Alternating torsions in a living '9+2' flagellum

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Live spermatozoa of the Japanese quail were observed as they swam in highly viscous salines. Under these conditions, torsions of the flagellum were readily seen. The torsions had a characteristic magnitude (nominally 180°) and pattern of incidence. As a cycle of bending propagated over it, each position on the flagellum experienced first a sinistral torsion and, later, a restoring dextral torsion. The two zones of torsion were each associated with bending; between them was a torsion-free zone that tended to be straight. The amount of interdoublet sliding needed to generate the torsions may be as little as 10 nm. These dynamic propagating torsions have been detected by following the angular displacements of individual (swollen) mitochondria lying adjacent to the axoneme. It is suggested that torque generation is a primary outcome when the unconstrained '9+2' axoneme is activated.

Keywords: flagellum; cilium; axoneme; spermatozoon; motility

1. INTRODUCTION

It is important to know whether the mechanical activity within the '9+2' axoneme of flagella and cilia can result in torsion of the axoneme. This is because torsion will affect the magnitudes of the sliding displacements that are believed to underlie the shape changes that result in motility. It has often been assumed that torsion does not occur in flagella (Gibbons 1982; Brokaw 1991), even in the case of helical bends (Gray 1953; Chwang & Wu 1971). Sliding filament theory has been developed, generally, with the 'no-torsion' assumption as one of its premisses. The best evidence that it is a valid assumption for planar-beating flagella is the work of Brokaw (1991). Yet instances of possible flagellar torsion have been reported (Gibbons 1975; Omoto & Brokaw 1983). Ideally, the question should be decided by observing living cells, thereby avoiding any possibility of artefact due to chemical fixation. The difficulty in detecting torsion is due to the small diameter of the axoneme (*ca.* 200 nm). The success we report in this paper has been possible because of certain features of the flagellum chosen for study, the sperm flagellum of the Japanese quail (*Coturnix coturnix* L. var *japonica*).

The midpiece of this flagellum is unusually long, with the mitochondria arranged around the axoneme as four chains parallel to the flagellar axis (Woolley 1995). A crucial fact is that some individual mitochondria within this sheath become swollen as the sperm age. These swollen mitochondria are visible as bright spots in dark-field illumination and, thus, they can be used as positional markers around the axoneme. They make it possible to detect torsions of the mitochondrial sheath—torsions which are assumed to be caused by torsions of the axoneme within the sheath. In this way, we have been able to see that a characteristic pattern of torsion does occur in the axonemes of living quail spermatozoa when low amplitude, three-dimensional bends propagate.

2. METHODS

Sperm were collected from each quail (Vernon & Woolley 1995) and diluted in 0.25 ml Hanks' balanced salt solution containing 1% bovine serum albumin and $800 \mu\text{g ml}^{-1}$ trypsin inhibitor (from soya bean). Under a supported cover-slip, a droplet of this suspension was juxtaposed against a droplet of the same saline but also containing 2% methyl cellulose (nominally either 1500 centipoises or 4000 centipoises). Observations were made at room temperature on sperm that had swum across the interface. An oil-immersion dark-field condenser and a $\times 100$ Achromat (with insert stop) were used. The lamp was a point source xenon tube (Chadwick-Helmuth Inc.) synchronized to a 50 Hz (CCD) camera. An ultraviolet barrier filter was used. By placing a projector lens in front of the camera, the magnification on the monitor was $\times 15,400$. For analysis the image contrast was reversed. Electron microscopy was by a standard method (Woolley 1995).

3. RESULTS

The spermatozoon of the Japanese quail has a flagellum that is $208 \mu\text{m}$ long. The structure of the '9+2' axoneme is conventional. The midpiece, which is $161 \mu\text{m}$ long, contains approximately 2500 mitochondria (Woolley 1995). The arrangement of the mitochondria as four chains means that there is a mitochondrion occupying each quadrant of the flagellum at any location along the midpiece. When the sperm suspension is kept on the bench for *ca.* 2 h at room temperature, some individual mitochondria swell (figure 1). The motility of the sperm is unaffected by this development. Individual swollen mitochondria are visible (as 'speckles') and can be followed reliably. The apparent movement of a mitochondrion from one edge of the midpiece to the other indicates that the mitochondrial sheath has rolled around its local axis. Our interpretation is that the axoneme within has also rolled and is the prime mover; we shall discuss the validity of this assumption later. A roll of the axoneme at

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Figure 1. Electron micrograph of a longitudinal section of the midpiece of a quail spermatozoon. Two of the mitochondria in this micrograph had become swollen as a result of ageing the sperm suspension prior to fixation. Swollen mitochondria were seen as bright speckles in dark-field illumination. Scale bar, $0.2\ \mu\text{m}$.

one location without the same roll at another location means that torsion has occurred in the intervening region.

The key observations were first made on sperm that were swimming in highly viscous saline; this slows and regularizes the activity. Under these conditions the flagellum screws through the medium as a sinistral helix of three to four gyres; when a sperm swims upwards and reaches the cover-slip, the helical motion is converted to a slow clockwise rotation but there is no close confinement of the flagellum against the cover-slip (Vernon & Woolley 1999). Superimposed on this major curved waveform are trains of bends. This study concentrates on such bends, particularly on those of the smallest amplitude. From observing these bends, we recognized the repeating unit of mechanical activity as a 'cycle of bending' consisting of a pair of small bends. Unfortunately we have not been able to specify the geometry of these low-amplitude bends except to note that each of the two bends is sigmoidal rather than a single arc and is not planar (figure 2*a–e*); possible bend geometries are considered in §4.

Torsions could be related to these cycles of bending as follows. We observed some position marked on its edge by a speckle. During the passage of the first bend, the speckle would roll through apparently *ca.* 180° to appear on the opposite edge, where it would remain until the passage of the second bend, during which it would again roll through apparently around 180° to reappear in its original position (figure 2*a–e*). The exact magnitude of the roll is uncertain but we are confident that the speckle rolls first to some position in the opposite quadrant and then to somewhere in its starting quadrant: we shall refer to this as 'nominally 180° ' of torsion.

From an analysis of 16 sequences (from ten spermatozoa) the mean ($\pm$ s.d.) propagation velocity of the cycle of bending was $53 \pm 23\ \mu\text{m s}^{-1}$. The mean time taken for the first roll was $118 \pm 71\ \text{ms}$, for the second roll $92 \pm 33\ \text{ms}$ and for the delay between first and second rolls $149 \pm 81\ \text{ms}$. The length of the axoneme containing that torsion was calculated from knowledge of the velocity of the bends and the time interval between the start and finish of a torsion. The average lengths of the first and second bends in the cycle were 6.3 and $4.9\ \mu\text{m}$, respectively, with *ca.* $8.0\ \mu\text{m}$ of relatively straight axoneme intervening. These values correlate with our approximate, direct estimates of 'bend length' ($r = 0.7467$). We found that we could determine the direction of roll by carefully

Figure 2. Video frames illustrating torsion of the midpiece in a living quail spermatozoon. Dark-field illumination was used for recording; it has been found helpful, however, to reverse the image contrast, so that the bright speckles (swollen mitochondria) are now dark features. (*a–e*) A sequence of frames with time intervals to show the passage of a cycle of bending. The focus is at mid-flagellar level, so that speckle is only visibly clearly when positioned laterally (on the edge). (*a*) A speckle (arrowed) is on the lower edge prior to the arrival of a bend. (*b*) An approximately sigmoidal bend is in transit, propagating from left to right and centred over the speckle which is lost to view (broken arrow) having rolled through *ca.* 90° . (*c*) After the passage of the first bend, the speckle is now on the upper edge (nominally 180° roll). (*d*) The second approximately sigmoidal bend is in transit and centred over the speckle, which is again lost to focus through having rolled *ca.* 90° . (*e*) After the passage of the second bend, the speckle has completed its second roll and has returned to its original position. The displacements of the speckle towards the left in (*a–e*) are due to the cell's forward progression. (*f*) and (*g*) The same flagellum now carefully focused on the upper surface to show that a displacement of a speckle over the axoneme (arrowed) can be distinguished from a displacement under the axoneme (speckle on right, not arrowed). Scale bar, $1\ \mu\text{m}$.

Figure 3. A simple diagram of the axonemal surface to show the torsions associated with a propagating cycle of bending or mechanical activity. The thickness of the axoneme has been exaggerated by a factor of $\times 16$ relative to its length. Because of the uncertainties over the exact three-dimensional geometry of the bends, the diagram shows only the torsions and not the bends usually associated with them; it therefore corresponds pictorially to those examples where torsion was not accompanied by bends of detectable magnitude. In the more common examples, where bending was visible, the cycle of bending was contained within the zone marked by vertical arrows. This zone should be imagined to be moving distally, i.e. to the right. The regions of sinistral and dextral torsion (T_s and T_d) each contained a bend and were separated by a torsion-free segment (T-F) that was relatively straight. A mitochondrion (m) is shown on the extreme right overlying part of the axoneme that will soon be involved in the propagating cycle of activity. The underlying activity will cause it to roll first clockwise then anticlockwise (as seen from a proximal viewpoint). The magnitude of the torsion is shown nominally as 180° , but it is possible, if slippage were occurring, that the torsion of the axoneme itself might be in excess of this. Heuristically, of course, the torsions of the axoneme have been inferred from the displacements of the mitochondria (see the text).

focusing on the upper surface of the flagellum (figure 2*f,g*). After analysing 12 such sequences, we report that the speckles roll clockwise first and then anticlockwise (as would be seen from the proximal end). We decided which movement came first and which second by concentrating on intermittent rather than continuous trains of bends. The rolling movement of locations on the midpiece was not accompanied by rolling movement of the base, i.e. all torsional movement occurred with respect to a fixed angular position of the basal body. Important variations to the scenario described above were (i) it was possible to witness this same movement of the speckle (i.e. nominally 180°) in the absence of any discernible bending and (ii) it was possible to see the entire sequence of events occurring more rapidly in saline of standard viscosity (i.e. without methyl cellulose).

4. DISCUSSION

Thus, propagating torsions of the mitochondrial sheath occur and are coincident with propagating bends where bending is occurring. However, the typical pattern of torsion has been seen where bends were not discernible. Two important conclusions are drawn from this. First, in the absence of measurable bending, torsions of the axoneme can be the only reasonable explanation for torsions of the mitochondrial sheath; therefore, in the following paragraphs, torsion of the axoneme is inferred from torsion of the sheath. Second, bending of the axoneme is the consequence of its torsion (not vice versa); perhaps bending begins when torsion exceeds some threshold, so as to develop tension. It is known that regions of tension develop locally and propagate meta-

chronally along quail sperm axonemes (Vernon & Woolley 1995).

It can be deduced from the observations that the propagating cycle of bending includes a segment of torsion-free axoneme bounded at each end by segments that have torsions of approximately equal magnitude and rate but opposite chirality (figure 3).

How are the torsions generated? We do not think that excessive viscous shear is causing them indirectly, because the torsions could be seen at low viscosity. By general consensus (Porter 1996), the axonemal dyneins are minus end-directed motors, which means that active shear between all nine arrays at any location would cause dextral torsion, as discussed by Woolley & Brammall (1987). Thus, referring to figure 3, T_d (dextral torsion), may be the active zone, with T-F (torsion-free segment) and T_s (sinistral torsion) as the passive consequences in inactive, downstream locations. This interpretation is concordant with the 'active zone' interpretation of the motility of split axonemes from quail sperm (Vernon & Woolley 1995). The sliding displacements need only be small: we calculate that a 180° torsion distributed over $\approx 5 \mu\text{m}$ of straight axoneme would require a tilting of the doublets equivalent to 10 nm of interdoublet sliding. This is similar to the displacement of the major subunit of the outer dynein arm between the 'rigor' and 'relaxed' states (Burgess 1995). Thus, the simplest interpretation is that the dyneins are in their activated state in zone T_d ; this explanation is also preferred on account of its consistency with our earlier work on propagating zones of tension development (Vernon & Woolley 1995). However, there are two other possible interpretations that we cannot rule out at present. The general symmetry of T_d and T_s and their spatial separation could mean that both the torsions are actively generated, invoking the unorthodox idea that the polarity of active sliding may alternate (Ishijima *et al.* 1996). Logically, the third possibility is that the T-F region may roll actively clockwise about its own axis ('self-spin') causing both torsions to occur passively—a conjectural mechanism for this might be a rotational interaction between the doublets and the central pair via the radial spokes. This highly unconventional third idea is only included for the sake of completeness and because the spermatozoa of some birds (passerine) and molluscs do seem able to achieve self-spin (Bishop 1958; Vernon & Woolley 1999; R. W. Berry, personal communication).

The restoration of the inactive, untwisted state following the passage of a cycle of bending is likely to be due to the torsional elasticity of the axonemal assembly. Strong resistance to dextral torsion was noted in a physical model of the axoneme which incorporated the sinistral-coiling tendency of the individual doublets (Woolley & Brammall 1987).

On the question of bend geometry, if the two bends in the cycle are the result of the torsions exceeding some shear threshold, then one would expect the developing bends to become helical curves each of the same chirality as its torsion. And, given that the overall torsion in each bend is nominally 180° , perhaps each bend is essentially half of one cycle of a helical wave. This interpretation awaits confirmation because of the difficulty in recovering the z -axis coordinates. Nevertheless, this speculation is

Figure 4. A video frame illustrating the compression of the waveform when the sperm head (out of view) became stuck. When this low-frequency, slowly propagating meander wave lay exactly planar against the cover-slip as illustrated, no torsions were evident, as judged by the constant positioning of the mitochondrial speckles as the waves passed over them. Scale bar, 5 μm .

attractive in that a train of such bends, when modelled, tends towards planarity when their chirality alternates. We also note that, if both torsions were developed actively, then a much shorter axoneme, such as in a cilium, might express them sequentially, with the cycle of bending (as described above) becoming equivalent to one beat cycle (as conventionally understood).

Previous studies of dynamic torsion in flagella have required careful chemical fixation followed by electron microscopy. Gibbons (1975) fixed rigor 'models' of echinoderm sperm and, by high-voltage electron microscopy, discovered two zones of torsion of opposite chirality in the bent regions bounding each straight section of axoneme. Omoto & Brokaw (1983) showed that, in surface replicas, bends in tunicate sperm flagella coincided with torsions. Because of the methods used, these previous studies have had to be regarded with caution. Yet, although the magnitude of torsion was smaller, those earlier results do compare reasonably well with our observations on quail spermatozoa, particularly with regard to Gibbon's (1975) conclusion that no net torsion would accrue because the chirality alternated. Dynamic torsion in cilia was reported by Omoto & Kung (1980), who used both serial thin sections and high-voltage electron microscopy. They found both sinistral and dextral torsions among the axonemal cylinders. In addition, ciliary torsion was strongly implied by Dentler & LeCluyse (1982) from their demonstration of restricted sliding at the ciliary tip. The running torsions on the quail sperm flagellum, however they are generated, are likely to involve torsion of the central pair of microtubules as well as of the peripheral doublets. Although we have no evidence on this point, we note from the literature that central pair torsion was apparent in an early paper by Bradfield (1955) and in Gibbon's (1975) micrographs and those of Omoto & Kung (1980). However, in the work of Omoto & Kung (1980), this torsion was always sinistral and this fact is consistent with other evidence for the idea that the central pair of the active cilium is spinning (continuously) through 360° per beat cycle in the clockwise direction, as seen from the base. There is evidence for continuous spin of the central pair in the flagella of other organisms (Jarosch & Fuchs 1975; Omoto & Witman 1981; Kamiya 1982). If continuous central pair spin occurs in quail

sperm, it would indicate that the torsions T_s and T_d are not equivalent in their action on the central pair.

We conclude with an additional observation which suggests that torsion may not always underlie bending. Under the conditions stated, some spermatozoa did become attached to the cover-slip by the head. The waveform became compressed into a meander form and sometimes the whole meander would lie against the cover-slip exactly planar (figure 4). This effect is familiar from studies of other flagella (Brokaw 1974; Tani & Kamimura 1998). The beat frequency was very low (ca. 0.1–0.2 Hz) as was the propagation velocity. We watched the speckles and saw no torsions associated with the passage of these large-angle bends. One could make the ad hoc hypothesis that the rate of axonemal torsion here is so slow that the torsional impulse is not transmitted to the mitochondrial sheath. Another possibility, however, is that the axoneme may indeed become torsion free through being confined strictly to a plane surface.

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