Oxidative Stress and the Mechanical Properties of Naturally Occurring Chimeric Collagen-Containing Fibers

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ABSTRACT The byssal threads of marine mussels are a fiber-reinforced composite material. Fibers are continuous, separated by matrix, and consist of chimeric collagens that encompass within the same primary protein structure domains corresponding to collagen, polyhistidine, and either elastin or dragline spider silk. The elastic modulus (stiffness) of the proximal portion of byssal threads was measured by cyclic stress-strain analysis at 50% extension. Before measurement, the threads were conditioned by various treatments, particularly agitation in aerated or nitrogen-sparged seawater. Stiffness can be permanently increased by more than two times, e.g., from 25 MPa to a maximum of 65 MPa, by simple agitation in aerated seawater. Much but not all of this stiffening can be prevented by agitation under nitrogen. Reversible strain stiffening would seem to be a useful adaptation to lower residual stresses arising from the deformation of two joined materials, i.e., distal and proximal portions with rather different elastic moduli. The permanent strain stiffening that characterizes proximal byssal threads subjected to oxidative stress is probably due to protein cross-linking. In the short term, this results in a stronger thread but at the expense of dynamic interactions between the molecules in the structure.

INTRODUCTION

Byssal threads are collagenous fibers used by marine mussels to attach to hard surfaces and provide shock absorption against buffeting by waves. Whereas they resemble the tendons of typical fusiform muscle structures in having distinct origination and insertion points, they are distally extended into seawater well beyond the confines of living tissue (Waite, 1992). There are no fibroblasts or other cells to maintain the extracellular matrix proteins in byssal threads once they are secreted (Bairati and Vitellaro-Zuccarello, 1976; Benedict and Waite, 1986).

As might be expected for tendons that must function and fend for themselves in high-energy seawater habitats, byssal threads have evolved some unusual strategies to cope with turbulent environments. Among the more obvious of these are the deposition of a protective cuticle around each thread (Vitellaro-Zuccarello, 1981), the addition of a substantial matrix component (Vitellaro-Zuccarello et al., 1983), and the "redesign" of collagen. Accordingly, byssal threads are a composite material in which anisotropic bundles of continuous collagen fibers are embedded in a microfibrillar matrix, all of which is surrounded by thin cuticle (diameter 5–10 μm). With respect to collagen redesign, recent studies have revealed that byssal collagens (250 kD) are chimeric in the sense that they have a "block copolymer" structure in which a central collagen domain is flanked by either silk-like or elastin-like domains and terminated at both ends by histidine- and DOPA-rich sequences (Coyne et al., 1997; Waite et al., 1998) (Fig. 1). Natural consequences of this redesign include the loss of quarter-stagger assembly, an ultimate strain 6 to 16 times greater (Fig. 1) than the breaking strain of 10% in mature rat tail tendon (Kastelic and Baer, 1980), and a histidine/metal-mediated assembly (Waite et al., 1998). Matrix proteins are particularly prominent in the proximal portion of the thread and can exceed 30% of the total protein (Waite et al., 2001). Characterization of the matrix proteins is just beginning.

Fig. 1 summarizes the known mechanical properties of the proximal and distal portions of a byssal thread and how these correlate with the distribution of the silk-like and elastin-like flanking domains in the byssal collagens or preCols. The distal portion, which is attached to a variety of hard surfaces by way of the adhesive pad, is much stiffer than the proximal moiety, which is fused to retractor muscles within the animal.

In this study, we set out to examine the modulus or stiffness of proximal byssal threads as modified by mechanical and oxidative stress factors. Our results show that whether repetitively loaded under tension or agitated in seawater, proximal byssal threads possess an inherent capacity to harden and stiffen. Most of the hardening connected with oxidative stress is permanent, whereas hardening associated with cyclic stress-strain or shaking in oxygen-depleted seawater is reversible.

MATERIALS AND METHODS

Marine mussels *Mytilus galloprovincialis* were collected from Goleta Pier (Goleta, CA) and transferred to aquaria with flowing raw seawater (15°C) after removal of the old byssus. The proximal portions of byssal threads deposited by individual mussels over a 4- to 5-day period were dissected from the distal portions and stem material. Each experimental data set was obtained using threads (80–100 threads/mussel) produced by a single mussel. Hydrated thread diameters (range 50–100 μm) were measured using a graticule-fitted stereomicroscope. All thread treatments were car-

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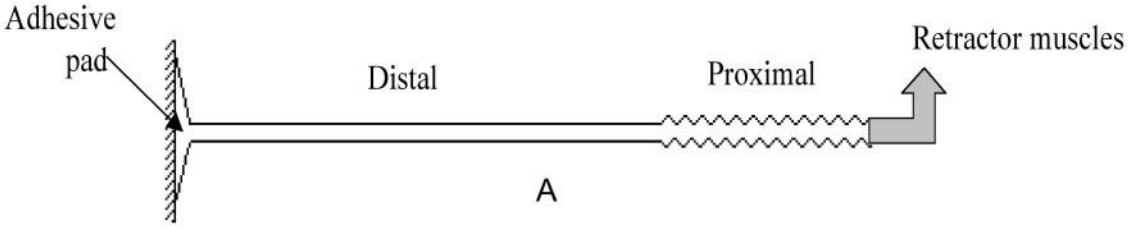

A

PORTION	STRAIN	STRESS MPa	MODULUS MPa	FLANK TYPE
Distal	0.6	90	300	dragline silk
Proximal	2.0	25	26	elastin

B

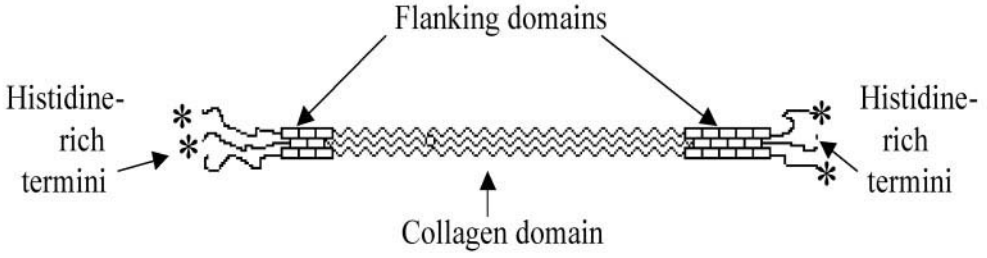

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FIGURE 1 (*A*) Schematic model of a mussel byssal thread (typical length 2–3 cm and 0.05- to 0.1-mm diameter) showing the distal and proximal portions as well as location of muscle fusion and adhesive pad attachment. (*B*) Correlation of breaking stress, strain, elastic moduli with preCol flanking structures for the distal and proximal portions of byssal thread from data by Bell and Gosline (1996) and Smeathers and Vincent (1979). (*C*) Block domain structure of byssal preCols. The length of the collagen domain is ~150 nm. *, At the end of the histidine-rich domains represents the location of DOPA side-chains.

ried out at 20°C and involved agitation or stationary incubation of thread sets in 10 mL of filtered seawater using 20-mL scintillation vials with loose-fitting caps. Concentration of oxygen in seawater at 20°C is given as 0.231 mol m^{-3} (Weiss, 1970).

Samples were agitated open to air at 200 rpm on an orbital shaker (Gyrotory, Model G2, New Brunswick Scientific, New Brunswick, NJ). Some sample sets were shaken or kept stationary under a positive nitrogen (ultrahigh purity) flow of 20 cc/min. Others were shaken or kept stationary in filtered natural seawater to which 20 units/mL of native or heat denatured phenoloxidase (Sigma, St. Louis, MO) were added. Recovery time for shaken and control samples was 24 h.

Cyclic tensile stress-strain tests were performed on a Bionix 200 instrument (MTS Systems, Cary, NC) equipped with a 10-N load cell set at a maximal strain of 50% and a cross-head speed of 5 mm/min. Gauge lengths ranged from 3 to 4 mm. The cyclic stress-strain tests were performed as follows: the cross-head was set to pull the specimens to 50% strain at 5 mm/min and then to return to zero strain. Thread ends were sandwiched between two layers of double-stick tape before fixation by sample grips. All samples were tested in filtered seawater, except for the phenoloxidase experiment, in which the same treatment solution (20 units/mL enzyme in seawater) was used to wet the specimens. The elastic modulus was estimated from the best slope fit of the stress-strain curves, usually between 45 and 50% strain. Stress and strain are used according to the engineering convention, which assumes that the cross-sectional area and the unstretched length of the specimen remain constant during testing.

RESULTS

The proximal portion of byssal threads was selected for testing because it is the weakest and most extensible link in the byssus (Bell and Gosline, 1996). Any increase in the strength of this portion has the potential to improve the tenacity of the entire structure. Because determining the strength, also known as ultimate tensile stress, is a one-time measurement that involves extending threads to breakage, we opted here instead for a cyclic measurement of stress and modulus at 50% extension. An increase in modulus con-

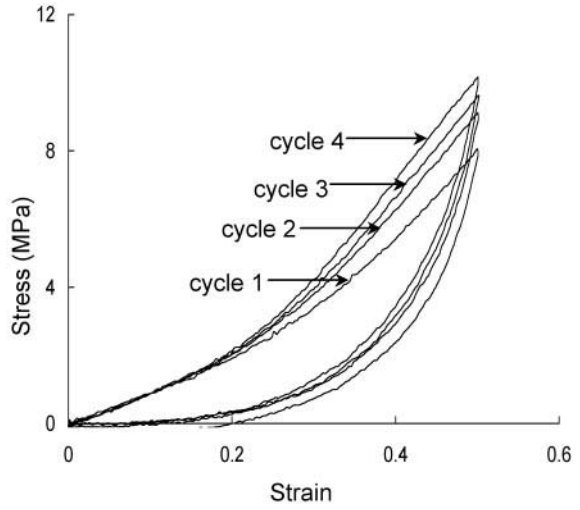

FIGURE 2 A family of stress-strain curves for a virgin proximal thread (cycle 1, $t = 0$; cycles 2–4 each 3 min after the previous cycle).

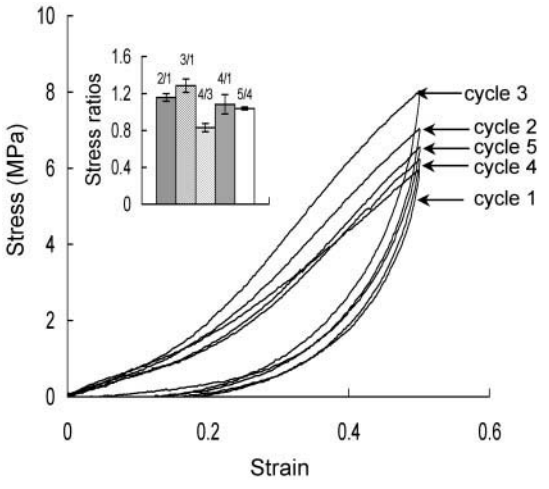

FIGURE 3 Family of stress-strain curves for a virgin proximal thread with rest interval (cycles 1–3, consecutive stress-strain cycles; cycle 4, 60 min after cycle 3; cycle 5, immediately after cycle 4). (*Inset*) Stress ratios at 50% strain for the five cycles ($n = 10$). The stress ratio after last pull (4/3) is significantly lower than all other ratios. Values are averages ± SE.

comitant with cyclic extension is referred to as "strain stiffening," whereas an increase in stress (but not modulus) at fixed extension is "strain hardening." Due to individual mussel-dependent thread diameter and the effect of strain history on the mechanical behavior of byssal threads (Vaccaro and Waite, 2001; Bell, 1998), collected proximal threads were not randomly pooled but were tested as "sets" from individual mussels *M. galloprovincialis*. A set of "virgin" byssal threads, that is, those threads with no prior strain history, were extended to a strain of 50% in a series of four cycles each at 3 min after the previous cycle (Fig. 2). As shown, there is strain hardening in which the maximal stress at 50% strain increases with each stress-strain cycle. Fig. 3 indicates that this behavior is reversible; this thread was cycled consecutively three times and then rested for 60 min. The post-rest 4th cycle shows a nearly complete return to the initial stress-strain pattern. It is again hardened, although to a lesser extent, if followed immediately by a 5th cycle to 50% strain (Fig. 3). The stress-strain curve at $t = 0$ (cycle 1) is representative of viscoelastic materials such as collagen and α-keratin with an initial "toe" region in which stress and strain are nearly independent of load, followed by a "heel," and then by a steep linear portion (Fratzl et al., 1997). The linear portion customarily provides the slope for estimation of Young's modulus in collagens (Bennet et al., 1986) and was adopted here for byssal threads as well.

The dependence of the steepest portion of the modulus on the rate of strain is shown in Table 1 for three cross-head speeds. These speeds translate to ~0.1 min^{-1}, 0.5 min^{-1}, and 2.5 min^{-1} given a typical initial length of 1 cm. Proximal threads were also tested at three different strain amplitudes: 0.25, 0.50, and 0.70 (Table 1). Within the range tested, proximal thread modulus does not show a strong dependence on the rate of strain. There may be a slight increase in modulus with increasing strain amplitude.

To explore the effect of aerated agitation, experimental thread sets were subjected to two different conditions: shaking in aerated seawater and shaking in aerated seawater with added phenoloxidase. Controls were unshaken threads with or without added phenoloxidase, both open to air. Threads shaken for 24 h appear to be conditioned or tempered to maximal stress and stiffness levels with no margin for change (Fig. 4) in contrast to unshaken threads. The moduli resulting from the different treatments in the first experiment are summarized in Fig. 5. Note that threads shaken in seawater open to air are more than twice as stiff as the unshaken controls. Threads shaken with phenoloxidase exhibit a dramatic and significant enhancement in modulus and strength at 50% strain relative to the unshaken controls (Fig. 5): stiffness increases to a maximum of three times the

TABLE 1 Dependence of proximal byssal thread modulus (final modulus) on strain rate and strain amplitude used in testing

	Strain amplitude			Strain rate % min^{-1}		
Modulus	25%	50%	70%	0.1	0.5	2.5
MPa	33.84 ± 2.36	37.36 ± 2.57	41.16 ± 4.16	26.12 ± 2.82	38.14 ± 4.61	33.57 ± 4.9

Mean, SE are as shown. $N = 10$. Strain rate is approximate and determined from cross-head speeds of 1, 5, and 25 mm min^{-1} and a sample length averaging ~10 mm.

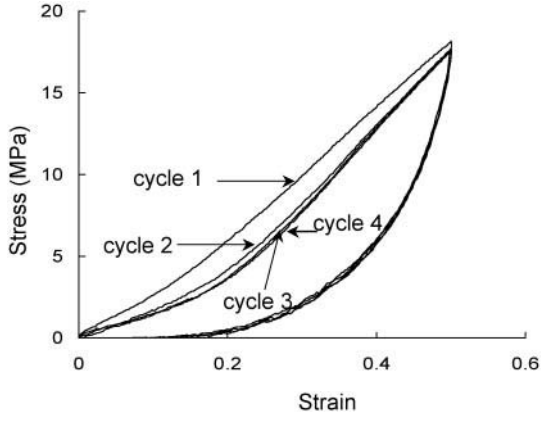

FIGURE 4 Family of stress-strain curves for threads shaken in aerated seawater (cycle 1, $t = 0$; cycle 2–4 each 3 min after the previous cycle).

unshaken control without enzyme. Shaking with heat-denatured enzyme was not different from shaking without enzyme (data not shown).

These results beg two interpretations: 1) threads are stiffened by mechanical strains associated with deformation during shaking and 2) tempering by shaking is facilitated by better oxygen mixing, which drives an oxidative curing process in the threads. To test which of these is more probable, a second set of threads was subjected to two conditions: shaken or shaken with nitrogen sparging (Fig. 6). Both controls were unshaken: one with and the other without nitrogen. This time, the threads shaken under nitrogen were slightly stiffer than the unshaken controls, but both were significantly less stiff than those shaken in air. The stress levels at 50% strain followed a similar pattern. Given

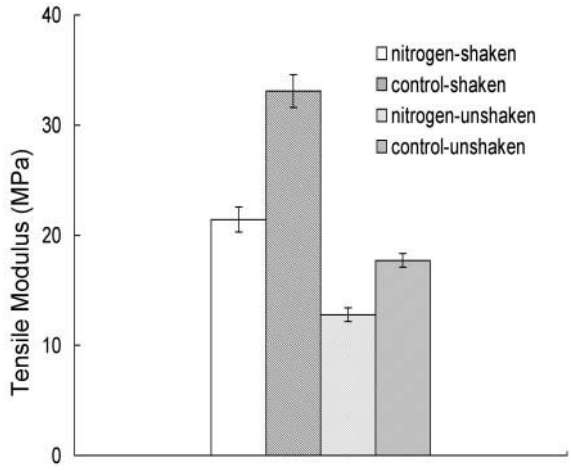

FIGURE 6 Effect of shaking with nitrogen sparging. (*Nitrogen-shaken*) Shaken for 24 h in seawater under a flow of nitrogen ($n = 24$); (*nitrogen-unshaken*) unshaken for 24 h under nitrogen ($n = 22$); (*control-shaken*) $n = 20$; (*control-unshaken*) $n = 24$: same as in Fig. 5. Values are averages ± SE.

that the unshaken controls placed under nitrogen had the lowest observed modulus, oxygen seems to have been effectively depleted by sparging.

In view of the stiffening effect of agitation on threads in aerated seawater, the rate of stiffening and capacity to recover initial modulus were of interest. Results of a third experiment with a fresh set of threads that was shaken for 1 and 24 h indicated that hardening by shaking was not a rapid transformation: the modulus changed by less than 10% after 1 h of shaking (Fig. 7). Moreover, even after 24 h of unagitated rest, the modulus in samples previously shaken

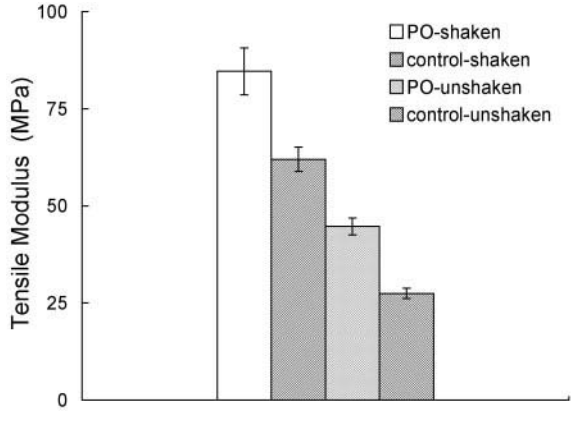

FIGURE 5 Effect of aerated shaking and phenoloxidase (PO) on thread modulus. (*PO-shaken*) Threads shaken for 24 h in the presence of phenoloxidase and in aerated seawater ($n = 31$); (*PO-unshaken*) threads immersed in the phenoloxidase/seawater solution and open to air for 24 h under stationary conditions ($n = 32$); (*control-shaken*) threads shaken for 24 h in seawater open to air ($n = 24$); (*control-unshaken*) threads under stationary conditions for 24 h in seawater open to air ($n = 40$). Values are averages ± SE.

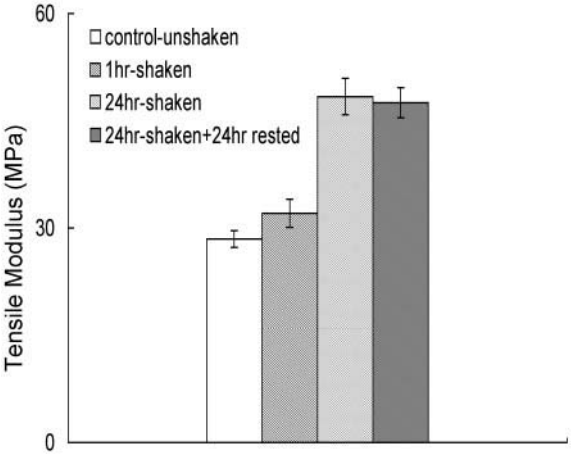

FIGURE 7 Irreversibility of stiffening induced by agitation in aerated seawater. (*24 hr-shaken*) Threads shaken for 24 h in aerated seawater ($n = 23$); (*24 hr-shaken+24 hr-rested*) threads shaken for 24 h in aerated seawater, followed by rest for 24 h open to air ($n = 24$); (*1 hr-shaken*) threads shaken for 1 h in aerated seawater ($n = 36$); (*control-unshaken*) $n = 24$: same as in Fig. 5. Values are averages ± SE.

in aerated seawater did not recover to the levels of the unshaken controls (Fig. 7).

DISCUSSION

The results reported here make two points: one expected and the other unexpected. The expected point is that proximal byssal threads subjected to repetitive loading show a strain hardening and/or stiffening tendency. This is apparent in control threads cycled to 50% extension at various intervals (Fig. 2). A similar trend is evident in threads shaken for 24 h in seawater sparged with N_2. Strain hardening has been reported in a variety of fiber-forming proteins. Type I collagen exhibits strain stiffening that is correlated with strain amplitude (Viidik et al., 1982). Actin in the presence of α-actinin (Xu et al., 2000) and keratin proteins IF-I, IF-II, and vimentin (Ma et al., 1999; Janmey et al., 1991) all exhibit strain-hardening that is shear dependent. In keratins, stiffness increases by an order of magnitude with 10 consecutive deformations before saturating (Ma et al., 1999). In contrast, the interaction of actin and α-actinin provides an intriguing model of fiber-matrix relationships: strain hardening is attributed to dynamic cross-linking of actin by α-actinin. Apparently cross-linking loci in actin are exposed by deformation. Actin and keratin both share with byssal threads a tendency to recover initial modulus and stress upon relaxation.

The unexpected point to emerge from the results is that aerated shaking of threads in seawater leads to a permanent hardening or sclerotization (Fig. 4). Oxidative stress seems a plausible explanation for this, i.e., increased mixing in the shaken solutions leads to thinner stationary boundary layers and better oxygen supply for oxidation-driven cross-linking processes. There is precedence for oxidative effects on the modulus of structural proteins: a brief treatment of elastin with 20% sodium hypochlorite increased the stiffness by a factor of eight (Urry et al., 1988). The modulus of tendon type I collagen increases with maturation (from 10 to 140 days) (Viidik et al., 1982) reflecting the oxidation-dependent formation of aldimine and aldol condensation cross-links. Such cross-links were not detectable in byssal threads (Van Ness et al., 1988). Disulfide bond formation is a common consequence of oxidative stress in cellular proteins (Åslund and Beckwith, 1999). Based on the cDNA-deduced byssal collagen (preCol) sequences, Cys is certainly present in the form of one Cys residue per α-chain in preCol-D and preCol-NG (Qin and Waite, 1997, 1998). However, most if not all of the Cys is detectable as disulfides in purified preCol precursors and hydrolysates of byssal threads (Qin and Waite, 1998; unpublished data).

Another redox-sensitive functionality in byssal threads is peptidyl-tyrosine, especially its hydroxylated analog, 3,4-dihydroxyphenyl-L-alanine (DOPA). In the chimeric byssal collagens, preCol-P and preCol-NG, one to five residues of DOPA are located in the N- and C-histidine-rich termini of each preCol (Coyne et al., 1997; Qin and Waite, 1998). The concentration of DOPA in the proximal portion of the thread averages $\leq$1 residue/1000, whereas Tyr ranges from 18 to 25 residues/1000 (Mascolo and Waite, 1986). Tyr and DOPA are susceptible to oxidation by intrinsic catecholoxidase activity in byssal threads (Waite, 1985) as well as by the high levels of Cu, Zn, and Fe ($>$0.1% dry weight) that mussels in the genus *Mytilus* sequester from seawater and incorporate into their threads (Coombs and Keller, 1981). With a supply of O_2, metals such as Cu and Fe can provide Fenton-type reaction centers that hydroxylate Tyr, whereas catecholoxidase can catalyze DOPA oxidation to dopaquinone (Kunai et al., 1986; Cohen et al., 1998). Dopaquinones undergo dismutation to form semiquinone radicals, which couple as diDOPA cross-links (Burzio and Waite, 2000).

Recent experiments using solid-state nuclear magnetic resonance detected increased DOPA and diDOPA cross-link formation in byssus corresponding to an increase in the ambient flow of seawater around the threads (McDowell et al., 1999). Assuming that some of the diDOPA cross-links are intermolecular and between preCols or preCols and matrix proteins, these are likely to permanently increase stiffness/hardness of the threads. However, the production of more DOPA from Tyr could also have a stiffening effect given the high affinity (stability constant $\sim 10^{40}$) that DOPA has for bound metal ions such as Fe(III) (Taylor et al., 1996). The stiffness-enhancing effect of adding extrinsic phenoloxidase, an enzyme that specifically catalyzes the oxidation of DOPA and tyrosine (k_{cat} for DOPA $> 10 \times k_{cat}$ for tyrosine; Vanni and Gastaldi, 1990), supports these concepts. Exposure of fibrillar matrices made from type I collagen to DOPA under oxidizing conditions is reported to enhance stability and tensile strength (Gade et al., 1991).

In summary, byssal threads possess an inherent capacity to become stiffer and harder. We have observed two types of stiffening in byssal threads. One is strain-dependent, short-lived, and reversible (Fig. 2). The other appears to be driven by oxidation during sustained mixing and is irreversible (Fig. 4). Assuming no loss in toughness, doubling byssal tenacity (at 50% strain) before reinforcement by the synthesis of new threads would seem to offer adaptive advantages to mussels facing increased flow in ambient seawater.

However, there is another biological perspective. The moduli of the distal and proximal portions of each thread differ by an order of magnitude (Fig. 1; Bell and Gosline, 1996). Ordinarily two joined materials containing a modulus mismatch of such magnitude subjected to repetitive loading would be expected to fail at their common interface due to the accumulation of residual stresses (Rabin et al., 1995; Kinloch, 1987). This does not happen in byssal threads perhaps because of "modulus management," i.e., during repetitive loading, the distal portion exhibits stress softening from 500 to $\sim$60 MPa (Vaccaro and Waite, 2001), whereas the proximal portion stiffens from 30 to 60 MPa. In

other words, under duress the two halves of the thread start to resemble one another. To appreciate the biological significance of this very odd behavior it is helpful to point out that a typical mussel is attached to a stone by 20 or more threads that are distributed in a radial fashion. In laminar flow, some of these threads will be loaded more than others. As the loaded threads soften due to "modulus management," the load is redistributed as previously unloaded threads are increasingly recruited into action (Bell and Gosline, 1996). Extensive sclerotization of either proximal or distal portion by oxidative stress, for example, could undermine load redistribution and determine in part when threads become functionally obsolete and need to be replaced.

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