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Cross-Linking of the 25- and 20-Kilodalton Fragments of Skeletal Myosin Subfragment 1 by a Bifunctional ATP Analogue†

Shinsaku Maruta,‡ Morris Burke,§ and Mitsuo Ikebe*,‡

Department of Physiology and Biophysics, School of Medicine, and Department of Biology, Case Institute of Technology, Case Western Reserve University, Cleveland, Ohio 44106

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ABSTRACT: The bifunctional photoreactive ATP analogue azidonitrobenzoyl-8-azido-ATP (ANB-8-N_3-ATP) was synthesized. This ATP analogue carriers photoreactive azido groups at the eighth position of the adenine ring and at the 3′ position of ribose. Photolysis of this analogue in the presence of skeletal muscle α-chymotryptic subfragment 1 (S-1) resulted in a new 120-kDa band, while photolysis in the presence of the tryptic S-1 produced a new 45-kDa band. The 45-kDa peptide was shown to be combined with the 25-kDa N-terminal and 20-kDa C-terminal fragments since it was labeled with a monoclonal antibody specific for the N-terminal 25-kDa segment of the S-1 heavy chain, and it was also found to retain the fluorescence of (iodoacetamido)fluorescein attached specifically to the SH-1 thiol of the C-terminal 20-kDa segment. These results indicate that the 25- and 20-kDa peptides are in close contact with the ATPase active site.

It is well established that muscle contraction is performed by the relative sliding of thick and thin filaments (Huxley, 1969) in which the energy is supplied by ATP hydrolysis catalyzed by myosin. Although this scenario is well established, the molecular mechanism of energy transduction is still obscure. To shed light on this problem, it is essential to characterize the structure of the myosin active site. The assignment of the functionally important domains has been developed by using limited proteolysis. Initially, it was found that the head region of the myosin molecule [subfragment 1 (S-1)][1] (Margossian & Lowey, 1973) contains both the ATPase and actin binding sites. The 95-kDa heavy chain of S-1 is further cleaved by trypsin into three substructures connected by two protease-sensitive hinges (Balint et al., 1978; Mornet et al., 1979; Yamamoto & Sekine, 1979). Beginning with the N-terminus of the heavy chain, the approximate sizes of these three fragments are 25, 50, and 20 kDa. The N-terminal 25-kDa peptide contains the reactive lysine residue (Mornet et al., 1981a; Hozumi & Muhlrad 1981), while an activity-critical carboxyl group (Korner et al., 1983) and part of the actin binding site (Mornet et al., 1981b; Sutoh, 1983) have been located on the 50-kDa peptide. The two reactive thiols of myosin, termed SH-1 and SH-2, are located on the 20-kDa peptide (Balint et al., 1978), and it has been known that the modification of these thiols significantly alters the ATPase activity of myosin (Sekine et al., 1962). For example, the cross-linking of these thiols by bifunctional cross-linking reagents in the presence of Mg^{2+}-ADP abolishes the ATPase activity (Burke & Reisler, 1977) which is due to the stable trapping of Mg^{2+}-ADP on the enzyme (Wells & Yount, 1979).

To localize the ATP binding site on the myosin molecule, several photoaffinity labeling ATP analogues have been used. Szilagyi et al. (1979) showed that arylazido-β-alanyl-ATP specifically labeled the 25-kDa tryptic peptide. The 25-kDa peptide was also labeled by NANDP, a photoaffinity analogue of ADP (Nakamaye et al., 1985; Okamoto & Yount, 1985) and Mant-2-N_3-ADP (Maruta et al., 1989). On the other hand, Mahmood et al. (1984) showed that Bz_2-ATP containing a photoprobe at the ribose ring labeled the 50-kDa peptide. Recently, Maruta et al. (1989) reported that Mant-8-N_3-ATP labeled the 20-kDa peptide of myosin and suggested the close proximity of the 20-kDa peptide to the ATP binding site. In this paper, the bifunctional photoreactive ATP analogue

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* Address correspondence to this author.
‡ Department of Physiology and Biophysics.
§ Department of Biology.

[1] Abbreviations: S-1, subfragment 1; ANB, azidonitrobenzoyl; NANDP, *N*-(4-azido-2-nitrophenyl)-2-aminoethyl diphosphate; Bz_2, 4-benzoylbenzoyl; IAF, 5-(iodoacetamido)fluorescein; Mant, methylanthraniloyl; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; TLC, thin-layer chromatography; IR, infrared; SH-1, reactive thiol 1; SH-2, reactive thiol 2; PBS, phosphate-buffered saline; DMF, dimethylformamide; MOPS, 4-morpholinepropanesulfonic acid.

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ANB-8-N_3-ATP was synthesized and used as a probe to study the topological orientation of the ATP binding site.

Materials and Methods

Synthesis of Azidonitrobenzoyl-8-azido-ATP (ANB-8-N_3-ATP). Coupling of 8-N_3-ATP and 5-azido-2-nitrobenzoic acid was carried out by the method of Guillory and Jeng (1977). Azidonitrobenzoic acid (300 μmol) and carbonyldiimidazole (1 mmol) were stirred for 20 min at room temperature in 2 mL of dry DMF. 8-N_3-ATP (30 μmol) was dissolved in 2 mL of water and added dropwise to the reaction mixture. The coupling reaction was allowed to proceed for 4 h at 35 °C in the dark. Five volumes of chilled acetone (−18 °C) were then added, and the precipitate was collected by centrifugation (4500 rpm) for 20 min and dissolved in 3 mL of water. The product was purified by DEAE-Sephacel column chromatography (1.5 × 16 cm). The elution was carried out with a linear gradient of ammonium acetate from 0.1 to 1.25 M (500 mL of each). Three major peaks were obtained. The first peak eluted was unreacted azidonitrobenzoic acid, and the second was the unreacted 8-N_3-ATP. The third peak contained ANB-8-N_3-ATP, and the yield was approximately 15%. The fractions were collected and lyophilized. The purity of the product was analyzed by TLC (cellulose) using two solvents. In both solvents, a single spot of the product was observed, and the R_f was 0.65 for 1-butanol/water/acetic acid (5:3:2) and 0.68 for isobutyric acid/ammonium hydroxide/water (75:1:24).

Preparation of Proteins. Myosin was prepared from chicken (hubbered-type) breast muscle and rabbit back muscle according to Perry (1952) and was digested by α-chymotrypsin to obtain subfragment 1 (S-1) as reported by Weeds and Taylor (1975). Tryptic digestion of S-1 was carried out according to Mornet et al. (1981b). Monoclonal antibody which recognizes the N-terminal 23 residues (Rajasekharan et al., 1989) of rabbit skeletal myosin heavy chain was kindly supplied from Dr. K. Rajasekharan. Smooth muscle myosin was prepared from turkey gizzards as described previously (Ikebe & Hartshorne, 1985). Papain S-1 and *Staphylococcus aureus* protease S-1 were prepared according to the methods of Ikebe and Hartshorne (1985) and Ikebe and Hartshorne (1986), respectively.

Photoaffinity Labeling. Photoaffinity labeling of S-1 or tryptic S-1 with ANB-8-N_3-ATP was performed by irradiating at 366 nm (UVL-56 16W, Ultra-Violet Products) for 3 min 10–20 μM S-1 in the presence of excess ANB-8-N_3-ATP at a distance of 2 cm above the surface of the stirred solution of 250–500 μL containing 0.5 M KCl, 30 mM Tris-HCl, pH 7.5, and 5 mM $MgCl_2$.

Chemical Modification. S-1 (100 μM) was labeled with a 10-fold molar excess of IAF in a solution of 0.15 M KCl and 50 mM MOPS buffer at pH 7.0 at 0 °C for 30 min in the dark according to the method of Takashi (1979). The labeling was terminated upon addition of a 100-fold molar excess of β-mercaptoethanol over IAF. Subsequently, the S-1 labeled with IAF was separated from free IAF by using a Sephadex G-25 column equilibrated with 0.15 M KCl and 30 mM Tris-HCl, pH 7.0 at 4 °C.

Gel Electrophoresis and Western Blots. Electrophoresis was performed in 7.5–20% polyacrylamide gradient slab gels in the presence of 0.1% SDS at 35 mA of constant current in the discontinuous buffer system of Laemmli (1970). The proteins were stained with Coomassie brilliant blue (CBB), and the optical densities of the bands and their mass distributions were determined with a scanning densitometer (Hoefer Scientific Instruments GS300) connected to the integrator (Perkin-Elmer LCI-100). The amount of the 120-kDa band was expressed as a percentage of the total amount of heavy chain; i.e., % 120 kDa = $(A_{120}/A_{120} + A_H) \times 100$, where A_{120} and A_H are the peak areas of the 120-kDa band and the un-cross-linked heavy chain, respectively. Molecular weights were estimated by using the following standards: smooth muscle myosin heavy chain (200000), β-galactosidase (116000), phosphorylase *b* (97000), bovine serum albumin (66000), ovalbumin (45000), carbonic anhydrase (29000), trypsin inhibitor (20100), and lysozyme (14300). Electrophoretic transfer of the polypeptide patterns from SDS–polyacrylamide gels to nitrocellulose (Schleicher & Schuell) was done as described by Towbin et al. (1979) for 3 h at 6 V/cm. The sheet was cut into vertical strips, and one strip was stained with Amido-Black. Other strips were impregnated in PBS–Tween buffer (PBS with 0.05% Tween 20) containing 2% bovine serum albumin for 2 h at room temperature to saturate nonspecific protein binding sites. The strips were then placed in PBS–Tween buffer containing 10–100 μg/mL purified monoclonal antibody for 1 h at room temperature. After a series of washings, the strips were soaked in PBS–Tween buffer containing 20 μg/mL peroxidase-conjugated anti-mouse IgG antibody for 30 min. After the subsequent washing, color was developed for 10 min in a fresh solution of 0.5% 4-chloro-1-naphthol, 50 mM Tris-HCl, pH 7.5, 200 mM NaCl, and 0.02% H_2O_2. The strips were washed with water to stop the reaction and were dried in the air.

Hydrolytic Cleavage of the Cross-Link. Fluorescence-labeled tryptic S-1 modified with ANB-8-N_3-ATP was subjected to SDS–PAGE. The fluorescent band corresponding to the 45-kDa fragment was cut out from gels under illumination with a UV lamp. The 45-kDa fragment was eluted out of gels by electrophoresis and collected into dialysis tubes. Isolated 45-kDa fragment in the dialysis tube was incubated in alkaline solution (0.5 M $NaHCO_3$, 1% SDS, and 0.1 M NaOH) at 37 °C for 24 h to hydrolyze the ester bond in ANB-8-N_3-ATP (Schafer et al., 1985). After dialysis against a running buffer of SDS–PAGE to remove the excess amount of alkaline reagent, the fragment was applied to the SDS–PAGE.

Other Procedures. One micromolar S-1 in 0.5 M KCl, 5 mM $MgCl_2$, $CaCl_2$, or EDTA, and 50 mM Tris-HCl, pH 7.5, was mixed with 1 mM ATP or ANB-8-N_3-ATP at 25 °C, and the liberated P_i was determined by the methods of Youngburg and Youngburg (1930).

Results and Discussion

ANB-8-N_3-ATP (Figure 1A) was synthesized by the coupling of 8-N_3-ATP and 5-azido-2-nitrobenzoic acid. The products were purified with DEAE-Sephacel chromatography as shown in Figure 1B. The first and second peaks were identified as 5-azido-2-nitrobenzoic acid [R_f = 0.31 for butane/acetone/H_2O, 65:25:15 (Lewis et al., 1977)] and 8-N_3-ATP [R_f = 0.29 for butanol/acetic acid/H_2O, 5:2:3 (Czarneck et al., 1979)], using silica gel and cellulose chromatography, respectively. The fourth peak (C) eluted at thin layer fractions 34–39 was found to be ANB-8-N_3-ATP because of the following reasons: (1) The R_f value from thin-layer chromatography was different from those of 5-azido-2-nitrobenzoic acid and 8-N_3-ATP. (2) The NO_2 group was found by IR spectrum analysis. (3) The UV spectrum showed the peaks at 280 nm due to 8-azidoadenosine (Maruta et al., 1989) and at 320 nm due to nitrobenzoyl group (Lewis et al., 1977) (Figure 1C). No obvious contamination was observed under the TLC analysis, yet myosin hydrolyzed this compound and released inorganic phosphate (see Table I). The hydrolysis of ANB-8-N_3-ATP by myosin under different conditions was measured, and the results were compared to the hydrolysis of

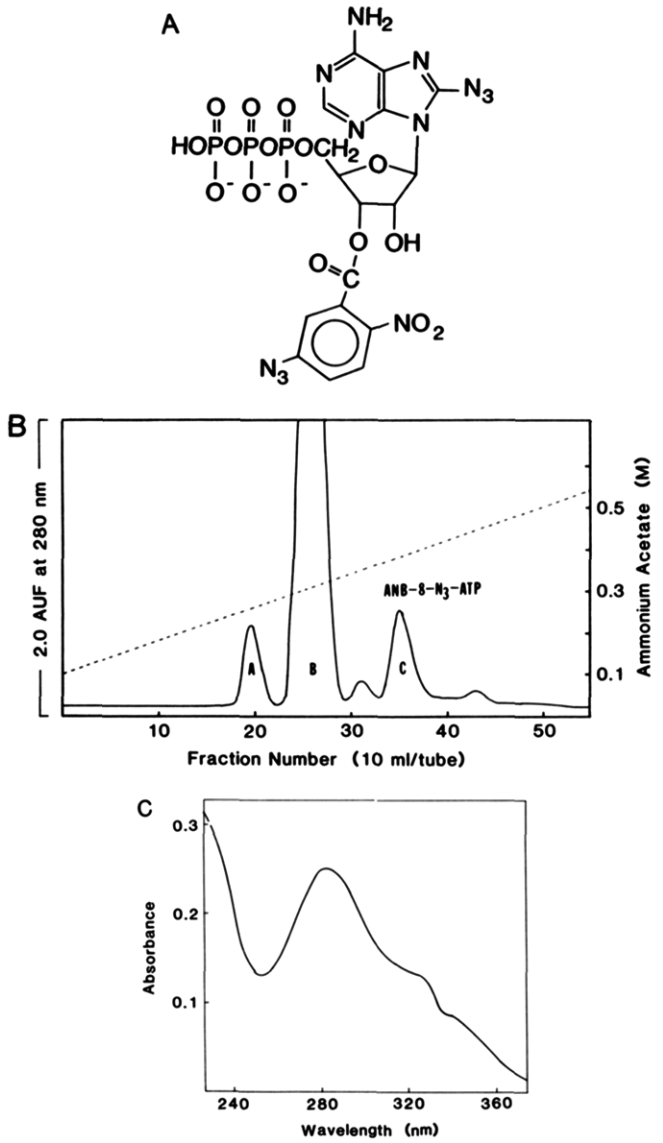

FIGURE 1: (A) Structural formula of azidonitrobenzoyl-8-N_3-ATP (ANB-8-N_3-ATP). (B) Purification of ANB-8-N_3-ATP by DEAE-Sephacel chromatography. The sample in water was applied on the column (1.5 × 16 cm) equilibrated with 0.1 M ammonium acetate. Fractions were eluted with a linear ammonium acetate gradient (0.1–1.25 M). Fractions (10 mL/tube) were collected at a flow rate of 100 mL/h. Peak A, unreacted azidonitrobenzoic acid; peak B, unreacted ATP; peak C, ANB-8-N_3-ATP. (C) Ultraviolet absorption spectrum of ANB-8-N_3-ATP. The spectrum was measured in 20 mM Tris-HCl, pH 7.5, using a Perkin-Elmer Lambda 4A UV/VIS spectrophotometer.

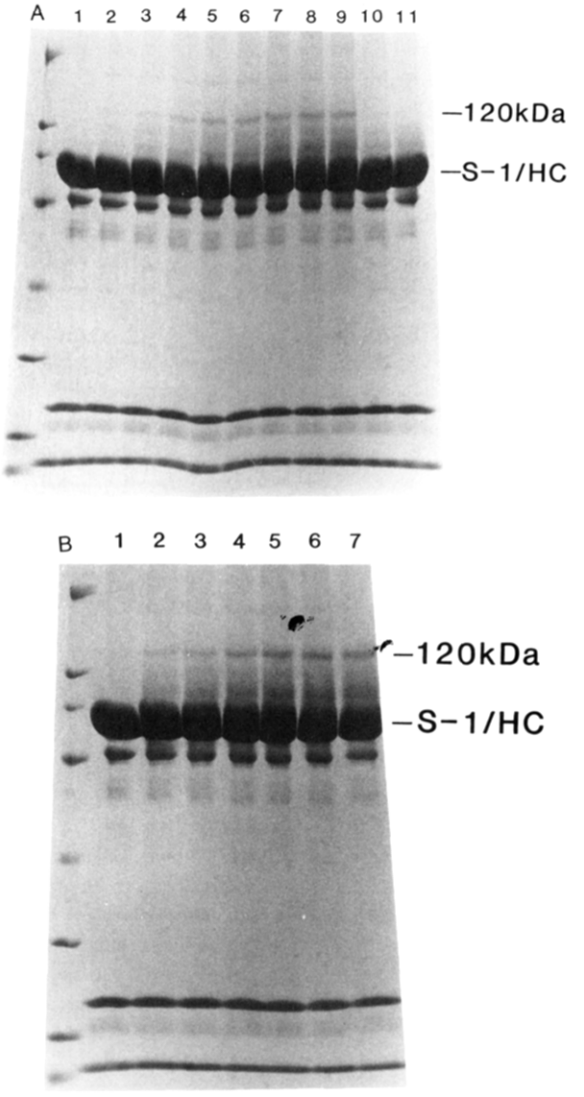

FIGURE 2: SDS–PAGE of skeletal muscle S-1 photolabeled with ANB-8-N_3-ATP. (A) Dependence of ANB-8-N_3-ATP concentration. 10 μM S-1 was photolabeled with 0, 10, 20, 30, 40, 50, 60, 70, and 80 μM ANB-8-N_3-ATP (lanes 1, 2, 3, 4, 5, 6, 7, 8, and 9, respectively) in a solution containing 0.5 M KCl, 30 mM Tris-HCl, pH 7.5, and 5 mM $MgCl_2$ for 5 min. Lane 10, in the presence of 30 μM ANB-8-N_3-ATP and 2 mM ATP. Lane 11, in the presence of 60 μM ANB-8-N_3-ATP and 2 mM ATP. Left lane, molecular weight markers. (B) Time course of photoirradiation. 10 μM S-1 was irradiated for 0, 1, 2, 3, 5, 7, and 10 min (lanes 1, 2, 3, 4, 5, 6, and 7, respectively) in a solution containing 0.5 M KCl, 30 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, and 50 μM ANB-8-N_3-ATP. Left lane, molecular weight markers.

Table I: Divalent Cation Dependence of ATPase Activity

	act. [mol of P_i·(mol of S-1)$^{-1}$·min^{-1}]	
	ANB-8-N_3-ATP	ATP
Mg^{2+}	23.7	1.2
Ca^{2+}	108.7	30.5
EDTA(K^+)	2.6	258.0

ATP (Table I). In the presence of Mg^{2+} and Ca^{2+}, S-1 hydrolyzed ANB-8-N_3-ATP significantly faster than ATP, while the hydrolysis of ANB-8-N_3-ATP in the absence of divalent cations was almost abolished.

It was reported previously (Takenaka et al., 1978) that the substitution of H at the eighth position of the adenine ring with bulky groups such as Br and N_3 altered the stereochemical conformation of ATP from the anti form to the syn form with respect to the adenine–ribose bond. It was also reported (Tonomura et al., 1967) that this change in the stereochemical conformation appeared to alter the ATPase activity [increase in the Mg^{2+}- and Ca^{2+}-ATPase activity and decrease in EDTA(K^+)-ATPase activity]. Therefore, these present ATPase data suggest that the stereochemical conformation of ANB-8-N_3-ATP is the syn form with respect to the adenine–ribose ring.

α-Chymotryptic S-1 was photolabeled with ANB-8-N_3-ATP which carries the photoreactive azido group on both adenine and ribose rings by irradiation with UV light at 366 nm. To reduce the nonspecific labeling, the photoreaction was carried out in the presence of high salt (0.5 M KCl). As shown in Figure 2, 120-kDa band was produced by photoreaction of ANB-8-N_3-ATP with S-1 (Figure 2). The formation of the 120-kDa band was increased with the irradiation time (Figure 2B). The amount of the 120-kDa band was estimated by using

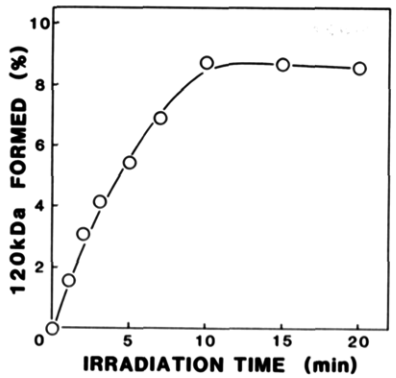

FIGURE 3: Irradiation time course of 120-kDa band formation in subfragment 1. The amount of 120-kDa band was determined by using a densitometer from the gel of Figure 2A (1–10 min) and the gel of samples irradiated for 10 and 15 min (data not shown).

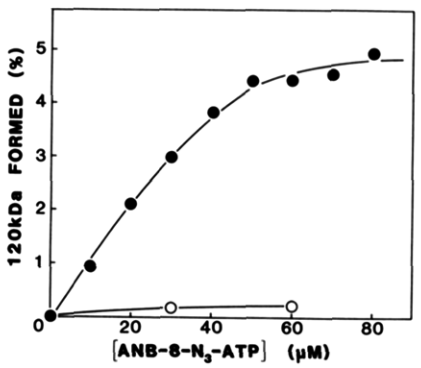

FIGURE 4: ANB-8-N_3-ATP concentration dependency of 120-kDa band formation in subfragment 1. The amount of 120-kDa band was determined from the gel of Figure 2B with the same methods of Figure 3. (●) In the absence of ATP; (○) in the presence of ATP.

densitometric analysis of the Coomassie brilliant blue stained gel. As shown in Figure 3, the formation of the 120-kDa product increased with radiation time and reached its maximum value (9%) at 10 min. The production of the 120-kDa band also increased with ANB-8-N_3-ATP concentration (Figures 2A and 4). In the presence of 2 mM ATP, the production of the 120-kDa band was almost completely abolished (Figures 2A and 4). This suggests that ATP interferes with the binding of the ANB-8-N_3-ATP to S-1 and abolishes the formation of the 120-kDa band. It should also be mentioned that the formation of the 120-kDa band was not observed without ANB-8-N_3-ATP (Figure 2A,B) or without irradiation (data not shown). Therefore, it was concluded that the production of the 120-kDa band was due to the specific-affinity cross-linking of the S-1 molecule by ANB-8-N_3-ATP. The cross-linking was much more efficient in the presence of Mg^{2+} than in the presence of Ca^{2+} (2 mM) or EDTA (2 mM) (data not shown). This is consistent with the fact that the K_M of Mg^{2+}-ATP is much lower than that of Ca^{2+}-ATP or K^+-ATP. The production of another band, observed just above the 95-kDa S-1 heavy chain, was dependent on the ANB-8-N_3-ATP concentration; however, it was not significantly affected by the addition of ATP (Figure 2A). Therefore, the formation of this band is likely to be due to nonspecific photolabeling of S-1.

To identify the cross-linking site of S-1 by ANB-8-N_3-ATP, tryptic S-1 was photo-cross-linked with ANB-8-N_3-ATP. The cross-linked sample was subjected to SDS–PAGE, and then the proteins in the gel were electroblotted to a nitrocellulose membrane. To reduce nonspecific binding, a low molar excess of ANB-8-N_3-ATP to tryptic S-1 (4:1) was used in these

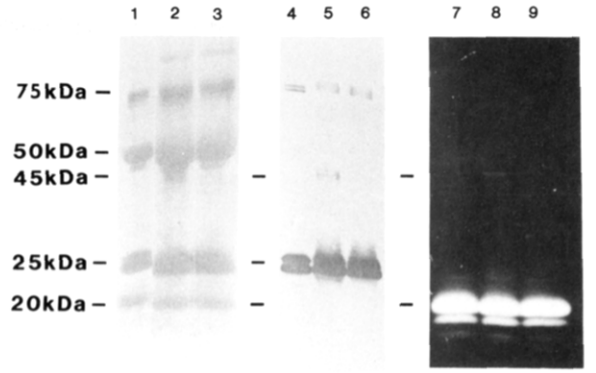

FIGURE 5: Cross-linking of tryptic S-1 with ANB-8-N_3-ATP. Tryptic S-1 was cross-linked with 4 times molar excess ANB-8-N_3-ATP. After the sample was subjected to SDS–PAGE, the peptides were transblotted to the nitrocellulose membrane. Lanes 1–3, amido black staining; lanes 4–6, immunostaining; lanes 1 and 4, tryptic S-1 photoirradiated without ANB-8-N_3-ATP; lanes 2 and 5, tryptic S-1 photolabeled with ANB-8-N_3-ATP; lanes 3 and 6, tryptic S-1 photolabeled with ANB-8-N_3-ATP in the presence of 2 mM ATP. Tryptic S-1 labeled with IAF was photolabeled with ANB-8-N_3-ATP and was subjected to SDS–PAGE. The gels were viewed under ultraviolet light (lanes 7–9). Lane 7, photoirradiated tryptic IAF–S-1; lane 8, tryptic IAF–S-1 photolabeled with ANB-8-N_3-ATP; lane 9, tryptic IAF–S-1 photolabeled with ANB-8-N_3-ATP in the presence of 2 mM ATP.

experiments. Under this condition, the yield of the 45-kDa band was estimated to be 2% by densitometric analysis of the stained gel (data not shown). The membrane was then immunostained by using an antibody which recognizes the N-terminal 23 amino acid residues of rabbit skeletal myosin (Rajasekharan et al., 1989). The antibody recognized 75-, 45-, and 25-kDa peptides (Figure 5, lane 4) but not 20- and 50-kDa peptides (Figure 5, lane 4). The 75-kDa peptide is thought to be the precursor of 25- and 50-kDa peptides. The production of the 45-kDa peptide was abolished when the photo-cross-linking reaction was carried out in the presence of excess ATP (Figure 5, lane 6). These results indicate that the 45-kDa peptide was the cross-linked product induced by the specific binding of ANB-8-N_3-ATP to the active site and contains the 25-kDa N-terminal fragment of S-1.

The peptide composition of the cross-linked 45-kDa peptide was also monitored by using the fluorescence labeled S-1 in which SH-1 in the 20-kDa peptide was labeled with 5-(iodoacetamido)fluoresceine (IAF). The modification is highly specific to SH-1 as shown in Figure 5 (lane 7), in which the 20-kDa band is the only peptide which is significantly labeled, although trace amounts of fluorescence can be detected at 50-kDa and at about 70–75 kDa. The trace of fluorescence associated with the 50-kDa band can be attributed to a very minor extent of nonspecific labeling of this segment of S-1 by IAF while that associated with the 70–75-kDa region is likely to be associated with the residual fragments of the precursor of the 20- and 50-kDa segments. Photolysis in the presence of ANB-8-N_3-ATP (Figure 5, lane 8) did not alter the pattern of fluorescence observed with the nonirradiated control (Figure 5, lane 7) except that a new fluorescent band of the 45-kDa peptide can now be observed. Furthermore, the fluorescence of this band was markedly reduced by addition of ATP during photolysis (Figure 5, lane 9). These results suggest that the 45-kDa band also contains the 20-kDa C-terminal peptide.

To obtain more conclusive evidence for the composition of the 45-kDa cross-linked product, hydrolysis of ANB-8-N_3-ATP in the 45-kDa peptide was attempted. In ANB-8-N_3-ATP, the azidonitrobenzoic acid moiety is linked to the ribose of 8-N_3-ATP through an ester bond (Figure 1A). Since ester

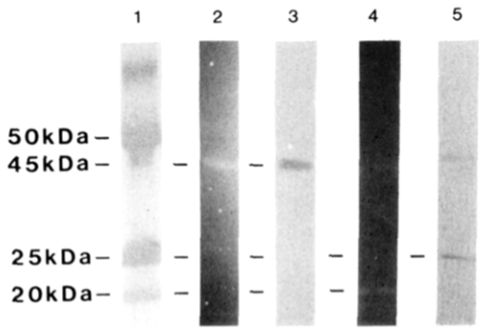

FIGURE 6: SDS–PAGE of the isolated 45-kDa fragment and the alkaline-treated 45-kDa fragment. The fluorescent band corresponding to the 45-kDa fragment was cut out from gels under illumination with a UV lamp. The 45-kDa fragment was eluted out of the gel by electrophoresis and collected into a dialysis tube. Half of the isolated 45-kDa fragment was again electrophoresed and transblotted to the nitrocellulose memblane. The other half of the 45-kDa fragment was treated with weak alkaline to cleave the ester bond in ANB-8-N_3-ATP and electrophoresed and transblotted to the nitrocellulose membrane. Lane 1, protein staining of tryptic S-1 modified with ANB-8-N_3-ATP. Lane 2, fluorescence of isolated 45-kDa fragment. Lane 3, immunostaining of isolated 45-kDa fragment with the 25-kDa specific antibody. Lane 4, fluorescence of alkaline-treated 45-kDa fragment. Lane 5, immunostaining of alkaline-treated 45-kDa fragment.

bonds can be readily cleaved by mild alkaline treatment without hydrolysis of peptide bonds (Schafer et al., 1985), the fluorescently labeled 45-kDa band was subjected to mild alkaline hydrolysis. As shown in Figure 6, the isolated 45-kDa peptide retained both fluorescence activity due to IAF (lane 2) and immunological reactivity against anti-N-terminal peptide antibody (lane 3). After alkaline hydrolysis of the isolated 45-kDa peptide, the fluorescence intensity was observed at the 20-kDa peptide (lane 4) while the immunoreactivity was observed at the 25-kDa peptide (Figure 5). These results indicate that the 45-kDa peptide was cleaved into 25- and 20-kDa fragments by hydrolysis of the covalently linked ANB-8-N_3-ATP.

The above data clearly show that the 20-kDa C-terminal peptide and the 25-kDa N-terminal peptide were cross-linked by ANB-8-N_3-ATP. Recently, it was demonstrated that the 20-kDa C-terminal peptide of skeletal muscle S-1 was labeled with Mant-8-N_3-ATP (Maruta et al., 1989). Since ANB-8-N_3-ATP has a similar structure to Mant-8-N_3-ATP [both carry an azido group at the eighth position of the adenine ring and a nitrobenzoic acid group in the ribose ring whose size is similar to the methylanthraniloyl (Mant) group in the ribose ring], it is reasonable to assume that the N_3 group in the adenine of ANB-8-N_3-ATP labeled 20-kDa peptide while the N_3 group in the ANB reacted with 25-kDa peptide.

It should be noted that these two affinity analogues (ANB-8-N_3-ATP and Mant-8-N_3-ATP) are the only ones yet tested that label the 20-kDa segment of S-1, and this observation raises the question of why this pattern of differential labeling arises with the various ATP analogues. Part of the answer may lie in the conformational state of the nucleotides. For example, it is well established that ATP is constrained in the anti conformation with respect to the N-glycoside bond (Takenaka et al., 1978), while analogues with a bulky group at the C8 position appear to be in the syn conformation (Takenaka et al., 1978). Both ANB-8-N_3-ATP and Mant-8-N_3-ATP should, therefore, have the syn structure, and consistent with this interpretation are the altered hydrolytic properties of these analogues exhibited with S-1 and acto–S-1 compared to those observed with ATP. Moreover, the inability of both of these analogues to support superprecipitation is consistent with a previous proposal that only analogues in the anti conformation are able to promote superprecipitation and exhibit actin activation (Takenaka et al., 1978). These results suggest that the hydrolysis of ANB-8-N_3-ATP by S-1 may not be coupled with the conformational change required for cross-bridge cycling. On the basis of these obsevations, we propose that the labeling of the 20-kDa peptide by these analogues may be directly attributed to their syn conformations.

The ability of ANB-8-N_3-ATP to act as a substrate and show ATP-dependent photo-cross-linking of the 20- and 25-kDa segments suggests that regions in both these segments of the heavy chain contribute to the ATPase site. The recent work of Cremo et al. (1989) has established that the consensus nucleotide sequence A of Walker et al. (1982) known as the "glycine-rich" loop, and which is in the 25-kDa segment of S-1 beginning at Gly-178, is indeed at the ATP site on the basis of the photooxidation of Ser-180 by Vi trapped together with Mg^{2+}-ADP in the protein. The proximity of regions in the 20-kDa segment to the 25-kDa segment has been demonstrated by a number of recent observations based on conventional and photo-cross-linking. For example, Lu et al. (1986) and Sutoh and Lu (1987) showed that SH-1 in the 20-kDa segment labeled with (iodoacetyl)benzophenone could be cross-linked to the 25-kDa segment in a nucleotide-dependent manner. Moreover, Sutoh and Hiratsuka (1988) demonstrated that SH-2 in the 20-kDa segment was between 0.3 and 0.45 nm from either Lys-184 and Lys-189 in the "glycine-rich" loop of the 25-kDa segment, but only when the ATP site was free of nucleotide. On the basis of these and other observations, primarily from fluorescence energy data, Botts et al. (1989) have proposed a three-dimensional lattice for the structure of S-1 which also indicates close proximity of parts of the 25- and 20-kDa segments of the protein. It is of interest to note that a segment close to SH-2 and incorporating SH-1 (Burke et al., 1990) bears a close resemblance to a second consensus sequence of some ATP-requiring proteins (Walker et al., 1982). In summary, these observations suggest that regions in the 20-kDa fragment are close to bound nucleotide, and the present work indicates that if these analogues are in the syn conformation then photolabeling of this segment of the heavy chain can also be observed.

It was previously shown that Mant-8-N_3-ATP labeled the N-terminal 29-kDa peptide of smooth muscle S-1 but not the 26-kDa C-terminal peptite (Maruta & Ikebe, 1989), in contrast to the labeling of skeletal muscle S-1. Consistent with this observation, we did not detect the cross-linked product of the N-terminal and C-terminal segments of smooth muscle S-1, both papain S-1 whose 20-kDa light chain is cleaved and *S. aureus* protease S-1 whose 20-kDa light chain is intact by irradiation with ANB-8-N_3-ATP (data not shown).

This result suggests that the conformation of the ATP binding site of smooth muscle myosin is somewhat different from that of skeletal muscle myosin.

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