Chemical Modification of Glycerinated Stalks Shows Tyrosine Residues Essential for Spasmoneme Contraction of Vorticella sp

Authors: Fang, Jie, Zhang, Bei, Chen, Ning, and Asai, Hiroshi

Source: Zoological Science, 21(5) : 527-532

Published By: Zoological Society of Japan

URL: https://doi.org/10.2108/zsj.21.527

ZOOLOGICAL SCIENCE **21**: 527–532 (2004)

Chemical Modification of Glycerinated Stalks Shows Tyrosine Residues Essential for Spasmoneme Contraction of *Vorticella* sp.

Jie Fang[1], Bei Zhang[2], Ning Chen[3] and Hiroshi Asai[1*,†]

[1]*Department of Bioscience and Biomedical Engineering, Graduate School of Science and Engineering, Waseda University, Tokyo 169-8555, Japan*
[2]*Nankai University, Tianjin, 300072, China*
[3]*Tianjin University of Science & Technology, Tianjin, 300222, China*

ABSTRACT—Chemical modification of glycerinated stalks of *Vorticella* with TNM is used to investigate the role of tyrosine residues in the Ca^{2+}-induced contraction of the spasmoneme. Tetranitromethane (TNM) is often employed as a specific reagent for the nitration of tyrosine residues in a protein at neutral and slightly alkaline pHs although TNM can also oxidize cysteine residues in the acidic and neutral pH range. Prior incubation with Ca^{2+} of stalks to be treated with TNM can protect the spasmoneme from irreversible denaturation. On the other hand, TNM treatment in the absence of free Ca^{2+} causes an irreversible denaturation of the spasmoneme. It was revealed by us that an isolated Ca^{2+}-binding protein called spasmin could not bind with Ca^{2+} after TNM treatment, even if the treatment was performed in the presence of Ca^{2+}. In an additional experiment, we confirmed that the chemical modification of cysteine residues in the spasmoneme with N-7-dimethyl-amino-4methyl- coumarinyl- maleimide (DACM) has no effect on the contractibility. These results suggest that tyrosine residues in spasmin are essential for spasmoneme contraction and are protected from TNM in the presence of Ca^{2+} when spasmin binds with its receptor protein in the spasmoneme.

Key words: tyrosine residue, spasmoneme, spasmin, tetranitromethane (TNM), *Vorticella* sp.

INTRODUCTION

Species of protozoa belonging to the Vorticellidae ciliates, such as *Vorticella*, *Carchesium* and *Zoothamnium*, possess a unique contractile system that is independent of ATP or other organic fuels (Hoffmann-Berling, 1958; Amos *et al*., 1975; Asai *et al*., 1978). The bundle of 3-nm-diameter contractile filaments in the stalk is named the spasmoneme. The major component of the spasmoneme is spasmin, an EF-hand calcium binding protein. Another protein in the spasmoneme related directly or indirectly to the Ca^{2+}-induced contraction is the putative spasmin-receptor protein (Asai *et al*., 1978). The contraction/ extension of the spasmoneme can be repeated many times *in vitro* by the addition/removal of Ca^{2+} (Amos *et al*., 1975 Asai *et al*., 1978;). Amos *et al*. (1975) suggested that spasmoneme contraction was due entirely to the spasmin molecule. However, mach indirect evidence has emerged that contradicts Amos' proposal (Yamada *et al*, 1985; Kono *et al*, 1997; Moriyama *et al*, 1999). Moriyama *et al* (1999) have revealed that the contraction is due to the molecular folding of spasmin and a putative spasmin - receptor protein. Our knowledge is still limited about the protein system performing the contraction. It is therefore worth verifying by a chemical method whether some amino acid residue(s) in a spasmoneme protein is essential for the contraction and whether the putative spasmin - receptor protein protects the active site of spasmin.

The chemical modification of amino acid residues with a special reagent is a good way to identify active sites in a protein. There are only a few reports on the amino acids essential for spasmoneme contraction (Kono *et al*., 1997; Fang *et al*. 2003). Tetraitromethane (TNM) is often used to investigate the role of tyrosine residues in protein (Nakata *et al*., 1986; Koffman *et al*., 1991; Beckingham *et al*., 2001). Fig. 1 shows the reaction with TNM. The rate of the reaction between TNM and the tyrosine residues in a protein increases as the pH is raised from 7.0 to 10.0. Nitration does not occur below pH 7.0 (Femfert *et al*., 1972; Koffman *et al*., 1991). In addition, TNM will decompose as the pH is raised (Sokolovsky *et al*., 1966). So the most suitable pH is

* Corresponding author: Tel. +81-424-24-8655;
FAX. +81-424-24-8655.
E-mail: asai@waseda.jp
† Present address: 4-16-7, Shimohouya, Nishitokyo 202-0004, Japan

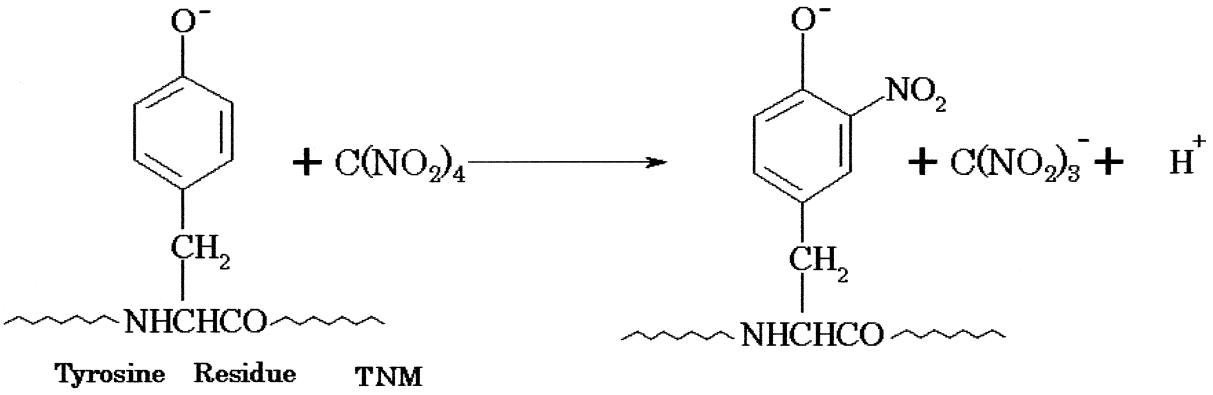

Fig. 1. Scheme of tyrosine residue that reacts with TNM.

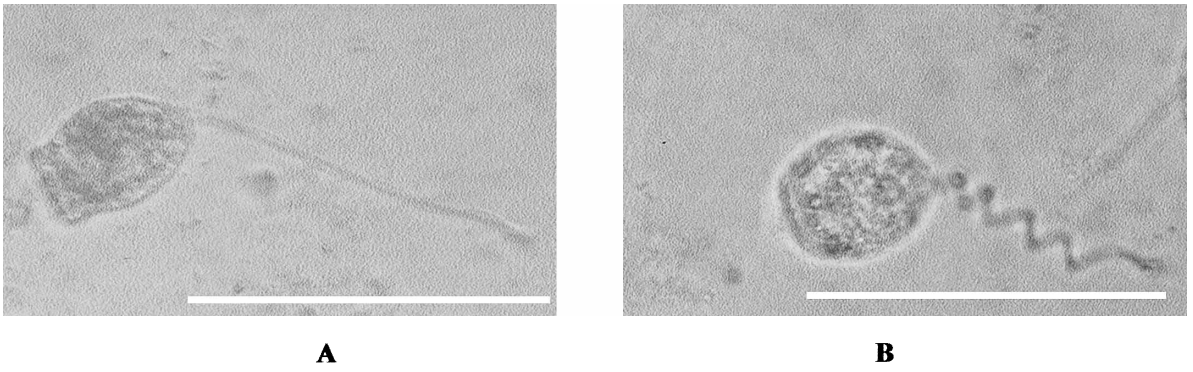

Fig. 2. Contraction and extension of stalk of *Vorticella* sp. A: Glycerinated stalks were incubated with Ca^{2+}-free solution (2 mM EGTA, 0.1 M KCl, and 20 mM imidazole, pH 6.8); B: Glycerinated stalks were incubated with Stalk Contraction Solution (2mM Ca^{2+}, 0.1 M KCl, and 20 mM imidazole, pH 6.8). Bar = 100 μm

8.0–9.0. The present study provides insight into the importance of tyrosine residues in the spasmoneme. It is also the first to report the tyrosine residues essential for spasmoneme contraction.

MATERIALS AND METHODS

Chemicals

TNM and DACM were purchased from Sigma Chemical Co., St. Louis. The solutions used for the chemical modification of TNM contained 4 mM EGTA-2Na, 0.1 M KCl, 20mM bufferincluding sodium phosphate at pH 6.0–7.5, Sodium borate at pH 8.0–9.0 and glycine at pH 9.5–10.0 and TNM (0~200 μM). The pH was adjusted according to different experiments. The DACM solution was freshly prepared in acetone and stored on ice in the dark (Ohyashiki *et al.*, 1994). It contained 4 mM EGTA-2Na, 0.1 M KCl, 20 mM imidazole and DACM (0~ 500 μM). The pH was controlled at pH 7.0.

Cell Culture and Glycerol Treatment

Vorticella sp. was found and collected at the Tianjin Jizhuanzi Sewage Facility, China. Fig. 2 shows the contraction and extension of glycerinated stalks on addition and removal of Ca^{2+}.

The collected colonies were cultured in large low-form flasks containing 0.25% vita-shrimp/cereal leaves infusion at 20°C (Ochiai *et al.*, 1979; Vacchiano *et al.*, 1991). Cover glasses were put on the culture liquid surface. A few days later, a suitable number of zooids would attach to the glass. Each cover slip with cultured *Vorticella* cells was treated in a solution consisting of 0.1% saponins, 4 mM EGTA-2Na, 0.1 M KCl, and 20 mM imidazole, at pH 6.8 and 0°C, for 45 min. Then, the cells were transferred to a new solution containing 35% glycerol, 4 mM EGTA-2Na, 0.1 M KCl, and 20 mM imidazole, at pH 6.8 and 0°C, for 60 min.

Procedure for the Chemical Modification of TNM

Before the chemical modification experiment, the cover glasses were washed (Washing Solution: 4 mM EGTA-2Na, 0.1 M KCl, and 20 mM imidazole, pH 6.8) for 20 min in order to remove the glycerol

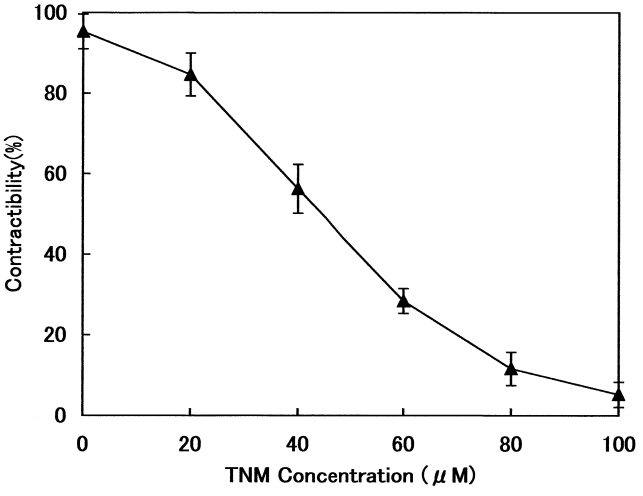

Fig. 3. Effect of TNM concentration on the contractibility of the spasmoneme. Data points are means ± standard errors (N = 100).

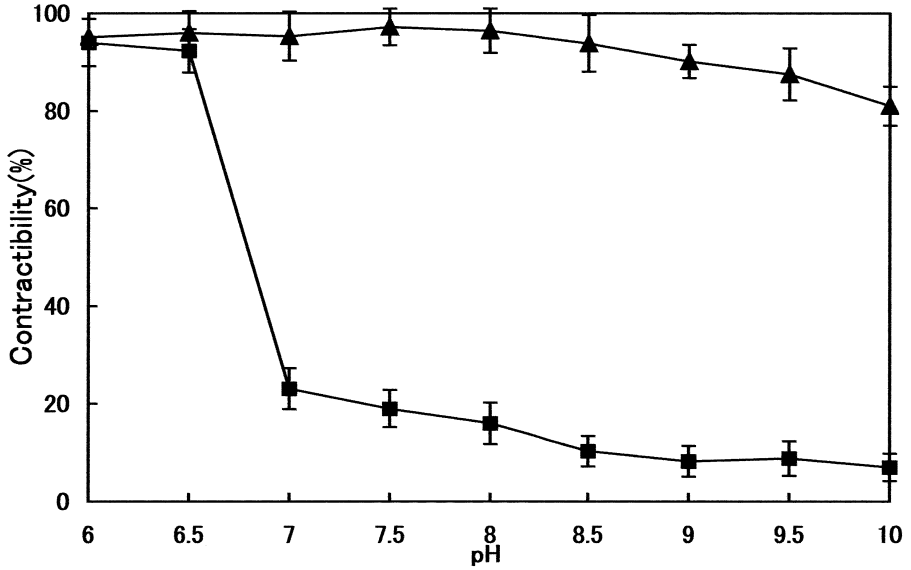

Fig. 4. Effect of pH in the presence of 200 μM TNM (■) and absence of TNM (▲). Data points are means ± standard errors (N = 100).

on the surface of the stalks. The stalks were incubated with contraction solution (2 mM Ca^{2+}, 0.1 M KCl, and 20mM imidazole, pH 6.8) for 10 min. Then, the cover glasses were photographed for counting the numbers of coils in stalks without TNM treatment and transferred to washing solution for 20 min in order to remove Ca^{2+}. After these experiments, the cover glasses with stalks were incubated in petri dishes containing the chemical modification solution under various conditions. Then, the stalks still attached to the cover glasses were washed with washing solution for 20 min in order to remove TNM and transferred to the contraction solution. After these experiments, the stalks were photographed again.

Experimental Procedure for Examining the Effect of DACM on Vorticella Stalks

The procedure used was the same as that for the chemical modification with TNM. The cover glasses with stalks were incubated in solutions of 100~500 μM DACM at 0°C and pH 7.0 for 60 min. In other experiments, the cover glasses with stalks were incubated in a solution of 500 μM DACM at 0°C, pH 7.0 for different periods of time.

Contractibility

The average numbers of coils in modified and unmodified stalks were estimated by counting the total for 100 individual stalks using the photos. Contractibility for modified stalks was taken as the ratio of the average number of coils in modified stalks to that in unmodified stalks. Namely,

$$\text{Contractibility} = \frac{\text{Average number after modification}}{\text{Average number before modification}} \times 100\%$$

Ca^{2+} Protection of Spasmoneme Contractibility against TNM

To investigate if preincubation with Ca^{2+} can protect the spasmoneme from denaturation, glycerinated stalks were pre-incubated with the contraction solution for 10 min. In control experiments, glycerinated stalks were not treated with the contraction solution before the TNM treatment. The stalks were modified with 20, 50 and 80 μM TNM solutions at pH 8.0 and 0°C for 60 min. The TNM was then removed by washing extensively. Next, the stalks were incubated with the contraction solution for 10 min and the coils were counted. Average numbers of coils in 100 stalks with and without pre-incubation with Ca^{2+} were calculated for determining contractibility.

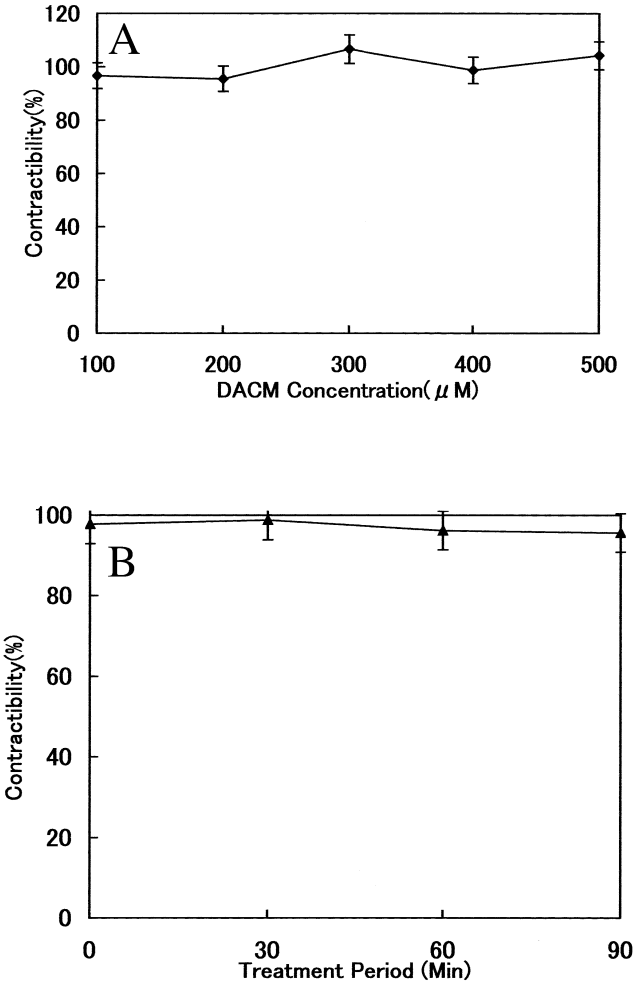

Fig. 5. Effect of DACM concentration (A) and period of treatment with 500 μM DACM (B) on spasmoneme contraction. Data points are means ± standard errors (N = 100).

Effect of TNM on Affinity of Spasmin for Ca^{2+}

The method used to purify spasmin was based on previous research (Fang *et al.*, 2003). The spasmin fraction in the absence of Ca^{2+} was modified with 100 μM TNM at 0°C for 60 min. Then, it was dialyzed against buffer (1 mM EGTA and 50 mM Tris-HCl, pH 7.5) and divided into two equal volumes and concentrated. 2 mM Ca^{2+} was added to one portion and 2 mM EGTA to the other. The spasmin fraction pre-incubated with Ca^{2+} was treated as described above. Native spasmins not treated with TNM, as controls, were incubated with 2 mM Ca^{2+} or 2 mM EGTA-2Na. These samples were resolved by 12.5% native polyacrylamide gel electrophoresis (Native-PAGE) (Ueno *et al.*, 1999). In this experiment, the TNM solution lacked EGTA-2Na.

RESULTS

Effect of TNM Concentration on Stalk Contraction

The effect of TNM concentration on the spasmoneme was investigated. The ability of *Vorticella* stalks to contract was examined at various TNM concentrations (Fig. 3). As shown in Fig. 3, the contractibility of the stalks decreased about 90% as the TNM concentration rose from 0 to 100 μM. The contraction was evidently inhibited when the TNM concentration was 80–100 μM.

Effect of TNM on Stalk Contraction at Various pH

The effect of TNM on contraction was examined at various pH. In consideration of the decomposition of TNM with rising pH, we used 200 μM of TNM, although 100 μM obviously inhibited spasmoneme contraction at 0°C, pH 8.0.

The contractibility of spasmonemes not treated with TNM was unaffected at pH 6.0 to 8.5, which suggests that the loss in Fig. 4 was caused by the chemical modification.

Effect of DACM Modification on *Vorticella* Stalks

As expected, the contractibility of the stalks was unaffected by the various concentrations of DACM (Fig. 5A). Therefore, experiments with different treatment times were performed. The results showed that the period of treatment with DACM had no effect on contraction, either (Fig. 5B). So, it was concluded that the modification of cysteine residues does not affect spasmoneme contraction in *Vorticella* sp.

Ca^{2+} Prevents Loss of Spasmoneme Contractibility

Preincubation with Ca^{2+} protected the spasmoneme against the loss of contractibility caused by the chemical

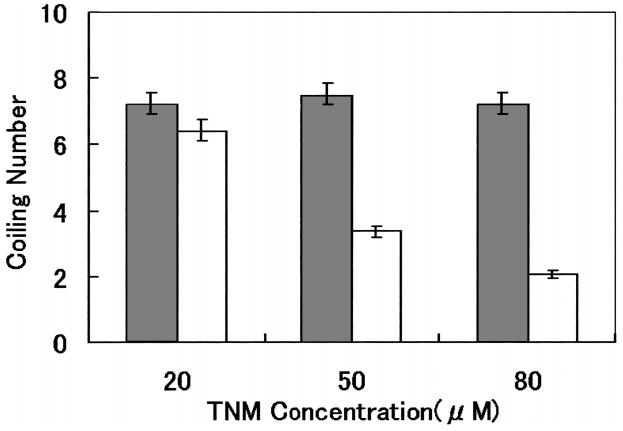

Fig. 6. Ca^{2+} protection of the spasmoneme against modification by TNM. Stalks were pre-incubated with 2 mM Ca^{2+} (■). Stalks were not pre-incubated with Ca^{2+}(□). Data points are means ± standard errors (N = 100).

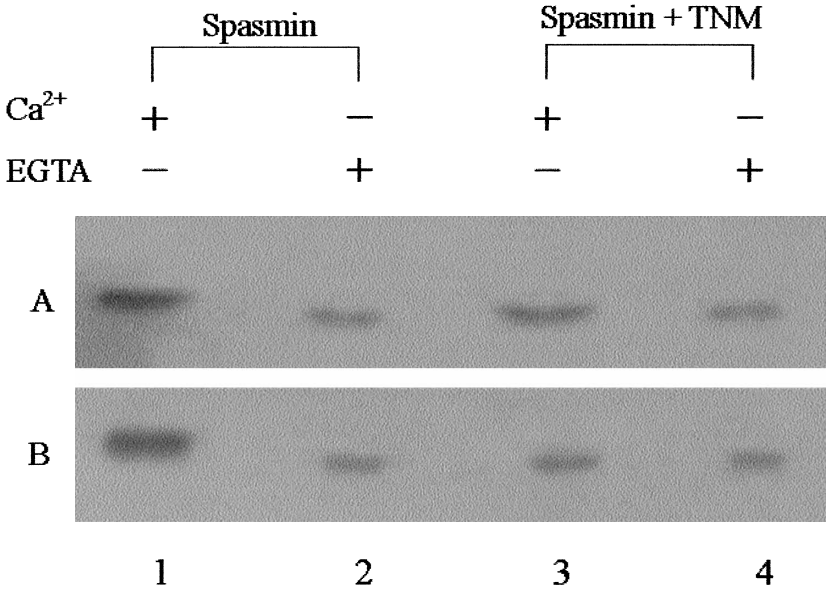

Fig. 7. Native PAGE (12.5%) of the spasmin in the presence and absence of Ca^{2+} without (A)and with Ca^{2+} pre-incubation (B). A: Lane 1: Native spasmin and 2 mM Ca^{2+}; Lane 2: Native spasmin and 2 mM EGTA; Lane 3: Native spasmin modified with 100 μM TNM and 2 mM Ca^{2+} (Ca^{2+} was added after TNM); Lane 4: Native spasmin modified with 100 μM TNM and 2 mM EGTA (EGTA was added after TNM). B: Lane 1: Native spasmin and 2 mM Ca^{2+}; Lane 2: Native spasmin and 2 mM EGTA; Lane 3: Native spasmin modified with 100 μM TNM and 2 mM Ca^{2+} (Ca^{2+} was added before TNM); Lane 4: Native spasmin modified with 100 μM TNM and 2 mM EGTA (EGTA was added before TNM).

modification with TNM (Fig. 6).

Effect of TNM on the Ability of Spasmin to Bind Ca^{2+}

Spasmin is the major component of spasmoneme and a Ca^{2+}-binding protein. Native spasmin has been shown to exhibit a Ca^{2+}-dependent shift on native PAGE (Yamada *et al*., 1982). In order to identify potential sites of the essential tyrosine residues in the spasmoneme, the effect of Ca^{2+} on spasmin was investigated. TNM-modified spasmin in the presence of Ca^{2+}(Fig. 7A Lane 3) migrated at the same rate as without Ca^{2+}(Fig. 7A Lane 4). The results of native PAGE of the chemically modified spasmin with and without Ca^{2+} implied that the affinity of spasmin for Ca^{2+} was reduced by TNM (Fig. 7A), which suggested that the loss of contractibility was due to the modification of spasmin. In Fig. 7B, preincubation with Ca^{2+} could not preserve the ability of spasmin to bind Ca^{2+}.

DISCUSSION

TNM has been shown to be a convenient reagent for the nitration of tyrosyl residues in proteins at pH 8.0 (Sokolovsky *et al*., 1966; Riordan *et al*., 1966), although specificity studies have shown that cysteine residues are also potentially reactive towards TNM (Riordan and Christen, 1968). In the present study, it was revealed that spasmoneme contraction depended on the TNM concentration. The contractibility decreased nearly 90% with a rise in the TNM concentration from 0 to 100 μM at 0°C, pH 8.0. It is generally recognized that TNM oxidizes thiol groups at pH of 6.0 to 9.0 (Sokolovsky *et al*., 1966). So it was necessary to explore the effect of TNM on spasmoneme contraction at various pH and the function of cysteine residues in order to confirm the role of these residues in the contraction. The spasmoneme contraction was unaffected at pH 6.0–8.5 and 0°C (Fig. 4), which indicated that the loss of contractibility was caused by the chemical modification. DACM is a highly specific fluorescent thiol reagent, often employed to determine the role of cysteine residues in a protein's function (Tezuka *et al*., 1978; Ida and Tokushige, 1985; Werneburg and Ash, 1993). DACM treatments had proved that the chemical modification of cysteine residues did not affect the contraction of *Vorticella* (Fig. 5). In Ca^{2+} protection experiment, the contraction was not affected by TNM treatment when the spasmoneme was incubated with Ca^{2+} before the chemical modification (Fig. 6). These results suggest that tyrosine residues are essential for spasmoneme contraction.

Spasmin is the main component of the spasmoneme and spasmoneme contraction is induced by Ca^{2+} binding. Therefore, the spasmoneme may have lost the ability to contraction because spasmin cannot bind with Ca^{2+} after TNM treatment. Maciejewski *et al*. has proved that native spasmin bound to Ca^{2+} migrates at a slower rate than that free from Ca^{2+} on native PAGE (Maciejewski *et al*., 1999). We investigated the ability of spasmin to bind Ca^{2+} by native PAGE. The results (Fig. 7) showed that tyrosine residues contributed to the binding (Fig. 7A) and preincubation with Ca^{2+} could not preserve the ability of spasmin to bind Ca^{2+} (Fig. 7B).

In the present study, the result that preincubation with Ca^{2+} did not preserve the affinity of spasmin for Ca^{2+} (Fig. 7) seemed to contradict the finding that the preincubation prevented a loss of contractibility caused by TNM (Fig. 6). The discrepancy could have two explanations. One possibility is that spasmin can be modified by TNM because it undergoes a change in structure when isolated from the spasmoneme. But the isolated spasmin not treated with TNM still bound with Ca^{2+} (Fig. 7, lane 1 and 2), which implied that the spasmin structure was unchanged. So, this possibility can be excluded. On the other hand, Yamada *et al* showed that there was another protein, namely the putative receptor protein, which played a complementary role in spasmoneme contraction (Yamada *et al*, 1982). In addition, the results of chemical modifications with tryptophan (Kono *et al*, 1997) and histidine (Fang *et al*, 2003) confirmed that the receptor protein was essential for spasmoneme contraction. So, the second possibility is that the receptor protein plays a complementary role in protecting tyrosine residues from TNM in the spasmoneme. If this hypothesis can be confirmed, our observations on tyrosine residues modification might be a good starting point for the investigation of spasmoneme contraction.

ACKNOWLEDGMENTS

This research was supported by grants from the Yamada Science Foundation (H.A.). We greatly appreciate the help, comments and suggestions provided by Dr. T. Itabashi and Dr. S.J. Jia. F.J. is a 21st Century COE research assistant of physics self-organization systems composed of multi-components.

REFERENCES

Amos WB (1975) Molecules and Cell Movement. Raven Press, New York, pp 411–436

Amos WB, Routledge LM, Yew FF (1975) Calcium binding proteins in a Vorticellid contractile organelle. J Cell Sci 19: 203–213

Asai H, Ninomiya T, Kono RI, Moriyama Y (1998) Spasmin and a putative spasmin binding protein(s) isolated from solubilized spasmonemes. J Euk Microbiol 45: 33–39

Asai H, Ochiai T, Fukui K (1978) Improved preparation and cooperative calcium contraction of glycerinated *Vorticella*. J Biochem 83: 795–798

Beckingham JA, Housden NG, Muir NM, Bottomley SP, Gore MG (2001) Studies on a single immunoglobulin-binding domain of protein L from Peptostreptococuus magnus: the role of tyrosine-53 in the reaction with human IgG. Biochem J 353: 395–401

Fang J, Zhang B, Asai H (2003) Chemical modification of contractile 3 nm diameter filaments in Vorticellidae spasmoneme by diethyl-pyrocarbonate and its reversible renaturation by hydroxylamine. Biochem Biophys Res Commun 310: 1067–1072

Femfert U, Cichocki P, Pfleiderer G (1972) On the Mechanism of Amide Bond Cleaveage Catalyzed by Aminopeptidase M. Enzymatic Proties of Nitroaminopeptidase M. FEBS Letter 26: 39–42

Hoffmann-Berling H (1958) Der Mechanismus eines neuen, von der Muskelkontraktion verchiedenen Kontraktionszyklus. Biochem Biophys Acta 27: 247–255

Ida N, Tokushige M (1985) Assignment of catalytically essential cysteine residues in aspartase by selective chemical modification with N-(7-dimethylamino-4-methl- coumaryl) maleimide. J Biochem (Tokyo) 98: 793–797

Koffman B, Modarress KJ, Beckerman T, Bashirelahi N (1991) Evidence for involvement of tyrosine in estradiol binding by rat uterus estrogen receptor. J Steroid Biochem Mol Biol 38: 135–139

Kono RI, Ochiai T, Asai H (1997) Chemical modification of amino acid residues in glycerinated *Vorticella* stalk and Ca^{2+}-induced contractility. Cell Motil Cytoskeleton 36: 305–312

Maciejewski JJ, Vacchiano EJ, McCutcheon SM, Buhse HE Jr (1999) Cloning and expression of a cDNA encoding a *Vorticella convallaria* spasmin: an EF-hand calcium-binding protein. J Euk Microbiol 46: 165–173

Moriyama Y, Okamoto H, Asai H (1999) Rubber-like elasticity and volume changes in the isolated spasmoneme of giant *Zoothamnium* sp. under Ca^{2+}-induced contraction. Biophys J 76: 993–1000

Nakata H, Regan JW, Lefkowitz RJ (1986) Chemical modification of alpha-2-adrenoceptors. Possible role for tyrosine in the ligand binding site. Biochem. Pharmacol 35: 4089–4094

Ochiai T, Asai H, Fukui K (1979) Hystersis of contraction-extension cycle of glycerinated *Vorticellla*. J Protozool 26: 420–425

Ohyashiki T, Sakata N, Matsui K (1994) Changes in SH reactivity of the protein in porcine intestinal brush-border membranes associated with lipid peroxidation. J Biochem (Tokyo) 115: 224–229

Riordan JF Christen P (1968) Reaction of Tetranitromethane with Protein Sulfhydryl Groups. Inactivation of Aldolase. Biochemistry 7: 1525–1530

Riordan JF, Sokolovsky M, Vallee BL (1966) Tetranitromethane. A reagent for the nitration of tyrosyl residues in proteins. J Am Chem Soc 88: 4104–4105

Sokolovsky M, Harell D, Riordan JF (1969) Reaction of tetranitromethane with sulfhydryl groups in proteins. Biochemistry 8: 4740–4745

Sokolovsky M, Riordan JF, Vallee BL (1966) Tetranitromethane. A reagent for the nitration of tyrosyl residues in proteins. Biochemisty 5: 3582–3589

Tezuka T, Hirai R, Ogawa H (1978) The fluorescent profiles of keratohyalin granules of newborn rat epidermis with a new fluorescent thiol reagent (DACM). Acta Derm Venereol 58: 391–394

Ueno Y, Kurano N, Miyachi S (1999) Purification and characterization of hydrogenase from the marine green alga, *Chlorococcum littorale*. FEBS Lett 443: 144–148

Vacchiano EJ, Kut J, Wyatt ML (1991) A novel method for mass-culture *Vorticlla*. J Protozool 38: 608–613

Werneburg BG, Ash DE (1993) Chemical modifications of chicken liver pyruvate carboxylase: evidence for essential cysteine-lysine pairs and a reactive sulfhydryl group. Arch Biochem Biophys 303: 214–221

Yamada-Horiuchi K, Asai H (1985) Circular dichroism studies of the Ca^{2+}-binding proteins form the spasmoneme of *Carchesium*. Comp Biochem Physiol 81B: 927–931

Yamada K, Asai H (1982) Extraction and some properties of the proteins, Spastin B, from the spasmoneme of *Carchesium polypinum*. J Biochem (Tokyo) 91: 1187–1195

(Received October 1, 2003 / Accepted February 12, 2004)