Formation of the Stable Myosin-ADP-Aluminum Fluoride and Myosin-ADP-Beryllium Fluoride Complexes and Their Analysis Using $^{19}$F NMR∗

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The effects of aluminum fluoride and beryllium fluoride on smooth muscle myosin and its subfragments were studied. Mg$^{2+}$-ATPase activity was inhibited in the presence of aluminum fluoride (beryllium fluoride). $^{[3]}$H$[^{}$ADP bound to heavy meromyosin (HMM) in the presence of aluminum fluoride (beryllium fluoride) and was not dissociated after 3 days of dialysis demonstrating that $^{[3]}$H$[^{}$ADP was trapped in HMM. These results suggest the formation of a stable HMM-ADP-fluorouruminate (fluoroberyllate) complex. The intrinsic tryptophane fluorescence intensity was increased in the presence of ADP and aluminum fluoride (beryllium fluoride). Acto-S1 was dissociated upon the formation of S1-ADP-fluoroberyllate and actin destabilized S1-ADP-fluoroberyllate complex, while S1-ADP-fluorouruminate failed to bind to actin. Furthermore, when S1 formed the complex with actin, nucleotide trapping did not occur in the presence of fluorouruminate. These results indicated that the myosin-ADP-fluoroberyllate complex resembles a weak binding state while myosin-ADP-fluorouruminate complex is a distinct conformation although the binding to actin was also weak. The structure of the ternary complex was investigated using $^{19}$F NMR. The $^{19}$F NMR spectrum of the S1-ADP-fluorouruminate complex showed a peak at $-66.7$ ppm which is due to the binding of fluorouruminate to S1. The peak was not observed when 5'-adenylylimidodiphosphate was substituted for ADP suggesting that aluminum fluoride plays a role as a phosphate analogue. The stoichiometry of the bound fluoride was determined to be 3.8 mol/mol S1 suggesting that the bound species is AlF$_2$.

Aluminum fluoride and beryllium fluoride have been shown to affect the activity of nucleotide-binding proteins, for example G-protein-regulated systems (Sternweis et al., 1982), the Na$^+$/K$^+$ ATPase (Robinson et al., 1986; Missiaen et al., 1988) the F$_{1}$/F$_{0}$ ATP synthase (Lunardi et al., 1988), and glucose-6-phosphatase (Lange et al., 1986). Recently, it has been shown that these compounds bind directly to the protein-nucleotide complex in the case of GDP-transducin (Bigay et al., 1985, 1987), GDP-tubulin (Carlier et al., 1988, 1989), and ADP-actin (Combeau et al., 1988). Tetrahedral fluorouruminate and fluoroberyllate analogues have been proposed to act as phosphate analogues (Bigay et al., 1987), binding tightly at the active site of the protein in the position normally occupied by the γ-phosphate of ATP or GTP. The net result is to mimic the presence of a non-hydrolyzable nucleotide triphosphate, for example, G-protein-regulated enzymes (e.g. adenylate cyclase) are persistently activated (Sternweis et al., 1982), whereas ATPases are inhibited (Lange et al., 1986; Robinson et al., 1986; Missiaen et al., 1988). In support of the phosphate analogue hypothesis, it was noted that the Al-F bond length is the same as the P-O bond length in phosphate (1.5-1.6 Å) and that fluorine in an ionic complex has a greater capacity than oxygen for accepting H-bonds from OH or NH donors (Chabre, 1990). These compounds are in many ways similar to vanadate, which can also act as a phosphate analogue (Goodno, 1979). Myosin and its active subfragments, heavy meromyosin (HMM)$^1$ and subfragment 1 (S1) are excellent candidates for interaction with aluminum and beryllium fluorides.

Muscle contraction is driven by the hydrolysis of ATP by myosin, although the mechanism through which the energy of hydrolysis is converted into mechanical work is not known (Cooke, 1986; Eisenberg and Green, 1980). Hydrolysis of Mg$^{2+}$ ATP occurs very rapidly at the active site of the enzyme, and an equilibrium is readily established (Bagshaw and Trentham, 1973). The release of products (ADP and P$_i$) is comparatively slow, but the rate of this step is greatly enhanced in the presence of actin. Myosin comprises two heavy chains and four light chains and has a total molecular weight close to 500,000. The N-terminal region of the heavy chain forms the globular myosin head, whereas the C-terminal regions of the two heavy chains are wound together in a long α-helical coiled coil, the myosin tail. The light chains are associated with the head-tail junction. Both the ATP hydrolysis site and the actin-binding site lie in the head region. Two enzymatically active fragments can be prepared from myosin by proteolytic cleavage: HMM possesses both myosin heads and a short region of tail whereas S1 consists of isolated globular heads.

In this series of experiments, we have determined the effects of both aluminum fluoride and beryllium fluoride on smooth muscle HMM and S1. The stable myosin-ADP-fluorouruminate (fluoroberyllate) complexes were isolated, and compared

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$^1$The abbreviations used are: HMM, heavy meromyosin; S1, myosin subfragment-1; S-2, myosin subfragment-2; DTT, dithiothreitol; 4 FB-ATP, 3′(2′)-O-(4-flourobenzoic)-ATP; TEA, triethylammonium; Vi, orthovanadate; AMP-PNP, 5′-adenylylimidodiphosphate; ppm, parts/million.
with the S1-ADP-P<sub>i</sub> state. The effect of actin on the ternary complex was also determined. The high sensitivity of <sup>31</sup>P NMR spectroscopy makes it an ideal technique for investigation of the structure of the complex, even for molecules as large as HMM and S1. <sup>31</sup>P NMR spectroscopy was used to test various aspects of the phosphate analogue hypothesis, most notably to determine the number of bound fluoride atoms. A preliminary form of this paper has been presented (Maruta et al., 1991).

**MATERIAL AND METHODS**

Smooth muscle myosin was isolated from frozen turkey gizzards (Ikebe and Hartshorne, 1985b). Smooth muscle myosin S1 and HMM were prepared by Staphylococcus aureus protease digestion according to Ikebe and Hartshorne (1986a). Skeletal muscle myosin S1 and S1 were prepared from rabbit back muscle (Margossian and Lowey, 1982).

ATPase assays were carried out in the presence of 1 mM ATP, 120 mM NaCl, 20 mM Tris-HCl, pH 7.5, 5 mM MgCl<sub>2</sub>, in the presence or absence of 1 mM BeSO<sub>4</sub> or AlCl<sub>3</sub>, 5 mM NaF, 1 mM ADP at 25°C. The reaction was stopped by addition of 10% trichloroacetic acid and the released P<sub>i</sub> was determined by the methods of Youngburg and Yount (1984). ATPase assays were  carried out in the presence of either aluminum fluoride or NaF (Goldstein, 1964) and that fluoroberyllate can be formed in the presence of BeSO<sub>4</sub> or NaF (Hogben et al., 1970). Fig. 1 shows the effects of fluorophosphate complexes on the Mg<sup>2+</sup>-ATPase activity of smooth muscle HMM. The ATPase activity was strongly inhibited in the presence of both ADP and fluoroberyllate in a time-dependent manner. Some inhibition was also observed in the absence of additional ADP. This inhibition was probably due to the ADP produced by the hydrolysis of ATP during the HMM-ATPase reaction. Inhibition of the ATPase activity was also observed when S1 was used (data not shown). Similar inhibition of the ATPase activity was also observed in the presence of fluoroberyllate and ADP (Fig. 2).

Previously, Goodno (1979) showed that the Mg<sup>2+</sup>-ATPase activity of myosin is inhibited by orthovanadate (Vi) in the presence of ADP in a similar time-dependent manner. ADP is trapped in the active site due to formation of the stable myosin-ADP-Vi complex which vanadate behaves as a phosphate analogue. The results shown in Figs. 1 and 2 suggest formation of the analogous stable HMM-ADP-fluoroaluminate (fluoroberyllate) complex in which ADP is also trapped at the active site.

The stable binding of ADP to HMM in the presence of fluoroberyllate and fluorobenzamide was shown by gel filtration of the protein-nucleotide complex. HMM was incubated with [3H]ADP in the presence of either aluminum fluoride or beryllium fluoride and subjected to Sephadex G-25 gel filtration. As shown in Fig. 3, [3H]ADP equilibrated with HMM from the integrals of the H8 and H1' proton resonances in an NMR spectrum in which a known volume of the derivative was added to a known concentration of ADP (determined by absorbance at 259 nm). The analogous compound, 3'(2')-O-(4-trifluoromethylbenzoic)-ATP was also synthesized as the three fluorine atoms would provide greater sensitivity. However, the electron-withdrawing effect of the trifluoromethyl group led to significant hydrolysis of the ester linkage under normal solution conditions.

3'(2')-O-(4'-Fluorobenzoic)-ATP was added to smooth muscle S1 in 36 mM Tris-HCl, 180 mM KCl, 1 mM DTT at a molar ratio of 0.75:1 4FB-ATP:S1, and left to hydrolyze on ice for 5 min. Premixed NaF and AlCl<sub>3</sub> were then added to final concentrations of 5 and 1 mM, respectively, to form the stable ternary complex. Any remaining unbound nucleotide was then removed by gel filtration on a Penefsky column (Penefsky, 1977), and the S1 was transferred immediately to an NMR tube in which the final concentrations of buffer and salts were 30 mM Tris-HCl, pH 7.5, 180 mM KCl, 1 mM AlCl<sub>3</sub>, 5 mM NaF, 5 mM MgCl<sub>2</sub>, and 1 mM DTT.

**RESULTS**

It is known that fluoroberyllate complexes can be formed in the presence of both AlCl<sub>3</sub> and NaF (Goldstein, 1964) and that fluoroberyllate can be formed in the presence of BeSO<sub>4</sub> or NaF (Hogben et al., 1970). 3'(2')-O-(4-trifluoromethylbenzoic)-ATP was added to smooth muscle S1 in 36 mM Tris-HCl, 180 mM KCl, 1 mM DTT at a molar ratio of 0.75:1 4FB-ATP:S1, and left to hydrolyze on ice for 5 min. Premixed NaF and AlCl<sub>3</sub> were then added to final concentrations of 5 and 1 mM, respectively, to form the stable ternary complex. Any remaining unbound nucleotide was then removed by gel filtration on a Penefsky column (Penefsky, 1977), and the S1 was transferred immediately to an NMR tube in which the final concentrations of buffer and salts were 30 mM Tris-HCl, pH 7.5, 180 mM KCl, 1 mM AlCl<sub>3</sub>, 5 mM NaF, 5 mM MgCl<sub>2</sub>, and 1 mM DTT.

**Fig. 1.** Inhibition of HMM Mg<sup>2+</sup>-ATPase activity by fluorobenzamide. 1 mM HMM was preincubated for 5 min in 120 mM NaCl, 5 mM MgCl<sub>2</sub>, 20 mM Tris-HCl, pH 7.5 (C), and 0.5 mM AlCl<sub>3</sub>, 2.5 mM NaF, 1 mM ADP (O), 0.5 mM AlCl<sub>3</sub>, 2.5 mM NaF (A), 1 mM ADP (o). The ATPase reaction was started by adding 2 mM ATP at 25°C.
Trapped in the active site roberyllate. 2.5 ADP osin-ADP-Pi intermediate, and this is characterized by an aluminate or fluoroberyllate. To address the question of whether ADP is quickly within 1 h. These results demonstrate that ADP is absence of fluoroaluminate (fluoroberyllate) was dialyzed out NaCl, 5 mM MgCl₂, 0.5 mM BeSO₄, 2.5 mM NaF, 1 mM ADP (●); 0.5 mM BeSO₄, 2.5 mM NaF (▲); 1 mM ADP (△). The ATPase reaction was started as described in Fig. 1.

The mixture was applied to 0.9 × 18-cm column of Sephadex G-25. Radioactivity of each fraction was measured by a Beckman LS 5000 scintillation counter. (monitored by the absorbance at 280 nm) in the presence of either fluoroaluminate or fluoroberyllate but not in their absence.

These results suggest that the stable ternary complex of HMM-ADP-fluoroaluminate (fluoroberyllate) is formed. The amount of bound ADP was approximately 0.7 mol/mol active site in the presence of fluoroaluminate and 0.8 mol/mol active site in the presence of fluoroberyllate. The stability of the complex was examined by measuring the bound [³H]ADP after dialysis of the complex (Fig. 4). More than 70% of the ADP initially bound to HMM in the presence of fluoroberyllate and more than 80% of the ADP initially bound to HMM in the presence of fluoroaluminate were still bound to HMM after 3 days of dialysis. ADP mixed with HMM in the absence of fluoroaluminate (fluoroberyllate) was dialyzed out quickly within 1 h. These results demonstrate that ADP is trapped in the active site of HMM in the presence of fluoroaluminate or fluoroberyllate.

Upon addition of Mg-ATP, myosin quickly forms the myosin-ADP-Pₐ intermediate, and this is characterized by an increase in the intrinsic tryptophan fluorescence intensity. We, therefore, examined the conformation of the myosin-ADP-fluoroaluminate and myosin-ADP-fluoroberyllate by measuring the change in the intrinsic tryptophan fluorescence as a probe. Upon the addition of ATP, the tryptophane fluorescence intensity of S1 increased in the presence of aluminum fluoride and 0.8 mol/mol active site in the presence of fluoroberyllate. The purified complex (5 μM HMM) was then dialyzed at 4 °C against 120 mM NaCl, 20 mM Tris-HCl, pH 7.5, 5 mM MgCl₂. After various times of dialysis, the sample was applied to Sephadex G-25 column. The amount of bound ADP was estimated by measuring the radioactivity of [³H]ADP.

Fig. 3. Separation of the bound ADP and free ADP by Sephadex G-25 gel filtration. 20 μM of HMM in buffer containing 120 mM NaCl, 20 mM Tris-HCl, pH 7.5, 5 mM MgCl₂ was preincubated for 30 min at 25 °C with 20 μM [³H]ATP. After ATP was completely hydrolyzed, 5 mM NaF, 1 mM AlCl₃ (▲), 5 mM NaF and 1 mM BeSO₄ (□) or H₂O (x) were added and incubated for 3 h at 25 °C. The mixture was applied to 0.9 × 18-cm column of Sephadex G-25. Radioactivity of each fraction was measured by a Beckman LS 5000 scintillation counter.

Fig. 5. Time course of increment of intrinsic tryptophan fluorescence intensity of S1 induced by ADP and fluoroaluminate (fluoroberyllate). To S1 (1 μM) in 50 mM KCl, 30 mM Tris-HCl, pH 7.5, 2 mM MgCl₂, 2.6 mM (△) NaF, and 0.5 mM AlCl₃ (□) or 0.5 mM BeSO₄ (▲) 10 μM ADP was added, and the fluorescence intensity was monitored. 10 μM ATP (●) was also added to the S1 solution in the absence of NaF, AlCl₃, and BeSO₄, and the change in the fluorescence intensity was monitored. Fluorescence intensity was monitored using an excitation wavelength at 300 nm and an emission wavelength at 335 nm.

In the presence of fluoroaluminate or fluoroberyllate and ADP. In the presence of fluoroaluminate, the intensity was almost identical to that of S1- ADP-X. The rate of fluorescence increase of S1 in the presence of aluminum fluoride and ADP was faster (approximately six times) than that in the presence of fluoroaluminate, however, the maximum intensity was lower than that of S1-ADP-Pₐ by 30%. These results suggest that S1 (myosin) forms a ternary complex in the presence of ADP and fluoroaluminate (fluoroberyllate) whose conformation is analogous to the myosin"
ADP-P\textsubscript{i} reaction intermediate in terms of the intrinsic tryptophane fluorescence. However, the intrinsic tryptophane fluorescence cannot distinguish the strong and weak actin binding state of S1, therefore, we examined the interaction of the complexes with actin to further characterize these ternary complexes.

Fig. 6 shows the dissociation of acto-S1 in the presence of ADP and various P\textsubscript{i} analogues. Acto-S1 dissociated quickly in the presence of ADP and beryllium fluoride and slowly in the presence of ADP and vanadate, whereas it did not dissociate at all in the presence of ADP and fluoroaluminate. These results suggested that myosin-ADP-fluoroberyllate is a weak binding state like myosin-ADP-Vi. On the other hand, two interpretations are possible for fluoroaluminate. Fluoroaluminate cannot bind to acto-S1 (and hence cannot dissociate the complex) or myosin-ADP-fluoroaluminate binds tightly to actin. To resolve this issue, the binding of S1-ADP-fluoroaluminate with actin was measured by centrifuge analysis (Fig. 7). In the presence of ADP but not fluoroaluminate, virtually all of the S1 coprecipitated with actin. However, when fluoroaluminate was present, most of the S1 remained in the supernatant. The results showed that S1-ADP-fluoroaluminate does not strongly bind to actin. The trapping of \([^{14}C]ADP\) into acto-S1 was also tested in the presence of fluoroaluminate, and it was found that acto-S1 failed to trap ADP (data not shown). These results suggest that myosin-ADP-fluoroaluminate is not a strong binding state. It is known that actin destabilizes the myosin-ADP-Vi complex (Goodno and Taylor, 1982) and, therefore, ADP can be trapped in the active site only in the absence of actin. In the further set of experiments, the effects of actin on the stability of the ternary complexes were examined by measuring the amount of \(^{3}H\)ADP retained by the S1-ADP-fluorometal complexes after addition of actin. As shown in Fig. 8, ADP was rapidly released from the S1-ADP-fluoroberyllate complex whereas ADP was not released from the S1-ADP-fluoroaluminate complex under the identical conditions. S1-ADP-fluoroberyllate thus appears to behave like a conventional weak binding state (like the S1-ADP-Vi complex). On the other hand, the stability of the S1-ADP-fluoroaluminate was unaffected by actin. Clearly, it is also a weak binding state, as shown by the direct binding assay, yet unlike fluoroberyllate, it cannot dissociate acto-S1. The best explanation of these data is that either actin or fluoroaluminate can bind to S1-ADP, but not both. It is not certain at this point that myosin-ADP-fluoroaluminate resembles what step of the myosin-ATPase reaction intermediate, but it is neither a strong binding state nor conventional weak binding state (like myosin-ADP-P\textsubscript{i}). To examine more quantitatively the binding of S1-ADP-fluoroberyllate to actin, the binding of S1-ADP-fluoroberyllate was examined as a function of actin concentration (Fig. 9). The affinity of S1-ADP-fluoroberyllate for actin is more difficult to measure in a centrifugation assay.
since actin destabilizes the complex. However, the binding can be compared directly to that of S1-ADP-P, (the major intermediate during ATPase cycle) in a light scattering experiment. The binding of S1-ADP-fluoroberyllate to actin was weak and indistinguishable from that of S1-ADP-P.

The structure of myosin-ADP-fluoroaluminate was investigated using $^{19}$F NMR spectroscopy. All spectra were recorded in the presence of 30 mM Tris-HCl, pH 7.5, 180 mM KCl, 1 mM DTT, 5 mM NaF, 1 mM AlCl$_3$, and 5 mM MgCl$_2$; S1 and ADP were included both separately and together. In the presence of S1 (0.08 mM but without ADP) (Fig. 10A) two peaks were observed; the downfield resonance at -44.3 ppm corresponds to free F\(^-\) ions whereas the upfield resonance at -79.2 ppm arises from fluoride ions complexed with alumnum. The F\(^-\) resonance undergoes extensive broadening in the presence of Mg\(^{2+}\) ions (Higashijima et al., 1991). Identical spectra are obtained in the absence of protein (data not shown). A similar sample (Fig. 10B) prepared with ADP (1 mM) but no protein yielded an almost identical spectrum. Otherwise identical solutions containing both S1 and ADP, however, showed a third peak at -66.7 ppm (Fig. 10C), indicating the formation of the S1-ADP-fluoroaluminate complex. All three components, S1, ADP, and aluminum fluoride are required for producing a stable ternary complex. The same results have been obtained with S1 isolated from skeletal muscle. For reasons that are not entirely clear, the analogous beryllium complex has proved much less amenable to study by $^{19}$F NMR. All further experiments focused exclusively on the aluminum species.

In order to establish that the new resonance at -66.7 ppm corresponds to genuine S1-bound fluoride, the protease trypsin was added at high concentration to thoroughly digest the protein. $^{19}$F NMR spectra were recorded as a function of time. As the digestion progressed, the intensity of the new resonance decreased, while that of the unbound aluminum-complexed fluoride simultaneously increased (Fig. 11) providing convincing evidence that the peak at -66.7 ppm does indeed correspond to bound fluoride. In a second series of experiments, the S1 was denatured by addition of solid urea (not shown). As the concentration of urea was increased, the intensity of the peak at -66.7 ppm decreased and was finally abolished at a urea concentration of 7 mM, where complete unfolding of the S1 is to be expected. A very similar resonance corresponding to bound fluoroaluminate was observed for the $\alpha$ subunit of transducin (Higashijima et al., 1991). It is interesting to note in this context that the $T_1$ relaxation time of the bound fluoroaluminate is very short (0.09 s at 25 °C) compared with the $T_1$ values of F\(^-\) and free aluminum-complexed fluoride (both about 1 s). The bound resonance is thus always fully relaxed and appears to be disproportionately large under normal acquisition parameters.

Experiments were also performed in which the non-hydrolyzable ATP analogue 5'-adenylylimidodiphosphate (AMP-PNP) replaced ADP. Under otherwise identical conditions (1 mM AMP-PNP), no bound fluoroaluminate was observed (spectrum not shown) suggesting that the complex will not form if the $\gamma$-phosphate site of S1 is occupied. This strongly supports the concept that aluminum fluoride plays a role as a phosphate analogue.

The precise nature of the bound complex is still unclear. Two structures frequently appear in the literature: ADP$^-$AlF$_3$ and ADP$-$O-AlF$_3$ (Combeau and Carlier, 1989; Dupuis et al., 1989; Antonny and Chabre, 1992). In the former, tetrahedral tetrafluoroaluminate anions substitute directly for inorganic phosphate at the active site, whereas in the latter, one of the F$^-$ ligands has been displaced by a $\beta$-phosphate oxygen atom of ADP, and the complex resembles a nucleotide triphosphate. In addition, the existence of a tetracoordinate aluminum species in aqueous solution has been called into question (e.g. Macdonald and Martin, 1988; Jackson, 1988) as Al$^{3+}$ ions in aqueous solution normally form a hexacoordinate octahedral complex. These issues were resolved by determining the ratio of bound ADP to aluminium-associated fluorine nuclei from the relative signal intensities in a $^{19}$F NMR spectrum of the fluoroaluminate complex formed in the presence of a $^{19}$F-labeled ADP derivative, 3'(2')-O-(4-fluorobenzoic)-ADP. The advantage of this experiment is that it does not require particularly accurate knowledge of the S1
concentration, and it contains an internal control against the possible presence of inactivated protein (i.e., molecules unable to bind nucleotide) since the bound fluoroaluminate complex exists only in the presence of ADP. 3’(2’)-O-(4-fluorobenzoic)-ADP is similar to the fluorescent derivative 3’(2’)-O-[(N-methyl anthraniloyl)ATP (Mant-ATP), and like Mant-ATP (Cremon et al., 1990a) both the 3’ (65%) and 2’ (35%) esters are present. The isomers are distinguished by their 19F chemical shifts (−29.4 and −29.1 ppm, respectively; see Fig. 12, inset A).

Substitutions on the ribose ring do not greatly affect the binding or hydrolysis of ATP by S1. Both the fluorescent derivate Mant-ATP, which is directly analogous to 4FB-ATP, and a spin-labeled cyclic ATP derivative modified on both the 3’ and 2’ positions are good substrates for S1 and support muscle contraction (Woodward et al., 1991; Alessi et al., 1992). Preliminary NMR experiments show that ADP and 4FB-ADP bind to S1 with similar affinities. However, it should be stressed that for the purpose of the determination of the number of bound fluoride atoms, the only prerequisite property of 4FB-ADP is that it should support formation of the fluoroaluminate complex. The 19F NMR spectrum of bound fluoroaluminate in the presence of 4-FB-ADP is indistinguishable from the spectrum obtained with ADP.

Fig. 12 shows the 19F NMR spectrum of smooth muscle S1 in the presence of 4-fluorobenzoic-ADP (as described under “Materials and Methods”) and excess AlCl3 (1 mM) and NaF (5 mM). A long delay between transients (>5*T1) was used to ensure full relaxation of the bound 4-(fluorobenzoic)-ADP (T1 = 0.85 s). The bound nucleotide gives rise to two broad but distinct resonances at −29.3 and −28.0 ppm in a ratio close to 2:1 (Fig. 12, inset B). The downfield resonance is significantly shifted from the frequencies of both isomers of the free compound, but the upfield peak is more or less coincident (Fig. 12 inset A). For this reason, great care was taken to eliminate the possibility of free nucleotide in the sample. The broad linewidths of the nucleotide resonances are fully consistent with protein-bound ADP. It is not known at this stage whether the two peaks arise from the two ADP isomers or from two conformations of the S1. Integration of the spectrum in Fig. 12 results in a fluoride to nucleotide ratio of 3.8:1 after taking the contribution of the unmodified ADP (8.6%) into account. Similar experiments on different samples have yielded values of 4.2 and 3.5, good evidence that the bound species is AlF7.

**DISCUSSION**

The results show that myosin forms a ternary complex of myosin-ADP-fluoroaluminate or myosin-ADP-fluoroberyllate. The complexes form slowly but they are very stable, as fluoroaluminate and fluoroberyllate both bind very much tighter than phosphate. However, they do dissociate and the incorporated nucleotide can be dialyzed out on a time scale of several days. Nucleotide can also be trapped in the active site of myosin and its subfragments using vanadate which is similar in many respects to fluoroaluminate and fluoroberyllate. Vanadate is also thought to behave as a phosphate

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2 G. D. Henry and B. D. Sykes, unpublished observations.
**Myosin-ADP-Aluminum Fluoride (Beryllium Fluoride) Complexes**

While this manuscript was being prepared, Phan and Reisler (1992) reported that skeletal muscle S1 formed a stable complex with ADP and fluoroberyllate, and S1-ADP-fluoroberyllate complex is analogous to the M-ADP-V, and M-ADP-P, states of myosin. This is consistent with the results of the present study. On the other hand, S1-ADP-fluoroaluminate did not dissociate acto-S1-ADP; neither are the bound nucleotide or fluoroaluminate released upon addition of actin. Furthermore, it was shown that the binding affinity of S1-ADP-AlF₃ for actin is quite low. Consequently, S1-ADP-fluoroaluminate was stable even in the presence of actin. It appeared that either S1-ADP-AlF₃ or S1-ADP-actin can exist, and the outcome is critically dependent on which complex was allowed to form initially. Clearly there must be a kinetic barrier preventing the interconversion of the complexes in the presence of fluoroaluminate which does not occur with fluoroberyllate. This might be biologically important or it might be comparatively trivial and involve the chemistry of formation of the fluoroaluminate complex itself.

Further experiments are in progress to characterize these phenomena. Both S1-ADP-AlF₃ and S1-ADP-fluoroberyllate are weak binding states with respect to actin and may be useful analogues of the kinetic intermediates in the actomyosin ATPase cycle. Fluoroaluminate and fluoroberyllate have other useful properties; for example, during photofinity labeling, it is helpful to trap the nucleotide in the active site. In the presence of Mg²⁺, the myosin-ADP-Vi complex is photooxidized, and the non-physiological Co²⁺ must be used instead (Garabedian and Yount, 1990). However, the fluoro-alkaline metals are not subject to this problem.

**¹⁹F NMR spectroscopy has clearly demonstrated the formation of a specific complex between S1-ADP and fluoroaluminate and has shown the presence of ADP to be an absolute requirement. The spectrum is very similar to that of the analogous transducin-ADP-fluoroaluminate complex (Higashijima et al., 1991). The **¹⁹F NMR experiments have also shown conclusively that four fluorine atoms are bound for each ADP, thus the complex is correctly written as myosin-ADP-AlF₃, not myosin-ADP-O-AlF₃. The AlF₃ species preserves the negative charge of orthophosphate. A similar fluoride to nucleotide ratio (i.e. 4:1) was obtained for actin-ADP-fluoroaluminate, tubulin-GDP-fluoroaluminate, and F₆ ATPase-ADP-fluoroaluminate after denaturation of the isolated protein complexes and measurement of the released F⁻ with an ion-selective electrode (Combeau and Carlier, 1989; Dupuis et al., 1989). These results contrast with recently published experiments on transducin (Antonny and Chabre, 1982) in which a nucleotide fluoride ratio of 1:3 was suggested by analysis of the association and dissociation kinetics as a function of pF. The fluoride-labeled nucleotide 3'(2')-O-(4'-fluoroberyllate)-ADP may prove to be very useful in the analysis of other systems.

We were unable to perform similar NMR experiments with the fluoroberyllate species. Combeau and Carlier (1989) suggest that both [Be(H₂O)₆F₂OH]⁻ and [Be(H₂O)₂F]⁻ may be bound to ADP-actin and GDP-transducin, the dominant species depending on pH and [F⁻]. This may account, in part, for the difficulties encountered in observing the S1-ADP-fluoroberyllate complex by NMR.
Myosin-ADP-Aluminum Fluoride (Beryllium Fluoride) Complexes

In view of the general nature and widespread applications of the fluoroaluminate and fluoroberyllate complexes as inhibitors of phosphotransfer reactions, it is important to understand the mechanism of interaction and to establish whether the phosphate analogue hypothesis of Charbre and co-workers is correct. Our results are completely consistent with AlF$_6^2$- binding at the active site. No bound fluoroaluminate can be observed by NMR when the γ-phosphate site is already occupied, as when the non-hydrolysable ATP analogue, AMP-PNP replaces ADP. These experiments, taken in conjunction with those of others (for example, the $^31$P and paramagnetic cation experiments performed by Higashijima et al. (1991) with transducin) provide strong support for the phosphate analogue model.

Although the beryllium complexes are known to adopt an exclusively tetrahedral geometry, the phosphate analogue hypothesis has been challenged with respect to aluminum on the grounds that a tetrahedral AlF$_4^-$ species cannot exist in aqueous solution (Martin, 1988; Jackson, 1988). Instead, an octahedral [AlF$_6$(H$_2$O)$_2$]$^-$ species is overwhelmingly preferred. However, the forces that determine the predominant species in aqueous solution do not necessarily apply at the active site of an enzyme where conditions are quite different from those of the bulk solution. Favorable steric and electrostatic interactions, the proximity of suitable hydrogen bond donors, and the partial exclusion of water may all contribute to the energy of stabilization of a tetrahedral species. The large difference in chemical shift (12.5 ppm) between the bound AlF$_6^-$ and the mixture of free hexacoordinated aluminum species also suggest a substantially different electronic environment for fluoride atoms in the S1-bound state and the fact that a single peak is observed indicates that all bound fluoride atoms are equivalent.

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