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

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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-4-methyl-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, much indirect evidence has emerged that contradicts Amos' pro-

posal (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). Tetranitromethane (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

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Effect of TNM on Affinity of Spasmin for Ca²⁺

The method used to purify spasmin was based on previous research (Fang *et al.*, 2003). The spasmin fraction in the absence of Ca²⁺ 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 Ca²⁺ was added to one portion and 2 mM EGTA to the other. The spasmin fraction pre-incubated with Ca²⁺ was treated as described above. Native spasmins not treated with TNM, as controls, were incubated with 2 mM Ca²⁺ 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.

Ca²⁺ Prevents Loss of Spasmoneme Contractibility

Preincubation with Ca²⁺ protected the spasmoneme against the loss of contractibility caused by the chemical

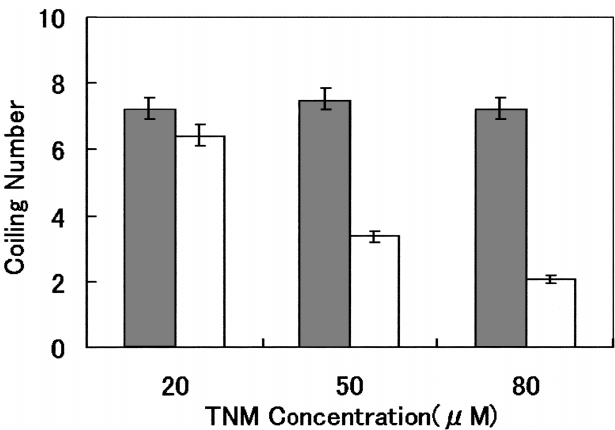


Fig. 6. Ca²⁺ protection of the spasmoneme against modification by TNM. Stalks were pre-incubated with 2 mM Ca²⁺ (■). Stalks were not pre-incubated with Ca²⁺ (□). Data points are means ± standard errors (N = 100).

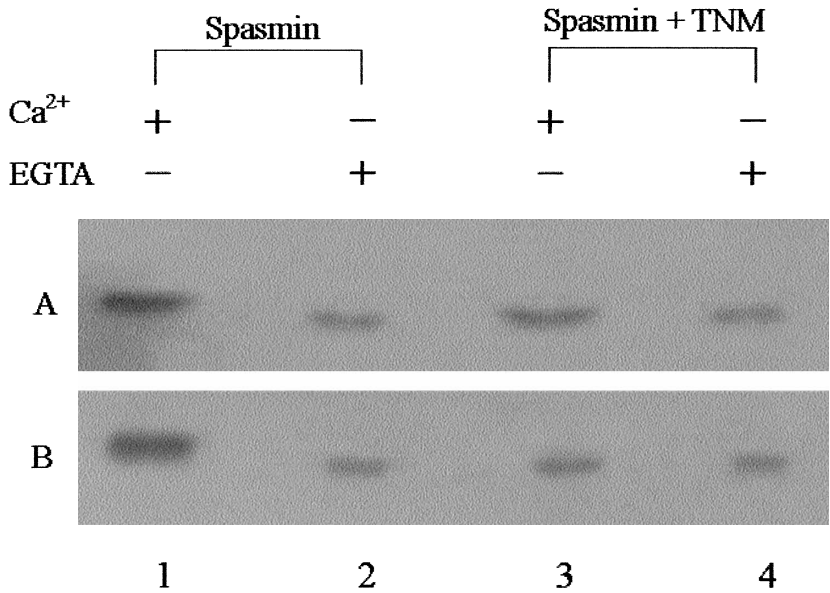


Fig. 7. Native PAGE (12.5%) of the spasmin in the presence and absence of Ca²⁺ without (A) and with Ca²⁺ pre-incubation (B). A: Lane 1: Native spasmin and 2 mM Ca²⁺; Lane 2: Native spasmin and 2 mM EGTA; Lane 3: Native spasmin modified with 100 μM TNM and 2 mM Ca²⁺ (Ca²⁺ was added after TNM); Lane 4: Native spasmin modified with 100 μM TNM and 2 mM EGTA (EGTA was added after TNM). B: Lane 1: Native spasmin and 2 mM Ca²⁺; Lane 2: Native spasmin and 2 mM EGTA; Lane 3: Native spasmin modified with 100 μM TNM and 2 mM Ca²⁺ (Ca²⁺ was added before TNM); Lane 4: Native spasmin modified with 100 μM TNM and 2 mM EGTA (EGTA was added before TNM).

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