Outer Arm Dynein from Trout Spermatozoa

PURIFICATION, POLYPEPTIDE COMPOSITION, AND ENZYMATIC PROPERTIES*

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Extraction of isolated axonemes from trout (Salmo gairdneri) sperm with 0.6 M NaCl removed 97% of the outer arms, ~12% of the protein, and ~50% of the MgATPase activity. Fractionation of this high salt extract by sucrose density gradient centrifugation yielded a single peak of ATPase activity with an apparent sedimentation coefficient of 19 S.

Electrophoretic analysis showed that this 19 S particle was composed of two heavy chains (termed α and β; M, 430,000 and 415,000, respectively), five intermediate molecular weight chains (IC1–IC5; M, 85,000, 73,000, 65,000, 63,000, and 57,000), and six light chains (LC1–LC6; M, 22,000–6,000). A similar complex was obtained following further purification by DEAE-Sephacel column chromatography. Quantitative densitometry of Coomassie Blue-stained gels indicated that the heavy and intermediate chains were present in equimolar amounts.

Electron microscopic examination of the 19 S particles revealed that it consisted of two globular heads joined together by a Y-shaped stem.

The 19 S particle had a specific MgATPase activity of 1.1 ± 0.3 μmol of phosphate released/min/mg and exhibited an apparent K_m for MgATP<sup>2+</sup> of 40 ± 16 μM. MnATP<sup>2+</sup> and CaATP<sup>2+</sup> were hydrolyzed at rates 100 and 80% that of MgATP<sup>2+</sup>, respectively. The MgATPase activity was inhibited by vanadate, but not by ouabain or oligomycin, and exhibited a high activity between pH 7.0 and 10.0 with a maximum at pH 9.0–9.5. ATP was the preferred nucleotide, although GTP and CTP (but not ITP) did interact with the dynein to a minor extent.

Based on its origin, sedimentation coefficient, polypeptide composition, and enzymatic properties, we conclude that this two-headed 19 S particle represents the entire trout sperm axonemal outer arm dynein. This dynein is probably exemplary of the outer arm dyneins of other vertebrates.

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Dyneins are multimeric ATPases which comprise the inner and outer arms associated with the outer doublet microtubules of eukaryotic ciliary and flagellar axonemes. During cycles of ATP binding and hydrolysis, dyneins are believed to interact with the adjacent doublet microtubule to produce active sliding which is the basis for axonemal bending (for reviews, see Gibbons, 1981; Satir, 1982; Witman, 1989a). Dyneins have been extracted from the axonemes of a wide variety of organisms by treatment with solutions of either high or low ionic strength (for review, see Witman, 1989a); however, our present understanding of dynein structure and function is based almost exclusively on the analysis of outer arm dyneins from Chlamydomonas and sea urchin sperm flagella and from Tetrahymena cilia.

Outer arm dyneins from these species are composed of several discrete subunits termed the α, β, and in some cases γ subunits. Each subunit exhibits an ATPase activity and contains a single high molecular weight polypeptide (named the α, β, and γ chains, respectively; M, > 400,000) associated with a variable number of intermediate and low molecular weight components (Pfister et al., 1982; Pfister and Witman, 1984; King and Witman, 1989; Porter and Johnson, 1983; Tang et al., 1982; see Witman, 1989a for review). Electron microscopic analysis of purified outer arm dyneins has demonstrated that they have two (sea urchin) or three (Chlamydomonas and Tetrahymena) globular domains connected by stems to a common base; in each case, the number of globular domains corresponds to the number of dynein heavy chains (Goodenough and Heuser, 1984; Johnson and Wall, 1983; Sale et al., 1985; Witman et al., 1983).

In contrast, less is known about the structure and composition of dyneins from vertebrates. Knowledge of vertebrate dyneins is important because in mammals the lack of dynein arms is associated with both male infertility and a group of severe respiratory diseases (Afzelius, 1979; Baccetti et al., 1986; Zamboni, 1987). Moreover, cytoplasmic dynein-like proteins have been purified from mammalian cells and seem to be involved in intracellular movements such as the transport of axonal vesicles (Pallini et al., 1982; Paschal et al., 1972b; Vallee et al., 1988). Several recent studies have examined mammalian dyneins extracted from ciliated tracheal cells and from sperm flagella (Pallini et al., 1982; Belles-Ibels et al., 1986; Hastie et al., 1986, 1988; McConnell et al., 1987), but unfortunately, due to low yields and proteolytic degradation during purification, only incomplete characterizations of these dyneins have been obtained. Indeed, these studies were unable to resolve definitively such fundamental questions as whether the isolated particles originated from the inner or outer arm and how many polypeptides comprise these vertebrate dyneins.

In order to learn more about vertebrate dyneins, we have initiated studies of dynein from the sperm of the rainbow trout.
trout (*Salmo gairdneri*). Trout sperm have several major advantages as a source of vertebrate dynein: 1) semen is available in large quantity; during the peak of the spawning season more than 20 ml of semen with 10⁸ sperm/ml can be collected from a single fish; 2) trout sperm do not possess an acrosomal vesicle (Billard, 1983), thereby reducing the potential for proteolysis during isolation; 3) unlike mammalian sperm, trout sperm exhibit a "simple" morphology, being composed of a round head containing the nucleus and mitochondrion, and a flagellum which contains a "9 + 2" axoneme but no dense fibers, mitochondrial or fibrous sheaths (Billard, 1983). The lack of such flagellar accessory structures may facilitate extraction of the dynein from the axoneme.

We report here the isolation of flagellar axonemes from trout spermatozoa, the selective and virtually complete removal of their outer arms by high ionic strength extraction, and the subsequent purification of the outer arm dynein. We have determined the composition, the structure, and some of the enzymatic properties of this macromolecular complex and compared them with the properties of dynein from other organisms.

**MATERIALS AND METHODS**

**Collection of Trout Semen**—The semen of adult male rainbow trout (*S. gairdneri*) weighing 2-3 kg was obtained by handstripping at the Sandwich State Fish Hatchery (Sandwich, MA) and kept on ice until use (within 2-3 h). Semen was collected in 50-ml polypropylene tubes, and special care was taken to avoid contaminating the sample with water or urine. Each fish produced 5-40 ml of semen containing 0.5-3.9 × 10⁸ sperm/ml. Semen from different fish were always treated separately.

Percent of motile sperm in each sample was assessed as described by Cosson et al. (1985). An aliquot of semen was diluted (1/100 to 1/ 200) with a high potassium saline solution (45 mM KCl, 80 mM NaCl, 20 mM Tris-HCl, pH 7.5) to avoid initiation of motility, and then diluted (1/100 to 1/200) into a drop of potassium-free saline solution (125 mM NaCl, 20 mM Tris-HCl, pH 8.0) or tap water on a microscope slide. The short burst of motility exhibited by the sperm was examined by dark-field microscopy. Only semen samples containing a high percent of motile sperm were used for the dynein isolation.

For some experiments, the dynein was prepared from sperm that had been frozen in the presence of 5% dimethyl sulfoxide and kept at liquid nitrogen temperature. No differences have been observed between dynein obtained from fresh or frozen material, even after several months of storage.

**Isolation and High Salt Extraction of the Trout Sperm Axonemes**—The procedures used to prepare trout sperm axonemes and to extract the outer arm dynein were derived from methods previously described for sea urchin sperm (Gibbons and Frøk, 1979; Sale et al., 1985). Briefly, 5 ml of semen were placed into a tight-fitting Dounce homogenizer and diluted with 10 ml of IMEN buffer (5 mM imidazole, 100 mM NaCl or KCl, 5 mM β-mercaptoethanol, 4 mM MgSO₄, 0.5 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, pH 7.0) supplemented with 1% Triton X-100 (0.66% final) to extract the membrane and components of the flagellar matrix. Sperm tails were detached by four strokes of the pestle, and an additional 10 ml of 1% Triton-buffer solution (brining the Triton X-100 concentration to 0.8% final) were added before the heads were pelleted by centrifugation at 750 × g for 5 min. Axonemes were subsequently collected from the supernatant by centrifugation (12,000 × g, 10 min), and the pellet washed three times by resuspension in 20 ml of IMEN buffer followed by centrifugation.

Outer arms were extracted by resuspending the pellet of axonemes from the third wash in an ice-cold high salt solution (IMEN buffer with the NaCl concentration raised to 0.6 M); 1 ml of extraction solution was layered onto a 2-ml × 10⁷ sperm/ml suspension in the initial sample. After a 15-min incubation on ice, the suspension was centrifuged at 40,000 × g for 10 min and the supernatant removed and loaded onto a sucrose density gradient.

**Sucrose Density Gradient Centrifugation**—High salt extract (0.8 M NaCl) was layered onto a 12-ml linear sucrose density gradients made in TEMK buffer (20 mM Tris-HCl, 0.5 mM EDTA, 5 mM β-mercaptoethanol, 4 mM MgSO₄, 50 mM KCl, pH 7.5) and centrifuged in a Beckman SW 41 rotor for 14.5 h (160,000 × g; ω²t = 6.8 × 10¹⁵ rad²/s). Fractions of 0.7 ml were collected from the bottom of each tube. When multiple gradients were run, the equivalent fractions from each gradient were pooled prior to analysis.

The apparent sedimentation coefficient of the outer arm dynein was estimated using thyroglobulin (19 S) and catalase (11.3 S) as standards.

**Protein and ATPase Assays**—Protein content and ATPases activity were determined as described previously (King et al., 1986; Pfister et al., 1982). The ATPase assay solution contained 25 mM KCl, 0.5 mM EDTA, 5 mM MgSO₄, 20 mM Tris-HCl, pH 7.5, except for the determination of pH dependence where the appropriate synthetic buffers were used.

**DEAE-Sephadex Chromatography**—Sucrose gradient-purified outer arm dynein was chromatographed on a DEAE-Sephacel column (Pharmacia LKB Biotechnology Inc.) as described previously for *Tetrahymena* dynein (Johnson, 1986). The column, previously equilibrated with TEMK buffer, was eluted with a 50-500 mM KCl gradient made in TEMK.

**Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis**—High molecular weight polypeptides were resolved by electrophoresis in 2-8 M urea, 3-5% acrylamide gradient gels; intermediate and low molecular weight components were separated using 0-2 M glycerol, 5-15% acrylamide gradient gels (King et al., 1986). Following electrophoresis, polyacrylamide gels were stained either with Coomassie Brilliant Blue or by the silver stain procedure of Merrill et al. (1981).

The relative molecular masses of the trout dynein heavy chains were estimated using *M* values of 480,000, 440,000, and 390,000 for the *Chlamydomonas* outer arm α and β heavy chains and the "band 1" outer arm fragment α, respectively; other *M* standards were as detailed previously (King and Wittman, 1987).

**Quantitative densitometry of Coomassie Blue-stained gels was performed using a Kontes model 800 scanning densitometer (Kontes, Vineland, NJ) as described by Otter et al. (1987).**

**Electron Microscopy**—Pellets of intact sperm, washed axonemes, and high salt extracted axonemes were fixed at room temperature with 1% glutaraldehyde in 0.1 M sodium cacodylate, pH 7.4. After four washes with cacodylate buffer, samples were postfixed with 1% osmium tetroxide and stained en bloc with 1% uranyl acetate, followed by dehydration with ethanol and embedding in Epon.

Sucrose gradient-purified trout outer arm dynein was prepared for negative-stain electron microscopy essentially as described by Marshese-Ragona et al. (1988). The dynein sample was diluted to ~20 μg/ml in 20 mM Tris-HCl, 0.5 mM EDTA, 25 mM KCl, pH 7.5. A thin carbon film was then floated from a mica support onto the surface of the dynein solution. The film was withdrawn after a few seconds, floated onto 1% uranyl acetate, and picked up on acetone-washed, uncoated, 400-mesh copper grids. Samples were examined at 80 kV in a Phillips CM-10 electron microscope.

**Chemicals**—Sodium (meta)vanadate was obtained from Fluka AG (Ronkonnok, NY); solutions were made just prior to use. Ouabain and oligomycin were from Sigma. Stock solutions of 10 μM ouabain and 10 μM oligomycin were prepared fresh in 0.1 M sucrose buffer; oligomycin stock was stored at ~30% in 50% ethanol, 50% dimethyl sulfoxide (v/v) and stored at ~20 °C until use. [γ-32P]ATP (specific activity ~35 Ci/mmol) was purchased from Du Pont-New England Nuclear. All other chemicals were of analytical or ultrapure grade and were obtained from Mallinckrodt (Bedford, MA) or Sigma.

**RESULTS**

**Isolation and High Salt Extraction of Trout Sperm Axonemes**—Trout spermatozoa were simultaneously demembranated with Triton X-100 and deflagellated by homogenization (see "Materials and Methods"). Heads were removed by differential centrifugation and the axonomes subsequently pelleted from the supernatant. After three washes with a buffer solution, a highly purified preparation of axonemes was obtained (~7 mg of protein/10¹⁵ sperm). These isolated axonomes appeared devoid of plasma membrane when examined by transmission electron microscopy (Fig. 1b), although in some preparations some membranous vesicles were observed. During the detergent treatment and the following washes, 4-28% of the outer dynein arms were removed from the axonomes (mean = 13%; 414 double microtubules scored from four different preparations). This effect was not due to a
fixation artifact since 99% of the outer arms were present in intact flagella (Fig. 1a; 235 outer arms present on 238 doublet microtubules; four different preparations). Following treatment with 0.6 M NaCl, 93–99% (mean = 97%) of the outer dynein arms were absent from the extracted axonemes (Fig. 1c; 895 outer arms missing from 927 doublet microtubules scored from four different preparations). Although some of the axonemes became splayed apart during these procedures, inner arms, nexin links, and radial spokes appeared intact after the high salt treatment (Fig. 1c).

High salt extraction of the axonemal preparation solubilized about 50% of the MgATPase activity and 12% of the protein (Table I). In some experiments, a minor loss of ATPase activity occurred at this step since the total activity recovered after extraction (total activity of the high salt extract and the remaining activity of the extracted axonemes) was 82 ± 17% (n = 5) of the MgATPase activity present in the last washed axoneme suspension.

Electrophoretic analysis of the axonemal proteins solubilized by the high salt treatment is shown in Fig. 2. The high molecular weight polypeptides of the intact axonemes were resolved into four distinct bands when stained by Coomassie Brilliant Blue; five to six bands, depending upon the loading on the gel, were observed when silver stain was used (not shown). Only two of these bands were found in the high salt extract. In some gels, the relative intensities of the two extracted chains were not equal (Fig. 2, right panel): the heaviest chain was less intensely stained than the lighter. This was due to an electrophoretic and/or sample preparation artifact which resulted in components from the slowest migrating band becoming smeared toward the top of the gel. This effect could be obviated by increasing the amount of sodium dodecyl sulfate in the sample and decreasing the protein loading (results not shown).

A number of intermediate and low molecular weight polypeptides (including variable amounts of tubulin) were also extracted (Fig. 2, left panel).
Purification and Composition of Trout Sperm Outer Arm Dynein—The axonemal high salt extract was fractionated by centrifugation in a 5–25% sucrose density gradient. Two peaks of protein with apparent sedimentation coefficients of 19 and 5 S were recovered from the gradient (Fig. 3). The MgATPase activity sedimented as a single peak corresponding to the 19 S protein peak; the specific MgATPase activity of the peak fraction was $1.2 \pm 0.3$ pmol of phosphate released/min/mg (mean $\pm$ S.D.; $n = 8$). We observed that the total amount of ATPase recovered from the gradient was always less than the amount loaded (Table I). This difference could be explained by a slow inactivation of the dynein by proteolysis or aggregation during the centrifugation, and/or by the inactivation of a contaminating membrane ATPase (see below).

The distribution of the two high molecular weight polypeptides paralleled that of the MgATPase activity throughout the sucrose gradient (Fig. 4, top panel). These chains had $M_r$ values of 430,000 and 415,000, respectively, and appeared to correspond to the $\alpha$ and $\beta$ heavy chains identified in other outer arm dyneins (Witman, 1989a). Five intermediate molecular weight chains (termed IC1–IC5) and six low molecular weight chains (termed LC1–LC6) also comigrated with the MgATPase activity (Fig. 4, lower panel). The apparent masses of these components are listed in Table II. Intermediate chains 3 and 4 migrated as a closely spaced doublet, and the relative intensities of the two bands varied somewhat from preparation to preparation. Intermediate chain 5 migrated close to the $\alpha$- and $\beta$-tubulin bands. However, this molecule was electrophoretically distinguishable from tubulin, and it was not recognized on protein blots by monoclonal antibodies (obtained from Amersham Corp.) directed against either $\alpha$- or $\beta$-tubulin (these antibodies did react with trout axonemal tubulin; results not shown). The 5 S protein peak contained mainly tubulin and several unidentified intermediate and low molecular weight polypeptides.

To confirm that the heavy, intermediate and light chains were all components of a multimeric dynein ATPase, the sucrose gradient-purified dynein was further purified by DEAE-Sephacel column chromatography (Fig. 5). A peak of MgATPase activity corresponding to the major protein peak was eluted from the column at low ionic strength. The specific

\[ \text{Fig. 3. Sucrose gradient fractionation of high salt extract.} \]

Profile of a 5–25% sucrose density gradient loaded with a high salt extract of trout sperm axonemes. The bottom of the gradient is at left. Fractions were assayed for protein content and ATPase activity. Arrows indicate the position of sedimentation for thyroglobulin (19 S; fraction 6) and catalase (11.3 S; fraction 11), respectively.

\[ \text{Fig. 4. Electrophoretic analysis of sucrose gradient fractions.} \]

Protein composition of the different fractions obtained by sucrose density gradient centrifugation of the high salt extract. Lanes on both gels were loaded with equal volumes of the fractions indicated at the top of the figure. Top, high molecular weight polypeptides separated in a 2–8 M urea, 3–5% acrylamide gradient gel. Bottom, intermediate and low molecular weight components separated in a 0–2.4 M glycerol, 5–15% acrylamide gradient gel. Both gels were silver-stained. Fractions 5–8 correspond to the 19 S dynein ATPase peak. At the left, a lane overloaded with protein from the 19 S peak fraction more clearly shows the low molecular weight polypeptides.

\[ \text{Table I.} \]

| Component | $M_r$ (kDa) |
|-----------|-------------|
| IC1        | 430,000     |
| IC2        | 415,000     |
| IC3        | 390,000     |
| IC4        | 370,000     |
| IC5        | 350,000     |
| LC1        | 320,000     |
| LC2        | 300,000     |
| LC3        | 280,000     |
| LC4        | 260,000     |
| LC5        | 240,000     |
| LC6        | 220,000     |

1 The abbreviations used are: IC, intermediate molecular weight chain; LC, low molecular weight chain.
TABLE II
Mass and stoichiometry of the component polypeptides of trout sperm outer arm dynein

| Polypeptide | \( M_r \times 10^3 \) | Stoichiometry* |
|-------------|-----------------|----------------|
| \( \alpha \)  | 430             | 1 (0.94)       |
| \( \beta \)   | 415             | 1 (1.00)       |
| IC1          | 85              | 1 (0.87)       |
| IC2          | 73              | 1 (1.17)       |
| IC3          | 65              | 1 (1.97)       |
| IC4          | 63              | 1 (1.06)       |
| IC5          | 57              | 1 (0.94)       |
| LC1          | 19              | ND             |
| LC2          | 11.5            | ND             |
| LC4          | 9               | ND             |
| LC5          | 7.5             | ND             |
| LC6          | 6               | ND             |

*Relative stoichiometries were determined with respect to the \( \beta \) heavy chain by quantitative densitometry of Coomassie Blue-stained gels. The numbers indicate the most probable number of chains per dynein arm based on the actual data shown in parentheses. ND, not determined.

**Fig. 5. Purification of the 19 S dynein by DEAE-Sephacel chromatography.** The 19 S dynein from the sucrose density gradient (~450 µg; specific activity 1.03 µmol of phosphate released/min/mg) was applied to a DEAE-Sephacel column and then eluted with a 0.05–0.5 M KCl gradient (dashed line). ATPase activity (●) and protein content (X–X) of the fractions are shown.

**Fig. 6.** Protein composition of DEAE-Sephacel purified dynein. Electrophoretic analysis of the proteins eluted from the DEAE-Sephacel column. Equal volumes of each fraction were loaded onto 2–8 M urea, 3–5% acrylamide (upper panel) and 0–2.4 M glycerol, 5–15% acrylamide (lower panel) gradient gels and silver-stained. The number of the fraction loaded is indicated above each lane; the positions of the dynein polypeptides are indicated at left. Some of the intermediate chains are partially obscured by artifacts created by electrophoretic conditions in the same region of the gel (Tasheva and Dessev, 1983; Pfister et al., 1984).

ATPase activity of the pooled peak fractions was 0.95 µmol of phosphate released/min/mg; this value is very close to that of the sample initially applied to the column (1.03 µmol of phosphate released/min/mg), indicating that no activation or inhibition of the dynein ATPase activity occurred during chromatography. The polypeptide composition of the DEAE-Sephacel-purified dynein was almost identical to that obtained from the sucrose gradient (Fig. 6). One light chain (LC2) was not detected in samples eluted from the column. This protein is not readily silver-stained, and its amount in this sample may have been insufficient for its detection. It is also possible that this light chain was dissociated from the dynein particle, although it was not observed in any other column fraction. In any case, the fact that the distribution of LC2 in the sucrose gradient exactly paralleled the distribution of the other dynein polypeptides suggests that this chain is also an integral component of the outer arm complex.

In some experiments, two polypeptide bands were observed between the heavy and intermediate chains (Figs. 4 and 6, lower panels). These polypeptides did not comigrate with the peak of ATPase activity in either the sucrose gradient or the column eluate, they varied in amount, and they only occurred in substoichiometric amounts relative to the heavy and intermediate chains. Therefore, they probably are not components
of the native 19 S particle.

Relative Stoichiometry of the Polypeptide Components of the 19 S Dynein Particle—The relative stoichiometries of the heavy and intermediate dynein chains in the 19 S peak fractions from the sucrose gradient were determined by quantitative densitometry of Coomassie Blue-stained gels (Table II). The α and β chains were present in a molar ratio of 0.94:1.

IC1, IC2, and IC5 were also present in an approximately 1:1 molar ratio with the α and β chains. Because IC3 and IC4 were not differentiated by the scanning densitometer, they appeared as a single peak with a ratio of ~2 per β chain. Insufficient amounts of the dynein light chains were obtained to allow for accurate quantitation; however, the observed differences in the intensity of stain associated with these bands suggests that some of them (especially LC6) may be present in multiple copies.

Negative Stain Electron Microscopy of 19 S Dynein—The purified 19 S particle was negatively stained with uranyl acetate and observed by electron microscopy (Fig. 7). The majority of particles were composed of two globular domains joined by a Y-shaped stem. Some one-headed particles, but present in multiple copies.

Enzymatic Properties of Trout Sperm Outer Arm Dynein—The purity of the outer arm dynein at different steps of preparation was investigated by examining the effects of several known ATPase inhibitors: ouabain, oligomycin, and vanadate (Table III). The in situ and extracted ATPases exhibited similar sensitivities toward the different inhibitors: the activity was reduced 50–65% by 25 μM vanadate, 35% by 0.5 mM ouabain, and was unaffected by 50 μM oligomycin.

This result indicates that the axonemes and high salt extract were slightly contaminated by a ouabain-sensitive membrane ATPase. In contrast, the 19 S sucrose gradient-purified dynein was almost insensitive to ouabain (13 ± 7% inhibition; mean ± S.D. for five experiments). The ATPase activity of the purified dynein was decreased by a maximum of 65% when 25 μM vanadate was present and increasing the vanadate concentration to 200 μM had only a slight effect.

A double-reciprocal plot of MgATP dependence of the trout outer arm dynein ATPase activity is shown in Fig. 8. Under the conditions of our assay, the trout outer arm dynein exhibited an apparent K_m for MgATP of 40 ± 16 μM and a V_max of 1.3 ± 0.8 μmol of phosphate liberated/min/mg (mean ± S.D.; n = 5).

Trot outer arm dynein exhibited a marked preference for ATP as nucleotide substrate: the rate at which 100 μM Mg-ATP was hydrolyzed decreased by only 35% in the presence of 5 mM MgCTP or MgGTP; the addition of 5 mM MgATP had no effect.

The substitution of other divalent cations for Mg^2+ in the ATPase assay had a slight effect on the dynein ATPase activity: MmATP or CaATP were hydrolyzed at 100 and 80% the rate of MgATP, respectively.

The MgATP activity of trout sperm dynein varied with pH; a high ATPase activity was observed between pH 7.5 and 10 with a maximum at pH 9.0–9.5 (not shown).

DISCUSSION

Previously, the detailed analysis of dyneins from vertebrate species has been hampered by difficulties inherent to the particular systems examined. These difficulties include: 1) the disruption of the ciliated or flagellated cells at an early stage during the isolation process, releasing proteases (for example those contained within the sperm acrosomal vesicle) into the medium; 2) the presence in sperm of accessory structures (mitochondrial and fibrous sheaths, dense fibers) within the flagellum which may physically impede the extraction of dyneins; and 3) the small amount of starting material. In all cases, only low yields of intact dyneins have been obtained under either high or low ionic strength conditions of extraction. To obviate these problems, we developed a model system in which vertebrate dyneins may be readily obtained and
analyzed: the sperm of the rainbow trout (S. gairdneri). These sperm can be obtained in very large amounts; they do not contain flagellar structures or an acrosomal vesicle; and, as demonstrated here, the outer arm dynein may be easily extracted and purified.

The single step Triton X-100 demembranation-homogenization procedure used here allowed a highly purified preparation of axonemes to be rapidly obtained. These axonemes were structurally intact as determined by thin section electron microscopy. Some membranous vesicles did remain associated with the axonemes and probably accounted for the minor amount of ouabain-sensitive ATPase activity observed. The subsequent extraction of the trout sperm axonemes with 0.6 M NaCl removed more than 95% of the outer arm dyneins as determined by electron microscopy. This result is comparable to those obtained when this procedure is applied to the axonemes of invertebrate species. However, with trout sperm axonemes only 50% of the MgATPase activity can be easily extracted. This value is slightly lower than that obtained with sea urchin sperm axonemes (Gibbons et al., 1976) and may reflect the presence of enzymatically more active inner arm dyneins in trout.

The high salt extract was fractionated by sucrose density gradient centrifugation, and the ATPase activity was recovered in a single peak which migrated with an apparent sedimentation coefficient of 19 S. The electrophoretic analysis of the sucrose gradient fractions revealed a striking similarity between the proteins extracted from trout sperm and those obtained from the sea urchin Strongylocentrotus purpuratus (compare Fig. 4 with Fig. 1b in Paschal et al., 1987a). The 19 S ATPase contained two heavy chains (α and β; M, 430,000 and 415,000), five intermediate chains (IC1-IC5; M, 85,000-57,000), and at least six light chains (LC1-LC6; M, 22,000-6,000). The masses obtained directly for the α and β chains were in agreement with those determined by the summation of the masses of the fragments generated by vanadate-dependent photolysis (V1 cleavage).2 The composition of the 19 S complex was confirmed following further purification of the sucrose gradient-purified 19 S particle by DEAE-Sepacel chromatography and also by direct purification of the dynein from the high salt extract by chromatography on a Sepharose CL-6B column (result not shown).

The intermediate and low molecular weight chains reported in Table II is a minimal estimate. Further studies of these chains by two-dimensional gel electrophoresis will be necessary to determine their precise number.

Using the values listed in Table II for the masses and stoichiometries of the various component polypeptides (and assuming that each light chain is present at one copy per dynein), we calculate a total mass for the trout outer arm dynein of 1.25 × 10^6 daltons. This value is very similar to the masses obtained previously for the two-headed dynein particles from Chlamydomonas (Witman et al., 1983) and sea urchin (Gibbons and Gibbons, 1987) by scanning transmission electron microscopy, and also to the total mass of the Chlamydomonas α-β dimer calculated from electrophoretic analysis of the individual components (King and Witman, 1987, 1989).

Negative stain electron microscopy revealed that trout sperm 19 S dynein is two-headed, as predicted by its sedimentation coefficient and polypeptide composition. Thus trout sperm outer arm dynein appears morphologically and structurally similar to the 21 S dynein of sea urchin sperm and comparable to the α-β dimer from Chlamydomonas.

A detailed comparison between the outer arm dynein from trout sperm and those from other vertebrates is more difficult due to the incomplete characterization of the latter; this includes uncertainty as to the origin of the dynein particles isolated. Three mammalian axonemal outer arm dyneins have been studied: two were derived from cilia, and the third from bull sperm flagella (for review, see Witman, 1989a).

Hastie et al. (1986) removed the outer arm and some inner arms of porcine tracheal ciliary axonemes by incubation at high ionic strength. Analysis of the extract by sucrose density gradient centrifugation revealed two peaks of MgATPase activity: the major peak migrated at 19 S and was associated with two heavy and two intermediate chains; the minor peak sedimented at 12 S and was composed of one heavy and two light polypeptides. Scanning transmission electron microscopic analysis indicated that the 19 S peak contained mainly a two-headed particle of 1.2 × 10^6 daltons (Hastie et al., 1988).

Sucrose gradient analysis of a low ionic strength dialysate of bovine tracheal and oviductal cilia revealed two peaks of ATPase activity: a minor one sedimenting at 18 S and containing two heavy chains, and a major one sedimenting at 12 S and containing the same two heavy chains as well as a third heavy chain (Pallini et al., 1982).

Two particles with ATPase activity were obtained from demembranated bull sperm after an overnight dialysis against a low ionic strength buffered solution. One migrated at 11–12 S and contained a single heavy chain; the other migrated at 19 S and contained two heavy chains and several intermediate chains (Pallini et al., 1982; Belles-Iles et al., 1986). This 19 S particle appeared as two globular domains joined together by a common base and had a mass reported to be 1.6 × 10^6 daltons; the 11–12 S dynein was a single globular particle with a mass of 400,000 daltons (Marchese-Ragona et al., 1987).

Therefore, in both mammalian ciliary and sperm axonemes, 19 S dynein particles are found that, based on our results with trout sperm axonemes, are likely to correspond to the outer arm dynein. The origin of the 11–12 S particle in the mammalian species is not yet clear, but it could be either a degradation product of the 19 S particle or derive from the inner arms as do the 10.5–11 S dyneins from Chlamydomonas (Piperno and Luck, 1981; Pfister et al., 1982). It is important to note also that the bovine brain cytoplasmic dynein (referred to previously as MAP 1C) is a 19 S particle composed of two globular domains connected by stems to a common base, with a total mass of 1.2 × 10^6 daltons (Vallee et al., 1988).

Most of the enzymatic properties exhibited by the trout sperm 19 S dynein particle are similar to those determined previously for the outer arm dyneins from Chlamydomonas (King and Witman, 1989; Pfister et al., 1982; Piperno and Luck, 1979), Tetrahymena (Johnson, 1983), sea urchin (Bell et al., 1982), and bull sperm (Belles-Iles et al., 1986). The K_m value is close to those reported for the 21 S sea urchin outer arm dynein (Gibbons and Frank, 1979; Bell et al., 1982) and bull sperm dyneins (Belles-Iles et al., 1986), but more than 10 times higher than those obtained for Chlamydomonas (Pfister et al., 1984, 1985) or Tetrahymena (Shimizu, 1987).

Unlike the dyneins from Chlamydomonas, Tetrahymena, and sea urchin, the trout dynein was not strongly inhibited by vanadate; only 65% of the ATPase activity was sensitive to vanadate even at high concentrations. Such insensitivity has been reported previously for bull sperm dyneins (Belles-Iles et al., 1986) and also for the bovine brain cytoplasmic dynein (Paschal et al., 1987b; Shpetner et al., 1988), suggesting that this may be a distinctive property of vertebrate dyneins.

However, it is interesting to note that 50% inhibition of trout dynein ATPase activity occurred at low concentrations of vanadate (~10 μM). More data will be needed to clarify this
phenomenon as the degree of vanadate inhibition of dynein ATPase activity may vary with the assay conditions (Gibbons et al., 1985).

In conclusion, we have described here a vertebrate model system in which to study the structure and function of flagellar outer arm dynein. Trout spermatozoa offer a number of distinct advantages over other potential systems. First, the sperm may be obtained in very large quantity at the peak of the spawning season, and the dyneins extracted and purified in biochemically manipulable amounts. Second, these sperm share some important physiological properties with mammalian sperm: for instance, the initiation of motility in both mammalian and trout sperm involves CAMP and Ca2+ (Garbers and Kopf, 1980), and the regulation of gene expression during spermiogenesis. Perhaps, also the regulation of gene expression during spermiogenesis.

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