Role of L-3,4-Dihydroxyphenylalanine in Mussel Adhesive Proteins

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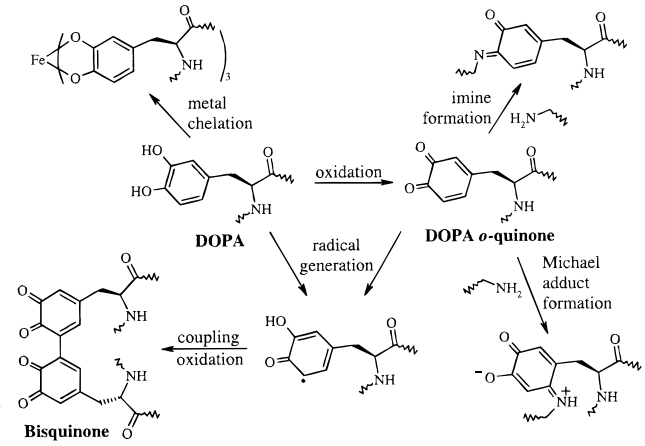
Mussel adhesive proteins (MAPs) have drawn interest for their ability to form strong adhesive bonds to a variety of substrates in wet environments.[1] There have been efforts to develop biomedical adhesives from these proteins, yet this has been hampered by the difficulty of isolation from biological sources.[2] Synthetic polymers are a potential source of functionally equivalent adhesives, yet little is known about how the MAPs function and therefore what must be incorporated into a synthetic analogue. The catechol functionality of L-3,4-dihydroxyphenylalanine, DOPA, residues is thought to be responsible for adhesion and cross-linking of the proteins; however, the mechanisms for these processes are unknown. The potential involvement of L-lysine and other polar residues in these reactions further complicates analytical efforts.[1] Speculation on the key components of these materials has been limited since no cross-link bond or specific bond to a substrate has been identified as yet. Through analysis of amino acid derivatives and simple copolypeptides under adhesive curing conditions, we have determined that DOPA is the only functional element required to reproduce the properties of MAPs. Furthermore, the primary roles of both catechol and *o*-quinone forms of DOPA can be assigned to adhesive bonding and cross-link formation, respectively.

The most detailed studies on MAPs have focused on the blue mussel, *Mytilus edulis*. This organism anchors itself to surfaces by means of plaques on the ends of fibrous threads. A considerable variety of adhesive proteins have been isolated from uncured plaques. These proteins range in mass from ca. 5 to 120 kDa, and all contain high levels of DOPA (ca. 5−20 mol %).[3] Variants also contain elevated levels of other polar amino acids such as hydroxylated prolines, lysine, and 4-hydroxyarginine. The variability of these proteins, in terms of their chain lengths, sequences, and compositions, has made it difficult to identify the important components responsible for adhesion.

It is known that catechol oxidase enzymes are present in MAP secretions that convert the catechol groups of DOPA into highly reactive *o*-quinone functionalities.[4] Numerous reactions have been proposed for cross-linking of the quinones (Scheme 1), yet none of these have been experimentally verified in MAPs.[1] The most often cited reaction is the Michael addition of side-chain amino groups of lysine residues to a DOPA−quinone residue.[5] Although all attempts to detect this product to date have been unsuccessful, the importance of the Michael addition in quinone chemistry has generated strong support for lysine cross-linking in MAPs.[6]

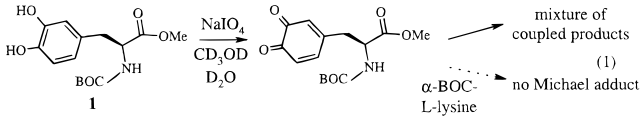
Our goal was to experimentally identify the roles of amino acids that are active in the adhesive chemistry of MAPs. Problems

Scheme 1. Hypothetical Cross-Linking Reaction Pathways for Peptidyl DOPA and DOPA *o*-Quinone Residues



associated with the complexity of polyfunctional proteins were avoided by studying small molecules and simple two-component copolypeptides. To connect the behavior of these model systems with that of MAPs, materials were analyzed using both molecular and macroscopic techniques (i.e., spectroscopy as well as measurement of bulk properties). Correlation of properties between small molecule and copolymer and then copolymer and protein thus connected the chemistry of individual amino acid components with the adhesive and cross-linking behavior of MAPs.

Initially, studies were focused on the oxidation and reactivity of single DOPA molecules. The methyl ester of *N*-BOC-DOPA, **1**, was reacted with $NaIO_4$ in pH 7.0 buffer, and the reaction was followed by ^{1}H NMR and UV/vis spectroscopy (eq 1). The



starting catechol was found to be quantitatively converted to the *o*-quinone within 5 min as expected (λ_{max} = 395 nm).[4] In the NMR reaction, a red DOPA-containing precipitate began to accumulate, as evidenced by a lack of aromatic protons in the NMR spectrum of the solution phase. In a dilute solution reaction followed by UV/vis spectroscopy, the quinone absorbance decreased in intensity over time and was gradually replaced by general absorption at higher wavelengths, also supportive of reaction of the quinone (Figure 1). It appeared that once the *o*-quinone groups were formed, they rapidly self-condensed to insoluble products.

When amines (e.g., α-*N*-BOC-L-lysine) were added to oxidized **1** in situ at pH 7.0, we observed no additional changes in the UV/vis spectra and no incorporation of amine into the product precipitates. Similar results were obtained when the reactions were performed under conditions similar to the tidal environment (pH 8.0). No evidence for Michael addition of primary amines to oxidized **1** was observed.[7] To determine the nature of the observed quinone self-condensation, we analyzed the precipitate formed in the oxidation of **1**. The product was found to be soluble in polar organic solvents (e.g., THF); however, ^{1}H NMR spectra in these solvents showed only numerous unassignable peaks. Size exclusion chromatography in THF showed that the products were low molecular weight compounds. These data were confirmed

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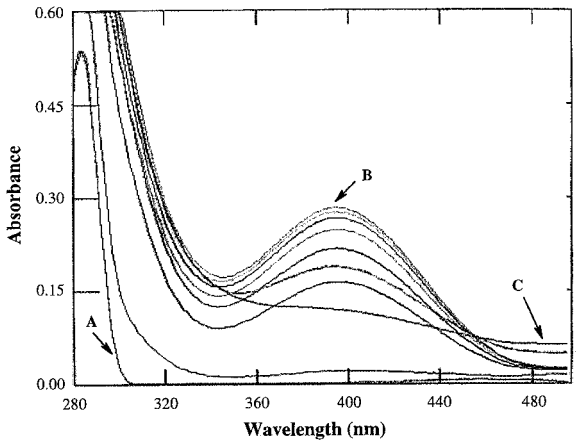

Figure 1. UV/vis spectrum of **1** mixed with $NaIO_4$ (10 equiv) in 0.025 M sodium phosphate at pH 7.0: (A) no oxidant; (B) 10 s after oxidant added; (C) 10 h after oxidant added.

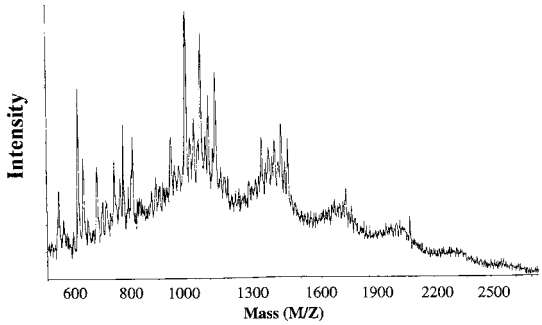

Figure 2. MALDI-TOF MS analysis of the oxidation products of **1**.

by MALDI-TOF mass spectra of the material. A series of DOPA oligomers were observed with masses ranging from 620 to 2620 Da, which correspond to dimers through octamers of the starting material (Figure 2). Thus, **1** couples to form bisquinone units (Scheme 1), which then condense with additional DOPA molecules to form higher oligomers.

To connect the chemistry of **1** with polymeric materials containing many DOPA residues, we studied simple copolymers of DOPA containing either L-lysine or L-glutamic acid. Statistical copolypeptides containing 5 mol % DOPA and the remainder either lysine or glutamic acid were prepared by standard procedures.[8] The resulting copolymers, poly(DOPA)$_{0.05}$(Lys·HBr)$_{0.95}$ (M_n = 148 000) and poly(DOPA)$_{0.05}$(Glu$^-$Na$^+$)$_{0.95}$ (M_n = 131 000), were both water soluble. DOPA reactivity in the copolymers was readily followed using UV/vis spectroscopy. Oxidation of each polymer with $NaIO_4$ in aqueous solution (pH 7) produced spectra that were intrinsically similar to one another, and to the data obtained for the DOPA model system. A quinone absorbance at 405 nm appeared initially, but over time decreased in intensity and was replaced by general absorption. With both copolymers, oxidation also resulted in gelation of the solutions. Viscosity measurements under oxidizing conditions showed that the copolymers formed networks of similar ultimate strength. This result indicated that the cross-link density, and thus the nature of the cross-links, was virtually identical for both copolypeptides. These data show conclusively that quinone curing in these copolypeptides does not require, and was not altered by, the presence of lysine residues.

We have previously shown that statistical copolypeptides of lysine and DOPA are able to form adhesive bonds in water comparable in strength to MAPs.[8] Bulk adhesive measurements on aluminum, where the bond was formed by oxidative curing of the polymer in water, were now undertaken for the DOPA−glutamate copolymer. It was found that this copolypeptide also formed moisture-resistant adhesive bonds (tensile strength 5.4(1.0) and 5.1(1.1) MPa for the lysine- and glutamate-containing copolymers at pH 7.0, respectively). As controls, poly(Lys·HBr) and poly(Glu$^-$Na$^+$) formed bonds of only negligible strength under similar curing conditions (0.6(0.2) and 0.6(0.4) MPa, respectively). Thus, it was evident that DOPA was the only residue required for both adhesion and cross-linking in these materials, which exhibit the key performance characteristics of MAPs.

To clarify the role of DOPA in the adhesion process, the bonding capability of a DOPA−lysine copolymer was measured in both the absence and presence of oxidant (H_2O_2). This experiment allowed separate evaluation of the abilities of DOPA−catechol and DOPA−quinone to adhere to wet surfaces. Bulk adhesive measurements of poly(DOPA)$_{0.05}$(Lys·HBr)$_{0.95}$ on aluminum under nitrogen with and without peroxide gave tensile strengths of 4.9(0.8) and 5.7(1.1) MPa, respectively. The bond strengths were similar, showing that the unoxidized catechol form of DOPA is primarily responsible for adhesion: addition of *o*-quinone groups did not improve, but rather weakened, bonding. By increasing the concentration of oxidant, the amount of catechol present for adhesion could be diminished. It was observed that rapid oxidation of copolymer solutions decreased adhesive-forming ability considerably (tensile strength 5.4(1.0) and 1.8-(0.7) MPa for 0.5 and 5.0% peroxide, respectively). Furthermore, rapidly oxidized adhesives formed bonds that failed at the interface, as opposed to cohesive failure for slowly oxidized samples, illustrating that catechol is the active form of DOPA in surface adhesion.

In summary, our experiments have shown that the adhesion and cross-linking chemistry of MAP model copolymers is due primarily to the DOPA residues in the materials. These studies also revealed that the catechol functionality is primarily responsible for moisture-resistant adhesion and that the oxidized *o*-quinone functionality is primarily responsible for cross-linking. These insights can be used to optimize the properties of DOPA-containing polymers to create highly effective moisture-resistant adhesives for both industrial and biomedical applications.

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Supporting Information Available: Details of all experiments, synthesis of materials, NMR and GPC of oxidation products of **1**, and UV/vis spectra and viscometry of oxidized polymers (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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