ATP-induced Opposite Changes in the Local Environments around Cys$^{697}$ (SH2) and Cys$^{707}$ (SH1) of the Myosin Motor Domain Revealed by the Prodan Fluorescence*

(Received for publication, May 10, 1999, and in revised form, July 20, 1999)

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To obtain a consistent view of the nucleotide-induced conformational changes around Cys$^{697}$ (SH2) and Cys$^{707}$ (SH1) in skeletal myosin subfragment-1 (S-1), the two thiols were labeled with the same environmentally sensitive fluorophore, 6-acryloyl-2-dimethylaminonaphthalene group, using 6-acryloyl-2-dimethylaminonaphthalene (acrylodan, AD) and 6-bromoacetyl-2-dimethylaminonaphthalene (BD), respectively. The resultant fluorescent derivatives, AD-S-1 and BD-S-1, have the same fluorescent properties as either SH2 or SH1, which was verified by inspection of changes in the ATPases and the localization of fluorescence after tryptic digestion and CNBr cleavage for the two derivatives. Especially, AD was found to be a very useful fluorescent reagent that readily reacts with only SH2 of S-1. Measurements of the nucleotide-induced changes in fluorescence emission spectra of AD-S-1 and BD-S-1 suggested that during ATP hydrolysis the environment around the fluorophore at SH2 is very different from that around the fluorophore at SH1, being defined as the former has a hydrophobic and closed character, whereas the latter is hydrophilic and open. The KI quenching study of the fluorescence of the two S-1 derivatives confirmed these results. The most straightforward interpretation for the present results is that during ATP hydrolysis, the helix containing SH2 is buried in hydrophobic side chains and rather reinforced, whereas the adjacent helix containing SH1 moves away from its stabilizing tertiary structural environment.

The structural element of the molecular motor myosin is the subfragment-1 (S-1) moiety that contains the sites responsible for the ATP hydrolysis and binding of actin (1). A striking feature of the S-1 structure is a long helix spanning 85 Å that is stabilized by interactions with the myosin light chains (2). It has been proposed that this light-chain-binding domain acts as a semi rigid “lever arm” to amplify and transmit conformational changes in the ATP and actin binding sites of S-1 (3–9).

Cys$^{707}$ (SH1) and Cys$^{697}$ (SH2) are located on the two different helices in the C-terminal segment of the S-1 catalytic domain and separated from one another by 19 Å (2). The two helices are kinked at a conserved glycine residue (Gly$^{699}$) found in a bend joining two helices. Recent experiments have led to the conclusion that the fulcrum point for the swinging motion of the lever arm is in the vicinity of the SH1-SH2 region (4–9). Furthermore, Gly$^{699}$ (close to SH2) and Gly$^{710}$ (close to SH1) have been proposed to act as pivot points or flexible hinges for such motion (8, 10, 11). These data show the importance of motion in the SH1-SH2 region in the force generation cycle of myosin. However, the precise conformational changes of this region that are involved in lever arm movement are still unknown.

One approach for understanding the involvement of the SH1-SH2 region in lever arm movement is a precise comparison of the individual conformational changes around SH1 and SH2 during ATPase cycle of S-1. Such a study could provide new insights into the mechanism of the lever arm movement in the generation of force. Although the conformational changes around SH1 have extensively been studied, little is known about those around SH2 (12–15). A recurrent question is whether SH2 is mobile or stationary. Several reports have suggested that SH2 is mobile (16–18). On the other hand, Xing and Cheung (19) have indicated that SH2 was relatively immobilized even in the presence of nucleotides. One approach to examine the mobility of SH2 is to attach a sensitive fluorescent probe covalently to SH2. However, little is known about a simple method for the fluorescent labeling of SH2 (20). Thus, I have initiated a study of covalent fluorescent probes that react specifically with SH2.

Several fluorescent probes were examined in an attempt to label only SH2. Of the compounds tested, 6-acryloyl-2-dimethylaminonaphthalene (AD) was chosen as a specific fluorescent label for SH2 (Scheme 1). Furthermore, an attempt at the labeling of SH1 with the same fluorophore as that labeled with SH2 was made to obtain a consistent view of conformational changes around the two thiols. Finally 6-bromoacetyl-2-dimethylaminonaphthalene (BD) was chosen as a fluorescent label for SH1. The resultant AD and BD derivatives of S-1 have the same fluorescent properties, the 6-acetyl-2-dimethylaminonaphthalene group, at either SH1 or SH2.

Unlike the vast majority of the more commonly used fluorophores, the noncovalent fluorescent probe prodan, the parent fluorophore of AD and BD (Scheme 1), should be particularly well suited to probe the surface of proteins by virtue of the exquisite sensitivity of the fluorophore to minor changes in the environment (21–24). It gives a 130-nm shift in the emission maximum from 401 nm in cyclohexane to 531 nm in water (21). Thus the emission maximum of the fluorophore would truly be a polarity sensor for proteins. Furthermore, the half-widths of the emission spectra of the prodan fluorophore provide an
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indication of the accessibility of solvent molecule to the fluorophore attached to proteins (21–23). Thus these remarkable properties of the prodan fluorophore are utilized in the present study to describe precise differences between SH2 and SH1 of S-1 in their environmental changes during ATPase cycle of S-1.

**EXPERIMENTAL PROCEDURES**

**Materials**—AD and BD were purchased from Molecular Probes. IAEDANS was from Aldrich. IAA was from Wako Pure Chemicals. α-Chymotrypsin, diphenylcarbamyl chloride-treated trypsin, soybean trypsin inhibitor, and AMP-PNP were from Sigma. ATP and ADP were from Kohjin. Other reagents were reagent or biochemical research grade.

**Protein Preparation**—S-1 was prepared from rabbit skeletal muscle (25), and the protein concentrations were determined from the absorbance at 280 nm as in my previous works (14, 16).

**Buffer Systems**—Experiments with protein samples were carried out in the following buffers: buffer A (25 mM HEPES, pH 8.0, 30 mM KCl, and 2 mM MgCl2), buffer B (50 mM Tris/Cl, pH 8.0, 0.5 mM KCl, and 5 mM CaCl2), and buffer C (50 mM Tris/Cl, pH 8.0, 0.5 mM KCl, and 5 mM EDTA).

**Fluorescent Labeling of S-1 with AD and BD**—Fluorescent labeling of S-1 with AD and BD was performed at 7 °C in buffer A in the dark. S-1 (2–2.5 mg/ml) was incubated with a 3-fold molar excess of the reagent over S-1. Because AD and BD were scarcely soluble in water, the reaction mixtures contained 0.3% dimethylformamide. The addition of 0.3% N,N-dimethylformamide and Ca2+ did not affect on the K+-, Mg2+-, and Ca2+-ATPases of S-1. The reaction was stopped by the addition of dithiothreitol at a final concentration of 2 mM. The labeled S-1 was then separated from the unreacted reagent and the reaction products with dithiothreitol by passage through 1.5 × 9 cm columns of Sephadex G-50 equilibrated with 25 mM HEPES, pH 8.0, and 30 mM KCl. For measurements of ATPase activities, the samples were compared with a control subjected to the same conditions but excluding the reagent. The enzymatic properties of the fluorescent derivatives of S-1, the S-1 samples were subjected to SDS-PAGE, and then relative amounts of fluorescence extracted from peptide bands were measured as described previously (34).

**ATPase Measurements**—The Ca2+- and K+-ATPase activities were measured at 25 °C in 1 mM ATP in buffers B and C, respectively. P liberated was determined by the method of Fiske and SubbaRow (35). The ATPase activities of the unlabeled S-1 in μmol of P/min/mg were: K+-ATPase, 10; Ca2+-ATPase, 1.2.

**Spectral Measurements**—All spectral measurements of the thiol-modified derivatives of S-1 were performed in buffer A. Absorption spectra were measured at room temperature with a Shimadzu MPS-2000 spectrophotometer. Corrected fluorescence emission spectra were recorded at 25 °C in a thermostated Hitachi fluorescence spectrophotometer (model MPF-4). All fluorescence measurements with S-1 samples (0.4–0.75 μM) were performed in the presence and absence of ligands (1 mM). Because fluorescence measurements were completed within 15 min, the data in the presence of ATP were regarded as those obtained during the steady state of ATP hydrolysis. Excitation wavelength was 390 nm. The slit widths on excitation and emission monochromators were 5 nm. For the measurements of the labeling rates of S-1 with AD and BD, increases in fluorescence at 500 nm were monitored at 7 °C.

**KI Fluorescence Quenching Measurements**—The freshly prepared KI solution was used. The stock solution (5 M) contained 10 × 10–4 M sodium thiosulfate to prevent oxidation of I-. All measurements were carried out at 25 °C with excitation and emission lights of 390 and 500 nm, respectively. Data were shown as Stern-Volmer plots to obtain the quenching constant, Ksv (36).

**Protein Determinations**—Concentrations of thiol-modified S-1 were determined by the biuret method, standardized against control S-1. Control experiments showed that the modification of S-1 did not interfere with protein determinations by the biuret method. S-1 was assumed to have an M of 120,000 (25).

**RESULTS**

**Properties of BD**—Alkylation of S-1 with IAA and IAEDANS invariably results in the modification of only SH1 (20, 26, 27). Although chemical properties of BD had scarcely been studied, the reagent was expected to be useful as a specific fluorescent label for SH1 of S-1. This is because the reactivity of the functional group of BD, the bromoacetyl group, is similar to those of functional groups of IAA and IAEDANS (Scheme 1).

On the other hand, the structure of the fluorophore moiety of BD, the 6-acyl-2-dimethylaminophenothalene group, is the same as those of another thiol reagent AD and the noncovalent probe prodan (21, 22). Thus BD can be expected to show fluorescence that is extremely sensitive to solvent polarity as well as prodan and AD (21–24).

**Reactions of S-1 with AD and BD**—The absorption spectra of BD and AD derivatives of S-1 exhibited a maximum at 278 nm together with those at 382 nm (AD-S-1) and 392 nm (BD-S-1) (Fig. 1). The maxima around 380–390 nm are associated entirely with the 6-acyl-2-dimethylaminophenothalene moiety of AD and BD (21, 22). Upon excitation at 390 nm, the S-1 derivatives fluoresced strongly with a single maximum at 502 nm (AD-S-1) and 508 nm (BD-S-1) (Fig. 1). On the other hand, the AD adduct of 2-mercaptopoethanol fluoresced at 535 nm (Fig. 1, inset). However, the fluorescence intensity of AD was less than 4% relative to that of the fluorescent adduct. Similar results were obtained in the case of the reaction of 2-mercaptopoethanol with BD (not shown). This indicates that the formation of the fluorescent derivatives of S-1 can be conveniently monitored by measurements of an increase in fluorescence that is associated with fluorophores attached to S-1 (see below).

**Enzymatic Properties of the Fluorescent Derivatives of S-1—**
Fig. 1. Absorption (1) and fluorescence emission (2) spectra of AD-S-1 (solid line) and BD-S-1 (dotted line) in which 0.9–1.0 thiol group/mol of S-1 had been labeled. After stopping the labeling reaction, the reaction mixtures were passed through a column of Sephadex G-50. The absorption and fluorescence spectra were then measured at 25°C in buffer A. Concentrations of samples were 8.5 μM for absorption and 0.7 μM for fluorescence spectra. The corrected fluorescence spectra were measured with 390-nm exciting light. Inset, fluorescence emission spectra of AD (a) and the AD derivative of 2-mercaptoethanol (b). AD (2 μM) was incubated at 25°C for 20 min in buffer A in the presence and absence of 2-mercaptoethanol (28 μM), and then fluorescence spectra were measured.

Fig. 2 shows the time courses of labeling of S-1 with AD, which occurred at 7°C with a 3-fold molar excess of AD over S-1. They were followed by measurements of the amount of labeled thiol groups and fluorescence increase at 500 nm. The time courses obtained by both measurements agreed well. The data indicate that the labeling of S-1 is relatively rapid for the first 30 min and much slower thereafter. The labeling reaction was completed in 1 h, resulting in the incorporation of 0.8–1.0 AD group/mol of S-1. It should be noted that the existence of 0.9 mol of the first-labeled thiol group is indicated by extrapolation of the slow labeling to zero time.

Fig. 2 (inset) shows the relationship between the percentage of remaining ATPase activities of AD-S-1 and the number of the labeled thiol groups/mol of S-1. The K⁺-ATPase activity was decreased linearly with increasing amount of the labeled thiol group. The decrease of 1 mol of thiol group led to a 70% loss of activity. On the other hand, the Ca²⁺-ATPase activity was scarcely affected by the labeling.

Fig. 3A shows the time course of the labeling of S-1 with BD, which has occurred under the same condition as that of the labeling with AD (Fig. 2). The rate of the labeling with BD was much faster than that of the labeling with AD. 1 mol of thiol group/mol of S-1 was labeled within 5 min. The existence of 1 mol of the first-labeled thiol group/mol of S-1 was indicated by extrapolation of the slow labeling to zero time. However, unlike the labeling with AD, another thiol group of S-1 was also labeled within 25 min (see below).

The effect of BD on the K⁺- and Ca²⁺-ATPase activities of S-1 was also followed. Fig. 3B shows that as thiol groups are labeled with BD the Ca²⁺-ATPase activity increases, while the K⁺-ATPase activity decreases. When 1.2 thiol group/mol of S-1 was labeled, the Ca²⁺-ATPase activity was increased to 220% of the original value. Thereafter, the activity began to plateau and then decreased. The linear relationship between the Ca²⁺-ATPase activity and the loss of K⁺-ATPase activity was observed (Fig. 3B, inset).

Identifications of Thiol Groups Labeled with AD and BD—The enzymatic properties of fluorescent derivatives of S-1 described above are indicative of the labeling of SH1 with BD and SH2 with AD (12–20, 26, 27). To ascertain this point, the locations of AD and BD attached to S-1 were defined by fragmenting the S-1 samples with both trypsin and CNBr (16, 32). All the samples were run on SDS-PAGE, followed by the examination under UV illumination. The S-1 derivative where SH1 had been fluorescently labeled with IAEDANS (26) was also used as a standard.

As shown in Fig. 4A, all fluorescent labels are predominantly localized in the heavy chain of S-1. However, some labeling of light chain-1 was found with BD. Relative amounts of fluorescence extracted from peptides bands of heavy chain and the combined light chains were 93 and 7% (IAEDANS-S-1), 78 and 22% (BD-S-1), and 91 and 9% (AD-S-1), respectively. For the tryptic digests, fluorescence was found only on the 20-kDa band (Fig. 4B). Upon CNBr cleavage for the samples, fluorescence was observed on the 10-kDa segment (Fig. 4C), showing that only thiol groups that had been labeled were either SH1 or SH2 for both the BD and AD derivatives of S-1 (32).

It is well established that hydroxylamine cleaves the 20-kDa tryptic peptide of S-1 at the single Asn-Gly bond to produce a C-terminal 13-kDa peptide containing SH1 and a 7-kDa peptide containing SH2 (31). However, the usual demonstration using this hydroxylamine cut is not possible with AD and BD because of the instability of the labels to the extreme pH and temperature conditions of the hydroxylamine treatment. I found that fluorescence of the samples of AD-S-1 and BD-S-1 disappeared by the treatment, although that of the sample of...
IAEDANS-S-1 remained unaltered. Because of this complication I tried an indirect approach where IAA-S-1 was further incubated with IAA and AD for 3 and 30 min, respectively. For control S-1, the amount of thiol groups labeled with BD was 1.05 mol/mol of S-1. However, for IAA-S-1, the amount was diminished to be only 0.19 mol/mol of S-1. Thus 0.86 thiol group/mol of S-1 (82% of the control value) was unlabeled with BD after treatment with IAA. This suggests that BD labels SH1 predominantly together with a slight labeling of light chain-1 (Figs. 3A and 4A). On the other hand, the labeling with AD was scarcely affected by the pretreatment with IAA. The amounts of labeled thiol groups were 0.90 and 0.82 mol/mol of S-1 for control S-1 and IAA-S-1, respectively. This indicates that AD can specifically label SH2. SDS-PAGE patterns for the samples of AD derivatives of S-1 and IAA-S-1 confirmed this result (Fig. 4D). In the case of labeling with AD, no difference in fluorescence intensities between S-1 and IAA-S-1 was observed on both bands of the heavy chain and the 20-kDa tryptic peptide. On the other hand, fluorescence was scarcely observed on the bands of IAA-S-1 in which 1.1 thiol groups/mol of S-1 had been modified was incubated with trypsin and fragment with CNBr as described under "Experimental Procedures." The samples were then subjected to SDS-PAGE using 7.5% (A, B, and D) and 15% (C) acrylamide gels according to Weber and Osborn (33). Lanes S, stained with Coomassie Brilliant Blue. Lanes D (IAEDANS-S-1), B (BD-S-1), and A (AD-S-1) were illuminated under UV lamp. HC, heavy chain; LC, light chain. A, before fragmentation; relative amounts of fluorescence extracted from peptide bands of heavy chain and the combined light chains were measured as described previously (34) and indicated under the electrophoretograms. B, tryptic digests. C, CNBr fragments. D, control S-1 (lanes C) and IAA-S-1 (lanes I) were incubated with IAEDANS (lanes D) and AD (lanes A), followed by tryptic digestion and subjected to SDS-PAGE. −, before digestion; +, tryptic digests.

**TABLE I**

Fluorescent labeling of IAA-S-1 with AD and BD

| Sample   | BD | AD | Amount of SH labeled | Amount of SH protected with IAA |
|----------|----|----|----------------------|---------------------------------|
| Control S-1 | +  | -  | 1.05                 |                                 |
| IAA-S-1   | +  | -  | 0.19                 | 0.86                            |
| Control S-1 | -  | +  | 0.90                 |                                 |
| IAA-S-1   | -  | +  | 0.82                 | 0.08                            |

* IAA-S-1 in which 1.1 thiol groups/mol of S-1 had been modified was used.
* Incubation times were 30 and 3 min for AD and BD, respectively.

Effects of Nucleotides and the Analog on Labeling Rates—The labeling of S-1 with AD and BD can be conveniently monitored by measurements of an increase in fluorescence of the fluorophore (Fig. 2). The rate constants of labeling of the two thiols were calculated from the slopes of plots of the 1n ([ΔF]) versus time (not shown). The results of these measurements are presented in Table II. To account for reagent-dependent differences in reactivities of the two thiols, the rate...
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Fluorescence emission spectra of BD-S-1 (A) and AD-S-1 (B) in the presence (solid line) and absence (dotted line) of ATP. Corrected fluorescence spectra of the samples (0.4 |m|) were measured at 25 °C in buffer A in the presence and absence of ATP (1 |m|) with 390-nm exciting light.

**Table II**

| Reagent/ligand | Ligand | \( k / k_d \) | F.I. |
|----------------|--------|---------------|------|
| AD             | ATP    | 3.7           | 0.97 |
|                | ADP    | 3.8           | 0.95 |
|                | AMP-PNP| 6.0           | 0.91 |
| IAA + AD       | ATP    | 2.2           | 0.97 |
| BD             | ADP    | 1.8           | 0.95 |
|                | AMP-PNP| 3.8           | 0.91 |

*IAA + AD; S-1 in which SH1 had been modified with IAA was further incubated with AD.
*All values are the average and standard deviation of three independent experiments.
*The values of \( k / k_d \) and \( k_d \) represent the rates of labeling in the presence and absence of ligands, respectively.

In the absence of ligands, the reactivity of SH1 toward BD was 2.2-fold higher than that of SH2 toward AD, in agreement with the results shown in Figs. 2 and 3A. In the presence of ligands, both reactivities were increased. However, the reactivity of SH2 was much more affected by the ligands than that of SH1 (4–6-fold for SH2 versus 2–4-fold for SH1). The reactivity not only of SH2 but of SH1 depends strongly on the ligand bound to S-1. In both cases of SH2 and SH1, the ratios of rates \( (k_d / k) \) in the presence of ATP are similar to those in the presence of ADP, suggesting the conformational analogy between these two complexes. On the other hand, reactivities of the two thiols are the highest in the presence of AMP-PNP, suggesting that the conformation of the complexes of AMP-PNP-S-1 are distinct from those of the complexes with ATP and ADP. For labeling of SH2 with AD, the rate of IAA-S-1 (0.60 \( \times \) 10^{-3} s^{-1}) was similar to that of control S-1 (0.79 \( \times \) 10^{-3} s^{-1}). This indicates that the preblocking of SH1 with IAA has no effect on the reactivity of SH2.

**Nucleotide-induced Fluorescence Changes**

The effects of nucleotides and the analog on the emission spectra of the two S-1 derivatives were examined in the presence of Mg^{2+}. As shown in Fig. 5A, ATP decreased the fluorescence intensity of BD-S-1 by 10%. This decrease was accompanied by a red shift of the emission maximum from 508 to 515 nm. Interestingly, ATP-induced changes in the emission spectrum of AD-S-1 differed significantly from those in the emission spectrum of BD-S-1. As shown in Fig. 5B, the emission maximum of AD-S-1 was blue-shifted from 502 to 485 nm upon addition of ATP, whereas the fluorescence intensity at the maximum was scarcely changed. It should be noted that ATP causes a shoulder around 460 nm in the emission spectrum of AD-S-1. Although the spectra are not shown, changes in emission spectra depended strongly on ligands added in both cases of BD-S-1 and AD-S-1 (Table III).

It is well established that the emission maximum of the prodan fluorophore can be used as a ruler of the polarity around its binding sites of proteins (21). Furthermore, additional information on the dipolar character of the prodan binding sites of proteins can be obtained from examination of the half-widths of the emission spectra (21, 22). Table III summarizes the spectral data containing the half-widths of the emission spectra of the two S-1 derivatives in the presence and absence of ligands. For the comparative purpose, the data of prodan in various solvents by Weber and Farris (21) are also presented. Their data clearly indicate that the emission half-width of prodan in viscous solvent ethylene glycol (3530 cm^{-1}) is much larger than those of prodan in nonviscous solvents (2525–2849 cm^{-1}).

Upon addition of ligands, the fluorescence intensities of both S-1 derivatives were changed by only less than 10%. On the other hand, the emission maxima of the two S-1 derivatives were shifted in the opposite direction from each other. The maximum of AD-S-1 was blue-shifted by 6–7 nm, whereas that of BD-S-1 was red-shifted by 5–7 nm. Furthermore, the half-widths of the emission spectra of both S-1 derivatives were increased upon addition of ligands. However, the ligand-induced increase in the emission half-widths showed a striking difference between the emissions of AD-S-1 and BD-S-1. The increases (238–353 cm^{-1}) in the emission half-width of the fluorophore at SH2 were 2.7–4 times larger than those (89–109 cm^{-1}) at the SH1 site.
cm$^{-1}$) of the fluorophore at SH1 in the presence of the corresponding ligands.

The spectral properties of AD-S-1 and BD-S-1 were compared with those of prodan in various solvents (21) to obtain information about environments around the fluorophores at SH2 and SH1. In comparison with the data of prodan in non-viscous solvents, the emission maxima of AD-S-1 and BD-S-1 in the absence of ligands (502–508 nm) were close to that of prodan in methanol (505 nm). However, upon addition of ligands, the emission maximum of BD-S-1 (508 nm) was red-shifted to 513–515 nm. These values fall between the emission maxima of prodan in methanol (505 nm) and water (531 nm), suggesting the hydrophobic characteristics of environment around the fluorophore at SH1 in the presence of ligands. On the other hand, the emission maxima of AD-S-1 in the presence of ligands (494–496 nm) were close to that of prodan in ethanol (496 nm), suggesting the hydrophobic characteristics of the environment around the fluorophore at SH2. However, the emission half-widths of AD-S-1 in the presence of ligands (3635–3750 cm$^{-1}$) were not close to that of prodan in ethanol (2525 cm$^{-1}$). It should be emphasized that these values were rather larger than that of prodan in viscous solvent ethylene glycol (3530 cm$^{-1}$). These results suggest that nucleotides make the fluorophore at SH2 hard to access to solvent molecule. In contrast, the emission half-widths of BD-S-1 (3291–3400 cm$^{-1}$) remained smaller than that of prodan in ethylene glycol, suggesting that solvent molecule is rather easy to access to the fluorophore at SH1 regardless of the presence and absence of nucleotides. Thus the present spectral data for AD-S-1 and BD-S-1 indicate that the nucleotide-induced change in environment around the fluorophore at SH2 differs significantly from that around the fluorophore at SH1, being rather opposite in its direction.

**KI Quenching of the Fluorophores Attached to SH1 and SH2**—The nucleotide-induced changes in the accessibility of the fluorophore at SH2 was compared with that of the fluorophore at SH1. Because acrylamide scarcely quenched the prodan fluorescence, the accessibility of the fluorophore was measured in the presence and absence of ligands with KI as a quencher. The data were analyzed by calculation of Stern-Volmer quenching constants ($K_{SV}$) from plots of relative fluorescence versus KI concentration (36) (not shown). Within the range of the KI concentrations studied, all data could be described by a straight line, indicating the absence of multiple components or of a significant level of static quenching. In all cases, the fluorescence emission spectra were found to be unaltered by KI.

The results of these measurements are presented in Table IV. To account for the effects of ligands bound to S-1 on KI quenching of the fluorescence, the quenching constant data for each sample are presented also in the form of $K_{SV}/K_{SV}$ ratios. The values of $K_{SV}(N)$ and $K_{SV}$ represent the quenching constants in the presence and absence of ligands with KI as a quencher. Thus $K_{SV}(N)/K_{SV}$ ratios reveal the effect that ligands have on the fluorescence quenching of the fluorophores at SH1 and SH2.

Comparison of $K_{SV}$ values in the absence of ligand suggests that the fluorophore at SH2 ($K_{SV} = 3.0$ m$^{-1}$) is more accessible than that at SH1 ($K_{SV} = 1.3$ m$^{-1}$). On the other hand, comparison of the values of $K_{SV}(N)/K_{SV}$ ratios suggests that AMP-PNP scarcely affects the accessibility of the fluorophore in both S-1 derivatives compared with ATP and ADP. By contrast, the effects of ADP and ATP on the accessibility of the fluorophore were strongly dependent on the labeled thiol. Upon addition of ADP, the accessibility of the fluorophore at SH2 was decreased greatly ($K_{SV}(N)/K_{SV} = 0.53$), whereas that of the fluorophore at SH1 was scarcely changed ($K_{SV}(N)/K_{SV} = 0.92$). ATP, on the contrary, caused a 2.2-fold increase in the accessibility of the fluorophore at SH1. This is in marked contrast to the fluorophore at SH2. For the fluorophore at SH2, ATP made it less accessible with a $K_{SV}(N)/K_{SV}$ ratio of 0.60. Thus ATP-induced changes in the accessibility of the fluorophores attached to SH1 and SH2 are again opposite in its direction; the fluorophore at SH2 becomes inaccessible, whereas that at SH1 becomes accessible.

**Discussion**

It has been shown that the SH1-SH2 region of the motor domain of myosin plays a key role in the energy transduction system of myosin (4–9, 12–19). In particular, the nucleotide-induced changes in the location of SH2 observed in the atomic structure of S-1 (4, 6) have suggested its involvement in signal transduction on S-1 from the binding sites of ATP and actin to the mechanically important lever arm region. Despite these obvious reasons for the interest in SH2, little is known about the nucleotide-induced changes around SH2.

Several environmentally sensitive fluorescent probes are known to label conveniently only SH1 (14, 20, 26). In contrast to this, thus far little has been known about such probes for SH2 (20). In previous studies with the intention of monitoring the conformational changes around SH2, the fluorescent labeling of SH2 was performed after the protection of SH1 by F-actin (13) and the reversible blocking agent 2,4-dinitro-1-fluorobenzene (16, 19). In the present study, I found that AD can easily label only SH2 of S-1. I verified the specificity toward SH2 under conditions in which the reaction was performed at pH 8.0 and 7°C with a 3-fold molar excess of AD over S-1. AD proved to be a very useful labeling reagent for SH2 because of its selectivity to SH2 and moderate reactivity. Furthermore, it has the same fluorophore as that of prodan, which has frequently been used as an extremely sensitive environmental probe for proteins (21–24). These properties allow ready use of AD not only as a specific fluorescent label for SH2 but as a sensitive probe to precisely describe the conformational changes around SH2.

In this study, I used another fluorescent thiol reagent BD. Thus far, no study has been reported on its application to the fluorescent labeling not only of proteins but of amino acids. BD was found to predominantly label SH1, as was to be expected from the structure of its functional group. Therefore I could conveniently obtain the fluorescent derivatives of S-1 that bear the same prodan fluorophore at either SH1 or SH2. These

**Table IV**

| Fluorophore $^a$ | $K_{SV}^b$ (m$^{-1}$) | $K_{SV}(N)/K_{SV}$ |
|------------------|----------------------|---------------------|
| AD at SH2        |                      |                     |
| −Ligand          | 3.0 ± 0.1            | 0.60                |
| +ATP             | 1.8 ± 0.05           | 0.60                |
| +ADP             | 1.6 ± 0.05           | 0.53                |
| +AMP-PNP         | 3.4 ± 0.1            | 1.1                 |
| BD at SH1        |                      |                     |
| −Ligand          | 1.3 ± 0.05           | 0.60                |
| +ATP             | 2.8 ± 0.05           | 2.2                 |
| +ADP             | 1.2 ± 0.05           | 0.92                |
| +AMP-PNP         | 1.6 ± 0.1            | 1.2                 |

$^a$ Excitation was at 390 nm, and emission was at 500 nm.

$^b$ All values are the averages and standard deviations of three independent experiments.

SH1 was scarcely changed ($K_{SV}(N)/K_{SV} = 0.92$). ATP, on the contrary, caused a 2.2-fold increase in the accessibility of the fluorophore at SH1. This is in marked contrast to the fluorophore at SH2. For the fluorophore at SH2, ATP made it less accessible with a $K_{SV}(N)/K_{SV}$ ratio of 0.60. Thus ATP-induced changes in the accessibility of the fluorophores attached to SH1 and SH2 are again opposite in its direction; the fluorophore at SH2 becomes inaccessible, whereas that at SH1 becomes accessible.

**Discussion**

It has been shown that the SH1-SH2 region of the motor domain of myosin plays a key role in the energy transduction system of myosin (4–9, 12–19). In particular, the nucleotide-induced changes in the location of SH2 observed in the atomic structure of S-1 (4, 6) have suggested its involvement in signal transduction on S-1 from the binding sites of ATP and actin to the mechanically important lever arm region. Despite these obvious reasons for the interest in SH2, little is known about the nucleotide-induced changes around SH2.

Several environmentally sensitive fluorescent probes are known to label conveniently only SH1 (14, 20, 26). In contrast to this, thus far little has been known about such probes for SH2 (20). In previous studies with the intention of monitoring the conformational changes around SH2, the fluorescent labeling of SH2 was performed after the protection of SH1 by F-actin (13) and the reversible blocking agent 2,4-dinitro-1-fluorobenzene (16, 19). In the present study, I found that AD can easily label only SH2 of S-1. I verified the specificity toward SH2 under conditions in which the reaction was performed at pH 8.0 and 7°C with a 3-fold molar excess of AD over S-1. AD proved to be a very useful labeling reagent for SH2 because of its selectivity to SH2 and moderate reactivity. Furthermore, it has the same fluorophore as that of prodan, which has frequently been used as an extremely sensitive environmental probe for proteins (21–24). These properties allow ready use of AD not only as a specific fluorescent label for SH2 but as a sensitive probe to precisely describe the conformational changes around SH2.

In this study, I used another fluorescent thiol reagent BD. Thus far, no study has been reported on its application to the fluorescent labeling not only of proteins but of amino acids. BD was found to predominantly label SH1, as was to be expected from the structure of its functional group. Therefore I could conveniently obtain the fluorescent derivatives of S-1 that bear the same prodan fluorophore at either SH1 or SH2. These
fluorescent derivatives of S-1 are the most suitable for the study on the comparison of the nucleotide-induced changes in environment around SH2 with those in environment around SH1. If the different fluorophores are used, they may have different orientations at the labeling sites and interact with side chains located at different distances from the thiols, resulting in the complicated spectral data. To obtain a consistent view of the conformational changes around SH1 and SH2, it is necessary to use the same fluorophore. In the present paper, AD-S-1 and BD-S-1 could provide a precise description of the local environments around SH2 and SH1, respectively. The results allow us to compare the properties of the two sites in a more direct manner because the same sensitive fluorophore prodan is used to probe both sites.

Previous works (21, 22) have shown that an increase in the half-widths of the emission spectra of prodan indicates incomplete dipolar relaxation of the environment around the fluorophore bound to proteins, which results from the inaccessibility of solvent molecule to the fluorophore. This was the case for the emission spectra of AD and BD derivatives of S-1. The values of AD-S-1 in the absence of nucleotides (3397 cm\(^{-1}\)) and BD-S-1 (3291–3400 cm\(^{-1}\)) were close to that of the papain derivative (3411 cm\(^{-1}\)) (22). In the case of the S-1 derivatives, nucleotides further increase their spectral half-widths, especially for AD-S-1. It should be emphasized that the nucleotide-induced increases in the emission half-widths of AD-S-1 were more than 2.7 times larger than those of BD-S-1. This suggests that in the presence of nucleotides the fluorophore at SH2 is much less accessible to solvent molecule than that at SH1. These results were consistent with those obtained with the KI quenching of the fluorescence.

The crystal structure of S-1 (2) shows that the sulfur atom of SH2 is almost van der Waals’ contacts with the side chains of three surrounding hydrophobic residues Ala\(^{585}\), Phe\(^{477}\), and Ile\(^{669}\). From the present data it would appear that ATP and ADP make the fluorophore at SH2 placed in a hydrophobic and closed environment, which cause the blue shifts in emission maxima, the increases in the half-widths of the emission spectra, and the decreases in Stern-Volmer constants for the KI quenching of the fluorescence. Thus it is highly likely that upon addition of nucleotides, the fluorophore at SH2 is buried in the three surrounding hydrophobic residues, resulting in the reinforcement of the helix containing SH2. This conformational change may in turn induce movements of other segments to bring about the lever arm movement (3–9).

The fluorescent maleimide reagent MIANS also reports large spectral changes in response to conformational changes around SH2 initiated by the binding of nucleotides (16, 19). However, the changes are in the opposite direction of those sensed by AD reported here. ATP induces a 10-nm red spectral shift and a 40% decrease in the fluorescence intensity for MIANS-S-1 (16, 19). Furthermore, the acrylamide quenching study of the fluorescence has shown that the fluorophore at SH2 is exposed upon binding of ATP and ADP (16). These results are also opposite to those from the KI quenching study of the fluorescence of AD-S-1. It should be noted that the two fluorophores of MIANS (2-anilinonaphthalene-6-sulfonate) and AD (6-acyl-2-dimethylaminonaphthalene) are different not only in their sizes but in their charges: about 12 Å and negative charge for MIANS versus about 7.5 Å and noncharge for AD. Thus the two fluorophores attached to SH2 may have different orientations and interact differently with adjacent side chains. The apparent opposite changes in the SH2 microenvironment upon binding of nucleotides may thus reflect the different properties of the two fluorophores of MIANS and AD. Taking into account the compact size and the noncharge of the AD fluorophore, AD-S-1 may prefer to report the changes in its interactions with the neighboring hydrophobic side chains of SH2, unlike MIANS-S-1.

The overall data reported here suggest that the nucleotide-induced changes in environment around SH2 differ significantly from those in environment around SH1. In particular, during ATP hydrolysis, the changes around the two thiols are just opposite in its direction. Gly\(^{699}\) (close to SH2) and Gly\(^{710}\) (close to SH1) have been proposed to act as pivot points or flexible hinges upon movement of the lever arm (8, 10, 11). Supposing the conformational changes in the ATP binding site is propagated from the SH2 region to SH1 region, Gly\(^{699}\) as a pivot point disrupts a direct transmission of such signal along the polypeptide chain. Consequently, the two helices containing SH1 and SH2 cannot move as a single cooperative unit but behave so independently to suffer opposite changes in its environment. The most straightforward interpretation for the present results is that during ATP hydrolysis, the helix containing SH2 is buried in the hydrophobic side chains and rather reinforced, whereas the adjacent helix containing SH1 moves away from its stabilizing tertiary structural environment. Thus, the SH2 helix seems to remain stable throughout the ATPase cycle unlike the SH1 helix.

Since the manuscript was submitted, a comparative study of the crystal structure of S-1-ADP with those of uncomplexed S-1 and S-1-ADP-AlF\(_4^–\) (corresponding to the transient state of S-1 during ATP hydrolysis) has been published (37). The structures clearly show that the SH2 helix is so intact as to be wound throughout the three conformational states of S-1. However, the SH1 helix shows significant differences in structure for the three states of S-1. For the complex with MgADP, the SH1 helix is unwound, whereas the helix remains wound for the complex with MgADP-AlF\(_4^–\). Therefore, the nature of spectral changes detected around AD at SH2 in the present paper are compatible with the crystallographic data (37). However, the nucleotide-induced spectral changes of BD at SH1 cannot be explained only from the unwinding of the SH1 helix revealed in the crystallographic data. It is highly likely that changes in interactions between the SH1 helix and the switch II and/or the relay (4, 6, 37) are also involved in the nucleotide-induced spectral changes around BD at SH1.

Acknowledgment—I am grateful to Kyoko Nakamura for typing the manuscript.

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