Ferric Ion Complexes of a DOPA-Containing Adhesive Protein from *Mytilus edulis*

Steven W. Taylor,[†,‡] D. Bruce Chase,[§] Mark H. Emptage,[§] Mark J. Nelson,[§] and J. Herbert Waite*[,†]

Department of Chemistry/Biochemistry and College of Marine Studies, University of Delaware, Newark, Delaware 19716, and Central Research & Development Department, E. I. du Pont de Nemours and Company, Inc., Wilmington, Delaware 19880-0328

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Iron(III) binding to the DOPA-containing *Mytilus edulis* adhesive protein (Mefp1) has been studied by spectrophotometric titrations, electron paramagnetic resonance (EPR), and resonance Raman spectroscopies. At pH 7.0, two different forms of the iron−protein complex exist: one purple (λ_{max} = 548 nm) and one pink (λ_{max} = 500 nm). The pink form is favored at high DOPA:Fe ratios and the purple at low DOPA:Fe ratios. Resonance Raman spectroscopy of both forms demonstrates that the chromophores are ferric catecholate complexes. EPR spectra of both forms of the protein measured at the same iron concentration reveal a $g \approx 4.3$ resonance of approximately 4 times the intensity in the spectrum of the pink complex compared with that of the purple form. On the basis of the collective evidence obtained here, a model for the purple form of the ferric Mefp1 involving bis(catecholato) coordination of ferric ions with most of the iron(III) complexed as EPR-silent μ-oxo- or μ-hydroxo-bridged binuclear clusters is suggested. In the pink form, in contrast, the ferric iron is EPR-active, mononuclear, and present in high-spin tris(catecholato) complexes. The biological implications of these complexes are discussed.

Introduction

Catecholato iron(III) complexes are widely distributed in biological systems in which at least three functional categories of these compounds are distinguishable. The first and best characterized examples are the siderophores, low molecular weight catecholamides, which are secreted by bacteria to selectively sequester iron.[1] The second consists of enzymes with ferric ion-containing active sites that bind low molecular weight catechols either as substrates, e.g., catechol dioxygenases,[2] as products, e.g., tyrosine 3-monooxygenases,[3] or as inhibitors with useful spectroscopic properties, e.g., soybean lipoxygenases.[4] A third emerging area is the coordination chemistry of high molecular weight proteins and peptides with catecholic functional groups present in the primary structure as peptidyl (3,4-dihydroxyphenyl)-L-alanine (DOPA). Examples of peptidyl-DOPA come from both artificial systems such as radiation- or radical-damaged proteins[5,6] or mutant recombinant ribonucleotide reductase[7] and natural systems in which DOPA is formed as a post-translational modification of tyrosine.

Certain marine invertebrates produce DOPA proteins which are believed to fulfill a range of functions including adhesion, sclerotization, wound repair, etc. Perhaps many of these functions result from properties attributable to the tendency of DOPA residues to oxidize and cross-link in a process known as quinone-tanning.[8] The presence of high concentrations of metal ions in the proximity of these proteins both intracellularly (such as in ascidian blood cells) and extracellularly (as in mussel byssus) implicates them as non-covalent cross-linking agents. This is particularly true of iron(III), which forms catechol complexes of exceptional stability.

Although they are not mineralized, byssal threads of the mussel *Mytilus edulis* have long been known to be associated with various metals including iron.[9] The extraction of iron, in particular, from mussel byssus resists treatment with 1 N NaOH, 1 N HCl, and 0.1 M ethylenediaminetetraacetic acid.[9] According to labeling studies with ^{59}Fe by George et al.,[10] mussels remove iron from the sea water by filtration through the gills, following which it is taken up by amebocytes and distributed to other tissues. After seven days, approximately 35% of the physiological burden had been transferred to the byssal glands from which it was secreted, probably bound to byssal precursor proteins. To determine to which protein(s) it is bound, we have attempted to characterize each of the byssal precursors and to study their iron-binding properties. Mefp1 (*M. edulis* foot protein 1) is deposited as a cuticular varnish over the entire byssus and consists of tandemly repeated hexa- and decapeptide sequences which contain DOPA.[11,12] Recently, Taylor et al.[13] reported that peptides derived from Mefp1 bind Fe(III) in bis- and tris(catecholate) coordination modes with log stability constants in excess of 38. In previous studies, the interaction of ferreascidin, a 10 kDa DOPA glycoprotein from the blood

* Corresponding author. FAX: (302) 831-6335. E-mail: hwaite@udel.edu.
† University of Delaware.
‡ Present address: Institut für Organische Chemie, Universität Tübingen, Auf der Morgenstelle 18, 72076 Tübingen, Germany.
§ E. I. du Pont de Nemours and Company, Inc.
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cells of the ascidian *Pyura stolonifera*, with ferric ion revealed that the protein adopts a bis(catecholato) coordination mode with possibly one tyrosine ligand.[14] Subsequently, it was shown that ferreascidin binds iron(III) as novel bi- and trinuclear clusters.[15] Here we have sought to probe the interaction of ferric ion with intact Mefp1 by spectrophotometric titrations, electron paramagnetic resonance (EPR), and resonance Raman spectroscopies.

Experimental Section

Materials. 2,2-Bis(hydroxymethyl)-2,2,2′-nitrilotriethanol (Bistris) was used as supplied from Sigma Chemical Co., St. Louis, MO. Iron(III) solutions were prepared from ferric chloride solution (1 mg/mL Fe) supplied as an atomic absorption standard by Sigma. All other reagents were of analytical grade. Water from a Milli-Q reagent water system was used at all times.

Protein Purification. The purification of Mefp1 has been described elsewhere.[16,17] Purity was assessed by acid urea polyacrylamide gel electrophoresis, amino acid analysis,[16] and matrix-assisted laser desorption ionization mass spectrometry with time-of-flight. Using the latter, a molecular mass (MH^+) of 108.6 kDa was obtained for Mefp1. HPLC-purified protein was lyophilized and stored at −80 °C until required.

Spectrophotometric Titrations. All spectra were recorded at room temperature with a Hewlett-Packard HP 8452A diode array spectrophotometer. Typically, a 2-mL aliquot of 40 μM ferric ion in Bistris buffer (pH 7.0) supplemented with 0.l M NaCl would be transferred to a quartz cuvette containing a stirrer "flea" and the reference spectrum recorded. After the buffer was sparged for 2 min with a stream of nitrogen, the cuvette was fitted with a rubber septum. Lyophilized Mefp1 would be resuspended in 200−300 μL of water that had been slightly acidified (pH 5.0), and after the concentration of Mefp1 was defined in terms of its DOPA content,[18] the protein was drawn into a 250-μL Hamilton gas-tight syringe which was inserted into the septum. Spectra were recorded after attainment of a constant absorbance reading at the visible maximum (typically 5−10 min early in the titration and 20−40 min for the last additions) following successive additions of protein. In reversed titrations, $FeCl_3$ (3.58 mM) with pH adjusted to 3.0 was added to Mefp1 in the Bistris buffer.

Electron Paramagnetic Resonance Spectroscopy. Two forms of the iron(III)−protein complex identified at pH 7.0 in the spectrophotometric titrations (i.e., purple ([Fe^{+3}] > [DOPA]) and pink ([DOPA] > [Fe^{+3}])) were prepared for EPR at a concentration of 0.15 mM in Fe^{+3} in Bistris buffer. Samples were frozen and stored in liquid nitrogen. EPR spectra were obtained using a Brucker EM-200 spectrometer with an Oxford Instruments ESR900 liquid helium cryostat. The conditions were microwave frequency, 9.46 GHz; modulation amplitude, 0.6 mT; microwave power, 10 mW; *T*, 20 K; scan rate, 2.4 mT/s.

Resonance Raman Spectroscopy. A Coherent DPSS532 doubled, diode-pumped Nd:YAG laser operating at 532 nm was used for sample excitation. The output of the laser was passed through a 50-μm optical fiber to a Kaiser Holoprobe filter head equipped with an f/1.3 telescopic collection lens. Raman spectra were recorded with a Kaiser Instruments Holospec f/1.8i spectrometer using a 180° backscattering configuration. Scattered radiation was collected and detected with an EEV 1511 CCD chip (256 × 1024 pixels) mounted in a Photometrics CH250 camera. The CCD was operated at −40 °C. The spectrometer was calibrated using reference spectra of 1,4-bis(2-methylstyryl)benzene, acetaminophen, and sulfur. Spectral resolution was 4−6 cm^{-1}, corresponding to 2−3 pixels on the CCD. Power at the sample was typically less than 5 mW.

Samples of both the pink and purple forms were prepared at a total concentration of 0.35 mM iron in 5 mM Bistris, pH 7.0. Mefp1 was added to give a final concentration of 0.32 mM DOPA equivalents in the purple sample and 1.2 mM DOPA equivalents in the pink sample. Samples were allowed to stand on ice for 1 h and then loaded into wells on a precooled (270 K) gold-plated copper cold finger. Samples were rapidly frozen and maintained *in vacuo* at 80 K with an Air Products LT3-110 cryostat using liquid nitrogen as the refrigerant. Evacuation was begun at a sample temperature of 200 K to minimize lyophilization. Peaks in spectra of the same sample obtained on the same day agreed to within 0.5 cm^{-1}; therefore, the spectra of pink and purple Mefp1 used for direct comparison (Figure 6) were obtained on the same day from samples loaded at the same time. The data were processed and displayed using Grams (Galactic Industries).

Results and Discussion

Spectrophotometric Titrations. In contrast to a previous investigation into iron(III) binding by ferreascidin,[14,19] spectrophotometric titrations were performed by adding the protein (Mefp1) in a slightly acidified water solution to ferric ion in Bistris buffer at pH 7.0. The approach has the advantage of minimizing the pH drop during the course of the titration and of better protecting the catecholic moieties from oxidation prior to complexation. Spectrophotometric titrations of Mefp1 resulted in the development of a chromophore at 550 nm which increased stepwise until the Fe:DOPA stoichiometry was 1:2, after which the maximum shifted to 500 nm with an isosbestic point at 584 nm (Figures 1 and 2). Early additions of Mefp1 produced a maximum at 510 nm initially which shifted to 550 nm over a 10-min equilibration period (Figure 1, inset). Later additions maintained the position of the absorption maximum during the course of the equilibration period but equilibration took much longer. This can be accounted for by the following scheme:

$$Fe^{3+} + \text{Mefp1} \rightarrow \text{pink} \rightarrow \text{purple}$$

$$\text{purple} + \text{Mefp1} \rightarrow \text{pink}$$

The titration of ferric ion with Mefp1 produces a transient pink complex which rearranges to a purple complex. When the purple is fully formed, i.e., all ferric ion is complexed, further additions of Mefp1 induce a rearrangement back to the pink complex. The type of ferric ion complex formed by Mefp1

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(19) Spectrophotometric titrations were initially performed as previously described for ferreascidin, i.e., ferric chloride was prepared at mM concentrations, the pH adjusted to 3.0, and the metal added to a buffered solution of protein at pH 7.0.[14] The problem with this approach is that, even at pH 3.0, ferric ion exists in a variety of hydrolyzed forms. A later Mössbauer investigation of the ferric−ferreascidin complex revealed that the iron was bound as a variety of bi- and trinuclear clusters which may have been added directly to the protein.[15] Adding ferric ion to Mefp1 in spectrophotometric titrations resulted in a visible chromophore being formed with a maximum at 510 nm shifting to 550 nm. The increase in absorbance was linear, indicating stoichiometric binding, but stopped at unusually high Fe:DOPA ratios. A comparison of the interaction of pyrocatechol with ferric iron under identical conditions revealed no development of a visible chromophore, indicating that there was no complexation whatsoever. While ferric ion is bound by Mefp1 under the conditions of the spectrophotometric titrations, it is completely inaccessible to pyrocatechol. It appears that Mefp1 and ferreascidin have the ability to extract low molecular weight iron from oligomeric ferric iron while pyrocatechol does not. This may be a reflection of the enhanced chelating potential conferred by having the catechols linked as DOPA residues on a polypeptide backbone. In contrast, the reverse titration, i.e., adding pyrocatechol to ferric ion in Bistris buffer solution, resulted in formation of a chromophore at 570 nm in agreement with that reported for biscatecholatoferrate.[20] Bistris appears to be fulfilling its role as a weak complexing agent, keeping the iron accessible to the chelate at pH 7 by preventing formation of high molecular weight aggregates.

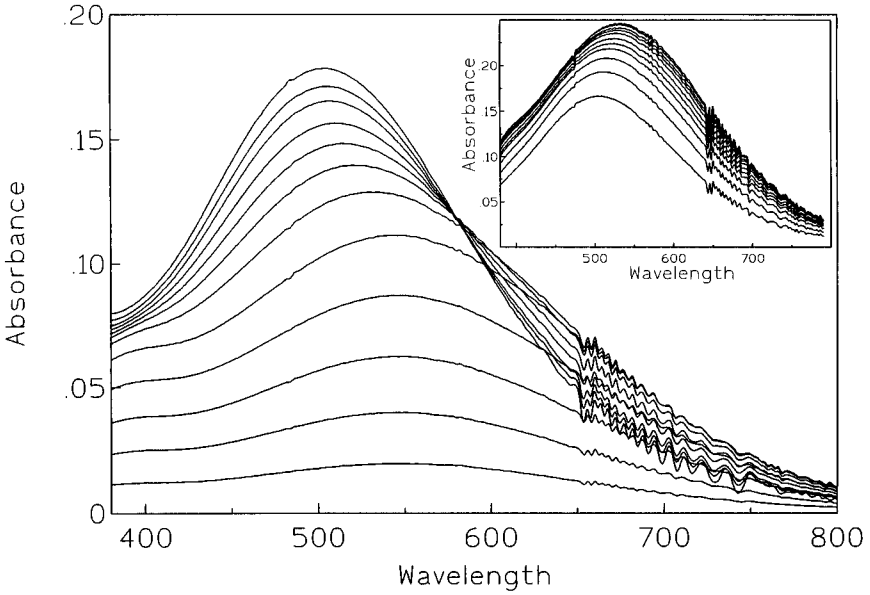

Figure 1. Spectrophotometric titration of 2 mL of 40 μM $FeCl_3$ in 5 mM Bistris-HCl buffer, pH 7.0, 0.1 M NaCl with 5 μL aliquots of Mefp1 ([DOPA] = 4.5 mM). Inset: Characteristic spectral changes observed for Mefp1 in excess Fe^{3+}. Spectra were recorded at 30 s intervals from t = 0 to 300 s, [DOPA] = 0.105 mM, [Fe^{3+}] = 0.15 mM.

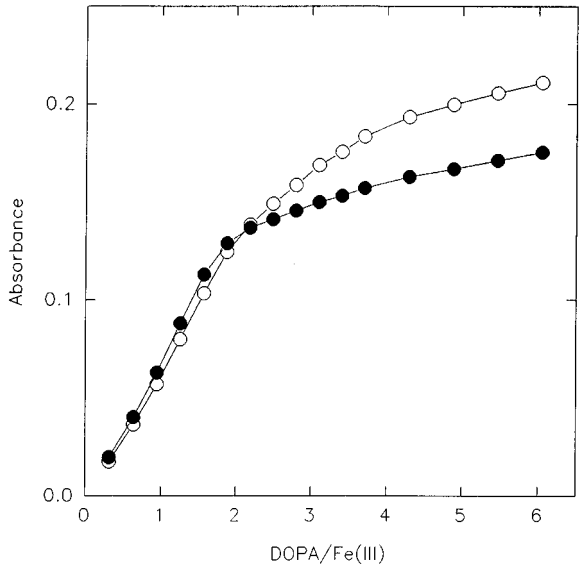

Figure 2. Binding curve for Figure 1: (○) 500 nm; (●) 550 nm.

appears to be governed by stoichiometry and pH. At lower pH values, the maximum in the visible region develops at 570 nm and at higher pH at 498 nm.[21] According to Avdeef et al.,[20] these maxima are indicative of bis- and tris(catecholato) coordination modes, respectively. At pH 7.0, in excess iron, the ferric−Mefp1 complex exists as a purple form with a λ_{max} (548 nm) closer to that reported for the bis(catecholato) coordination mode. In excess Mefp1, the λ_{max} (500 nm) of the pink complex is closer to that reported for tris(catecholato) coordination. In excess iron there is more of the metal to be shared between catechols, so bis(catecholato) coordination may be favored while increasing concentrations of Mefp1 induce a change to tris(catecholato) coordination as more catechols compete for a decreasing amount of "free" iron. The results of analysis by resonance Raman and EPR spectroscopies allow further insights into the nature of the pink and purple complexes.

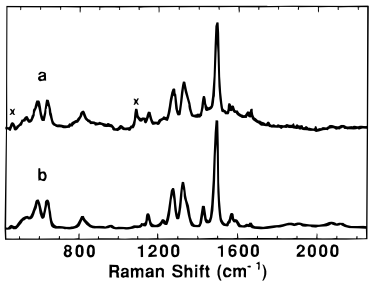

Figure 3. Resonance Raman spectra of (a) pink and (b) purple Mefp1 obtained with 532 nm excitation. Features present in the buffer are labeled "x".

Resonance Raman Spectroscopy. Raman spectra of frozen samples of the pink and purple forms of Mefp1 were obtained using excitation at 532 nm (Figure 3). The spectra of the two forms are virtually identical in the number and relative intensity of the peaks observed. Control spectra were obtained at 785 nm to enable identification of resonance-enhanced features; these are listed in Table 1. The Raman spectra of both pink and purple Mefp1 are very similar to those of synthetic iron−catecholate complexes of non-heme iron enzymes, including tyrosine hydroxylase, phenylalanine hydroxylase, and lipoxygenase.[2,22−26] Previous work has shown that the features in the 1100−1600 cm^{-1} region arise from catechol ring vibrations, whereas those in the 500−700 cm^{-1} region arise from vibrations involving the Fe−O bonds.[27] The Raman spectra are diagnostic for the presence of iron−catechol complexes in both the pink and purple forms of Mefp1.

Elegant ^{18}O-labeling experiments have led to the assignment of the peaks in the low-energy region of the resonance Raman

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Table 1. Resonance Raman Shifts and Catechol-to-Iron Charge Transfer Maxima for Catechol Complexes of Non-Heme Iron Proteins and Models

sample	λ_{max} (nm)	Raman shifts (cm^{-1})										ref
$Fe(cat)_3^{3-}$	500	533		621	800	1154	1262	1322		1487	1572	Salama et al., 1978[c]
$Fe(HDA)cat^{2-}$	582[a]			630	795	1158	1258	1320		1480	1576	Cox 1988[d]
$Fe(NTA)cat^{2-}$	618[a]	526		630	794	1154	1254	1312		1473	1573	Cox et al., 1988[e]
tyr hydroxylase + catechol		528		619		1150	1257	1314		1466	1566	Michaud-Soret et al., 1995[f]
tyr hydroxylase + dopamine		528	592	631			1275	1320	1425	1475		Michaud-Soret et al., 1995[f]
Mefp1												
pink	500[b]	531	591	638	815	1152	1274	1326	1426	1491	1571	this work
purple	548[b]	533	594	639	818	1152	1274	1324	1427	1490	1570	this work

[a] Methanol. [b] 5 mM Bistris, pH 7.0. [c] Reference 26. [d] Reference 29. [e] Reference 23. [f] Reference 24.

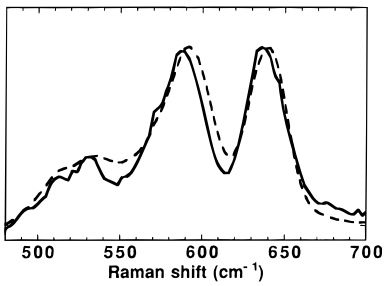

Figure 4. Resonance Raman spectra of pink (—) and purple (- - -) Mefp1, obtained with 533 nm excitation. Peak positions were determined by deconvoluting the spectrum into a set of peaks with a mixture of Gaussian and Lorentzian line shapes.

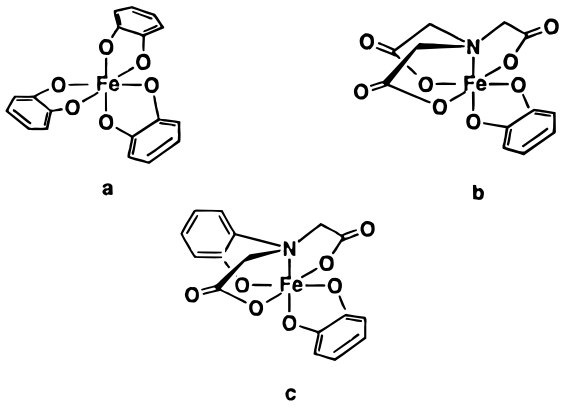

Figure 5. Synthetic complexes used as spectroscopic models: (a) $Fe(cat)_3^{3-}$; (b) Fe(NTA) cat^{2-}; (c) Fe(HDA) cat^{2-}.

spectra of the iron−catechol complexes.[24] Bidentate iron−catecholate complexes have a feature at approximately 530 cm^{-1} that arises from an iron−catecholate ring mode.[24,26] The presence of a feature at 531 and 533 cm^{-1} in the spectra of the pink and purple forms demonstrates that both contain bidentate catecholate complexes. Peaks around 620 cm^{-1} in the spectra of the iron−catecholate complexes have been assigned to the Fe−O stretching interaction;[24] in complexes of substituted catechols, this feature is split into two, at approximately 590 and 640 cm^{-1}. These peaks are assigned to vibrations in the bonds between the iron and the catecholate C3 and C4 oxygens.[24,28] Features in Figure 4 at 591 and 638 cm^{-1} (pink Mefp1) and 594 and 639 cm^{-1} (purple Mefp1) are therefore assigned to the Fe−O (C3) and Fe−O (C4) stretches of the iron−catechol complex. The average frequency of these peaks is 2 cm^{-1} greater in the purple form than in the pink form (Figure 4), suggesting that the Fe−O bonds are stronger in the purple form of Mefp1. An increase in average Fe−O bond strength is consistent with greater Lewis acidity of the ferric ion in the purple form. That conclusion is also suggested by the appearance of the catechol-to-iron transfer band at longer wavelength in the purple form than in the pink form.[23]

Similar changes in the energies of the charge transfer band and the Fe−O vibration have been observed in model complexes. For example, $Fe(cat)_3^{3-}$ (cat = catechol) shows a charge transfer band at 500 nm and an Fe−O stretch at 621 cm^{-1} [26] while the charge transfer band in $Fe(NTA)cat^{2-}$ (NTA = nitrilotriacetic acid) is at 618 nm and the Fe−O stretch is at 630 cm^{-1}.[23] These two complexes have very different iron coordination environments, with two bidentate catecholate ligands in the former being replaced by an amine and three carboxylate ligands in the latter (Figure 5), leading to a much greater Lewis acidity of the iron in $Fe(NTA)cat^{2-}$. A more subtle difference, changing a phenoxide ligand to a carboxylate in going from $Fe(HDA)cat^{2-}$ (HDA = *N*-(*o*-hydroxybenzyl)-iminodiacetic acid) to $Fe(NTA)cat^{2-}$, is not sufficient to result in a change in the Fe−O stretching energy and leads only to a 36-nm shift in the charge transfer band.[29] The difference in Lewis acidity of the metal ion in the pink and purple forms of Mefp1 is apparently between these two examples and leads to intermediate differences in both the visible and Raman spectra of these complexes.

The spectroscopic and titration data are consistent with the pink form of Mefp1 containing primarily $Fe(DOPA)_3$ units. The energies of the corresponding Raman features are significantly different in the spectra of $Fe(cat)_3^{3-}$ and the pink form of Mefp1, but changes of similar magnitude and direction are seen in substitution of DOPA for catechol in complexes of tyrosine hydroxylase.[24] The data are consistent with the purple form containing predominantly $Fe(DOPA)_2XY$ units, where X and Y represent ligands other than a bidentate DOPA catecholate.

The data of Enemark and Stack[30] also agree with this model. During the course of their investigations into model siderophore bis(catecholamido)metal complexes, two coordination modes were observed. The first was a bis(μ-hydroxo)-bridged dimer involving bis(catecholato) coordination of the two metal centers bridged by the two ligands and two hydroxides. The second coordination mode involved tris(catecholato) coordination of the two metal centers bridged by three ligands in a dinuclear triple helix. These modes were interconvertible depending on pH or metal:ligand stoichiometry as suggested by the spectroscopic data for ferric Mefp1.

The visible and Raman spectra show that the non-catechol ligands X and Y in the purple form of Mefp1 are less basic ligands than is DOPA catecholate. Candidate ligands that could bridge between ferric ions include either bridging terminal tyrosine phenoxides and aspartate or glutamate carboxylates, as well as bridging μ-catecholato, μ-hydroxo, and μ-oxo ligands.

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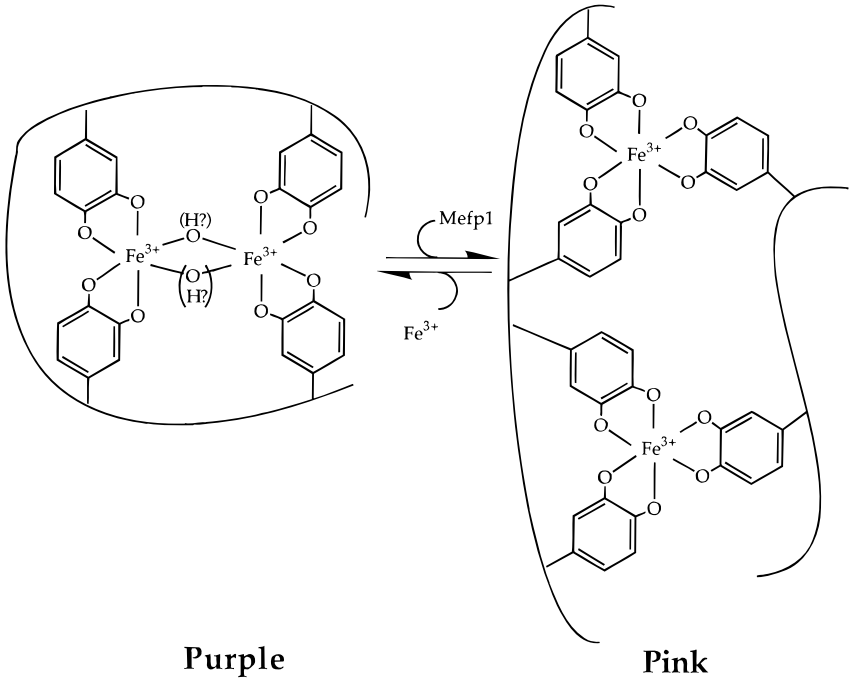

Figure 6. Proposed iron(III) coordination modes for purple and pink FeMefp1 complexes.

A bridging DOPA or tyrosine phenoxide should give rise to additional Fe−O vibrations in the Raman spectra at lower energy;[31] these were never observed for the present case. A terminal tyrosine phenoxide ligand is also not supported by the data. The excitation energy used in the Raman experiments (532 nm) is appropriate for tyrosine-to-iron charge transfer absorptions[22] and should have given rise to resonance-enhanced Raman bands for coordinated phenoxide at approximately 1170, 1270, 1500, and 1600 cm^{-1}.[22,27] In particular, the sharp band found in iron−tyrosinate complexes around 1600 cm^{-1} was never observed in the spectra of purple Mefp1. Carboxylate ligation from glutamate or aspartate is also unlikely because there are only two such residues in the mature protein, far less than sufficient for stoichiometric coordination to the iron centers. Consequently, we conclude that the most likely choices for X and Y are μ-oxo or μ-hydroxo ligands, and we suggest the model shown in Figure 6, in which the purple form of Mefp1 contains μ-oxo or μ-hydroxo-bridged dimers of bis(catecholato) Fe^{3+}.

In favorable cases, it is possible to observe μ-oxo ligands directly by Raman spectroscopy, but excitation wavelengths shorter than 450 nm (inaccessible with our apparatus) are required for resonance enhancement.[32] In addition, the resonance enhancement of the μ-oxo Fe−O stretch is greater in complexes with imidazole (or other unsaturated N-donor heterocycle) ligands *trans* to the bridging oxo ligand.[33] The model for the iron site in the purple form of Mefp1 (Figure 6) does not include histidine imidazole ligation; there is no evidence in the Raman spectra for an Fe−imidazole stretch between 220 and 280 cm^{-1}. Therefore, we predict that it would be difficult to observe the μ-*o*xo Fe-stretch even with the appropriate excitation wavelength.

It is not possible at this stage to determine whether the catechol coordination is intramolecular, intermolecular, or both as Mefp1 and its ferric complex adhere tenaciously to most surfaces at pH 7.0, making the study of protein−protein−ferric ion interactions extremely difficult. A previous unpublished study of the bis- and tris(catecholato) coordination modes of selected Mefp1-derived peptides using electrospray ionization mass spectrometry was unable to detect any intermolecular complexes.[34] This needs to be pursued in future research.

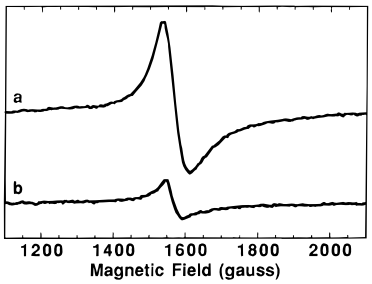

Figure 7. EPR spectra of (a) pink and (b) purple Mefp1. Both samples were prepared with 0.15 mM Fe^{3+} by addition of different amounts of apoprotein.

EPR Spectroscopy. EPR spectra of both the pink and purple complexes of Mefp1 were obtained at a ferric ion concentration of 0.15 M and temperature of 20 K. Both solutions gave a $g \approx 4.3$ signal characteristic of high-spin ferric ion in an orthorhombic environment. Similar spectra have been reported for a variety of synthetic ferric catechol complexes of non-heme iron proteins.[4,23]

The signal intensity in the spectrum of the pink form was about 4-fold higher than in the spectrum of the purple form (Figure 7). This suggests that a large proportion of the iron in the purple sample was not EPR-active perhaps by virtue of being present as antiferromagnetically coupled dimers. Indeed, such dimers were revealed by Mössbauer spectroscopy of ferreascidin prepared from excess iron under similar conditions.[15] Such dimers might be revealed in EPR spectra obtained at higher temperatures. For example, in the case of $(Ph_3\ P)_2[Fe_2(cat)_2(H_2O)_2]\cdot 6H_2O$, the ferric ions are bridged by catecholato oxygens resulting in a weak antiferromagnetic coupling ($-J = 9.7\ cm^{-1}$).[35] At temperatures as low as 20 K, the $S = 1$ excited

(31) Assuming for a terminal catecholate $r_{Fe-O} = 1.9$ Å and $\nu_{Fe-O} = 620\ cm^{-1}$, and for a bridging catecholate $r_{Fe-O} = 2.05$ Å, Badger's rule predicts $\nu_{Fe-O} \approx 450\ cm^{-1}$ for the bridging catecholate.

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state is sufficiently populated to give rise to EPR absorption in the $g = 2$ region. Ferric ion dimers bridged by μ-hydroxo ligands also give relatively small coupling ($7 < -J < 17$ cm^{-1}),[36] so one might expect to see features arising from the excited state at temperatures below 100 K in that case. On the other hand, ferric ion dimers bridged by μ-oxo ligands show strong coupling ($-J > 80$ cm^{-1}), and the excited states are insufficiently populated at reasonable temperatures to yield EPR spectra.[36] We observed no protein-derived signals other than those at $g \approx 4.3$ in spectra obtained between 5 and 100 K. Although this might suggest that the postulated diferric cluster in the purple form of Mefp1 has strong antiferromagnetic coupling and therefore probably μ-oxo rather than μ-catecholato bridging ligands, it has been pointed out that EPR spectroscopy is an unreliable tool for determining the identity of bridging ligands in these systems.[36]

We also attempted to detect the semi-met state of the predicted clusters by EPR after reduction. Degassed samples of both the purple and pink forms of Mefp1 were reduced by anaerobic addition of 0.5−1.5 equiv of sodium dithionite with a trace of methyl viologen. EPR spectra demonstrated partial reduction in intensity in the $g \approx 4.3$ signal in both cases, but no increase in intensity in the $g \approx 2$ region resulting from production of a spin-coupled semi-met ($Fe^{3+}-Fe^{2+}$) cluster in either case. Again, this negative evidence is not very meaningful; although the semi-met forms of hemerythrin, methane monooxygenase, and the purple acid phosphatases are relatively easy to generate and have been characterized by EPR,[37,38] those of the μ-oxo-bridged diferric clusters in ribonucleotide reductase[39−42] and fatty acid-CoA desaturase[43] have proven to be very difficult or impossible to obtain.

Conclusions

These results may cast some light on how mussels contrive chemically to enrich their byssus with iron. With as many as 150 DOPA residues per molecule of Mefp1, the protein has the capacity to bind between 50 and 75 tightly complexed ferric ions depending on whether it adopts a tris- or bis(catecholato) coordination mode, respectively. Since Mefp1 is applied to the byssus as a lacquer-like coating, the addition of ferric iron may render it an "iron−clad" finish and contribute to the notorious intractability of this material. It goes without saying that, in the presence of ferric iron, Mefp1 forms an extremely complicated macromolecular complex even without invoking the possibility of higher molecular weight aggregates due to the *intermolecular* coordination of ferric ion. Notwithstanding this, we have found in the current study that Mefp1 behaves very similarly to low molecular weight catecholates. Like the siderophores, the protein can solubilize ferric iron even when present in polynuclear hydrolytic species inaccessible to simple complexing agents. The pink and purple complexes described herein may represent two important types of ferric ion complexation, the predominant form being determined by the relative concentrations of iron and Mefp1 at physiological pH with the tris(catecholato) coordination mode being favored in sea water at pH 8.0. Admittedly, the formation of μ-oxo/hydroxo-bridged, antimagnetically coupled bis(catecholato) Fe(III) dimers is tentative at this stage and cited only because it is the only reasonable configuration to fit the experimental data. Further Mössbauer studies are expected to determine the exact nature of the EPR-silent iron centers but are presently constrained by the availability of only limited amounts of protein. Notably, μ-hydroxo bridging has recently been reported in a low molecular weight synthetic bis(catecholato) Fe(III) dimer. In this case, however, significant antimagnetic coupling occurred only in the solid state; coupling in solution was weak at best.[44,45]

It is hoped that this and future studies will illuminate how iron coordination stabilizes proteins in the byssus and, perhaps, be able to explain some of the remarkable anticorrosive properties exhibited by Mefp1 adsorbed onto stainless steel.[46]

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