Electric Potential at Regions near the Two Specific Thiols of Heavy Meromyosin Determined by the Fluorescence Quenching Technique

I. Effect of ATP

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Electric potentials at regions near the two specific thiol groups, SH₁ and SH₂, of the heavy meromyosin (HMM) molecule were studied by the fluorescence quenching technique. The effects of binding of ATP to HMM upon the electric potentials were also studied. N-(p(2-Benzimidazolyl)phenyl)maleimide (BIPM) was used as a thiol-directed fluorescent reagent. Prior to the labeling of SH₂ with BIPM, the SH₁ group was blocked with N-ethylmaleimide (NEM). Iodide ions (I⁻), thallium ions (Tl⁺), and acrylamide were used as quenchers of fluorescence. The sign of the electric potential was collectively determined from the dependence of the Stern-Volmer constants upon the ionic strength of solutions.

1. The region near SH₁ was at a negative electric potential, whereas the electric potential at the region near SH₂ was almost zero.

2. On the addition of ATP, the fluorescence intensity of BIPM bound to SH₁ was unchanged, whereas that of BIPM bound to SH₂ was greatly decreased to about 50% of the original level. The fluorescence intensity recovered as the added ATP was split into ADP and orthophosphate, and became saturated. The saturated level of the fluorescence intensity was, however, smaller than the original one, due to binding of the produced ADP to HMM.

3. On the addition of ATP, the negative electric potential at the region near SH₁ was unchanged, whereas a negative electric potential with large gradient was newly introduced at the region near SH₂. The value of the newly introduced electric potential was calculated on the basis of various assumptions. These results are discussed in connection with the functions of myosin.

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Abbreviations: HMM, heavy meromyosin; BIPM, N-(p(2-benzimidazolyl)phenyl)maleimide; N-Ac-Cys, N-acetyl-cysteine; 2-ME, 2-mercaptoethanol; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; NEM, N-ethylmaleimide.
The electric properties of muscle proteins, especially myosin and actin, may play an important role in the molecular mechanism of force generation in muscles. However, few studies have been reported on this.

Kobayashi et al. studied the electric properties of F-actin (1) and myosin subfragments (2) by using the method of electric birefringence. This method allows us to measure the dipole moment of whole protein. Although the electric properties of the whole protein must be important, local electric properties appear to be rather more important, taking into consideration that interactions among F-actin, myosin, and ATP occur at specific sites. Prior to the present report, we have shown, in simple systems without protein, that application of fluorescence quenching allows us to study the electric potential at the local region where the fluorophore is located (3). In the present study, this method was applied to investigations of electric potentials at two regions within HMM which contain the specific thiol groups, SH₁ and SH₂.

As these two of the 44 thiol groups which myosin contains (4, 5) are particularly susceptible to chemical modification, they have been studied in detail. Blockage of SH₁ inactivates the EDTA-ATPase and concomitantly activates the Ca²⁺-ATPase (6, 7). When SH₂ is blocked subsequently to blocking SH₁, the Ca²⁺-ATPase is inactivated (8, 9). Furthermore, binding of the two thiol groups with a bifunctional reagent inhibits the interaction of the modified protein with F-actin (10). The reactivities of SH₁ and SH₂ have also been used as a probe for myosin conformation. The binding of ATP or ADP to myosin enhances the reactivity of SH₁ in the presence of Mg²⁺ (8, 9), and F-actin further enhances the reactivity in the presence of Mg²⁺-ATP (11). Formation of a complex of F-actin and myosin heads blocks the reactivity of SH₁ (12). As a result of these investigations, it has been suggested that one or both thiol groups (13) may be located at or near sites essential for the functions of myosin. If both thiol groups or either are involved in the mechanism by which the functions of myosin proceed, it would be valuable to study the electric potentials in the vicinity of the thiol, because it is quite probable that the electric properties are involved in the mechanism.

The earliest study on the effects of electric charge (ultimately, the effects of electric potential) carried by the fluorophore upon the fluorescence quenching was made by Stoughton and Rollefson in 1939 (14). In 1971, Lehrer observed the charge effects using various kinds of model compounds (positively charged, negatively charged, and uncharged) with an indole ring, using iodide and hydrogen ions as quenchers (15). In 1974, Pownall and Smith also observed the effects on quenching of anthracene in charged micelles by pyridinium and iodide ions (16). These studies were, however, made on model compounds or systems with known electric properties, and have not yet been applied for studies on unknown electric properties of proteins. For such studies, three kinds of appropriate quenchers carrying positive, negative, and zero charge, appear to be required in order to obtain valid conclusions from the quenching data. Quenching of a fluorophore attached to a protein by an ionic quencher may be influenced by not only the exposure of the fluorophore but also by charges near it. When an uncharged quencher is used, quenching is affected only by the exposure of the fluorophore. Therefore, the use of an uncharged quencher is important. Recently, Eftink and Ghiron found acrylamide be an effective uncharged quencher (17). Although hydrogen and pyridinium ions have been used as positively charged quenchers, they tend to denature proteins (3). We found thallium (I) ions to be excellent positively charged quencher (3). Thus, three kinds of quenchers are available for proteins, i.e., I⁻, Tl⁺, and acrylamide.

From measurements of quenching of BIPM separately bound to SH₁ and SH₂ by the three kinds of quenchers, the following conclusions were obtained. The region near SH₁ is at a negative electric potential, whereas the electric potential at the region near SH₂ is zero. On the addition of ATP, the negative electric potential at the region near SH₁ is unchanged, whereas a negative electric potential is newly generated at the region near SH₂. These results are discussed in connection with the functions of myosin, and in the light of the primary structure in the vicinity of SH₁ and SH₂.
ELECTRIC POTENTIAL AT REGIONS NEAR SH₁ AND SH₂

MATERIALS

HMM was prepared from myosin by cleavage with trypsin which had been treated with tosyl-phenylalanine chloromethyl ketone (trypsin-Tos-PheCH₂Cl, Worthington Biochemicals), according to Highsmith (18).

N-Acetylcysteine (N-Ac-Cys), NEM, BIPM, KI, CH₃COOT₁, 2-mercaptoethanol (2-ME), and acrylamide were obtained from Wako Junyaku Co. ATP and ADP were purchased from Sigma Chemical Co.

METHODS

Modification of the SH₁ group of HMM with BIPM was carried out according to the method of Takamori et al. (19) with slight modifications: 3.6 mg/ml of HMM was incubated with a three-fold molar excess of BIPM in 0.5 M KCl, 20 mM Tris-maleate buffer (pH 6.0) for 45 min at 0°C with continuous stirring using a magnetic stirrer. The reaction was stopped by adding a large excess of 2-ME.

Selective modification of the SH₂ group of HMM was achieved by prior blocking of SH₁ with NEM by the method of Sekine and Yamaguchi (8) and subsequent reaction of SH₂ of the SH₁-NEM-HMM with BIPM. SH₁-NEM-HMM (4.4 mg/ml) was incubated with a two-fold molar excess of BIPM in 50 mM KCl, 20 mM Tris-maleate buffer (pH 7.0), 1 mM ADP for 5 min at 0°C with continuous stirring using a magnetic stirrer, then a large excess of 2-ME was added to the reaction mixture. Purification of SH₁-BIPM-HMM and SH₂-BIPM-HMM to remove the unreacted reagent was achieved using a Sephadex G-25 column equilibrated with 25 mM Tris-histidine (pH 8.0) and 1 mM MgCl₂ at 4°C.

Modification of N-Ac-Cys with BIPM was carried out as follows: 0.2 mM N-Ac-Cys dissolved in 25 mM Tris-histidine (pH 8.0) and 1 mM EDTA was mixed with 0.3 mM BIPM at room temperature. EDTA protects thiol groups against air oxidation (20). The unreacted BIPM was not removed, because it is non fluorescent (21) and causes no interference.

The adduct of BIPM with SH groups was not stable and the succinimide ring gradually opened to form the corresponding succinamic acid, which is more strongly fluorescent and is stable (22). Therefore, prior to various measurements, the BIPM-labeled HMM and N-Ac-Cys were kept in the dark at 0°C for three days after labeling.

According to the method of Yamamoto et al. (22), the extent of BIPM binding to HMM was determined by comparing the fluorescence intensity of the denatured form of BIPM-HMM in 1% sodium dodecyl sulfate (SDS) and 20 mM Tris-HCl (pH 8.0), with that of N-Ac-Cys-BIPM in the same solution.

The distribution of bound BIPM on heavy chains and the set of light chains was examined by measuring the fluorescence intensity of extracts from corresponding protein bands separated by SDS-polyacrylamide gel electrophoresis (PAGE). SDS-PAGE was carried out as described by Weber and Osborn (23) using 7% gel in 8-cm glass tubes. The total number of tubes was twelve. About 100 µg of modified HMM per tube was applied to eight tubes. Protein was not charged in the remaining tubes, which were used for measurements of the fluorescence of extracts from the polyacrylamide gel itself (22). Since the fluorescence of BIPM adduct within a gel would not be visible, four of the eight gels containing protein were stained with Coomassie Brilliant Blue. Two regions within the unstained gels, corresponding to heavy chains and the set of light chains, were dissected out using a pair of scissors. After crushing the dissected gels in a test tube, 2% SDS was added to extract the labeled protein, and the mixture was left at 37°C for 48 h with occasional mixing. After filtration of the suspension to remove the gel fragments, HCl was added to the filtrate to 30 mM in order to enhance the fluorescence intensity (21). The fluorescence intensity was measured at 380 nm with excitation at 320 nm. At the same time, the fluorescence intensity of the extract from the same amount of gel without protein was measured, and this value was subtracted from that obtained above.

The Ca²⁺- and EDTA-ATPase activities of native, SH₁-NEM-, SH₁-BIPM-, and SH₂-BIPM-HMM were assayed in 0.5 M KCl, 20 mM Tris-HCl (pH 7.4), 1 mM EDTA or 5 mM CaCl₂, and 1 mM ATP, with 0.03-0.08 mg protein per ml at 37°C. The amount of P₂ liberated was determined by the method of Gomori (24) with slight modifications.

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Quenching measurements at constant temperature, 25°C, were made on solutions containing 25 mM Tris-histidine buffer (pH 8.0), 1 mM MgCl₂, 0.5 μM SH₁ or SH₂-BIPM-HMM, various amounts of KCl or CH₃COOK and various amounts of a given quencher. When the measurements were made in the presence of ATP, 3 μl of 40 mM ATP was added to 3 ml of test solutions. A small amount of S₂O₃⁻ (ca. 10⁻⁴M) was added to the stock solution of KI (2 M) to prevent I₃⁻ formation (15). All stock solutions of KI, CH₃COOTl, acrylamide, KCl, CH₃COOK, ATP, SH₂-BIPM-HMM, and SH₂-BIPM-HMM contained 25 mM Tris-histidine buffer (pH 8.0) and 1 mM MgCl₂. As the ionic strength of solutions increases on the addition of KI or CH₃COOTl, the ionic strength was adjusted to a constant value with KCl or CH₃C00K. The fluorescence intensity was measured at 400 nm with excitation at 335 nm. Relative quantum yields of SH₁ and SH₂-BIPM-HMM solutions in the absence of quenchers were determined by dividing the fluorescence intensity by the optical density at 335 nm.

A Shimadzu RF-503 spectrofluorometer was used for fluorescence measurements, with an L-40 cutoff filter (Ohnaga Kogaku Inc.) for which T% was 0.01% at 360 nm.

The concentration of BIPM was determined in ethanol solution using a molar extinction coefficient of 996 at 310 nm (21). The concentration of HMM was determined by the method of Lowry et al. (25) taking a value of 3.4 x 10⁶ for the molecular weight.

**ANALYSIS**

A collisional quenching of fluorescence can be described by the familiar Stern-Volmer equation;

\[ \frac{F_0}{F} - 1 = K_Q[Q], \]

where \( F_0 \) is the fluorescence intensity in the absence of quencher, \( F \) is the fluorescence intensity in the presence of quencher at a concentration \([Q]\), and \( K_Q \) is the Stern-Volmer constant. \( K_Q \) is equal to the product of the rate constant \( k_q \) of the quenching reaction and the fluorescence lifetime \( \tau_F \) in the absence of quencher. The fluorescence lifetime is generally proportional to the quantum yield \( \phi \) of fluorescence. Thus, \( k_q \) is proportional to \( K_Q/\phi \).

The rate constant \( k_q \) for the quenching reaction between the quencher and a fluorophore bound to protein is influenced by the degree of exposure of the fluorophore. When the quencher carries an electric charge \( Z_qe_0 \), the rate constant is also influenced by the product of \( Z_qe_0 \) and the electric potential \( \phi \) at the position where the fluorophore is located (3).

The sign of \( \phi \) (denoted by \( S_e.\phi \)) can be determined from the dependence of \( K_Q/\phi \) on the ionic strength \( \mu \) of solutions, if the exposure of the fluorophore is not significantly influenced by ionic strength. The equation for determination of the sign is

\[ S_e.\phi = S_q \times S_{\mu}, \]

where \( S_q \) is the sign of the charge carried by the quencher and \( S_{\mu} \) is the sign of \( \partial(K_Q/\phi)/\partial \mu \) (3). If the value of \( K_Q \) in the case of \( Z_qe_0\phi = 0 \), \( K_Q^0 \), is given, the value of electric potential can also be calculated from the value of \( K_Q \) in the case of \( Z_qe_0\phi \neq 0 \), by using the following equation;

\[ \phi = \frac{k}{Z_qe_0} \ln \left( \frac{K_Q^0}{K_Q} \right), \]

where \( k \) is Boltzman's constant and \( T \) is the absolute temperature (3).

**RESULTS**

Amount and Distribution of Reacted BIPM—Modification of SH₁ with a thiol reagent leads to enhancement of the Ca²⁺-ATPase activity and a concomitant decrease of the EDTA-ATPase activity of myosin or its enzymatic subfragments. Subsequent modification of SH₂ results in a decrease of the Ca²⁺-ATPase activity of the SH₁-modified myosin or its enzymatic subfragments. These changes in the ATPase activity have been well established. On the basis of these established facts, it is possible to confirm whether selective modifications of SH₁ and SH₂ with BIPM have been achieved or not. Table I shows the results, confirming successful selective modification. Table I also shows that large amounts of unreacted SH₁ remained in the SH₁-BIPM-HMM. This is due to the adoption of pH 6.0 for the modification of SH₁. The adoption of this pH was a compromise strategy for the selective modification of SH₁.
TABLE I. The EDTA-ATPase and Ca²⁺-ATPase activities of native and modified HMM. Assays: 0.5 M KCl, 20 mm Tris-HCl (pH 7.4), 1 mm EDTA or 5 mm CaCl₂, 1 mm ATP and 0.03-0.08 mg protein per ml at 37°C.

<table>
<thead>
<tr>
<th>Protein</th>
<th>EDTA-ATPase (μmol P₁/min/mg protein)</th>
<th>Ca²⁺-ATPase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native-HMM</td>
<td>1.08</td>
<td>0.14</td>
</tr>
<tr>
<td>SH₁-NEM-HMM</td>
<td>0</td>
<td>1.34</td>
</tr>
<tr>
<td>SH₂-BIPM-HMM</td>
<td>0.58</td>
<td>0.8</td>
</tr>
<tr>
<td>SH₃-BIPM-HMM</td>
<td>0</td>
<td>0.27</td>
</tr>
</tbody>
</table>

TABLE II. Numbers of BIPM molecules reacted per HMM molecule and their distribution into heavy and light chains.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Amounts of BIPM reacted</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total</td>
</tr>
<tr>
<td>SH₁-BIPM-HMM</td>
<td>0.9</td>
</tr>
<tr>
<td>SH₂-BIPM-HMM</td>
<td>1.36</td>
</tr>
</tbody>
</table>

* Values are numbers of BIPM molecules incorporated into total heavy or total light chains in one HMM molecule, and are averages of 8 gels in two experiments.

because SH₁ and SH₂ are modified simultaneously at more alkaline pH (19).

Although the method utilizing the specific changes in the ATPase activity is convenient, it does not allow us to examine the bound BIPM on light chains of HMM. Yamamoto and Sekine reported the distribution of a thiol reagent, N-(7-dimethyl-amino-4-methyl-coumarinyl)maleimide, on heavy and light chains of myosin (26). Therefore, the distributions of BIPM within SH₁-BIPM-HMM and SH₂-BIPM-HMM on heavy chains and the set of light chains were studied by SDS-PAGE. The total numbers of BIPM molecules bound to SH₁ and SH₂ were 0.9 and 1.36 per HMM molecule, respectively (see Table II). The former was distributed in the ratio of 7 : 3 on heavy and light chains. In the case of SH₂-BIPM-HMM, the 1.36 molecules of BIPM were distributed in a ratio of about 9 : 1 on heavy and light chains.

Effects of ATP on the Fluorescence of SH₂-BIPM-HMM—Since specific changes in the ATPase activity of myosin are induced by chemical modifications of SH₁ and SH₂, it has been suggested that one or both of SH₁ and SH₂ are located at or near the active site of myosin. Therefore, we studied whether changes in the fluorescence of BIPM incorporated into SH₁ or SH₂ were induced by the binding of ATP to the modified proteins. The addition of ATP to SH₁-BIPM-HMM did not induce any change in the fluorescence of the BIPM adduct. On the other hand, as shown in Fig. 1, the addition of ATP to SH₂-BIPM-HMM induced a large decrease in the fluorescence intensity of the BIPM adduct. The wavelengths of the emission and excitation maxima, however, were not changed, and the optical absorption was also unchanged. As shown in Fig. 2, the fluorescence intensity initially decreased on adding ATP and then gradually increased as the added ATP was consumed by the Mg²⁺-ATPase of the modified HMM. It was saturated at a level lower than the original one, due to binding of ADP produced by the splitting of the added ATP. Indeed, as the amount of ATP was increased, the saturated level after the consumption of the added ATP gradually became smaller. The extent of the maximum decrease in the fluorescence intensity produced by excess ATP was same as that caused by excess ADP.

Fluorescence Quenching of Free SH₂-BIPM—Prior to measurements of quenching of BIPM bound to protein, the fluorescence quenching of free N-Ac-Cys-BIPM was studied. The Stern-Volmer plots are shown in Fig. 3. The slopes of the straight lines represent each Stern-Volmer constant. The slopes with I⁻, acrylamide and Tl⁺ increased in that order. Although the quenching measurements were made at about 0.6 ionic strength, the effects of negative charge carried by the carboxyl group of N-Ac-Cys-BIPM appear to be still significant.

Whether a collisional quenching mechanism is dominant over a static one in these quenchings can be judged from the value of the quenching rate constant, k₉ (=K₀/τ₀). The fluorescence lifetime of the adduct of BIPM with SH groups is known to be about 1 ns (27). Using the K₀ value with acrylamide (5.45 M⁻¹) and the fluores-
Fig. 1. Excitation and emission spectra of 0.5 μM SH2-BIPM-HMM in 25 mM Tris-histidine buffer (pH 8.0), 1 mM MgCl₂ at 25°C. The excitation spectra were measured at 365 nm and the emission spectra were obtained by excitation at 320 nm. The stronger and weaker signals were measured in the absence and presence of ATP (40 μM), respectively.

Fig. 2. The time course of fluorescence intensity of 0.28 μM SH2-BIPM-HMM in 0.2 M KCl, 1 mM MgCl₂, and 25 mM Tris-histidine buffer (pH 8.0) at 25°C. 1.5 μM ATP was added at arrow 1, 3 μM ATP was further added at arrow 2, and 4.5 μM ATP was further added at arrow 3. The fluorescence was measured at 400 nm with excitation at 335 nm.

cence lifetime, $k_q$ is calculated to be $5.45 \times 10^4 \text{M}^{-1} \text{s}^{-1}$. This $k_q$ value is almost equal to the rate constant of diffusion-controlled reactions in solutions (3, 28). Therefore, the type of quenching of N-Ac-Cys-BIPM by acrylamide appears to be collisional with a quenching efficiency of about 1. The same conclusion can be reached for quenching by $\text{I}^-$ and $\text{Th}^+$, considering the effects of negative charge.

*J. Biochem.*
Electric Potential at Regions Near SH1 and SH2

The Stern-Volmer plots of quenching of SH1-BIPM-HMM by I−, Ti+, and acrylamide are shown in Fig. 4 (a, b, c). The plots do not follow the simple Stern-Volmer relationship and are nonlinear. A downward deviation of the plots generally occurs when several classes of fluorophore having different Stern-Volmer constants are present (15, 17). As mentioned above, BIPM was distributed to the light chains as well as SH1. Alternatively, the conformation in the vicinity of SH1 might be changed by higher concentrations of quencher, and BIPM bound to SH1 might be buried in a region of HMM which is inaccessible to the quencher. In fact it has been reported that conformational changes of myosin are induced by high concentrations of LiBr, KI, and KCNB (29). Whatever the mechanism, it is clear that the initial slope of the Stern-Volmer plots represents the average of Stern-Volmer constants weighted by the appropriate fractions of the total fluorescence intensity of BIPM bound to SH1 and light chains (3). The initial slopes with I− and acrylamide increased with increase of the ionic strength of the solutions, while in contrast, the slope with...
Fig. 5. Dependence of the relative quantum yield of SH$_1$-BIPM-HMM upon ionic strength ($\mu$). Conditions: 25 mM Tris-histidine buffer (pH 8.0), 1 mM MgCl$_2$, 0.5 $\mu$m SH$_1$-BIPM-HMM, and various amounts of KCl (○) or CH$_3$COOK (□) at 25°C. The relative quantum yield was obtained by dividing the fluorescence intensity at 400 nm with excitation at 335 nm, by the optical density at 335 nm. The value at 0.5 M KCl was normalized to unity.

Tl$^+$ decreased. These changes in the slope do not directly represent changes in the corresponding rate constant, $k_q$ ($\propto K_0/\phi$), because the quantum yield of fluorescence of SH$_1$-BIPM-HMM increased with increase of the ionic strength, as shown in Fig. 5. Therefore, plotting $K_0/\phi_{rel}$ vs. ionic strength ($\mu$), we obtain Fig. 6. Since $K_0/\phi_{rel}$ with acrylamide was independent of ionic strength, it was concluded that exposure of the BIPM adduct did not change with ionic strength.

As mentioned in “ANALYSIS,” the sign of the electric potential at the position where the fluorophore is located can be determined from the sign of the quencher’s charge and the sign of $\partial(K_0/\phi_{rel})/\partial \mu$, through Eq. 2. The data in Fig. 6 for quenching by Tl$^+$ and I$^-$ clearly indicate a negative sign of the electric potential. It is, however, difficult to conclude definitely that the sign of the electric potential at the region near SH$_1$ is negative, because BIPM within SH$_1$-BIPM-HMM was also incorporated into light chains to some extent. However, we can conclude that one or both of the region near SH$_1$ and the light chains region are in an environment of negative electric potential. If the observed negative electric potential is present only at the light chains region, however, the absolute value of the potential must be very large.

It should be noted here that the dissociative group, imidazole, within the BIPM adduct is not charged at pH 8.0, which was adopted for quenching measurements.

The Stern-Volmer plots of quenching of SH$_2$-BIPM-HMM by I$^-$ (○), Tl$^+$ (□), and acrylamide (△) upon the ionic strength ($\mu$) of the solutions. The values of the relative rate constants were obtained from the data in Figs. 4 and 5.
Fig. 7. Stern-Volmer plots of quenching of 0.5 μM SH2-BIPM-HMM by I− (a), Tl+ (b), and acrylamide (c) in the absence and presence of ATP. Conditions: 1 mM MgCl₂, 25 mM Tris-histidine buffer (pH 8.0), various amounts of KCl (a, c) or CH₃COOK (b) and 0 (○,△,□) or 40 μM (●, ■, ■) ATP, at 25°C. The ionic strengths were adjusted by adding KCl or CH₃COOK. The ionic strengths were about 0.115 (○,●), 0.215 (△,■), and 0.415 (□,■). The fluorescence was measured at 400 nm with excitation at 335 nm.

Fig. 8. Dependence of the relative quantum yield of SH2-BIPM-HMM upon ionic strength (μ), and on ATP. Conditions: 25 mM Tris-histidine buffer (pH 8.0), 1 mM MgCl₂, various amounts of KCl (○,●) or CH₃COOK (□,■) and 0 (○,□) or 40 μM (●,■) ATP, at 25°C. The relative quantum yield was obtained by dividing the fluorescence intensity at 400 nm with excitation at 335 nm, by the optical density at 335 nm. The value at 0.4 M KCl in the absence of ATP was normalized to unity.

Fig. 9. The dependence of the relative rate constants (KQ/φrel) of quenching of SH2-BIPM-HMM by I− (○,●), Tl+ (□,■), and acrylamide (△,■) in the absence (○,△,□) and presence (●,■,■) of 40 μM ATP upon ionic strength (μ). The values of the relative rate constants were obtained from the data in Figs. 7 and 8.
action between BIPM bound to SH₂ and the ionic quenchers. As about 90% of BIPM was incorporated into SH₂, it can be concluded that the electric potential at the region near SH₂ was zero.

As mentioned above, if the negative electric potential observed on SH₁-BIPM-HMM is present only at the light chains region, the absolute value of the potential must be very large. Assuming that this is so, a negative electric potential should also be observed on SH₂-BIPM-HMM, since 0.12 molecules of BIPM per SH₂-BIPM-HMM molecule was incorporated into the light chains. As since this was not observed in practice, the above assumption does not appear to be correct. Therefore, it seems reasonable to conclude that the region near SH₁ is at a negative electric potential.

**Effects of ATP on the Electric Potential**—Mg²⁺-ATP carries a double negative charge. If SH₁ and SH₂ are located at or near the active site of HMM, it is quite probable that the binding of Mg²⁺-ATP to HMM would introduce some electric potential at the regions near SH₁ and SH₂. Therefore, the effects of ATP on the electric potential were studied.

Regardless of the presence of any quencher, the addition of ATP to SH₁-BIPM-HMM did not induce any change in the fluorescence of the BIPM adduct. That is, the negative electric potential observed in the absence of ATP did not change on adding ATP.

As mentioned above, the quantum yield of fluorescence of SH₂-BIPM-HMM was significantly decreased (see Fig. 8). Further, the Stern-Volmer plots of quenching by I⁻, Tl⁺, and acrylamide were also significantly changed, as shown by filled symbols in Fig. 7 (a, b, c). Plots of $K_Q/\phi_{rel}$ vs. ionic strength are shown in Fig. 9 (closed symbols). $K_Q/\phi_{rel}$ with acrylamide in the presence of ATP was almost the same as that in the absence of ATP, which indicates that the binding of ATP to the modified HMM induced no change in the exposure of the BIPM bound to SH₂. $K_Q/\phi_{rel}$ with I⁻ in the presence of ATP was almost the same as that in the absence of ATP, which indicates that the binding of ATP to the modified HMM induced no change in the exposure of the BIPM bound to SH₂. $K_Q/\phi_{rel}$ with Tl⁺ increased on the addition of ATP, which indicates that a repulsive force and an attractive force arose between I⁻ and the BIPM adduct and between Tl⁺ and the BIPM adduct, respectively. This is strongly supported by the dependence of $K_Q/\phi_{rel}$ with I⁻ and Tl⁺ in the presence of ATP upon ionic strength. The value of $K_Q/\phi_{rel}$ with I⁻ increased and that with Tl⁺ decreased with increase of the ionic strength. Therefore, it can be concluded that a negative electric potential was newly generated at the region near SH₂ on adding ATP. As the exposure of BIPM bound to SH₂ was scarcely changed by adding ATP, and the electric potential at the region near SH₂ was zero in the presence of ATP, the value of the negative electric potential in the presence of ATP can be calculated by using Eq. 3. From the data with Tl⁺ in Fig. 9, the electric potential was calculated to be $-0.035 \text{ V}$, and from the data with I⁻, it was calculated to be $-0.018 \text{ V}$. The difference between these values appears to arise from the so-called "size effect" (3). That is, when a large gradient of negative electric potential exists in the range of size of the fluorophore, a quencher carrying a negative charge tends to collide with the fluorophore at positions of higher electric potential, while a quencher carrying a positive charge tends to collide with the fluorophore at positions of lower electric potential. This "size effect" causes the value of electric potential obtained from the data with Tl⁺ to be lower than that from the data with I⁻.

The negative electric potential newly introduced at the SH₂ region might be generated by the charge carried by Mg²⁺-ATP or an intermediate product. Alternatively, it might be generated by charged side chains in the vicinity of SH₂ which moved in response to changes in the conformation of the active site of the modified HMM. If the former mechanism is correct, the distance between BIPM bound to SH₂ and the negative charge carried by Mg²⁺-ATP or the intermediate product can be calculated in the manner used previously to calculate the distance between the ethenoadenine ring and phosphate groups in 1,N⁶-ethenoadenosine oligophosphates (3). In this calculation, some assumptions must be made, i.e., that the dielectric constant and ionic strength of medium near the active site are the same as those of the external solution, and that the negative charge of Mg²⁺-ATP or the intermediate product is bivalent. On these assumptions, the distance can be calculated by means of the Debye-Hückel equation as follows,

$$
\phi \approx \frac{2e_0 e^{a/\lambda}}{D} \frac{e^{-<r>/\lambda}}{1 + a/\lambda} \langle r \rangle.
$$

where $D$ is the dielectric constant of the solvent, $a$ is the mean distance of closest approach of ions, J. Biochem.
\( \lambda \) is the so-called Debye radius, \( \langle r \rangle \) is the ensemble average of distance between the bivalent negative charge and a quencher which forms an encounter complex with BIPM bound to SH. The value of \( \lambda \) for an aqueous solution of ionic strength 0.125, at 25°C, is about 8.7 Å. Introducing this value of \( \lambda \), the estimated value of \( a \), 4 Å, and the value of the electric potential obtained above into Eq. 4, we obtained values of \( \langle r \rangle \) of 5.8 Å and 8.2 Å, corresponding to \(-0.035\) V and \(-0.018\) V electric potentials, respectively. Therefore, the distance between the center of the BIPM molecule bound to SH and the bivalent negative charge may be about the average of the two values, \( i.e., \), 7 Å.

**DISCUSSION**

It was shown in this report that the electric potential at the region near SH of modified HMM is negative. The electric potential at a certain position within a protein is determined by the configuration of negatively and positively charged amino acid residues. The amino acid sequences of an SH-containing peptide and an SH-containing peptide have been studied by Yamashita et al. (30, 31). Recently, Elzinga and Collins determined the sequence of a myosin fragment that contains both SH1 and SH2 (32). According to these studies, two arginines and one lysine are within four amino acids adjacent to SH1. These amino acids bear positively ionizable groups, guanidyl and \( \varepsilon \)-amino groups, respectively. These groups appear to be positively ionized in practice, because of the extremely low \( pK_a \) value of SH1, 6.28, which was measured by Takamori et al. (19). Although they speculated that this low \( pK_a \) value might be due to hydrogen bonding or to the surrounding hydrophobic amino acids, this does not seem likely. SH groups are generally poor in forming hydrogen bonds (33), and the \( pK_a \) value of acidic groups increases with decrease of the dielectric constant of solvents (34). One of the factors causing the SH groups to have a low \( pK_a \) is positive electric potential (35). Therefore, it is very possible that the electric potential at SH1 is positive. This does not necessarily conflict with our observations, because it is not at SH1 but in the neighbourhood of SH1 where the luminous group of the BIPM adduct is located. Indeed the size of the luminous group is quite large, and further, it is connected with SH1 through a short succinamic acid chain (22). Therefore, some carboxylic groups must be in the vicinity of SH1 in the tertiary structure of myosin heads.

Although Seidel and Gergely previously reported changes in the spectra of SH1-spin-labeled HMM on adding ATP (36), we could not observe any change in the fluorescence of BIPM bound to SH1 or in the negative electric potential. On the other hand, the addition of ATP greatly decreased the fluorescence intensity of BIPM bound to SH2. Quite similar changes in fluorescence were observed by Sekine et al., using \( N-(p-(2-benzoxazolyl)phenyl)maleimide \) as an SH-directed reagent (37). Furthermore, we observed that a negative electric potential with a large gradient was newly generated at the region near SH2 on adding ATP. Although SH1 is located near SH2 and the distance between them is 12–14 Å in the presence of Mg\(^{2+}\)-ATP (38), the striking contrast between the above phenomena strongly suggests that Mg\(^{2+}\)-ATP interacts more strongly with amino acid side chains in the vicinity of SH2 than with those in the vicinity of SH1.

It is probably premature at present to conclude that the electric potential generated at the region near SH2 on adding ATP arises directly from the negative charge carried by the substrate. We propose here the following mechanism for the potential change. The charge carried by the substrate induces changes in the degree of dissociation of the ionizable residual groups located close to the substrate binding site. The changes in the degree of dissociation result in a new arrangement of the electric charges carried by the residues. Finally, the newly arranged electric charges induce a negative electric potential at the region near SH2. The rearrangement of electric charges in such a scheme must cause a change in the inherent ability of myosin to bind strongly to F-actin (rigor binding), which might result in the dissociation of myosin from F-actin. In practice, electrostatic interaction must play an important role in the ternary interactions among myosin, F-actin and ATP, because the actin-activated ATPase activity is sensitive to the ionic strength of solutions.

We have thus shown that the fluorescence quenching technique is very useful for the determination of electric potential at a local region of
a protein. Further studies on electric potential changes during the interactions among muscle proteins may permit further elucidation of the molecular mechanism of muscle contraction.

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