BBA 46250

REACTION OF 2-BROMOACETAMIDE-4-NITROPHENOL WITH HEAVY MEROMYOSIN ATPase

K. UCHIDA, K. TANAKA AND T. HIRATSUKA

Department of Chemistry, Faculty of Science, Hokkaido University, Sapporo (Japan)

(Received May 24th, 1971)

SUMMARY

2-Acetamide-4-nitrophenolic groups were introduced into the reactive cysteine residues of heavy meromyosin. Incorporation of the nitrophenolic groups occurred in two phases, characterized by an initial rapid rate followed by a slow rate. When 3.5 nitrophenolic groups per mole of heavy meromyosin were incorporated during the initial phase, a 4-fold activation of Ca^{2+}-activated ATPase was observed. Changes in the ATPase activity of the labeled enzyme are qualitatively the same as those observed with heavy meromyosin modified by other sulfhydryl (SH) reagents.

Difference spectra of the labeled enzyme, induced by ATP and its analogues, were measured around 445 nm. ATP, ADP and pyrophosphate produced a specific shift in the absorption spectrum toward a higher wavelength when interacting with the labeled enzyme. This suggests that binding of these compounds to the active site of the labeled enzyme produces a shift of the nitrophenolic groups bound to heavy meromyosin to a less polar environment. The difference absorbance at 440 nm was measured as a function of ADP concentration. Spectral changes appear to reflect certain structural consequences of nucleotide binding to the active site of the enzyme.

INTRODUCTION

A number of observations indicate that sulfhydryl (SH) groups are implicated in the catalytic activities of myosin and heavy meromyosin and are sensitive sites of action of various chemical modifiers of ATPase activity[1–11]. Activation of Ca^{2+}-activated ATPase of myosin is observed at low molar ratios of the SH reagent to enzyme, although inhibition is seen at high molar ratios. This characteristic of modification has been interpreted in terms of conformational changes in the myosin molecule[6].

It has been reported that 2-bromoacetamide-4-nitrophenol reacts selectively with SH groups at the active site of triosephosphate dehydrogenase to introduce the environmentally sensitive *p*-nitrophenolic group into protein[12]. Binding of the chromophore to SH groups of heavy meromyosin permits the study of the effect of changes

Abbreviations: SH, sulfhydryl; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); PMB, *p*-mercuribenzoate.

in the environment of a specific region of the enzyme. The present communication describes the activation of heavy meromyosin ATPase with 2-bromoacetamide-4-nitrophenol and a means of assessing the role of the cysteinyl moiety in the catalytic activity of heavy meromyosin. The results suggest that ATP alters the chemical environment of the nitrophenolic group attached to SH groups which exert a large influence on the ATPase activity of heavy meromyosin.

EXPERIMENTAL

Materials

Heavy meromyosin was prepared by the procedure described previously[8,11]. 2-Bromoacetamide-4-nitrophenol was prepared as reported by HILLE AND KOSHLAND[13] and recrystallized from 99% ethanol, m.p. 215°. 2-Acetamide-4-nitrophenol was prepared according to the method of HEWITT AND KING[14]. A product with a melting point of 279° was obtained. 5,5'-Dithiobis(2-nitrobenzoic acid) (DTNB) was obtained from the Aldrich Chemical Co. ATP and ADP were obtained from Sigma Chemical Co.

Methods

Chemical modification of heavy meromyosin with a 200-fold molar excess of 2-bromoacetamide-4-nitrophenol over the enzyme was carried out with magnetic strirring at 25°. A solution of the enzyme in 4 mM borate buffer (pH 8.0) was added to the reagent suspended in 0.5 M KCl and 50 mM borate buffer (pH 8.0). The final concentration of protein in the reaction mixture was 10–15 mg/ml in all experiments. After a given period the reaction mixture was quickly chilled in an ice bath, followed by filtration to eliminate insoluble materials. Then, gel filtration with a column of Sephadex G-50 (1.7 cm × 28 cm) was used to separate the labeled enzyme from unreacted reagent. The gel was equilibrated with 4 mM borate buffer (pH 8.0) and 0.1 M KCl at 4°. The control heavy meromyosin was treated in the same manner but without addition of the reagent unless otherwise stated.

The number of 2-acetamide-4-nitrophenol groups incorporated into heavy meromyosin was determined using the extinction coefficient of 17600 $M^{-1} \cdot cm^{-1}$ for absorption at 417 nm and at pH 9.0. The extinction coefficient was determined from the absorbance of the labeled enzyme at 417 nm and moles of 2-amino-4-nitrophenol liberated from the labeled enzyme by acid hydrolysis as reported by HILLE AND KOSHLAND[13].

Protein concentration was determined by measuring the absorbance at 280 nm using a factor of 0.63/mg per ml and also by the biuret method. The molecular weight of heavy meromyosin was taken as $3.5 \cdot 10^5$ (ref. 15).

Ca^{2+}-activated ATPase assay was performed as described in previous papers[8,11].

SH groups were determined according to the method of ELLMAN[16] in the presence of urea[11]. Titration with *p*-mercuribenzoate (PMB) was also carried out[17].

Spectra were measured with a Hitachi recording spectrophotometer, Type 124, at room temperature. The measurement of difference spectra was performed in four matched 1-cm cells; two reference cells and two sample cells were used in each experiment[18]. A full-scale sensitivity of 0.1 absorbance unit was used.

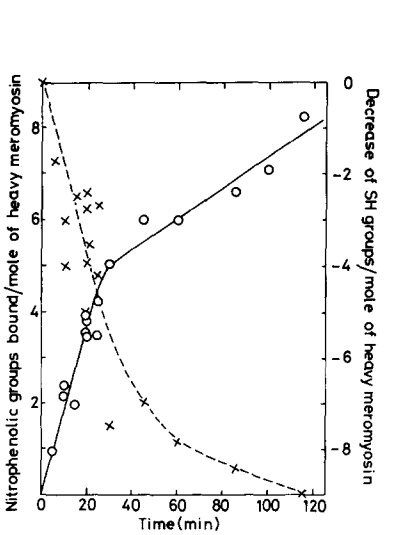
RESULTS

Modification and its effect on ATPase activity

Since the side reaction, the intramolecular cyclization of 2-bromoacetamide-4-nitrophenol, occurring in neutral and basic aqueous solution[13] results in the depletion of the reagent, a large excess of solid reagent was added into the reaction mixture. The labeled enzyme obtained has an absorption maximum at 417 nm at neutral and basic pHs. The labeled enzyme was hydrolyzed in 6 M HCl for 24 h in a sealed tube at 110°. The hydrolyzate was verified to contain 2-amino-4-nitrophenol from its spectrum in 0.5 M NaOH with an absorption maximum at 445 nm[13].

Incubation of heavy meromyosin with a 200-fold molar excess of the reagent resulted in increased incorporation of nitrophenolic groups as a function of time. Subsequent to the removal of excess reagent, an aliquot of the labeled enzyme was exposed to 0.1 M Tris–HCl buffer (pH 9.0) and 0.5 M KCl to determine the number of nitrophenolic groups incorporated. Another aliquot was used for the SH determination. It can be seen in Fig. 1 that incorporation of nitrophenolic groups occurred in two phases, characterized by an initial rapid rate followed by a slow rate. In the initial rapid phase, 4 nitrophenolic groups were incorporated per mole of heavy meromyosin, concomitant with a proportional decrease in the number of SH groups.

As shown in Fig. 2, incorporation of nitrophenolic groups into heavy mero-



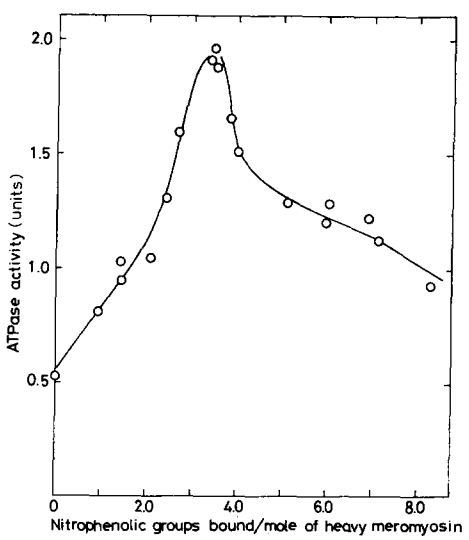


Fig. 1. Incorporation of 2-acetamide-4-nitrophenolic groups into heavy meromyosin as a function of time. Heavy meromyosin (50–80 mg) in 5 ml of 50 mM borate buffer (pH 8.0) and 0.5 M KCl was treated with a 200-fold molar excess of 2-bromoacetamide-4-nitrophenol at 25°. The degree of incorporation was determined after gel filtration as described under EXPERIMENTAL (○). The decrease of SH groups in the labeled enzyme was obtained by subtraction of the SH content of the control heavy meromyosin, treated in the same manner but without the reagent, from that of the labeled enzyme (×).

Fig. 2. Change in Ca^{2+}-activated ATPase activity of heavy meromyosin and number of nitrophenolic groups incorporated. Reaction times ranging from 5 to 115 min were taken to obtain the various degrees of incorporation. The ATPase activity was expressed in units representing μmoles P_i liberated/min per mg protein.

myosin resulted in a marked increase in the Ca^{2+}-activated ATPase activity. The activity rose to a peak value with an incorporation of 3.5 groups (4-fold activation) and then progressively fell to a level of 2-fold activation with 8 groups incorporated. K^+-activated ATPase of the labeled enzyme containing 3.5 nitrophenolic groups was still active in the presence of 5 mM EDTA (specific activity was 0.2 μmole P_i/ liberated per min per mg protein).

Up to a 3.5-fold activation of Ca^{2+}-activated ATPase of the control, heavy meromyosin was initially observed on reaction with PMB at about one-half the concentration of free SH groups whereas inactivation was observed at higher concentrations of PMB (Fig. 3). However, only progressive inhibition, and no activation, was found when the labeled enzyme containing 3.5 nitrophenolic groups was titrated with PMB. The activation and inhibition were further confirmed and quantitated with respect to the number of SH groups in two preparations reacted by spectrophotometric titration with PMB. A total of 26 SH groups per mole of the control heavy meromyosin, which was treated with 2-acetamide-4-nitrophenol instead of 2-bromoacetamide-4-nitrophenol, was found, compared with 22.8 SH groups in the labeled enzyme. It is clear that nitrophenolic groups are bound covalently to the reactive SH groups which are essential for activation of Ca^{2+}-activated ATPase.

The pH–activity curve of heavy meromyosin ATPase has a maximum at pH 6.5 and a minimum at pH 7.5 in the presence of 5 mM $CaCl_2$ and 0.5 M KCl. As shown

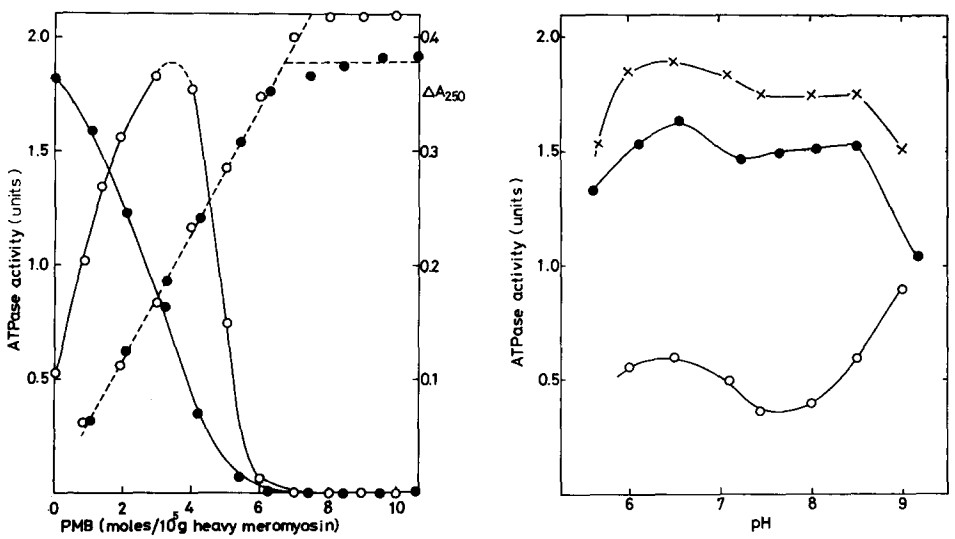


Fig. 3. Spectrophotometric titration of SH groups with PMB and Ca^{2+}-activated ATPase activity of the labeled enzyme and the control. The labeled enzyme contains 3.4 nitrophenolic groups per enzyme (●). In this case, heavy meromyosin as the control was treated with 2-acetamide-4-nitrophenol in place of 2-bromoacetamide-4-nitrophenol (○). Incubation with PMB was carried out in 0.5 M KCl and 50 mM Tris–HCl buffer (pH 8.0) for 20 h at 4°. ———, ATPase activity; – – – – –, difference absorbance at 250 nm.

Fig. 4. pH and activity profile of the control and the labeled enzyme. The labeled enzymes contain 2.4 (●) and 3.5 (×) nitrophenolic groups, respectively. The control (○) was prepared as described in the legend to Fig. 3. ATPase activity was measured in 0.5 M KCl, 5 mM $CaCl_2$, 2 mM ATP and 0.1 M Tris–acetate or 0.1 M Tris–maleate buffer. Reaction was initiated by addition of the enzyme.

in Fig. 4, the curve of the labeled enzyme containing 2.4 or 3.5 nitrophenolic groups was different from that of the control. Activation was considerable at the neutral pH.

Changes in the ATPase activities of the labeled enzyme are qualitatively the same as those observed with heavy meromyosin modified by other SH reagents[3,8,11]. These results strongly suggest that 2-bromoacetamide-4-nitrophenol reacts selectively and rapidly with 3.5–4 SH groups per mole of heavy meromyosin which are more reactive than the others in heavy meromyosin.

Spectrophotometric studies of the labeled enzyme

The absorption spectrum of the labeled enzyme containing 3.5 nitrophenolic groups was measured at various pH values. 2-Acetamide-4-nitrophenol was used for control spectra. Free nitrophenol showed an absorption maximum at 410 nm in the pH range from 5.5 to 9.4. The protein-bound moiety at pH values from 6.05 to 9.4 has an absorption maximum at 417 nm. Measurement at pH values lower than 6.0 was not attempted because of the aggregation of the enzyme. When the fractional absorbances of 2-acetamide-4-nitrophenol and the labeled enzyme at 420 nm were plotted to indicate the fraction of ionized and unionized forms, Fig. 5 was obtained in which points represent the experimental values and the curves are calculated for a single ionization with pK values of 6.12 and 6.6, respectively. Binding to heavy meromyosin, the pK of nitrophenolic groups shifted to that of a weaker acid. Such a shift suggests that the protein-bound nitrophenolic group is in an environment more hydrophobic than an aqueous one.

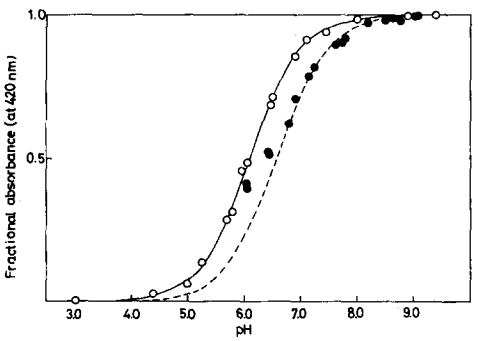


Fig. 5. Spectrophotometric titration of 2-acetamide-4-nitrophenol (○) and the labeled enzyme (●). The experimental points were measured at 420 nm. The curves are calculated for the ionization of nitrophenolate with a pK of 6.12 (———) and 6.6 (– – – –), respectively. The following buffers were used: 0.1 M Tris–HCl, 0.1 M Tris–maleate, 0.1 M Tris–acetate, 50 mM potassium phosphate and 0.17 M glycine–HCl in the presence of 0.5 M KCl. 40 μM 2-Acetamide-4-nitrophenol was used. The labeled enzyme contains 2.4 nitrophenolic groups.

The effect of changing the solvent on the spectrum of 2-acetamide-4-nitrophenol was compared to that of ATP on the spectrum of the labeled enzyme containing 2.4 nitrophenolic groups (Fig. 6). Fig. 6A showed the solvent effect, red shift, of ethylene glycol on a spectrum of 2-acetamide-4-nitrophenol. The difference spectrum with a positive peak at 445 nm was obtained. The λ_{max} of the absorption spectrum of the labeled enzyme shifted from 417 to 419 nm when 68 μM ATP was added (Fig. 6B).

The difference spectrum due to substrate interaction showed a positive peak at 440 nm which is very similar to that of the model compound observed in Fig 6A. However, the spectrum depicted in Fig. 6B is slightly different from that obtained by the addition of a higher concentration of ATP as shown in Fig. 7, although it is similar to that obtained by addition of ADP. Thus, the difference spectrum shown in Fig. 6B should be more likely due to interaction between ADP and heavy meromyosin.

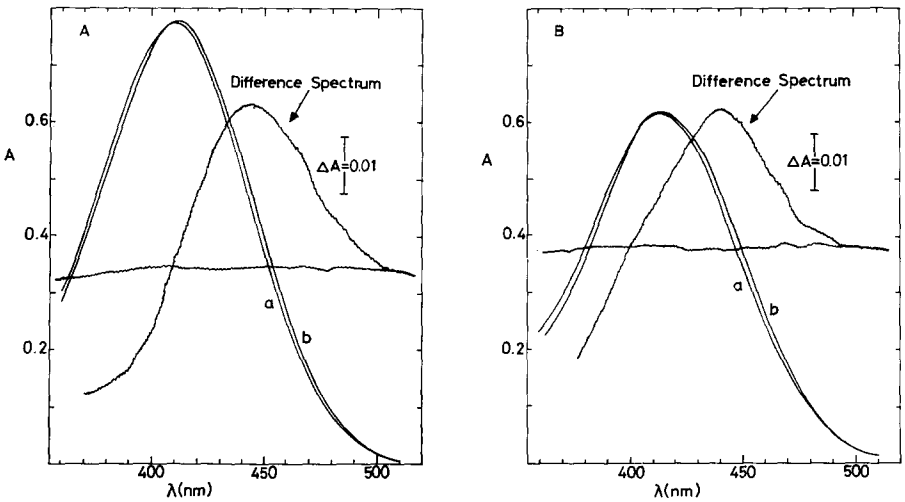


Fig. 6. A. Absorption spectra of 2-acetamide-4-nitrophenol (42 μM) in aqueous solution (a) and in 20% ethylene glycol (b). The difference spectrum was obtained by using four matched 1-cm cells. Reference Cell 1 contains 0.5 M KCl, 0.1 M Tris–HCl buffer (pH 8.0), 10 mM $MgCl_2$ and the reagent; Cell 2 contains 0.5 M KCl, 0.1 M Tris–HCl buffer, 10 mM $MgCl_2$ and ethylene glycol. Sample Cell 1 contains salts, buffer, the reagent and ethylene glycol in the same concentration as those in the reference cell; Cell 2 contains salts and buffer. B. Absorption spectra of the labeled enzyme (a) and a mixture of the labeled enzyme and ATP (b). The labeled enzyme contains 2.4 nitrophenolic groups. For experiments, 2.93 mg per ml of the enzyme, 0.5 M KCl, 10 mM $MgCl_2$, 0.1 M Tris–HCl buffer (pH 8) and 68 μM ATP were used. The difference spectrum was obtained by the method described for A.

The difference spectrum with a peak at 450 nm of the labeled enzyme induced by addition of ATP at a concentration of 0.5 mM was shown in Fig. 7. ADP and pyrophosphate also caused the difference spectrum with a peak at 445 nm. Adenosine was not found to cause such a difference spectrum.

Titration of heavy meromyosin with DTNB in the presence of urea showed 30 SH groups per mole of the enzyme (Fig. 8). All the SH groups were titrated within 10 min. In the absence of urea, reaction of heavy meromyosin with DTNB occurred in two phases, characterized by an initial rapid rate followed by a slow rate, although complete titration of SH groups was prolonged beyond 60 min or more. The reaction with a rapid rate was slowed down by addition of ATP, while the reaction with a slow rate was accelerated by ATP. This agrees with the fact that reaction of the SH reagent with the S_2 SH groups (where S_2 is functionally the slowly reacting SH groups in myosin and S_1 rapidly reacting groups) of myosin is accelerated by ADP[5,10]. The results strongly suggest that the environment of the SH groups is changed indirectly as a result of a conformational change in the protein molecule caused by substrate interaction, and that their reactivities toward DTNB are then affected.

The magnitudes of the spectral differences of the labeled enzyme induced by addition of nucleotide were found to be dependent on its concentration. When the observed change in absorbance at 440 nm was plotted against the total concentration of ATP or ADP, the points shown in Fig. 9 were obtained. The change is essentially that induced by ADP, since measurement was carried out after ATP was hydrolyzed.

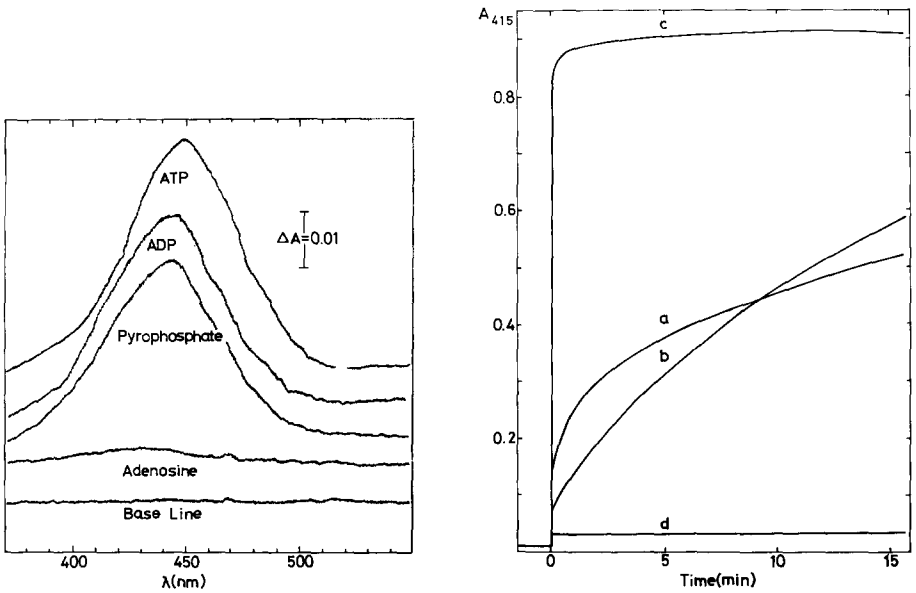


Fig. 7. Difference spectra of the labeled enzyme induced by ATP and its analogues. The traces are displaced vertically from the base line. For each measurement, 4.9 mg per ml of the labeled enzyme containing 3.4 nitrophenolic groups and 500 μM ATP or its analogues were used. Other conditions and methods are the same as in Fig. 6B.

Fig. 8. Reaction of SH groups in heavy meromyosin with DTNB. The reaction was carried out in 2.8 ml of 0.5 M KCl, 50 mM Tris–HCl buffer (pH 7.7) containing 2.2 mg heavy meromyosin in the presence (b) and absence (a) of 2 mM ATP. The reaction was started by adding 0.2 ml of 2 mM DTNB. (c), The same as (a) except for the presence of 5 M urea; (d) the same as (a) except for the absence of heavy meromyosin.

The data are consistent with a curve predicted for one ADP binding site per $3.5 \cdot 10^5$ g of the labeled enzyme and a dissociation constant of 0.57 μM. The dissociation constant is of the same order of magnitude as the value obtained from the data of ADP-induced difference spectra of the ultraviolet absorption of heavy meromyosin[18]. It seems likely that ADP binds strongly to heavy meromyosin even when the reactive SH groups are blocked and that these SH groups are not directly involved in ADP binding.

The effect of ADP on the labeled enzyme was also measured under conditions different from those shown in Fig. 9. Care was particularly taken to keep the pH of the mixture constant, since changes in pH have a large effect on the absorbance of the labeled enzyme. In all the spectrophotometric experiments, the pH of the reaction mixtures was measured by means of a glass electrode immediately after each spectrophotometric measurement. No significant change of the desired pH was found. The difference absorbance at 440 nm induced by addition of ADP was plotted as a function

of the total concentration of ADP added as shown in Fig. 10. Under the conditions used here, 0.5 M KCl, 1 mM $MgCl_2$ and 0.1 M Tris–HCl, the minimum concentration of ADP needed to give the maximum difference absorbance was 0.4–0.5 mM and a simple saturation curve was not obtained. The result suggests a weaker binding of ADP to the labeled enzyme than under the conditions given in Fig. 9. It is also possible to assume that the enzyme contains two binding sites with different affinities.

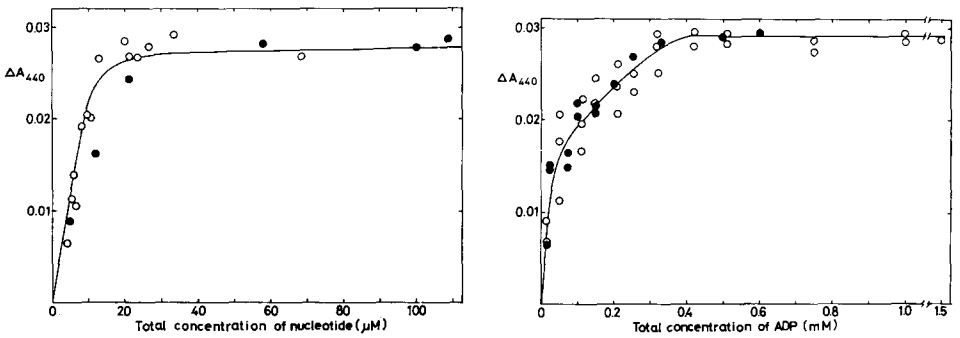


Fig. 9. Dependence of the magnitude of the spectral difference of the labeled enzyme on the concentration of added ATP (○) and ADP (●). In all experiments, 2.93 mg/ml of the labeled enzyme containing 3.5 nitrophenolic groups, 0.1 M KCl, 10 mM $MgCl_2$ and 50 mM Tris–HCl buffer (pH 8.0) were used. Each measurement was performed 10 min after the nucleotide was added. Calculated dissociation constant for nucleotide is 0.57 μM.

Fig. 10. Dependence of the magnitude of the spectral difference of the labeled enzyme on the concentration of added ADP. For the measurements, 5.1 mg per ml of the labeled enzyme containing 3.9 nitrophenolic groups (○) and 4.6 mg per ml of the labeled enzyme containing 4.1 groups (●) in 0.5 M KCl, 1 mM $MgCl_2$ and 0.1 M Tris–HCl buffer (pH 8.0) were used. All difference spectra were measured after the mixtures were immersed in a water bath at 25° for 5 min.

DISCUSSION

The environmentally sensitive protein-bound chromophore, recently termed reporter group, has been extensively discussed in reports dealing with protein structure[12,13,19,20]. Heavy meromyosin possesses the reactive SH groups which have been implicated in its ATPase activity and are known as the most reactive groups in the molecule[4,9]. By using the reagent 2-bromoacetamide-4-nitrophenol it is possible to insert the environmentally sensitive groups into a specific region of heavy meromyosin containing the reactive SH groups implicated in its ATPase activity. The agreement between the number of nitrophenolic groups incorporated and the loss of SH groups as measured by the DTNB method and PMB titration, as well as the fact that Ca^{2+}-activated ATPase is markedly activated, strongly supports a conclusion that the reactive SH groups which are involved in the change in ATPase activity, are readily attacked by the reagent. However, since K^+-activated ATPase of the labeled enzyme was not completely abolished even when the activity of Ca^{2+}-activated ATPase rose to a peak value, it could not be concluded that the reagent reacts only with the S_1 SH groups[5,10].

The difference spectrum resulting from the interaction of ATP with the labeled enzyme showed a positive peak typical for a spectral red shift in the absorption of nitrophenolate anion. It suggests that the substrate binding produces a shift of the

nitrophenolate anion to a less polar environment. In order to account for the effect of ATP binding on the spectrum of the labeled enzyme, the possibility can be considered that the spectral red shift is due to juxtaposition of the nitrophenolate anion with a nonpolar region of ATP. It seems unlikely that juxtaposition is responsible since pyrophosphate, being negatively charged, shows the same red shift and adenosine does not. It seems more likely, therefore, that the environment of the anion is changed indirectly as a consequence of a conformational change in which the anion is buried in the interior of the protein molecule. This explanation might be supported by the result that initial rate of reaction of SH groups with DTNB is prevented by addition of ATP as shown in Fig. 8. The fact that ATP or ADP binding results in a conformational change in the heavy meromyosin molecule has been reported by MORITA AND YAGI[21] and MORITA[18,22].

In contrast to the above explanation, SEIDEL *et al.*[23] and STONE[24] have recently reported that ATP produces a change in electron spin resonance spectra of the spin-labeled myosin and heavy meromyosin, which indicates an increase in the mobility of strongly immobilized spin labels. In these experiments, a nitroxide derivative of iodoacetamide was introduced as a spin label into the reactive SH groups of myosin and heavy meromyosin.

MORITA[18,22] demonstrated by means of the difference spectra at 280–290 nm that the stoichiometry obtained from the minimum concentration of ATP to give the maximum difference absorbance is 2 with the same affinities in 0.1 M KCl and 2 mM $MgCl_2$ at 10°, and the stoichiometry of ADP is 1 mole/mole of heavy meromyosin in 0.06 M KCl and 8.3 mM $MgCl_2$ at 25°. Recently, she concluded that only 1 mole of ADP of 2 moles bound is responsible for inducing the difference spectrum[25]. Results of many investigators[26–30] indicate binding of 2 moles of nucleotide or pyrophosphate per mole of myosin or heavy meromyosin with both binding sites having the same affinity.

The present studies with the labeled heavy meromyosin show the different saturation curves representing the changes in the difference absorbance produced by ADP, under conditions which differ in respect to KCl concentration and $MgCl_2$ concentration. It is suggested that two nucleotide-binding sites of heavy meromyosin have different affinities for ADP under certain conditions (Fig. 10) and that only one site is involved in the generation of the difference spectrum under other conditions (Fig. 9). A further study on nucleotide binding is in progress.

ACKNOWLEDGEMENT

We are indebted to Dr. K. Yagi for his discussions and support. This work was supported by a grant from Muscular Distrophy Association of America Inc.

REFERENCES

1 W. W. KIELLEY AND L. B. BRADLEY, *J. Biol. Chem.*, 218 (1956) 653.
2 J. J. BLUM, *Arch. Biochem. Biophys.*, 97 (1962) 309.
3 T. SEKINE, L. M. BARNETT AND W. W. KIELLEY, *J. Biol. Chem.*, 237 (1962) 2769.
4 T. SEKINE AND W. W. KIELLEY, *Biochim. Biophys. Acta*, 81 (1964) 336.
5 T. YAMASHITA, Y. SOMA, S. KOBAYASHI AND T. SEKINE, *J. Biochem. Tokyo*, 57 (1965) 460.
6 P. RAINFORD, K. HOTTA AND M. MORALES, *Biochemistry*, 3 (1964) 1213.
7 E. GAETJENS, T. THERATTIL-ANTONY AND M. BÁRÁNY, *Biochim. Biophys. Acta*, 86 (1964) 554.

8 K. YAGI AND Y. YAZAWA, *J. Biochem. Tokyo*, 60 (1964) 450.
9 G. BAILIN AND M. BÁRÁNY, *Biochim. Biophys. Acta*, 168 (1968) 282.
10 J. C. SEIDEL, *Biochim. Biophys. Acta*, 180 (1969) 216.
11 K. UCHIDA AND T. HIRATSUKA, *Biochim. Biophys. Acta*, 229 (1971) 310.
12 M. E. KIRTLEY AND D. E. KOSHLAND, JR., *J. Biol. Chem.*, 245 (1970) 276.
13 M. B. HILLE AND D. E. KOSHLAND, JR., *J. Am. Chem. Soc.*, 89 (1967) 5945.
14 L. F. HEWITT AND H. KING, *J. Chem. Soc.*, (1926) 817.
15 H. MUELLER, *J. Biol. Chem.*, 239 (1964) 797.
16 G. L. ELLMAN, *Arch. Biochem. Biophys.*, 82 (1959) 70.
17 P. D. BOYER, *J. Am. Chem. Soc.*, 76 (1954) 4331.
18 F. MORITA, *J. Biol. Chem.*, 242 (1967) 4501.
19 M. BURR AND D. E. KOSHLAND, JR., *Proc. Natl. Acad. Sci. U.S.*, 52 (1964) 1017.
20 M. E. KIRTLEY AND D. E. KOSHLAND, JR., *Biochem. Biophys. Res. Commun.*, 23 (1966) 810.
21 F. MORITA AND K. YAGI, *Biochem. Biophys. Res. Commun.*, 22 (1966) 297.
22 F. MORITA, *Biochim. Biophys. Acta*, 172 (1969) 319.
23 J. C. SEIDEL, M. CHOPEK AND J. GERGELY, *Biochemistry*, 9 (1970) 3265.
24 D. B. STONE, *Arch. Biochem. Biophys.*, 141 (1970) 378.
25 F. MORITA, *J. Biochem.*, 69 (1971) 517.
26 L. H. SCHLISEFELD AND M. BÁRÁNY, *Biochemistry*, 7 (1968) 3206.
27 B. KIELY AND A. MARTONOSI, *J. Biol. Chem.*, 243 (1968) 2273.
28 B. KIELY AND A. MARTONOSI, *Biochim. Biophys. Acta*, 172 (1969) 158.
29 K. M. NAUSS, S. KITAGAWA AND J. GERGELY, *J. Biol. Chem.*, 244 (1969) 755.
30 S. LOWEY AND S. M. LUCK, *Biochemistry*, 8 (1969) 3195.