Trinitrophenylation of Akazara Scallop Striated Adductor Myosin

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When akazara scallop striated adductor myosin reacted with 2,4,6-trinitrobenzenesulfonate, one mol of trinitrophenyl(TNP)-group was rapidly incorporated into one mol of myosin. The amount of the TNP-group incorporated in the myosin was not influenced by the presence of 2 mM sodium pyrophosphate (PPI), however, the effects of trinitrophenylation on myosin ATPase activities were remarkably affected by the presence of PPI. Thus, the Ca-ATPase activity was decreased by trinitrophenylation to 80% of its original in the absence of PPI, but to 40% in the presence of PPI. The Mg-ATPase activity both in the presence and absence of Ca^{2+} was increased by trinitrophenylation in the absence of PPI, maintaining a relatively high Ca^{2+} -sensitivity. But, in the presence of PPI the activity in the absence of Ca^{2+} was significantly increased and that in the presence of Ca^{2+} was slightly decreased, losing its Ca^{2+} -sensitivity.

Both the decreased ATPase activity and Ca^{2+} -sensitivity of TNP-myosin were found to recover by the subsequent dithiothreitol (DTT)-treatment of the myosin. Moreover, the reactivity of the SH_2 -type thiol of the myosin with N-(iodoacetyl)-N'-(5-sulfo-1-naphthyl)-ethylenediamine was found to be remarkably lowered by trinitrophenylation in the presence of PPI but recovered by the DTT-treatment, indicating that trinitrophenylation blocked the SH-group(s) of myosin.

From these results, it is concluded that the trinitrophenylation of akazara scallop myosin, especially in the presence of PPI, is accompanied by the modification of the SH-group(s) and that the modification of SH-group causes a decrease not only in myosin ATPase activity but also in Ca^{2+} -regulating ability.

Contraction of the molluscan muscle is regulated by myosin-linked regulatory light chains,¹⁻³⁾ although the troponin-tropomyosin system is additionally present on the actin filaments.⁴⁻⁷⁾ The myosin-linked regulation mechanism had been considered to act as a result of the inhibition of interaction between myosin and actin depending on the Ca^{2+} concentration, similar to the actin-linked tropomyosin-troponin system.¹⁻³⁾ However, it became evident that Mg-ATPase activity of myosin from clam,⁸⁾ akazara scallop,⁹⁻¹¹⁾ squid and ezo-giant scallop¹²⁾ showed Ca^{2+} -sensitivity even in the absence of actin. Thus it has been proposed that the primary role of the regulatory light chain may not be to regulate the myosin-actin interaction-site but rather to regulate the ATPase active-site. Hence, it becomes important to obtain structural information around the active-site in order to understand the mechanism of molluscan myosin-linked Ca^{2+} -regulation.

In 1960, Kubo *et al.*¹³⁾ attempted to determine

the chemical structure of the ATPase active-site of rabbit skeletal myosin using 2,4,6-trinitrobenzenesulfonate (TNBS) as a modifying reagent, and showed that two lysine ϵ -amino groups per one mol of myosin which were intimately related to the ATPase activity were specifically modified. Furthermore, Muhlrad *et al.*¹⁴⁾ showed by trinitrophenylation that one ATPase active-site is present in each of the two heads of rabbit myosin. On the other hand, Miyanishi *et al.*¹⁵⁾ found that the amount of TNBS which reacted rapidly with rabbit myosin was different depending on the presence and absence of PPI, and proposed that the chemical structures of the two heads of myosin were different with each other. Recently, Kodama *et al.*¹⁶⁾ reported that when TNP-rabbit myosin was treated with DTT, the absorbance at 345 nm was decreased making it seem as if the TNP-groups bound to the myosin were removed. This was accompanied by the restoration of the inhibited Ca-ATPase and EDTA-ATPase ac-

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Abbreviations: PPI, sodium pyrophosphate; TNP, trinitrophenyl; DTT, dithiothreitol; EGTA, ethyleneglycol-bis (β -aminoethylether)-N,N'-tetraacetate; DPCC, diphenylcarbonyl chloride; HMM, heavy meromyosin; S1, subfragment-1; 1,5-IAEDANS, N-(iodoacetyl)-N'-(5-sulfo-1-naphthyl)ethylenediamine.

tivities.¹⁶⁾

In the present paper, we report the effects of trinitrophenylation and successive treatment with DTT, and 1,5-IAEDANS on the biochemical properties of akazara scallop myosin.

Materials and Methods

Myosins from striated adductor muscles of akazara scallop and rabbit skeletal muscles were prepared by the method of Nishita *et al.*³⁾ and Perry,¹⁷⁾ respectively. Trinitrophenylation of myosin was carried out in accordance with the method of Miyanishi *et al.*¹⁵⁾ as follows; myosin (4–5 mg/ml) in 0.5 M KCl, 0.1 M imidazole-HCl (pH 7.0), 2 mM MgCl₂, and 0.1 mM EDTA was allowed to react with 0.25 mM TNBS in the presence and absence of 2 mM PPI at 15°C. At various intervals, aliquots were taken out and diluted with 10 volumes of cold 2 mM MgCl₂ to stop the reaction. The formed precipitates were collected by centrifugation at 10,000 × g for 10 min, resuspended in 0.04 M KCl and 2 mM MgCl₂. The precipitates were spun down, and dissolved in and dialyzed against 0.6 M KCl and 20 mM imidazole-HCl (pH 7.0). Trinitrophenylation proceeded insignificantly during these procedures. The number of bound TNP-groups was calculated using a molar extinction coefficient of TNP-lysine as 1.45×10^4 ¹⁸⁾ and the molecular weight of myosin as 4.8×10^5 .¹⁹⁾ The difference absorption spectrum of TNP-myosin was scanned by using a Hitachi UV 200-10 spectrophotometer. EDTA- and Ca-ATPase activities of myosin were measured at 15°C in a medium containing 0.6 M KCl, 25 mM Tris maleate (pH 7.0), 1 mM ATP, and 5 mM EDTA and 5 mM CaCl₂, respectively. Mg-ATPase activity of myosin and actomyosin was measured at 15°C in a medium containing 0.03 M KCl, 20 mM Tris maleate (pH 6.8), 2 mM MgCl₂, 1 mM ATP and 0.2 mM EGTA (in the absence of Ca²⁺) and 0.2 mM EGTA plus 0.3 mM CaCl₂ (in the presence of Ca²⁺). The inorganic phosphate liberated by ATP hydrolysis was determined according to the method of Youngburg and Youngburg.²⁰⁾ The Ca²⁺-binding of myosin was measured by the equilibrium dialysis method.^{21,22)} Akazara scallop myosin S1 was prepared according to the method of Weeds and Taylor²³⁾ with a slight modification as follows; akazara scallop myosin (10 mg/ml) in 0.12 M KCl, 20 mM imidazole-HCl (pH 7.0), 1 mM EDTA and 5 mM 2-mercaptoethanol was digested with 0.05 mg/ml

α-chymotrypsin at 15°C for 30 min. The digestion was stopped by the addition of 0.2 mM phenylmethylsulfonylfluoride. After dialyzing against 30 mM KCl and 20 mM imidazole-HCl (pH 7.0), the digests were centrifuged at 100,000 × g for 30 min to remove the insoluble materials, then solid ammonium sulfate was added to the 60% saturation. The thus formed S1 precipitates were collected by centrifugation at 10,000 × g for 15 min, and dialyzed against 30 mM KCl and 20 mM imidazole-HCl (pH 7.0). The dialyzates were purified by gel filtration on a Sephacryl S-300 column (2.5 × 75 cm) preequilibrated with the above dialysis buffer. The reaction of S1 and trinitrophenylated S1 with 1,5-IAEDANS was carried out according to the method of Hozumi and Mühlrad²⁴⁾; 1,5-IAEDANS (20 μM) was added to S1 (20 μM) in 30 mM KCl, 20 mM imidazole-HCl (pH 7.0) and 1 mM MgATP and was allowed to react at 0°C for 12 h. The reaction was stopped by the addition of 5 mM 2-mercaptoethanol. The mixture was dialyzed against 30 mM KCl, 20 mM imidazole-HCl (pH 7.0) and 5 mM 2-mercaptoethanol to remove unreacted 1,5-IAEDANS. Tryptic digestion of akazara scallop S1 was carried out essentially by the method of Yamamoto and Sekine.²⁵⁾ Thus, S1 (2 mg/ml) in 30 mM KCl, 20 mM imidazole-HCl (pH 7.0) and 5 mM 2-mercaptoethanol was digested with the 1/50 weight of DPCC-treated trypsin (Sigma Chem. Co.). At various intervals, 0.08 ml of the reaction mixture was taken out and mixed with an equal volume of 10 M urea, 1% SDS, 50 mM Tris-HCl (pH 8.9), 0.02% bromophenolblue and 0.6 M 2-mercaptoethanol. The mixture was then heated at 80–100°C for 2 min, and subjected to SDS-polyacrylamide gel electrophoresis.²⁶⁾ AEDANS-peptide fragments in the gel were detected under long wave UV-light (340 nm). Urea-polyacrylamide gel electrophoresis was carried out according to the method of Perrie and Perry.²⁷⁾ Protein concentration was determined by the biuret method²⁸⁾ using bovine serum albumin fraction V as a standard protein.

Results

Trinitrophenylation of Myosin

As shown in Fig. 1A and B, the reaction of akazara scallop and rabbit myosins with TNBS proceeded biphasically, *i.e.*, an initial rapid phase and a latter slow phase. As can be seen in Fig. 1B, the number of rapidly incorporated TNP-

Fig. 1. Trinitrophenylation of akazara scallop and rabbit myosin with 2,4,6-trinitrobenzenesulfonate (TNBS). Akazara scallop myosin (A) and rabbit myosin (B) (4 mg/ml) were allowed to react with 0.25 mM TNBS at 15°C in 0.5 M KCl, 0.1 M imidazole-HCl (pH 7.0), 2 mM $MgCl_2$, 0.1 mM EDTA, and either in the presence or absence of 2 mM PPI (+PPI and -PPI, respectively). The amount of trinitrophenyl (TNP)-groups bound was estimated from the absorbance at 345 nm.¹⁸⁾

groups into rabbit myosin was determined by the extrapolation of the latter slope as 1 mol and 2 mol/mol myosin in the presence and absence of PPI, respectively, in accord with the results of Miyanishi *et al.*¹⁵⁾ However, the TNP-group incorporated into akazara myosin was 1 mol/mol myosin regardless of the presence or absence of PPI (Fig. 1A).

Difference Absorption Spectrum of TNP-Myosin

Mühlrad²⁰⁾ reported that the UV-absorption spectrum of TNP-group bound to rabbit skeletal myosin (and its HMM and S1) blue-shifted by the addition of ADP or PPI, suggesting that the environment of the functional lysine residue becomes less polar upon binding of ATP analogue to myosin, *i.e.*, it becomes buried in the hydrophobic core of the molecule. Then, the effect of PPI on the UV-absorption spectrum of the TNP-group attached to the akazara scallop myosin was studied. As shown in Fig. 2A, akazara scallop myosins which trinitrophenylated in the presence and absence of PPI were both incapable of showing a difference spectrum by the addition of PPI. On the other hand, rabbit myosin trinitrophenylated in the absence of PPI showed a difference spectrum upon the addition of PPI, while, that modified in the absence of PPI did not (Fig. 2B).

Fig. 2. Difference spectra of TNP-myosin induced by the addition of PPI. Difference spectra of TNP-myosin (2.5 mg/ml) prepared by the trinitrophenylation in the presence (+PPI) or absence (-PPI) of 2 mM PPI were induced by the addition of 1 mM PPI in 0.6 M KCl, 20 mM Tris-HCl (pH 7.5), and 2 mM $MgCl_2$. A) akazara scallop TNP-myosin. B) rabbit skeletal TNP-myosin. Dotted lines indicate the base lines.

Fig. 3. Effect of trinitrophenylation on the EDTA-ATPase activities of akazara scallop and rabbit myosin. Akazara scallop and rabbit myosin were reacted with TNBS in the presence (●) or absence (○) of 2 mM PPI for various times indicated on the abscissa as described in the text. The EDTA-ATPase activities of akazara scallop (A) and rabbit (B) TNP-myosins were assayed at 15°C in a medium containing 0.6 M KCl, 5 mM EDTA, 25 mM Tris maleate (pH 7.0), and 1 mM ATP.

Effect of Trinitrophenylation on EDTA- and Ca-ATPase Activities

Figs. 3 and 4 show the changes in EDTA- and Ca-ATPase activities of akazara scallop and rabbit myosins during the trinitrophenylation.

The EDTA-ATPase activity of akazara scallop

Fig. 4. Effect of trinitrophenylation on the Ca-ATPase activities of akazara scallop and rabbit myosin. The myosins were reacted with TNBS in the presence (●) and absence (○) of 2 mM PPI for various times indicated on the abscissa as described in the text. The Ca-ATPase activity of akazara scallop TNP-myosin (A) and rabbit TNP-myosin (B) was assayed as in Fig. 3 except that 5 mM CaCl_2 was present in the reaction mixture instead of 5 mM EDTA.

myosin was repressed by trinitrophenylation depending on the presence or absence of PPI, *i.e.*, it was repressed to about 30% of its original in the absence of PPI and less than 5% in its presence. On the other hand, the EDTA-ATPase activity of rabbit myosin was repressed to about 5% irrespective of the presence or absence of PPI (Fig. 3B).

The decrease in Ca-ATPase activity of akazara scallop myosin during trinitrophenylation was also affected by the presence of PPI, *i.e.*, the activity of akazara scallop myosin modified in the absence of PPI was repressed to only 80% of its original but that modified in the presence of PPI was repressed to about 40% (Fig. 4A). The activity of rabbit myosin was repressed to about 30% regardless of the presence or absence of PPI (Fig. 4B).

Effect of Trinitrophenylation on Ca^{2+} -Sensitivity of Myosin Mg-ATPase Activity

Since molluscan myosin has Ca^{2+} -dependent Mg-ATPase activity unlike the vertebrate striated muscle myosin,⁸⁻¹² we examined the effect of trinitrophenylation on myosin Mg-ATPase activity and its Ca^{2+} -sensitivity.

As shown in Fig. 5A, when akazara scallop myosin was modified in the absence of PPI, the Mg-ATPase activity both in the presence and absence of Ca^{2+} increased as the modification

Fig. 5. Effect of trinitrophenylation on the Mg-ATPase activities of akazara scallop and rabbit myosin. The Mg-ATPase activities of akazara scallop (A) and rabbit (B) TNP-myosins which were obtained by reacting with TNBS for various times indicated on the abscissa either in the absence (○) or presence (●) of 2 mM PPI were measured at 15°C in a medium containing 30 mM KCl, 20 mM Tris maleate (pH 6.8), 2 mM MgCl_2 , and 1 mM ATP. The activity of akazara scallop TNP-myosin was assayed either in the presence (—) or absence (---) of 0.1 mM Ca^{2+} . The urea-gel electrophoretic patterns of akazara scallop untreated myosin (CM) and akazara scallop TNP-myosin modified in the presence of 2 mM PPI for 30 min (TNP-M (+PPI)) are shown in inset of the figure. RLC and SHLC were regulatory and sulfhydryl light chains of myosin, respectively.

proceeded showing a slight decrease in Ca^{2+} -sensitivity. On the other hand, when the myosin was modified in the presence of PPI, the Mg-ATPase activity in the presence of Ca^{2+} decreased slightly but that in the absence of Ca^{2+} significantly increased, resulting in a decrease in Ca^{2+} -sensitivity. This decrease in Ca^{2+} -sensitivity was shown not to be due to the dissociation of regulatory light chain (Fig. 5A, inset).

On the other hand, Mg-ATPase activity of rabbit myosin was increased markedly by the modification in the absence of PPI but slightly in its presence (Fig. 5B).

Effect of Trinitrophenylation on the Ca^{2+} -Binding of Akazara Scallop Myosin

It has been proved that the Ca^{2+} -binding of myosin is involved in the Ca^{2+} -regulation of the molluscan myosin.^{1,2,12,30} Recently, Kishimura *et al.*²² reported that 1 mol of akazara scallop myosin bound 2 mol of Ca^{2+} but its desensitized myosin, having had their regulatory light chains

completely removed, bound no Ca^{2+} . In order to investigate the cause for the decrease in Ca^{2+} -sensitivity during trinitrophenylation, the Ca^{2+} -binding of unmodified and modified akazara scallop myosins was measured by the equilibrium dialysis method using ^{45}Ca . Fig. 6 shows that the myosins modified in the presence and absence of PPi could bind about 1.2–1.7 mol Ca^{2+} /mol myosin at 10^{-4} M Ca^{2+} , indicating that the decrease in Ca^{2+} -sensitivity was not due to the alteration of its Ca^{2+} -binding ability.

Recovery of ATPase Activities and Ca^{2+} -Sensitivity by DTT-Treatment of TNP-Myosin

It has already been reported that when TNP-rabbit myosin was treated with DTT, its absorbance at 345 nm was decreased.¹⁰⁾ Therefore, we examined the effects of DTT-treatment on the Mg-ATPase activities and Ca^{2+} -sensitivity of TNP-akazara scallop myosin modified in the presence of 2 mM PPi. The TNP-myosin was dialyzed against 0.6 M KCl, 20 mM imidazole-HCl (pH 7.0) and 1 mM DTT, and then aliquots were taken at various times to measure the Mg- and EDTA-ATPase activities and to determine the number of bound TNP-group. As seen in Fig. 7A, the Mg-ATPase activities and also Ca^{2+} -sensitivity which had decreased by the trinitrophenylation

Fig. 6. Ca^{2+} -binding of akazara scallop TNP-myosin. Ca^{2+} -binding of akazara untreated myosin ($\circ$), TNP-myosins modified in the absence ($\triangle$) and presence ($\blacktriangle$) of 2 mM PPi were measured at 10°C by the equilibrium dialysis method using ^{45}Ca in the medium of 0.5 M KCl, 20 mM Tris maleate (pH 6.8), and 5 mg/ml myosin. Free Ca^{2+} concentration was determined by using Ca-EGTA buffer adopting the apparent association constant $8.45 \times 10^5 \text{ M}^{-1}$.³³⁾

Fig. 7. Effect of dithiothreitol (DTT)-treatment on the Mg- and EDTA-ATPase activities of akazara scallop TNP-myosin. TNP-myosin was prepared by trinitrophenylation of akazara scallop myosin in the presence of 2 mM PPi for 30 min. The TNP-myosin was dialyzed against 0.6 M KCl, 20 mM imidazole-HCl (pH 7.0), 1 mM DTT for various times, then diluted to 60 mM KCl and collected by centrifugation ($10,000 \times g$, for 10 min). The DTT-treated TNP-myosin was dissolved in 0.6 M KCl, 20 mM imidazole-HCl (pH 7.0) and used for ATPase assay and bound TNP determination. Amount of bound TNP ($\triangle$) was expressed by mol of TNP-group/mol of myosin. The Mg-ATPase activities in the presence ($\bullet$) and absence ($\circ$) of 0.1 mM Ca^{2+} were measured under the same conditions as in Fig. 5. Ca^{2+} -sensitivity of the Mg-ATPase activity ($\times$) was calculated by the following equation:

$$\text{Ca}^{2+}\text{-sensitivity} = \left[1 - \frac{\text{Activity}(-\text{Ca}^{2+})}{\text{Activity}(+\text{Ca}^{2+})} \right] \times 100 (\%)$$

EDTA-ATPase activity ($\square$) was measured as in Fig. 3. Co, activities of untreated myosin.

Fig. 8. SDS-gel electrophoresis of AEDANS-labeled TNP-S1 and DTT-treated TNP-S1. Trinitrophenylation and DTT-treatment of akazara scallop S1 was carried out as described in Table 1. Control S1 (untreated S1), TNP-S1s, and DTT-treated TNP-S1s were incubated at 0°C overnight with equimolar amount of 1,5-IAEDANS in 30 mM KCl, 20 mM imidazole-HCl (pH 7.0). The AEDANS-labeled S1s (a) and those digested by trypsin (b) were analyzed by SDS-gel electrophoresis according to the method of Porzio and Pearson.²⁰ Photographs of the gel were taken under UV illumination (A) or after staining with Coomassie Brilliant Blue (B). 1, control S1; 2, TNP-S1 (−PPi); 3, TNP-S1 (+PPi); 4, DTT-treated TNP-S1 (−PPi); 5, DTT-treated TNP-S1 (+PPi). About 80 μg of sample was applied to each well.

recovered rapidly by the DTT-treatment. In this case, about 0.2 mol TNP-group/mol myosin was released in 3 h of DTT-treatment, and about 0.5 mol TNP-group/mol myosin (from 1.2 mol to 0.7 mol TNP-group/mol myosin) was released in 12 h. This was accompanied by the recovery of EDTA-ATPase activity as well as the Ca^{2+} -sensitivity of Mg-ATPase activity (Fig. 7B).

Reaction of TNP-Akazara Scallop Myosin with 1,5-IAEDANS

In order to investigate whether the trinitrophenylation and the DTT-treatment of akazara scallop myosin affect reversibly on the reactivity of SH-group(s) similar to SH_1 of vertebrate skeletal myosin which is known to be essential for ATPase activity, the reactivity of TNP-S1 with 1,5-IAEDANS, a fluorescent reagent, was examined before and after the DTT-treatment. In this experiment, we used S1 instead of intact myosin since the modified site can be followed more readily. After the reaction with 1,5-IAEDANS, the modified S1 was digested with trypsin and subjected to SDS-polyacrylamide gel electrophoresis. Upon exposing the electrophoretogram to ultraviolet light, its fluorescence was observed on the 19 kDa band alone, suggesting that reactive SH-groups analogous to rabbit myosin SH_1 , which is known to locate in the 20 kDa fragment region of rabbit S1, was modified with 1,5-IAEDANS (Fig. 8). Whereas the 19 kDa fragment from

akazara scallop S1 trinitrophenylated in the absence and presence of PPi showed weak and practically no fluorescence, respectively, suggesting that reactivity of the SH-group(s) decreased by trinitrophenylation especially in the presence of PPi. Moreover, it became evident that DTT-treatment of the TNP-S1 enabled its 19 kDa

Table 1. Effect of dithiothreitol (DTT)-treatment on the EDTA-ATPase activity of akazara scallop TNP-S1. Akazara scallop S1 (3 mg/ml) was reacted at 15°C with 0.25 mM TNBS for 30 min in a solution containing 0.5 M KCl, 0.1 M imidazole-HCl (pH 7.0), 2 mM MgCl_2 , 0.1 mM EDTA, and either in the presence or absence of 2 mM PPi (−PPi and +PPi, respectively), and dialyzed against 30 mM KCl, 20 mM imidazole-HCl (pH 7.0). The TNP-S1 (−PPi) and TNP-S1 (+PPi) were then dialyzed against 30 mM KCl, 20 mM imidazole-HCl (pH 7.0) containing 1 mM DTT for 12 h. The DTT-treated TNP-S1s were dialyzed against the same buffer without DTT, and their EDTA-ATPase activities were measured under the same condition as described in the legend for Fig. 3

	EDTA-ATPase activity (μmol Pi/min·mg)	
	−PPi	+PPi
Control* ¹	0.328	
TNP-S1* ²	0.071	0.033
DTT-treated TNP-S1	0.125	0.168

*¹ untreated akazara scallop S1. *² trinitrophenylated akazara scallop S1.

fragment region to react with 1,5-IAEDANS. In addition, the alteration of the EDTA-ATPase activity was accompanied by an alteration in the reactivity of SH-group(s) (Table 1).

Discussion

In order to obtain information concerning the chemical structure of the molluscan Ca^{2+} -regulatory myosin, akazara scallop striated adductor myosin was reacted with TNBS, DTT and 1,5-IAEDANS successively, and their effects on ATPase activities, Ca^{2+} -sensitivity and tryptic fragmentation were investigated.

Akazara scallop myosin was shown to possess 1 mol of reactive residue per mol of myosin which can be readily trinitrophenylated. The TNP-group attached to akazara scallop myosin was found to be incapable of showing the difference spectrum upon addition of PPi, suggesting that its environment was not altered by the addition of PPi. These properties were similar to those reported in porcine cardiac and chicken gizzard myosins, *i.e.*, these myosins were shown to incorporate only 1 mol of the TNP-group rapidly, and the TNP-groups were incapable of showing the difference spectrum.³¹⁾ Srivastava *et al.*³¹⁾ proposed that the reactive lysine residue only in head Bs of cardiac and gizzard myosins, that catalyzing the initial burst of inorganic phosphate liberation, was trinitrophenylated regardless of the presence or absence of PPi, thus the difference spectrum was not observed.

On the contrary, rabbit skeletal myosin was reported to have 2 mol and 1 mol of lysine residue per mol of myosin that can be specifically trinitrophenylated in the absence and presence of PPi, respectively,¹⁵⁾ and the TNP-group attached to reactive lysine residue showed a considerable blue shift in its absorption spectrum by the addition of PPi, suggesting that the environment of the reactive lysine residue becomes buried in the hydrophobic core of the molecule.²⁰⁾ The spectral change was interpreted to occur from the TNP-group attached to the non-burst head (head A) of the two TNP-heads by the addition of ADP or PPi.¹⁵⁾ Consequently, rabbit myosin was interpreted to have two equimolar kinds of head with a reactive lysine residue but the residues behave differently to trinitrophenylation because of the difference in the two heads' chemical structures.¹⁵⁾

It was therefore assumed that only one of the two heads of akazara scallop myosin has a reac-

tive residue, *i.e.*, the chemical structures of the two heads are different based on the previous interpretation.^{15, 31)}

Both EDTA- and Ca-ATPase activities and Ca^{2+} -sensitivity of myosin were decreased by the trinitrophenylation depending on the presence of PPi (Figs. 3-5). However, the decreased activities were recovered when the TNP-myosin was treated with DTT (Fig. 7). Generally, it is hardly explicable that the TNP-group attached to lysine residue can be removed by the DTT-treatment. Moreover, the Mg- and EDTA-ATPase activities of the TNP-myosin were restored by the DTT-treatment, but was not necessarily stoichiometric with the amount of TNP-group removed (Fig. 7). We therefore assumed that SH-modification might occur in akazara scallop myosin depending on the presence of PPi during trinitrophenylation, and that such a modification could be readily reversed by the DTT-treatment. Recently, Nakata *et al.*³²⁾ found that nitrite production occurred when chicken breast and porcine cardiac myosins were trinitrophenylated and subsequently allowed to stand at 20°C in an alkaline pH, but did not occur when the SH-group had been blocked with 2-nitro-5-thiocyanobenzoate. From these findings, they proposed that the TNP-group bound to the reactive lysine residue might react with SH₁.

Thus using the modification with 1,5-IAEDANS, we examined whether or not the SH-group remained reactive even after the trinitrophenylation. As a result, the SH-group of TNP-akazara scallop myosin corresponding to rabbit myosin SH₁ was found to hardly react with the fluorescent reagent. Moreover, the repressed reactivity was recovered after the subsequent DTT-treatment. These facts may indicate that the trinitrophenylation was accompanied by the oxidation of the SH-group. We therefore consider that the difference in the effects of trinitrophenylation on the ATPase activities between akazara scallop and vertebrate skeletal myosins may be attributed to the difference in the subsequent SH modification by the trinitrophenyl group(s) attached to lysine residue.

As already stated, trinitrophenylation of akazara scallop myosin was associated with the decrease in Ca- and EDTA-ATPase activities and Ca^{2+} -sensitivity of Mg-ATPase activity. It was shown that the decrease in Ca^{2+} -sensitivity was not due to the dissociation of the regulatory light chains from myosin nor the decrease in Ca^{2+} -binding ability of myosin. Therefore, the trinitrophenylation may directly cause the decrease in

not only Ca- and EDTA-ATPase activities but also Ca^{2+} -sensitivity. This suggests that the site involved in Ca^{2+} -regulation may be close to the ATPase active-site or on the site communicating with the active-site for Ca^{2+} -regulation.

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