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Changes in Conformation and Subunit Assembly of Cod Myosin at Low and High pH and after Subsequent Refolding

HORDUR G. KRISTINSSON*,† AND HERBERT O. HULTIN‡

Laboratory of Aquatic Food Biomolecular Research, Aquatic Food Products Program, Department of Food Science and Human Nutrition, University of Florida, Gainesville, Florida 32611, and Massachusetts Agricultural Experiment Station, Department of Food Science, University of Massachusetts−Amherst, Marine Station, Gloucester, Massachusetts 01930

Conformational and structural changes of cod myosin at pH 2.5 and 11 and after subsequent pH readjustment to pH 7.5 were studied. Results suggest that on acid unfolding, the myosin rod may fully dissociate due to electrostatic repulsion within the coiled coil, while it does not dissociate at alkaline pH. Both pHs led to significant conformational changes in the globular head fraction of the myosin heavy chains, suggesting that it takes on a molten globular configuration. A large part of the myosin light chains are lost on both pH treatments. On pH readjustment to neutrality, the heavy chains take on a structural form similar to the native state with the coiled-coil rod reassociating from acid pH while leaving the globular head less packed, more hydrophobic and structurally less stable. The irreversible change brought about in the globular head region leads to the failure of light chains to reassemble onto it, a drastic loss in ATPase activity, and more exposure of reactive thiol groups. The acid and alkali processes therefore lead to substantial changes in the globular part of the myosin molecule and perhaps more importantly to different molecular changes in myosin, depending on which pH treatment is employed.

KEYWORDS: Cod myosin; acid pH; alkaline pH; conformational changes; molten globule; unfolding; refolding

INTRODUCTION

To address the global need for better utilization of harvested fish species and byproducts, a process was developed in our laboratory to economically produce functional protein isolates from muscle sources of low value. This process utilizes the pH-dependent solubility properties of fish muscle proteins for their separation and recovery from other components of muscle not desirable in a final product, such as the oxidatively unstable lipids in cellular membranes. The process involves subjecting a diluted homogenized muscle tissue to a low (2−3) or high (10.5−11.5) pH. At these pH values, the muscle proteins are soluble due to electrostatic repulsion. A large drop in solution viscosity under these conditions enables the cellular membranes to be separated from the soluble proteins by centrifugation (*1*−*2*). Membrane- and lipid-free soluble proteins are then recovered by isoelectric precipitation by adjusting the pH to ca. 5.5. The resulting isolate can be used for various purposes (e.g., as a functional food ingredient or directly used to produce value added fish products such as surimi).

Muscle tissue is composed of a number of different proteins which account for 15−22% of its total weight. The myofibrillar proteins, which make up ca. 60−70% of all the proteins in muscle, have been found to play the major structural and functional role of meat and meat derived products in food processing as they have a tendency to interact with each other and other nonprotein ingredients (*3*). Myosin is the single most abundant protein of the myofibrils, and a number of studies have found it to be largely responsible for the functional properties of muscle tissue, including water binding, gelation, emulsification, and foaming (*3*−*4*). Myosin has a complex architecture, as it is a very large ∼520 kDa hexamere. The two largest polypeptides have each a globular head flexibly joined to a rodlike tail. Myosin has both a structural and catalytic role in muscle. The tail is more generally referred to as the myosin rod, and it consists of two α-helixes that are wound around one another in a left-handed coiled coil (*6*−*7*). This coil, which has a typical seven-residue heptad repeat, is stabilized by alternating hydrophobic residues in the coil interface and alternating oppositely charged residues on the surface of the coil, making it highly charged (*8*−*9*). Destabilizing residues bearing the same charge are located within the helix interface (*6*). This high charge on the myosin rod is believed to dictate its arrangement into thick filaments at physiological ionic strengths and its solubility at high ionic strength. At the amino end, the myosin rod folds up to a globular structure, thus endowing myosin the characteristics of both a fibrous and globular protein. The large

* To whom correspondence should be addressed. Fax: (352) 392-9437. Tel.: (352) 392-1991. E-mail: hgkristinsson@mail.ifas.ufl.edu.
† University of Florida.
‡ University of Massachusetts/Amherst.

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polypeptides are referred to as the myosin heavy chains, and each has a molecular mass of ca. 220 kDa. The globular head of myosin has ATPase activity and actin binding capability and is believed to play the dominant role in muscle contraction along with the part of the rod nearest to the globular head (*10*). On each of the globular heads, two small light chains are non-covalently attached. One is thought to be essential for ATPase activity (the essential light chain), and the other is not (the regulatory light chain), with both having a molecular weight between 18 and 25 kDa.

Altering the pH of a medium is one of the oldest known methods used to unfold proteins. As pH is increased or decreased away from the isoelectric point, the ionizable groups in proteins become increasingly charged up to a point where the charge repulsion causes the protein molecule to unfold as the unfolded configuration becomes more energetically favorable under these conditions. This is because the electrostatic free energy of the charged protein is reduced on unfolding, because charge repulsion is reduced as charges are distributed over a larger volume of the unfolded protein (*11*). Under these conditions, the hydrophobic forces, which normally account for most of protein stability, would not be strong enough to counteract the electrostatic free energy brought about at low or high pH. In contrast to previous beliefs that proteins are unfolded to a random coil at extremes of pH, many recent reports suggest that many proteins may only be partly unfolded under extreme pH conditions (primarily done at low pH) still retaining a relatively compact state (i.e., retention of a large part of secondary structure) while losing most of its side-chain interactions (e.g., tertiary structure) (e.g., *12*−*14*). The compact state, often referred to as a "molten globule", is primarily stabilized by hydrophobic forces that cause contraction and is opposed by excluded volume entropy effects or charge repulsion, which promote expansion. Different intermediate states at low pH can be explained by differences in contribution from charge repulsion, which acts to expand the protein, and hydrophobic forces, which work to contract the structure and stabilize it (*11*). According to this theory, it is evident that different unfolding pHs and different unfolding solvents may bring about different protein structures that could have modified functionalities over the native proteins.

The large changes observed in muscle cell structure, viscosity, and protein solubility in the acid/alkali process (*15*) suggest the major molecular changes occur with the muscle proteins. This is not unlikely, because the pHs employed are either very low or very high. The role acid and alkaline unfolding has on myosin's subunit structure and conformation, and ultimately its functionality, is largely unknown. Only a handful of studies have been conducted on what occurs with myosin at extremes of pH, all of which have been conducted on rabbit myosin and mostly on its rod fraction (*7*, *16*−*19*). These studies indicate that the helical structure of myosin may not be much affected at low and high pH, while little information exists on its globular head, and even less is known about what occurs on refolding. It remains unknown whether heavy chains dissociate at extremes of pH, while it is documented that part of the light chains dissociate at high pH (*18*). These are all important questions for the acid and alkaline process as it will enable us to understand what happens to myosin on the molecular level in the process. It is of much interest to study and understand on a fundamental level what occurs with the proteins in the acid/alkali processes in terms of their conformation and functionality and how their behavior is affected as the pH is readjusted to neutrality. An understanding of how the muscle proteins unfold at acid or alkali pH and refold on subsequent pH readjustment may provide us with important information enabling us to produce different protein structures with different functional properties by employing different unfolding and refolding schemes.

In this paper, we report on what occurs with the conformation and subunit assembly of myosin, the single most abundant protein in any muscle raw material used in the acid or alkali processes, as it experiences acid and alkali pHs and is readjusted back to the neutral pH range.

MATERIALS AND METHODS

Preparation of Myosin. Freshly caught Atlantic cod (*Gadus morhua*) was obtained from day boats operating out of Gloucester, MA. Muscle was filleted immediately upon arrival at the laboratory, and only white muscle was collected, which was trimmed of most visible connective tissue. The method described by Kristinsson (*20*) was used to isolate cod myosin. Myosin purity was analyzed by 4−20% linear gradient pre-cast SDS−PAGE gels (ICN Biomedicals, Costa Mesa, CA) and fixed and stained with Pro-blue reagents (OWL Scientific, Portsmouth, NH), as described previously by Kelleher and Hultin (*2*). Muscle protein bands were identified according to Stefansson and Hultin (*21*) with a standard curve constructed using high-molecular weight SDS−PAGE standards. Samples of cod myosin were of over 97% purity according to densitometry. Protein content was measured according to Lowry et al. (*22*). Myosin samples (∼15−20 mg/mL in 0.5 M KCl, 5 mM β-MCE, 20 mM Tris-HCl at pH 7.5) were kept on ice and typically used within 2 days. This storage period did not change the electrophoretic pattern, ATPase activity or secondary/tertiary structure of myosin (data not shown). For some of the conformational studies, myosin was stored in 50% glycerol at −20 °C (∼5 mg/mL) and used within 2 months, a period which did not change the properties of myosin hitherto mentioned.

Unfolding and Refolding Conditions of Myosin at Low and High pH. Myosin was unfolded at low and high pH by mixing an aliquot of myosin with either HCl−H_2O (at pH 1.5−3.5) or NaOH−H_2O (at pH 10.5−12), followed immediately by clarification at 10 000*g* and readjustment of protein concentration to a desired value (depending on experiments performed). Samples were kept for 30 min under these conditions to allow sufficient unfolding. This unfolding time is typical of the acid and alkali aided process. Longer unfolding times (up to 24 h) did not result in any more changes according to tryptophan fluorescence and circular dichroism spectra, and 30 min was used in all subsequent experiments. In the refolding studies, myosin (280 μg/mL) was unfolded at pH 2.5 (HCl−H_2O) and pH 11 (NaOH−H_2O) for 30 min, and refolding was accomplished by rapid dilution of the samples with KCl-Tris-EDTA buffer to give a final pH and salt concentration of 7.5 and 0.6M KCl (and 20 mM Tris and 1 mM EDTA). Samples were held for 60 min to allow sufficient time for refolding. Samples were clarified at 10 000*g* for 15 min after refolding, and protein concentration was adjusted to ∼150 μg/mL.

Conformational Changes in Myosin on Unfolding and after Refolding. *Spectroscopic Studies.* Clarified myosin solutions at pH 2.5 (HCl−H_2O), pH 7.5 (0.6M KCl, 20 mM Tris, 1 mM EDTA), and pH 11 (NaOH−H_2O) were studied for conformational changes. Myosin refolded from pH 2.5 and 11, as described above, was also studied for recovery of conformation.

Changes in intrinsic tryptophan fluorescence was investigated by recording the emission spectra from 300 to 400 nm of unfolded and refolded myosin (∼30 μg/mL) excited at 297 nm in a Hitachi F-2000 spectrofluorometer (Hitachi Instruments).

Changes in secondary and tertiary structure were monitored using circular dichroism in a Jasco J-715 spectropolarimeter at 5 °C. Secondary structure determination was performed by scanning clarified unfolded and refolded myosin solutions (∼30 μg/mL) from 260 to 190 nm in a 2 mm quartz cell. Tertiary structure was studied by scanning clarified myosin solutions (∼1 mg/mL) from 350 to 260 nm in a 1 cm quartz cell. Resolution was set at 0.2 nm, bandwidth was 1 nm, sensitivity 20 mdeg, response 1 s and scanning speed was 20 mdeg/

min. Five spectra were acquired for each sample and results averaged in one spectra.

Hydrophobicity of refolded myosin was studied from its ability to bind a hydrophobic dye, 8-anilino-1-naphthalene-sulfonic acid (ANS). To increasing concentrations of clarified myosin, 10 uL of 10 mM ANS was added and ANS-myosin emission spectra from 400 to 550 nm recorded after 15 min in a Hitachi F-2000 spectrofluorometer (Hitachi Instruments). Excitation was set at 380 nm.

Viscosity Studies. Viscosity of Myosin at pH 2.5, 11, and 7.5. Myosin solutions were adjusted to pH 2.5 (HCl− H_2O), pH 7.5 (0.6M KCl, 20 mM Tris, 1 mM EDTA), and pH 11 (NaOH−H_2O) and kept at these pH values for 30 min. The samples were then clarified for 10 min at 10 000*g* in a Beckman L8−55M ultracentrifuge, and protein concentration was adjusted to 1.35 mg/mL. The viscosity of the myosin solution was studied using an Ostwald viscometer (Fisher Scientific). To the Ostwald viscometer, 5 mL of myosin solution was added, and flow time (sec) of the solution was recorded. Experiments were performed in triplicate. The density of the solutions was determined by weighing 1 mL of the respective solutions. Relative viscosity (unitless) of the solutions was calculated according to the following equation:

$$\frac{n2}{n1} = \frac{\rho_2 \cdot t_2}{\rho_1 \cdot t_1}$$

where n = solution viscosity, ρ = solution density (g/cm^3), and t = flow time (sec) through the Ostwald viscometer. The numbers 1 and 2 refer to the buffer and the myosin solution, respectively.

Viscosity of Refolded Myosin. Myosin was unfolded at pH 2.5 and 11 for 30 min and refolded to pH 7.5 as described previously. This solution was clarified for 30 min at 10 000*g*, and protein concentration was adjusted to 0.84 mg/mL with buffer (0.6M KCl, 20 mM Tris, 1 mM EDTA; pH 7.5). Solution viscosity was determined as described above.

Conformational Stability of Refolded Myosin. Myosin was denatured at pH 2.5 and 11 for 30 min and subsequently refolded as described above. An aliquot of refolded myosin and native myosin was then added to increasing concentrations of guanidine hydrochloride (0−6 M Gu-HCl), to give ∼30 μg/mL protein, and kept for 1 h at 5 °C. Extent of unfolding was assessed by monitoring secondary structure (222 nm), intrinsic tryptophan fluorescence (from shift in maximum peak wavelength), and ANS binding/fluorescence (at 470 nm) of the samples, as described above. The apparent fraction of unfolded protein was estimated based on a two-state unfolding mechanism

$$f_{app} = \frac{f_U - f}{f_U - f_N}$$

where f_{app} represents the apparent unfolded fraction of myosin, f the observed ellipticity or fluorescence intensity under the given conditions, and f_U and f_N are the corresponding values in the unfolded and native state, respectively (*23*).

Determination of Subunit Dissociation and Reassembly on Unfolding and Refolding. *Dissociation of Light Chains.* An aliquot of myosin was added to pH 2.5 (HCl−H_2O), pH 7.5 (0.6M KCl, 20 mM Tris, 1 mM EDTA), pH 11 (NaOH−H_2O) or 5M Gu-HCl. Samples were held under these conditions for 30 min and 24 h at 5 °C and then clarified at 10 000*g* for 25 min at 5 °C. Sediment was analyzed for protein composition using SDS−PAGE with 4−20% linear gradient gels and was found to be of the same composition as the soluble myosin samples. Clarified samples were analyzed for protein content by absorbance at 280 nm using an extinction coefficient of 5.60 cm^{-1}M^{-1} (Weeds, 1976). Samples were then adjusted to give the same protein concentration for all solutions (∼1 mg/mL). To Centricon (Millipore Corporation, Bedford, MA) centrifugal filters (50 000 and 100 000 Da molecular weight cutoff), 1 mL of the above solutions was added, and the filter and filtrate collector were weighed. Samples were filtrated at 2200*g* (5 °C) for 30−60 min. Filtrate was then weighed and collected, and retentate was reconstituted with the same volume of buffer as went through the filter. On average, 70−80% of the sample went through the filter. Samples were then prepared for electrophoresis, as described earlier in the Materials and Methods, and run on 4−20% linear gradient SDS−PAGE gel and analyzed by densitometry using a Hoefer GS-300 Densitometer (Hoefer Scientific, San Francisco, CA). The amount of dissociated light chains was quantified by integrating individual peaks and calculating the percent of total area assuming equal dye binding of subunits and also taking into account the solution that did not go through the filter, which also contained dissociated light chains.

For evaluation of light chain reassembly on refolding, myosin was unfolded at pH 2.5 and 11 for 30 min and then refolded as described previously. This sample was clarified and run through the Centricon filters, and the amount of reassembled light chains was determined from SDS−PAGE analysis of the retentate and filtrate as described above.

Dissociation of Heavy Chains. Cross-linking of myosin with glutaraldehyde (GA) was performed to establish if myosin heavy chains dissociate on unfolding. Cross-linking experiments essentially followed those described by Azem et al. (*24*) with minor modifications. Preliminary trials were conducted at various concentrations of myosin and GA. Aggregation of myosin in the presence of GA called for working at low protein and GA concentrations. Myosin, at pH 2.5, 7.5, and 11, was kept at ∼0.2 mg/mL, and less than 1 uL of 25% GA in 1 mL myosin solution was used. Myosin was left to cross-link for 10 min to 24 h at 5 °C, and the reaction stopped by addition of a one-third volume of 1 M tris-glycine (pH 8.8), 4% SDS, and 10% 2-mercaptoethanol. The samples were then heated at 100 °C for 2.5 min and submitted to SDS−PAGE as described above.

Reactive Sulfhydryls on Refolded and Native Myosin. Myosin refolded from pH 2.5 or 11 and native myosin was assayed for the exposure of reactive sulfhydryl groups (−SH) employing the method of Lin and Park (*25*). To 2.25 mL of clarified solution of native and refolded myosin (0.04 mg/mL), 15 μL of 10 mM 5,5′-dithiobis(2-nitrobenzoic acid) was added. The reaction was followed at 5 °C at 420 nm, using a molar extinction coefficient of 13 600 M^{-1}cm^{-1}, and values were read when they stabilized.

ATPase Activity of Isolated Myosin. Myosin refolded from pH 2.5 and 11 (after 30 min unfolding) and native myosin (at pH 7.5) were assayed for ATPase activity according to the method of Perry (*26*), as modified by Ang and Hultin (*27*). This method measures inorganic phosphate liberated by the myosin ATPase spectrophotometrically at 363 nm. Myosin concentration was ∼0.45 mg/mL. ATPase activity of the samples was reported as a relative percentage, with native myosin representing 100% activity.

RESULTS

Conformational and Structural Changes of Cod Myosin at Low and High pH. Several structural probes were employed to gain insight into the unfolding of different regions in the complex cod myosin molecule. Subjecting myosin to either pH 2.5 or 11 did not lead to significant changes in the secondary structure of the protein compared to the native protein at pH 7.5 (**Figure 1**a). At pH 2.5, myosin experienced ∼10% loss in helical structure, while at pH 11, no change in secondary structure was observed. On the other hand, denaturing the protein in 6M Gu-HCl led to complete loss of the secondary structure spectrum of myosin, suggesting it had fully unfolded. There were notable changes observed in the near-UV spectra of myosin under acid and alkaline conditions, indicating changes in tertiary structure (**Figure 1b**). At pH 2.5 and 11 and in 6M Gu-HCl, the protein had apparently lost all its tertiary interactions. Changes in the environment of myosin tryptophan residues showed a notable quenching of fluorescence on acid and alkali unfolding; however, the wavelength of maximum fluorescence was not shifted (**Figure 1**). The fully unfolded molecule in 6M Gu-HCl exhibited more quenching of tryptophan fluorescence and was significantly red shifted (∼350 nm), similar to that of tryptophan fully exposed to solvent. The conformational changes at pH 2.5 and 11 were accompanied by total loss of ATPase activity (**Table 1**).

The changes in conformation assessed by the spectroscopic methods were complemented by studying the hydrodynamic

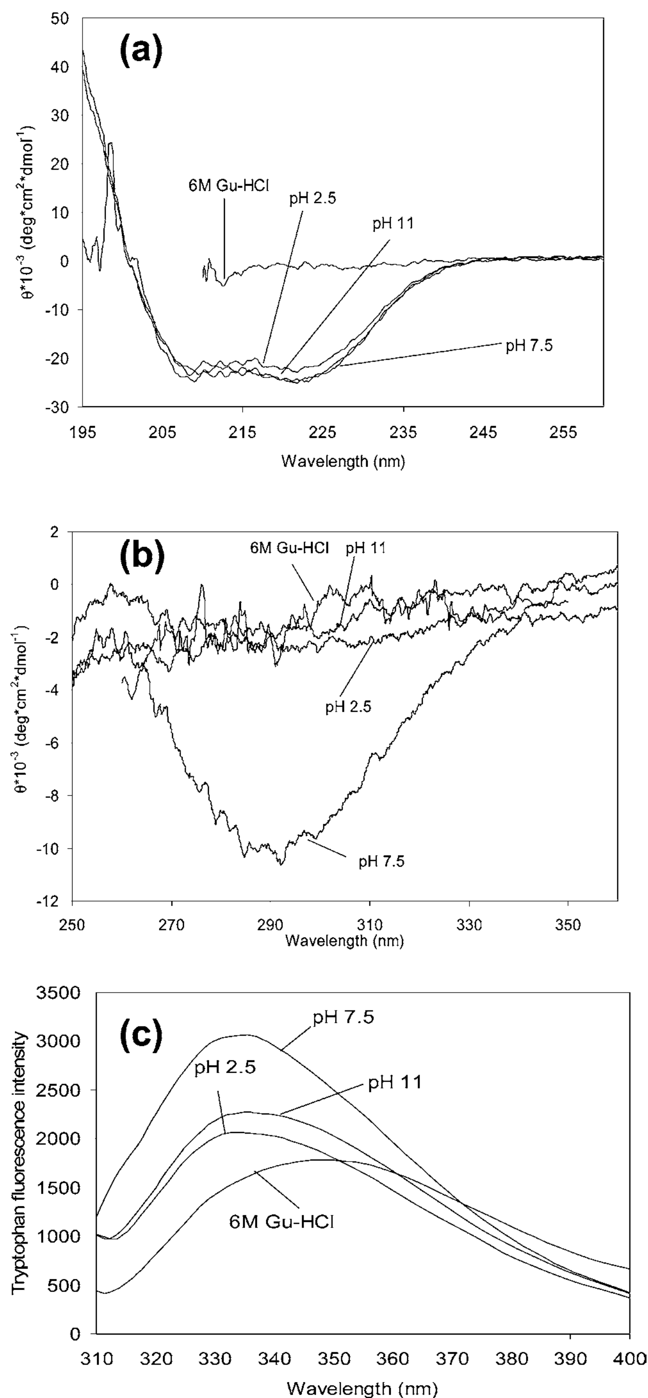

Figure 1. Conformational change in myosin at pH 2.5, 7.5, 11, and in 6M Gu-HCl. (a) Far-UV CD spectra, (b) Near-UV CD spectra, and (c) Intrinsic tryptophan fluorescence.

Table 1. ATPase Activity of Cod Myosin after Acid and Alkaline Treatment for 30 min

pH treatment	ATPase activity (%)[a]
pH 7.5	100
pH 2.5	0
pH 11	0
pH 2.5→7.5	9.6 ± 2.9
pH 11→7.5	13.8 ± 3.2

[a] Myosin concentration was 0.45 mg/mL in 0.6M KCl, 20 mM Tris, pH 7.5.

Table 2. Viscosity of Cod Myosin under Native and Denaturing Conditions

pH treatment	relative viscosity[a]
pH 7.5	1.4165 ± 0.003
pH 2.5	2.6883 ± 0.006
pH 11	1.7740 ± 0.015

[a] Myosin concentration was 1.35 mg/mL.

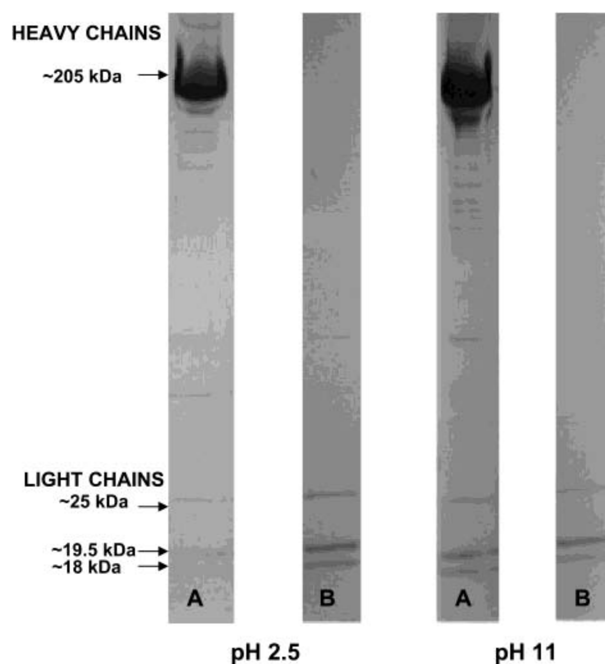

Figure 2. Dissociation of cod myosin light chains at pH 2.5 and 11 determined using ultrafiltration and SDS-PAGE. Filter MW cutoff was 100 kDa and samples were filtered at 1 mg/mL, and retentate and filtrate fractions applied to the gel after reconstitution of the volume that went through the filter to put them on the same basis. A represents the retentate and B the filtrate from the ultrafiltration. There was no dissociation of light chains for control myosin samples at pH 7.5 (see Figure 7).

behavior of myosin. The relative viscosity of myosin at pH 2.5 was notably higher than that of native myosin at pH 7.5 (**Table 2**). Viscosity at pH 11 was also higher than that of native myosin, but was considerably less than that at pH 2.5. This indicated that the structure of the protein may be significantly different at pH 2.5 than pH 11, something that the spectroscopic probes did not indicate.

As myosin is a multimeric protein, these changes in conformation and especially hydrodynamic behavior raised questions about the structural assembly of the protein. The potential dissociation of the four light chains and the two heavy chains were studied separately. According to ultrafiltration of myosin at pH 2.5 and 11 and analysis of the collected fractions by SDS–PAGE and densitometry, between 70 and 80% of myosin light chains were found to have dissociated at pH 2.5 and ca. 60% at pH 11 (**Figure 2**). Full dissociation may have occurred, but since all of the protein solution did not go through the ultrafiltration device, it was difficult to determine this. No light chains went through the filter at pH 7.5, indicating no dissociation. In 6M Gu-HCl, light chains were also found to have dissociated (data not shown). There was no change observed in dissociation even after 24 h. **Figure 2** also clearly shows that there was no hydrolysis observed at pH 2.5 and pH 11, because the heavy chains and light chains were intact. Potential myosin hydrolysis at pH 2.5 and 11 (5 °C) was tested for 3 days, and no hydrolysis was seen during this period (data not shown).

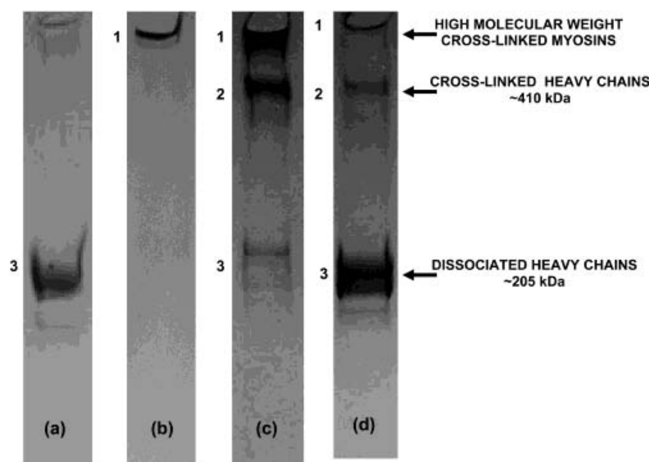

Figure 3. Potential dissociation of cod myosin heavy chains at pH 2.5 and 11 as determined by glutaraldehyde (GA) cross-linking. (a) Myosin control without GA cross-linking. (b) Myosin at pH 7.5. (c) Myosin at pH 11. (d) Myosin at pH 2.5. Number 1 refers to cross-linked myosin dimers or higher molecular weights, number 2 refers to cross-linked heavy chains, and number 3 refers to dissociated heavy chains. The top 1/3rd of the SDS-PAGE gel is shown to emphasize the myosin heavy-chains.

Using a glutaraldehyde cross-linking assay, it could be determined if myosin heavy chains dissociated at low and high pH. The premise of the method is that if protein subunits are associated, the GA covalently cross-links these units, and the cross-link is not broken during electrophoresis. On the other hand, if units are dissociated, the GA is unable to cross-link them because it works over a short distance. Results with the GA assay and native myosin show that a high molecular species just entered the gel, possibly a dimerized myosin (**Figure 3b**). At pH 11, three high molecular weight bands appeared, one just below the loading well, one slightly below it, representing a nondissociated heavy chain, and one very faint band representative of dissociated heavy chains (**Figure 3c**). At pH 2.5, however, two bands were observed, one faint band possibly representing a nondissociated heavy chain, and a stronger band representing dissociated heavy chains (**Figure 3d**). In 5M Gu-HCl, only dissociated heavy chains were found, as has been observed by others (*17*). Our preliminary studies using native tube gel electrophoresis supported these findings.

Conformational and Structural Changes of Cod Myosin Refolded from Low and High pH. Changes in both conformation and potential reassembly of the myosin molecule were studied after refolding the protein from pH 2.5 and 11. Secondary structure of myosin was largely retained on refolding, as it was little affected at pH 2.5 and 11 (**Figure 4**a). It was remarkable that refolding myosin from 6M Gu-HCl resulted in almost full renaturation of the helix, and presumably the coiled coil. However, only partial recovery in tertiary structure was found on refolding, more for myosin refolded from pH 2.5 compared to pH 11 (**Figure 4b**). Increased exposure of hydrophobic clusters was found on the refolded protein compared to the native protein, as assessed by ANS binding (**Figure 5**), similarly reflecting a partial recovery in the native environment of myosin. ANS binding was most pronounced for the protein refolded from pH 11. Refolding myosin from acid or alkaline conditions led to low recoveries of ATPase activity (**Table 1**). An increase in reactive myosin thiol groups of the refolded protein was also observed, more for the alkali unfolded species than the acid unfolded one (**Table 3**). Furthermore, on refolding, the myosin molecule reverted to a form with hydrodynamic properties essentially the same as the native state (**Table 4**), suggesting possible reassembly of its subunits.

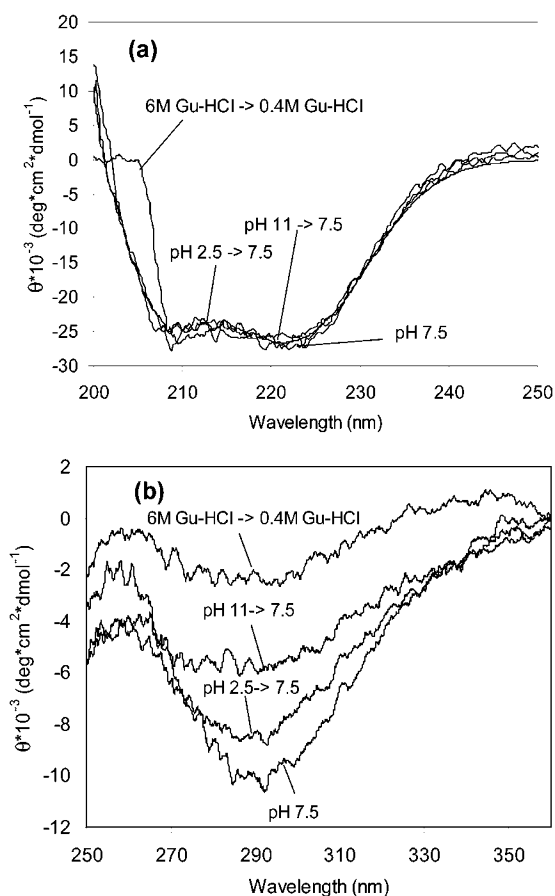

Figure 4. Recovery of secondary and tertiary structure on refolding. (a) Far-UV circular dichroism spectra reflecting myosin secondary structure. (b) Near-UV circular dichroism spectra reflecting myosin tertiary structure.

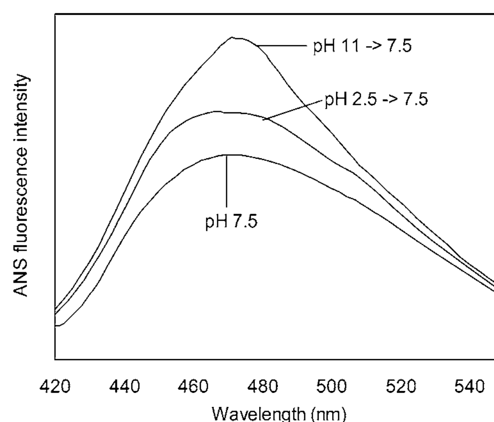

Figure 5. Exposure of hydrophobic residues of native and refolded myosin. (a) ANS binding/fluorescence emission spectra of myosin. Fluorescence excitation was at 390 nm and emission wavelength was 420–550 nm. The temperature was 5 °C, and samples were measured 15 min after ANS (10 mM) was added.

Potential myosin reassembly on refolding was therefore investigated. Ultrafiltration showed that light chains did not reassemble onto the myosin head region when the protein was refolded from pH 2.5 or 11 (**Figure 6**), because close to equal amounts of dissociated light chains were found after refolding compared to pH 2.5 and 11. This also indicates that the light chains contribute little to the hydrodynamic behavior of myosin, because the refolded myosin had similar viscosity as the native

Table 3. Relative Increase in Reactivity of Accessible Thiol Groups on Myosin

pH treatment	relative increase in reactive thiols[a]
pH 7.5	0
pH 2.5→7.5	10%
pH 11→7.5	29%

[a] Myosin concentration was 0.04 mg/mL in 0.6M KCl, 20 mM Tris, pH 7.5. Relative increase was calculated as absorbance of refolded myosin in 5,5′-dithiobis(2-nitrobenzoic acid) at 420 nm over the absorbance of native myosin.

Table 4. Viscosity of Refolded Myosin

pH treatment	relative viscosity[a]
pH 7.5	1.253 ± 0.002
pH 2.5→7.5	1.229 ± 0.061
pH 11→7.5	1.249 ± 0.013

[a] Myosin concentration was 0.84 mg/mL in 0.6M KCl, 20 mM Tris, pH 7.

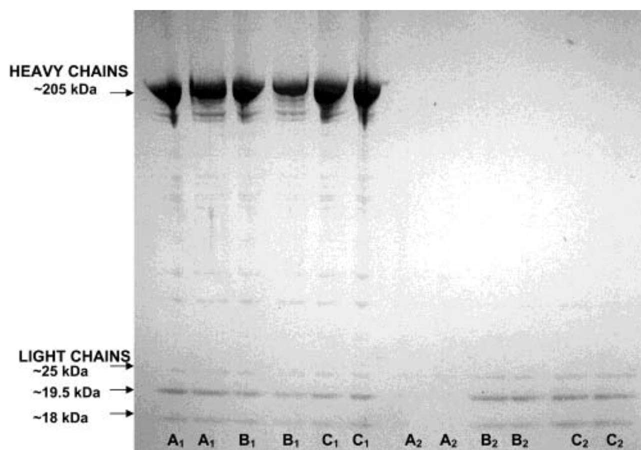

Figure 6. Fate of myosin light chains on refolding from acid and alkaline pH. Solutions containing refolded or native myosin were ultrafiltrated in Centricon filtration devices and reconstituted retentate (Lanes A_1–C_1) and filtrate (Lanes A_2–C_2) run on SDS–PAGE to elucidate if light chains went through the filter. Dissociated light chains would show up in the filtrate. Lanes represent the following treatments: $\mathbf{A_1}$ Retentate of native myosin at pH 7.5; $\mathbf{B_1}$ Retentate of myosin unfolded at pH 2.5 and refolded to pH 7.5; $\mathbf{C_1}$ Retentate of myosin unfolded at pH 11 and refolded to pH 7.5; $\mathbf{A_2}$ Filtrate of native myosin at pH 7.5; $\mathbf{B_2}$ Filtrate of myosin unfolded at pH 2.5 and refolded to pH 7.5; $\mathbf{C_2}$ Filtrate of myosin unfolded at pH 11 and refolded to pH 7.5. Experiments were done in duplicate as represented by two lanes for each letter.

myosin. Using low concentrations of both protein and GA, it was possible to get an indication if the refolded species had reassembled. Cross-linking results with the refolded protein led to a species migrating just below the loading well at a similar location as myosin at pH 7.5 (**Figure 7**). This band could represent cross-linking of the coiled-coil to a higher polymeric form. For the protein refolded from pH 2.5, a faint band representing that of dissociated heavy chains was also observed, although it was only a fraction of the protein applied on the gel. No such band appeared for myosin refolded from pH 11.

Conformational Stability of the Refolded Myosin. One approach to get information on the structural integrity of a protein is by investigating its conformational stability under denaturing conditions. This was accomplished by (a) unfolding the native and refolded proteins in 0−6M Gu-HCl and following secondary structure, tryptophan fluorescence, and ANS binding

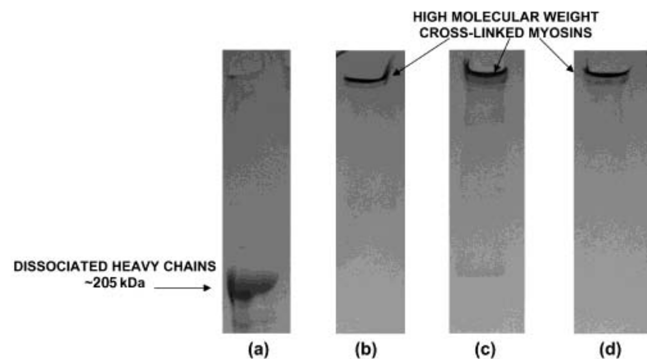

Figure 7. Re-assembly of myosin heavy chains on refolding from acid and alkaline pH assessed by glutaraldehyde (GA) cross-linking. (a) Myosin control without GA cross-linking. (b) Myosin at pH 7.5 after GA cross-linking. (c) Myosin refolded from pH 2.5 and then cross-linked with GA. (d) Myosin refolded from pH 11 and then cross-linked with GA. The cross-linked and dissociated myosin heavy chains (MHC) are labeled on the figure. The top 1/4th of the SDS–PAGE gel is shown to emphasize the myosin heavy-chains.

(**Figure 8**). With increasing amounts of Gu-HCl, the native and refolded myosins exhibited almost identical unfolding behavior, according to changes in secondary structure (**Figure 8a**). The shift in tryptophan fluorescence wavelength on Gu-HCl unfolding was also similar for all treatments, but myosin refolded from pH 11 appeared slightly more sensitive to unfolding (**Figure 8b**). Exposure of hydrophobic sites as measured by ANS binding on Gu-HCl denaturation suggested easier unfolding of the refolded proteins (**Figure 8c**). According to the ANS assay, the midpoint of the unfolding transition was lowest for myosin unfolded at pH 11, ∼1.9 M Gu-HCl, followed by myosin unfolded at pH 2.5, ∼2.2 M Gu-HCl, and with native myosin unfolding at 2.8 M Gu-HCl.

DISCUSSION

Myosin has been the subject of much study in the past as to its biological function and its role in muscle food chemistry. There have however been very few studies conducted on myosin's conformation and structural assembly at very low or high pHs and after refolding from acid and alkaline conditions. Most of the studies conducted so far at extremes of pH were focused on the light meromyosin (LMM) fraction, which represents a large part of the myosin rod (**Figure 9**a). Both the rods and heads region are thought to play important and separate roles in various functional properties of myosin as a food molecule. As myosin is subjected to highly acid and alkaline conditions in the acid and alkali protein isolation processes, it is of interest to understand what conformational changes occur and what the subunit composition of myosin is under conditions similar to that used in the acid and alkaline solubilization processes. A knowledge at the molecular level will give us insight into the molecular changes that occur and hopefully an ability to understand what molecular changes are responsible for the functionality of the modified proteins.

Conformation and Assembly of Myosin Under Low and High pH Conditions. As myosin is endowed with properties of both a globular and fibrous protein (*10*), one can employ different spectroscopic techniques to obtain information on what may be occurring with the different regions of the protein as they unfold. The myosin rod is almost 100% helical, while the globular head fraction has less than 50% α-helix (*9*). The rod has therefore been found to represent the great majority of the secondary structure spectra of myosin (*16*, *28*). On the other

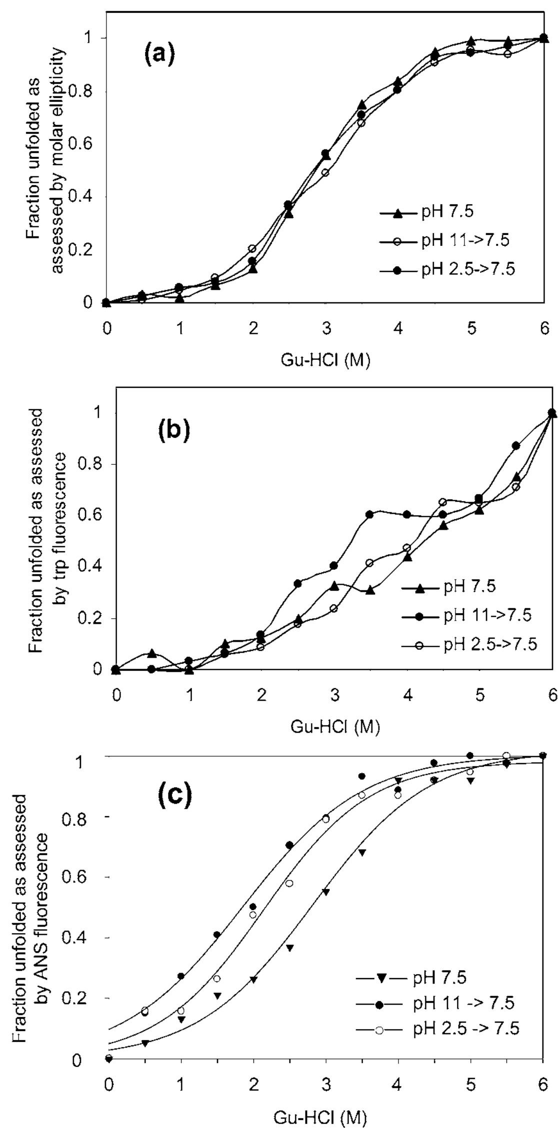

Figure 8. Conformational stability of native and refolded myosin as assessed by unfolding in 0–6M Gu-HCl. (a) Unfolding of secondary structure by following changes in molar ellipticity at 222 nm. (b) Exposure of tryptophan residues on unfolding assessed by wavelength shift. (c) Exposure of hydrophobic clusters on unfolding determined by ANS binding/fluorescence. All experiments were performed at 5 °C after 1 h in 0–6M Gu-HCl. Myosin was left to refold for 1 h prior to measurements.

hand, the globular configuration of the head region is expected to represent most of the tertiary structure. Because myosin retained almost all its secondary structure at low and high pH (**Figure 1a**), it can be concluded that the α-helixes making up the rod, and possibly a large part of the globular region as well, were largely unaffected. The retention of helical structure in rabbit myosin rod has been demonstrated previously at low (pH 2) and high pH (pH 11) (*7*, *16*). Lowey reported that most of the changes in secondary structure of myosin were found in the heavy meromyosin (HMM) (*16*). Stracher (*29*), on the other hand, observed an increase in helicity up to pH 10.5, followed by a large decrease to pH 11 and then an enormous increase at pH 12. This could have been due to the fact that he studied the protein at alkaline pH in 0.6M KCl, which leads to protein aggregation and will greatly interfere with spectroscopic analysis of the protein (Kristinsson, unpublished observations).

The loss in tertiary structure as seen from the near-UV CD spectra of cod myosin (**Figure 1b**) is a strong indication that the globular head region of the protein has taken on a distorted form because interactions between the helixes (which still may be somewhat intact) are lost (*30*). In fact, the evidence that the secondary structure is largely native but not the tertiary structure may suggest that the globular part of refolded myosin has the characteristics of a molten globular protein (*31*). These changes in the myosin head may be responsible for the loss of light chains at low and high pH (**Figure 2**). In the native state, the light chains (two on each head) are wrapped around a long α-helix in the neck region of the head (where it links with the rod) mostly via polar and electrostatic interactions, as the head and the light chains are of opposite polarity where they interact (*9*). The light chains may have lost their contact with the head as the result of electrostatic repulsion and distortion of the neck region. Alkali dissociation of light chains has been previously documented for rabbit myosin (*17*, *32*). Dissociation of light chains from rabbit myosin was also seen at acid pH in 1−10 M acetic acid (*19*). The lower percentage of light chain dissociation at alkaline conditions (60 vs 70−80% at pH 2.5) found with cod myosin in the current study may be due to the fact that high pH only releases the two "essential" light chains (*33*). Acid pH may thus release both the essentia and "regulatory" chains.

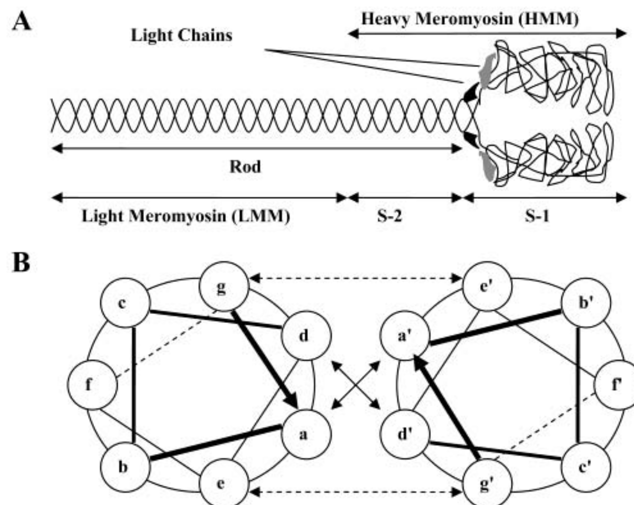

Figure 9. (A) Structure of sarcomeric muscle myosin II. The figure is not drawn to scale. (B) Helical representation of the α-helical coiled-coil myosin rod seen from the N-termini. Interactions between a–a and d–d are mostly hydrophobic, whereas interactions between g–e are electrostatic.

Myosin has tryptophan residues both in its rod and head region (*34*). The results with cod myosin suggest that these residues may be still shielded from the solvent, as they are not shifted to a higher emission wavelength compared to the fully denatured protein in 6M Gu-HCl (**Figure 1c**). The fluorescence quenching seen here with cod myosin at low and high pH has been observed with myosin rods on thermal unfolding (*28*, *35*) and myosin heads on chemical unfolding (*34*). Chang and Ludescher (*35*) explained this quenching as a result of ionization of nearby tyrosines, which leads to the transfer of energy from exited tyrosines to tryptophans exposed on unfolding. The above, therefore, could indicate that the globular region of myosin may not be in a random coil state at low and high pH, as the emitted fluorescence of tryptophan is still nativelike, which strengthens our proposal that the globular head takes on a molten globular configuration at low and high pH. The large increase in hydrodynamic volume at low and high pH, however, suggests that the protein may take on an extended configuration, in contrast to the idea that the protein headgroups remain compact.

Kato and Konno (*34*) concluded that tryptophan fluorescence was not a particularly sensitive and informative structural probe for the myosin head region but was so for the rod. The lack of wavelength shift may thus be giving evidence primarily on the conformation of the rod as the rod appears to retain its structure at low and high pH.

The coiled coil of myosin has a characteristic regular 7-residue pattern, *a*, *b*, *c*, *d*, *e*, *f*, *g*, where the hydrophobic residues are concentrated at alternate intervals along the length of the chain at positions *a* and *d* and tightly pack and stabilize the helix (**Figure 9b**). The surface of the helix is highly charged with alternating acidic and basic residues clustered in the outer positions *b*, *c*, and *f* (*6*, *9*). At positions *e* and *g*, there are charged amino acids that are believed to participate in salt bridges that stabilize the coil (*6*). Because of the high charge density of myosin and particularly the occurrence of positively charged residues in the rod at low pH oppositely facing each other, it would perhaps not be surprising that cod myosin may have dissociated at low pH as indicated from the lack of cross linking in glutaraldehyde (**Figure 3**). Furthermore, the great increase in viscosity at pH 2.5 could have been a result of the dissociation, as each dissociated helix would have an effective hydrodynamic volume about the same as the coiled coil, thereby doubling the effective volume of the protein. Ozog and Bechet (*7*) demonstrated that the rabbit myosin rod became more resistant to Gu-HCl denaturation at pH 2 vs pH 7 but had no data as to whether it had dissociated. Noelken and Holtzer (*37*) had previously obtained the same results with tropomyosin and paramyosin, both made up of coiled-coils, when subjecting them to high concentrations of urea or Gu-HCl at pH 2. Lowey (*16*) reported that these two proteins became more heat resistant at low vs neutral pH. As secondary structure of cod myosin was retained at pH 2.5, it would suggest that if it was dissociated, its free α-helixes were intact. One previous study (*19*) demonstrated dissociation of rods at acid pH in 1−10 M acetic acid. So by what mechanism did the myosin rods dissociate at low pH? A clue for this can be obtained from Lowey's early work (*38*). Lowey (*38*) found that HMM likely dissociates at acidic conditions (pH 2) while LMM appears intact. The increase in viscosity may thus be a result of partial unwinding of the rod in the S-2 region of the HMM. This unwinding may then lead to a cooperative unwinding of the whole rod, thus dissociating the protein. An expansion of the head region (as indicated by loss of tertiary interactions) and the unwinding of the rod could partly account for the increase in viscosity as the hydrodynamic volume would increase.

At pH 11, there was also an increase in viscosity, but much less than at pH 2.5, which may have been partly due to distortion of the head region, but not unwinding of the rod, as the protein did not show signs of rod dissociation at this pH. Several studies have indeed indicated no heavy chain dissociation with rabbit myosin at alkaline pH (*17*−*19*, *32*). Part of the increase in viscosity at pH 2.5 and 11 could also be due to the increased electrostatic repulsion between the proteins at this pH.

Structural and Conformational Recovery on Refolding. Refolding of many proteins can be demonstrated by placing the unfolded protein under conditions in which the folded state is stable. This situation is however not simple for large multimeric proteins such as myosin, and no general scheme for folding and assembly exists for such proteins. The refolding in vitro of multiple domain or multiple polypeptide proteins generally occurs at a considerably lower rate and with a lower yield in native structure than folding in vivo (*39*−*40*). Kinetic barriers are typically high in complex protein folding (*41*), which may lead to misfolded protein structures being stuck in their respective energy wells. Refolding of myosin from acid or alkaline conditions would involve folding of its individual subunits followed by their association (i.e., those that are dissociated). The various segments of a polypeptide chain seem to interfere with each other in folding, and association of folded chains is a slow process (*39*). In the case of cod myosin, this study showed that the acid and alkali unfolding led to a partly misfolded species when readjusted to pH 7.5.

It seems reasonable to assume that the myosin rod reverted to its native state on refolding, as secondary structure was almost identical to native myosin on refolding (**Figure 4a**). This is not surprising, considering that the secondary structure was little affected at low or high pH. The absence of dissociated heavy chains on GA cross-linking is also an indication that the coiled coil was either fully or partly reassociated on refolding (**Figure 7**). The similar apparent viscosities of refolded and native myosins (**Table 4**) support the conclusion that the molecule reverted to a nativelike configuration. That the fully dissociated and distorted heavy chain in a high concentration of Gu-HCl fully regained its native secondary structure on refolding is evidence of the thermodynamic stability of this structure (**Figure 4**). This phenomenon has been observed previously with isolated myosin rods (*42*−*43*). The low percentage of dissociated heavy chains from the acid unfolded/refolded protein seen on SDS−PAGE analysis (**Figure 7**) may have been a result of incomplete cross-linking rather than dissociation. It was however somewhat surprising that the cross-linked species formed had an estimated molecular mass well above ∼400−450 kDa, which would be the size of intact heavy chains (without the light chains). It has been reported by some workers that in its native state in high salt, myosin is present as a dimer rather than a monomeric protein, with rapid equilibrium between the two species (*8*), which could be the case here.

The conformation of the myosin head did not revert to its native state on refolding in contrast to the conformation of the rod. This can be concluded from several of our structural probes. First, the near-UV spectra of the protein represented lack of tertiary interactions (**Figure 4b**), which are predominantly in the globular head. This lack of structural recovery in the myosin head was characterized by an increased exposure of hydrophobic clusters as indicated by the protein's increased ability to bind ANS (**Figure 5**). The head of myosin has been found to represent most of the ANS-binding capability of myosin under denaturing conditions, while the rod is unable to bind ANS under native or denaturing conditions (*34*), as it lacks a sizable hydrophobic core to do so (*44*). Furthermore, the greater ability of the alkali unfolded/refolded protein to bind ANS compared to the acid unfolded/refolded protein was correlated with less recovery in tertiary structure. This strongly suggests that alkali treatment led to less recovery in the native structure of the globular head region than acid treatment. Myosin (rabbit), reportedly, has ∼42 thiol groups, about 26 of which are on its globular head and 6 in the light chains (*45*). The increased reactivity of the SH groups after refolding (especially for the alkaline treated protein) is further evidence that the globular head is not properly folded. That myosin refolded from pH 11 had more reactive SH groups than myosin refolded from pH 2.5 is further evidence that the native conformation of its globular head was less recovered than the globular head of the acid treated protein.

The improper refolding of the head may also explain why the light chains did not reassemble onto it, as the association of the small myosin light chains onto the globular head is very

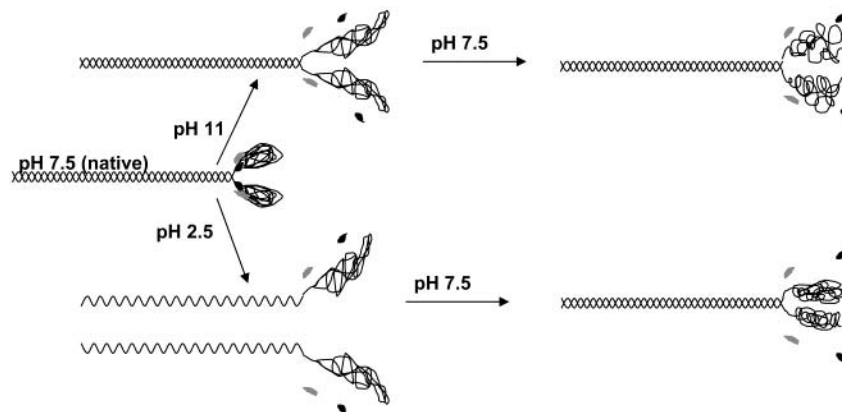

Figure 10. Proposed structural change in the myosin molecules at acid and alkaline pH and after refolding to pH 7.5. Experimental data suggests that at high pH, myosin heavy chains are still associated while a fraction of the light chains dissociate. At low pH, results suggest that heavy chains dissociate along with a large fraction of the light chains. On refolding to pH 7.5, it is proposed that the heavy chains reassociate from low pH, while light chains remain dissociated for both pH treatments. As indicated in the drawing, there is more recovery in the tertiary structure of the myosin head region on refolding from pH 2.5 compared to refolding from pH 11.

specific (*10*). Part of the lack in tertiary structure and enhanced ANS binding may also stem from the lack of light chain reassembly and/or their improper refolding. As two of the light chains (the "alkali" or "essential" light chains) appear essential for ATPase activity (*33*), their dissociation and lack of reassociation would explain why only a small part of the ATPase activity was recovered after refolding myosin from acid or alkaline pH. Previous studies have found ATPase activity to be lost on alkaline, heat, urea, and Gu-HCl treatment, presumably as light chains were removed (*18*, *32*, *46*). Light chains were also found to have dissociated in 1−10 M acetic acid (*19*), but no reports are available on their reassembly or activity recovery after acid unfolding to the best of our knowledge.

The conformational integrity of the rod versus the head fraction of the refolded protein can be differentiated by looking at how the molecule responds to chemical denaturation by different molecular probes. Information of the molecule's stability will provide information on the state of its different subunits after refolding, and may provide insight into any functional changes observed (see following paper). That almost identical unfolding behavior was observed for the secondary structure of native and refolded myosin (i.e., molar ellipticity at 222 nm) in 0−6M Gu-HCl (**Figure 8a**) is a confirmation that the coiled-coil structure was intact or had reversibly refolded after extreme pH treatment. That ANS binding occurred at a lower Gu-HCl concentration for the refolded proteins (**Figure 8c**) is a strong indication that the myosin head was structurally unstable after refolding, as it did not refold properly. From the ANS binding results, the alkaline-treated protein appeared least stable, in good agreement with its lower recovery in native structure on refolding. The ease of unfolding of the alkaline-treated protein was also demonstrated by tryptophan fluorescence wavelength shift (**Figure 8b**) but not for the acid treated protein. Most tryptophan residues reside in the myosin head, but a good fraction of tryptophan fluorescence also comes from the rod (*34*). The greater ease of unfolding of the alkaline treated protein indicated by tryptophan fluorescence compared to unfolding of the acid-treated and native protein indicates that tryptophan fluorescence is most likely a combined response of the rod and the head and is less informative on structural changes occurring in specific regions of the protein, in contrast to molar ellipticity at 222 nm (rod portion) and ANS binding (head portion).

To summarize the above: our data therefore strongly suggest that at low pH, myosin is likely completely dissociated, and on refolding, the heavy chains reassociate, while the light chains remain dissociated. At pH 11, on the other hand, only half of the light chains are dissociated, and the heavy chains stay intact. This proposal is depicted in **Figure 10**. These results also suggest that myosin takes on a distinctly different conformation depending on whether it is exposed to acid or alkaline conditions. It may thus be possible via controlled unfolding and refolding conditions to direct myosin into different conformational states of one's choice and thus manipulate its functional properties.

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LITERATURE CITED

(1) Kelleher, S. D.; Hultin, H. O. Functional chicken muscle protein isolates prepared using low ionic strength, acid solubilization/precipitation. *Reciprocal Meat Conf. Prooc.* **2000**, *53*, 76−81.
(2) Kelleher S. D.; Feng Y.; Kristinsson H. G.; Hultin H. O.; McClements D. J. 2002. Functional fish protein isolates prepared using low ionic strength, acid solubilization/precipitation. *Fisheries Sci.*, in Print.
(3) Xiong, Y. L. Structure−function relationships of muscle proteins. In *Food Proteins and Their Applications*; S. Damodaran and A. Paraf, Eds.; Marcel Dekker: New York, 1997; pp 341−392.
(4) Offer, G.; Knight, P. The structural basis of water-holding in meat. Part 1: General principles and water-uptake in meat processing. In *Developments in Meat Science*; R. Lawrie, Ed.; Elsevier Applied Science Publishers: London, 1988; pp 63−172.
(5) Foegeding, E. A.; Lanier, T. C.; Hultin, H. O. Characteristics of edible muscle tissue. In *Food Chemistry*; O. R. Fennema, Ed.; Marcel Dekker: New York, 1996; pp 879−942.
(6) McLachlan, A. D.; Karn, J. Periodic charge distribution in the myosin rod amino acid sequence match cross-bridge spacings in muscle. *Nature* **1982**, *299*, 226−234.
(7) Ozog, A.; Bechet, J.-J. The effect of pH on the folding and stability of the myosin rod. *Eur. J. Biochem.* **1995**, *234*, 501−505.

(8) Sellers, J. R. *Myosins*, 2 ed.; Oxford University Press: Oxford, UK, 1999.

(9) Harrington, W. F.; Burke, M. Geometry of the myosin dimer in high-salt media. I. Association behavior of rod segments from myosin. *Biochemistry* **1972**, *11*, 1448−1455.

(10) Harrington, W. F.; Rodgers, M. E. Myosin. *Annu. Rev. Biochem.* **1984**, *53*, 35−73.

(11) Dill, K. A.; Shortle, D. Denatured states of proteins. *Annu. Rev. Biochem.* **1991**, *60*, 795−825.

(12) Goto, Y.; Fink, A. L. Conformational states of β-lactamase: Molten-globule states at acidic and alkaline pH with high salt. *Biochemistry* **1989**, *28*, 945−952.

(13) Goto, Y.; Calciano, L. J.; Fink, A. L. Acid-induced folding of proteins. *Proc. Natl. Acad. Sci.* **1990**, *87*, 573−577.

(14) Fink, A. L.; Calciano, L. J.; Goto, Y.; Kurotsu, T.; Palleros, D. R. Classification of acid denaturation of proteins: Intermediates and unfolded states. *Biochemistry* **1994**, *33*, 12504−12511.

(15) Kristinsson, H. G. Conformational and functional changes of hemoglobin and myosin induced by pH: Functional role in fish quality. Ph. D. dissertation. University of Massachusetts at Amherst.

(16) Lowey, S. Comparative study of the alpha-helical muscle proteins. *J. Biol. Chem.* **1965**, *240*, 2421−2427.

(17) Gershman, L. C.; Dreizen, P.; Stracher, A. Subunit structure of myosin II. Heavy and light alkali components. *Proc. Natl. Acad. Sci. U. S. A.* **1966**, *56*, 966−973.

(18) Gershman, L. C.; Stracher, A.; Dreizen, P. Subunit structure of myosin. *J. Biol. Chem.* **1969**, *244*, 2726−2736.

(19) Szuchet, S.; Zobel, C. R. The dissociation of myosin in acid. *Biochemistry* **1974**, *13*, 1482−1491.

(20) Kristinsson, H. G. Evaluation of different methods to isolate cod (*Gadus morhua*) muscle myosin. *J. Food Biochem.* **2001**, *25*, 249−256.

(21) Stefansson, G.; Hultin, H. O. On the solubility of cod muscle proteins in water. *J. Agric. Food Chem.* **1994**, *42*, 2656−2664.

(22) Lowry, Q. H.; Rosenbrough, N. J.; Farr, L. A.; Randall, R. J. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **1951**, *193*, 265−275.

(23) Haezebrouck, P.; Joniau, M.; Van Dael, H.; Hooke, S. D.; Woodruff, N. D.; Dobson, M. An equilibrium partially folded state of human lysozyme at low pH. *J. Mol. Biol.* **1995**, *246*, 382−387.

(24) Azem, A.; Kessel, M.; Goloubinoff, P. Characterization of a functional $GroEL_{14}(GroES_7)_2$ chaperonin hetero-oligomer. *Science* **1994**, *265*, 653−656.

(25) Lin, T. M.; Park, J. W. Solubility of salmon myosin as affected by conformational changes at various ionic strengths and pH. *J. Food Sci.* **1998**, *63*, 215−218.

(26) Perry, S. V. Myosin adenosine triphosphatase. *Methods Enzymol.* **1955**, *2*, 582−589.

(27) Ang, J. F.; Hultin, H. O. Denaturation of cod myosin during freezing after modification with formaldehyde. *J. Food Sci.* **1989**, *54*, 814−818.

(28) King, L.; Lehrer, S. S. Thermal unfolding of myosin rod and light meromyosin: Circular dichrosim and tryptophan fluorescence studies. *Biochemistry* **1989**, *28*, 3498−3502.

(29) Stracher, A. Effect of pH and urea on the optical rotation, viscosity, and adenosine triphosphatase activity of myosin A. *J. Biol. Chem.* **1961**, *236*, 2467−2471.

(30) Ptitsyn, O. B. Protein folding: hypotheses and experiments. *J. Protein Chem.* **1987**, *6*, 273−293.

(31) Hirose, M. Molten globule state of food proteins. *Trends Food Sci. Technol.* **1993**, *4*, 48−51.

(32) Gershman, L. C.; Dreizen, P. Relationship of structure to function in myosin. I. Subunit dissociation in concentrated salt solutions. *Biochemistry* **1970**, *9*, 1677−1687.

(33) Harrington, W. F. Contractile proteins of muscle. In *The Proteins*; H. Neurath and R. L. Hill, Eds.; Academic Press: New York, 1979; pp 245−409.

(34) Kato, S.; Konno, K. Isolation of carp myosin rod and its structural stability. *Nippon Suisan Gakkaishi* **1993**, *59*, 539−544.

(35) Chang, Y. C.; Ludescher, R. D. Tryptophan fluorescence quenching in rabbit skeletal myosin rod. *Biophys. Chem.* **1993**, *48*, 49−59.

(36) Bandman, E.; Arrizubieta, M.-J.; Wick, M.; Hattori, A.; Tablin, F.; Zhang, S.; Zhang, Q. Functional analysis of the chicken sarcomeric myosin rod: Regulation of dimerization, solubility, and fibrillogenesis. *Cell Struct. Funct.* **1997**, *22*, 131−137.

(37) Noelken, M.; Holtzer, A. M. The denaturation of paramyosin and tropomyosin by guanidine hydrochloride. In *Biochemistry of Muscle Contraction*; J. Gergely, Ed.; Little, Brown, and Co.: Boston, MA, 1964; pp 374−378.

(38) Lowey, S. Myosin substructure: isolation of a helical subunit from heavy meromyosin. *Science* **1964**, *597*, 145−147.

(39) Jaenicke, R. Folding and association of proteins. *Prog. Biophys. Mol. Biol.* **1987**, *49*, 117−237.

(40) Price, N. C. Conformational issues in the characterization of proteins. *Biotechnol. Appl. Biochem.* **2000**, *31*, 29−40.

(41) Kumar, S.; Ma, B.; Tsai, C.-J.; Sinha, N.; Nussinov, R. Folding and binding cascades: Dynamic landscapes and population shifts. *Protein Sci.* **2000**, *9*, 10−19.

(42) Kerwin, B.; Bandman, E. Assembly of avian skeletal muscle myosins: Evidence that homodimers of the heavy chain subunit are the thermodynamically stable form. *J. Cell Biol.* **1991**, *113*, 311−320.

(43) Nozais, A.; Bechet, J.-J. Unfolding/refolding studies of the myosin rod. *Eur. J. Biochem.* **1993**, *218*, 1049−1055.

(44) Harvey, S. C.; Cheung, H. C. Fluorescence depolarization studies on the flexibility of the myosin rod. *Biochemistry* **1977**, *16*, 5181−5187.

(45) Smyth, A. B.; Smith, D. M.; O'Neill, E. Disulfide bonds influence the heat-induced gel properties of chicken breast muscle myosin. *J. Food. Sci.* **1998**, *63*, 584−588.

(46) Frederiksen, D. W.; Holtzer, A. The substructure of the myosin molecule. Production and properties of the alkali subunits. *Biochemistry* **1968**, *7*, 3935−3940.

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