The Mechanism of Force Generation in Myosin: A Disorder-to-Order Transition, Coupled to Internal Structural Changes

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ABSTRACT We propose a molecular mechanism of force generation in muscle, based primarily on site-specific spectroscopic probe studies of myosin heads in contracting muscle fibers and myofibrils. Electron paramagnetic resonance (EPR) and time-resolved phosphorescence anisotropy (TPA) of probes attached to SH1 (Cys 707, in the catalytic domain of the head) have consistently shown that most myosin heads in contracting muscle are dynamically disordered, undergoing large-amplitude rotations in the μs time range. Some of these disordered heads are bound to actin, especially in the early (weak-binding, preforce) phase of the ATPase cycle. The small ordered population (10–20%) is rigidly oriented precisely as in rigor, with no other distinct angle observed in contraction or in the presence of intermediate states trapped by nucleotide analogs. These results are not consistent with the classical model in which the entire head undergoes a 45° transition between two distinct orientations. Therefore, it has been proposed that the catalytic domain of the myosin head has only one stereospecific (rigor-like) actin-binding angle, and that the head's internal structure changes during force generation, causing the distal light-chain-binding domain to rotate. To test this model, we have performed EPR and TPA studies of probes attached to regulatory light chains (RLCs) in rabbit and scallop myofibrils and fibers. The RLC results confirm the predominance of dynamic (μs) rotational disorder in both relaxation and contraction, and show that the different mechanisms of calcium regulation in the two muscles produce different rotational dynamics. In rabbit myofibrils, RLC probes are more dynamically disordered than SH1 probes, especially in rigor and contraction, indicating that *the light-chain-binding domain undergoes rotational motions relative to the catalytic domain when myosin heads interact with actin.* An SH1-bound spin label, which is sensitive to myosin's internal dynamics, resolves three distinct conformations during contraction, and time-resolved EPR shows that these transitions are coupled to specific steps in the ATPase cycle. We propose that *force is generated during contraction by a disorder-to-order transition,* in which myosin heads first attach weakly to actin in a nonstereospecific mode characterized by large-scale dynamic disorder, then undergo at least *two conformational transitions* involving large-scale structural (rotational) changes within the head, culminating in a highly ordered strong-binding state that bears force.

INTRODUCTION

Spectroscopic probes have played an essential role in testing models of muscle contraction, by providing direct information about myosin head orientation and rotational motion. In most of these studies, probes have been attached to a reactive sulfhydryl group, SH1 (Cys 707), on the myosin head of rabbit skeletal muscle. Electron paramagnetic resonance (EPR) has played a central role in this field, because of its unequaled orientational resolution, combined with sensitivity to rotational motions from the ps to ms time scales. Early EPR experiments established a maleimide spin label (MSL) attached to SH1 as a useful probe of myosin head orientation (by conventional EPR; Thomas and Cooke, 1980) and μs rotational dynamics (by saturation transfer EPR (STEPR); Thomas et al., 1980). Two surprising results were obtained from these early studies. 1) Most of the heads in contracting myofibrils or fibers were found to be *dynamically disordered* in their axial orientation on the μs time scale (Thomas et al., 1980; Barnett and Thomas, 1989). This was later confirmed by time-resolved phosphorescence anisotropy (TPA) of phosphorescent dyes bound to SH1 in skinned fibers (Stein et al., 1990). 2) The small fraction (10–20%) of distinctly ordered heads in contraction was found to have precisely the *same orientation as in rigor* (Cooke et al., 1982; Fajer et al., 1990). These EPR observations did not seem consistent with the classical model for force generation in which heads undergo a 45° transition between two distinct orientations. Even intermediate states trapped by nucleotide analogs have not produced any new angles (Pate and Cooke, 1988; Fajer et al., 1988, 1991, 1995; Raucher and Fajer, 1994).

These EPR results suggested that either 1) the catalytic domain of the myosin head has only one mode of binding to actin, in a rigor-like orientation, while heads are dynamically disordered only when detached from actin; or 2) heads do have two modes of binding, one in which the catalytic domain is rigor-like, and another in which heads are dynamically disordered even when bound to actin (Huxley and Kress, 1985; Thomas, 1987).

To distinguish between these two models, we performed STEPR under conditions where the fraction of actin-bound heads was known, based on direct biochemical measurements in solution or in myofibrils. We showed that *actin-bound heads undergo microsecond rotational motion* during steady ATPase activity in solution (Berger et al., 1989) and

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in myofibrils (Berger and Thomas, 1993). In similar studies with nucleotide analogs in solution (Berger and Thomas, 1991) and in myofibrils (Berger and Thomas, 1994), we found that *actin-bound heads are mobile in weak-binding states* (simulated with ATPγS) but not in strong-binding states (simulated with AMPPNP). This led us to propose that force is produced by a *disorder-to-order transition*, in which heads bind initially to actin in a nonstereospecific, dynamically disordered, weak-binding state; later in the ATPase cycle, they increase their affinity to actin and assume a rigor-like (45°) orientation (Berger and Thomas, 1994; see Fig. 1). As shown in Fig. 1, this would be sufficient to produce force and a net rotation of about 45°, as long as the mean orientation in the disordered weak-binding states was centered about a 90° angle.

Despite the wealth of information provided by SH1-bound probes, it has become clear that definitive testing of these and other models requires probes attached to other sites on myosin. One reason for this is that SH1 blocking modifies the actomyosin ATPase cycle and decreases force (Crowder and Cooke, 1984; Svensson and Thomas, 1986), although the fundamental mechanism of force generation is probably not changed (Matta et al., 1992; Bell et al., 1995). A more serious limitation arises from the possibility that the SH1 site is not representative of the entire myosin head. Indeed, because specific orientational changes were not detected in active muscle with probes attached to SH1, it was proposed that the actin-bound catalytic domain of the myosin head does not change in orientation while the light-chain-binding domain rotates during contraction (Huxley and Kress, 1985; Cooke, 1986; Rayment et al., 1993a, b). Therefore, there is a clear need to probe the rotation of the light-chain-binding domain.

ROTATIONAL DYNAMICS OF LIGHT CHAIN PROBES

Conventional EPR studies of rabbit psoas fibers have generally found more disorder for regulatory light chain (RLC) labels than SH1 labels (Arata, 1990; Hambly et al., 1991), with no difference detectable between relaxation and contraction (Hambly et al., 1992; Roopnarine et al., 1994). Steady-state fluorescence polarization studies yielded similar results (Shrimpton et al., 1990). However, these studies do not provide any information on μs mobility or resolve different motional states. We have addressed this by using two different techniques, STEPR and TPA, which are both sensitive to μs rotational motion, and can thus be used to resolve different rotational states of proteins even in the presence of rotational disorder. We studied both scallop and rabbit skeletal muscle to compare the rotational dynamics of muscle systems with different mechanisms of calcium-dependent activation, based on calcium binding to myosin light chains in scallop, and to troponin C in rabbit. Moreover, in scallop muscle all of the endogenous light chains can be easily removed under mild conditions, eliminating the calcium sensitivity of ATPase activity, and the functional binding of labeled RLC can be demonstrated clearly from the resulting resensitization to calcium (Sellers et al., 1980).

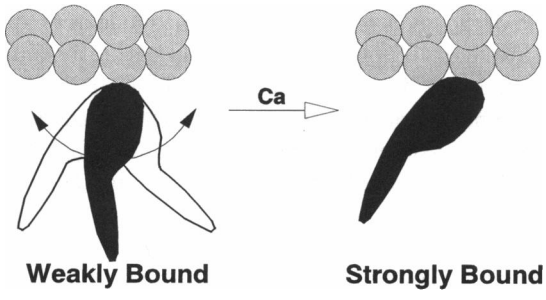

FIGURE 1 Disorder-to-order model (Berger and Thomas, 1994), in which force is generated by a transition from a weakly bound state, which is dynamically disordered and has a mean orientation of 90°, to a strongly bound state, which is highly ordered and rigor-like (45°).

In the EPR studies, an indane-dione spin label was used to label either rabbit RLC, which was exchanged into skinned rabbit psoas fibers, or clam (*Mercenaria*) RLC, which was exchanged into skinned scallop fibers (Roopnarine et al., 1994, 1995). While the conventional EPR spectra of both preparations showed so much disorder that little or no difference could be detected between rigor, relaxation, or contraction, STEPR showed clearly that relaxation and contraction have much more dynamic disorder than rigor in the μs time range (Roopnarine et al., 1994, 1995). Thus spin labels show that *ATP (relaxation or contraction) induces μs rotational motion in both the catalytic and light-chain-binding domains* of myosin in both vertebrate and invertebrate muscle. The STEPR studies showed that probes on the RLC in rabbit muscle were more rotationally mobile than both the RLC in scallop and the catalytic domain in rabbit (Roopnarine et al., 1995).

To obtain more detailed information about the rotational dynamics of the RLC in fibers, it is necessary to use a time-resolved spectroscopic technique. Therefore, we performed TPA experiments on phosphorescent-labeled RLC, exchanged into either rabbit or scallop myofibrils. Smooth-muscle (chicken gizzard) myosin RLC was used, because 1) it has a single cysteine to facilitate site-specific labeling with probes, and 2) it can be exchanged into either scallop or rabbit muscle, producing fibers or myofibrils with normal calcium-sensitive ATPase and tension. Rabbit myofibrils were prepared from psoas muscle stored in 50% glycerol at −20°C (Fajer et al., 1988; Roopnarine and Thomas, 1994b). Striated scallop (*Placopecten magellanicus*) myofibrils were prepared from muscle stored in 50% ethylene glycol at −20°C (Sellers et al., 1980). RLC was prepared from chicken gizzard (Sobieszek et al., 1975), then labeled with excess erythrosin iodoacetamide (ErIA) or eosin maleimide (EoM) overnight at 4°C, resulting in complete and specific labeling of the single Cys residue (Cys 108). Native RLC were removed by incubation in either 10 mM EDTA (scallop) or 10 mM CDTA (rabbit). Extracted troponin C from the latter preparation was replaced by overnight incubation with

1 mg/ml troponin C (provided by Dr. Albert Gordon, University of Washington, Seattle, WA) at 4°C. RLC-extracted myofibrils were incubated overnight with labeled RLCs. Gel analysis showed that labeled gizzard RLCs replaced 90–100% and 50–55% of the native RLC in scallop and rabbit myofibrils, respectively. Labeled RLCs were fully functional, as evidenced by complete restoration of Ca^{+2} sensitivity of myofibril ATPase activity.

TPA experiments were performed at 4°C with an instrument designed and built in our laboratory (Ludescher and Thomas, 1988). The sample was excited by vertically polarized light from an excimer-pumped dye laser at 530 nm. The vertically (I_{vv}) and horizontally (I_{vh}) polarized emitted light was detected by a gated photomultiplier tube (PMT), digitized, and signal averaged. The acquisition of a typical signal-averaged anisotropy decay required 5–10 min. Anisotropy was calculated as $r(t) = (I_{vv} - I_{vh})/(I_{vv} + 2I_{vh})$ and analyzed by least-squares fitting to determine rotational correlation times and amplitudes (Ludescher and Thomas, 1988). Solutions (150 mM ionic strength, pH 7) for relaxation and contraction included an ATP-regenerating system to ensure ATP saturation (Stein et al., 1990).

Fig. 2 shows the TPA results for ErIA-gizzard RLC in rabbit (*left*) and scallop (*right*) myofibrils, in rigor, relaxation, and contraction. In both systems, the highest anisotropy (lowest rotational mobility) was observed in rigor (*top curves*), and 5 mM ATP induced substantial microsecond decay (indicating large-amplitude rotational motion; i.e., dynamic disorder) in both relaxation (Fig. 2, *REL*) and contraction (Fig. 2, *CON*). There was no effect of Ca in rigor (not shown), but the addition of Ca to relaxed rabbit myofibrils, initiating contraction (Fig. 2 *left*, *CON*), decreased the residual anisotropy relative to relaxation, indicating decreased order (larger rotational amplitude). In scallop, an opposite effect of Ca was observed: the residual anisotropy was higher in contraction than in relaxation, indicating more order (smaller rotational amplitude) in contraction (Fig. 2, *right*). In addition, the anisotropy decays (rotational motions) in scallop were considerably slower than in rabbit. Another way to describe the difference between rabbit and scallop in Fig. 2 is that the two systems have quite similar RLC dynamics when myosin is detached from actin (relaxation, REL), *but in the actin-bound states of rigor (RIG) and contraction (CON), scallop shows much less motion (slower, smaller amplitude) than rabbit.*

We conclude that *there are substantial differences in myosin head rotational dynamics between scallop and rabbit muscle, especially in their response to calcium activation.* Since the same labeled light chains were used in both systems, these differences must be due either to 1) different interaction of RLC with the myosin head or 2) different rotational dynamics of the neck region of the myosin head. In the recently solved crystal structures of skeletal myosin S1 (Rayment et al., 1993a, b) and scallop regulatory domain (Xie et al., 1994), the scallop structure shows more complete light chain structures (suggesting more order), including the region of interaction between the essential and regulatory light chains (Xie et al., 1994). These observations in crystals, combined with the present TPA results in functional myofibrils, support the proposal that the light-chain-binding domain is more ordered in scallop than in rabbit, possibly because of more stable interactions of the light chains with each other and with the heavy chain.

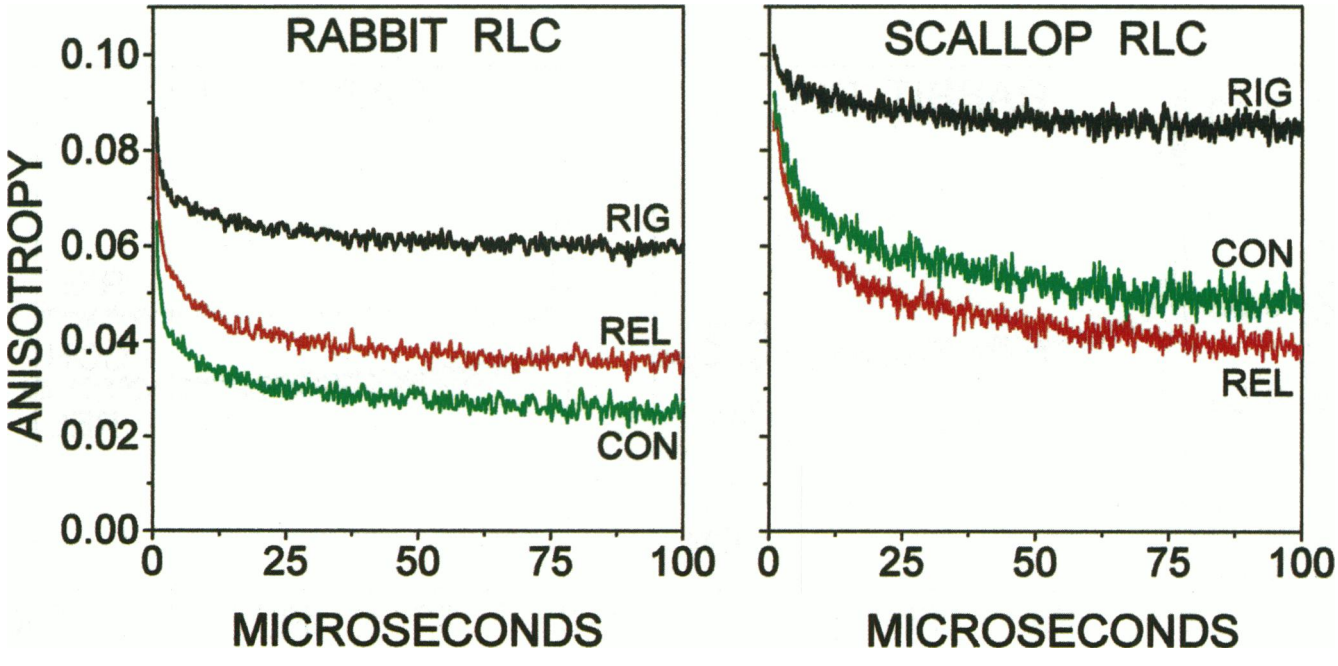

FIGURE 2 TPA decays at 4°C of ErIA bound to Cys 108 on gizzard RLC, which was bound to myosin heads in fully functional myofibrils prepared from rabbit psoas (*left*) or scallop (*right*) muscle, in rigor (*RIG*), relaxation (*REL*, rigor plus 5 mM ATP), and contraction (*CON*, relaxation plus 0.1 mM Ca^{2+}).

We also compared the motion of the RLC in rabbit muscle with that at SH1. Fig. 3 shows the results for rabbit myofibrils containing EoM-gizzard RLC (*left*) and myofibrils labeled at SH1 with the same dye (*right*). The results from EoM-gizzard RLC (Fig. 3, *left*) were very similar to those obtained from ErIA-gizzard RLC (Fig. 2, *left*), but the SH1 results (Fig. 3, *right*) were very different from the RLC results (Fig. 3, *left*), and these differences are illustrated schematically in Fig. 4. In all three physiological states, more motion (lower anisotropy in Fig. 3) was seen in the RLC than at SH1, indicating increased dynamic disorder (larger amplitude rotations in Fig. 4) in the light-chain-binding domain relative to the catalytic domain. The most dramatic differences between the two sites were seen upon activation by Ca, inducing the transition from relaxation (Fig. 3, *REL*) to contraction (Fig. 3, *CON*). While activation decreased the anisotropy (increased the amplitude of rotation) at the RLC (Fig. 3, left), it increased the anisotropy at SH1 (Fig. 3, *right*), consistent with previous studies of SH1 labels by EPR (Cooke et al., 1982; Barnett and Thomas, 1989; Roopnarine and Thomas, 1995) and TPA (Stein et al., 1990). These results support the proposal that *the RLC domain rotates relative to the catalytic domain during contraction* (Fig. 4). When troponin C was not added back to the RLC-labeled rabbit myofibrils, no change in the anisotropy decay was seen upon addition of Ca^{2+} (data not shown), showing that the effect of Ca in rabbit was linked to the thin filament regulatory apparatus, not to direct binding of Ca to myosin.

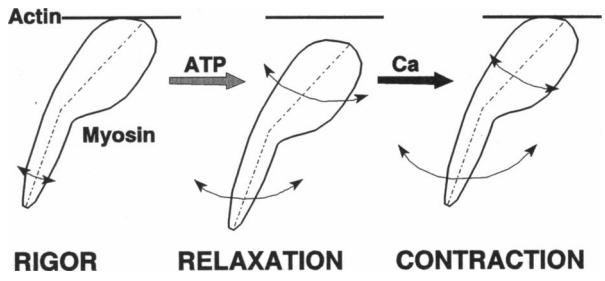

FIGURE 4 Illustration of the main conclusions from the TPA experiments on rabbit myofibrils (shown in Fig. 3). The arrows indicate the angular range of the μs rotational motion detected for the SH1-bound probe in the catalytic domain (*top*, nearest to actin) and the RLC-bound probe in the light-chain-binding domain (*bottom*, farthest from actin). In rigor (*left*) the amplitude of rotation was negligible at SH1, greater at the RLC. In relaxation (*center*), both domains had rotational amplitudes of ±40 to 50°. The addition of Ca to produce contraction (*right*) decreased the rotational amplitude in the catalytic domain but increased the amplitude in the RLC.

RESOLUTION OF MYOSIN CONFORMATIONAL TRANSITIONS WITH EPR

To probe in more detail the global and internal structural changes that are proposed to accompany the ATPase cycle in muscle (Rayment et al., 1993a, b), we have used two spin labels bound to SH1. InVSL, which resolves the global orientational distribution of the myosin head tilt relative to the fiber axis (Roopnarine and Thomas, 1994b, 1995), and IASL, which resolves three internal structural states of the myosin head, on the basis of changes in the ns rotational dynamics of the probe within the head (Barnett and Thomas, 1987; Ostap et al., 1993). The InVSL work showed even more clearly than the earlier observations (made with MSL; Cooke et al., 1982) that the axial orientations of heads in contraction are either dynamically disordered (80%) or oriented and rigor-like (20%). Quantitative analysis, using computer simulations, ruled out more rigorously the presence of either 1)

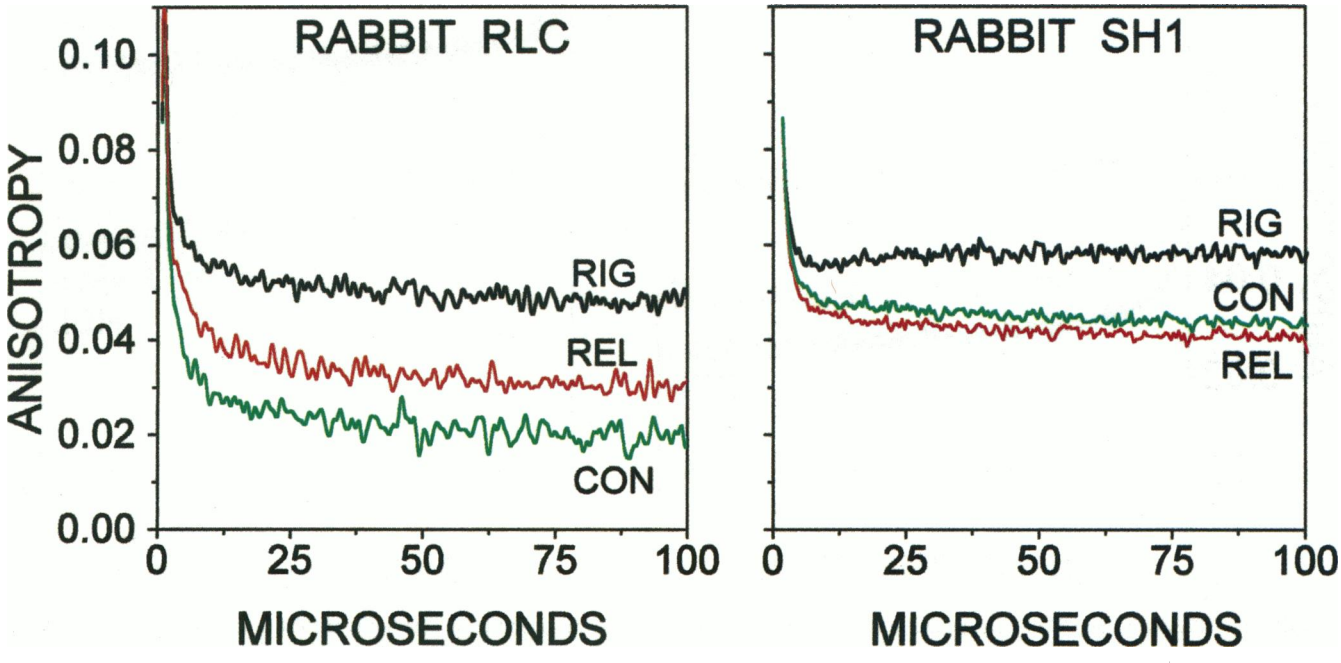

FIGURE 3 TPA decays at 4°C of rabbit myofibrils, labeled with EoM on gizzard RLC (*left*) or SH1 (Cys 707, *right*), in rigor (*RIG*), relaxation (*REL*, rigor plus 5 mM ATP), and contraction (*CON*, relaxation plus 0.1 mM Ca^{2+}).

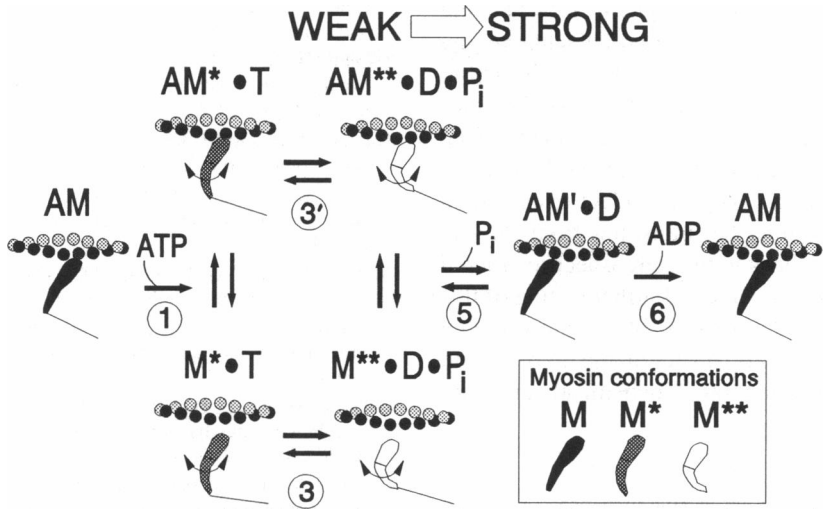

FIGURE 5 A schematic model of muscle contraction (Ostap et al., 1995) that incorporates the results discussed in this paper, in which the transition from weak to strong binding is accompanied by a transition from dynamic disorder (*curved arrows*) to rigor-like order, coupled to conformational changes (different head *shading* and *shape*) that are coupled to biochemical transitions. See text for further discussion.

an ordered population or 2) a disordered population of heads in contraction that has a mean orientation substantially different from that of rigor. This not only argues against the classic model of a 45-to-90° rotation; it also suggests that *the simple disorder-to-order transition of a rigid head is not sufficient to generate force*, because that would require a mean orientation different from that of rigor (see Fig. 1).

The three internal structural states (conformations) of the myosin head resolved by EPR of IASL in solution (Barnett and Thomas, 1987) are also detected in relaxed and contracting muscle fibers, where their signals can be resolved and quantitated to determine their relative populations in different physiological states (Ostap et al., 1995). Correlation with trapped intermediates and with rapid biochemical kinetics (Ostap et al., 1993) showed that these resolved conformations are identical to the (A)M, (A)M*.ATP, and (A)M**.ADP.P states (Fig. 5). This supports the model that the internal structure of the myosin head is changed only slightly upon ATP binding, and much more profoundly upon ATP hydrolysis (Rayment et al., 1995), and it shows that these structural changes occur in the contracting muscle fiber as well as in solution.

To obtain further resolution of the global and internal structural states of myosin during the ATPase cycle, we have carried out time-resolved EPR experiments using flash photolysis of caged ATP, in solutions of spin-labeled S1 and in spin-labeled fibers. The global orientational changes of the head, detected by transient EPR of InVSL (Roopnarine and Thomas, 1994a), show that *the ATP-induced transition from order to disorder precedes ATP hydrolysis*, confirming that weak-binding states are disordered very early in the ATPase cycle. The internal conformational changes detected by transient EPR of IASL (Ostap and Thomas, 1995) provide further support to the model that the two conformational transitions leading to force generation occur during the weak-binding phase of the ATPase cycle, in which heads are dynamically disordered, and force is generated by a subsequent reordering step (Fig. 5).

CONCLUSIONS

Taken together, our spectroscopic probe results support the following conclusions: 1) The catalytic domain of myosin, as probed by labels on SH1, has only one stereospecific orientation with which it binds to actin during muscle contraction. This rules out the classical model in which the entire head rotates between two distinct orientations on actin. 2) Most myosin heads in contracting muscle are dynamically disordered on the μs time scale, including actin-attached heads in weak-binding (preforce) cross-bridge states that are produced both before and after the ATP hydrolysis step. 3) The light-chain-binding domain of the myosin head, as probed by labels on the RLC, is even more dynamically disordered than the catalytic domain, especially in actin-bound cross-bridge states. Thus the light-chain-binding domain rotates relative to the catalytic domain during the actin-bound phase of the cross-bridge cycle. 4) The addition of Ca^{2+} to relaxed myofibrils has opposite effects on the dynamics of the RLC in scallop and rabbit muscle: the amplitude of μs rotation decreases in scallop but increases in rabbit. This indicates differences in the molecular dynamics of the neck region in response to activation, probably due to different activation mechanisms between scallop and skeletal muscle. 5) Two distinct internal structural changes within the myosin head, coupled to ATP binding and hydrolysis, occur in the dynamically disordered weak-binding (preforce) phase of the cross-bridge cycle in contracting muscle.

Therefore, we propose the mechanism of force generation depicted schematically in Fig. 5. Before ATP binds, myosin heads bind rigidly and stereospecifically to actin at an angle of about 45° (*far left*). (Step 1) ATP binding induces an

internal structural change within myosin (M*), weakening the interaction with actin, producing rapid detachment and reattachment, resulting in dynamic disorder of the entire actin-bound head, and causing rotational motion of the light-chain-binding region relative to the catalytic domain. (Step 3′) ATP hydrolysis (producing M**) accentuates the internal structural change of the head. (Step 5) Product release (or a step just preceding it) permits the transition from the dynamically disordered state back to the stereospecific ordered state, producing force and movement. Further testing of this model will require continued efforts to obtain time-resolved dynamic information from multiple sites on the myosin head and actin, coupled with structural, biochemical, and mechanical measurements.

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DISCUSSION

Session Chairperson: Ivan Rayment
Scribe: Alexander L. Friedman

IVAN RAYMENT: What's the binding constant? You've exchanged the light chains. Do they have the same binding constants for the corresponding heavy chains you've exchanged in the gizzard?

DAVID D. THOMAS: They're extremely tight. We know what the spectrum from the free light chain would be, and we're not seeing any of that. I think in fact it's been shown that the gizzard light chain, at least the unlabeled one, actually binds more tightly than the rabbit light chain to rabbit myosin. So we don't have a problem with that, and as I said, in the scallop we have very clear data that we've restored the calcium sensitivity. Also we do experiments in fibers where we flow solutions through and the light chains don't wash out.

KINOSITA: I think the difference in the phosphorescence anisotropy you have shown could be explained by a difference in local mobility in the ns time range. I guess you measured the prompt flourescence to exclude that possibility.

THOMAS: Yes, there is a difference in the ns mobility, which is revealed by the differences in initial anisotropies that we see. But it's not enough to account for the μs motions that we see.

KINOSITA: Basically your phosphorescence anisotropies are parallel to each other, all three curves.

THOMAS: Well actually, ATP induces larger amplitudes in the several-μs time domain. But . . . if you look at the rigor in the scallop and the rabbit, you can see that the scallop starts from a higher level and doesn't go down so far, indicating that the probe is held more rigidly in the scallop in rigor. You see a similar effect in contraction, which is to say, in scallop in both of the states where there should be interactions with actin, the light chain appears to be more rigidly held in scallop, more tightly coupled to the actin. I think that might actually be an important part of the regulatory mechanism, where this light chain must talk to the actin more directly than in the rabbit.

REISLER: I have a question that is related to Ivan [Rayment]'s question and maybe Susan [Lowy] can comment on that also. Is anything known about the binding of light chain to heavy chain in the presence of MgATP? The reason I am asking that question is because the original experiments that were done on stripping light chains required elevated temperature but also the presence of MgATP. So the question is whether there is any weakening of that interaction in the presence of MgATP?

THOMAS: There may be, but we can wash solutions through these fibers, say relaxing solution for 20, 30 minutes, and we don't lose any light chains, we don't lose any signal. There could be some loosening of the structure. That's possibly what we're seeing. That might be a way to explain the difference, for example, between the SH-1 and the light chain here. We're not necessarily looking at global domain movements. But we see the same motions for four different sites at opposite ends of this regulatory light chain, so we are observing the motion of the whole light chain.