CONFORMATIONAL CHANGE IN THE OUTER DOUBLET MICROTUBULES FROM SEA URCHIN SPERM FLAGELLA

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ABSTRACT

Dark-field microscopy with a high-powered light source revealed that the outer doublet microtubules (DMTs) from sea urchin (*Pseudocentrotus depressus* and *Hemicentrotus pulcherrimus*) sperm flagella assume helically coiled configurations (Miki-Noumura, T., and R. Kamiya. 1976. *Exp. Cell Res.* **97:** 451.).

We report here that the DMTs change shape when the pH or Ca-ion concentration is changed. The DMTs assumed a left-handed helical shape with a diameter of 3.7 ± 0.5 μm and a pitch of 2.8 ± 0.7 μm at pH 7.4 in the presence of 0.1 mM $CaCl_2$, 1 mM $MgSO_4$, and 10 mM Tris-HCl. When the pH was raised to 8.3, the helical diameter and pitch decreased to 2.1 ± 0.1 μm and 1.3 ± 0.3 μm, respectively. This transformation was a rapid and reversible process and was completed within 1 min. Between pH 7.2 and 8.3, the DMTs assumed intermediate shapes. When the Ca-ion concentration was depleted with EGTA, the helical structure became significantly larger in both pitch and diameter. For instance, the diameter was 3.8 ± 0.4 μm at pH 8.3 in the presence of 1 mM EGTA and 2 mM $MgSO_4$. Using a Ca-buffer system, we obtained results which suggested that this Ca-induced transformation took place at a Ca concentration of $\sim 10^{-7}$ M. These results were highly reproducible. The conformational changes in the DMT may play some role in the bending wave form of flagellar movement.

KEY WORDS outer doublet microtubule · Ca · pH · helical conformation

We have reported the visualization of individual microtubules by dark-field microscopy with high-powered illumination, and shown that the outer doublet microtubules (DMTs) from sperm tails assumed a coiled appearance in solutions whereas the microtubules reconstituted from mammalian brain tubulin were straight (8). Although several authors (12, 14) have already reported the coiling tendency of DMT, there has been no quantitative investigation of the coiled form. In the present study, we measured the helical parameters of coiled DMTs under different environmental media, and found that the helical shape varies reversibly when the pH value or concentration of Ca ion is changed, whereas it is fairly uniform under a given set of conditions.

MATERIALS AND METHODS

Preparation of DMTs

Spermatozoa of the sea urchins, *Pseudocentrotus depressus* and *Hemicentrotus pulcherrimus*, were obtained by introducing 0.5 M KCl into the body cavity. The DMTs were prepared by the methods of Summers and Gibbons (12) and Renaud et al. (10), with some modifications. Approx. 2 ml of dry sperm was added to 100 ml of extracting solution containing 1% (vol/vol) Triton

X-100, 0.15 M KCl, 0.5 mM EDTA, 2 mM $MgSO_4$, 1 mM dithiothreitol (DTT), 1 mM ATP, and 10 mM Tris-HCl buffer (pH 8.1). The sperm tails were separated from the heads by stirring in the extracting solution for 10 min. After centrifugation at 5,000 *g* for 10 min, axonemes that were almost the length of intact tails (~40 μm) were recovered in the supernatant fraction. The axonemes were washed with the buffer solution described above without Triton X-100, and suspended to give a concentration of ~1 mg protein/ml in 30 mM Tris-HCl buffer solution (pH 7.8) containing 0.1 mM DTT and 2.5 mM $MgSO_4$. Trypsin was added to this solution at a protein weight ratio of 1:1,000, and the solution was left at room temperature. The proteolysis was followed by observing the optical density at 660 nm and stopped by adding soybean trypsin inhibitor when the turbidity was reduced to 80% of the initial value. The digested axonemes were pelleted by centrifugation and resuspended in 1 mM Tris-HCl buffer solution (pH 8.0) containing 0.1 mM DTT and 0.1 mM EDTA, followed by dialysis against the same buffer solution to remove dynein arms (3). After centrifugation at 20,000 *g* for 30 min, the pellet was suspended in 10 mM Tris-HCl buffer solution (pH 8.0) containing 0.05 mM $MgSO_4$, 0.1 mM EDTA, and 0.1 mM DTT. This solution was used as the stock solution of DMTs.

The DMTs could be prepared by dialysis of the demembranated axonemes against 1 mM Tris-HCl buffer (pH 8.0) containing 0.1 mM DTT and 0.1 mM EDTA for 2 d without trypsin digestion. The DMTs had the same helical shape as those prepared with trypsin digestion and dialysis, although some DMTs in the dialyzed preparations were occasionally grouped as clusters of two or three, and were not separated into individual DMT.

Observation by Dark-Field Microscopy

Specimens for microscopic observation were prepared by mixing the stock solution of DMTs with a large volume of the appropriate solution to give the desired salt concentration and pH value. The final concentration of DMTs was ~0.05 mg protein/ml. Sometimes, the pH value of the specimen was adjusted by adding a small volume of 1 N HCl or 1 N NaOH while the pH value was being monitored by a pH meter.

The optical system used has been described previously (7, 8).

Measurement of Helical Parameters

The pitch and the diameter of the coiled helix of DMT were measured on a number of enlarged photographs. Generally, ~40 helices with more than two helical turns were used. Care was taken to exclude helical images which had apparently suffered deformation by interaction with the glass surface of the slide or coverslip. Helical handedness was determined by the optical sectioning method originally used for bacterial flagella (11, 13).

RESULTS

General Appearance of DMT Under the Light Microscope

As reported in the previous paper, the DMT prepared as described above always had a coiled shape when observed under the microscope. Although the coiled structure was flexible, in that it was easily distorted by apparent interaction with the glass surface, its gross shape seemed to be fairly constant from one preparation to another as long as the structures were free-floating in solution (8). The DMTs had not changed shape when observed after storage at 4°C for a few days. After prolonged storage, however, some of the coiled DMTs became somewhat loose and elongated. Observation by electron microscopy of negatively stained specimens showed that such DMTs contained many deficient units whose characteristic doublet structure was broken. Hence, we concluded that the close-coiling structure is consistently preserved as long as the doublet structure is intact.

The helical handedness of the coiled DMT was determined by the optical sectioning method: a series of photographs (Fig. 1) taken with successively varied focusing revealed that this coiled helix is left-handed. We have not observed any right-handed helices in the present study.

We had noticed that the coiled structure of the DMT varied somewhat when the environmental conditions were changed (8); we therefore surveyed various factors which might affect the shape of the DMT, including ionic strength, pH value,

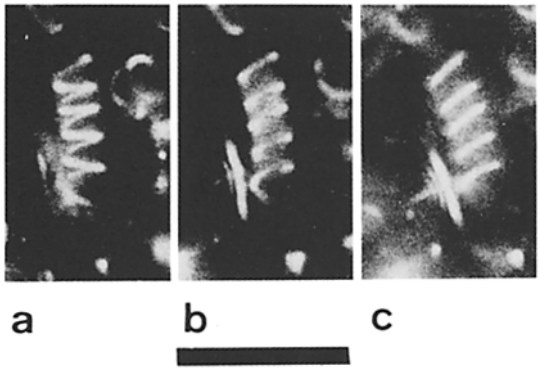

FIGURE 1 Dark-field micrographs of the helical shape of DMT in 10 mM Tris-HCl buffer solution (pH 8.0) containing 1 mM $MgSO_4$ and 0.1 mM $CaCl_2$. This set of pictures indicates that the helix is left-handed. Focus was on (*a*) the upper surface, (*b*) the middle portion, and (*c*) the lower surface of the helix. Bar, 10 μm.

the concentration of divalent cations, and the presence or absence of nucleotides. Among these, the pH value and the Ca^{2+} concentration were found to have the most striking effect on the coiling conformation. We describe below the transformation of the DMT induced by these factors. The helical parameters of DMT in the given conditions (described below) were observed to remain constant from one preparation to another, and even in the two different species of sea urchin.

pH-Dependent Conformation of DMT

Fig. 2 shows light micrographs of DMTs taken at pH 7.4 and 8.3 which clearly demonstrate that the coil formed at pH 7.4 was considerably larger than that at pH 8.3 in both pitch and diameter. Because some of the coils appeared with the helical axis normal to the view, we could measure the pitch and diameter of some helices simultaneously. Fig. 3 presents the results of such measurements, showing the quantitative differences between the coils found at pH 7.4 and 8.3. It shows that the coils are highly uniform at pH 8.3, whereas their dimensions are somewhat scattered at pH 7.4. This might reflect a difference in the flexibility of the DMT at different pH values, although it could partly be because a helical structure with a larger diameter is more easily distorted by external forces.

This pH-induced conformational change was a reversible and rapid process which was completed within 1 min. When the pH value of the medium was raised to pH 8.3 after microscopic observation at pH 7.4, the DMT again assumed a coiled shape indistinguishable from that found in the original specimen at pH 8.3 (Table I).

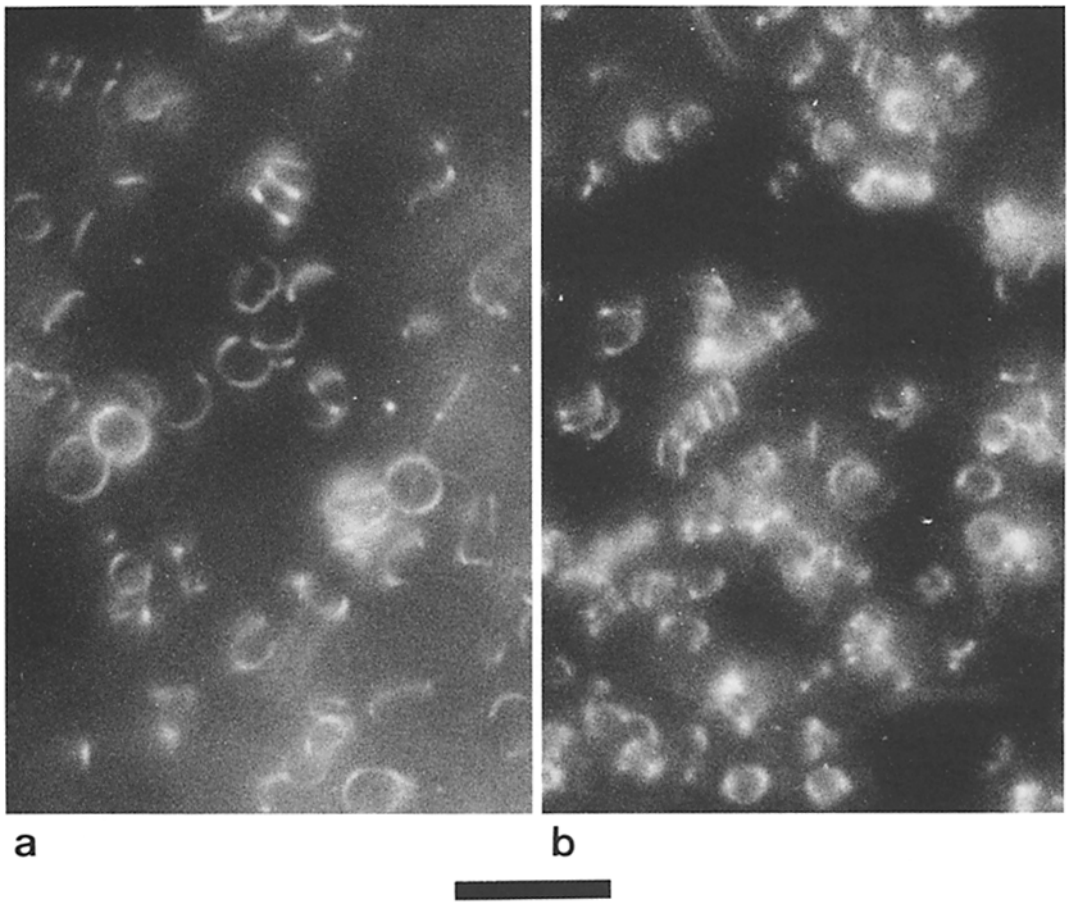

FIGURE 2 Dark-field micrographs of helical DMTs at (*a*) pH 7.4 and (*b*) pH 8.3. Solution composition: 1 mM $MgSO_4$, 0.1 mM $CaCl_2$, and 10 mM Tris-HCl buffer at pH 7.4 or pH 8.3. Bar, 10 μm.

Fig. 4 shows that the helical parameters of the DMT change continuously with the pH values between 7.2 and 8.3, and that a constant value can be obtained reproducibly from one preparation to another at a given value of pH. At pH <7.2 or >8.3, however, we could not obtain reproducible results because the DMTs became less stable and lost their characteristic doublet structure. Sometimes, many aggregates of DMTs were found under these conditions.

Effect of Ca on the Gross Structure of DMT

All of the observations described in the preceding section were made on specimens in media containing 0.1 mM $CaCl_2$. However, we found that the conformation of the DMT varied with minute changes in the concentration of Ca ion. As Table II shows, the diameter of the DMT helices became significantly greater when the concentration of free Ca^{2+} was depleted with EGTA at pH 8.3. This increase in diameter also occurred in the presence of $MgSO_4$ (Table IId), and could be reversed back to the smaller diameter by the addition of an excess amount of $CaCl_2$. When both EGTA and $CaCl_2$ were absent (Table IIb), the DMTs took the form of the smaller helices, similar to those found in the presence of 1 mM $CaCl_2$. This suggests that the presence of a minute amount of Ca^{2+} ion could prevent the DMTs from assuming the larger helical form. To estimate the concentration of Ca^{2+} ion at which the conformational change of the DMT occurs, we measured the helical diameter of the DMT coils in the presence of controlled concentrations of free Ca^{2+} ion, using a Ca-buffer system at pH 8.0 (9). The result is shown in Fig. 5, where we can see that the transition between the larger and smaller coiled structures occurs at a Ca concentration of $\sim 10^{-7}$ M.

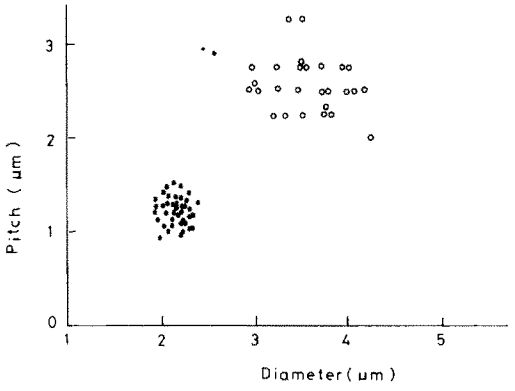

FIGURE 3 The pitch and diameter of DMT helices found at pH 7.4 (○) and at pH 8.3 (●). The solution composition was the same as in Fig. 2.

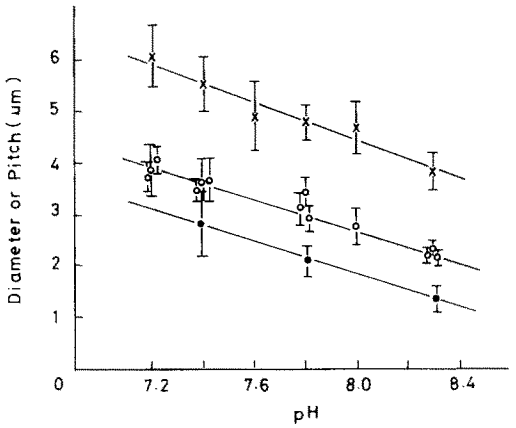

FIGURE 4 The change in pitch (●) and diameter (○) of the DMT helices as pH varies from 7.2 to 8.3. Three different preparations of DMTs were used in the measurements of diameter. Because the number of helical turns decreased when the diameter became larger, we could not measure the helical pitch at pH 7.2. Composition of the solution was the same as in Fig. 2 at each pH. (×) is the length of the diameter when Ca^{2+} concentration was depleted with 1 mM EGTA (see Effect of Ca). Solution composition: 1 mM $MgSO_4$, 0.1 mM $CaCl_2$, 1 mM EGTA, 10 mM Tris-HCl buffer at each pH. The bars represent the SD.

TABLE I

Reversibility of the pH-Induced Transformation

	pH 8.3 →	pH 7.4 →	pH 8.3 →	pH 7.4
			μm	
Diameter	2.2 (0.1)	3.4 (0.3)	2.1 (0.3)	3.4 (0.3)
Pitch	1.2 (0.3)	2.5 (0.7)	1.3 (0.3)	2.3 (1.0)

Arrows indicate successive exchanges of the pH media from pH 8.3 to 7.4.
Numbers in parentheses are SD.
Media used: 1 mM $MgSO_4$, 0.1 mM $CaCl_2$, 10 mM Tris-HCl buffer at both pHs.

TABLE II

Calcium Dependency of the DMT Helix Diameter

	a 1 mM $CaCl_2$	b 1 mM $MgSO_4$	c 1 mM EGTA	d 2 mM $MgSO_4$ 1 mM EGTA
			μm	
pH 8.3	2.2 (0.2)	2.2 (0.4)	4.1 (0.3)	3.8 (0.4)
pH 7.4	3.5 (0.3)	3.8 (0.4)	5.3 (0.9)	5.5 (0.8)

The numbers in parentheses are SD.
10 mM Tris-HCl buffer at pH 7.4 or 8.3 containing the chemicals listed in the heading was used as the media.

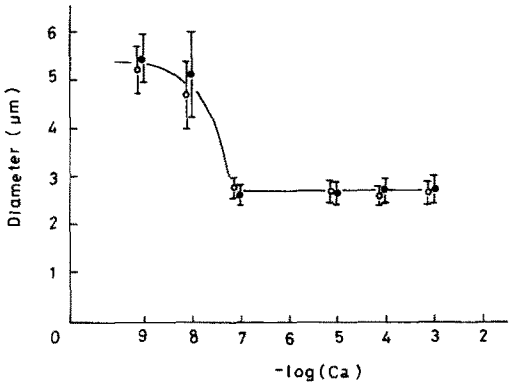

FIGURE 5 Ca dependence of the helical diameter of the DMT. Ca concentrations $<10^{-7}$ M were controlled by a changing ratio of $CaCl_2$ and $MgSO_4$ to EGTA (1 mM) in medium containing 20 mM Tris-HCl buffer (pH 8.0). Concentrations $>10^{-6}$ M were obtained by simple addition of $CaCl_2$ to 20 mM Tris-HCl buffer solution (pH 8.0).

The configuration of the DMT was found to be pH dependent in the absence as well as in the presence of Ca ion (Fig. 4). This implies that H^+ and Ca^{2+} ions govern the conformation of the DMT, at least in part, through different mechanisms.

DISCUSSION

The coiling tendency of the DMT has been reported by several authors (12, 14). However, there has remained a doubt that the coiling might fail to occur under physiological conditions (4). Our present study dispels this doubt.

The fact that the DMT takes a close-coiled form under physiological conditions suggests that the DMT is a mechanically stressed structure in the axoneme, and that the state of the stress can change depending on the internal condition of the axoneme (2, 14). On the other hand, several lines of evidence show that the motility of the flagellar axoneme is controlled by a low concentration of Ca^{2+} ion, as is the case in many contractile systems. In *Crithidia,* the direction of flagellar wave propagation in detergent-extracted cells was dependent on the Ca concentration in the reactivating solution (6). In the sea urchin sperm-model, Brokaw et al. (1) found that the flagellar wave form became more asymmetric when the Ca concentration was increased from 10^{-9} to 10^{-3} M at pH 8.0. Goldstein (5) also showed that flagella in the Triton model of sea urchin spermatozoa exhibited highly asymmetric wave forms in reactivating solutions of low Ca concentration at pH 7.4–7.6, whereas they showed normal wave forms and motility at pH 8.0. Although the mechanism of the Ca control of axonemal motility is entirely unknown, it is possible that it is exerted through some change in the mechanical properties of the axoneme, and that the Ca-induced change in the curvature or rigidity of the DMT might play an important role in the bending wave form of flagellar movement.

In electron micrographs of negatively stained DMTs, the A tubules are always observed to lie on the outer side of the coiled structure (2): i.e., the mean length per pitch of the A subtubule (L_A) is slightly greater than that of the B subtubule (L_B). The relative difference between L_A and L_B, $(L_A - L_B)/(L_A + L_B)$, is proportional to the curvature of the gross helical structure, which can be calculated from the pitch and diameter of the helix. Assuming the center-to-center distance between the A and B tubules to be 0.02 μm, we can consider L_A as being ~1.4% greater than L_B at pH 8.3. Also, from the same assumption, it is inferred that the local axes of the A and B subtubules tilt at an angle of 0.2° with respect to each other. This seems not to depend very much on the pH (see Fig. 4). Because the helices of the DMT were always found to be left-handed, this local twisting of the structure is also left-handed.

In the axoneme, the DMT appears to run fairly straight along its length. Then the left-handed torsional force of each DMT must give the axonemal structure a left-handed twist. In some detergent-extracted models of sea urchin sperm, we often observed that the axoneme as a whole became helically coiled after incomplete digestion by trypsin and the addition of ATP, and found that the helical structure was always left-handed. This fact might reflect the internal torsional force of the DMTs in the flagellar structure.

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