Are Conformational Changes, Induced by Osmotic Pressure Variations, the Underlying Mechanism of Controlling the Adhesive Activity of Mussel Adhesive Proteins?

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The mussel adhesive protein Mefp-1, under physiological conditions, presumably has a self-avoiding random walk conformation with helix-like or turned deca-peptide segments. Such a conformation may coil up under osmotic pressure induced by surrounding macromolecules. As a consequence, the orientation of the 3,4-dihydroxy-phenylalanine groups (dopa), essential for the adhesive strength as well as the cohesive strength in Mefp-1, will be altered. Changing the concentration of the protein itself or of different-type surrounding macromolecules may therefore be a tool to control the protein's adhesive activity. The effect of osmotic pressure on the conformation and dopa reactivity of Mefp-1 is studied by the addition of (poly)-ethylene oxide (PEO) as a model macromolecule (M_w = 100 kD). From UV-spectroscopy measurements, it can be concluded that dopa reactivity in Mefp-1 changes with increasing PEO concentration. Fitting of the measured absorbance intensity data of the oxidation product dopaquinone versus time with a kinetic model points to the decreased accessibility of dopa groups in the Mefp-1 structure, a faster oxidation, and diminished cross linking under the influence of increasing PEO concentration up to 2.4 g/L, corresponding to an osmotic pressure of ∼73 Pa. At higher PEO concentrations, the accessibility of the dopa groups for oxidation as well as cross-link formation decreases until about 20% of the dopa groups are oxidized at a PEO concentration of 3.8 g/L, corresponding to an osmotic pressure of ∼113 Pa. FTIR measurements on the basis of amide I shifts qualitatively point to a transition to a more continuously turned structure of Mefp-1 in the presence of PEO. Therefore, it seems that conformational changes caused by variations of osmotic pressure determine the extent of steric hindrance of the dopa groups and hence the adhesive reactivity of Mefp-1.

Introduction

Marine mussels, such as the common blue mussel *Mytilus edulis*, protect themselves against being thrown ashore by tidal movements and waves by spinning a set of anchoring byssus threads. They are able to glue these byssal threads to a range of (hydrophilic) surfaces by using a mix of proteins. Byssal thread and glue bond are synthesized within 2 to 5 min. From the viewpoint of adhesion theory, it is quite an achievement to create underwater strong durable adhesive bonds on hydrophilic surfaces in such a short time without the use of harsh, high-affinity chemical reactions, especially if it is realized that the only solvent the mussel can use to store and transport its adhesive is water! Finding the secrets behind the mussel's remarkable adhesion technology may help us to design a synthetic polymer with the same essential properties for surgical applications.

Until now, six adhesive proteins, varying in type and molecular weight, have been isolated from the phenolic glands of the blue mussel's foot.[1] They are indicated by Mefp-1 to 6, the abbreviation of *Mytilus edulis* foot protein. All of these proteins contain the peculiar amino acid 3,4-dihydroxy-phenylalanine, called dopa. Dopa groups appeared to play an essential role in the adhesive strength[2] as well as the cohesive strength of the mussel glue.[3,4] Dopa is easily oxidized into dopaquinone (Figure 1).

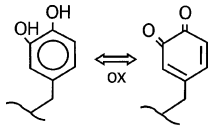

Figure 1. Oxidation of dopa to dopaquinone.

The oxidation of dopa has been found to have strong effects on the strength of the interactions with oxide and metallic surfaces[5−7] and on the degree of cross linking[8] resulting from a reverse dismutation reaction between dopa and dopaquinone (Figure 2). Therefore, the oxidation of dopa to dopaquinone is the first important step in the process of making the mix of proteins active as a glue.

The adhesive proteins are not synthesized during the adhesion process but are stored, ready for use, in densely packed, membrane-bound vesicles. The adhesive becomes active as soon as it is secreted but remains inactive as long it is stored. The question that then arises is if the method of storage in granules enables the preservation of adhesive power until it is needed. Compared to seawater pH (∼8), the intragranular pH of secretory granules in regulated segretion is relatively low (around 5),[9,10] which will reduce the tendency of dopa to oxidize. At pH 4.5, however, considerable dopaquinone formation and ag-

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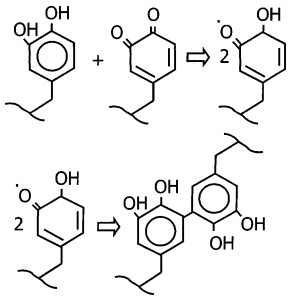

Figure 2. Reverse dismutation reaction between dopa and dopaquinone, leading to the formation of cross links.

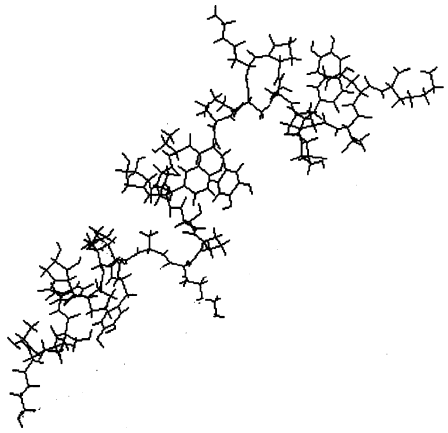

Figure 3. Conformation of a molecule composed of three Mefp-1 decapeptides, generated with Insight II (Biosym Technologies Inc.). The result of dynamic energy minimization with an Amber force field in vacuum at 300 K and a dielectric constant of 1. Potential function atom types have been fixed. The 10-2 and the 10-10 amino acids of lysine both have charges of +1.

gregation were still found under aerobic conditions.[11,12] Oxidation may be (further) suppressed by the presence of a reductant. For instance, in the case of norepinephrine secretion, the chromaffin granules contain high levels of ascorbic acid (~20 mM).[13] It is also posssible that the mussel adhesive storage vesicles contain reducing agents. This is as yet unknown. Another tool used to protect the dopa groups against oxidation may be the dense packing of the granules. It is likely that dense packing influences the conformation of the high-molecular-weight mussel adhesive proteins. In a previous paper, it was concluded that Mefp-1 (115 kD), mainly consisting of a repeating uniform deca-peptide, has a random coil conformation under dilute and physiological conditions.[14] In the same paper, strong indications for a helical or turned shape of the deca-peptide segment are presented. Such conformation may coil up under an applied osmotic pressure induced by surrounding macromolecules. As a consequence, the reactivity of the essential dopa groups will be influenced via less or more steric hindrance (Figure 3). Therefore, provoking a conformational change by changing the concentration might be a way to control adhesive activity.

In this article, the effect of osmotic pressure on the conformation and dopa reactivity of Mefp-1 is studied by the addition of PEO ((poly)ethylene oxide) (100 kD) as a model water-soluble macromolecule.

Experimental Section

The reactivity of dopa was measured at room temperature using UV−vis spectroscopy (Unicam 500 double-beam spectrometer) by following the formation of dopaquinone with time at a wavelength λ of 390 nm. As an oxidation agent, 0.001 M $NaIO_4$ was added. The experimental pH was 6.5. Under these conditions, dopa oxidation is fast and easily measurable. In some cases, time-dependent spectra of the solutions were monitored at wavelengths from 250 to 600 nm in intelliscan mode (scan speed is varied according to the absorbance of the spectrum) with a data interval of 1 nm. Samples were measured in small-volume quartz cells with a path length of 10.00 mm and were blanked against the buffer + $NaIO_4$ + PEO if used.

In addition, conformational changes were qualitatively determined by FTIR (Fourier transform infrared) spectroscopy on the basis of amide I absorption shifts. Measurements were performed with a Perkin-Elmer Spectrum 1000 spectrometer equipped with a KBr beam splitter and a DSTG detector. A 6 μm path length Spectra Tech demountable cell equipped with CaF_2 windows and Mylar spacers was used. The resolution was set at 2 cm^{-1} for 256 scans. These were averaged using Happ-Ganzel apodization to obtain each spectrum. The amide I absorption peaks were determined by deconvolution as well as the second-derivative method. Spectra were corrected for the solvent (buffer solution including PEO if applied) in an interactive manner. The samples were dissolved in D_2O to eliminate vibrational mixing between amide I absorption and water bending modes.

M. Qvist (Biopolymer Products AB, Alingsås, Sweden) kindly supplied the samples of Mefp-1. These samples had a purity of 95% and were delivered in 1% citric acid at concentrations of 1.1, 1.2, and 1.6 g/L. These stock solutions were stored in the dark at 4 °C. Poly(ethylene oxide) (PEO, $M_w = 100$ kD) was purchased from Aldrich (mp 5−160 °C) and was used without further purification. L-Dopa (3,4 di-hydroxy-L-phenylalanine) was obtained from Fluka (37 830).

For the UV spectroscopy measurements, samples were prepared by 1:1 dilution of the Mefp-1 stock solution with an aqueous solution containing 0.08 M Na_3PO_4, 0.002 M $NaIO_4$, and 0−7.6 g/L PEO. This resulted in a 0.55−0.6 g/L Mefp-1 measuring sample at pH 6.5 and an ion strength of 0.1 M NaCl, as was controlled by measuring the conductivity. In case of the measurements with l-dopa, identical conditions were used. The applied L-dopa concentration was 0.08 g/L in the cell (4×10^{-4} M), resulting in a dopa concentration comparable to that in the Mefp-1 measuring samples. Because the Mefp-1 stock solutions contain 1% citric acid, this was also added to the l-dopa samples.

The first FTIR spectra of Mefp-1 were collected at a concentration of 0.8 g/L. To obtain a higher intensity of the amide I absorbance peak, the Mefp-1 samples were concentrated about 8 times by repeatedly centrifuging (2720 rpm) in a Centriprep YM-30 tube (Micon Bioseparations) at 4 °C. Prior to the FTIR measurements, Mefp-1 samples were dissolved in a D_2O phosphate buffer (pH 6.5/pD 6.9) or in such a buffer solution containing 4.6 g/L PEO, resulting in a Mefp-1 concentration of ~2.4 g/L and a PEO concentration of 3.5 g/L. Degassed solutions were used throughout. During the sample handling, the samples were kept in ice under an argon blanket to prevent the oxidation of the dopa groups.

The pD values were obtained by using the method of Glasoe and Long[15] to correct the values determined by pH electrode measurements.

Results and Discussion

UV Spectroscopy. *Mefp-1.* Figure 4 shows UV−vis spectra of Mefp-1 obtained before (thick line) and during the $NaIO_4$-mediated oxidation reaction at pH 6.5 (thin lines). After $NaIO_4$ addition, first a decrease in the dopa peak intensity ($\lambda = 270$ nm) was observed (measurement

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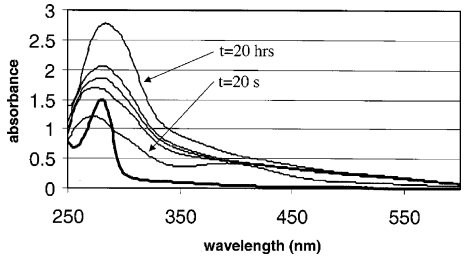

Figure 4. UV−vis spectra during the 0.001 M $NaIO_4$-mediated oxidation of 0.6 mg/mL Mefp-1 at pH 6.5. The thick line shows the spectrum before the oxidation.

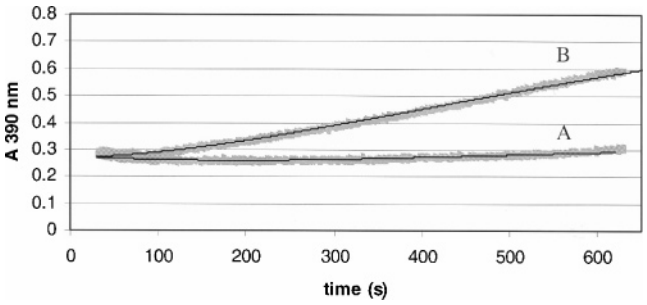

Figure 5. Absorbance intensity at 390 nm as a function of time (s) during the $NaIO_4$-mediated oxidation of Mefp-1 at pH 6.5: (A) without PEO and (B) in the presence of 2.4 g/L PEO. Thick gray lines: experimental results; thin black lines: fitting results.

20 s after mixing the solutions), followed by a significant increase and broadening. Around $\lambda = 390$ nm, an absorption peak is developed because of dopaquinone formation.[16,17]

The data agree with the proposed reaction process of oxidation of the dopa groups (decrease of 270 nm peak) into dopa-quinone (emergence of 390 nm peak) and subsequent cross linking via a reverse dismutation reaction, resulting in phenol-coupled polymer products (increase and broadening of 270 nm peak, together with increasing absorption around 450 nm).

Mefp-1 + PEO. PEO proved to be an appropriate model compound for the UV experiments because it does not interfere with the measurements. Its UV absorption spectrum shows no absorption at wavelengths >300 nm. Furthermore, no reaction occurs between PEO and $NaIO_4$, as was checked by the quantitative determination of IO_4^- according to ref 18 before and after exposure to PEO.

The effect of PEO addition on the Mefp-1 oxidation reaction process was followed by monitoring the intensity of the 390 nm peak during the first 600 s of measuring time. Figure 5 shows representative measurements of Mefp-1 during its oxidation at pH 6.5 without PEO (A) and in the presence of 2.4 g/L PEO (B). About 300 s after the mixing of the solutions, the absorption intensity starts to increase slowly without PEO, whereas in the presence of PEO the absorption intensity increase is more rapid and is already noticeable after about 80 s of running time. Different PEO concentrations resulted in different absorption versus time slopes.

L-Dopa. The single amino acid L-dopa demonstrated a different behavior. About 5 s after the start of the measurement (~20 s after the mixing of the solutions), the absorption intensity decreased, whereafter a linear

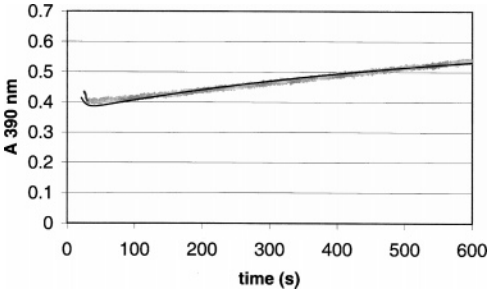

Figure 6. Absorbance intensity at 390 nm as a function of time (s) during the $NaIO_4$-mediated oxidation of L-dopa at pH 6.5. Thick gray line: experimental results; thin black line: fitting results. Absorbance vs time data obtained without and with PEO addition coincide.

increase in the 390 nm absorbance with time was observed, as shown in Figure 6.

The addition of PEO in the concentration range of 0.2−3.8 g/L did not have any effect on the measured absorption versus time data. Because formed dopaquinone in L-dopa easily reacts further to form leukodopachrome and dopachrome by internal cyclization and subsequent rearrangements,[17,19] additional experiments were performed at $\lambda = 302$ nm (absorbance maximum of dopachrome). Again, no effect of PEO was noticed. Because L-dopa has, of course, no possibility of conformational chances, these findings support the proposed idea that the effect of PEO on Mefp-1 kinetics is related to conformational changes in the Mefp-1 molecule.

Osmotic Pressure. The osmotic pressure (π) induced by the different PEO concentrations was determined by osmometry (semipermeable membrane) for concentrations between 5 and 15 g/L. Osmotic pressure data for lower PEO concentrations were obtained by extrapolation of the fitted data and by calculation. For the calculations of π, according to

$$\frac{\pi}{C} = \frac{RT}{M}(1 + B_2C) \qquad (1)$$

with C = concentration (kg m^{-3}), R = molar gas constant (8.31 J mol^{-1} K^{-1}), and M = molecular mass, the second virial coefficient B_2 (mol m^{3}kg^{-2}) was estimated from

$$B_2 = \frac{2R_gN_A}{M^2} \qquad (2)$$

with a radius of gyration $R_g = 1.55R_H$ (good solvent) and N_A being Avogadro's number. The hydrodynamic radius R_H of PEO ($M = 10^5$ g/mol) was measured by dynamic light scattering (DLS), giving a value of 16.8 nm. The obtained data, given in Table 1, agree well with literature values.[20−22]

Because of the relatively large amounts of material needed for the osmometry measurements, these could not be done for Mefp-1. An estimation of the osmotic pressure contribution by Mefp-1 had to be made by calculation. Also, for Mefp-1 B_2 was derived from the DLS measured hydrodynamic radius ($R_H = 10.5$ nm) using eq 2. The calculated osmotic pressure data are listed in Table 2.

Analysis. In a first attempt to analyze the obtained data, the derivative of the dopaquinone absorbance at λ

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Table 1. Osmotic Pressure Data for Different PEO Concentrations

C_{PEO} (g/L) $M_w = 10^5$ g/mol	osmotic pressure (Pa) (1)[a]	(2)[b]	(3)[c]
0.5			12
1.0			25
1.5		34	37
2.0		48	50
2.4		59	60
2.5		61	62
3.0		76	75
3.5		98	88
3.8		104	96
4.6	123		117
5.0	135		127
10.0	300		265
15.0	470		414

[a] (1) Measured data. [b] (2) Values obtained by extrapolation of the best fit of the measured data. [c] (3) Values calculated by incorporation of a second virial coefficient derived from the measured (DLS) hydrodynamic radius of PEO in water (16.8 nm).

Table 2. Osmotic Pressure Values for Different Mefp-1 Concentrations, Calculated by Incorporation of a Second Virial Coefficient Derived from the Measured (DLS) Hydrodynamic Radius of Mefp-1 in Water (10.5 nm)

C_{Mefp-1} (g/L) $M_w = 115$ kg/mol	osmotic pressure (Pa)
0.5	11
0.6	13
0.8	17
2.4	51

= 390 nm with respect to time, $dAbs_{390}/dt$, was taken by linear regression between 350 and 600 s. The results are presented in Figure 7A and B for Mefp-1 and L-dopa, respectively. In case of Mefp-1, first a decrease in $dAbs_{390}/dt$ with increasing PEO concentration is seen until $C_{PEO} \approx 0.5$ g/L. Subsequently, at $0.5 < C_{PEO} < 2.4$ g/L $dAbs_{390}/dt$ increases until a maximum of 0.0007 s^{-1} is reached. At higher PEO concentrations (from ~2.4 g/L), $dAbs_{390}/dt$ decreases and even reaches zero at a PEO concentration of 3.8 mg/mL for a measuring time of 600 s. In the case of L-dopa, a constant $dAbs_{390}/dt$ value of about 0.0002 s^{-1} was found for the whole PEO concentration range of 0−3.8 g/L. At $\lambda = 302$ nm, a constant $dAbs_{302}/dt$ value of about 0.0003 s^{-1} was measured.

From the data in Tables 1 and 2, the actual experimental osmotic pressures were estimated and included in Figure 7, part C. Optimal reactivity of the dopa groups toward dopaquinone formation occurs at an osmotic pressure $\pi \approx 73$ Pa, which corresponds with a Mefp-1 concentration of ~3.5 g/L based on the above estimation of the osmotic pressure induced by Mefp-1. The dopaquinone concentration does not change, anyway not during the measuring time of 600 s, at $\pi \approx 113$ Pa. Such osmotic pressure will be reached at a Mefp-1 concentration of ~5.4 g/L.

Because the $dAbs_{390}/dt$ data for Mefp-1 are the result of the simultaneous reactions of the oxidation of dopa (H_2Q) to dopaquinone (Q), the reduction of Q to H_2Q, and aryloxy radical formation by a reverse dismutation reaction between H_2Q and Q leading to cross-linked material,[8] more detailed information on the kinetics is necessary. Assuming first-order rates, quinone formation with time can be expressed as

$$\frac{d[Q]}{dt} = k_{ox,NaIO_4}[H_2Q][IO_4^-] + k_{ox,O_2}[H_2Q][O_2] - k_{red}[Q][H^+][IO_3^-] - k_{xl}[H_2Q][Q] \quad (3)$$

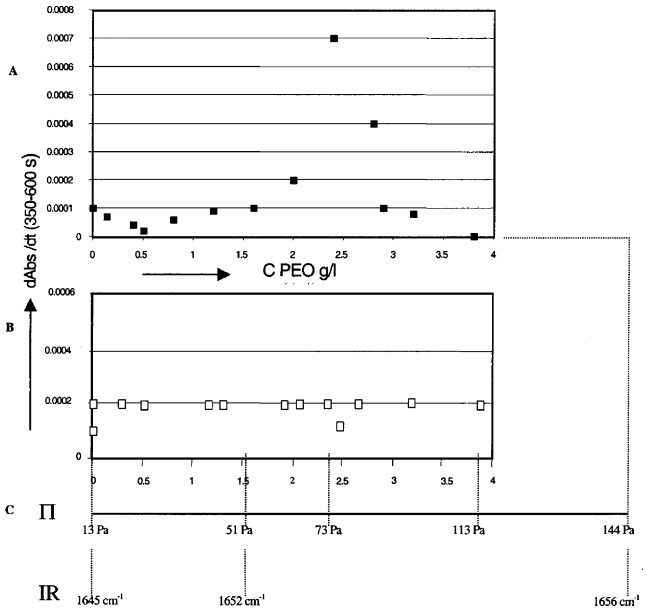

Figure 7. Effect of different PEO concentrations (A and B) and corresponding osmotic pressures (C) on the dopa reactivity toward oxidation at pH 6.5. Plots of the derivative of the absorbance at 390 nm with respect to time in the 350−600 s interval as a function of C_{PEO} (g/L) for Mefp-1 (A) and L-dopa (B). The given amide I wavenumbers correspond to the osmotic pressure data as indicated.

in which $k_{ox,NaIO_4}$, k_{ox,O_2}, k_{red}, and k_{xl}, are the effective rate constants of dopa oxidation by $NaIO_4$ and dissolved (activated) oxygen, of dopaquinone reduction, and of radical formation leading to cross links, respectively. When the molar extinction coefficient of quinone at 390 nm (ϵ^Q_{390}) is known, the measured A390 versus time data can be described by eq 3. To determine ϵ^Q_{390}, calibration lines of L-dopa and of several synthetic dopa-containing polymers were measured for dopa concentrations ranging from 2.7×10^{-4} to 1.3×10^{-3} M at pH 6.5 and a $NaIO_4$ concentration of 4×10^{-3} M. Although it was not possible to determine ϵ^Q_{390} accurately because of proceeding subsequent reactions, the ϵ_{390} values that were found, derived from absorption plateau values, were higher (2200 to 3500 M^{-1} cm^{-1} for the synthetic polymers, 1600 M^{-1} cm^{-1} for L-dopa) than reported in the literature[23] for enzymatically prepared quinone products, which vary between ±1000 and 1400 M^{-1} cm^{-1}. However, in L-dopa the formed dopaquinone appeared to be very unstable under the applied measuring conditions. It almost immediately reacts further to form leukodopachrome, followed by fast conversion to dopachrome (as was evidenced by the occurrence of the 473 nm characteristic peak). The measured ϵ_{390} value of 1600 M^{-1} cm^{-1} therefore represents the molar extinction coefficient ϵ^{DC}_{390} of dopachrome at 390 nm. To estimate ϵ^Q_{390} of L-dopa, the course of A390 of a 0.0004 M L-dopa solution at pH 6.5 after the addition of 0.001 M $NaIO_4$ versus time was fitted by

$$\frac{d[Q]}{dt} = k_{ox,NaIO_4}[H_2Q][IO_4^-] + k_{ox,O_2}[H_2Q][O_2] - k_{red}[Q][H^+][IO_3^-] - k_{LC}[Q] - k_{DC}[LC] \quad (4)$$

in which k_{LC} and k_{DC} are the effective rate constants of leukodopachrome and dopachrome formation, respectively, and [LC] is the leukodopachrome concentration.

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Table 3. Fitting Parameters and Input Data of the Experiments with L-Dopa and Mefp-1

	ϵ_{390}^{Q}	ϵ_{390}^{DC}	ϵ_{390}^{XL}	$k_{ox,NaIO_4}$	k_{ox,O_2}	$k_{red\bullet}[H^+]$	k_{xl}	k_{LC}	k_{DC}	k_c
	$M^{-1}\,cm^{-1}$	$M^{-1}\,cm^{-1}$	$M^{-1}\,cm^{-1}$	$M^{-1}\,cm^{-1}$	$M^{-1}\,s^{-1}$	$M^{-1}\,s^{-1}$	$M^{-1}\,s^{-1}$	s^{-1}	s^{-1}	s^{-1}
L-dopa	3000	1600		1.2×10^2	5.3×10^2	1.3×10^{-3}		1.9×10^{-1}	1.7×10^{-3}	-
Mefp-1 no PEO	3000		1100	0.5	2.2	1.3×10^{-3}	19			8.3×10^{-4}
Mefp-1 0.5 g/L PEO	3000		1100	0.5	2.2	1.3×10^{-3}	19			2.0×10^{-3}
Mefp-1	3000		1100	1.0	4.4	1.3×10^{-3}	1.9			2.0×10^{-3}
2.4 g/L PEO	3000		1100	2.0	8.8	1.3×10^{-3}	1.9			1.0×10^{-3}

After correcting for dopachrome absorption at 390 nm using $\epsilon_{390}^{DC} = 1600\ M^{-1}\ cm^{-1}$, for L-dopa a ϵ_{390}^{Q} value of $\gg 3000\ M^{-1}\ cm^{-1}$ was found, which is within the range found for the dopa polymers. The result of the fit is presented in Figure 6. Detailed fitting data are given in Table 3.

Quinone formation by synthetic dopa-containing polymers could be successfully numerically fitted by applying eq 3 and by incorporating a correction for spectral overlapping due to cross-link formation, as will be presented in a forthcoming paper. However, such fitting of the measured Mefp-1 data appeared to be impossible without the assumption that only part of the dopa groups in Mefp-1 are accessible at the start of the oxidation reaction and that dopa groups "hidden" in the protein structure become gradually accessible for oxidation and/or cross linking. The substitution of $[H_2Q]$ in eq 3 by $[H_2Q]_{total}$ according to

$$[H_2Q]_{total} = [H_2Q] + [H_2Q]_{hidden}(1 - \exp(-k_c t)) \quad (5)$$

(k_c = rate constant of conformational changes and t = time) resulted in a satisfying fit of the measured data, as shown in Figure 5A. In these Mefp-1 fits, molar extinction coefficients of 3000 and 1100 $M^{-1}\ cm^{-1}$ were used for the absorption of quinone (ϵ_{390}^{Q}) and cross-linked products (ϵ_{390}^{XL}) at 390 nm, respectively. It was thus found that about 20% of the dopa groups in Mefp-1 are directly available for oxidation, and for Mefp-1, oxidized by 0.001 M $NaIO_4$ and dissolved oxygen at pH 6.5, a k_c value of 0.0083 s^{-1} was derived. Detailed fitting data are incorporated in Table 3.

The observed $dAbs_{390}/dt$ minimum occurs at $C_{PEO} = 0.5$ g/L, which means at an osmotic pressure equal to that of the Mefp-1 stock solution (~23 Pa). Fitting results of the A390 versus time data in the PEO concentration range <0.5 g/L point to a decreasing k_c value with increasing C_{PEO}, whereas the other rate constants in eq 3 remain unchanged. Apparently the Mefp-1 coils expand after 1:1 dilution because of their new environment of lower osmotic pressure. This expansion is increasingly hampered with increasing PEO concentration until the osmotic pressure before and after 1:1 dilution is the same. A k_c value of 0.002 s^{-1} is then found.

Best fits of the data of the C_{PEO} range from 0.5 to 2.4 g/L revealed increases in $k_{ox,NaIO_4}$ and k_{ox,O_2} with increasing C_{PEO}. No information on the progress of k_c could be obtained from these fits. For k_c = constant = 0.002 s^{-1} with $k_{ox,NaIO_4}$ and k_{ox,O_2} increasing by a factor 2 and k_{xl} decreasing by 1 order of magnitude at C_{PEO}=2.4 g/L, equal accuracy was acquired as for k_c decreasing until 0.001 s^{-1} with $k_{ox,NaIO_4}$ and k_{ox,O_2} increasing by a factor 4 and k_{xl} decreasing by 1 order of magnitude at C_{PEO} = 2.4 g/L. At higher PEO concentrations (>2.4 g/L), lower values for k_c, $k_{ox,NaIO_4}$, and k_{ox,O_2} are found. It is therefore reasonable to assume that k_c decreases with increasing PEO concentration over the whole PEO concentration range.

Because it is generally accepted to describe reversible folding−unfolding processes by a kinetic expression as incorporated in eq 5 (e.g., refs 24 and 25), k_c will also include the effect of the coiling up of the protein chains on the accessibility of the dopa groups. The decrease in k_c may therefore be attributed to the increased steric hindrance of the dopa groups by coiling up of the Mefp-1 segments under osmotic pressure. The found increase in the rates of oxidation until a PEO concentration of 2.4 g/L is reached can be explained by the increased concentration of dopa groups in the interior of the Mefp-1 coils due to their compression. The less open structure will, at the same time, reduce the ability to form cross links, which agrees with the lower k_{xl} values that were found. The decreases in all rate constants at $C_{PEO} > 2.4$ g/L can be understood as an (almost) effective locking up of the dopa groups. At the highest measured PEO concentration of 3.8 g/L ($\pi \approx 113$ Pa), only the easily accessible dopa groups at the outside of the coil (~20%) are quickly oxidized; the others are hidden in the Mefp-1 structure, as was concluded from a constant A390 value during the experiment.

FTIR Experiments. Although D_2O solutions of PEO show little or no absorbance in the amide I region (1600−1700 cm^{-1}),[26] first a possible effect of PEO on the position of a protein amide I peak was checked by measurements with the globular protein β-lactoglobulin. In view of the compact structure of β-lactoglobulin, no PEO effect on its conformation is expected. The obtained spectra of β-lactoglobulin in D_2O were consistent with the reported values in the literature.[27] At pH 6.5 (pD 6.9), the main amide I peak was found at 1635 cm^{-1}, and another was found at 1645 cm^{-1}, corresponding to a 60% β-sheet and 22% α-helix conformation, respectively. The addition of PEO (3.5 and 5 g/L) did not produce any shifts in the spectrum. It can therefore be concluded that PEO itself does not disturb the FTIR measurements in the amide I region. In the case of Mefp-1, the FTIR measurements revealed a shift of the amide I absorption by increasing the Mefp-1 concentration and by adding PEO. Without PEO and a Mefp-1 concentration of 0.8 g/L, a wavenumber of 1645 ± 4 cm^{-1} was found. Without PEO and a Mefp-1 concentration of 2.4 g/L, the amide I wavenumber shifted to 1652 ± 2 cm^{-1}, whereas in the presence of PEO (3.5 g/L) a shift to 1656 ± 2 cm^{-1} was observed.

Figure 8 shows the transmission spectra of 2.4 g/L Mefp-1 in D_2O with and without PEO between 1300 and 1950 cm^{-1}. Inserted is a detail of the absorption spectra between 1600 and 1700 cm^{-1}, obtained after deconvolution and smoothing, to illustrate the shift due to PEO addition (not on the same scale).

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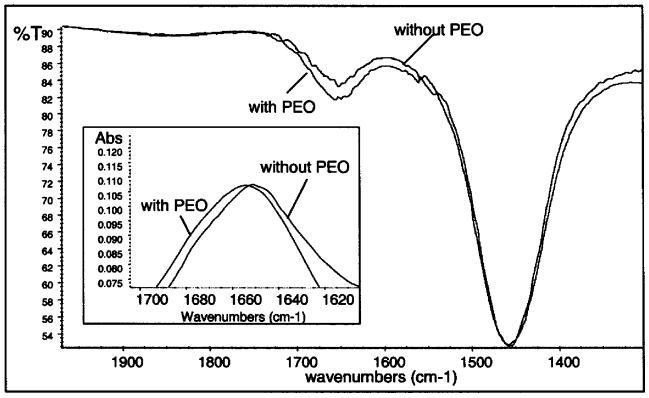

Figure 8. FTIR transmittance spectra of 2.4 g/L Mefp-1 without and in the presence of 3.5 g/L PEO. The inlay shows a detail of the absorbance vs wavenumber spectra, indicating the amide I shift.

Although the utmost care was taken to prevent oxidation, possible interference of the νC=O absorption of dopaquinone should be taken into account. However, in an FTIR study on mixtures of various quinones and their hydroquinone forms,[28] the νC=O absorption band is found between 1645 and 1652 cm^{-1}. It is therefore reasonable to assume that the absorption found at 1656 cm^{-1} is not due to dopaquinone formation. Therefore, although relatively low Mefp-1 concentrations had to be used because of the scarcity of the protein and thus low-intensity amide I absorbances were obtained, we found (qualitative) indications for an effect of PEO (and possibly of increasing Mefp-1 concentration) on the conformation of Mefp-1.

On the basis of empirical rules for the correspondence between the frequencies of the amide I bands and the secondary structure (α-helix, β-sheet, random coil and turns), the amide I wavenumber found for the (very) low Mefp-1 concentration lies within the random coil region (1640−1650 cm^{-1}).[29,30] This is in agreement with the results of far-UV−circular dichroism (CD) measurements at similar concentrations.[31] According to these "classical" rules, the wavenumbers found for the higher Mefp-1 concentration without and with PEO are both situated within the α-helix region (1650−1660 cm^{-1}), which is in conflict with the earlier conclusion drawn that Mefp-1 under physiological and dilute conditions has a random coil conformation with the possibility of turned segments.[14] However, an increasing number of studies have shown that analyzing the amide I bands of proteins by this empirical rule procedure is not free from shortcomings (e.g., refs 32 and 33). It is, for example, suggested that β-turns may give rise to IR bands over a wider frequency region than 1660−1695 cm^{-1}, which overlaps with the region assigned to α-helix bands.[34] Another shortcoming of the empirical rules is that loops and 3_{10}-helices are not included. Loops may contribute to IR intensities in the 1650−1660 cm^{-1} region.[35−37] Malin et al.[38] assign a separate category termed large loop and helix for the elements centered at 1656 cm^{-1} (solvent D_2O).

It is not clear yet what types of turns occur in Mefp-1. The most obvious types are polyproline turns and type III β-turns.[14,39] Polyproline-rich polypeptides, as Mefp-1, as well as random coil proteins, also as Mefp-1, are often known to adopt the PPII or left-handed 3_1-helix conformation.[40,41] Tiffany and Krimm[42] suggest that poly(L-lysine) and poly(L-glutamate) are not truly random but contain elements of PPII. Two spectral features of these polypeptides at neutral pH are IR bands between 1652 and 1656 cm^{-1}.[43]

According to Krimm and Baldekar,[34] strong amide I absorption for a type III β-turn should be found at 1650 cm^{-1} and for a 3_{10}-helix at 1656 cm^{-1}. A 3_{10}-helix is formed from consecutive type III β-turns, so apparently a shift to a higher wavenumber occurs when several type III β-turns form a 3_{10}-helix.

In summary, although the measured values are not absolute and much caution has to be taken with the assignments of the bands, the wavenumber shift that is found is indeed an indication of a transition to a more continuously turned structure under osmotic pressure induced by surrounding macromolecules.

Conclusions

Osmotic pressure, induced by surrounding macromolecules, changes the conformation of the mussel adhesive protein Mefp-1. Increasing osmotic pressure probably results in a coiling up of the turned decapeptide segments of the random coil protein. Because of these conformational changes, the accessibility of the dopa groups and their reactivity toward oxidation and cross linking are altered. At pH 6.5 and an ion strength of 0.1 M NaCl, optimal dopa reactivity toward oxidation is found at an osmotic pressure of ∼73 Pa. Increasing osmotic pressure leads to diminished cross linking and inhibits the accessibility kinetics of the dopa groups inside the structure. At osmotic pressures above ∼113 Pa, the reactivity of dopa groups inside the Mefp-1 coils toward oxidation and cross linking is strongly retarded or even blocked. The dense packing of the mussel adhesive proteins may therefore be an important mechanism for preserving their adhesive power until it is needed.

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Supporting Information Available: Formation of highly reactive radicals via a reverse dismutation reaction between dopa and an adjacent dopa-quinone. Additional details

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on the numerical fitting of the experimental data (Mefp-1 without and in the presence of 2.4 g/L PEO). Absorbance at 390 nm versus time. Comparison between experimental data and numerical fitting results. The process of dopa groups becoming accessible and the actual dopa concentration during the oxidation and cross-linking reactions. Cross-link formation and dopa-quinone formation for Mefp-1 without and with 2.4 g/L PEO. This material is available free of charge via the Internet at http://pubs.acs.org.

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