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Electron microscopic visualization of the ATPase site of myosin by photoaffinity labeling with a biotinylated photoreactive ADP analog

(myosin head/avidin–biotin interaction/rotary shadowing)

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ABSTRACT **An ADP analog carrying a biotin moiety and a photoreactive group was synthesized. In the presence of vanadate ion (V_i), the analog was tightly trapped into the ATPase site of heavy meromyosin (HMM) or myosin subfragment 1 (S1) in an ADP analog/ATPase site molar ratio of 1:1. UV illumination on the HMM (or S1)–V_i–ADP analog complex resulted in covalent incorporation of the analog into the ATPase site. About 15% of the trapped analog was crosslinked to HMM or S1. Mapping of the crosslinking site of the analog showed that the N-terminal M_r 25,000 segment of the heavy chain participated in binding the ADP analog. The biotin moiety of the analog covalently incorporated into the ATPase site was visualized in electron microscopy by attaching an avidin oligomer. Rotary-shadowed images of the HMM–avidin complex revealed that the crosslinked ADP analog was located about 140 Å from the head–rod junction on the head. The result indicates that the ATPase site of myosin is about 140 Å apart from the head–rod junction along the head.**

Myosin head has two essential sites for force generation in muscle: one is the ATPase site and the other is the actin-binding site. It is of vital importance to identify spatial locations of these sites on the head and also to locate these sites along polypeptides of the head in order to understand muscle contraction at the molecular level.

As for the actin-binding site, three-dimensional reconstitution of electron microscopic images of the rigor complex of actin and myosin head (subfragment 1 or S1) showed how the head binds to F-actin (1–7). Locations of the actin–head interface were identified along polypeptide chains of actin as well as S1 by the use of chemical crosslinkings (8–11). As for the ATPase site, several groups tried to map its location along polypeptides of myosin by affinity labeling with photoreactive ATP analogs (12–14). These studies showed that part of the M_r 25,000 segment and part of the M_r 50,000 segment of S1 heavy chain were in proximity of the ATPase site. Spatial location of the site was investigated by a fluorescence energy-transfer experiment (15), which showed that the relative distance between the ATPase site and one of the most reactive thiols of myosin (SH_1) was about 40 Å. Since we have located the SH_1 thiol about 130 Å from the head–rod junction on the myosin head by electron microscopy with a recently developed avidin–biotin system (16), it seems that the ATPase site of myosin is in the distal half of the head.

In this study, we tried to identify spatial location of the ATPase site by electron microscopy. For the purpose, we synthesized a novel photoreactive ADP analog carrying a biotin moiety for use in affinity labeling. After photoaffinity labeling of the ATPase site with the analog, the biotin moiety was visualized by attaching avidin (16). Analysis of rotary-shadowed images indicates that the site is located about 140 Å apart from the head–rod junction on the head.

MATERIALS AND METHODS

Synthesis of a Biotinylated Photoreactive Analog of ADP for Affinity Labeling. An ADP analog carrying a biotin moiety and a photoreactive group (Fig. 1) was synthesized as follows. N^6-[(6-aminohexyl)carbamoylmethyl]-ADP (ACM-ADP) was synthesized by the method of Lindberg and Mosbach (17). ACM-ADP was then biotinylated with the *N*-succinimide ester of biotin by a method previously described (18). The *N*-succinimide ester of biotin (1.2 mmol) in 10 ml of dimethylformamide was added dropwise to ACM-ADP (0.6 mmol) in 10 ml of water. The pH of the mixture was maintained at 9.0 during the reaction. The extent of biotinylation was followed by the ninhydrin reaction. After completion of the biotinylation reaction, 5 vol of cold acetone was added to the mixture. The resulting precipitate was collected by low-speed centrifugation and dissolved in 50 ml of water. After the pH was adjusted to 3.2, the solution was loaded onto a Dowex-1 column (2 × 10 cm) equilibrated with 0.5 M LiCl (pH 2.0). The biotinylated ACM-ADP was eluted from the column by a linear gradient of LiCl from 0.5 M to 1.0 M at pH 2.0. Fractions of the major peak were collected and neutralized. Pooled fractions were concentrated to 50 ml by a rotary evaporator at 40°C. To the concentrated solution was added cold acetone/ethanol, 1:1 (vol/vol). The resulting precipitate was collected by centrifugation and again dissolved in 50 ml of water. Acetone/ethanol precipitation was repeated twice. The product was finally dissolved in 50 ml of water. Purity of the product was checked by HPLC with a DEAE-5PW column (Toyo-Soda, Japan). When monitored at 260 nm, neither the starting material nor by-product was detected. Coupling of the biotinylated ACM-ADP and 5-azido-2-nitrobenzoic acid (19) was carried out by the method of Guillory and Jeng (20). The biotinylated ACM-ADP (15 μmol) in 1 ml of water was mixed with azidonitrobenzoic acid (150 μmol) in 1 ml of dimethylformamide after activation by carbonyldiimidazole. The coupling reaction was allowed to proceed for 4 hr at 35°C. Five volumes of cold acetone was then added, and the precipitate was collected by low-speed centrifugation and dissolved in 5 ml of water. The product was purified by HPLC with the DEAE-5PW column. Elution was carried out with a linear gradient of ammonium acetate from 0.1 M to 0.65 M and monitored at 260 and 320 nm (due to its azidonitrobenzoyl group, the product has absorption maxima at both 252 and 320 nm). Collected fractions corresponding to the product were lyophylized. It showed a single

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Abbreviations: HMM, heavy meromyosin; S1, myosin subfragment 1; V_i, vanadate ion in an unspecified degree of protonation (i.e., VO_4^{-3}, HVO_4^{-2}, or $H_2VO_4^{-3}$; DACM, *N*-[7-(dimethylamino)-4-methyl-3-coumarinyl]maleimide; ACM-ADP, N^6-[(6-aminohexyl)-carbamoylmethyl]-ADP.

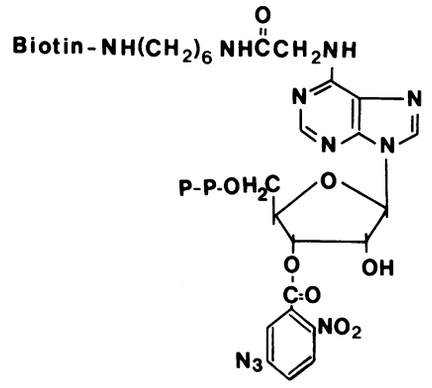

FIG. 1. Chemical structure of the biotinylated photoreactive ADP analog.

peak when its purity was checked by HPLC (DEAE-5PW). All procedures after introduction of the photoreactive group were carried out in the dark.

Incorporation of the ADP Analog into HMM or S1. HMM (or S1) prepared from rabbit skeletal muscle myosin (21) (≈4 mg/ml) in 0.1 M NaCl/20 mM imidazole/2 mM $MgCl_2$, pH 7.0, was mixed with the biotinylated photoreactive ADP analog in an analog/ATPase site molar ratio of 2:1. Then 0.01 vol of 70 mM vanadate ion (V_i) was added to trap the ADP analog into the ATPase site of HMM (or S1) (22). After incubation at 0°C for 16 hr, the mixture was loaded on a HPLC column (G4000SW, Toyo-Soda) to separate the HMM (or S1)–V_i–ADP analog complex from unbound V_i and the ADP analog. Elution was carried out with 0.1 M NaCl and 20 mM imidazole (pH 7.0) and was monitored at 280 and 320 nm. The HMM (or S1)–V_i–ADP analog complex was identified by its strong absorption at 320 nm due to the azidonitrobenzoyl group in the analog (19). The complex was eluted at the same position as HMM (or S1). Unbound ADP analog and V_i were eluted after the column volume because they were slightly adsorbed by the column matrix. The eluted complex (≈1 mg/ml) was illuminated with a UV lamp (356 nm, 16 W) at the distance of 2 cm for 5 min on ice to activate the photoreactive group in the ADP analog. The resulting solution was exhaustively dialyzed against 0.5 M NaCl/20 mM imidazole, pH 7.0.

Estimation of the Number of ADP Analog Molecules Trapped into HMM or S1. The number of ADP analog molecules trapped into HMM or S1 was estimated as follows. The mixture of HMM (or S1), V_i, and analog was loaded on the G4000SW HPLC column and eluted as above. Absorbance of the peak corresponding to the HMM (or S1)–V_i–ADP analog complex (plus free HMM or S1, if any) was recorded at 320 nm (A_{320}) and at 280 nm (A_{280}). As a control, a mixture in which the ADP analog was replaced by ADP was loaded on the column. Absorbance of the HMM (or S1)–V_i–ADP complex peak was recorded at 320 nm (A°_{320}) and at 280 nm (A°_{280}). No significant difference was observed between A_{280} and A°_{280} while A°_{320} was much smaller than A_{320}. The number of the trapped analog was then estimated as

$$\frac{(A_{320} - A^\circ_{320})/\varepsilon_{320}}{A_{280}/\varepsilon_{280}},$$

where ε_{280} was the molar extinction coefficient of HMM (or S1) and ε_{320} was that of the azidonitrobenzoyl group (19).

Titration of the Covalently Crosslinked ADP Analog with Fluorescent Avidin. Avidin was labeled with a fluorescent dye *N*-[7-(dimethylamino)-4-methyl-3-coumarinyl]maleimide (DACM) as described (16). Complex formation between the fluorescent avidin and HMM (or S1) covalently labeled with the ADP analog was followed by HPLC (G4000SW column) equipped with a fluorescence monitor (16). The number of avidin molecules bound to the labeled HMM (or S1) was estimated from elution profiles of the DACM fluorescence as described (16).

Electron Microscopy. The fluorescent avidin (2 mg/ml) in 0.5 M NaCl/20 mM imidazole, pH 7.0, was mixed with divalent biotin (16, 23) (2 mM in dimethyl sulfoxide) in a divalent biotin/avidin molar ratio of 2:1 to generate the fluorescent avidin oligomer (16, 23). The resulting avidin oligomer was loaded on a G3000SW HPLC column (Toyo-Soda) and eluted with 0.5 M NaCl/20 mM imidazole, pH 7.0. The fraction corresponding to the trimer of avidin was collected. Rechromatography of the fraction showed that this contained other avidin species such as the dimer or tetramer, although the trimer was most abundant.

The HMM–V_i–ADP analog complex, which was illuminated with UV light and then dialyzed against 0.5 M NaCl/20 mM imidazole, pH 7.0, was mixed with the trimer fraction of avidin (≈0.4 mg/ml) in a HMM/avidin monomer molar ratio of 1:6. After incubation at 0°C for 4 hr, the mixture was loaded on the G4000SW column. Elution was carried out with 0.65 M ammonium acetate. Fluorescence of the eluant was monitored. The HMM–avidin complex was detected as a broad fluorescent peak just before a broad peak corresponding to the trimer fraction of avidin. The fraction corresponding to the HMM–avidin complex was collected and used for electron microscopic observation. When absorption of the eluant was monitored at 280 nm, however, it was observed that the tailing part of the HMM–avidin peak overlapped with a large peak of free HMM (this peak was not fluorescent). Thus, the HMM–avidin complex obtained as above contained free HMM.

The HMM–avidin complex was finally diluted to 10 μg/ml in 0.65 M ammonium acetate/50% (vol/vol) glycerol (24–26) and sprayed onto a freshly cleaved mica surface (27). The mica was then dried under vacuum and rotary-shadowed with platinum/carbon at an angle of 10° by using an electron beam gun with a freeze-etching apparatus (JFD-7000, JEOL) as described (28). The replica was examined in a JEOL 100-CX electron microscope at 100 kV. Special precaution was taken in shadowing the specimen and also in developing negative films to increase the contrast between the myosin head and the avidin oligomer on it.

Blotting. S1 was photochemically labeled with the ADP analog as above and then was digested with trypsin at a trypsin/S1 weight ratio of 1:50 for 20 min at 25°C. The cleavage product was electrophoresed on an acrylamide gel in the presence of $NaDodSO_4$ (29) in duplicate and then was transferred onto a nitrocellulose membrane (30). One of the duplicate blots was stained with Coomassie blue, and the other was stained with avidin–peroxidase complex to detect the biotin moiety (16).

RESULTS

Incorporation of the Biotinylated Photoreactive ADP Analog into HMM or S1. HMM was incubated with the biotinylated photoreactive ADP analog in the presence of V_i (22) to trap the analog into the ATPase site. The mixture was loaded on the G4000SW HPLC column. Since the azidonitrobenzoyl group in the ADP analog had an absorption maximum at 320 nm (19), it was expected that the HMM–V_i–ADP analog complex would be detected by monitoring the eluant at 320 nm. In fact, a peak whose retention time was just the same as that of HMM was observed at 320 nm. This peak was observed only when all of the constituents (HMM, V_i, and analog) were present, indicating that it corresponded to the HMM–V_i–ADP analog complex. Based on the known extinction coefficient of HMM at 280 nm and that of the azidonitrobenzoyl group at 320 nm (19), the number of the ADP analog molecules trapped into HMM was estimated for the peak fraction. From several independent measurements,

it was shown that about one (1.0 ± 0.1) ADP analog molecule was trapped into one myosin head when more than the equivalent amount of the analog per ATPase site was present. When an excess amount of ADP (10 molar excess) was present, no incorporation of the analog into HMM was detected. These results indicated that the ADP analog was actually trapped into the ATPase site as ADP (22).

The association of the ADP analog with HMM was very tight. The HMM–V_i–ADP analog complex eluted from the HPLC column released only a few percent of the trapped ADP analog, even after 24 hr at 0°C, when checked by HPLC (G4000SW).

In order to covalently crosslink the trapped analog to HMM, the HMM–V_i–ADP analog complex eluted from the HPLC column was illuminated with UV light. The azidonitrobenzoyl group in the ADP analog was activated, and the resulting nitrene inserted covalently into a polypeptide chain in its proximity. When the illuminated complex was mixed with DACM-avidin, association between the complex and the fluorescent avidin was detected by HPLC (16). On the other hand, no association was detected when the fluorescent avidin was mixed either with HMM or with the HMM–V_i–ADP analog complex without UV illumination. Thus, it seems that only the covalently incorporated ADP analog could bridge HMM and avidin. ADP analog noncovalently trapped into HMM would be stripped off by avidin because affinity between avidin and the biotin moiety in the ADP analog was so strong. The notion was supported by the finding that no association of the fluorescent avidin with the HMM–V_i–ADP analog complex was observed when the complex was illuminated after mixing avidin and the complex.

Since only the covalently crosslinked ADP analog could bridge HMM and avidin, the extent of covalent labeling of HMM with the analog was estimated by titration with the fluorescent avidin as described (16). The result showed that about 15% of the ADP analog trapped into the ATPase site was crosslinked covalently after UV illumination. It is very likely that the crosslinking site of the analog was the ATPase site of HMM because no migration of the analog was expected during illumination, considering the following facts. (*i*) When HMM was illuminated before addition of V_i and the ADP analog, the resulting HMM could trap the ADP analog just as nonilluminated HMM. (*ii*) After UV illumination on the HMM–V_i–ADP analog complex, no dissociation of the photolyzed ADP analog from HMM was detected.

When S1 was used in place of HMM, results were just the same as those above.

Location of the Covalently Crosslinked ADP Analog Along Polypeptide Chains of S1. Location of the covalently incorporated ADP analog along polypeptide chains of S1 was examined by peptide maps. S1 photochemically labeled as above with the analog was electrophoresed in the presence of $NaDodSO_4$ and then was blotted on a nitrocellulose membrane (30). Peptides carrying the crosslinked ADP analog were stained with the avidin–peroxidase complex (16). Only the M_r 95,000 S1 heavy chain was heavily stained with the avidin–peroxidase complex on the nitrocellulose membrane, indicating that the analog was crosslinked to the heavy chain, not to light chains. For further mappings, S1 was digested with trypsin to cleave the heavy chain into three fragments (M_rs 20,000, 25,000, and 50,000) (31, 32). When the blot of the tryptic digest was stained with the avidin–peroxidase complex, only the M_r 25,000 fragment and its breakdown product (M_r 22,000) were stained (Fig. 2). This result indicated that the ADP analog was covalently incorporated into the N-terminal M_r 25,000 segment of the S1 heavy chain.

Electron Microscopic Examination of the HMM–Avidin Complex Bridged by the Analog. HMM photochemically labeled with the ADP analog was mixed with the DACM-

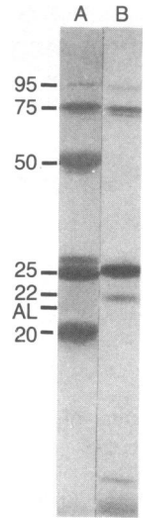

FIG. 2. Mapping of the crosslinking site of the ADP analog along the S1 heavy chain. S1 photochemically labeled with the analog was digested with trypsin. The tryptic digest was electrophoresed on an acrylamide gel in the presence of $NaDodSO_4$ in duplicate and then was transferred on a nitrocellulose membrane. One of the duplicate blots was stained with Coomassie blue (lane A), and the other was stained with avidin–peroxidase complex (lane B). Note that the M_r 95,000 S1 heavy chain was digested into three fragments (M_rs 20,000, 25,000, and 50,000) (lane A) and that the M_r 25,000 fragment and its M_r 22,000 breakdown product were stained with the avidin–peroxidase complex (lane B). On lane B, a faint M_r 95,000 band corresponds to the intact heavy chain remaining after the tryptic digestion, while the M_r 75,000 band corresponds to a precursor of the M_r 25,000 and M_r 50,000 fragments. Alkaline light chain 1 (AL) was faintly visible on the Coomassie-stained membrane, while alkaline light chain 2 could not be detected under the present blotting conditions. Molecular weights are shown × 10^{-3}.

labeled avidin oligomer (16). The fluorescent avidin oligomer bound tightly to the biotin moiety of the ADP analog. Thus, the covalently incorporated ADP analog bridged HMM and avidin, generating the HMM–avidin complex. Since no HMM–avidin complex was generated when avidin was mixed either with HMM or with the HMM–V_i–ADP analog complex before UV illumination, it is evident that avidin bound to HMM only through the crosslinked ADP analog.

The HMM–avidin complex was freed from unbound avidin by the use of the G4000SW HPLC column and then was examined by an electron microscope after rotary shadowing. A gallery of shadowed images of the HMM–avidin complex is shown in Fig. 3. Avidin oligomers are observed as elongated particles on "pear"-shaped myosin heads as described (16). Although the high density of platinum particles on avidin oligomers easily smeared the shape of heads as previously observed (16), we could obtain more satisfactory images of the HMM–avidin complex by carefully controlling the contrast between avidin oligomer and myosin head. Usually the density of platinum particles was higher on avidin than on the head. Therefore, the attachment site of avidin oligomer on the head could be identified for many complexes. For these HMM–avidin complexes, the distance between the head–rod junction and the attachment site of avidin was measured on the head as follows. A myosin head was divided into 30-Å sections from the junction to the tip. The number of myosin molecules with bound avidin in a particular section was counted. Fig. 4 shows the histogram of the distribution. Center of the distribution (average of 52 particles) is located at 140 Å (± 35 Å SD), while the average length of the head is 200 Å. Since avidin attached to the ADP analog that was crosslinked to the ATPase site of HMM, the result indicates

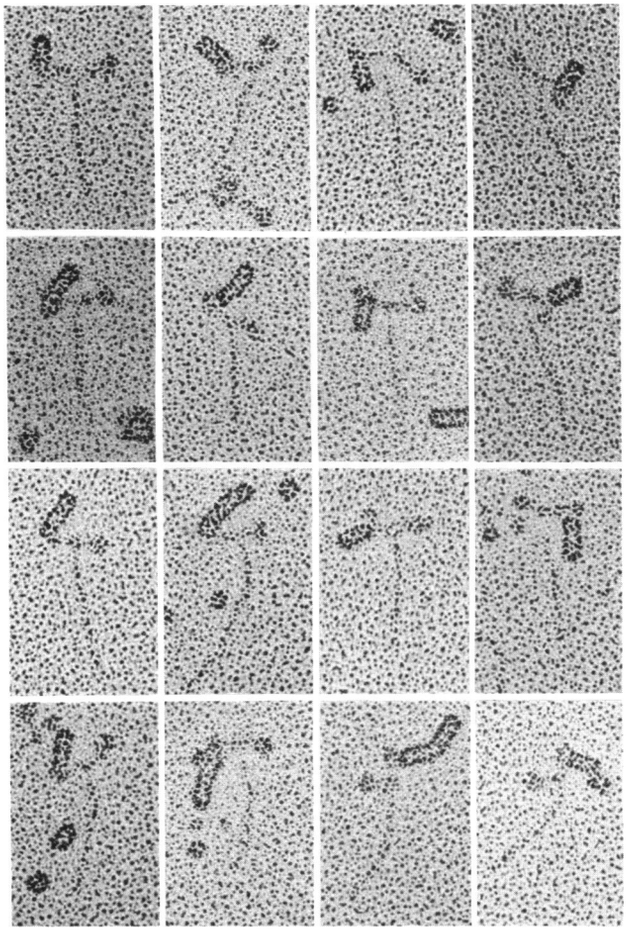

FIG. 3. A gallery of shadowed images of the HMM–avidin oligomer complex. Elongated particles on myosin heads are linear avidin oligomers. Density of the platinum particles is higher on avidin than on myosin head. (×250,000.)

that the ATPase site of the head is located about 140 Å apart from the head–rod junction.

We also tried to use avidin monomer in place of avidin oligomer as an electron microscopic probe (16). Unfortunately, however, many particles showed only increase of the size of the head, possibly because of attachment of avidin on the head. Contrast between the head and avidin on it was not significant enough to discern the avidin probe from the head.

DISCUSSION

By using a biotinylated photoreactive ADP analog for affinity labeling, we succeeded in visualizing the ATPase site of myosin. The site is located about 140 Å from the head–rod junction on the head. We previously had located the SH_1 thiol about 130 Å from the head–rod junction by using an avidin–biotin system similar to that used here (16). As far as the distance along the head is concerned, these two sites are in proximity with each other. On the other hand, previous fluorescence energy transfer experiment (15) has shown that the relative distance between these sites is 40 Å. One explanation for the apparent discrepancy would be that the SH_1 thiol and the ATPase site of myosin are on opposite sides of the head. We used rather large probes (avidin monomer and avidin oligomer) in order to directly visualize these sites by an electron microscope. Large probes introduce some uncertainty in locating their attachment site on the head. Therefore, we think it premature to compare our results with fluorescence energy transfer experiment in a quantitative way.

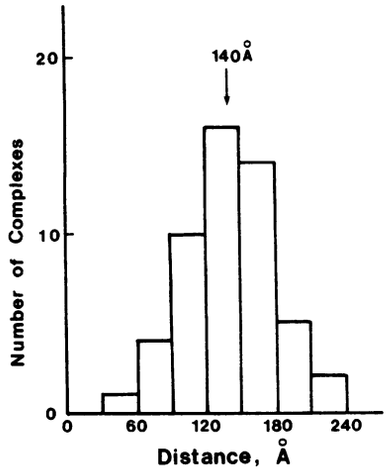

FIG. 4. A histogram of the distance between the attachment site of avidin and the head–rod junction on the head. A myosin head was divided into 30-Å sections from the junction to the tip. The number of myosin molecules with bound avidin in a particular section was counted. For these measurements, size of the head was not normalized to its average value (200 Å). Therefore, for some HMM–avidin complexes in which the length of the head was >200 Å, the measured distance exceeded 200 Å. The average distance between the junction and the attachment site was 140 Å (± 35 Å SD). Fifty-two particles in which the attachment site of avidin on the head was clearly discerned were examined.

In the previous paper (16), we used avidin monomer as an electron microscopic probe to visualize the SH_1 site. In the present study, we also tried avidin monomer to visualize the ATPase site. Unfortunately, however, we failed to collect enough of the HMM–avidin monomer complexes in which bound avidin was clearly discernable from the head. We do not know the exact reason why the contrast between the bound avidin and the head was not good enough in the HMM–avidin monomer complex bridged by the ADP analog. As shown above, however, avidin oligomer could be satisfactorily used as an electron microscopic probe for locating the ATPase site. Careful control of contrast between the probe and the head was necessary for obtaining satisfactory electron microscopic images.

In the ADP analog, the biotin moiety was some distance from ADP, separated by a spacer arm; this spacer arm, —NH—$(CH_2)_6$—NH—, was introduced in the analog to form a stable avidin–biotin complex because the biotin binding sites are located in deep clefts in avidin (23). The length of the arm was selected so that its tail just reached the surface of avidin (23). Therefore, it is unlikely that the spacer arm, although rather long, introduced significant error in locating the ATPase site.

The biotinylated photoreactive ADP analog was covalently crosslinked to the N-terminal M_r 25,000 segment of S1-heavy chain upon UV illumination. Since the azidonitrobenzoyl group attached to the ribose ring of ADP spans only several Angstroms, it is very likely that a part of the M_r 25,000 segment participates in ATP binding. This result is consistent with the previous observation that a photoreactive ATP analog, arylazido-β-alanyl ATP (20), affinity-labeled the M_r 25,000 segment (12). Participation of the segment in the ATPase site was also demonstrated by another ADP analog, *N*-(4-azido-2-nitrophenyl)-2-aminoethyl diphosphate (14). Recently, however, Mahmood and Yount showed that the M_r 50,000 segment of the heavy chain was also labeled with 3′-*O*-(4-benzoyl)benzoyl ADP (13). It seems likely that a part of the M_r 25,000 segment and a part of the M_r 50,000 segment are in proximity with each other at the ATPase site.

The biotinylated photoreactive ADP analog was successfully used in affinity labeling to map the spatial location of the ATPase site of myosin by electron microscopy and also to map a peptide segment participating in ATP binding by the blotting technique. The same methodology would be applicable to other ATPases such as dynein.

Various groups have mapped functional sites on the myosin head by the use of the fluorescence energy-transfer technique (for example, see ref. 33 and references cited therein). The fluorescence energy-transfer experiments provide us with detailed information on the relative distance between two points on the head. On the other hand, locations of functional sites can be directly visualized by the avidin–biotin system as described above, although spatial resolution is rather low when compared to the fluorescence energy-transfer technique. By combining these two techniques, however, we may be able to construct a detailed three-dimensional map of functional sites on the myosin head.

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