Photoaffinity labelling of smooth-muscle myosin by methylantraniloyl-8-azido-ATP

Shinsaku MARUTA* and Mitsuo IKEBE†

Department of Physiology and Biophysics, School of Medicine, Case Western Reserve University, Cleveland, OH 44106, U.S.A.

Methylantraniloyl-8-azido-ATP (Mant-8-N₃-ATP), which binds to the 20 kDa C-terminal tryptic fragment of skeletal-muscle myosin subfragment-1 [Maruta, Miyanishi and Matsuda (1989) *Eur. J. Biochem.* **184**, 213–221], was synthesized and used as a probe of the conformational change of smooth-muscle myosin. Mant-8-N₃-ATP, like ATP, induced the formation of the 10 S conformation at low ionic strength. In the presence of vanadate, smooth-muscle myosin formed a stable complex with Mant-8-N₃-ADP, and this complex showed the 10 S→6 S transition of myosin. ATP-binding sites for 6 S (extended state) and 10 S (folded state) myosin were studied by photolabelling of myosin with Mant-8-N₃-ADP. For both 6 S and 10 S myosin, Mant-8-N₃-ATP was incorporated into the 29 kDa N-terminal

tryptic fragment of myosin heavy chain. This is unlike the labelling of skeletal-muscle myosin, in which the 20 kDa C-terminal fragment is labelled. The labelling of 29 kDa fragment was diminished significantly by addition of ATP. These results suggest that the conformation of the ATP-binding site of smooth-muscle myosin is different from that of skeletal-muscle myosin. To examine further the possible differences in the labelling site between 6 S and 10 S myosin, the affinity-labelled 29 kDa fragment was subjected to complete proteolysis by lysylendopeptidase. The fluorescent-labelled-peptide map suggested that the Mant-8-N₃-ADP-binding sites for 6 S and 10 S myosin were identical.

INTRODUCTION

Contractile apparatus of smooth muscle is regulated by the phosphorylation of the 20 kDa light chain of myosin, which is catalysed by the calmodulin-dependent myosin-light-chain kinase [1]. Phosphorylation of the 20 kDa light chain of myosin leads to an increase in the actin-activated ATPase of myosin, and it is assumed that this corresponds to the initiation of smooth-muscle contraction.

The mechanism of activation of the ATPase activity by phosphorylation is unclear, but some progress has been made in defining the molecular changes induced by phosphorylation. It was found that monomeric smooth-muscle myosin can exist in two different conformations, namely the folded (10 S) and extended (6 S) states [2–5]. An important point is that these conformations are characterized by distinct enzymic properties [6], and it has been suggested that some component of the 10 S→6 S transition is involved in the regulation of ATPase activity [6,7]. Although several parameters can affect the conformational transition [6], the most important finding is that the phosphorylation of myosin favours the 6 S conformation [4–6,8]. These findings led to the hypothesis that the 10 S→6 S transition, or some associated conformational change, could be a component of the regulatory mechanism in smooth muscle and that phosphorylation alters the enzymic activity via a change in myosin conformation. However, myosin is thought to exist only in the filamentous state *in vivo* [9], and it is unlikely that a large change in the conformation occurs in filamentous myosin.

Information to resolve these problems was subsequently obtained, and we proposed that a conformational transition at

the subfragment-1/subfragment-2 (S-1/S-2) junction is important for dictating the ATPase activity, as shown by the following evidence. (1) The resistance to the proteolysis at the S-1/S-2 junction is markedly altered with the conformational transition of monomeric and filamentous myosin [7]; (2) the proteolytic lability at the S-1/S-2 junction is markedly different between phosphorylated (active) and dephosphorylated (inactive) heavy meromyosin (HMM), which does not form a folded structure because of its lack of the tail portion [7,10]; (3) the actin activated Mg²⁺-ATPase activity of S-1, which retains an intact 20 kDa light chain is not regulated by phosphorylation, whereas HMM is [10]; (4) the antibody which prevents the formation of the 10 S conformation activates the actin-activated Mg²⁺-ATPase activity of dephosphorylated HMM [11]; (5) the position of the heads are distinct in the 7.5 and 9 S conformations of HMM [12,13]; (6) the flexibility at the S-1/S-2 junction is markedly decreased in the 10 S conformation [14,15]. The change in the conformation at the S-1/S-2 junction results in a large change in the shape of the myosin molecule in solution.

However, the possible change in the conformation at the active site of smooth-muscle myosin during the transition between the active and inactive conformations is still obscure. To study the active-site structure, photoaffinity labelling of myosin by an ATP analogue gives useful information. For skeletal-muscle myosin, several photoaffinity-labelling ATP analogues have been used. The 25 kDa N-terminal tryptic fragment of S-1 is labelled with Arylazido-β-alanyl-ATP [16], NANDP [17,18] and Mant-2-N₃-ATP [19]. The 50 kDa tryptic fragment of S-1 is labelled with Bz₂-ATP [20]. On the other hand, Maruta et al. [19] reported that Mant-8-N₃-ATP labelled the 20 kDa C-terminal tryptic fragment

Abbreviations used: Mant-8-N₃-ATP, methylantraniloyl-8-azido-ATP; S-1(-2), subfragment-1(-2); HMM, heavy meromyosin; SAP-S-1, subfragment-1 prepared by digestion with *Staphylococcus aureus*; Bz₂-ATP, 4-benzoylbenzoyl-ATP; NANDP, N-(4-azido-2-nitrophenyl)-2-aminoethyl diphosphate.

* Present address: Department of Bioengineering, Faculty of Engineering, Soka University, Hachioji, Tokyo 192, Japan.

† To whom correspondence should be addressed.

of S-1, and it was suggested that the difference in the labelled sites is due to the difference in the conformation of ATP analogues. That is, arylazido- β -alanine ATP, Mant-2-N₃-ATP and 4-benzoylbenzoyl-ATP (Bz₂-ATP) adopt the *anti* conformation with respect to the adenine-ribose bond, whereas Mant-8-N₃-ATP has the *syn* conformation.

For gizzard myosin it was reported that Bz₂-ATP labelled the 50 kDa tryptic fragment of S-1 [21,22], as is found in skeletal muscle S-1 [20], and UDP in the presence of vanadate labelled Glu-185 of smooth muscle S-1 [23], as is found in *Acanthamoeba* myosin [24]. In the present study Mant-8-N₃-ATP was used as a probe of active-site conformation of smooth-muscle myosin.

MATERIALS AND METHODS

Myosin was isolated from frozen turkey gizzards [25]. Smooth-muscle myosin S-1 was prepared by *Staphylococcus aureus* proteinase digestion as described by Ikebe and Hartshorne [10]. Trypsin (Type VII) and papain were obtained from Sigma. Lysyl endopeptidase was obtained from Boehringer Mannheim. Papain was activated in 50 mM Tris/HCl (pH 7.5)/0.5 mM EDTA/20 mM dithiothreitol at 30 °C for 1 h before use.

Mant-8-N₃-ATP was prepared from 8-N₃-ATP (Sigma) as described by Maruta et al. [19]. Photoaffinity labelling of smooth-muscle myosin or S-1 with Mant-8-N₃-ATP was performed by irradiating 10–20 μ M myosin or S-1 with a 4-fold molar excess of Mant-8-N₃-ATP in 0.5 M (or 0.2 M) KCl/30 mM Tris/HCl (pH 7.5)/2 mM MgCl₂ at 366 nm by using a UVL-56 16 W (Ultra-Violet Products) lamp for 3 min at a distance of 2 cm above the surface of stirred solution at 0.5 ml. Modified myosin was digested with papain (enzyme/substrate 1:50, w/w) for 10 min at 25 °C, and the reaction was stopped by adding 5 mM of iodoacetamide. The digested protein was dialysed against 10 mM MgCl₂/20 mM Tris/HCl, pH 7.5, and centrifuged at 100 000 *g* for 60 min to remove undigested myosin and rods. The S-1 obtained was digested with trypsin (1:100, w/w) at 25 °C for 30 min, and the peptide composition was monitored by SDS/PAGE.

Electrophoresis was carried out on 7.5–20% (w/v) polyacrylamide gradient slab gels using the discontinuous buffer system described by Laemmli [26]. Molecular masses were estimated by using the following standards: smooth-muscle myosin heavy chain (200 kDa), β -galactosidase (116 kDa), phosphorylase *b* (97 kDa), BSA (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (29 kDa), trypsin inhibitor (20 kDa) and lysozyme (14.3 kDa). Fluorescence in the gels was detected under a 366 nm u.v. lamp (UVL-56), photographed and stained in 0.06% Coomassie Brilliant Blue R-250 (Sigma).

The 29 kDa N-terminal tryptic fragment was purified as follows. Myosin was photolabelled with Mant-8-N₃-ATP and digested with papain. The obtained S-1 was then digested with trypsin (1:100, w/w) at 25 °C for 5 min. The sample was freeze-dried, then dissolved in 2 ml of 6 M guanidinium chloride/2 mM EDTA/2 mM dithiothreitol at 25 °C for 60 min. After 4 vol. of ethanol had been added, the solution was left for 24 h at –18 °C. The 29 kDa fragment was selectively precipitated. The solution was centrifuged at 10 000 *g* for 30 min and the precipitates were dissolved in 1 ml of 6 M guanidinium chloride. The obtained 29 kDa fragment was dialysed against distilled water and freeze-dried. For further analysis the purified 29 kDa fragment labelled with the analogue was digested with lysyl endopeptidase (1:200, mol/mol) in a solution containing 4 M urea and 20 mM Tris/HCl, pH 7.5, at 30 °C for 5 h. The peptides were subjected to the aquapore RP-300 C-8 reverse-phase column attached to a

Perkin–Elmer Series 410 h.p.l.c. system. The peptides were eluted with linear acetonitrile gradient (0.1% trifluoroacetic acid, 0–90% acetonitrile, 0.1% trifluoroacetic acid) at a flow rate of 0.8 ml/min.

ATPase activity was estimated by measuring the liberated P_i by the methods of Youngburg and Youngburg [27]. Viscosity was measured at 25 °C in Cannon–Ubbelohde viscometers with water flow of approx. 25 s [6].

RESULTS

The hydrolysis of Mant-8-N₃-ATP by smooth-muscle myosin under three different conditions was measured and the results were compared with the hydrolysis of ATP (Table 1). The hydrolysis of Mant-8-N₃-ATP was significantly faster than that of ATP in the presence of Mg²⁺, whereas it was almost abolished in the presence of EDTA. Similar results were observed previously using skeletal muscle S-1 [19], and it was proposed that this characteristic bivalent cation dependence of Mant-8-N₃-ATP is due to the *syn* conformation of the analogue with respect to the N-glycosidic bond [28].

It is known that ATP induces the 10 S conformation at low ionic strength, and this conformational change can be monitored by viscosity measurements [6]. Figure 1 shows the KCl concentration-dependence on the relative viscosity of smooth-muscle myosin. As was found in the presence of ATP, the viscosity markedly decreased below 0.35 M KCl in the presence of Mant-8-N₃-ATP. This indicates that Mant-8-N₃-ATP also induces the 10 S myosin conformation at low ionic strength. It was shown previously [6] that the Mg²⁺- and Ca²⁺-ATPase activity of smooth-muscle myosin is paralleled by changes in the myosin conformation. As shown in Figure 2, the hydrolysis of Mant-8-N₃-ATP in the presence of Mg²⁺ markedly decreased below 0.3 M KCl, the point at which the myosin conformation changed from 6 S to 10 S.

It is known that myosin–ADP–P_i is the stable reaction intermediate in the presence of Mg²⁺-ATP; therefore, it is reasonable to assume that the myosin–ADP–P_i intermediate changes the conformational from 6 S to 10 S with decreasing ionic strength. As shown in Figure 3, in the presence of Mant-8-N₃-ADP and vanadate the viscosity of myosin showed the same ionic-strength-dependence as in the presence of ATP. The change in the viscosity of myosin was markedly reduced in the absence of vanadate. It is known that vanadate is a phosphate analogue, and myosin forms, in the presence of vanadate, a stable myosin–ADP–vanadate complex that is thought to be a transition-state analogue of myosin [29]. These results therefore suggest that Mant-8-N₃-ADP and vanadate form a myosin–ADP–vanadate-type complex, and this exhibits the conformational transition of

Table 1 Bivalent-cation-dependence of ATPase activity

The incubation mixture contained 0.5 μ M myosin, 0.5 mM ATP (or ATP analogue), 0.5 M KCl and 30 mM Tris/HCl pH 7.5. (a) 5 mM MgCl₂; (b) 5 mM CaCl₂; (c) 5 mM EDTA.

	Activity (mol of P _i /min per mol of myosin head)	
	Mant-8-N ₃ -ATP	ATP
(a) Mg ²⁺	9.6	1.1
(b) Ca ²⁺	13.0	14.0
(c) EDTA (K ⁺)	0.5	66.5

Figure 1 KCl-dependence of the relative viscosity of myosin in the presence of Mant-8-N₃-ATP

Myosin (2 mg/ml) and 0.5 mM ATP (○) or Mant-8-N₃-ATP (▲) was incubated at 25 °C in the solution containing 2 mM MgCl₂, 30 mM Tris/HCl pH 7.5, and various concentrations of KCl, and the viscosity of the solutions was measured as described in the Materials and methods section.

Figure 2 KCl-dependence on Mant-8-N₃-ATPase activity in the presence of Mg²⁺

Myosin (0.5 mg/ml) in 2 mM MgCl₂/30 mM Tris/HCl, pH 7.5, was incubated at 25 °C and the reaction was started by adding 0.5 mM ATP (○) or Mant-8-N₃-ATP (▲).

myosin, although the KCl-concentration-dependence of the transition is shifted to a lower KCl concentration as compared with ATP (Figure 3).

Figure 3 KCl-dependence of the relative viscosity of myosin in the presence of nucleotide and vanadate

Conditions: 4 mg/ml myosin, 8 μM ADP (○) or Mant-8-N₃-ADP (▲), 0.2 mM vanadate, 2 mM MgCl₂ and 30 mM Tris/HCl, pH 7.5, at 25 °C. Δ, 8 μM Mant-8-N₃-ADP in the absence of vanadate; □, 80 μM Mant-8-N₃-ADP + 1 mM vanadate.

Figure 4 Gel filtration of the myosin-Mant-8-N₃-ADP-vanadate complex

(a) Myosin, 5 μM in 0.5 M KCl/30 mM Tris/HCl (pH 7.5)/2 mM MgCl₂, was preincubated 20 min at 25 °C with 10 μM Mant-8-N₃-ATP to effect quantitative conversion into Mant-8-N₃-ADP. Vanadate at 0.2 mM was then added and reaction was allowed to proceed for 2.5 h at 25 °C. —, Absorbance at 280 nm; ----, relative fluorescence intensity in the presence of vanadate; — — —, relative fluorescence intensity in the absence of vanadate. Excitation was at 366 nm and emission was at 445 nm. (b) Myosin, 5 μM in 0.2 M KCl/30 mM Tris/HCl (pH 7.5)/2 mM MgCl₂, was trapped with 10 μM Mant-8-N₃-ATP according to the method described in (a). —, Absorbance at 280 nm; ----, relative fluorescence intensity in the presence of vanadate; — — —, relative fluorescence intensity in the absence of vanadate. Excitation was at 360 nm and emission was at 455 nm.

The difference in the KCl-dependence of the conformational transition between ADP-vanadate and Mant-8-N₃-ADP-vanadate may be due to the difference in the dissociation constant of the myosin-nucleotide-vanadate complex. Actually, at high concentrations of Mant-8-N₃-ADP, the viscosity at 0.25 M KCl decreased, as shown in Figure 3. The amount of Mant-8-N₃-ADP that bound to 6 S myosin and 10 S myosin was estimated by gel filtration (Figure 4). In the presence of stoichiometric nucleotide concentrations, only 6.5% of Mant-8-N₃-ADP was bound to 6 S myosin in the presence of vanadate, which is significantly lower than for the binding of ADP (70%). The bound Mant-8-N₃-ADP increased at higher nucleotide concen-

Figure 5 SDS/PAGE of the tryptic fragment of S-1 labelled with Mant-8- N_3 -ATP

SAP-S-1 (15 μ M) was labelled with 80 μ M Mant-8- N_3 -ATP in 120 mM NaCl/5 mM $MgCl_2$ /30 mM Tris/HCl, pH 7.8, in the absence of ATP (lanes 2 and 5) or in the presence of 2 mM ATP (lanes 3 and 6) as described in the Materials and methods section. S-1 labelled with Mant-8- N_3 -ATP was digested with trypsin (1:100, w/w). Lanes 1 and 4, Coomassie Brilliant Blue staining of S-1 (lane 1) and S-1 digested by trypsin (lane 4); lanes 2 and 3, fluorescence of S-1 modified by Mant-8- N_3 -ATP; lanes 5 and 6, fluorescence of S-1 digested by trypsin after modification with Mant-8- N_3 -ATP. Abbreviation: *M*, molecular mass.

Figure 6 SDS/PAGE of the purified 29 kDa fragment

29 kDa fragment was purified as described in the Materials and methods section. Myosin was labelled with Mant-8- N_3 -ATP in the 6 S or 10 S conformation, and the tryptic fragments were prepared as described in Figure 5. Lane 1, tryptic S-1 modified in the 10 S condition; lane 2, tryptic S-1 modified in the 6 S condition; lane 3, 29 kDa fragment purified from lane 1; lane 4, 29 kDa fragment purified from lane 2; lane 5, molecular-mass (*M*) standards.

trations (results not shown). On the other hand, 28.0% of nucleotide was bound to 10 S myosin in the presence of vanadate. This suggests that the dissociation constant for the formation of the ternary complex might be significantly lower when myosin forms the 10 S conformation. This is consistent with previously obtained findings [30], which showed that ATP is trapped by 10 S myosin. However, only 3% of Mant-8- N_3 -ADP was bound to the 10 S myosin in the absence of vanadate; therefore, a high level of ADP binding of 10 S myosin in the presence of vanadate is due to both the formation of 10 S myosin and the presence of vanadate. These results suggest that Mant-8- N_3 -ATP has a similar effect to that of ATP on the smooth-muscle myosin conformational transition, although the conformation of Mant-8- N_3 -ATP in solution is different from ATP with respect to the adenine-ribose bond.

To identify the binding site of Mant-8- N_3 -ATP, subfragment-1 prepared by *S. aureus* digestion (SAP-S-1) was allowed to

Figure 7 Elution profiles of C-8 reverse-phase chromatography

Purified 29 kDa fragment (1.2 mg) labelled with Mant-8- N_3 -ATP was digested by lysyl endopeptidase and the peptides produced were separated by C-8 reverse-phase chromatography (solvent A: 0.1% TFA; solvent B: 90% acetonitrile/0.1% trifluoroacetic acid). The peptides were eluted by 0–90% linear gradient of acetonitrile at a flow rate of 0.8 ml/min. The full-scale absorbance is 0.5. (a) Lysyl-endopeptidase digests of 29 kDa fragment modified in the 6 S conformation by Mant-8- N_3 -ATP; (b) lysyl-endopeptidase digests of 29 kDa fragment modified in the 10 S conformation by Mant-8- N_3 -ATP.

photoreact with Mant-8- N_3 -ATP. The photolabelling was carried out in the presence of a 4-fold molar excess of Mant-8- N_3 -ATP. After excess nucleotide had been removed on a Dowex 1-X8 column, the amount of the labelled nucleotide was estimated by measuring the fluorescence intensity in the presence of 5 M guanidinium chloride using the fluorescence intensity of Mant-8- N_3 -ATP as a standard. It was previously shown that SAP-S-1 is composed of the 68 kDa N-terminal fragment and the 26 kDa C-terminal fragment [10]. Mant-8- N_3 -ATP was incorporated into the 68 kDa fragment, and this was diminished in the presence of excess ATP (Figure 5). The labelled S-1 was then digested with trypsin. During the course of digestion, the 68 kDa N-terminal fragment was cleaved into the 29 kDa N-terminal fragment and a 50 kDa fragment, and the 26 kDa C-terminal fragment was further degraded into a 25 kDa fragment. As shown in Figure 5, Mant-8- N_3 -ATP was incorporated predominantly into the 29 kDa fragment, and the incorporation was significantly reduced when ATP was added. To study the effect of conformational change of 10 S to 6 S myosin on the ATP-binding site, photoaffinity labelling was performed on the 6 S and 10 S conformations. Fluorescence was observed in both the myosin heavy chain and 17 kDa light chain, but not in the 20 kDa light chain (results not shown). The photoaffinity-labelled myosin was then digested with papain and trypsin, and labelled fragments were analysed by SDS/PAGE. The fluorescence of Mant-8- N_3 -ATP was found to be predominantly associated with the 17 kDa light chain and 29 kDa fragment. The fluorescence incorporated into the 29 kDa fragment was markedly reduced when the labelling was carried out in the presence of excess ATP, whereas the fluorescence incorporated in the 17 kDa light chain was not significantly reduced in the presence of excess ATP. This suggests that the labelling of Mant-8- N_3 -ATP in the 29 kDa fragment was due to

the specific binding of Mant-8-N₃-ATP to myosin, while the labelling at the 17 kDa light chain was due to the non-specific binding of Mant-8-N₃-ATP. The non-specific labelling on the 17 kDa light chain was not observed when SAP-S-1 was used, although the 17 kDa light chain was present in S-1. This result further supports the notion that the labelling of the 17 kDa light chain is non-specific (since S-1 contains an ATPase active site) and suggests that the heavy-chain structure at the head-rod junction may contribute to the non-specific labelling of the 17 kDa light chain. The labelling of Mant-8-N₃-ATP was not significantly influenced by the myosin conformation (6 S→10 S), and the 29 kDa fragment was labelled to a similar extent in both conformations of myosin, although the fluorescence intensity in the gel was a little stronger in 29 kDa fragment obtained from 10 S myosin than that from 6 S myosin. To investigate further a possible change in the labelling site between the two conformations, the labelled 29 kDa fragment was isolated (Figure 6) and subjected to the complete lysyl endopeptidase digestion. The obtained peptides were analysed by C-8 reverse-phase chromatography. As Figure 7 shows, the peptides which were obtained from the labelled 6 S and 10 S myosin showed the same elution profile, as monitored by the fluorescence; however, the incorporated fluorescence intensity was 30% higher in the peptides obtained from 10 S myosin than those from 6 S myosin as measured by the fluorimeter. This suggests that the Mant-8-N₃-ATP-binding site was not significantly different in the 6 S and 10 S myosins.

DISCUSSION

It is known that the ATP analogue which modifies at the eighth position of the adenine ring with a bulky group has a *syn* conformation with respect to the N-glycosidic bond [28]. Mant-8-N₃-ATP, with a bulky group at the eighth position, is also thought to adopt the *syn* conformation with respect to the N-glycosidic bond [19]. The ATP analogues having a *syn* conformation show a characteristic bivalent-cation-dependence, i.e. high Mg²⁺-ATPase and very low EDTA(K⁺)-ATPase [28], and the results obtained here (Table 1) are consistent with this criterion.

The interesting finding of the present study is that, although Mant-8-N₃-ATP retains the *syn* conformation, it can also induce the conformational transition of myosin from 6 S to 10 S at low ionic strength. Since this change in conformation can also be brought about by other nucleotides, such as CTP, GTP and ITP (M. Ikebe, unpublished work), and since these effects are enhanced by divalent cations [6], it is likely that the triphosphate moiety, but not the base moiety, is important in inducing the 6 S→10 S transition.

It is known that ADP is trapped in the ATPase active site of skeletal-muscle myosin in the presence of vanadate, forming a myosin-Mg²⁺-ADP/vanadate complex [29]. In the presence of vanadate, ADP was trapped in the active site of smooth-muscle myosin, and the complex showed the same 6 S→10 S transition as that seen in the presence of ATP (Figure 3).

It is proposed that myosin-Mg²⁺-ADP-vanadate complex corresponds to the transition-state analogue of myosin ATPase; therefore, it is reasonable to suppose that myosin-Mg²⁺-ADP-vanadate complex showed the 6 S→10 S transition. It is not clear, however, whether or not the formation of the transition state is required to express the 6 S→10 S transition. It has been shown that a 6 S→10 S transition occurs in the presence of Ca²⁺-ATP or Mg²⁺-adenosine 5'-[βγ-imido]triphosphate [6,13], but a different conformation from that of myosin-Mg²⁺-ADP-P_i is formed. Therefore, it may be concluded that the 6 S→10 S

transition does not depend on the existence of a myosin-Mg²⁺-ADP-P_i state, but does require bivalent cation and triphosphate binding. Although some binding of Mant-8-N₃-ADP to myosin and S-1 in the presence of vanadate was observed, the binding was significantly lower than ADP and vanadate. This can be explained by the high dissociation constant for 6 S myosin, Mant-8-N₃-ADP and vanadate. Actually the bound nucleotide was increased at higher nucleotide concentration (Figure 3). On the other hand, the binding of Mant-8-N₃-ADP to 10 S myosin in the presence of vanadate was significantly higher than to 6 S myosin, and this suggests a lower nucleotide-10 S myosin dissociation constant. In the presence of vanadate and Mant-8-N₃-ADP, myosin also showed the 6 S→10 S transition, although the KCl-dependence was shifted to a lower concentration. This shift can be explained by myosin-Mant-8-N₃-ADP-vanadate having two different dissociation constants for 6 S and 10 S myosin:

where K_{d1} is significantly higher than K_{d2} , and $K_{d'}$ is dependent on the KCl concentration. When ADP is used instead of Mant-8-N₃-ADP, K_{d1} and K_{d2} are very low; therefore, the KCl-dependence of the 6 S→10 S transition (viscosity data) exactly paralleled the KCl-dependence of $K_{d'}$. However, the case of Mant-8-N₃-ADP, the equilibrium shifted to the left side (6 S conformation) at relatively high ionic strength. At low ionic strength, where

shifts completely to the right, the predominant amount of myosin forms the 10 S conformation, since K_{d2} is low enough to make the 10 S myosin-Mant-8-N₃-ADP-vanadate complex.

Previously it was reported that Mant-8-N₃-ATP labels the 20 kDa C-terminal fragment of skeletal-muscle S-1 [19], unlike other photoaffinity-labelling analogues, which label the 27 kDa N-terminal fragment of skeletal-muscle S-1 [16,18]. It was suggested that this is due to the *syn* conformation of the analogue and the hydrophobic group in the ribose moiety of Mant-8-N₃-ATP. In the present study, Mant-8-N₃-ATP labelled the 29 kDa N-terminal fragment of smooth muscle in contrast with the labelling of skeletal-muscle myosin. This suggests the difference between the active-site conformation of smooth-muscle myosin and that of skeletal-muscle myosin. This is consistent with the recent observation [32] that azidonitrobenzoyl-8-N₃-ATP, which has a similar structure to Mant-8-N₃-ATP, cross-linked the 20 kDa C-terminus and 25 kDa N-terminus fragments of tryptic skeletal-muscle myosin S-1, but did not cross-link the 29 kDa N-terminus and 25 kDa C-terminus tryptic fragments of smooth-muscle myosin S-1. It was reported [33,34] that ATP analogues which form the *syn* conformation with respect to their N-glycosidic bond can alter their conformation to *anti* when they bind to protein. Since ATP analogues which have the *anti* conformation are bound to the N-terminal fragment of skeletal muscle S-1 [19], Mant-8-N₃-ATP may change its conformation from *syn* to *anti* when it binds to the smooth-muscle-myosin active site, but not in the skeletal-muscle-myosin active site. Quite recently, the three-dimensional structure of skeletal-muscle S-1 was determined by X-ray crystallography, and results suggest that the nucleotide binds in a cleft formed by the 25 kDa and

50 kDa region of the heavy chain. The end of the cleft is capped by a segment of the 20 kDa region (I. Rayment, personal communication). The present results therefore suggest that the way in which nucleotide binds in this cleft may differ between smooth and skeletal muscle. It is known that smooth-muscle myosin forms two distinct conformations (6 S and 10 S) which are characterized by distinct enzymic activities [6]. Furthermore it has been proposed that the 6 S→10 S conformational transition is regulated by phosphorylation, and therefore the 6 S→10 S transition, or some associated conformational change, could be a component of the regulatory mechanism of smooth muscle. In the present study the Mant-8-N₃-ATP-binding sites of 6 S and 10 S myosin were investigated; however, no difference in the location of the binding site was detected. Recently, Cole and Yount [22] reported that the dibenzoyl-ADP-binding site of gizzard myosin was Pro-324 for both the 6 S and the 10 S myosin. Since Mg²⁺-triphosphate binding may be important for the 6 S→10 S transition, as discussed above, the conformational change essential for the regulation may occur at the phosphate-binding sites, but not at adenine or ribose sites. The identification of such a conformational change in the active site needs further study.

This work was supported by The National Institutes of Health (grant no. AR38888). M.I. is an Established Investigator of the American Heart Association, and S.M. is a recipient of a Postdoctoral Research Fellowship from the American Heart Association, North-East Ohio Affiliate.

REFERENCES

- Hartshorne, D. J. (1987) in *Physiology of the Gastrointestinal Tract* (Johnson, L. R., ed.), vol. 1, 2nd edn., pp. 423–482, Raven Press, New York
- Suzuki, H., Kamata, T., Onishi, H. and Watanabe, S. (1982) *J. Biochem. (Tokyo)* **91**, 1699–1705
- Onishi, J. and Wakabayashi, T. (1982) *J. Biochem. (Tokyo)* **92**, 871–879
- Trybus, K. M., Huiatt, T. M. and Lowey, S. (1982) *Proc. Natl. Acad. Sci. U.S.A.* **79**, 6151–6155
- Craig, R., Smith, R. and Kendrick-Jones, J. (1983) *Nature (London)* **302**, 436–439
- Ikebe, M., Hinkins, S. and Hartshorne, D. J. (1983) *Biochemistry* **22**, 4580–4587
- Ikebe, M. and Hartshorne, D. J. (1984) *J. Biol. Chem.* **259**, 11639–11642
- Onishi, H., Wakabayashi, T., Kamata, T. and Watanabe, S. (1983) *J. Biochem. (Tokyo)* **94**, 1147–1154
- Somlyo, A. V., Butler, T. M., Bond, M. and Somlyo, A. P. (1981) *Nature (London)* **294**, 567–569
- Ikebe, M. and Hartshorne, D. J. (1985) *Biochemistry* **24**, 2380–2387
- Higashihara, M., Young-Frado, L. L., Craig, R. and Ikebe, M. (1989) *J. Biol. Chem.* **264**, 5218–5225
- Suzuki, H., Stafford, W. F., III, Slayter, H. S. and Seidel, J. C. (1985) *J. Biol. Chem.* **260**, 14810–14817
- Hartshorne, D. J. and Ikebe, M. (1987) in *Platelet Activation* (Yamazaki, H. and Mustard, J. F., eds.), pp. 3–16, Academic Press, Tokyo
- Ikebe, M. and Takashi, R. (1990) *Biophys. J.* **57**, 331a
- Morita, J., Takashi, R. and Ikebe, M. (1991) *Biochemistry* **30**, 9539–9545
- Szilagyi, L., Balint, M., Streter, F. A. and Gergely, J. (1979) *Biochem. Biophys. Res. Commun.* **87**, 936–945
- Nakamaye, K. L., Wells, J. A., Bridengaugh, R. L., Okamoto, Y. and Yount, R. G. (1985) *Biochemistry* **24**, 5226–5235
- Okamoto, Y. and Yount, R. G. (1985) *Proc. Natl. Acad. Sci. U.S.A.* **82**, 1575–1579
- Maruta, S., Miyashita, T. and Matsuda, G. (1989) *Eur. J. Biochem.* **184**, 213–221
- Mahmood, R. and Yount, R. G. (1984) *J. Biol. Chem.* **259**, 12956–12959
- Srivastava, S., Cable, M. B. and Driska, S. P. (1986) *Eur. J. Biochem.* **156**, 447–451
- Cole, D. G. and Yount, R. G. (1990) *J. Biol. Chem.* **265**, 22537–22546
- Garabedian, T. E. and Yount, R. G. (1990) *J. Biol. Chem.* **265**, 22547–22557
- Atkinson, M. A., Robinson, E. A., Appella, E. and Korn, E. D. (1986) *J. Biol. Chem.* **261**, 1844–1848
- Ikebe, M. and Hartshorne, D. J. (1985) *J. Biol. Chem.* **260**, 13146–13153
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- Youngberg, G. E. and Youngberg, M. V. (1930) *J. Lab. Clin. Med.* **16**, 158–166
- Takenaka, H., Ikehara, M. and Tonomura, Y. (1978) *Proc. Natl. Acad. Sci. U.S.A.* **75**, 4229–4233
- Goodno, C. C. (1979) *Proc. Natl. Acad. Sci. U.S.A.* **76**, 2620–2624
- Cross, R. A., Cross, K. E. and Sobieszek, A. (1986) *EMBO J.* **5**, 2637–2641
- Ikebe, M. and Hartshorne, D. J. (1986) *Biochemistry* **25**, 6177–6185
- Maruta, S., Burke, M. and Ikebe, M. (1990) *Biochemistry* **29**, 9910–9915
- Garin, J., Vignais, P. V., Gronenborn, A. M., Clore, G. M., Gao, Z. and Baeuerlein, E. (1988) *FEBS Lett.* **242**, 178–182
- Abdallah, M. A., Biellmann, J. F., Nordstrom, B. and Branden, C. I. (1975) *Eur. J. Biochem.* **50**, 475–481