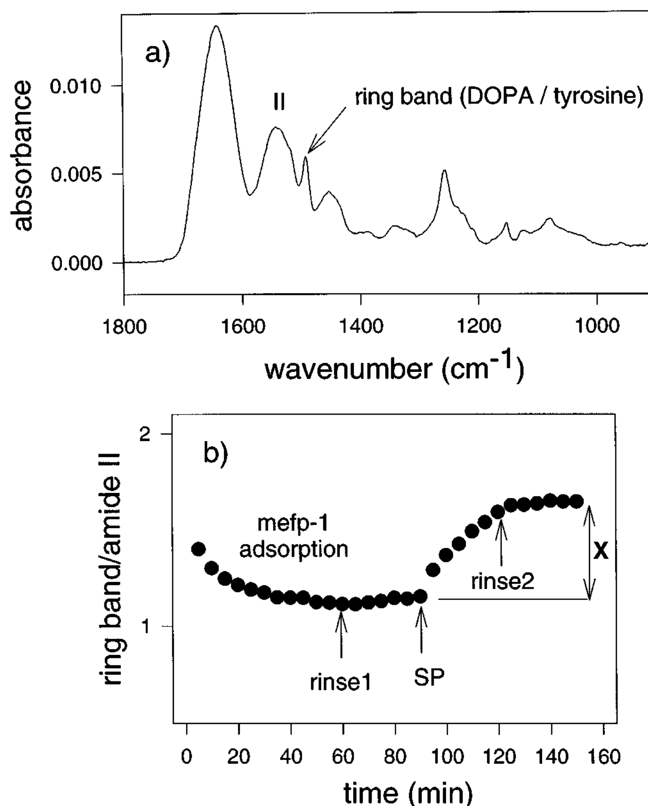


FIG. 4. (a) Spectrum of Mefp-1 adlayer on Ge. Amide II band and small band that may originate from the L-dopa residues are indicated. (b) Time course of change in area of ring band indicated in (a) normalized to amide II band. Time of introduction of sodium periodate into flow chamber (SP) and beginning of rinse 1 and 2 are indicated. X is quantity used to test correlation between change in putative L-dopa band and binding of alginate to lysine residues measured by algII/II (Fig. 5).

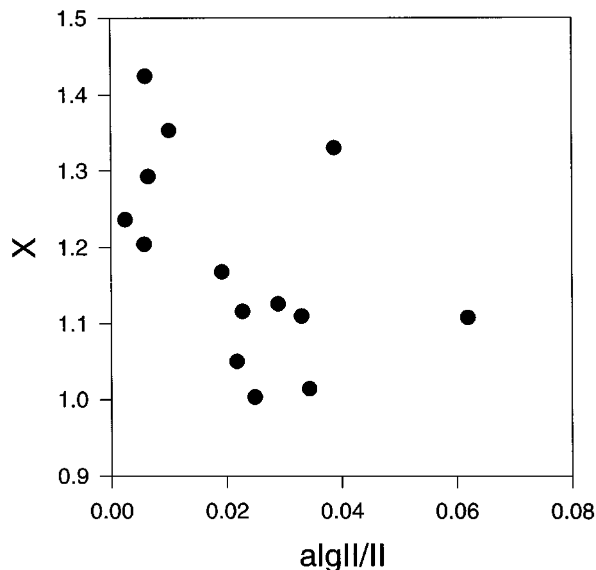


FIG. 5. Quantity indicated in Fig. 4b (X) plotted versus alg II/II at end of rinse 2 for experiments used to acquire data presented in Fig. 3.

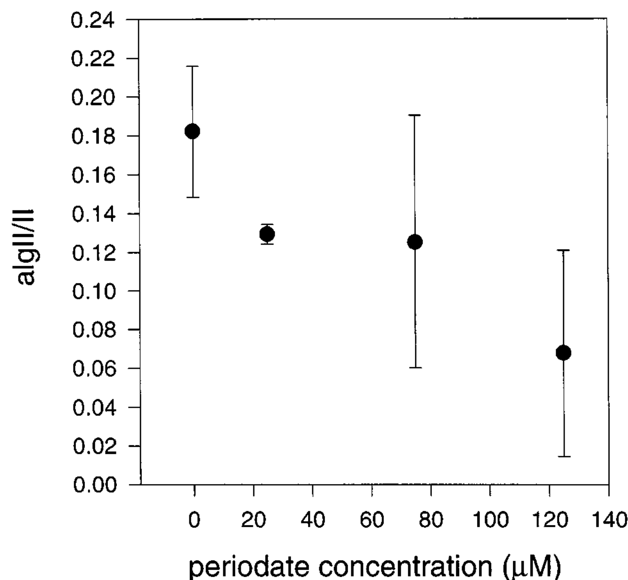


FIG. 6. Similar to Fig. 3, but data are for polylysine adlayers instead of Mefp-1 adlayers. Means and standard deviations are for three experiments except data for exposure to 75 μM sodium periodate (five experiments).

in order to determine whether the moderation in loss of ε-amino groups induced by addition of the proline was due simply to reaction of the sodium periodate with the proline. It can be seen that in both sets of experiments the loss of amines is moderated by exposure to the mixture of proline and sodium periodate (means are significantly different at the 0.05 level using unpaired Student's t test).

Difference spectra acquired at the beginning of exposure of Mefp-1 adlayers to proline reveal that there is a small amount of proline that remains at the surface at the end of the 30-min rinse (data not shown). It is presumably bound to the Mefp-1 adlayer since proline is reversibly rinsed from clean Ge (data not shown). The spectral features of this residue correspond to those of bulk proline with no apparent modification by reaction with the Mefp-1 adlayer.

If the loss of ε-amino groups of lysine induced by exposure to sodium periodate is due exclusively to reaction with Mefp-1 quinones then adlayers of polylysine should not exhibit this loss. Results of exposure of adlayers of polylysine to sodium periodate, following an identical protocol to that used to obtain results for adlayers of Mefp-1 (Fig. 3), are shown in Fig. 6. As in Fig. 3, data points are means and error bars are standard deviations based on the results of three experiments, with the exception of data for 75 μM sodium periodate, which is based on the results of five experiments. Although comparison of the shape of the data curves of Figs. 4 and 7 suggests that the reactions responsible for the loss of ε-amino groups are different for Mefp-1 and polylysine, the hypothesis that the reaction involves L-dopa residues is weakened considerably.

For oxygenated solutions, sodium periodate and mushroom tyrosinase are both used as the oxidant for Michael addition (9, 10). Mushroom tyrosinase has the advantage that it can be thermally inactivated to provide a control (20). A spectrum of mushroom tyrosinase is shown in Fig. 7. The spectrum presented was acquired for adsorption from a 0.5 mg/ml mushroom tyrosinase solution onto a clean Ge substratum. Spectra of both active and inactive enzymes adsorbed to adlayers of Mefp-1 have features essentially identical to the spectrum presented in Fig. 7, i.e., at the S/N of our system there were no consistent differences in spectral features (data not shown). The kinetics of adsorption of mushroom tyrosinase onto adlayers of Mefp-1 are shown in Figs. 8 and 9. Data points are means and standard errors for three experiments. The amide II band used to follow the kinetics is indicated in Fig. 7. (Limits for the area calculation were 1481–1590 cm^{-1}). Figure 8 shows data for a relatively dilute bulk solution of mushroom tyrosinase (0.01 mg/ml), i.e., comparable

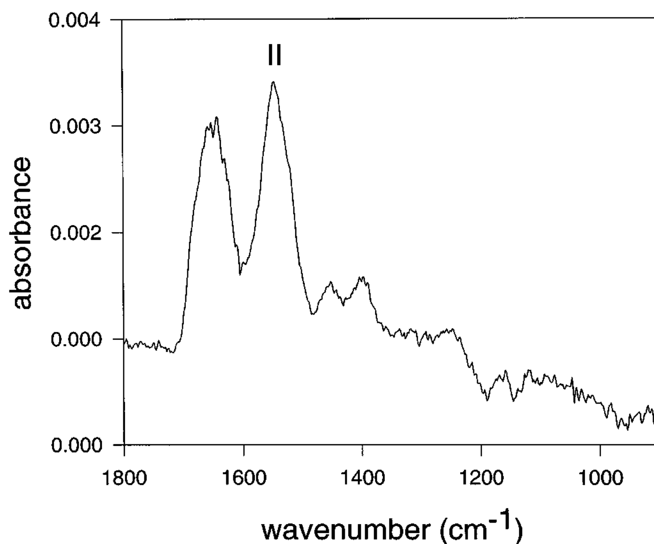


FIG. 7. Spectrum of adlayer of mushroom tryrosinase on Ge. Amide II band (II) used to follow kinetics of adsorption is indicated.

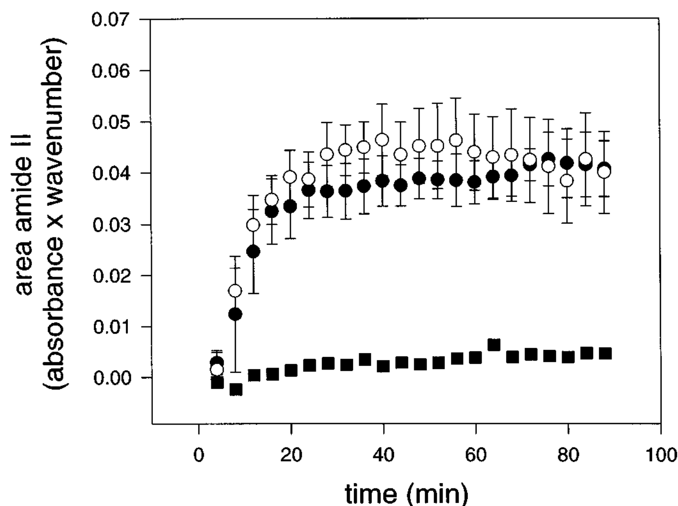


FIG. 8. Kinetics of adsorption of mushroom tyrosinase onto adlayer of Mefp-1 (circles) or clean Ge (squares) from a 0.01 mg/ml bulk solution. Open circles are thermally inactivated enzyme. Means and standard errors of three experiments are presented for adsorption onto the Mefp-1 adlayers.

to catalytic concentrations used for solution studies (10). Data for Fig. 9 were obtained using bulk concentrations 50× higher (0.5 mg/ml). This latter bulk concentration was used in a study in which catalyzed cross-linking of Mefp-1 adlayers was observed using atomic force microscopy (18). In this study cross-linking of Mefp-1, catalyzed by mushroom tyrosinase, was reported to be complete after a 20-min exposure period. In addition to kinetics of adsorption of the active enzyme onto adlayers of Mefp-1, Figs. 8 and 9 present data for the thermally deactivated enzyme and data for adsorption of mushroom tyrosinase onto a clean Ge substratum. This latter data set indicates that adsorption of mushroom tryrosinase is primarily due to binding to the adlayer of Mefp-1 at the lower bulk concentration (Fig. 8). For these experiments it can be estimated that roughly one molecule of

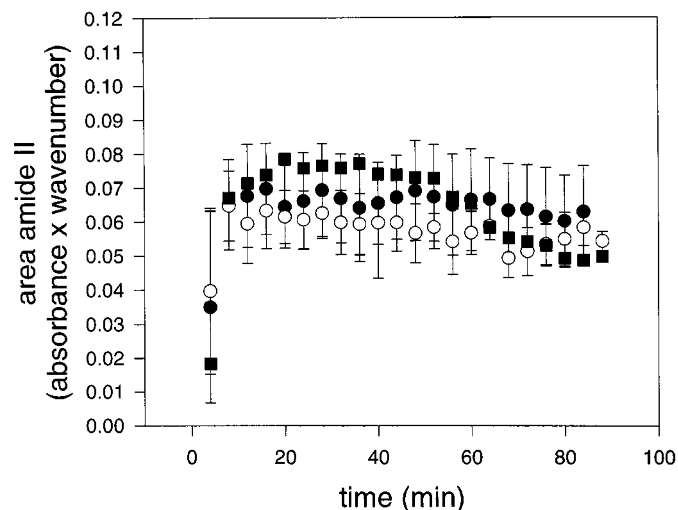


FIG. 9. Similar to Fig. 8 but adsorption of mushroom tyrosinase was from a 0.5 mg/ml bulk solution.

TABLE 1
Values of algII/II for Mefp-1 Adlayers Reacted with Mushroom Tryrosinase

Row	Adsorption condition[a]	algII/II ($\times 10^2$)
1	Mefp-1(0.05)/Ge(no exposure to mt)	5.48 (1.23)[b]
2	mt(0.01)/Mefp-1/Ge	4.67 (0.047)
3	mt-inact(0.01)/Mefp-1/Ge	5.01 (0.049)
4	mt(0.5)/Mefp-1/Ge	3.72 (1.92)
5	mt-inact(0.5)/Mefp-1/Ge	4.57 (1.94)
6	mt(0.5)/Ge	0.029[c]
7	Mefp-1(0.05) + mt/Ge(0.01)	6.23 (0.064)

[a] mt, mushroom tyrosinase; mt-inact, thermally inactivated enzyme. Numbers in parenthesis are bulk concentrations in mg/ml; subsequent exposure of substratum to substances is from right to left; e.g., mt(0.01)/Mefp-1/Ge is for the adlayer of Mefp-1 on Ge exposed to 0.01 mg/ml mushroom tyrosinase. Mefp-1(0.05) + mt/Ge(0.01), adlayer formed from a mixture of Mefp-1 and mt in bulk solution.
[b] Mean (standard deviation).
[c] Two experiments.

mushroom tryrosinase is adsorbed for each four molecules of Mefp-1 in the pre-adsorbed adlayer with an average Mefp-1 surface coverage of 0.17 μg/cm^2. There appears to be no significant difference in the kinetics of adsorption of the active and inactive enzyme onto adlayers of Mefp-1.

For each experiment used to obtain the data presented in Figs. 8 and 9 alginate was adsorbed to the composite adlayer of Mefp-1 and mushroom tyrosinase. This was followed by a rinse period. During this adsorption and rinse period (adsorption 3 and rinse 3) difference spectra were acquired as described above for adsorption 2 and rinse 2 (Fig. 1). Table 1 shows the mean values and standard deviations (three experiments) of alg II/II at the end of rinse 3 for the different conditions used to obtain data presented in Figs. 8 and 9 and for Mefp-1 adsorbed to clean Ge substratum for comparison (data of Fig. 3 for 0 μM periodate). The amide II band used for the normalization was that obtained at the end of rinse 1 in all cases. Adlayers of mushroom tyrosinase have almost negligable ε-amino groups accessible for alginate binding as indicated by the data for adsorption of alginate to adlayers of mushroom tyrosinase on clean Ge at 0.5 mg/ml bulk concentration (mt(0.5)/Ge). This is not surprising since the lysine content of mushroom tyrosinase is only 4.9% (21). Thus, the appearance of alg II upon exposure of composite Mefp-1–mushroom tyrosinase adlayers to alginate is due to binding of alginate to the ε-amino groups of Mefp-1. There is a slight reduction of alg II/II caused by the reaction of mushroom tyrosinase with Mefp-1 adlayers, especially at the higher bulk concentration (compare row 1 with rows 2–5). The means that are most significantly different in rows 1–5 are the pair for Mefp-1 not exposed to mushroom tyrosinase (Mefp-1/Ge) and Mefp-1 exposed to 0.5 mg/ml (active) mushroom tyrosinase (mt(0.5)/Mefp-1/Ge); however, the p-value of the Student's t test is well above 0.05 ($p = 0.1331$). For both high and low mushroom tyrosinase concentrations the reduction in alg II/II values compared to unexposed Mefp-1 is slightly greater for the active enzyme; however, the difference between means for

these pairs of experiments (active versus inactive mushroom tyrosinase) is still less significant ($p = 0.3007$ and $p = 0.6186$ for rows 2, 3 and 4, 5 of Table 1, respectively). This suggests that the small reduction in alg II/II for adlayers of Mefp-1 exposed to mushroom tyrosinase (compared to unexposed adlayers) is due to masking of the alginate binding sites on the Mefp-1 adlayer by the adsorbed mushroom tyrosinase (e.g., some form of steric hindrance), rather than reduction in available ε-amino groups resulting from a catalyzed Michael addition. In Table 1 are also presented alg II/II data for adlayers formed by exposing Ge to a mixture of Mefp-1 (0.05 mg/ml) and mushroom tyrosinase (0.01 mg/ml) (row 7 of Table 1). Again, the difference between the means of rows 1 and 7 is not significant at the 0.05 level (0.3591).

DISCUSSION

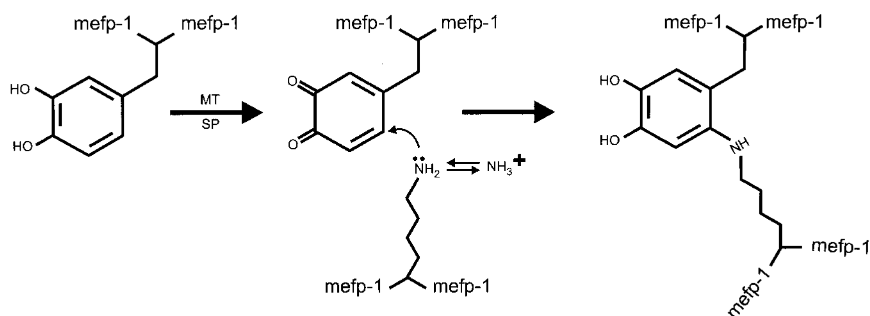
Although Mefp-1 is sometimes referred to as the mussel adhesive protein, it is only one of a number of proteins that are present in the adhesive plaque of *M. edulis*. Of the other plaque components, two L-dopa containing proteins (Mefp-2,3) have been purified to homogeneity (22, 23) and two catechol oxidases from *M. edulis* have been well characterized (7). In addition, at least one collagen is present in the adhesive plaque (24). The functional role of these biopolymers in plaque formation has not been determined.

The adhesive plaque serves to anchor byssal threads to a chosen substratum. Although the molecular interactions responsible for plaque formation have not been characterized, it is obvious that components of the plaque must form bonds to the surface and with each other. For both these functions a number of possible roles of the L-dopa residues of Mefp-1 have been suggested (1). Among these possibilities are many types of chemical reactions that result in covalent bonds (5, 6). The catechol itself is not very reactive. However, the oxidized form (quinone) can participate in numerous reactions that are sometimes collectively referred to as quinone tanning reactions (5). One of the subgroups of possible reactions is nucleophilic addition (a form of Michael addition) into the aromatic ring.

One potential nucleophile that is available in large abundance in Mefp-1 is the ε-amino group of the lysine residues. Of the decapeptide amino acid resides, lysine and L-dopa are the most faithfully conserved for protein variants found within the species (25). An inevitable hypothesis is that cross-linking of proteins (specifically Mefp-1) in the adhesive plaque involves the addition of lysine residues into the oxidized L-dopa residues. The possible reaction sequence, based on solution studies, is shown in Scheme 1.

Results presented here (Fig. 3) indicate that the number of ε-amino groups of the lysine residues of a Mefp-1 adlayer, accessible for binding to alginate, is diminished by exposure to sodium periodate. This suggests formation of aryl-lysine bonds by reaction of lysine and L-dopa residues. The moderation of loss of ε-amino groups by inclusion of proline in the reaction mixture lends support to this explanation, since proline would be expected to add into the quinone rings of L-dopa, competing with lysine residues. However, the results for adlayers of polylysine (Fig. 6) detract from this interpretation, since exposure to sodium periodate causes loss of ε-amino groups in this case and L-dopa is absent. These latter results suggest that sodium periodate reacts directly with the amines. The nature of this reaction is not apparent. Regions of the spectrum of adlayers of polylysine in which primary amines or corresponding amine salts are expected to produce bands (stretch, deformation, and wag regions) exhibit no reproducible changes during exposure of adlayers of polylysine to sodium periodate (data not shown). However, failure to detect changes in these regions could be due to insufficient sensitivity. The oxidation of glycols having adjacent hydroxyl groups by sodium periodate is a well-known reaction (26). The reaction is used to specifically modify carbohydrate moieties of proteins at periodate concentrations in the mM range (27, 28). Sodium periodate (15 mM) has also been used as an oxidant for regeneration of lysines from adducts, with no suggestion of any subsequent modification of the ε-amino groups by the excess periodate (29). The assumption of the specificity of sodium periodate oxidation is also implicit in solution studies of Michael addition. Therefore, its apparent reaction with primary amines for results here is unanticipated.

Quinones are involved in a number of biological processes including melanin formation and cuticle sclerotization and therefore enzymes having tyrosinase activity are fairly widespread.



SCHEME 1. Possible cross-linking reaction between Mefp-1 residues through Michael addition. Oxidation of catechol functionality of L-dopa by mushroom tyrosinase (MT) or sodium periodate (SP) is followed by nucleophilic addition of ε-amino groups of lysine residues of Mefp-1 into the ring.

Enzymes from *Agaricus bisporus* (used here) and *Neurospora crassa* (30) have been most well characterized. Mushroom tyrosinase is expected to bind to the catechol functionality of L-dopa residues by coordination of the oxygens to a binuclear copper site (30, 31). Adlayers of Mefp-1 promote binding of mushroom tyrosinase (compared to a clean Ge substratum). However, there is no evidence that binding to Mefp-1 adlayers is enhanced or even altered by the integrity of the active site (Figs. 8 and 9). Although exposure to high temperature destroys the activity of mushroom tyrosinase (20), its precise effect on the structure of the protein has not been investigated.

Binding of mushroom tyrosinase onto adlayers of Mefp-1 causes lysines to become slightly less available for alginate binding (Table 1). However, the differences are not significant. It may be that with more repetitions of the experiment these differences would become significant. However, more importantly, the differences between active and inactive enzyme are quite slight; for catalytic amounts of enzyme (Table 1, rows 2 and 3), even though standard deviations are reasonably small, means are not significantly different even at the 0.30 level. This suggests that the moderate reduction in ε-amino groups in Mefp-1 adlayers by exposure to mushroom tyrosinase is due to obstruction of alginate binding sites on Mefp-1 (some form of steric hindrance), rather than the result of a reaction with quinones. Results of Table 1, row 6 indicate that binding of mushroom tyrosinase to Mefp-1 adlayers contributes only a slight portion of ε-amino groups to the composite adlayer and this is consistent with the relatively low lysine content of mushroom tyrosinase (4.9%) (21).

Addition of the ε-amino group of lysine into a quinone has been proposed as the mechanism of formation of a cross-linked enzyme cofactor (32). However, the prevalence of lysine participation in quinone tanning in general was questioned in an early exhaustive review (5). More recently, evidence was presented suggesting that the addition of the ε-amino group of lysine into the quinone ring of L-dopa is an improbable reaction (33). A study of threads and plaque using nuclear magnetic resonance failed to detect any aryl-amine bonds (34). A similar study found evidence for diphenolic cross-links in adhesive plaques produced by mussels subjected to a laminar flow regime during growth (35). Formation of diphenolic bonds could be responsible for the spectral changes in Mefp-1 induced by exposure to sodium periodate (Fig. 4).

There are other possible roles of the lysines of Mefp-1, besides participating in aryl-lysine cross-links. Formation of inter- or intramolecular hydrogen bonds might make L-dopa residues of Mefp-1 less accessible to other reactive plaque components. The abundance of lysine residues in Mefp-1 might serve to prevent hydrogen bond formation, thus preserving an open conformation (36). The disruption of the secondary structure of polylysine for pH below the pK_a of the side group is well known (37). Lysine ε-amino groups may serve to cross-link plaque components through bonds with acidic functionalities. Ionic bonds are comparable to covalent bonds in strength (38). The specific plaque components (Mefp series) that have been most well characterized all have very positive isoelectric points. However, the plaque proteins as a whole are much more balanced in terms of having acidic, neutral, and basic residues (39, 40). Mefp-2 contains C and N terminal regions with acidic residues (22). In addition all the byssal collagens characterized thus for contain an acidic cluster of residues (41–43). The catechol oxidase of the mussel is present in large amounts suggesting that it may be a structural, as well as a catalytic component (25). The amino acid composition of the pro-enzymes in the mussel foot suggests that the acidic amino acid content of the catechol oxidase in the plaque may be relatively large (28%) (7). In a relatively early study of the mussel byssus it was suggested that a significant portion of the plaque might consist of a mucous (polysaccharide) substance (44). Recently, it was found that Mefp-1 forms a complex with pig gastric mucin in solution (45). It has been suggested that binding of plaque components to an acidic polysaccharide would neutralize the positive charge carried by the lysine residues, facilitating condensation (2). Alginate might serve as a relevant model compound for studies aimed at ferreting out possible interactions between relatively well-characterized basic proteins of the adhesive plaque and components that are primarily composed of acidic subunits or have acidic segments.

SUMMARY

The *M. edulis* adhesive plaque is composed of a mixture of biopolymers (perhaps exclusively proteins) that have associated to form a number of condensed phases. The most well-characterized protein in the mixture is Mefp-1. Mefp-1 is heavily laden with lysine residues and the unusual residue L-dopa. Oxidation of the L-dopa residues to the quinone offers many possibilities for reactions that form covalent bonds. One possibility is the addition of the lysine residues into the ring to form aryl-amine cross-links. The results presented here suggest that while exposure of adlayers of Mefp-1 on Ge to sodium periodate may induce formation of aryl-amines, exposure to mushroom tyrosinase does not.

ACKNOWLEDGMENTS

This study was supported by a grant from the Office of Naval Research (N00014-97-1-1062) (Geesey) and the National Science Foundation (EEC8907039) (Center for Biofilm Engineering).

REFERENCES

1. Waite, J. H., *Int. J. Adhesion Adhesives* **7,** 9 (1987).
2. Laursen, R. A., *in* "Biopolymers" (S. T. Case, Ed.), pp. 55–74, Springer-Verlag, Berlin, 1992.
3. Benedict, C. V., and Waite, J. H., *J. Morphol.* **189,** 171 (1986).
4. Waite, J. H., Housley, T. J., and Tanzer, M. L., *Biochemistry* **24,** 5010 (1985).
5. Sugumaran, M., *in* "Advances in Insect Physiology" (P. D. Evans and V. B. Wigglesworth, Eds.), Vol. 21, pp. 179–231. Academic Press, New York, 1988.
6. Rzepecki, L. M., and Waite, J. H., *Bioorg. Marine Chem.* **4,** 119 (1991).
7. Burzio, L. A., "Catechol Oxidase Associated with Byssus Fromation in the Blue Mussel *Mytilus Edulis*." M. S. thesis, University of Delaware, 1996.
8. Mason, H. S., and Peterson, E. W., *Bioch. Biophys. Acta* **111,** 134 (1965).

9. Cabanes, J., Garcia-Canovas, F., and Garcia-Carmona, F., *Bioch. Biophys. Acta* **914,** 190 (1987).
10. Valero, E., Escribanop, J., and Garcia-Carmona, F., *Phytochemistry* **27,** 2055 (1988).
11. Kalyanaraman, B., Premovic, P. I., and Sealy, R. C., *J. Biol. Chem.* **262,** 11080 (1987).
12. Suci, P. A., and Geesey, G. G., *J. Colloid Interface Sci.* **172,** 347 (1995).
13. Waite, J. H., *Methods Enzymol.* **258,** 1 (1995).
14. Dousseau, F., Therien, M., and Pezolet, M., *Appl. Spectrosc.* **43,** 538 (1989).
15. Fink, D. J., Hutson, T. B., Chittur, K. K., and Gendreau, R. M., *Anal. Biochem.* **165,** 147 (1987).
16. Knutzen, K., and Lyman, D. J., *in* "Surface and Interfacial Aspects of Biomedical Polymers, Vol. 1, Surface Chemistry and Physics" (J. D. Andrade, Ed.), pp. 197–247. Plenum Press, New York, 1985.
17. Bhosle, N., Suci, P. A., Baty, A. M., Weiner, R. M., and Geesey, G. G., *J. Colloid Interface Sci.* **205,** 89 (1998).
18. Hansen, D. C., Corcoran, S. G., and Waite, J. H., *Langmuir* **14,** 1139 (1998).
19. Rzepecki, L. M., and Waite, J. H., *Anal. Biochem.* **79,** 375 (1989).
20. Lissitzky, S., and Rolland, M., *Bioch. Biophys. Acta* **56,** 95 (1962).
21. Strothkamp, K. G., Jolley, R. L., and Mason, H. S., *Bioch. Biophys. Res. Comm.* **70,** 519 (1976).
22. Rzepecki, L. M., Leszek, M., Hansen, D. C., Karolyn, M., and Waite, J. H., *Biol. Bull.* **183,** 123 (1992).
23. Papov, V. V., Diamond, T. V., Biemann, K., and Waite, J. H., *J. Biol. Chem.* **270,** 20183 (1995).
24. Qin, X., and Waite, J. H., *J. Exp. Biol.* **198,** 633 (1995).
25. Waite, J. H., *Int. J. Biol. Macromol.* **12,** 139 (1990).
26. Morrison, R. T., and Boyd, R. N., "Organic Chemstry," 3rd ed., p. 538. Allyn and Bacon, New York, 1973.
27. Wenneras, C., Neeser, J. R., and Svennerholm, A. M., *Infect. Immun.* **63,** 640 (1995).
28. Klein, B. S., and Jones, J. M., *Infect. Immun.* **62,** 3890 (1994).
29. Acharya, A. S., and Manjula, B. N., *Biochemistry* **26,** 3524 (1987).
30. Lerch, K., *Mol. Cell. Biochem.* **52,** 125 (1983).
31. Yong, G., Leone, C., and Strothkamp, K. G., *Biochemistry* **29,** 9684 (1990).
32. Wang, S. X., Mure, M., Medzihradszky, K. F., Burlingame, A. L., Brown, D. E., Dooley, D. M., Smith, A. J., Kagan, H. M., and Klinman, J. P., *Science* **273,** 1078 (1996).
33. Yu, M., Hwang, J., and Deming, T. J., *J. Amer. Chem. Soc.* **121,** 5825 (1999).
34. Klug, C. A., Burzio, L. A., Waite, J. H., and Schaefer, J., *Arch. Biochem. Biophys.* **333,** 221 (1996).
35. McDowell, L. M., Burzio, L. A., Waite, J. H., and Schaefer, J., *J. Biol. Chem.* **274,** 20293 (1999).
36. Williams, T., Marumo, K., Waite, J. H., and Henkens, R. W., *Arch. Biochem. Biophys.* **269,** 415 (1989).
37. Doty, P., *in* "Biophysical Science" (J. L. Oncley, Ed.), p. 108. Wiley, New York, 1959.
38. Israelachvili, J. N., "Intermolecular and Surface Forces," p. 32. Academic Press, New York, 1992.
39. Benedict, C. V., and Waite, J. H., *J. Morphol.* **189,** 261 (1986).
40. Waite, J. H., and Tanzer, M. L., *Biochem. Biophys. Res. Commun.* **96,** 1554 (1980).
41. Qin, X. X., Coyne, K. J., and Waite, J. H., *J. Biol. Chem.* **272,** 32623 (1997).
42. Qin, X. X., and Waite, J. H., *Proc. Natl. Acad. Sci. USA* **95,** 10517 (1998).
43. Coyne, K. J., Qin, X. X., and Waite, J. H., *Science* **277,** 1830 (1997).
44. Tamarin, A., Lewis, P., and Askey, J., *J. Morph.* **149,** 199 (1976).
45. Deacon, M. P., Davis, S. S., Waite, J. H., and Harding, S. E., *Biochemistry* **37,** 14108 (1998).