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Measurement of protein structure change in active muscle by hydrogen–tritium exchange

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Abstract

A hydrogen–tritium exchange method was developed to study protein structure changes at the molecular level in active muscle. Skinned rabbit psoas fibers mounted on a specially designed holder were selectively tritium labeled at peptide group NH sites that change from a highly protected form in rigor to an easily exchangeable, essentially random coil condition when muscle is activated. The number of sites found to show this behavior varies linearly with thick filament–thin filament overlap, and would correspond to 83 amino acids per myosin molecule in the muscle, although the experiments do not yet place these sites in any given protein. Half of the sensitive sites respond to relaxing conditions as well to activation.

Keywords: Hydrogen exchange; Muscle function; Force generation; Proteins, structure change

1. Introduction

Muscle contraction is a multimolecular process that converts chemical energy into mechanical force by way of interacting protein structure changes. The availability of molecular structures of some of the major muscle proteins [1–3] now makes it possible to consider the detailed protein structure changes that generate tension and movement.

The present work was motivated by the hypothesis of William F. Harrington [4,5] that force generation in muscle might involve a helix to coil transition in the subfragment-2 (S-2) region of the myosin molecule. Harrington postulated that "the S-2 link is helical in the resting state where it is bound to the thick filament surface. After attachment of the myosin head to a neighboring thin filament in a crossbridge cycle, the S-2 link swings away from the surface. A part of this structure, the force-generating element, is destabilized and undergoes a helix to coil transition" [5]. Melting of the helical force-generating element, proposed to be in the hinge region of the myosin rod, leads to a retractive force since the end to end distance of a polypeptide chain is shorter in the random coil than in the helical conformation.

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Although the myosin head alone is able to generate tension and movement [6–8], Harrington and his colleagues and others have found evidence that a helix to coil transition in the myosin hinge does occur during active contraction and may play an important role in muscle function. These studies generally covered three areas. (1) The stability and physical properties of the myosin rod showed a relative instability in the hinge region detected by differential scanning calorimetry [9,10], proteolytic sensitivity [11], optical rotation and light scattering studies [12], and electron microscopic investigations [13–17]. (2) Crosslinking [18–20] and proteolysis studies [21–23] showed that S-2 can be induced to move away from the thick filament core under conditions that correspond to the contractile cycle. (3) Enzyme probe studies showed that proteolytic cleavage of the hinge region is enhanced by 100-fold in active muscle [23,24], correlating with isometric tension and the fraction of active crossbridges. Studies using antibodies to the S-2 region of myosin have also suggested a role for S-2 in force generation but not in unloaded shortening in both myofibrils and single fibers [25–27].

To study protein structure changes in an insoluble multimolecular system, special methodologies are required. Here we demonstrate a method, based on hydrogen–tritium exchange (HX) of peptide group NH hydrogens, that is able to detect and localize protein structure changes in a working single muscle fiber. It has been universally observed that when proteins change their functional state, the exchange rate of some of their peptide group NH hydrogens changes [28]. Presumably these sensitive NH sites mark the parts of the molecule that participate in the structural change. One can take advantage of this differential rate property to selectively label, locate, and study the behavior of the particular protein segments that are actively engaged in the function studied [29]. One earlier attempt to detect protein structure changes in contracting muscle [30] by use of direct hydrogen–tritium exchange suffered from very high levels of tritium incorporation, making identification of specific functional elements impractical. The approach described here is designed to selectively label only functional elements.

In the present work, a single muscle fiber or fiber bundle was mounted on a small stainless steel wire using aluminum T-clips. The maneuverability of the holder allows the functional state of the muscle to be switched in seconds simply by moving the holder and its carried fiber between test tubes containing the requisite biochemical agents (ATP, Ca^{2+}). This capability was used to promote the selective tritium labeling of molecular sites involved in activation-related structure change. The number of sites measured would correspond to 83 NH hydrogens per myosin molecule in the muscle, although the localization of the sites to a given molecular type has not yet been accomplished.

2. Methods

2.1. Preparation of muscles

Young adult rabbits (ca. 2 kg) were anesthetized (ketamine at 50 mg and xylazine at 10 mg per kg body weight) and sacrificed. Psoas muscle strips were prepared and skinned [31,32]. Skinning solution contained 150 mM potassium propionate, 5 mM potassium phosphate, 5 mM magnesium acetate, 3 mM vanadium-free ATP, 5 mM ethylene glycol-bis(β-aminoethyl ether) N,N,N',N'-tetraacetic acid (EGTA), 1 mM D,L-dithiothreitol (DTT) and 0.2 mM sodium azide at pH 7.1 and 25°C. Muscle bundles (ca. 1 mm) were separated from the anterior lateral edge of the psoas, tied to sticks at their in vivo length, cut free from the muscle, placed in skinning solution at 0°C in a 100-ml beaker, and gently stirred on ice for 1 h. The solution was replaced by fresh skinning solution [containing 0.5% (w/v) Brij 58 and 0.1 mM phenylmethylsulfonyl fluoride (PMSF)] and allowed to stir gently on ice for an additional 1–1.5 h. Finally, each stick was transferred to a separate tube containing 15 ml skinning solution and stored at 0°C for up to 1 week until needed. The skinning solution was changed daily. In some early experiments, glycerinated psoas muscle, prepared as described previously [33] was used. No significant differences in results were obtained using glycerinated as compared to non-glycerinated fibers.

Experimental fibers were dissected according to the method of Yu and Brenner [31]. The length (ca. 1.1 cm) of each fiber and the diameter measured at

both ends were recorded. The average diameter of the 53 fibers used in these experiments was 0.08 ± 0.005 mm. Aluminum T-clips [34] were attached to the ends of a fiber or bundle of two fibers and the length of the fiber between the T-clips was measured. The fiber was mounted in a stainless steel holder (described below) and the sarcomere spacing was measured, as follows. The holder with its mounted fiber was suspended in a cuvette containing skinning solution. The fiber was illuminated with a 3 mW HeNe laser, and the position of the first order diffraction maximum was recorded at five different positions along the length of the fiber. From these numbers the average sarcomere length was calculated. Fibers with large variations in sarcomere length or poor diffraction patterns were rejected. To obtain longer sarcomere spacings, the aluminum T-clips were initially attached to a shorter fiber (< 1.1 cm) so that the fiber was stretched when mounted on the stainless steel holder. Fibers were then placed in either rigor or relaxing buffer, depending on the experiment, and stored at 0°C until needed.

2.2. Fiber holders

A major problem in early experiments was high tritium count background due to contamination by tritiated water inadvertently carried through the washing steps by the device used to mount the fiber. Any fiber holder using adhesives, fabricated of multiple pieces, or having a handle that extends above the solution meniscus was found to carry unacceptably high levels of tritiated water through the wash procedures. This problem was solved by designing a fiber mount from a single piece of stainless steel wire (Type 304, 0.3 mm diameter, ca. 5 cm long). The wire ends were electrochemically etched to a sharp point in fuming nitric acid to a diameter of ca. 0.08 mm at a position 3 mm from the tip, then bent as shown in Fig. 1 to form the fiber mount. The 11.5 mm opening allows for a 1 cm fiber with a 0.75 mm T-clip on each end. The unit can be transferred between tubes containing appropriate wash solutions using either a long stainless steel hook or fine stainless steel forceps. This design resulted in negligible levels of carried tritium.

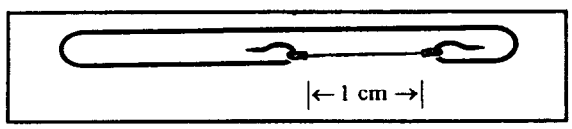

Fig. 1. Illustration of the stainless steel holder with a mounted fiber held by aluminum T-clips.

2.3. HX methods

The design of the stainless steel mount allowed rapid and clean transfer of the muscle fiber from one functional state to another in seconds. To accomplish this, small plastic microcentrifuge tubes (8 × 30 mm) were filled with 0.5 ml of the appropriate solutions and held at 22°C. The tritium-labeled muscle fiber was moved from tube to tube at predetermined time intervals. The bound tritium label exchanged out into the buffer in each test tube during this time period was determined by liquid scintillation counting. Initial exchange-in of the tritium label was done in a smaller tube (6 × 45 mm) with only 0.2 ml of buffer present to minimize the amount of high level tritiated water needed (20 mCi/0.2 ml). All tritium labeling and exchange-out was done at pH 6 and 22°C unless otherwise indicated.

2.4. Solutions used

Rigor buffer contained 124 mM propionic acid, 20 mM β-glycerophosphate, 0.1 mM dithiothreitol (DTT), 2 mM magnesium acetate, 5 mM 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid (BAPTA). Relaxing buffer contained 118 mM propionic acid, 20 mM β-glycerophosphate, 0.1 mM DTT, 3 mM magnesium acetate, 5 mM BAPTA and 1 mM ATP. Activating buffer contained 102 mM propionic acid, 20 mM β-glycerophosphate, 0.1 mM DTT, 3 mM magnesium acetate, 5 mM BAPTA, 1 mM ATP, 5.1 mM calcium acetate. The chelator BAPTA retains high selectivity for Ca over Mg but is markedly less sensitive to pH than EGTA [35,36]. Calcium rigor buffer was rigor buffer with 5.1 mM calcium acetate added. The double-pulse experiment (Fig. 5) employed rigor buffer with 1 mM sodium pyrophosphate added. All buffers were titrated to pH 6.0 with KOH.

2.5. Data analysis

Eq. 1 was used to translate the measured number of tritium counts into equivalent peptide group NH hydrogens per myosin molecule [29].

$$\mathrm{NH/myosin} = (111C/C_0)/(\mathrm{myosin/vol}) \qquad (1)$$

The number 111 is the molar concentration of hydrogen in the aqueous exchange-in solution. C is the total tritium counts released into the 0.5 ml of exchange-out buffer above the background of non-responsive hydrogens (see e.g. Fig. 2). C_0 is the tritium count level in the initial labeling solution obtained by diluting the exchange-in mix by a factor of 2×10^5 and counting 0.5 ml. Myosin refers to the moles of myosin present in the experimental fiber, calculated from the concentration of myosin in fibers (77 μM $\pm$ 10% [37]) and the diameter and pre-mounting length assuming a cylindrical cross-section. Vol is the volume (l) of solution that the fiber was placed in for exchange-out measurements and serves to convert the myosin in the fiber to its effective molarity in the experimental solutions.

All results were corrected for the overlap of thick and thin filaments (except for the zero overlap system) since only active cross bridges in the overlap region contribute to the signal (see below). The fraction of overlap was calculated as $(4 - \langle SL \rangle)/1.45$, where $\langle SL \rangle$ is the average sarcomere length. This assumes zero overlap at a sarcomere length of 4 μm and a thick filament bare zone length of 0.15 μm. For $\langle SL \rangle$ values less than 2.55, the fraction overlap was assumed to be 1.

2.6. Tension measurements

To ensure functional integrity of the fibers under our experimental conditions, isometric tensions were measured on control fibers prepared identically from the same psoas bundle. (Control experiments were also done at pH 7.3.) Tensions were measured using a Model 404 force transducer (Cambridge Technologies, Cambridge, MA). The measurement cell was thermostated with a circulating water bath and had dimensions $2 \times 2 \times 15$ mm. Sarcomere lengths were

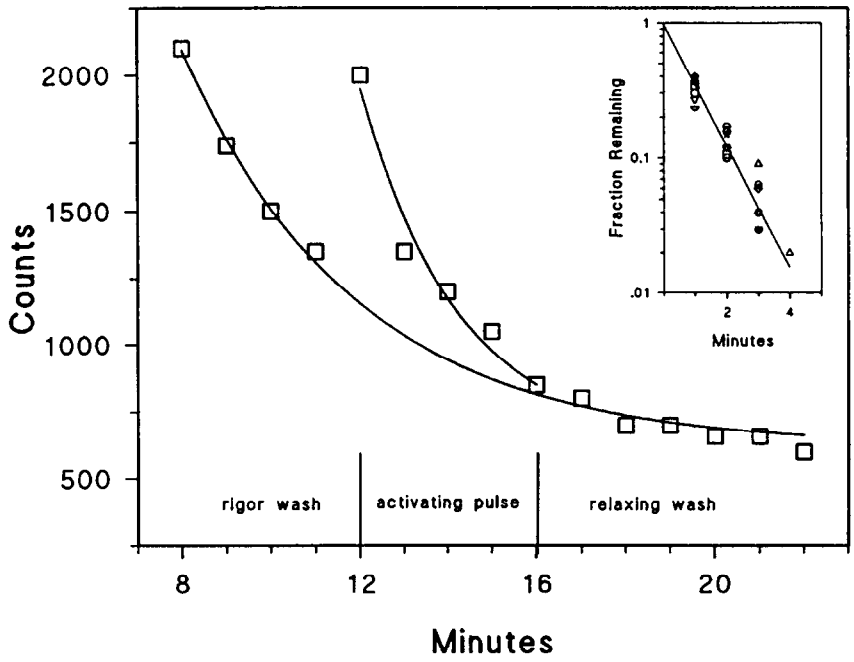

Fig. 2. Exchange-out of tritium label in a psoas muscle fiber initially labeled in the activated form. Data points represent total counts (10 min) measured in individual wash test tubes in the steps indicated. The inset displays the exchange-out behavior of the sensitive hydrogens in nine different fibers. When a fiber was used more than once the mean value is plotted.

adjusted to 2.6 μm using the first order diffraction maximum produced by illuminating the fiber with a HeNe laser. In a typical isometric tension measurement we found values of 132 and 148 kN/m^2 for two different fibers at 10 and 20°C both at pH 7.3. These are close to the values reported by Goldman et al. [38]; the differences are likely due to inaccuracies in our measurement of fiber diameter. We generally find that isometric tension at pH 6 is ca. 50–70% of that at the pH 7.3 value. This is comparable to the 45% decrease at pH 6 relative to pH 7 reported previously [39].

3. Results

To selectively label peptide group NH sites at positions involved in structure change, one can take advantage of the fact that these sites are fast exchanging in one protein form and slow in the other [29]. The present experiments were designed to selectively label and quantitate responsive hydrogens that exchange near the free peptide rate in activated muscle and much more slowly in the rigor state.

Individually mounted muscle fibers (Fig. 1) were exposed to tritium exchange labeling by equilibrating in the high-level tritiated buffer for a short period (usually 1 min) in the fast exchanging, activated form. Exchange-out of the tritium label was initiated by moving the muscle fiber into rigor buffer and passing it through a series of tritium-free rigor washes. This wash sequence removes tritium label that does not become locked into a much slower exchanging condition in rigor. The selectively labeled muscle can then be returned to the activating buffer to initiate contraction and fast exchange-out of responsive hydrogens.

Fig. 2 shows the results of a typical experiment in which a muscle fiber was initially labeled in the activated form. A fiber clipped to the holder was immersed in 0.2 ml activating buffer with 20 mCi tritiated water added. The exchange of tritium label into the muscle was allowed to proceed for 1 min. The exchanging peptide NH groups that become labeled with tritium during this period include functionally sensitive as well as insensitive hydrogens. To free the muscle of ATP and Ca^{2+} before passing the fiber through the washing sequence (rigor buffer containing no tritium), the tritiated fiber was placed in 0.2 ml rigor buffer with 20 mCi tritium added for 0.5 min. During this locking step, ATP and Ca^{2+} are washed out but the tritium label remains. In order to reduce the tritium label on insensitive sites to an acceptable level (< 1000 cpm/min), the fiber was then passed through a series of rigor buffer washes (0.5 ml of tritium-free rigor buffer in sequential test tubes). The tritium label on sites that are unaffected by structure change is lost at the same rate at which it exchanged-in, but the activation-sensitive NH groups exchange at a much lower rate in rigor. Thus the muscle fiber selectively retains tritium label on sites actively involved in the contraction process. The fiber was then placed in activating buffer. The tritium label that was locked in the slow form

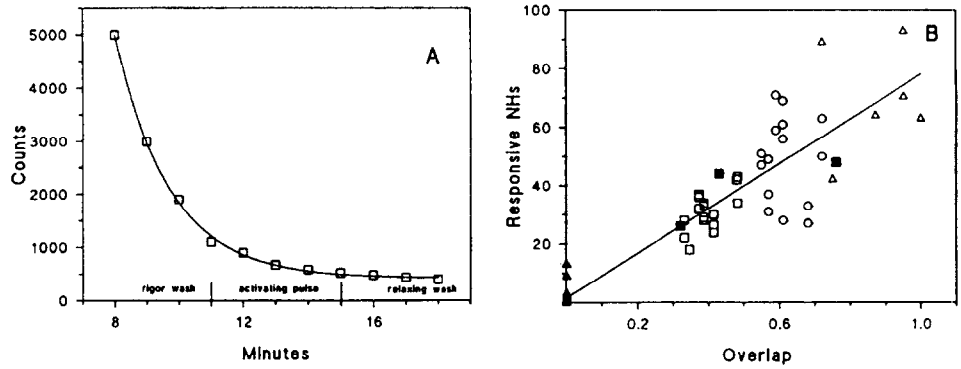

Fig. 3. The effect of thick filament–thin filament overlap. (A) Data for a fiber stretched to non-overlap. (B) Number of responsive NH sites as a function of fractional overlap (calculated on a per myosin molecule basis). Each set of symbols represents fibers prepared on the same day. For fibers used more than once the mean value is plotted.

switches back to the fast conformation, exchanges-out at the fast rate, and can be specifically captured and measured. The tritium label released from sites sensitive to activation was captured by successive 1-min washes in activating buffer. Finally the fiber was transferred through several changes of relaxing buffer to further delineate the background exchange-out level of insensitive hydrogens.

The inset in Fig. 2 shows data for nine different fibers that were labeled and exchanged-out as just described. Counts are plotted as the fraction of total activation-sensitive counts that has not yet exchanged-out. The halftime for exchange-out of the sensitive hydrogens in the fast form, activated state is 42 s.

The muscle fibers used here are held at constant length (Fig. 1), therefore at constant thick filament–thin filament overlap. Filament overlap, measured for each fiber as described in Materials and Methods, determines the fraction of cross bridges that participate in the myosin–actin interaction. When fibers stretched to the point of no filament overlap were passed through the standard protocol, activation of the fiber did not generate a pulse of tritium (Fig. 3A). This specifically rules out a significant contribution due to troponin C–Ca^{2+} interaction that is independent of the myosin–actin interaction. Fibers with differing filament overlap exhibited a linear relationship between the number of responsive sites measured and the fractional overlap (Fig. 3B). This suggests that the slow exchange–fast exchange transition signals an event in the crossbridge cycle. An alternative possibility is that molecules in the non-overlapped region experience the change equally but the tritium label there is not protected by rigor interactions and so is lost during the wash period. Against this interpretation is the fact that non-overlapped myosin does not actively hydrolyze ATP and also that Ca^{2+} alone has no effect (see below).

To obtain the true number of activation-sensitive sites, the tritium label measured in the activating pulse must be corrected for incomplete initial labeling during the 1-min, activated state exchange-in. Since exchange in the activated state has a halftime of 42 s (Fig. 2), only 63% of the total sites are labeled during the 1-min exchange-in. A correction is also necessary for the amount lost during the rigor wash. To determine the rate of loss of the sensitive

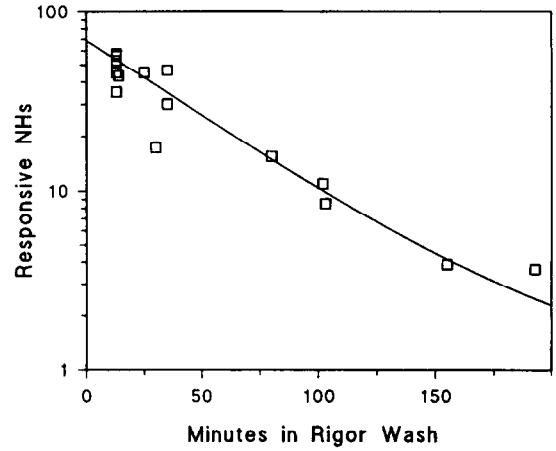

Fig. 4. Loss of the sensitive label as a function of time spent in the rigor wash. The label measured is expressed on a per myosin molecule basis.

label during the rigor buffer wash, fibers were labeled in activating buffer, locked in rigor as before, and washed for increasing time periods before the muscle was switched to the activated state. Results in Fig. 4 show that the halftime for exchange-out of the sensitive NH hydrogens in the slow exchanging rigor form is 40 min. Thus 20% of the sensitive label is lost during the standard 13-min rigor wash. The overall correction factor is then $(1/0.63) \times (1/0.8) = 1.98$. Longer or shorter wash times require modified corrections. All data were also corrected by normalizing to 100% overlap.

We have seen that a pulse of tritium is released when sites initially labeled during activation are again activated by exposure to Ca^{2+} plus ATP. Experiments were done to test the effects of ATP and Ca^{2+} separately. Fibers were labeled, locked, and washed as usual, and then exposed to 1 mM sodium pyrophosphate, which mimics ATP in that it dissociates the actomyosin complex but cannot support active contraction. Fig. 5 shows that this treatment causes a partial release of the tritium accumulated during activation. Subsequent exposure of the muscle to activating buffer (ATP and Ca^{2+}) elicits a second pulse of tritium. The insets in Fig. 5 show seven repetitions of this experiment. The pulse splits into two nearly equal portions. The measured exchange-out halftime of the two pulses, 43 s and 50 s, are in good agreement with the 42 s found for the

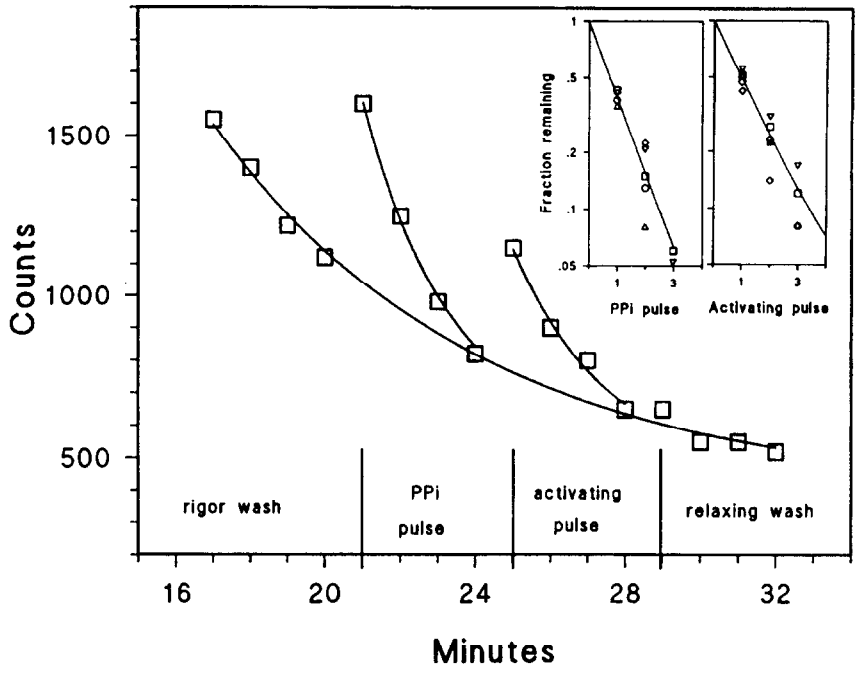

Fig. 5. Response of the sensitive NH sites to ATP analogue (PPi) alone and to ATP plus Ca^{2+}. The inset represents analogous results from five different fibers.

single activating pulse in Fig. 1, and the same total number of responsive NH sites are found as in ATP + Ca^{2+} excitation. When the order was re-

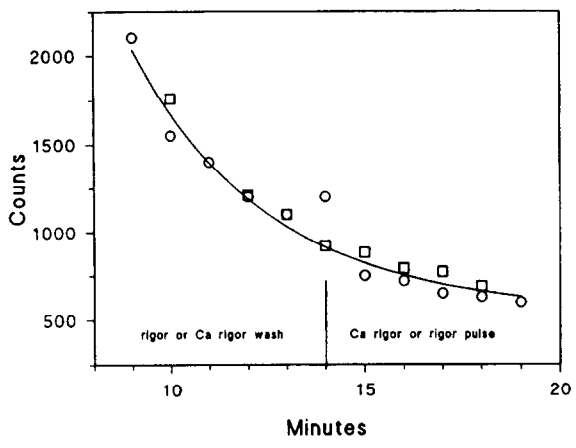

Fig. 6. Dependence of the responsive NH sites on Ca^{2+} alone. Initial labeling and final release of the label used either rigor buffer alone (○) or rigor buffer containing 5.1 mM Ca^{2+} (□).

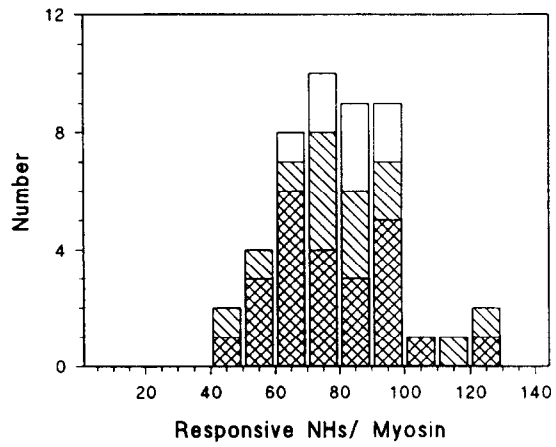

Fig. 7. Aggregate of 46 data sets from experiments like those in Figs. 2–5. In all cases the muscle fiber was labeled in activating buffer, then locked and exchanged-out in the rigor state before final activation. The cross-hatched areas denote experiments in which the initial labeling was done for 1 min at pH 6 and 22°C (as in Figs. 2–4). Back slashes represent experiments that used more intense initial labeling conditions (pH 7 or 36°C). Open bars represent the sum of the two pulses obtained on exposure to pyrophosphate–rigor and then activating conditions (as in Fig. 5).

versed so that the fiber was first exposed to ATP and Ca^{2+}, the entire pulse was released and further exposure to buffer containing ATP alone elicited no tritium release above background.

Fig. 6 shows experiments to test the effect of Ca^{2+} alone. Fibers were labeled in Ca^{2+} rigor buffer, then locked in tritiated rigor buffer and washed in the usual way (rigor buffer without Ca^{2+}). The final challenge in Ca^{2+} rigor buffer produced only a minimal pulse of tritium corresponding to four sensitive sites. Reciprocal experiments in which the presence or absence of Ca^{2+} was reversed similarly produced a negligible pulse of tritium.

Fig. 7 brings together 46 different data sets obtained as in Figs. 2–5. Results from the different kinds of experiments are indicated by different shading. Experiments are also included in which the exchange-in was modified to fully label the sensitive sites by raising the pH to 7 (10 times faster exchange) or the temperature to 36°C (5 times faster). The total number of sensitive NH/myosin was the same as that found for exchange-in at pH 6 and 22°C after appropriate correction for incomplete exchange-in, losses during the rigor wash, and incomplete overlap. The mean number of responsive NH/myosin found is 83 on a per myosin molecule basis.

4. Discussion

4.1. Experimental design

This work devised a hydrogen-exchange labeling method that can detect functionally related structure changes in actively contracting muscle. This study was inspired by the proposal of William F. Harrington that the tension generating event in muscle contraction involves a helix to coil transition in the hinge region of the myosin rod. Such a change in the hydrogen bonding state of a significant number of residues should be detectable by hydrogen-exchange labeling techniques [29] which exploit the fact that one can selectively incorporate and release tritium label in structured elements that experience the transition.

Experiments were designed to search for structure changes during isometric contraction that transiently generate random coil. The hydrogen exchange rate characteristic for non-hydrogen-bonded peptide NH groups is much faster than the hydrogen bonded rate. Accordingly labeling times were kept relatively short in order to label transiently generated random coil structure while minimizing non-specific labeling. The 1-min exchange labeling time used (pH 6, 22°C) is longer than the 1 s free peptide halftime characteristic of an unprotected Ala–Ala peptide NH and covers the range expected for some other more slowly exchanging amino acid sequence combinations [40–42]. We used the rigor state for the wash-out period since rigor can be expected to stabilize the slowly exchanging helical form in the Harrington model and thus conserve label at the targeted sites while non-specific background label is chased.

A crucial part of the experiment was the design of the fiber holder (Fig. 1). The count level in the initial labeling solution (C_0) is 4×10^{10} cpm/0.5 ml. The signal obtained from the activated muscle fiber after the wash period is less than 100 cpm/0.5 ml for each sample tube (Fig. 2). Thus the wash method must not only reduce background counts in the muscle itself to low levels but must also allow a thorough purge of extraneous tritiated water that may be carried on the mounting device. The mounting described here (Section 2.3 and Fig. 1) accomplishes this purpose.

4.2. Initial results and interpretation

The sensitive NH groups detected in these experiments switch from a condition with exchange halftime of 40 min in the rigor state to 42 s in active contraction. The measured 42 s halftime is somewhat slower than expected for the exchange of freely exposed peptide group NH [41,42], namely about 3 s for an average amino acid sequence at the experimental conditions used (pH 6, 22°C). However, muscle spends only a fraction of its time in each of the states that compose the contraction cycle. Recent in vitro studies [8,43,44] suggest that the force transient consumes ca. 10 ms compared to the overall cross-bridge cycle time of 120 ms (calculated from the pH 7, 20°C value and corrected to pH 6 according to Cooke et al. [39]). According to the Harrington model, the sensitive myosin S-2 region would then be expected to spend 1/12 of its time in the random coil form during active contraction, leading to an

exchange halftime of ca. 40 s, as was observed. Thus the sensitive NH sites found reveal a transition to the random coil state if it is assumed that the change occurs only during the contraction power stroke.

The data obtained measure 83 responsive peptide group NH hydrogens, corresponding to 1.9% of the number of amino acids in the myosin molecule. This is lower by up to a factor of three than the number expected from the calculations and experimental results considered by Harrington. It is possible that the number of NH sites measured underestimates the true number of amino acid residues in the active region, especially due to potential losses in bound label during the rigor wash step.

We have not yet been able to localize the responsive sites. Results from the filament overlap experiments, which show that the number of sensitive NH groups measured scales with the actin–myosin interaction, point to the actomyosin complex as the likely source. Other results independently tend to rule out the calcium-dependent regulatory proteins since calcium alone has no effect. Thus these experiments point to a transition to a near random coil in the active tension generating crossbridge structure, involving myosin and actin. It appears that the label measured arises from two separable components. Half is released when the fiber is either relaxed by the addition of MgPPi or activated by the addition of both Ca^{2+} and MgATP. The other half is made fast exchanging only when the fiber is activated.

5. Conclusions

Elucidation of the mechanism for force generation in active muscle and its regulation will require the determination of the molecular changes that lead to contraction. The hydrogen-exchange labeling method demonstrated here can detect, characterize, and perhaps localize protein structure changes in intact, functioning muscle. Initial experiments were designed to detect residues that change from a hydrogen bonded to a random coil conformation, as proposed in the helix–coil mechanism of Harrington. The model suggests that about 200–250 residues in the myosin S-2 region experience a helix to coil transition concomitant with contraction. Experiments done so far document a number of sites that show this behavior. The number of sites found would correspond to 83 residues per myosin molecule, although it should be stressed that the labeled residues remain to be located. The number of labeled hydrogens could be underestimated if some exchange in rigor at a somewhat faster rate than we used to calculate losses. About half of the peptide group NH sites measured become fast exchanging either upon calcium induced dissociation of the actomyosin complex or on active force generation.

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