Myosin Catalytic Domain Flexibility in MgADP†

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ABSTRACT: Conventional EPR studies of muscle fibers labeled with a novel α-iodoketo spin label at Cys-707 of the myosin head revealed substantial internal domain reorganization on the addition of ADP to rigor fibers. The spin probes that are well-ordered in the rigor state become disordered and form two distinct populations. These orientational changes do not correspond to rotation of the myosin catalytic domain as a whole because other probes (maleimide and iodoacetamide nitroxides attached to the same Cys-707 of myosin head) report only a small (5-10°) torsional rotation and little or no change in the tilt angle [Ajtai et al. (1992) Biochemistry 31, 207–17; Fajer (1994) Biophys. J. 66, 2039–50]. In the presence of ADP, the labeled domain becomes more flexible and executes large-amplitude microsecond motions, as measured by saturation-transfer EPR with rates (τr = 150 μs) intermediate between the rotations of detached (τr = 7 μs) and rigor heads (τr = 2500 μs). This finding contrasts with an absence of motion of the myosin head in ADP (τr = 2200 μs) as reported by the maleimide spin label. Our results imply that the myosin head in a single chemical state (AM-ADP) is capable of attaining many internal configurations, some of which are dynamic. The presence of these slow structural fluctuations might be related to the slow release of the hydrolysis products of actomyosin ATPase.

The cyclic interaction of myosin and actin filaments forms the basis for muscle contraction (Huxley, 1969, 1974). The sequence of structural events beginning with the attachment of myosin heads to actin, followed by a structural change in the actomyosin complex, and subsequent detachment of the heads allows for force generation and length change of the cell. This mechanical cycle is driven by the chemical cycle of actomyosin ATPase. Actin and myosin form a strong complex in the absence of nucleotides, which can be dissociated by the binding of MgATP† to a site on the myosin head. The MgATP is subsequently cleaved by myosin, and the products of this hydrolysis are released after the myosin head has rebound to actin. It is natural to assume that each of the chemical states might induce a different structure of the actomyosin complex, and much effort has been devoted to the structural characterization of muscle proteins in different chemical states. A recent determination of the atomic structure of actin (Kabsch et al., 1990) and myosin (Raymont et al., 1993a) and subsequent “docking” experiments in which actin and the myosin head were brought together to fit a 3D reconstruction of the complex from electron microscopy have suggested large shape changes of the head that might occur on nucleotide and actin binding (Raymont et al., 1993b). Such changes have been observed by a comparison of the length of the myosin head in rigor and ADP using low-angle X-ray scattering (Wakabayashi et al., 1992) and electrical birefringence (Highsmith & Eden, 1992). ADP is known to induce changes in optical probes (IATR dichroism) (Borejdo et al., 1982; Burghardt et al., 1983) which most probably correspond to local changes in the head internal structure, because EPR has suggested that the head does not substantially reorient as a rigid body (Fajer et al., 1990; Fajer, 1994a).

Internal fluctuations of myosin heads induced by nucleotide binding have been inferred previously from changes of sulphydryl reactivity (Burke & Reisler, 1977; Schaub et al., 1975), cross-linking studies (Wells & Yount, 1979; Lu & Wong, 1989), NMR (Shriver & Sykes, 1982), and EPR (Barnett & Thomas, 1987). Most of these studies were confined to isolated myosin in solution and were not suited to resolving or observing the heterogeneity of the structures within a complex of myosin and nucleotide.

In this paper, we report on local domain changes induced by ADP in muscle fibers labeled with a novel iodoketo spin label [IKSL: 3-(2-iodopropionyl)-1-oxyl-2,5-dihydro-2,2,5,5-tetramethyl-1H-pyrroline]. The observation of two distinct populations while attached to the actin filament strongly implies that a multitude of local structures correspond to a single chemical state. Furthermore, these conformations are not static as in rigor; there is considerable domain mobility in the 100–200-μs range. Because MSL, which is more sensitive to global reorientation of the head and attached at the same Cys-707, does not sense these motions, we believe that these motions correspond to nucleotide-induced slow structural fluctuations that might be important in the release...
of products during the actomyosin ATPase cycle. The presence of different conformational substates in myoglobin has been linked to different reaction rates (Hong et al., 1990; Frauenfelder et al., 1991).

EXPERIMENTAL SECTION

Muscle fibers from rabbit psoas were harvested and glycerinated as described by Fajer et al. (1988). The glycerinated fibers were dissected into 0.5-mm bundles and washed with labeling buffer, LB (60 mM KAc, 20 mM MOPS, 5 mM MgCl₂, 1 mM EGTA, 1 mM Na₃H, pH 6.5). In order to block cysteines other than Cys-707 of myosin, we pretreated the fibers in rigor with 120 μM DTNB (reversible sulfhydryl reagent) in LB for 120 min at pH 8.0 (Naber and Cooke, private communication). The labeling with 0.25 mM IKSL (α-iodopropionyl)pyrrole spin label or MSL [N-(1-oxy-2,2,6,6-tetramethylpiperidin-4-yl)maleimide] was accomplished in LB at pH 6.5 in the presence of 5 mM MgATP, which detaches the myosin heads increasing the reactivity of Cys-707. Incubation time was 180 min for IKSL and 20 min for MSL. After labeling, the TNB blocking groups were removed by repeated thiolysis with 30 mM DTT in the presence of 5 mM MgATP at 20 °C for 30 min. The extent of labeling as estimated from the integrated intensity of the EPR signal and protein determination was 0.55 spin label per myosin head. The efficiency of TNB thiolysis was 80% as determined colorimetrically.

The rigor buffer (RB) consisted of 130 mM KPr, 2 mM MgCl₂, 1 mM EGTA, 20 mM MOPS, and 1 mM Na₃H, pH 7.0. Relaxation was achieved by the addition of 5 mM MgATP to the rigor solution. ADP solution was made from 5 mM MgADP plus 100 μM ATP. ADP solution was made from 5 mM MgATP to the rigor solution. ADP solution was made from 5 mM MgADP plus 100 μM ATP. All chemicals were obtained from Sigma Chemical Co. (St. Louis, MO).

Myosin subfragment 1 (S1) was prepared from myosin according to the method of Weeds and Taylor (1975) and was labeled as described by Fajer et al. (1988). Labeled S1 was covalently attached to isothiocyanate glass beads (Machleidt & Wachter, 1977). The beads were incubated overnight with labeled S1 at an equimolar ratio of protein and isothiocyanate in S1 buffer, 120 mM KCl, 10 mM MOPS, and 1 mM EDTA, pH 7.0. The unbound S1 was removed by repeated washes. Fiber decoration experiments were performed by a 20-min infusion of 10 mg/mL. IKSL-S1 in RB, followed by the washout of unbound molecules with rigor solution.

EPR and ST-EPR spectra were obtained as previously described (Raucher & Fajer, 1994) with a Bruker ECS 106 spectrometer (Bruker Instruments, Billerica, MA). Conventional spectra of fibers parallel to the magnetic field were obtained in a modified TM₁₀₀ cavity; ST-EPR spectra of samples perpendicular to the field were collected in a TE₁₀₂ cavity. The experiments were performed at 20 °C. Effective rotational correlation times (τₑ) were obtained by comparison to spectra of hemoglobin tumbling in media of known viscosity (Thomas et al., 1976). This comparison was accomplished on normalized integrated intensity of ST-EPR spectra which circumvents orientational effects in the spectra (Squire & Thomas, 1988).

Synthesis of α-Iodoketone Spin Label. A new spin label has been designed to achieve the specificity of the commonly used iodoacetamide spin label (IASL) but with steric restrictions of the IASL librational motion. The spacer between the cysteine reacting group (iodine) and the nitroxide has been reduced by one bond length; the methyl group was introduced next to the protein attachment point hindering cis-trans isomerization of S—C bond, and finally the six-membered piperidine ring of nitroxide was substituted with the five-membered pyrrole ring, which is more rigid (Hideg & Hankovszky, 1989). This iodoketone spin label is more reactive than the corresponding iodoacetamide since the NH group neutralizes the electron-withdrawing effect of the spin label (see Figure 1).

The synthesis was accomplished as follows: to a solution of 3-(2-bromopropionyl)-2,5-dihydro-2,2,5,5-tetramethyl-1H-pyrrol-1-yloxy radical (1.0 mmol, 275.2 mg) in dry acetone (50 mL), NaI (2.0 mmol, 300.0 mg) was added and refluxed for 30 min (Hankovszky et al., 1990). The mixture was then diluted with water, extracted with chloroform, dried (Na₂SO₄), and evaporated to dryness. The solid yellow residue was purified by preparative TLC with hexane-EtOAc (4:1) on Merck Silica Gel 60 F-254 plates to give 273.8 mg (85%) of 3-(2-iodopropionyl)-2,5-dihydro-2,2,5,5-tetramethyl-1H-pyrrol-1-yloxy radical as yellow crystals, mp 110–112 °C. Anal. Calcd for [C₁₁H₁₁I₂N₂O₅ (322.17)]: C, 41.01; H, 5.32; N, 4.30; I, 39.39%. Found: C, 40.89; H, 5.22; N, 4.28; I, 39.30%.

RESULTS

Orientation. The spectra of IKSL-labeled fibers in rigor and relaxation (Figure 2) display the same characteristic features as the spectra of fibers labeled with MSL, implying a high degree of order in rigor and dynamic relaxation in ATP. In rigor, the spectra show three sharp resonances due to interaction with the ¹⁴N nucleus of the nitroxide. The splitting of the resonances, 2₁ₑₑ = 17.9 ± 0.2 G, is similar to that of MSL-labeled fibers (18.0 ± 0.2 G), suggesting that the orientation of the z-axis of the nitroxide is similar for the two labels [MSL spectra have been extensively published; e.g., Thomas and Cooke (1980), Fajer et al. (1991), and Raucher et al. (1994)]. The width of the low-field resonance at half-height is 3.9 ± 0.2 versus 3.6 ± 0.2 G for MSL, because of the high order of the labeled domains in rigor. The addition of ATP detaches the actin-bound heads, and the ensuing spectrum has a characteristic, nearly isotropic line shape indicating large head disorder (Thomas & Cooke, 1980; Thomas et al., 1980). The similarity of the reported changes is not coincidental since both MSL and IKSL are covalently bound to the same site (Cys-707) on the myosin head. The identification of the IKSL-modified
site was accomplished by competition with SH1-specific probes: IASL, IAA, and FDNB (Raucher et al., 1994).

The spectral line shape in the presence of 4 mM MgADP is significantly different from the rigor or relaxed spectra. There is substantial spectral intensity at both P1 (disordered peak) and P2 (ordered peak), implying that a fraction of the probes is ordered at an average angle similar to that observed in rigor, while the remainder of the probes are disordered. These populations are, however, not identical to rigor and relaxation. The ADP spectrum cannot be simulated by a linear composition of relaxed and rigor spectra. A close inspection of the P1 peak (Figure 2, upper inset) reveals that the ordered population is distinct from the rigor orientation. Likewise, a comparison of the P1 regions of the disordered component and the relaxed spectrum in the lower inset of Figure 2 shows the differences between the disordered fraction in ADP and the relaxed heads.

Because disordering of the heads is often associated with detachment, e.g., PP, and AMPPNP (Pate & Cooke, 1988; Fajer et al., 1988), we have measured the detachment of IKSL-labeled S1 decorating unlabeled fibers. As for any other label, the spectrum of decorated IKSL-S1 in the absence of any nucleotide is identical to that of intrinsically labeled fibers in rigor (data not shown). The addition of ADP to decorated fibers resulted in changes identical to those in intrinsically labeled fibers (Figure 3, inset). Importantly, subsequent perfusion of the fiber with ADP solution resulted in little change of total intensity, indicating that the labeled heads remained bound in ADP. Over a period of 35 min, only 10% of the spins were lost to detachment and washed out from the muscle fiber (Figure 3). The myosin heads were not trapped with the fiber as the addition of 5 mM MgATP washed out all but 15% of the labeled S1 from the fiber within a 10-min period.

Therefore, the difference in the line shape at P1, along with the strong binding of all of the heads in the presence of ADP implies that the labeled domain of the attached myosin heads is undergoing significant disordering. This behavior can be contrasted with that of the MSL- and IASL-labeled heads; in both cases a high degree (±8°) of order is maintained in ADP (Fajer et al., 1990; Fajer, 1994a). The difference is not due to possible structural changes induced by the iodoketo label since both the tension, steady-state ATPase, and the kinetic rates of the cycle were not affected by labeling (Raucher et al., 1994). Thus, we conclude that the IKSL label is sensitive to local disordering of the immediate probe environment, whereas the other labels report the global behavior of the head.

Mobility. The saturation-transfer EPR spectra in Figure 4 demonstrate that the disordering of the IKSL probes is accompanied by the onset of rotational motion. The spectra in ADP are intermediate between the immobilized rigor heads, correlation time \( \tau = 2-3 \) ms, and the rapidly rotating relaxed heads, \( \tau = 3-10 \mu s \) (Table 1) (calculated from the integrated intensities which avoids orientational effects in ST-EPR spectra). Because neither of the two distinct orientational populations is rigor- or relaxed-like, no rigorous attempt was made to assign any particular mobility to the ordered and disordered fractions. However, if we equate the conventional line shapes of the ordered and disordered fractions to rigor and relaxation respectively, then we can estimate the disordered fraction to be 60–70 mol %; similar analysis of ST-EPR spectra indicates the mobile (relaxed-like) fraction to be 40 mol %. The difference between the orientational and motional values points to a difference between the mobility of the disordered fraction and that of the relaxed heads. Because the heads in the ordered fraction are unlikely to be more immobilized than the rigor heads, the mobility of the disordered heads must be lower than that of the heads in the presence of ATP.

In contrast to IKSL, MSL-labeled fibers in the presence of ADP show no change compared to rigor spectra (Figure 4), which restricts the global movement of the head to the millisecond time range. At least parts of the head or the myosin head as a whole is very immobilized.

The question arises whether the disordering and the flexibility arise from librational motion of the label with respect to the protein. A standard method of assessing librational motions is to immobilize the protein on glass beads and look at the mobility of the label, as was done for the myosin head (Bottomley & Trayer, 1980; Thomas et al., 1980), myosin light chains (Hambly & Cooke, 1990), troponin C (Li & Fajer, 1994), and tropomyosin (Szczezysna and Fajer, private communication). The conventional EPR...
FIGURE 4: Saturation-transfer EPR spectra of IKSL fibers (left) and MSL-labeled fibers (right). Top: In the absence of nucleotides, rigor; middle: in 4 mM ADP; bottom: relaxed with 5 mM ATP. Second harmonic, out-of-phase spectra (dotted) and their integrals (solid). The height of the arrows denotes relative restriction on mobility in each state.

Table 1: Rotational Correlation Times ($\tau$) of IKSL- and MSL-Labeled Fibers

<table>
<thead>
<tr>
<th>Sample</th>
<th>IKSL (μs)</th>
<th>MSL (μs)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rigor</td>
<td>2000–3000</td>
<td>2000–3000</td>
</tr>
<tr>
<td>ADP</td>
<td>100–200</td>
<td>1800–2800</td>
</tr>
<tr>
<td>ATP</td>
<td>5–10</td>
<td>3–8</td>
</tr>
</tbody>
</table>

$\tau$, in microseconds.

DISCUSSION

Conventional EPR spectroscopy of IKSL-labeled myosin in skinned muscle fibers revealed significant ADP-induced local changes in both orientation and mobility of the domain containing Cys-707. The addition of ADP to rigor fibers induced the appearance of a bimodal orientational population with components of varying degrees of order, intermediate between rigor and relaxation. The more-ordered component was similar to that observed in rigor but had a wider orientational distribution; the disordered component was more ordered than the distribution observed for relaxed fibers. The mobility of the labeled domain as measured by ST-EPR was also intermediate between the microsecond mobility of relaxation and the millisecond mobility of rigor. The observed changes are consistent with a number of findings implying structural changes on the binding of ADP to the myosin head in solution. Our findings extend the presence of these structural changes to heads interacting with actin in muscle fibers and characterize these changes in terms of the orientation and mobility of the labeled domain.

Other Studies. In solution, the binding of ADP to myosin heads causes a number of well-documented changes [re-
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FIGURE 5: Conventional EPR spectra (left) and ST-EPR of IKSL-S1 covalently bound to glass beads. Top: in the absence of nucleotides, rigor; middle: 4 mM ADP; bottom: 5 mM ATP.

Table 2: Mobility of IKSL-S1 Bound to Glass Beads

<table>
<thead>
<tr>
<th>sample</th>
<th>STEPR intensity* (%)</th>
<th>L''/L*</th>
<th>H''/H*</th>
<th>2Teff (G)</th>
</tr>
</thead>
<tbody>
<tr>
<td>no nucleotide</td>
<td>100</td>
<td>0.74</td>
<td>0.86</td>
<td>69.9</td>
</tr>
<tr>
<td>ADP</td>
<td>101</td>
<td>0.75</td>
<td>0.86</td>
<td>69.9</td>
</tr>
<tr>
<td>ATP</td>
<td>101</td>
<td>0.75</td>
<td>0.87</td>
<td>69.9</td>
</tr>
</tbody>
</table>

* Double-integrated intensity of ST-EPR spectra normalized to rigor, error is ±5%. * Error = ±0.05. * Error = ±0.06. * Error = ±0.1 G.

viewed in Cooke and Highsmith (1983). The reactivity of Cys-707 and Cys-697 increases in the presence of ADP (Reisler et al., 1977), the distance between these two cysteines decreases by 4–7 Å as probed by chemical cross-linkers (Burke & Reisler, 1977; Wells & Yount, 1979) and by fluorescence energy transfer (Dalbey et al., 1983). The mobility of some spin probes (IASL) is sensitive to ADP binding, albeit not as much as in the M-ATP and M-ADP-Pi states (Seidel et al., 1970; Barnett & Thomas, 1987). Since Cys-707 is near the active-site pocket in the crystal structure of the myosin head (Rayment et al., 1993a), it should not be surprising that the probe environment is changing in the presence of nucleotides. Indeed, a change of shape due to the closure and opening of the active site induced by different states of bound nucleotide was postulated to result in force generation by muscle (Highsmith & Eden, 1992; Wakabayashi et al., 1992; Rayment et al., 1993b). Using low angle X-ray diffraction (Wakabayashi et al., 1992) and electrical birefringence (Highsmith & Eden, 1992) changes were observed on ADP binding in the chord length of the myosin head in solution.

Whether the changes observed in solution studies also occur in fibers has been less certain. No changes have been observed in X-ray diffraction patterns (Rodger & Tregear, 1984), EM (Katayama, 1989), birefringence (Oboriah & Irving, 1989), or linear dichroism of a probe attached to SH2 (Ajtai & Burghardt, 1989). Our own and others EPR studies using the maleimide and iodoacetamide derivatives of nitroxides attached to SH1 indicated a small torsional reorientation of the Cys-707 domain, putting an upper bound of 5–10° on the rotation of the myosin head as a rigid body (Fajer et al., 1990; Ajtai et al., 1992). However optical probes, especially IATR, did show reorientation of the probe axis on ADP binding (Borejdo et al., 1982; Burghardt et al., 1983). The lack of time correlation of tension change and spectroscopic change (Tanner et al., 1992) and our recent EPR modeling studies, which excluded the possibility that the spin probe orientation might make EPR insensitive to head rotation (Fajer et al., 1994a, b), both indicate that the optical probe movement is associated with an internal structural change rather than head rotation as a rigid body. The present study confirms this assertion since (a) two orientationally resolved conformations were observed; (b) one of them displayed a large orientational distribution; (c) the onset of microsecond fluctuations was observed. Any one of these phenomena would account for a change in the time- and spatially-averaged orientation of the optical probe. Why different labels attached to the same site are behaving differently is unclear (Fajer, 1994a). The size of the linkage arm might play a role; in the case of MSL, the labeled cysteine is separated by a succinamide ring from the reporting...
nitrooxide, whereas in IKSL the nitrooxide is separated by three C—C bonds. However, IASL is similar in linkage structure to IKSL (Figure 1), and it also does not report any significant changes in ADP (Fajer et al., 1990; Fajer, 1994a). This question might be answered when the atomic structure of the myosin head is made available, and steric restraints on different labels can be established.

In addition to identifying the nature of the structural change induced by ADP, we have established the coupling of this change to the interaction with actin. The myosin bound to glass beads did not reveal increased increased domain mobility in ADP as was found in the fibers. Apart from a possible change in the structure induced by binding to the glass beads, the difference between fibers and beads is the strong binding to actin. The flow experiment of Figure 3 indicates that the heads remain strongly bound in fibers in the presence of ADP.

The presence of two resolved populations of the probe signifies that the myosin head does not have a singular structure even in a single chemical state, which cautions against simplistic interpretation of static structures as derived from EM and crystallography. Conformational heterogeneity of the myosin head was first noted by Shriver and Sykes (1981a,b, 1982), who observed two resolved NMR resonances of the $\beta$-phosphate of ADP and two limiting values of chemical shift of a fluorine label attached to SH1. It was suggested that the equilibrium between these two different populations of the myosin head was determined by the chemical state of the nucleotide, and the transition between the two forms was responsible for force generation (Shriver & Sykes, 1981a). It is not clear whether the two orientational populations observed here correspond to the conformations observed in NMR because no change in mobility was observed in isolated myosin heads and no NMR was done in fibers. We would argue that the structural changes observed here correspond to optical probe changes which are not related to force generation (Tanner et al., 1992) but which assist the release of ATP hydrolysis products.

**Internal Mobility.** The observed structural changes indicate that the head has a considerable flexibility, manifesting itself in the large orientational spread and the presence of microsecond mobility. The labeled-domain "breathing" motions are slower than the mobility of the detached myosin heads ($\tau_c = 100-200$ and $5-10 \mu s$, respectively). An alternative explanation to structural fluctuations is that the pocket around Cys-707 created by ADP allows the label to orient itself at many different angles with respect to the protein. This possibility is unlikely because the accompanying mobility is 4–5 orders of magnitude slower than the rotation about the C–S bond of cysteine. Such a rotation is predicted to be in the range of 0.05–0.1 ns (Oton et al., 1981). Because there is little steric hinderance (wide spread of orientations in the disordered component), there should be equally little restriction on the rate of reorientation.

Proteins have been described previously as dynamic systems (Linderstrom-Lang & Shellman, 1959; Weber, 1975) with many structural substates (Hong et al., 1990; Frauenfelder et al., 1991). For example, CO dipoles in carbonmonoxyhemoglobin can have three different angles with respect to the heme plane. The transitions between these substates depend on the energy landscape of the conformational surface. Substates separated by low activation energies will exhibit fast transitions, slowing down with increasing activation energies. Thus, the internal protein dynamics might exhibit a full range of time scales. The side chain motions might be on the picosecond time scale (Karplus & Petsko, 1990), but correlated motions involving large segments of protein structure are considerably slower in the nanosecond to millisecond regime (Frauenfelder et al., 1991).

Most domain motions are in the nanosecond range, e.g., in hemoglobin (Oton et al., 1981), troponin C (Wang et al., 1993; Potter et al., 1975; Li & Fajer, 1994), tropomyosin (Graceff & Lehrer, 1984; Szczesna and Fajer, private communication), or gramicidin A (North & Cross, 1993), but slower motions are also observed, e.g., disulfide bond isomerization; cis-trans isomerization of proline peptide bonds and aromatic ring flips are on the millisecond time scale (Otting et al., 1993; Wutrich, 1976). The motions responsible for hydrogen exchange are thought to be even slower (Englander & Mael, 1972). Internal motions in myosin have been described previously, but all of these were in the nanosecond region (Seidel et al., 1970; Barnett & Thomas, 1987), and then are inhibited by the interaction with actin (highsmith et al., 1979). The internal motions observed here are qualitatively different, they are considerably slower (correlation times in the microsecond region), and they are only observed in the presence of actin.

These motions and the presence of conformational substates most certainly have functional implications. The "induced fit" model proposed by D. Koshland (1958) explicitly treated protein conformations and transitions between them as being at the core of catalytic activity. More recently, Frauenfelder and colleagues have shown that the conformational substates of myoglobin display different binding kinetics (Hong et al., 1990). The oxygen affinity of hemoglobin has been correlated with the conformations and internal flexibility of the hemoglobin subunits (el Antri et al., 1990). We can only speculate that the microsecond structural fluctuations observed here are of importance to enzymatic activity. ADP release, which is thought to limit the actomyosin ATPase in the fibers, has been shown to be 10 times slower than the rate of structural fluctuations. It is likely that the structural changes observed here assist ADP release (Siemanskowski & White, 1984).

In conclusion, we have observed slow ($\tau_c = 150 \mu s$) internal motions of the catalytic domain of the myosin head bound to actin, which that are present in ADP, but absent in the rigor state. These motions result in a wide range of local conformations, as probed by the attached spin label.

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**REFERENCES**


