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The role of metals in molluscan adhesive gels

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Summary

Several gastropod molluscs produce glues that are interesting because they are dilute gels and yet they produce strong adhesion. Specific glue proteins have been identified that play a central role in this adhesion, possibly by crosslinking other polymers in the gel. This study investigates the role of metals in the action of these glue proteins. Atomic absorption spectrometry showed that glue from the slug *Arion subfuscus* contains substantial quantities of zinc (46±7 p.p.m. and 189±80 p.p.m. in two different sets of experiments) and also iron, copper and manganese (2–7 p.p.m.). Iron-specific staining demonstrates that iron is bound specifically to the 15 kDa glue protein. Several approaches were used to show that these metals have important functional effects. Adding iron or copper to dissolved glue causes the proteins to precipitate rapidly, although zinc has no effect. Removing iron and related transition metals with a chelator during secretion of the glue causes a sixfold increase in the solubility of the glue. Once the glue has set, however, removing these metals has no effect. Finally, the gel-stiffening activity of the glue proteins was measured in the presence and absence of the chelator. The chelator eliminated the gel-stiffening effect of the proteins, suggesting that transition metals were necessary for the proteins to act on the gel. Thus, the glue contains transition metals and these metals play an essential role in glue function.

Key words: iron, zinc, adhesion, glue, gel, gastropod, slug, *Arion subfuscus*.

Introduction

Biological adhesives are interesting because they have useful properties that differ substantially from those of artificial adhesives (Smith and Callow, 2006). Most strikingly, many biological adhesives form strong attachments underwater. They adhere strongly and non-specifically, even to highly irregular and fouled surfaces. Much could be gained from learning how they create such adhesion. To do this, it is necessary to know the structure of the glues and how that structure gives rise to the physical properties.

Recent work has made substantial progress in determining the biochemical structure of several biological adhesives (Smith and Callow, 2006). It is clear that many animal glues depend on crosslinked proteins to provide cohesive strength; these animals include mussels, barnacles, sea stars, sea cucumbers, frogs and gastropod molluscs (Sagert et al., 2006; Kamino, 2006; Flammang, 2006; Graham et al., 2006; Smith, 2006). The number and strength of the crosslinks in the glue probably play an essential role in its strength (Denny, 1983; Smith, 2002).

The glues of gastropod molluscs are particularly interesting because they are gels (Smith, 2002; Smith, 2006). These dilute gels consist of more than 95% water, yet provide strong attachment forces. The ability to make the gel into an effective adhesive depends on glue proteins (Smith et al., 1999; Smith and Morin, 2002; Pawlicki et al., 2004). These are proteins that are common in the adhesive form of the gel, but not in the similar, non-adhesive gels from the same animal. The glue proteins have been shown to stiffen gels non-specifically (Pawlicki et al., 2004). This suggests that they function by crosslinking the other polymers in the gel or crosslinking to each other, thus forming a much stiffer material. They may also be involved in adhesive interactions with the substrate.

A central question revolves around the nature of the crosslinks. Understanding the bonds between the polymers will go a long way towards explaining the functional properties of the glue. There are a wide variety of possible crosslinking mechanisms, but the utility of some mechanisms is markedly reduced in the marine environment. The ability to form strong crosslinks underwater is impressive because water weakens many common chemical interactions. As described by Waite et al. (Waite et al., 2005), the high dielectric constant of water impairs electrostatic interactions. Because of their polarity, water molecules reorient around charges, effectively neutralizing and masking them. Similarly, water interacts strongly with other polar surfaces. Thus, although hydrogen bonding can often join molecules effectively, this bonding is complicated by the presence of

water. If the adhesive molecules cannot compete with water for hydrogen bonding sites on a surface, or if they cannot exclude water in the area of the bond, the presence of water will substantially impair the bond strength. Covalent bonds work well underwater, but they often have a high specificity for particular substrates. Because of these considerations, artificial glues rarely work in the presence of water. In comparison, the ability of biological glues to adhere under these conditions is intriguing.

Recent work has identified several bonding mechanisms that provide good cohesive strength underwater. Hydrophobic interactions are important for barnacle cement, and these interactions would clearly be effective underwater (Kamino, 2006). Solubility-driven changes in the interactions between calcium and phosphate appear responsible for the properties of some tube-worm cements (Stewart et al., 2004). Another mechanism that is particularly interesting is the use of metal-mediated crosslinks. These appear to play a significant role in mussel byssus formation. Mussels have been shown to sequester iron and use it to form strong crosslinks (Waite et al., 2005; Sagert et al., 2006). Amino acids such as dihydroxyphenylalanine (DOPA) can chelate metals, particularly ferric iron (Waite et al., 2005). The ability to complex with iron oxides may promote adhesion to rocky surfaces, and the ability of several DOPA residues to bind to iron simultaneously can effectively link them tightly despite the presence of water. This mechanism is used by the tough, water-insoluble outer varnish of mussel byssal threads (Waite et al., 2005). Sever et al. similarly show that mussel adhesive proteins can bind iron (Sever et al., 2004), and this can play a role in byssal crosslinking, and may be involved in coupling the proteins to the surface. Other invertebrates may use different transition metals. Zinc and copper have been found to play a role in the mechanical properties of polychaete jaws (Lichtenegger et al., 2003), with zinc-mediated crosslinks between proteins controlling the stiffness and hardness of the jaws of the polychaete *Nereis* (Broomell et al., 2006).

Metal-based interactions can provide strong bonds that are stable underwater, and can act relatively non-specifically. Furthermore, metal binding is not uncommon among proteins, as it can occur with several structural motifs, often involving amino or carboxyl side groups (Lippard and Berg, 1994). Thus, it may turn out to be an important way of crosslinking marine bioadhesives. Previous work by Smith et al. found that limpet adhesive mucus has significantly elevated levels of iron (Smith et al., 1999). One to three percent of the inorganic content of the glue was iron, with several samples having much more (Smith et al., 1999). The rest of the inorganic content was typical of seawater. Thus, metals such as iron may be important for gastropod glues.

This study investigates the role of metal-based interactions in gastropod adhesive gels. The goal of the study is to determine whether transition metals are present in significant amounts in the adhesive gel of the slug *Arion subfuscus*, and to test the hypothesis that these metals are involved in the functional properties of the glue.

Materials and methods

Animals

Experiments were performed using glue from the terrestrial slug *Arion subfuscus* (Draparnaud), which was collected in Ithaca, NY, USA. The glue appears to be a defensive secretion, as Mair and Port found for a similar terrestrial slug (Mair and Port, 2002). The slug was used because it is readily available locally, and it produces a large amount of glue when handled. The glue was collected on the same day the animals were collected. Collection and storage of glue samples was performed as described by Pawlicki et al. (Pawlicki et al., 2004). Note that the glue was never dried, as this makes it difficult to redissolve.

Quantification of metal content

Glue samples were analyzed by inductively coupled plasma (ICP) atomic absorption spectrometry and graphite furnace atomic absorption spectrometry. The graphite furnace provided greater sensitivity, whereas the ICP could quantify several metals simultaneously. The slugs were rinsed with distilled water before collection of the glue. Each sample contained glue from one individual slug. For the ICP analysis, glue samples were hydrolyzed under vacuum at 110°C for 24 h in 6 mol l^{-1} HCl (10 μl per mg of glue). The acid was flash evaporated at 50°C under vacuum and the samples were redissolved in 0.02 mol l^{-1} HCl. They were centrifuged to remove insoluble material and analyzed on the spectrometer. Blanks were treated identically, but did not contain glue. The standard was ICP analytical mixture 6 purchased from High Purity Standards (Charleston, SC, USA), diluted in reverse osmosis (RO)-purified water to 100 and 200 parts per billion (ppb). Three glue samples and two blanks were tested. In addition, five other glue samples and six blanks were tested using shorter hydrolysis without flash evaporation. All values are given in p.p.m. of the initial wet mass of the sample.

For the graphite furnace atomization, glue samples from separate slugs were diced with a razor blade and soaked overnight in RO water (200 μl water per mg glue). The tubes containing the swollen samples were then placed in a sonicator bath for 20 min. The samples were centrifuged to remove insoluble material, and the supernatants were run on the spectrometer. It should be noted that this procedure typically only extracts approximately a quarter to a third of the protein. This is based on Bradford assays of samples dissolved in water *versus* samples dissolved in urea and non-ionic detergent, as described by Pawlicki et al. (Pawlicki et al., 2004). The iron standard was purchased from Sigma-Aldrich (St Louis, MO, USA) and was diluted in RO water. Blanks were RO water, kept overnight in glass tubes and sonicated. Finally, the Bradford assay (Bradford, 1976) was used to measure the concentration of protein dissolved in RO water for comparison with the iron concentration, and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (as described below) was used to determine the relative proportions of the proteins that had dissolved.

The glue was also analyzed with a scanning electron

microscope outfitted for energy dispersive spectroscopy (EDS-SEM). Two separate samples were glued with carbon tape onto a stub, dried overnight at 37°C, and sputter coated with gold for 30 s under vacuum. With the EDS-SEM, it was possible to scan for a wide range of metals, and quantify them roughly, although it was much less sensitive than the atomic absorption spectrometer.

Demonstration of iron binding: electrophoresis and blotting

If there is metal in the glue, it is important to know if the metal is associated with a specific protein. Iron binding by proteins in the glue was measured because initial experiments suggested the importance of iron and because there is a commercially available stain for iron. Samples of glue were dissolved with heat and sonication in 2× sample buffer for SDS-PAGE (0.125 mol l^{-1} Tris-Cl, pH 6.8, 4% SDS, 10% 2-mercaptoethanol, 1.6 mol l^{-1} urea). For this and other experiments, glue was dissolved at a ratio of 1 mg wet glue per 10 μl buffer unless otherwise noted. Samples were centrifuged at 14 000 ***g*** for 10 min to remove undissolved material. They were run on discontinuous 15% gels using the method of Laemmli (Laemmli, 1970) and the protocols of Hames (Hames, 1990). Bovine serum albumin (BSA) was used for comparison. BSA is not known for strong iron binding, but it is capable of binding to iron (Lippard and Berg, 1994; Løvstad, 1995). Ferritin was tested as a positive control in several trials. Part of the gel containing one sample and one control lane was stained with Coomassie Blue R-250. The rest of the gel was electroblotted onto nitrocellulose in Towbin buffer (25 mmol l^{-1} Tris-Cl, 0.192 mol l^{-1} glycine, 0.1% SDS and 20% methanol) using a semi-dry transfer apparatus. After blotting, the gel was stained with Coomassie Blue to determine the efficiency of transfer.

The membrane was divided in two, with each half containing a sample and control lane. Both halves were then stained with the Ferene-S stain described by Chung (Chung, 1985) and Ishikawa et al. (Ishikawa et al., 2003). This consists of 1 mmol l^{-1} 3-(2-pyridyl)-5,6-bis(2-[5-furyl sulfonic acid])-1,2,4-triazine (Ferene S), 15 mmol l^{-1} thioglycolic acid and 2% (v/v) acetic acid. The inclusion of thioglycolic acid prevents interference from copper (Derman et al., 1989). The staining procedure followed the general approach of Chen and Drysdale (Chen and Drysdale, 1993), with one half being incubated with iron, and the other not. Thus, it was possible to distinguish between the ability to bind iron that is present in solution and the presence of iron bound to proteins in the original glue. The staining procedure involved three washes in rinse buffer (20 mmol l^{-1} Tris-Cl, pH 7.0, 150 mmol l^{-1} NaCl) for 10 min each, a 45 min incubation with either rinse buffer or 1 mmol l^{-1} ferrous ammonium sulfate in rinse buffer, three washes with rinse buffer for 10 min each, and then staining with the Ferene-S solution until bands became visible (10–20 min). The membranes were allowed to incubate for an additional 10–20 min after that point. The membranes and stained gels were photographed with a Kodak EDAS-290 digital imaging system (Rochester, NY, USA), and the net intensity of staining was quantified with Kodak 1D software. This experiment was replicated several times using glue from different slugs.

The specificity of the Ferene-S stain was tested by adding different dissolved metals to the stain and measuring the absorbance at 594 nm, as recommended by Qian et al. (Qian et al., 1998). The metals were diluted to approximately 1 p.p.m. in the stain. The following metals were tested: ferrous ammonium sulfate, cupric sulfate, aluminum chloride, zinc sulfate, nickelous chloride, chromium potassium sulfate, cobalt chloride and manganese chloride.

The effect of metals on dissolved glue: precipitation and solubility

If metal binding is important for gastropod glue, then adding metals to glue extracts may result in easily observable changes to the glue, and/or changes in the mobility of proteins on SDS-PAGE. It has been shown that divalent metal binding can cause such changes in mobility (Cartaud and Ozon, 1980). Glue was dissolved in 50 mmol l^{-1} Tris-Cl (pH 7.4) with 150 mmol l^{-1} NaCl with homogenization, heating and sonication. Note that throughout this study, slightly different versions of this basic buffer were used. These differences reflect minor adjustments in procedure over time, but they do not substantially change the results. The sample was centrifuged to remove undissolved material. This extract was divided and different metals were added to each fraction up to a final concentration of 10 mmol l^{-1}. The chloride salts of iron, manganese, cobalt, nickel, copper, zinc, magnesium and calcium were tested. All were in the +2 oxidation state except iron at +3. In addition, ferrous ammonium sulfate was tested to see the effect of Fe^{2+}, and 1 mmol l^{-1} EDTA was tested to see the impact of removing divalent ions. One fraction was left as a control. All samples were vortexed briefly, then centrifuged at 14 000 ***g*** for 10 min. The protein remaining in the supernatant was assayed by SDS-PAGE. This experiment was replicated using ferric chloride concentrations of 1 mmol l^{-1}, 0.1 mmol l^{-1}, 0.01 mmol l^{-1} and 0.001 mmol l^{-1} in a 20 mmol l^{-1} Tris-Cl (pH 8.2) buffer with no sodium chloride. Finally, the experiment was repeated, testing the effect of the transition metals on BSA.

The importance of metals to the integrity of the glue after it has set was investigated by measuring the effect of a metal chelator on glue solubility. Metals were removed or bound by deferoxamine mesylate, which is a high-affinity iron chelator (Lederman et al., 1984; Zanninelli et al., 1997). This has a binding constant of 10^{31} for iron, but also has substantial affinity for other transition metals such as zinc and copper (binding constants of 10^{11} and 10^{14}) (Keberle, 1964; Hider et al., 1999; Maclean et al., 2001). Six trials were performed, using glue from separate slugs. The glue was collected with a spatula and allowed to set for several minutes, by which time it had solidified into a firm, elastic mass. Each glue sample was separated into two roughly equal pieces, which were immediately weighed. Each piece was immersed in either Tris (50 mmol l^{-1} Tris-Cl, pH 7.4) or Tris with 1 mmol l^{-1} deferoxamine mesylate at a ratio of 1 mg glue to 20 μl solution. Samples were dissolved with an identical protocol of repeated

heating (to 60°C) and vortexing. They were then centrifuged at 14 000 ***g*** and the supernatant was analyzed using SDS-PAGE stained with Coomassie Blue R-250. The net staining intensity of each of the major bands on the gel was quantified using the EDAS 290 digital imaging system. For quantification, the bands on SDS-PAGE were categorized into three groups that represented most of the common proteins in the glue, and which were easy to quantify accurately. These were around 61 kDa, 40 kDa and 15 kDa. The 61 and 15 kDa groups were proteins that are predominantly found in the glue, and include one main band and several neighboring bands of slightly different mobility. Several less intense bands of 116 kDa and greater were not included, because they were prone to more background distortion. Nevertheless, the results for these bands were similar to those of the other bands. A similar set of three trials was performed using 10 mmol l^{-1} deferoxamine and homogenization in a rotor-stator homogenizer.

The importance of metals to the curing of the glue was tested by chelating metal as the glue was secreted. Sixteen freshly caught slugs were placed in 15 ml tubes containing 2 ml of either 20 mmol l^{-1} Tris (pH 8) or 10 mmol l^{-1} deferoxamine in Tris. They were then shaken on a vortexer on a low setting for 1 min. The vortexing provided mechanical stimulation to trigger glue secretion; the slugs typically secrete glue whenever they are handled or physically disturbed. The washing solution was then collected and analyzed by SDS-PAGE. In addition, several samples of glue were collected with a spatula directly into Tris or Tris with deferoxamine. It should be noted that the pH of the control and treatment solutions was the same.

Because pH affects the stability of some metal interactions, the effect of pH on the solubility of the glue was tested. A glue sample pooled from several individuals was divided into five roughly equal portions. They were then dissolved in different buffers maintaining a ratio of 1 mg glue per 15 μl buffer. The buffers were acetate (pH 4), phosphate (pH 6 and 8) and sodium hydroxide/glycine (pH 10 and 11), all at concentrations of 0.2 mol l^{-1}. All samples were dissolved with the same protocol of repeated heating to 70°C and vortexing (3×1 min for each heating and vortexing step). Samples were then sonicated for 30 s. They were centrifuged and the supernatant was run on SDS-PAGE and quantitated as described above.

The effect of metals on glue protein function: rheometry

The ability of glue proteins to stiffen gels was measured with and without metal chelation. Eight samples of glue from eight different slugs were dissolved in 100 mmol l^{-1} Tris-Cl (pH 7.4) with heat, vortexing and sonication. They were centrifuged to remove insoluble material. Previous work had demonstrated that purified glue proteins can stiffen gels formed from polymers such as agar and citrus pectin (Pawlicki et al., 2004). Whole glue extracts containing glue proteins are also able to do this, and bypassing the purification step greatly simplifies the experiment, but more importantly avoids the possibility of interference from residual urea and Tween, which are necessary in the purification procedures. Also, Tris was used instead of a phosphate buffer, because phosphate can interact with the calcium. Because of these differences, the stiffness values are not directly comparable to those of Pawlicki et al. (Pawlicki et al., 2004).

Four treatments were set up: (1) a pectin control; (2) a control to ensure that the chelator does not directly affect pectin; (3) pectin with glue proteins to quantify the normal gel-stiffening effect; (4) chelation and glue proteins, to determine the gel-stiffening ability in the absence of metals. Eight sets of these four treatments were run, with glue from different slugs used in each set. All samples had citrus pectin (2%), 33 mmol l^{-1} Tris-Cl (pH 7.4), and 50 mmol l^{-1} $CaCl_2$ to assist gelling. The treatment groups had either 1 mmol l^{-1} deferoxamine mesylate, dissolved slug glue (approximately 0.5 mg ml^{-1} total protein), or both deferoxamine and slug glue. Samples (0.5 ml) were mixed using concentrated stock solutions, vortexed briefly, and then their storage modulus was measured with a dynamic rheometer (ARES; TA Instruments, New Castle, DE, USA) as described by Pawlicki et al. (Pawlicki et al., 2004). These experiments were repeated with 0.5 mmol l^{-1} deferoxamine. In addition, qualitative observations of the stiffening effect of glue extracts were observed with different concentrations of deferoxamine.

Results

Quantification of metal content

In one set of trials, samples of glue from three different slugs had zinc contents ranging from 70–340 p.p.m. (Fig. 1). Assuming the glue contains more than 95% water, which is consistent with estimates based on the Bradford assay, a zinc concentration of 200 p.p.m. corresponds to 0.4% or more of the dry weight of the glue. In addition, there was between 2–7 p.p.m. of iron, manganese and copper (Fig. 1). There was no detectable nickel or cobalt in the samples, with the results from these elements indistinguishable from the blanks. The error among repeated trials from each sample was less than 2%.

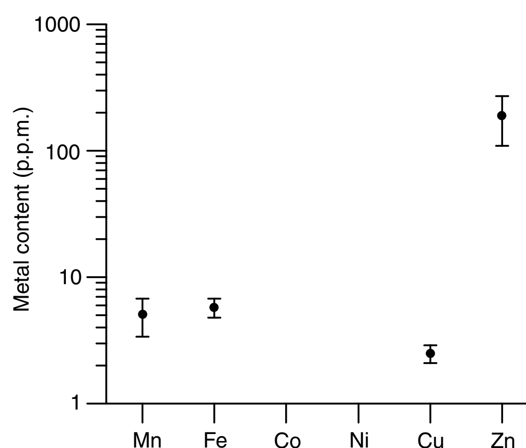

Fig. 1. The metal content of the glue of *A. subfuscus* for three samples using the longest hydrolysis, as determined by the ICP atomic absorption spectrometer. There was no detectable cobalt or nickel. Values are mean ± s.e.m.

The blanks gave readings that would have corresponded to less than 0.6 p.p.m. of each element in the original sample except zinc; one of the blanks had substantial contamination from zinc (115 p.p.m.). Because of the risk of contamination by zinc, the measurements were repeated without flash evaporation. These results were similar with approximately 5 p.p.m. iron and manganese, 0–2 p.p.m. copper and no nickel or cobalt. The zinc levels were lower and more consistent, with a mean value of 46.0±6.9 p.p.m. (mean ± s.e.m., *N*=5) (estimated to be 0.1% by dry mass) (Fig. 1). All six of the blanks for these trials had zinc contents that would correspond to less than 0.6 p.p.m..

The EDS-SEM also found zinc, with concentrations from 0.1 to 0.3% of the dry weight. Thus, it was consistent with the atomic absorption spectrometer, although less precise as 0.1% is near the detection limit of this method.

The atomic absorption results for iron using the graphite furnace attachment for the spectrometer were similar, giving an iron content of intact glue of 8.1±3.3 p.p.m. (mean ± s.e.m., *N*=8). Because the glue was dissolved in water for this test, and only a fraction of the glue dissolves in water, this may underestimate the true iron content of the glue. A gram of slug glue normally contains approximately 30–40 mg of protein (A.M.S., unpublished data), as is true of limpet glue (Smith et al., 1999), but only 12±2 mg was extracted per gram of glue with water. Furthermore, SDS-PAGE showed that the glue proteins made up a relatively small fraction of the dissolved protein, and it is the glue proteins that bind to iron (see below).

To put the metal concentrations in context, it is useful to consider the ratio of metal to protein in the glue. The 15 kDa glue proteins will be considered, as they make up the majority of proteins by molar fraction. If the glue contains 30 mg protein per gram (3% protein), and the 15 kDa glue proteins are approximately one-quarter of the total mass, as Pawlicki et al. (Pawlicki et al., 2004) found, then their concentration is approximately 0.5 mmol l^{-1}. The same mass of larger proteins would correspond to a lower molarity. For the transition metals from iron to zinc, a concentration of 0.5 mmol l^{-1} would correspond to 28–33 p.p.m.. Thus, the ratio of metal atoms to glue proteins is greater than 1:1.

Demonstration of iron binding

The 15 kDa glue protein bound iron strongly, as did several associated proteins of slightly greater mobility on the gel (Fig. 2). In most of the trials these were the only proteins from the adhesive to bind iron detectably, although in two trials the 61 kDa proteins that Pawlicki et al. (Pawlicki et al., 2004) also identified as glue proteins stained as well. In blots that were stained without being preincubated with iron, staining was still positive, although lighter (data not shown). Thus, the proteins had iron that was still bound after electrophoresis in SDS, and could bind more.

The ability of the glue proteins to bind iron was substantially greater than that of BSA. The glue proteins stained far more darkly for iron, despite having less protein in the original gel. In two separate experiments, the intensity of iron staining relative to the amount of protein in the original Coomassie-

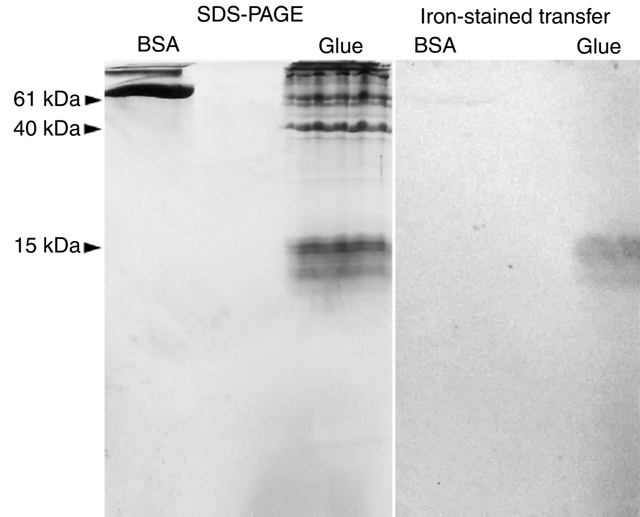

Fig. 2. Electroblot showing the iron-binding ability of proteins in the glue of *A. subfuscus*. The left side shows the Coomassie Blue-stained gel and the right side shows the corresponding ferene-stained membrane. Bovine serum albumin (BSA) is in the left lane of each as a control, whereas the dissolved glue is in the right lane. Molecular masses are indicated in the left margin.

stained band was 8 and 12 times stronger for the glue protein than BSA. The difference is actually greater when one takes into account the poor transfer of the glue proteins to the membrane. Based on staining of lanes before and after transfer, the efficiency of transfer of the glue protein was approximately three times less than that for BSA. In other words, whereas 75% of the BSA transferred, only 26% of the glue protein did. This slow transfer is typical of glue proteins from other molluscan species (Smith et al., 1999). Thus, relative to the amount of protein on the membrane, the glue proteins bound approximately 24–36 times as much iron as BSA. The staining was roughly similar in intensity to the staining of ferritin (data not shown).

The Ferene-S stain was specific for iron. Of all the metals tested, only iron and copper caused a detectable color change at 594 nm. Copper only gave a weak result, however, and it was brown rather than blue. An iron concentration of 1 p.p.m. gave an absorbance of 0.504, whereas copper gave an absorbance of 0.034. All the other metals gave absorbances that were indistinguishable from blanks, although some turned slightly brown or yellow after several hours.

The effect of metals on dissolved glue: precipitation and solubility

The addition of 10 mmol l^{-1} ferric chloride caused rapid precipitation of all the proteins dissolved in the glue extract (Fig. 3). Cupric chloride had the same effect, but none of the other tested metals affected the proteins. A concentration of 1 mmol l^{-1} ferric chloride also caused substantial precipitation, whereas lower concentrations had little effect. It is worth noting that the actual concentration of dissolved iron would be much

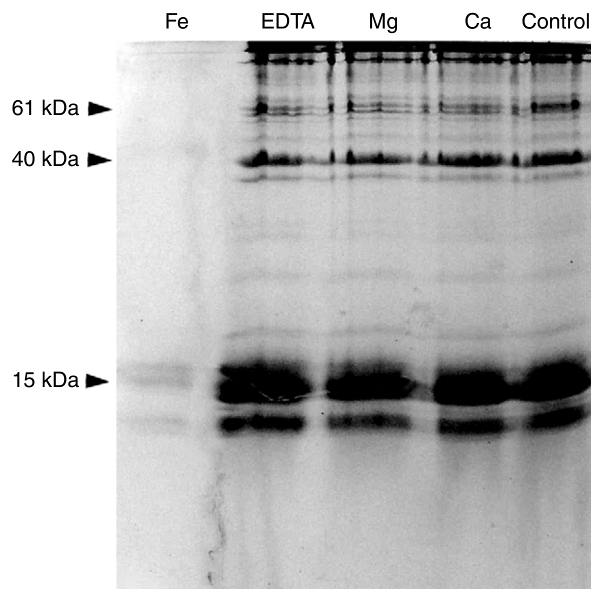

Fig. 3. The effect of specific ions on the proteins in the adhesive gel from *A. subfuscus*. The same amount of dissolved glue was initially present in each sample. From left to right, the lanes had either 10 mmol l^{-1} $FeCl_3$, 1 mmol l^{-1} EDTA, 10 mmol l^{-1} $MgCl_2$, 10 mmol l^{-1} $CaCl_2$ or no extra ions added. Molecular masses are indicated in the left margin.

less than the stated values, because of the low solubility of ferric iron in water (Sun and Waite, 2005). Ferrous ammonium sulfate also caused some precipitation, but not as strongly as ferric iron. None of the proteins showed a shift in mobility in the presence of magnesium or calcium, and these ions caused no precipitation (Fig. 3). In the BSA controls, zinc chloride caused complete precipitation, but this was only apparent after centrifugation and SDS-PAGE. Iron did not cause precipitation that was immediately obvious, but it did cause a noticable decrease in the concentration of dissolved BSA based on SDS-PAGE; roughly half or more of the BSA remained in solution, however. None of the other metals had a detectable effect.

Once the glue had set, removal of transition metals with the iron chelator had no effect on solubility. There were no significant differences between the intensities of Coomassie-stained bands resulting from glues that were dissolved with 1 mmol l^{-1} deferoxamine compared with the controls with just buffer (Fig. 4A). Similar results were found when the samples were homogenized with a higher deferoxamine concentration.

By contrast, when deferoxamine was present while the glue was being secreted, the resulting concentration of dissolved proteins in the washing solution was 6.2 times greater than the control (Wilcoxon rank sum test, P=0.014). The staining intensity of the primary groups of bands showed a quantitatively similar pattern (6–8 times greater staining intensity), although the deferoxamine only caused a 1.8-fold increase with the 61 kDa protein (Fig. 4B). These differences were significant for all three groups of bands (t-test, P<0.03 for all). The protein concentration of the controls was similar to what was typically extracted with the same volume of Tris using heat and vortexing of glue samples that had already set. The deferoxamine extracts were often visibly different as well. When slugs were vortexed with Tris, the buffer remained unchanged and visibly indistinguishable from water, even though the slug was coated with a layer of slime. When the slugs were vortexed with deferoxamine, the buffer usually became yellowish and often viscous.

The glue samples were markedly more soluble in basic buffers, and relatively insoluble at low pH. The biggest change in solubility occurred as the pH rose above neutral. The total net staining intensity in arbitrary units of the bands that were quantified was 8 (pH=4), 12 (pH=6), 79 (pH=8), 68 (pH=10) and 127 (pH=11), respectively. Notably, the acidic pH had no visible effect on the integrity of the gel, even after several days. The gels shrank somewhat, but were completely intact. At basic pH the gels broke up and were mostly solubilized.

The effect of metals on glue protein function: rheometry

Chelating iron and related metals blocked the ability of the glue proteins to stiffen gels (Fig. 5). Without chelation, the glue extract caused a twofold increase in the storage modulus (stiffness) of pectin gels (paired Student's t-test, N=8, P=0.003). Adding 1 mmol l^{-1} deferoxamine with the glue proteins completely blocked this effect, so that the samples were not significantly different from the controls (P=0.15). Deferoxamine alone had no effect on pectin (P=0.99). When these experiments were repeated with 0.5 mmol l^{-1}

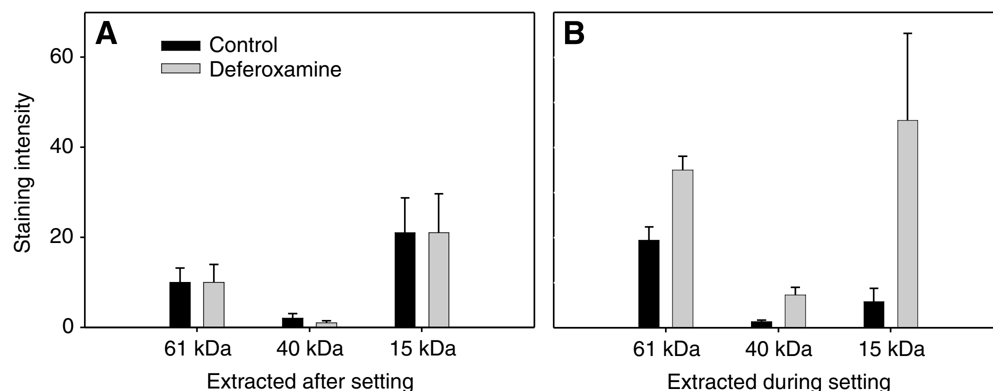

Fig. 4. The effect of deferoxamine on the solubility of *A. subfuscus* glue. Bars show the relative staining intensity of three primary groups of bands on the gel. Proteins were extracted by either a neutral Tris buffer or the same buffer with deferoxamine. (A) Extraction of proteins from gels that have already set. (B) Extraction of proteins from gels as they are being secreted (during setting).

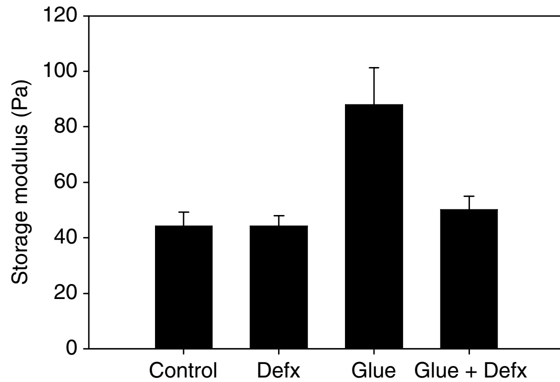

Fig. 5. The effect of iron on the ability of *A. subfuscus* glue proteins to stiffen 2% citrus pectin. Stiffness (storage modulus) was measured with a dynamic rheometer. Approximately 0.5 mg ml^{-1} total protein was used from the glue, and 1 mmol l^{-1} deferoxamine (defx).

deferoxamine, the same results were seen. The only difference was that the mean stiffness of the samples with glue and deferoxamine did not completely return to the control value, but the difference was only marginally significant (P=0.06), and it was still less stiff than the sample with glue alone (P=0.04). In several qualitative tests, deferoxamine concentrations of 0.1 mmol l^{-1} did not always affect the ability of the glue proteins to stiffen gels, but higher concentrations caused greater and more reliable inhibition.

The concentrations of deferoxamine that were effective were what we would expect given the previously determined amount of metal in the glue. Assuming that the intact glue contains a total transition metal concentration of approximately 200 p.p.m., the diluted extracts used in this test would have approximately 3 p.p.m., giving a metal concentration of approximately 0.05 mmol l^{-1}. Deferoxamine binds to metals at a 1:1 molar ratio, according to the information provided by the supplier. In other words, at least 0.05 mmol l^{-1} deferoxamine would be necessary to chelate all the metal. More would probably be necessary because it is competing with the metal-binding sites on proteins and chelation is unlikely to be perfectly efficient.

Discussion

The glue of the slug *Arion subfuscus* contains a significant amount of transition metals, and these metals appear to be necessary for the glue to function. Chelation of the metals blocks the ability of the glue proteins to function in a gel-stiffening assay. Furthermore, the solubility experiments suggest that chelation blocks setting of the actual glue. At present, the exact role of the different metals is unclear. Although zinc is the most prevalent metal, the 15 kDa glue protein specifically binds to iron, and iron and copper have a marked effect on dissolved glue extracts, triggering rapid precipitation. This presumably occurs through binding to the proteins and crosslinking them or changing their solubility. Further work is needed to determine whether the zinc is associated with specific molecules and whether or not all the metals have the same role.

Metal content

The metal content found in *A. subfuscus* glue is far above the levels normally seen in the environment. The zinc in particular is high, but the quantities of iron, copper and manganese are also well above the levels that are naturally present in water. For example, although the iron content of water from natural sources varies considerably, a level of 0.3 p.p.m. is considered the maximum contaminant level for potable water and the median value for drinking water tested in US cities is 0.02 p.p.m. (Pais and Jones, 1997). Head reports values of 0.01 p.p.m. for a clear river and 0.6 p.p.m. for an iron-rich river showing reddish deposits (Head, 1971).

The concentration of metals in slug glue is close to that of several other metal-containing biomaterials. Iron plays a key role in mussel byssus formation (Sever et al., 2004; Waite et al., 2005), and the iron content of the byssal threads and plaques is 20–2000 p.p.m. depending on the environment (George et al., 1976; Coombs and Keller, 1981). Previous work on the adhesive gel of limpets using a scanning electron microscope with an energy-dispersive X-ray system found that 1–3% of the inorganic content of most samples was iron (Smith et al., 1999). There was considerable variation, however, and the possibility of contamination could not be ruled out in that study. Also, that was as a percentage of inorganic content, which made up roughly half of the total dry weight. Finally, the jaws of the polychaete *Nereis* contain approximately 2% zinc by dry weight (Broomell et al., 2006). In general, these concentrations amount to several metal atoms per protein.

Metal binding by components of the glue

The ferene stain, which is highly specific for iron, showed that the iron was found specifically attached to the glue proteins. The location of the other metals in the glue is an essential question for further study. It is possible that the iron-binding ability of the 15 kDa glue protein indicates a general ability to bind transition metals. Alternatively, this may be a specific interaction and zinc may be bound to other components of the glue. It will be interesting to see if the different transition metals are functionally interchangable, or if they play separate roles.

The results of the precipitation experiment suggest that the metals are not completely interchangable. Iron and copper caused marked precipitation of soluble glue extracts, whereas zinc did not (even though zinc could precipitate BSA). These results suggest that proteins in the glue are capable of binding to iron or copper, and this may trigger crosslinking and thus precipitation. Nevertheless, this does not necessarily mean that the smaller amounts of these metals that are naturally present play a similar role *in vivo*. The lack of effect of zinc is also interesting. It may mean that there is already sufficient zinc, and it is fully participating in crosslinking reactions so that excess zinc does not matter. Alternatively, it may imply that

zinc binds less strongly or does not link the proteins as strongly, or that it plays a different role from iron and copper.

The importance of metals to glue function: possible mechanisms of metal-mediated crosslinking

As suggested previously, the most likely way that metals could function in a glue is by crosslinking the polymers in the secretion (Smith, 2002; Smith, 2006). There are two related questions to address if metals are involved in crosslinking. First, does the metal form a structural part of the crosslink, or does it catalyze crosslinking? Second, what is the mechanism involved in crosslink formation? Although the data in this study does not conclusively answer these questions, it is consistent with the hypothesis that metals catalyze crosslinking. The effect of transition metal chelation during glue secretion and in the rheometry assays strongly suggests that transition metals are necessary for the glue to set. Once the glue is set, however, deferoxamine has no effect on solubility. This could mean that the metals are not part of the final crosslinks.

An alternative interpretation of the solubility experiments is that metals are structural parts of the crosslinks but deferoxamine may not be able to remove them after the glue sets. It is possible that deferoxamine does not have sufficient affinity to outcompete the glue proteins for iron once they have linked, or perhaps it cannot penetrate aggregated clumps of protein. There may also be other types of interaction that act in addition to iron crosslinks, and which must be broken as well to separate the proteins. These possibilities are not specifically excluded by our experiments.

When using treatments such as metal chelation, it is also important to consider the possibility of non-specific effects. For example, in the solubility experiments it is possible that the deferoxamine somehow stimulates the secretion of more glue, rather than dissolving more of what is secreted. There are several reasons to believe that this is not the case. First is the consistency of these results with the rheometry experiments, which controlled for non-specific effects. Also, the slugs are quick to respond to any mechanical stimulation by secreting the adhesive. Being vortexed in a small tube virtually assures substantial release of glue. Despite this, little protein went into solution when only a Tris buffer was used, even though the slugs were clearly coated with their secretion. By contrast, the samples collected in deferoxamine achieved good extractions; these were substantially better than was achieved with heating and vortexing of samples that had been already set. Finally, it should be noted that deferoxamine is a standard clinical treatment for humans suffering from iron overload (Lederman et al., 1984; Zanninelli et al., 1997). It is regarded as relatively safe (Lederman et al., 1984) and it is not lipophilic, so it doesn't penetrate cells (Zanninelli et al., 1997). Thus, it is not absorbed across mucus membranes and must be injected when used therapeutically.

The second question focuses more specifically on the mechanism. The most likely possibilities are electrostatic interactions, ionic interactions driven by low solubility of two components of the gel, coordinate covalent bonds and metal-catalyzed oxidations. Electrostatic interactions involving divalent ions are used in gel-forming polymers such as pectin (Smith, 2002). They would seem unlikely to provide substantial strength in water, however, because of water's ability to mask charge. Furthermore, electrostatic interactions involving transition metals would probably be disrupted easily by strong chelators such as deferoxamine.

It is also possible that the metals form insoluble complexes with other groups, thus forming strong ionic bonds that are inaccessible. This is what seems to occur between calcium and phosphate in tube worm cement (Stewart et al., 2004). The sudden precipitation of the proteins from slug glue when exposed to iron or copper would be consistent with this mechanism.

Coordinate covalent bonds are also possible. These are interactions in which the metal is a Lewis acid, with vacant orbitals that can share electron pairs that are available on Lewis bases such as amino groups (Lippard and Berg, 1994). Each metal may be coordinated by several ligands in this way, thus linking them together. Such interactions would be stable underwater. Nevertheless, they would be more soluble at low pH, which was not the case with the slug glue. In fact, the exact opposite was true. Similarly, Smith et al. note that limpet glue is more soluble in basic than acidic solutions (Smith et al., 1999). The low solubility could be explained by the low isoelectric points of the proteins, which are between 4.7 and 5.3 (Smith, 2006). These proteins may be expected to be insoluble at low pH. This would not explain why the integrity of the glue was unaffected by acid, however. If coordinate covalent bonds were essential for the structure of the gel, one would expect acid to break down the gel.

The previous mechanisms involve the metals as structural components of the crosslink, but it is also possible that they catalyze crosslinking instead. One mechanism by which they could do so is through redox chemistry. Sever et al. show that iron in mussel adhesives is associated with organic radicals (Sever et al., 2004). They propose that iron that has been chelated by the adhesive protein triggers protein oxidation and production of reactive radical species. This may play a key role in forming interactions between the protein and the substrate. Such metal-catalyzed oxidations are relatively common (Stadtman and Berlett, 1991). They would also be effective in modifying a variety of functional groups, particularly carboxyl groups. Such modifications can often make proteins more likely to crosslink. This would explain the finding that the glue proteins are relatively non-specific (Pawlicki et al., 2004). Pawlicki et al. had suggested that the glue proteins did not play a catalytic role because of this non-specificity (Pawlicki et al., 2004), but the results of this study suggest that such a role is possible. Nevertheless, the predominant metal in the glue is zinc, which is not as active as other metals in oxidation reactions (Fenton, 1995). Iron and copper are much more redox active, and they are present in smaller but still significant quantities. It may be that they work together with zinc, or that they act by one mechanism while zinc is involved in another. Finally, it is worth noting that electrostatic interactions and

coordinate covalent bonds may still play a role in metal-catalyzed oxidations, as they could account for the glue proteins' ability to bind to metal in the first place, and they may be involved in the initial interactions during formation of the crosslink.

In conclusion, there are several possible metal-mediated crosslinking mechanisms that would work well underwater. It is also possible that several work in concert. Thus, the finding that metals play an essential role in glue function opens up a variety of possible areas of investigation that may help guide the design of artificial glues.

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