Initiation of the power stroke in muscle: Insights from the phosphate analog AlF_4

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Motile forces in muscle are generated by the so-called “power stroke,” a series of structural changes in the actomyosin cross-bridge driven by hydrolysis of ATP. The initiation of this power stroke is closely related to phosphate release after ATP cleavage and to the change of the myosin head from weak, nonstereospecific actin attachment to strong, stereospecific binding. The exact sequence of events, however, is highly controversial but crucial for the mechanism of how ATP hydrolysis drives structural changes in the head domain of myosins and related NTPases like kinesins and small G proteins. Here, we show that the phosphate analogue AlF_4 can form two ADP-phosphate analog states, one with weak binding of myosin to actin and the other with strong binding of myosin to actin. Thus, change from weak to strong binding (i.e., the initiation of the power stroke) can occur before phosphate is released from the active site.

actomyosin cross-bridge | phosphate release | weak binding cross-bridge states | strong binding cross-bridge states | force generation

Muscle contraction results from cyclic interactions of the myosin head with actin filaments as ATP is hydrolyzed. Within each cycle, a multistep power stroke drives actin filaments several nanometers past the myosin filaments or generates motile forces when filament sliding is prevented. According to the concept of Lymn and Taylor (1), the power stroke is driven by the release of the ATP-hydrolysis products ADP and phosphate from the active site. From subsequent work, it was proposed that the initiation of the power stroke is closely related to the release of phosphate (2) and to the change of the myosin head from a conformation of weak, nonstereospecific actin binding to a conformation of strong, stereospecific actin attachment (3, 4). The exact sequence of events [i.e., how structural changes in the actomyosin cross-bridge are coupled to changes in actin affinity and to the release of phosphate from the active site] is still unclear. This coupling, however, is crucial for the mechanism of how ATP hydrolysis drives structural changes in the head domain not only of myosins but also of kinesins and small G proteins. Although it is clear that intermediates with MgADP and phosphate in the active site (ADP·P_i intermediates) are central to the initiation of the power stroke, it is still controversial whether, for example, phosphate release has to occur before the transition to the strong binding conformation (5, 6) or whether a strong binding ADP·P_i intermediate is formed before phosphate release. A second ADP·P_i intermediate has been postulated before (7–11), but its properties in terms of weak or strong actin-binding conformation remained unclear. One difficulty in characterizing the ADP·P_i intermediates is their transient nature during active cross-bridge cycling, specifically of an ADP·P_i intermediate postulated to bind strongly to actin before the release of phosphate (8–10). To elucidate events around phosphate release, we searched for approaches to accumulate myosin heads, with one and the same phosphate analog, in different ADP·P_i analog states. We selected AlF_4 as the phosphate analog because (i) a previously described ADP· AlF_4 analog state (12) (referred to hereafter as ADP· AlF_4 -I) showed properties characteristic of the weak binding conformation of the myosin head, consistent with the closed, pre-power-stroke

structure of the catalytic domain found by x-ray crystallography for this state (13) and (ii) AlF_4 allowed us to trap in demembrated, skinned fibers of rabbit psoas muscle essentially all cross-bridges in this ADP· AlF_4 -I state. In our attempt to form an ADP· AlF_4 analog of the postulated second, strong binding ADP·P_i intermediate, we exposed nucleotide-free skinned muscle fibers first to MgADP and then to MgADP plus AlF_4 . We hypothesized that the tight binding of AlF_4 may allow us to trap cross-bridges in a state equivalent to the postulated second ADP·P_i intermediate, which, presumably due to unfavorable equilibria, so far has never been accessible (e.g., by just adding MgADP plus inorganic phosphate to a nucleotide-free rigor fiber). By this strategy, we could eventually accumulate myosin heads in a second ADP· AlF_4 analog state, hereafter referred to as ADP· AlF_4 -II. Cross-bridges became trapped in this second intermediate, which was stable for several hours even after removal of AlF_4 and MgADP from the solution, and no interchange between the two ADP· AlF_4 intermediates was detectable. To classify the ADP· AlF_4 -II intermediate in terms of weak vs. strong actin-binding properties, we characterized kinetics and stereospecificity of its binding to the activated (high Ca^{2+} concentration; pCa 4.5) vs. inactive (low Ca^{2+} ; pCa 8–9) actin filament. This distinction between weak and strong binding properties is based on the fact that, in contrast to the weak binding conformations of myosin, attachment of myosin in a strong binding conformation to the activated thin filament is stereospecific and much stronger than binding to the inactive actin filament (for review, see ref. 14).

Materials and Methods

Fiber Preparation, Solutions, and Incubation with AlF_4 . Single chemically skinned fibers from rabbit psoas muscle were prepared as described in refs. 15–18. Solutions, including Mg rigor solution, were composed as described in refs. 17, 19, and 20. For all solutions, pH was adjusted to 7.0 and ionic strength was adjusted to 80 mM, except for experiments shown in Fig. 2. Chemicals were from Sigma, except where noted. Quick removal of MgATP for change to nucleotide-free rigor conditions was achieved by transient incubation in a “quick-rinse” solution containing EGTA and EDTA (20). MgATPγS (Mg-adenosine 5'-[γ-thio]triphosphate) solutions were prepared as described in refs. 20 and 21. Solutions for incubation with AlF_4 were prepared according to Chase *et al.* (12) with $\text{Al}(\text{NO}_3)_3$ and NaF. Ionic strength was adjusted by sodium propionate, and pH was adjusted by NaOH or HCl. The ADP· AlF_4 -I trapping solution contained 2 mM $\text{Al}(\text{NO}_3)_3$ and 10 mM NaF and was otherwise the same as the activating solution, including creatine phosphate and creatine kinase as ATP backup (17). The ADP· AlF_4 -II trapping solution contained 10 mM imidazole, 3 mM EGTA or CaEGTA, 2 mM MgCl_2 , 200 mM glucose, 0.25 mM diadenon-

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Abbreviations: ATPγS, adenosine 5'-[γ-thio]triphosphate; ALL, actin-based layer line; MLL, myosin-based layer line; 1.ALL, first ALL; 1.MLL, first MLL.

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sine-pentaphosphate, 5 mM MgADP, 0.1 unit/ml hexokinase, 5 mM $\text{Al}(\text{NO}_3)_3$, and 25 mM NaF. The ADP·AlF₄-II trapping solutions, including MgADP, glucose, and hexokinase but without $\text{Al}(\text{NO}_3)_3$ and NaF, were kept for at least 1 h at room temperature to allow degradation of contaminant MgATP. $\text{Al}(\text{NO}_3)_3$ and NaF were added, and pH was adjusted immediately before incubation of fibers. All solutions, except rigor solution for mechanical studies, contained 5 mM DTT, 10 mM glutathione (reduced), and 1,000 units/ml catalase as described in ref. 19.

For the ADP·AlF₄-I state, AlF₄ was trapped at 3°C by incubating fibers in ADP·AlF₄-I trapping solution (12). For the ADP·AlF₄-II state, fibers were first kept in quick-rinse and rigor solution as described in ref. 19 to remove contaminant ATP before incubation of the fibers in Mg rigor solution with 5 mM MgADP. Subsequently, trapping of AlF₄ was allowed in ADP·AlF₄-II trapping solution at 3°C at high or low Ca^{2+} concentration. Trapping was complete after ≈ 1 h, as judged from the disappearance of the lattice sampling on the first actin-based layer line (1. ALL) (Fig. 4*b*, asterisks) in 2D x-ray diffraction patterns. Change in Ca^{2+} concentration after trapping in the AlF₄-II state resulted in identical properties, as seen when directly trapping at high or low Ca^{2+} , provided that reequilibration was allowed for some 30–60 min.

Mechanical Measurements. Fiber stiffness was measured with ramp-shaped stretches of different speeds (3×10^{-1} to $1\text{--}2 \times 10^4$ nm per half-sarcomere·s⁻¹) with sarcomere length recorded by laser light diffraction (22). Stiffness was determined as the force increase for a sarcomere length increase of 2 nm per half-sarcomere (23, 24). Actively contracting fibers were stabilized by a release/restretch protocol (15).

X-Ray Diffraction. 2D x-ray diffraction patterns were recorded on Fuji imaging plates at station A2 of the Deutsches Elektronen Synchrotron (Hamburg, Germany) (Figs. 3 and 4*b*) and with a 2D multiwire detector at station 2.1 of the Synchrotron Radiation Source (Daresbury, U.K.) (Fig. 4*a*). The specimen-to-detector distance was 2.85 m, and arrays of some 30 single fibers, mounted side by side, were used (19, 20). Solutions and incubation procedures were the same as for mechanical experiments. To reduce damage by the x-ray irradiation, 1,000 units/ml catalase was added to solutions for x-ray diffraction, and fiber arrays were scanned vertically in the x-ray beam (19). Diffraction patterns were normalized to the intensity of the incoming beam, rotated, centered, and folded, and profiles in the horizontal and vertical direction were obtained for further analysis (19, 20).

Results

We characterized both ADP·AlF₄ intermediates in terms of weak vs. strong binding conformation first by kinetics of dissociation from actin. We previously showed that fiber stiffness, recorded by application of small stretches to one end of the fibers, is sensitive to the speed of these stretches (23). We further had demonstrated that dependence of fiber stiffness on speed of stretch is a measure for the probability of myosin dissociation from actin (24, 25). In this work, we show that fiber stiffness with myosin heads in the ADP·AlF₄-I state has essentially identical behavior as we found previously in the presence of MgATP γ S (Fig. 1*a* and *b*). At low Ca^{2+} (pCa 8) when actin filaments are inactive, fiber stiffness with MgATP γ S or with the ADP·AlF₄-I complex is very low, and only at the highest speeds of stretch is some increase in stiffness detectable (Fig. 1*a* and *b*, open symbols). At high Ca^{2+} (pCa 4.5; filled symbols), when myosin heads can also interact with tropomyosin-controlled binding sites on actin, increase in stiffness can already be seen at speeds of stretch >100 -fold slower than at low Ca^{2+} , and stiffness continues to increase with faster stretches. Thus, cross-bridges in the

Fig. 1. Fiber stiffness vs. speed of stretches applied to one end of fibers. (*a*) Results in the presence of 10 mM MgATP γ S. Circles, low Ca^{2+} ; triangles, high Ca^{2+} . Data of 11 single fibers normalized to rigor stiffness at fastest speed of stretch are shown. (*b*) Results in the ADP·AlF₄-I state. $n = 10$ fibers; symbols and normalization are as in *a*. (*c*) Results in the ADP·AlF₄-II state. $n = 6$ fibers. Open circles, low Ca^{2+} ; filled inverted triangles, high Ca^{2+} . For comparison, stiffness in the presence of MgADP at low (open upright triangles) and high (filled upright triangles) Ca^{2+} , without nucleotide (rigor, squares), and under relaxing conditions (filled circles) is shown. Data points are means $\pm$ SEM (error bars are often smaller than symbols). Lines are drawn to guide the eye. Experimental temperature was 3°C; results at 20°C are essentially identical (see Supporting Text).

ADP·AlF₄-I state have essentially identical dissociation kinetics from both active and inactive actin filaments as cross-bridges with MgATP γ S (21), a nucleotide analog for generating the weak binding conformation of myosin heads. In contrast, cross-bridges in the ADP·AlF₄-II state show different behavior (Fig. 1*c*). At low Ca^{2+} (Fig. 1, open circles), fiber stiffness is essentially the same as that of relaxed fibers (filled circles) or in the ADP·AlF₄-I state (Fig. 1*b*). At high Ca^{2+} (pCa 4.5; Fig. 1*c*, filled inverted triangles), however, even at the slowest stretches we can apply, stiffness is only $\approx 30\%$ less than for the fastest stretches. This stiffness–speed dependence indicates slow cross-bridge detachment, as is typical for a strong binding conformation of the myosin head. Stiffness of fibers with cross-bridges in the ADP·AlF₄-II state at high Ca^{2+} is only about half of the fiber stiffness in rigor or with MgADP (Fig. 1*c*, open squares and filled upright triangles), which might indicate that due to lower actin affinity of the ADP·AlF₄-II state compared with MgADP or rigor conditions, only about one of the two heads of each myosin molecule is attached to actin. That stiffness with ADP·AlF₄-II at high Ca^{2+} is less than rigor stiffness is very likely not due to incomplete thin filament activation. In muscle fibers, at saturating Ca^{2+} concentrations (pCa 4.5), rigor-like interactions make rather limited additional activation of thin filaments (cf. ref. 26). Also note that, in contrast to the ADP·AlF₄-II state, in the presence of MgADP or in rigor, the much higher fiber stiffness with small sensitivity to speed of stretch is also found at low Ca^{2+} (Fig. 1*c*, open vs. filled upright triangles). Thus, the ADP·AlF₄-II-myosin head behaves quite differently from the myosin head with MgADP or no nucleotide.

Fig. 3. 2D x-ray diffraction patterns of the two ADP·AIF₄ states. (a and b) Half of the 2D pattern is shown for each condition. Low Ca^{2+} is shown at the top; high Ca^{2+} is shown at the bottom. (a) ADP·AIF₄-I state. (b) ADP·AIF₄-II state. Total exposure time for patterns in a is 120 s; exposure time in b is 90 s. Experimental temperature is 20°C. For the ADP·AIF₄-II state, cross-bridge attachment to activated actin filaments ($+Ca^{2+}$) results in much weaker MLLs and some enhancement of the 5.9-nm ALL. (c and d) Integrated intensity profiles across the closely spaced 1. ALLs and 1. MLLs. The range of horizontal integration is indicated by the box in a. (c) ADP·AIF₄-I state. (d) ADP·AIF₄-II state. Profiles are normalized to maximum intensity at low calcium. Heavy solid line, high Ca^{2+} ; gray solid line, low Ca^{2+} ; thin solid line in d, nucleotide-free rigor state. Vertical dashed line in c indicates the position of the 1. ALL in rigor. Dashed and dotted lines in d show Gaussian functions fitted to the data in the presence of Ca^{2+} to separate the myosin and actin components, respectively. The actin component is 34% of the myosin component at high Ca^{2+} and 14% at low Ca^{2+} (fitted curves not shown). For the ADP·AIF₄-I state, the actin component is 6% and 9% for low and high Ca^{2+} , respectively (fitted curves not shown), presumably reflecting the baseline originating from the actin filaments alone.

a rigor pattern (Fig. 5, filled symbols). Thus, dissociation of AIF₄ from the ADP·AIF₄-I or ADP·AIF₄-II state is very slow and far from being complete within 1 h. Note that despite the apparently long lifetime of the two myosin·ADP·AIF₄ complexes, the two trapping procedures result in two distinct cross-bridge populations, myosin·ADP·AIF₄-I and myosin·ADP·AIF₄-II, with no detectable convergence of their actin-binding properties or structural features even when observed for up to 2 h.

Discussion

In summary, cross-bridges in the ADP·AIF₄-I state show all characteristic properties of the weak binding conformation of the myosin head, whereas cross-bridges in the ADP·AIF₄-II state assume a conformation with tighter and stereospecific binding to the activated thin filament. Assuming that the two ADP·AIF₄ analog states at least qualitatively reflect properties of two ADP·P_i intermediates of the ATPase cycle, our data imply that the structural elements of the myosin head can change from weak, nonstereospecific binding to a conformation of tight, stereospecific binding to the activated thin filament while phosphate is still in the active site. The very long lifetime of both

myosin·ADP·AIF₄ states seen when AIF₄ and ADP are removed from the bathing medium, together with the lack of detectable convergence of properties (e.g., toward a common diffraction pattern), imply very slow kinetics for interchange between the two ADP·AIF₄ states such that equilibration is too slow to be detected.

Accumulation of Cross-Bridges in Two Distinct Analog States. Because our two procedures to form ADP·AIF₄ intermediates yield two distinct populations of cross-bridge states, myosin·ADP·AIF₄-I vs. myosin·ADP·AIF₄-II, the two states obviously cannot be formed by binding of AIF₄ to the same intermediate of the cross-bridge cycle. It is at present not clear to which intermediate of the cross-bridge cycle AIF₄ binds when the ADP·AIF₄-I state is formed upon addition of AIF₄ to actively contracting fibers (see *Supporting Text*). Formation of the ADP·AIF₄-II state occurs when AIF₄ binds to an actin·myosin·ADP complex. Substantial binding of phosphate to a myosin·ADP complex or actin·myosin·ADP complex formed by addition of MgADP to a nucleotide-free rigor complex has not been found before in fibers (34) or in solution (35). It was argued that phosphate can

Fig. 4. 2D x-ray diffraction patterns of relaxed and actively contracting muscle and muscle in the presence of MgADP. (a) The top half shows relaxing conditions (2 mM MgATP and low Ca^{2+}); the bottom half shows active isometric contraction (2 mM MgATP and high Ca^{2+}). (b) Results for 5 mM MgADP and no AlF_4 at low and high Ca^{2+} . Total exposure time in a is 120 s; exposure time in b is 90 s. Experimental temperature for all patterns was 20°C. Patterns in a were recorded at beamline 2.1 of the Synchrotron Radiation Source with a 2D multiwire detector.

only bind to an A·M'·ADP state with higher free energy than the predominant A·M·ADP state formed by binding of MgADP to myosin heads in rigor. Thus, an unfavorable equilibrium for the A·M'·ADP state prevents substantial formation of an ADP·P_i intermediate upon addition of phosphate plus MgADP to nucleotide-free myosin heads. The tight binding of AIF₄ to the active site with the very slow dissociation, however, might allow AIF₄ to bind to this A·M'·ADP intermediate and trap, and thus accumulate, cross-bridges in a state equivalent to an A·M'·ADP·P_i state despite an unfavorable equilibrium for formation of the A·M'·ADP state when rigor fibers are exposed to MgADP.

Nevertheless, if both cross-bridge states to which AIF₄ can bind and form the ADP·AIF₄-I and ADP·AIF₄-II states are on the normal cross-bridge pathway, one expects presence of both intermediates during active cross-bridge cycling. Thus, in our first procedure to bind AIF₄ to the active site, at least some of the myosin heads should be trapped in the ADP·AIF₄-II state, depending on the steady-state distribution of the two cross-bridge states to which AIF₄ binds and depending on the kinetics

Fig. 5. Change in the intensity ratio 1.ALL/1.MLL as a signature of AlF_4 dissociation from the ADP- AlF_4 -I and ADP- AlF_4 -II states upon transfer to nucleotide- and AlF_4 -free Mg rigor solution. Intensities of 1.ALL and 1.MLL from profiles parallel to meridian are shown (for range, compare Fig. 3a). Intensities were obtained by fitting Gaussian functions to the data points with ORIGIN software (Microcal, Amherst, MA). Error bars are SE of parameters. For comparison of time courses at high (filled symbols) vs. low (open symbols) Ca^{2+} and the ADP- AlF_4 -I (circles) vs. ADP- AlF_4 -II (triangles) state, the 1.ALL/1.MLL ratio for the ADP- AlF_4 -I and ADP- AlF_4 -II states in the presence of AlF_4 were set to zero, and 1.ALL/1.MLL of the corresponding Mg rigor condition of each data set was set to 1.0. To reduce overlap in the graph, symbols for the ADP- AlF_4 -I and ADP- AlF_4 -II states are displaced sideways. X-ray patterns were recorded at 20°C; the temperature during the waiting period was 3°C.

of AlF_4 binding to these two states. When AlF_4 is added during active cross-bridge cycling, our data show that AlF_4 preferentially, but possibly not exclusively, accumulates cross-bridges in the $\text{ADP}\cdot\text{AlF}_4\text{-I}$ analog state. Similarly, when ADP is added to a rigor system, some of the myosin heads should be trapped in the $\text{ADP}\cdot\text{AlF}_4\text{-I}$ state. Our data show that in the second approach, AlF_4 preferentially accumulates cross-bridges in the $\text{ADP}\cdot\text{AlF}_4\text{-II}$ state. Although it is expected that for both conditions, eventually the same distribution of $\text{ADP}\cdot\text{AlF}_4\text{-I}$ and $\text{ADP}\cdot\text{AlF}_4\text{-II}$ states should be reached, no evidence for similar or the same distribution is seen experimentally. This observation, however, can be accounted for by very slow equilibration kinetics between the two intermediates in which AlF_4 is very tightly bound to the active site, as indicated by the very slow dissociation in solutions without AlF_4 and MgADP (compare Fig. 5). The obviously very slow interchange between the $\text{ADP}\cdot\text{AlF}_4\text{-I}$ and $\text{ADP}\cdot\text{AlF}_4\text{-II}$ states implies a high activation energy barrier between the two analog states, presumably due to tight binding of AlF_4 but to somewhat different locations in the active site. Protein crystallography of myosin so far has not revealed two different structures with AlF_4 , although for KIF1A, two distinct binding sites for phosphate analogs were described (36). In myosin, a second structure with AlF_4 may have escaped detection because (predominant) formation of the $\text{ADP}\cdot\text{AlF}_4\text{-II}$ intermediate may require binding of AlF_4 to the strong binding conformation of the myosin head with the cleft between upper and lower 50-kDa subdomains closed. This conformation is only formed when myosin is tightly bound to actin. So far, however, crystals can only be grown in the absence of actin.

Implications for Events at the Initiation of the Power Stroke. From the findings presented here, together with previous work, the following picture emerges for the events around the transition from weak to strong binding of myosin to actin at the initiation of the power stroke: Interaction of cross-bridges in weak binding states with the activated thin filament (i.e., including interaction with the tropomyosin-controlled binding sites on actin) destabilizes the nucleotide (21) or, after nucleotide cleavage, destabilizes the inorganic phosphate in the active site, as seen by the accelerated untrapping of AlF_4 from the $\text{ADP}\cdot\text{AlF}_4\text{-I}$ state at high Ca^{2+} in the presence of the weak binding analog

MgATP γ S.[†] These observations are accounted for if interactions of cross-bridges in their weak binding conformation with the activated thin filament tend to close the cleft between the upper and lower 50-kDa subdomains of the myosin head, which in turn is associated with opening of switch I (5, 6, 37). Opening of switch I, however, reduces the contribution of switch I to the coordination of phosphate, or of phosphate analogs in the active site, accounting for destabilization of phosphate or phosphate analogs. Destabilization upon cleft closure may then trigger rearrangement of the inorganic phosphate with change of the myosin head domain to the strong binding conformation as represented by the ADP·AlF₄-II state. In the presence of AlF₄ instead of P_i, this rearrangement with change to the strong binding conformation is very slow due to the much tighter binding of AlF₄ to the active site, such that we cannot detect it as an interchange from the ADP·AlF₄-I state to the ADP·AlF₄-II state. Nevertheless, this rearrangement of myosin from weak and nonstereospecific binding (ADP·AlF₄-I) to stereospecific and much tighter binding to the activated thin filament (ADP·AlF₄-II) reflects the initiation of the power stroke. The strong and stereospecific binding of the myosin head to actin seems to finally drive the release of phosphate. Whether upon tight and ste-

reospecific binding of the ADP·AlF₄-II state and, in analogy, upon tight binding of the second ADP·P_i intermediate the lever arm has already started to move from its pre-power-stroke (“tail up”) conformation toward the post-power-stroke (“tail down”) conformation remains to be clarified.

Rearrangement from weak and nonstereospecific to strong and stereospecific binding of myosin to actin at the initiation of the power stroke has previously been proposed to be the first contribution to force generation and movement (4). Whether and how much the rearrangement from a weakly bound myosin head to a strongly bound one can contribute to force generation or filament sliding compared with subsequent changes in the orientation of the light chain-binding domain (38), however, cannot be answered from the present study and will require further structural studies of the two ADP·AlF₄ intermediates.

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