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Identification of Restricting Factors That Inhibit Swelling of Oxidized Myofibrils during Brine Irrigation

Zelong Liu,[‡] Youling L. Xiong,*[,§] and Jie Chen[‡]

[‡]State Key Laboratory of Food Science and Technology, Jiangnan University, Wuxi 214122, China, and [§]Department of Animal and Food Sciences, University of Kentucky, Lexington, Kentucky 40546

Porcine *longissimus* myofibrils were exposed to a hydroxyl radical-oxidizing system (10 μM $FeCl_3$, 100 μM ascorbic acid, 1 mM H_2O_2) at pH 6.2 for 1−12 h. Chemical analyses (sulfhydryls, disulfide bonds, carbonyls) indicated mild protein oxidation along with almost 40% loss of protein extractability in low-ionic-strength brines (≤0.4 M NaCl, 10 mM pyrophosphate, pH 6.2). Upon graded brine irrigation (0.2→0.6 M NaCl) with pyrophosphate, the swelling of myofibrils and the dissolution of the A-band of oxidized myofibrils were less pronounced than those of nonoxidized. This restriction of myofibril swelling, caused largely by disulfide cross-linkages formed between oxidized myosin tails, was positioned on the transversely expansible thick filaments, reflecting a significant role and susceptibility of intra- as well as intermyofilamental structures.

KEYWORDS: Myofibrils; protein oxidation; hydration; phase contrast microscopy

INTRODUCTION

The ability of myofibril lattices to withhold endogenous water upon cooking and to imbibe exogenous water during brine pumping, injection, or marination is a key functionality of meat because it directly impacts palatability (juiciness and tenderness) and economy (yield) of finished products. Water-binding properties of meat are influenced by a variety of processing factors, notably pH, salt concentration (ionic strength), divalent cations, and phosphates (*1*−*4*).

The mechanism of water-binding and hydration of meat has been extensively researched. It is generally agreed that the majority of endogenous water in fresh muscle is held within myofibrils in the space between myofilaments via capillarity or tightly associated with protein through hydrogen bonds. During marination, added salt (NaCl or KCl) and phosphate cause myofilaments to depolymerize and the actomyosin complex to dissociate, thereby promoting transverse expansion of the myofibrils and entrapment of exogenous water (*5*−*7*). Significant muscle fiber swelling occurs as the NaCl concentration is raised from the physiological level (~0.15 M) to about 0.6 M in the absence of phosphate, or 0.4 M in the presence of pyrophosphate or tripolyphosphate (*8*). At the same time of hydration, myofibril remnants, together with extracted myosin, form microscopic composite gels capable of immobilizing water (*9*). Limited postmortem proteolysis, which leads to loosening of the myofibril structure, also improves water-binding in aged meat (*10*, *11*). NaCl- and phosphate-induced myofibril expansion, thus transverse swelling, can be vividly viewed with a high-magnification microscope.

Abundant with lipids and various prooxidants, including unsaturated phospholipids and metal ions (iron, copper, etc.), postmortem muscle tissue depleted of natural antioxidants is highly susceptible to oxidation (*12*). The loss of water-binding capability is one of the main consequences commonly observed in oxidatively stressed meat; however, the mechanism has not been clearly defined. Indirect evidence that dietary vitamin E was capable of reducing drip loss in postmortem pork (*13*) and goat (*14*) muscle suggested that myofibrillar proteins, the principal water-binding substances in meat, were protected from oxidation. This hypothesis seems valid in light of the remarkable susceptibility of muscle proteins to reactive oxygen species (*15*−*18*). Using a low-field proton NMR, Bertram et al. (2007) confirmed that oxidatively stressed myofibrils from porcine muscle had reduced water-binding activity (*19*). It was also shown that an oxygen-enriched packaging system promoted protein oxidation (loss of sulfhydryls) and drip loss of fresh pork muscle (*20*).

In spite of the previous investigations, the main physicochemical and structural factors that restrict hydration and moisture-immobilization in oxidatively stressed meat remain poorly understood. There is little doubt that common oxidants readily generated in muscle foods, e.g., the highly reactive hydroxyl radicals (•OH) and ferryl [iron(IV)-oxy] species, can cause protein damage and structural changes (*21*, *22*). However, oxidative modification of individual protein amino acid residue side chains and peptide bonds does not provide a clear clue for structural and morphological alterations at the myofibril level. Yet, the specific changes of the myofibril architecture are known to profoundly influence the degree of water-binding and hydration by the myofibril matrix. Hultin et al. (1995) noted an increase in myosin and actin extraction from myofibrils after C-protein, X-protein, and α-actinin were removed (*23*). For oxidatively stressed myofibrils, the roles of these and perhaps other restricting factors are conceivably more complicated. The elucidation of the impact of reactive oxygen species on the morphology of the myofibril

*Corresponding author. Phone: 859-257-3822. Fax: 859-257-5318. E-mail: ylxiong@uky.edu.

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matrix in relation to its water-binding potential would improve the understanding of meat hydration under various processing conditions.

Hence, the objective of the present study was to demonstrate brine-induced ultrastructural changes and protein extraction from oxidatively stressed myofibrils. Our goals were to elucidate the roles of major myofibril components and structural segments in the hydration process. Emphasis was placed on the identification of restricting factors that were involved in the alteration of hydration properties of oxidized myofibrils.

MATERIALS AND METHODS

Preparation of Myofibrils. Center loins were obtained from 4 d postmortem carcasses of commercial size pigs humanely harvested at the University of Kentucky Meat Lab, a USDA-approved facility. Loins were trimmed to retain only the *longissimus* muscle, sliced into 1.5 cm thick chops, individually packaged with a vacuum seal, and stored in a −30 °C freezer until use. Myofibrils were isolated from thawed muscle (24 h at −4 °C) using a rigor buffer containing 0.1 M KCl, 2 mM $MgCl_2$, 1 mM EGTA, and 10 mM K_2HPO_4 (pH 7.0), as described elsewhere (*7*). After determination of protein concentration by the Biuret method, myofibril pellets were kept on ice and used within 18 h.

Oxidation of Myofibrils. Myofibril pellets were suspended (25 mg/mL protein) in a 15 mM piperazine−*N*,*N*-bis(2-ethane sulfonic acid) (PIPES) buffer (pH 6.2) containing 0.1 M NaCl. The suspension was subjected to oxidation at 4 °C by incubation 6 h (unless specified otherwise) with a •OH-generating system consisting of 10 μM $FeCl_3$, 100 μM L-ascorbic acid, and 1 mM H_2O_2 (all final concentrations). Oxidation was terminated by adding propyl gallate/Trolox C/EDTA (1 mM each) (*15*). The time of 6 h was chosen because significant, yet nonextensive, levels of protein oxidation (as indicated by the disulfide bond formation) were produced in this oxidizing system, as established in our preliminary study. Myofibril suspensions in the same buffer solution but without the oxidizing agents were used as the control. There were no detectable chemical changes (e.g., carbonyls, disulfides, sulfhydryls, and myosin ATPase activity) in the control samples when kept on ice for 24 h.

Measurement of Protein Oxidative Changes. The chemical changes of proteins in oxidatively stressed myofibrils were measured by the analysis of protein carbonyls, free sufhydryls, and disulfide linkages. In addition, protein solubility and cross-linking were determined.

Protein Carbonyls. Carbonyl contents in control (nonoxidized) and oxidized myofibril samples were determined using the procedure of Levine et al. (*24*) with some modifications. Carbonyl groups were reacted with 2,4-dinitrophenylhydrazine (DNPH) to form protein hydrazones. An aliquot of 100 μL of myofibrils (25 mg/mL protein) was suspended in a 1.5 mL microcentrifuge tube with 500 μL of DNPH, 2 N HCl and incubated at room temperature for 40 min to derivatize. The same amount of myofibrils was also incubated with 2 N HCl without DNPH as the control. This mixture was then precipitated with 10% TCA (final concentration) and centrifuged at 4000*g* for 10 min. The pellet was washed three times with 1 mL of ethanol:ethyl acetate (1:1 v/v) and centrifuged after every washing at the above condition. The residual solvents were evaporated by blow drying with a hair dryer at low heat (about 35 °C), and the pellet was dissolved in 1 mL of 6 M guanidine hydrochloride with 20 mM sodium phosphate buffer at pH 2.3. Absorbance was measured at 370 nm. The difference in absorbance between the DNPH treated sample and the control (in 2 N HCl without DNPH) was used for the calculation of carbonyl content. Protein concentration was calculated at 280 nm in the HCl control using a standard bovine serum albumin (BSA) in the same guanidine solution. The carbonyl content (nmol per mg protein) was calculated using an absorption coefficient of 22,000 M^{-1} cm^{-1} for protein hydrazones.

Sulfhydryls (SH). Total SH content was determined using 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB) according to Ellman (*25*) with some modifications. An aliquot of 25 μL of myofibrils (25 mg/mL) was precipitated with 10% TCA (final concentration) and centrifuged at 4000*g* for 10 min. The pellet was washed twice with 1 mL of ethanol:ethyl acetate (1:1 v/v) and centrifuged after every washing as indicated above. The residual solvents were evaporated by blow drying with a hair drier at low heat, and the pellet was dissolved in1000 μL of 50 mM Tris-HCl buffer (pH 8.3) containing 6 M guanidine chloride and 1 mM EDTA. Subsequently, 10 μL of 10 mM DTNB in 100 mM Tris-HCl buffer (pH 7.6) was added. The mixture was incubated at room temperature for 25 min, and absorbance at 412 nm was then measured. Sample blank was the assay solution only. Total sulfhydryl content was calculated using the molar extinction coefficient of 13,600 M^{-1} cm^{-1} and expressed as total SH nmol per mg protein. The protein concentration was measured using the Bradford assay.

Disulfide Linkages. Disulfide bond content was determined using the 2-nitro-5-thiosulfobenzoate (NTSB) assay as described by Thannhauser et al. (*26*). Dried myofibril pellets, prepared as above, were dissolved in 950 μL of Tris-HCl buffer (pH 9.5) containing 6 M guanidine and 1 mM EDTA. NTSB assay solution was then added, and the mixture was kept in the dark at room temperature for 25 min to allow the reaction to reach equilibrium. Absorbance at 412 nm was measured; the assay solution was used as the blank. Disulfide bond content was calculated using the molar extinction coefficient of 13,600 M^{-1} cm^{-1} and expressed as nmol per mg protein.

Protein Solubility. Myofibril pellets were suspended (5 mg/mL) in various concentrations of NaCl (0.2−0.8 M), 2 mM $MgCl_2$, 10 mM sodium pyrophosphate, 10 mM PIPES (pH 6.2). After incubation at 10 °C for 1.5 h, the protein suspensions were centrifuged (2 °C) at 5000*g* for 15 min. Protein solubility was defined as the protein concentration in the supernatant divided by the protein concentration of the original myofibril suspension. The protein concentration was determined by the Bradford method (*27*).

Hydration Properties of Myofibrils. To investigate the impact of oxidation on water-binding and hydration in myofibrils, control and •OH-stressed myofibrils were subjected to irrigation with different brine solutions that either disrupt or promote protein−protein interactions. Morphological changes associated with the dissolution and removal of myofibril components were examined using phase contrast microscopy and electrophoresis as outlined below.

Irrigation of Myofibrils. Myofibrils were irrigated according to Xiong et al. (*7*). An irrigation channel was devised as such (**Figure 1**): two 12 mm wide strips of a double-sided adhesive tape were stuck to the upper face of a 76 × 26 mm glass microscope slide to form a well approximately 2 mm wide and about 200 μm deep. A 24 × 24 mm coverslip was pressed into position on the tape strips. The superfluous tape was trimmed off with a surgical blade along the edge of the coverslip. On one end of the channel,

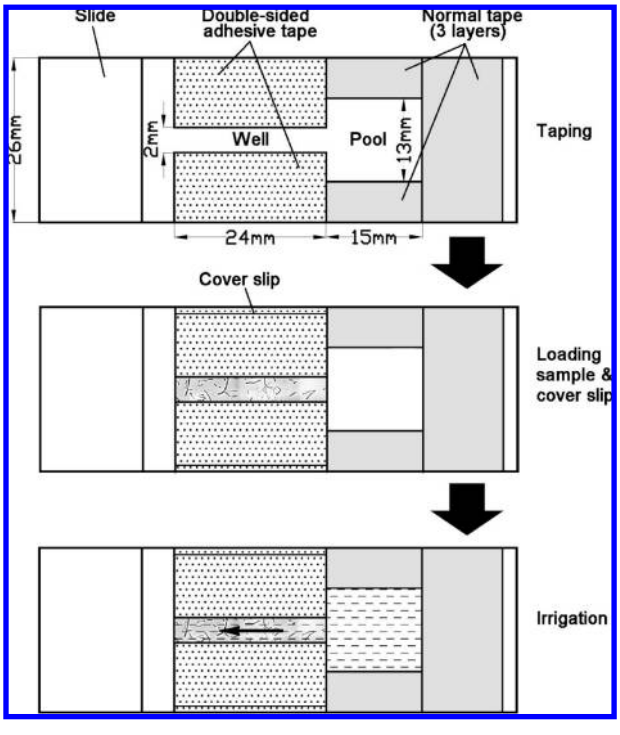

Figure 1. Schematic illustration of the device for myofibril irrigation.

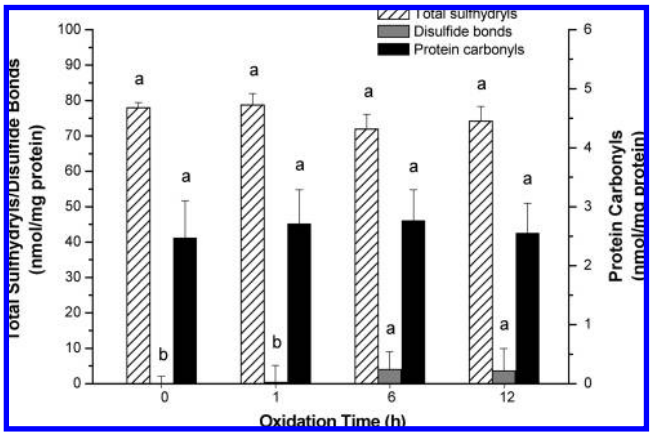

Figure 2. Total sulfhydryl, disulfide bond, and carbonyl contents in myofibrils after oxidation at 4 °C for 0 (control), 1, 6, and 12 h. Means ($n = 3$) with different letters differ significantly ($P < 0.05$). Error bars denote standard deviations.

a pool of about 13 mm wide, 15 mm long, and 500 μm deep was made with tape, which was used as the reservoir of the irrigation buffers.

Myofibril samples were diluted to a final concentration of 1−2 mg/mL with rigor buffer. One drop of suspended sample plus a drop of rigor buffer were applied directly to the middle of each half well. A glass coverslip was pressed against the slide and inverted for 2 min to allow the myofibrils to settle onto the glass slide. Once the myofibrils were settled and found under the microscope the irrigation began at room temperature. The following irrigation brines were used: (1) graded series of NaCl concentrations at 0.1, 0.2, 0.3, 0.4, 0.5 M with 2 mM $MgCl_2$ and 10 mM sodium pyrophosphate in 10 mM PIPES buffer (pH 6.2) to determine the effect of ionic strength on myofibril extraction; irrigation with decreasing NaCl concentrations was also done to determine partial reversibility of myofibril swelling; (2) the same extraction solutions as above except that ±5% β-mercaptoethanol (βME) was also used to establish the possible restricting effect of disulfide bonds on myofibril extraction; (3) graded series of NaCl concentrations of 0.2, 0.3, 0.4, 0.5 M with 4 mM EGTA, 4 mM $MgCl_2$, and 2 mM ATP in 10 mM PIPES buffer (pH 6.2) to compare relaxation patterns of myofibrils; and (4) a 10 mM PIPES buffer (pH 7.0) with 0.1 M KCl, 2 mM $MgCl_2$, 0.1 mM $CaCl_2$, and 0.1 mM ATP to examine myofibril contraction. All irrigation buffers were kept on ice prior to use. In our preliminary study, we noted that structural changes, including myofibril swelling, were completed in 3 min (180 s) during irrigation with the respective salt solutions. Therefore, unless specified otherwise, the results reported here represent those of 3 min irrigation.

Morphological changes in the myofibril architecture were observed using a model MICROPHOT-FXA Nikon photomicroscope equipped with a 100× oil immersion phase-contrast objective. Images were recorded with a digital camera. To measure the diameter of a myofibril, camera-captured images of myofibrils were enlarged and the width of the A-band was treated as the myofibril diameter (*7*). Relative myofibril swelling (%) was expressed as the myofibril diameter after irrigation divided by that before irrigation then multiplying by 100. Mean values from 6−9 individual myofibrils per irrigation treatment were reported.

Electrophoresis. To identify the myofibrillar proteins that were extracted with different extraction buffers or under different extraction conditions, both extracted (supernatant) and nonextracted (remnant or sediment) samples from the graded series of salt concentration treatments as described above were subjected to sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE). Myofibril remnants from the 0.3, 0.4, and 0.5 M NaCl extraction buffers were obtained as the sediments after centrifugation for 15 min at 5000*g*. Samples were treated with or without 5% βME, and proteins were concentrated in a 3% acrylamide stacking gel and separated in 10% acrylamide resolving gel. An equal volume (25 μL) of samples (whole myofibril samples, the extracts, or the remnants) was loaded to each lane.

Statistical Analysis. Unless specified otherwise, a minimum of three independent trials ($n = 3$) with triplicate sample analyses were performed.

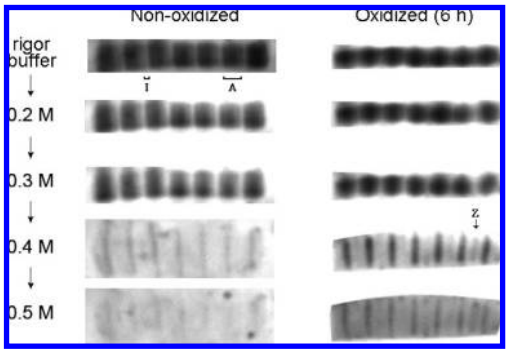

Figure 3. Phase contrast micrographs of nonoxidized (control) and oxidized myofibrils after sequential 3 min irrigations with different concentrations of NaCl as indicated. All irrigation solutions also contained 10 mM pyrophosphate, 2 mM $MgCl_2$, and 10 mM PIPES at pH 6.2.

Data (protein solubility, degree of myofibril swelling, etc.) were subjected to analysis of variance using the general linear model's procedure of the Statistix software 9.0 (Analytical Software, Tallahassee, FL). When a treatment effect (e.g., oxidation) was found significant, Tukey HSD all-pairwise multiple comparisons were performed to identify significant differences between individual means.

RESULTS AND DISCUSSION

Protein Oxidation. When exposed to an oxidizing environment, many amino acid residue side chain groups exposed to the surface of proteins can be readily modified and cleavage of the peptide bond can also occur, leading to the production of carbonyls (*28*). Furthermore, sulfhydryls (thiol) from cysteine residues are remarkably susceptible to radicals and form disulfide linkages under various oxidizing conditions (*29*). Hence, the disappearance of sulfhydryls and the formation of protein carbonyl groups and disulfide bonds provide sensitive assessment of the extent of protein oxidation. In the present study, oxidatively stressed myofibril samples exhibited a tendency to produce protein carbonyls and lose sulfhydryls when oxidized up to 6 h (**Figure 2**); however, these changes were nonsignificant ($P > 0.05$). On the other hand, myofibrils oxidized for more than 6 h contained about 5 nmol of disulfide (cystine) per mg of protein whereas nonoxidized control myofibrils contained none (**Figure 2**). These results indicated that myofibrils exposed to the •OH-generating system underwent relatively mild oxidation. The oxidizing system we employed (10 μM $FeCl_3$/100 μM ascorbic acid/1 mM H_2O_2) was known to be mild and considered to be close to many *in situ* conditions (*30*). We have previously shown that •OH produced from such systems do not effect substantial changes in myofibrillar proteins (*31*). It must be noted that reaction of carbonyls with electron-dense groups, such as amines, could contribute to the low carbonyl content detected in oxidized myofibril samples (*32*−*34*). Because prolonged oxidation (i.e., >18 h) in this •OH-oxidizing system could cause extensive protein structural changes and aggregation (*15*, *18*, *35*), which was confirmed in our preliminary trials in the present study, the subsequent hydration experiments were conducted on samples oxidized for 6 h.

Ultrastructural Changes in Oxidized Myofibrils During Salt Irrigation. Myofibrils viewed under the microscope varied in apparent diameter due to the presence of both well-separated and overlapped or associated myofibrils. However, any radial or axial changes during irrigation treatments could be quantitatively measured. **Figure 3** presents the micrograph of typical single myofibrils from nonoxidized (control) and oxidized samples. There was no remarkable differentiation between control and

Table 1. Degree of Swelling of Myofibrils Treated with Different Irrigation Solutions (pH 6.2)[a]

irrigation solution	effect of NaCl concentration				effect of βME		effect of ATP	
	0.1 ›0.4 M NaCl		0.4 ›0.1 M NaCl		0.4 M NaCl		0.2 ›0.4 M NaCl	
	non-oxidized	oxidized	non-oxidized	oxidized	non-oxidized	oxidized	non-oxidized	oxidized
0.1 M NaCl	100% a	100% a	109±2.0% b	112±1.1% a	–	–	–	–
0.2 M NaCl	105±0.4% a	102±2.9% a	129±5.5% a	126±2.5% b	–	–	130±4.5% a	112±3.8% b
0.3 M NaCl	109±4.9% a	105±6.8% a	166±5.3% a	152±2.3% b	–	–	159±17.7% a	127±2.7% b
0.4 M NaCl	203±18.7% a	158±3.3% b	197±2.9% a	161±1.5% b	–	–	209±6.4% a	155±13.2% b
sample (βME) 0.4 M NaCl	–	–	–	–	211±1.7% a	163±3.4% b	–	–
0.4 M NaCl +βME					211±3.3% a	210±14.9% a		
sample (+βME) 0.4 M NaCl	–	–	–	–	214±7.2% a	206±11.7% a	–	–

[a] The values, expressed as the means ± standard deviations, are obtained from 6−9 individual myofibril measurements from 2−3 replications. Means with different letters in the same row within the same irrigation treatment group differ significantly ($P < 0.05$). No quantitative data were collected from samples irrigated at >0.4 M NaCl due to the loss of definition of the A-band.

oxidized myofibrils in the rigor buffer (with 0.1 M NaCl). In both, a thin, albeit faint, dark band could be seen at the position of the M-line that divided each A-band into two equal parts, forming a lighter rectangular region in the middle of one sarcomere. However, the H-zone and Z-line could not be visualized in these myofibrils most likely due to postmortem degradation because the *longissimus* muscle from which myofibrils were isolated had been aged for 4 days.

As the NaCl concentration of the irrigation solution in the presence of 10 mM pyrophosphate was increased from 0.2 to 0.5 M, where maximum structural changes occurred, two distinct morphological results were observed: the transverse expansion (swelling) throughout the myofibrils and the longitudinal narrowing of the A-band. The length of the sarcomeres did not change, however. By 0.5 M NaCl, the A-band largely diminished, leaving only a fine line at the center of the original A-band in the remnant myofibrils. These ultrastructural changes were typical of myofibrils treated with similar salt brines as reported in the literature (*5*−*8*). However, the comparison between nonoxidized and oxidized myofibrils revealed marked differences. In particular, the transverse expansion of oxidized myofibrils was significantly reduced when compared with nonoxidized myofibrils at equal ionic strengths (**Table 1**). At ≥0.4 M NaCl, while the remnant A-band faded in nonoxidized samples, that in the oxidized myofibrils remained salient (**Figure 3**). The results indicated a lower degree of myofibril swelling as well as reduced myosin extraction in oxidatively stressed samples. The Z-lines in oxidized myofibrils, which were hardly detectable, became visible after 0.5 M NaCl treatment, presumably due to the removal of the masking thin filaments.

Quantitative comparisons of the amount of extracted proteins from control and oxidized myofibrils were made by measuring the protein solubility in the respective irrigation solutions. Myofibrillar proteins generally are soluble at ≥0.5 M NaCl, but significant solubility can be obtained at an ionic strength of 0.3 or less in the presence of both pyrophosphate and magnesium ion or with a prolonged muscle aging time (*8*, *23*, *36*−*38*). As shown in **Figure 4**, a substantial amount (~38%) of protein was soluble even at 0.2 M NaCl. However, between 0.2 and 0.4 M NaCl, the oxidized samples had reduced protein solubility compared to control samples ($P < 0.05$), suggesting that oxidatively stressed proteins were more difficult to be extracted. The result was consistent with the discrepant myofibril morphological changes illustrated above where the myosin band (A-band) from oxidized

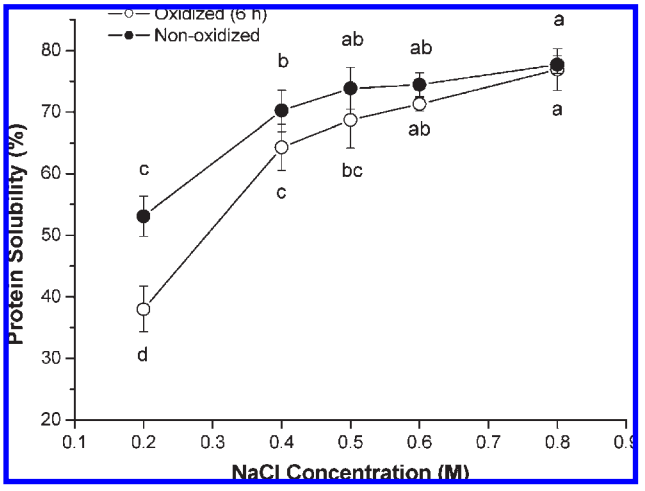

Figure 4. Protein solubility of nonoxidized (control) and oxidized myofibrils at various NaCl concentrations in 10 mM pyrophosphate, 2 mM $MgCl_2$, and 10 mM PIPES at pH 6.2. Means ($n = 3$) without a common letter differ significantly ($P < 0.05$). Error bars denote standard deviations.

myofibrils was more resistant to extraction (i.e., dissolution) than that from control myofibrils.

The solubility difference gradually diminished at increasing NaCl concentrations until 0.8 M NaCl, where control and oxidized samples had identical protein solubility (77%). Since oxidation causes hydrophobic groups to be exposed and charged amino acid side chain groups to be modified (*29*, *39*−*42*), the decrease of protein solubility in low salt concentration solutions possibly resulted from a shift of charge distribution on protein molecule surface to a less polar status. Notwithstanding, the loss of protein charges could be compensated for by Na^+ and Cl^- at high salt concentration solutions (≥0.5 M NaCl), thereby restoring repulsive forces between individual protein molecules and regaining a strong protein−water interaction. This would explain why a convergent curve pattern was seen in the solubility of nonoxidized versus oxidized myofibrils.

In order to identify the proteins that were readily extracted and those that are resistant to extraction or play a role in restricting total protein extraction from oxidatively stressed myofibrils, both extracted (with sequential 0.3, 0.4, and 0.5 M NaCl) and nonextracted (remnant) proteins were subjected to SDS−PAGE.

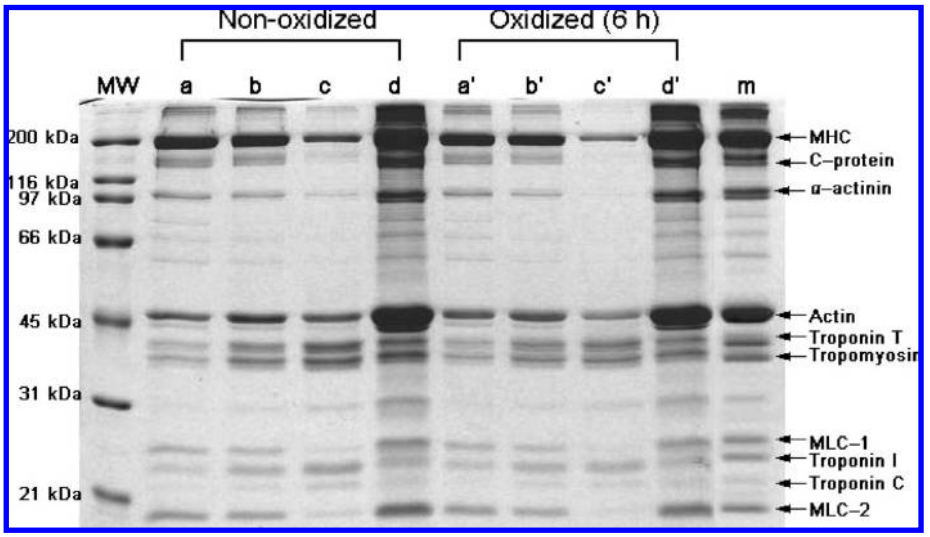

Figure 5. SDS−PAGE patterns of nonoxidized (control) and oxidized myofibrils after sequential salt extractions in the presence of 5% β-mercaptoethanol at pH 6.2. Lanes a and a′: supernatants extracted with 0.3 M NaCl (1st extraction). Lanes b and b′: supernatants of residues (from the 1st extraction) re-extracted with 0.4 M NaCl (2nd extraction). Lanes c and c′: supernatants of residues (from the 2nd extraction) re-extracted with 0.5 M NaCl. Lanes d and d′: final residues after extraction with 0.5 M NaCl. All irrigation solutions also contained 10 mM pyrophosphate, 2 mM $MgCl_2$, and 10 mM PIPES at pH 6.2. MW, molecular weight markers; m, nonoxidized original myofibrils; MHC, myosin heavy chain; MLC-1, myosin light chain-1; MLC-2, myosin light chain-2.

It was observed that substantial amounts of myosin heavy and light chains, C-protein, α-actinin, and actin were extracted with low-salt solutions (0.3 and 0.4 M NaCl) (**Figure 5**, lanes a, b, a′ and b′), leaving most troponins (T, I, C) and tropomyosins to be extracted by 0.5 M NaCl (c and c′). The final 0.5 M extraction was ineffective on myosin, C-protein, α-actinin, and actin, as the residue (remnant) retained significant amounts of these myofibril components (d and d′).

These results were consistent with the findings reported by Offer and Trinick (1983) (*5*) that, after substantial swelling of myofibrils upon high concentrations of NaCl irrigation with or without the presence of pyrophosphate, more troponin, tropomyosins, and α-actinin were extracted and the Z-line became weak or obscure. While the removal of troponins and tropomyosin was not a prerequisite for myosin extraction (e.g., at 0.3 M NaCl), it seemed to be involved with the abrupt swelling of the myofibrils (at 0.4 M NaCl) as observed in **Figure 3**. Comparison of nonoxidized and oxidized samples indicated slightly reduced band intensity for myosin heavy chain, corresponding to a lower C-protein and α-actinin band intensity in the latter when treated with 0.3 and 0.4 M NaCl. The finding suggested that oxidation of C-protein and α-actinin might have restricted the dissolution of the myosin filaments and myosin extraction.

As demonstrated by Offer and Trinick (*5*), brine-induced myofibril swelling can be reversed when swollen myofibrils are irrigated with reducing concentrations of NaCl. The reversibility is made possible because the Z-line is a pliable structure. To investigate the influence of oxidative treatment on myofibril structural flexibility, we initially irrigated myofibrils with 0.4 M NaCl and subsequently with 0.3, 0.2, and 0.1 M NaCl in the presence of 10 mM pyrophosphate (**Figure 6**). For nonoxidized myofibrils, the drastic transverse expansion (by 97%) upon treatment with 0.4 M NaCl was gradually reduced by irrigation with descending concentrations of NaCl; after the final irrigation with 0.1 M NaCl, the myofibril diameter was almost restored (**Table 1**). For oxidized myofibrils, the initial swelling (by 61% in 0.4 M NaCl) was also gradually diminished upon irrigation with decreasing concentrations of NaCl. However, the final recovery (in 0.1 M NaCl) from the original myofibril diameter (in rigor buffer) was still less ($P < 0.05$) than that in nonoxidized samples

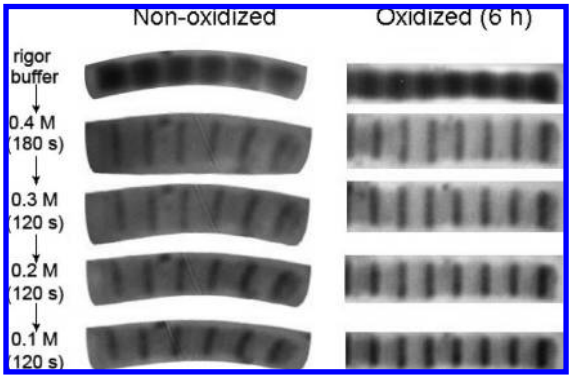

Figure 6. Reversibility of brine-induced swelling of nonoxidized (control) and oxidized myofibrils. Myofibrils were sequentially irrigated for 3 min with different concentrations of NaCl (as indicated) in a buffer consisting of 10 mM pyrophosphate, 2 mM $MgCl_2$, and 10 mM PIPES at pH 6.2.

(**Table 1**). The result suggested that Z-disks (lines), which keep myofibrils in register, were affected by •OH. Any of the Z-disk constituents, including α-actinin, could be a target of •OH, and, when modified, they would reduce the elasticity of the Z-disks, thereby limiting the expansion and shrinkage of I- and A-bands.

Influence of Disulfide Linkages on Myofibril Structural Changes. As indicated above (**Figure 2**), oxidation produced disulfide linkages in myofibrils. Disulfide cross-linking has been shown to occur within myosin (intramolecular) and between myosin molecules (*18*,*34*). When •OH is the oxidizing agent, cross-linking through disulfide bonds both between myosin S1 (head) groups and between the rod subfragments (tails) occurs, but the latter is favored (*15*). Nonetheless, it was not clear how •OH-induced disulfide linkages would affect the hydration and structural changes of myofibrils upon salt irrigation. Hence, experiments were performed to treat oxidized myofibrils with βME, a disulfide bond breaking agent, prior to irrigation or during irrigation to shed light on the conjecture that disulfide cross-linking was a restricting factor for myofibril hydration.

As shown in **Figure 7**, while irrigation beyond 3 min (180 s) with 0.4 M NaCl produced no further change in myofibrils

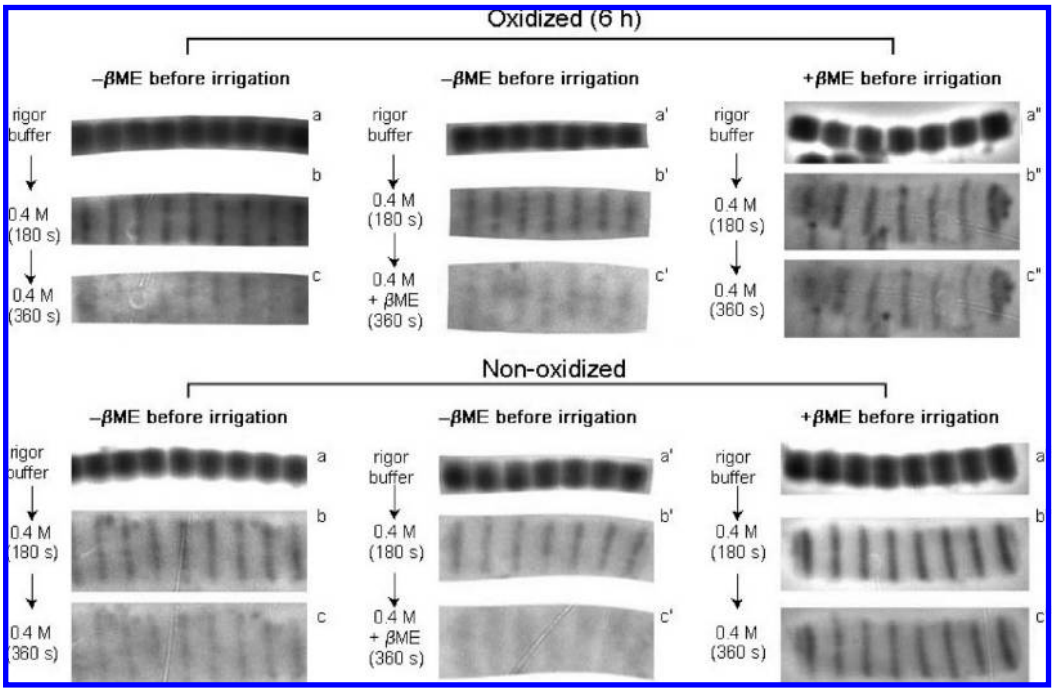

Figure 7. Swelling of oxidized and nonoxidized (control) myofibrils induced by 3 min irrigation with 0.4 M NaCl solution containing 10 mM pyrophosphate, 2 mM $MgCl_2$, and 10 mM PIPES at pH 6.2. Myofibril samples were treated with (+βME) or without (−βME) 5% β-mercaptoethanol before irrigation.

(a−c, upper panel), an additional 3 min of irrigation with the same NaCl solution but also with 5% βME effected further swelling (a′−c′, upper panel), bringing the final degree of swelling, i.e., enlargement of the diameter of A-band segments, from 163% to 210% ($P < 0.05$) (**Table 1**). The same additional βME treatment did not affect the extent of swelling (211%, **Table 1**) in nonoxidized myofibrils; however, morphologically, the salient remnant center line of the A-band seen at 0.4 M NaCl (b′, lower panel) became quite faint or diffuse after the βME irrigation (c′, lower panel). The latter result was unexpected because native myosin is devoid of disulfide bonds, and they were nondetected in fresh myofibrils (**Figure 2**). It is possible that residual disulfides that were beyond the sensitivity level of detection would still play an appreciable role in controlling the dissolution and extraction of myosin filaments upon brine irrigation. Moreover, though not measured, sulfenic acid (Cys-SOH) could form during myofibril isolation as it is readily produced in mild oxidative environments even under physiological conditions via Cys-SOH/Cys-SH redox cycles (*43*). Sulfenic thiols in peptides have been shown to cross-link with βME through disulfide bonds (*44*). Therefore, it is highly possible that, through binding to sulfenic acid, the added βME in control myofibril samples introduced new hydroxyl groups, and the result was a stronger hydrogen bonding interaction of proteins with the solvent leading to an enhanced protein solubility. However, this hypothesis is subject to future investigation.

The above results were evidence that oxidation-induced disulfide linkages were an important restricting factor limiting myofibril hydration. To further substantiate the constraining role of disulfide bonds, we pretreated oxidized myofibrils with 5% βME before irrigation with 0.4 M NaCl. The result showed essentially the same recovery of maximum swelling (206%) as in samples irrigated with 0.4 M NaCl plus βME (211%) (**Table 1**). On the other hand, the degree of swelling for nonoxidized myofibrils was not affected by this reducing agent treatment. Interestingly, however, irrigation of oxidized myofibrils that were treated with 5% βME prior to irrigation with 0.4 NaCl only slightly weakened the remnant A-band (a″−c″, upper panel in **Figure 7**). This structural response to βME was different from that when oxidized myofibrils were irrigated with 0.4 M NaCl plus 5% βME throughout the course of irrigation. Thus, the maintenance of a certain critical dosage of βME during irrigation seemed to be important. Notwithstanding, the present findings further confirmed that the removal of disulfide cross-linkages was crucial for oxidized myofibrils to achieve or regain maximal swelling or hydration.

Consistent with the augmented morphological changes, protein solubility (extractability) of oxidized myofibrils after treatment with 5% βME in 0.4 M NaCl was significantly improved. Specifically, the solubility decreased progressively during the course of oxidation, which was significant after 12 h ($P < 0.05$), but the loss was completely recovered after treatment with the reducing compound (**Figure 8**). When the oxidized (6 h) and control myofibril samples were subjected to electrophoresis, we noted prominent, further extraction of the following myofibrillar proteins when the brine (0.4 M NaCl) contained βME: C-protein, α-actinin, actin, troponins, and tropomyosins (lanes b and b′ in **Figure 9**). Although also improved, the extraction of these particular protein components was not as remarkable when myofibrils were pretreated with βME before irrigation with 0.4 M NaCl containing no βME (lanes c and c′ in **Figure 9**).

This improvement of extractability, as aforementioned, was caused by the exchange of the thiolate anions between the sulfhydryl reductant βME and oxidized or control proteins and/or by its binding to proteins, notably on M-line, Z-line, and I-band proteins. It is of interest to note that the increased Z-line (α-actinin) and I-band (actin, troponins, tropomyosin) protein extraction (lane b and b′ in **Figure 9**) coincided with the disappearance of the fine dark line bisecting the A-band in the position of M-line, creating a wider gap between adjacent A-segments (b′ and c′ in **Figure 7**). However, as demonstrated in the upper panel of **Figure 7**, the disappearance of the remnant A-segments occurred only after further transverse swelling had occurred, which was clearly seen in oxidized myofibrils irrigated with βME. This extraction pattern was also observed in our preliminary kinetic study where we irrigated myofibrils with

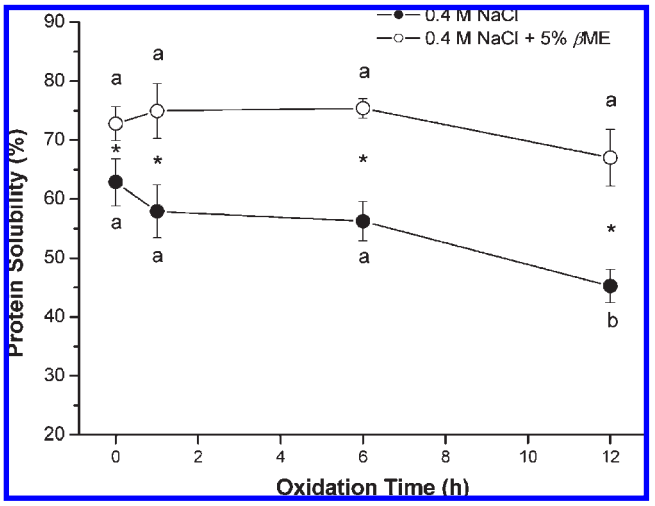

Figure 8. Time course of oxidation-induced changes of myofibrillar protein solubility in 0.4 M NaCl, 10 mM pyrophosphate, 2 mM $MgCl_2$, and 10 mM PIPES at pH 6.2, with or without 5% β-mercaptoethanol (βME). Means ($n = 3$) with different letters differ significantly ($P < 0.05$). Error bars denote standard deviations. The asterisks indicate significant difference ($P < 0.05$) between samples with or without βME.

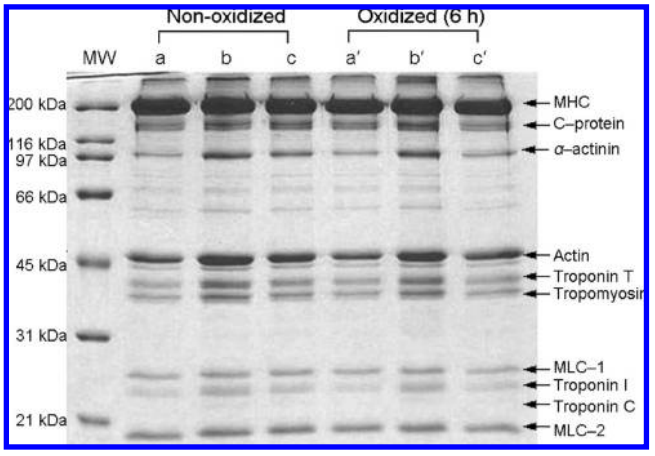

Figure 9. Influence of β-mercaptoethanol (βME) treatments on the SDS−PAGE patterns of protein extracts (supernatants) obtained from nonoxidized and oxidized myofibrils. The following basal extraction buffer was used for all samples: 0.4 M NaCl, 10 mM pyrophosphate, 2 mM $MgCl_2$, and 10 mM PIPES (pH 6.2). Lanes a and a′ are extracts of myofibrils with the basal extraction buffer; b and b′ are extracts of myofibrils with the basal extraction buffer plus 5% βME; c and c′ are extracts of 5% βME-treated myofibrils with the basal extraction buffer. Lane MW: molecular weight markers. MHC: myosin heavy chain. MLC-1: myosin light chain-1. MLC-2: myosin light chain-2.

0.4 M NaCl plus βME and recorded changes every 30 s (data not shown). Thus, disulfide cross-linking seemed to be the ultimate restricting factor limiting the hydration and swelling of oxidized myofibrils. Structural proteins, as indicated above, served as physical hindrances for myosin extraction, but their effect was largely diminished by the cleavage of disulfide linkages.

Changes in Myosin Filaments as Affected by Actomyosin Cross-Bridging. The results from the preceding experiments suggested that the main site of influence by the restricting factors was on the A-band region, i.e., myosin or the thick filaments. It has been proposed that weakening of thick (myosin) and thin (actin) filament interaction due to charge repulsion and depolymerization of myosin filaments at elevated NaCl concentration,

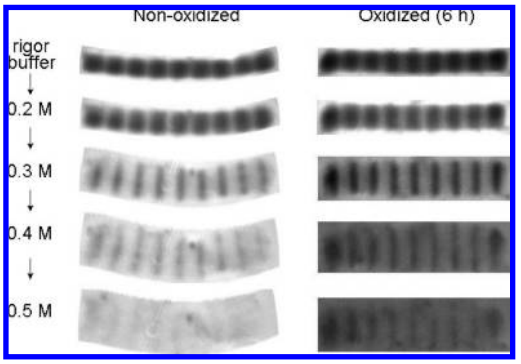

Figure 10. Phase contrast micrographs of nonoxidized (control) and oxidized myofibrils after sequential 3 min irrigations with different concentrations of NaCl (as indicated) in a relaxation buffer consisting of 2 mM ATP, 4 mM EGTA, 4 mM $MgCl_2$, and 10 mM PIPES at pH 6.2. The "rigor buffer" myofibril samples were not treated with the relaxation solution.

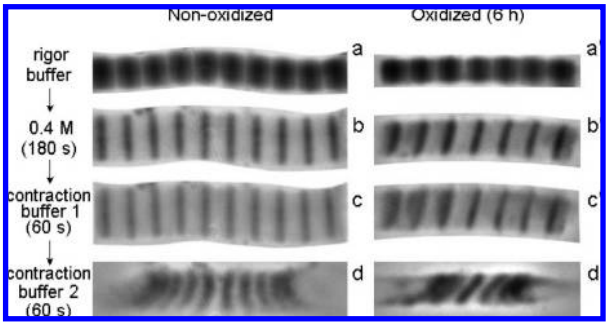

Figure 11. Responses of nonoxidized and oxidized myofibrils to two contraction solutions during sequential irrigation. Myofibrils in rigor buffer (a, a′) were first irrigated 180 s with 0.4 M NaCl, 10 mM pyrophosphate, 2 mM $MgCl_2$, and 10 mM PIPES at pH 6.2 (b, b′), then irrigated 60 s with contraction buffer 1 (0.3 M NaCl, 0.1 M KCl, 2 mM $MgCl_2$, 0.1 mM $CaCl_2$, 0.1 mM ATP, 10 mM PIPES, pH 7.0) (c, c′), and finally irrigated 60 s with contraction buffer 2 (0.1 M KCl, 2 mM $MgCl_2$, 0.1 mM $CaCl_2$, 0.1 mM ATP, 10 mM PIPES, pH 7.0) (d, d′).

regardless of the presence of pyrophosphate, is the main restricting factor for myofibril hydration (*5*). This stipulation is valid, but it does not explain the situation when myofibrils are oxidatively stressed. We hypothesize that cross-links formed within myosin filaments had a major influence on myofibril hydration. To test this hypothesis, we conducted two additional experiments that demonstrated the consequence of the removal or enforcement of actomyosin cross-bridges to highlight the role of myosin filaments.

In the first experiment, nonoxidized and oxidized myofibrils were sequentially irrigated with graded NaCl solutions in a "relaxation" environment where ATP was used to unlock the actomyosin ionic cross-bridges (**Figure 10**). For both nonoxidized and oxidized myofibrils, the detachment of actin from myosin (by ATP) resulted in progressive transverse expansion beginning at 0.2 M NaCl. Irrespective of oxidation, myofibril samples at 0.2 and 0.3 M NaCl exhibited more extensive swelling ($P < 0.05$) than those without the ATP treatment (**Figure 3**, **Table 1**). As expected, the ATP treatment did not alter the length of the sarcomere since the irrigation was done at elevated salt concentrations. The result indicated that the dissociation of the actomyosin complex promoted lateral expansion of the A-segment. However, even with ATP, the degree of swelling of oxidized myofibrils remained less compared with that of nonoxidized samples, suggesting that other cross-linkages, including disulfide

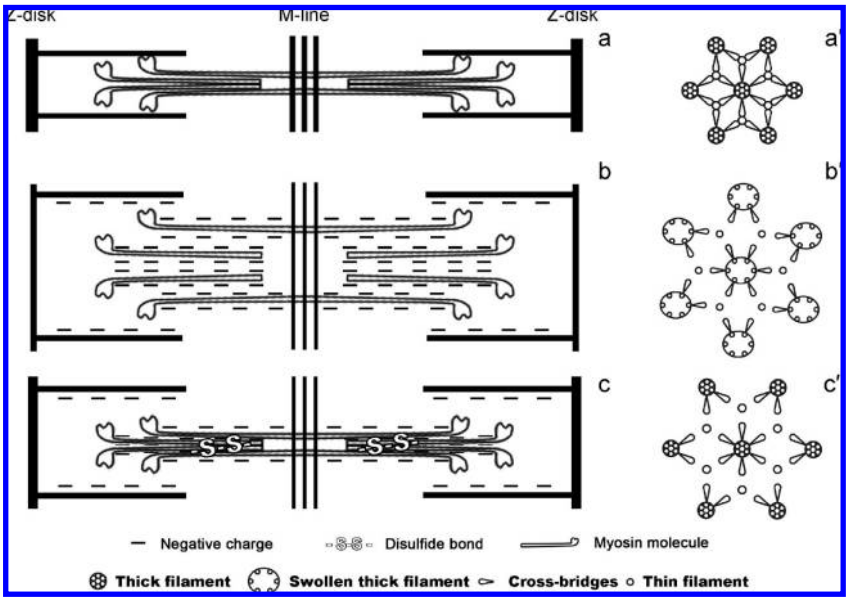

Figure 12. Schematic diagram illustrating how disulfide cross-linkages on myosin tail would restrict the swelling of myofibril. (a) a sarcomere and (a′) end-on view of the unswollen myofibril lattice; (b) a sarcomere and (b′) end-on view of the completely swollen myofibril lattice after effective salt irrigation; (c) a sarcomere and (c′) end-on view of the partially swollen myofibril lattice which is restricted by disulfide cross-linkages on the thick filaments after effective salt irrigation.

bonds as noted previously, restricted swelling of myosin filaments.

In the second experiment, hydrated control and oxidatively stressed myofibrils (0.4 M NaCl), which showed substantial swelling and removal of the myosin filaments, were irrigated with a contraction solution at the same salt concentration (0.4 M) condition, followed by irrigation with the same contraction solution but at a reduced ionic strength (0.1 M KCl). As illustrated in **Figure 11**, irrigation with the ATP contraction buffer containing 0.4 M salt produced no substantial structural change in the remnant myofibrils (b and c; b′ and c′). The lack of significant change was due to the enlarged interfilamental spaces (i.e., the gap) created by the ionic repulsion. On the other hand, subsequent irrigation with the contraction buffer at the 0.1 M KCl level affected drastic shortening of the myofibrils, especially on both ends (c and c′). The result indicated that residual thin (actin) and thick (myosin) filaments were still able to slide past each other causing the calcium-activated, ATP driven super contraction. However, the contraction was more severe in oxidized myofibrils. This can be explained because there were more residual myosin and possibly actin molecules left after the extraction with 0.4 M salt. These residual proteins or myofilaments, apparently retaining some native structural assembly, served as the scaffold to retain moisture during hydration.

Based on the above experiments, a model which emphasizes the role of disulfide linkages was developed to explain the hydration of oxidatively modified myofibrils. As sketched in **Figure 12**, disulfide bonds formed between myosin molecules inside the thick filaments serve as a primary restricting factor for the lateral expansion of myofibrils upon brine irrigation. Where intrafilamental disulfides are limited, swelling occurs in both intra- and intermyofilaments (b and b′). Because inhibition of swelling occurred in oxidized myofibrils even when the actomyosin cross-bridges had been removed, disulfide linkages mainly through myosin tails (*15*) would be generated between myosin molecules within the thick filaments (c and c′). This model would advance the previous proposition put forth by Offer and Trinick (*5*) that delineates the structural changes and interactions between myosin filaments rather than within them.

In summary, results from this study suggested that disulfide cross-linkages between staggered myosin tails are a major constraint restricting myofibril radial swelling during salt marination. The developed myofibril swelling model helps explain the physical and biochemical processes of water uptake and how radical-mediated protein oxidation impacts water-holding capacity in myofibrils. Further studies are needed to establish the role of oxidatively induced linkages other than disulfide bonds in the hydration behavior of myofibrils and to test the applicability of the results under *in situ* conditions.

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