Structure of the Dynein-1 Outer Arm in Sea Urchin Sperm Flagella

I. ANALYSIS BY SEPARATION OF SUBUNITS*

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The 21 S latent activity dynein-1 (LAD-1) extracted from flagellar axonemes by a 0.6 M NaCl/MgCl2 solution at pH 7.0 is dissociated into smaller particles upon dialysis against 5 mM imidazole/HCl, pH 7.0, 0.5 mM EDTA, 7 mM 2-mercaptoethanol. Zonal centrifugation separates this dissociated dynein-1 into two major fractions: one, containing the Aβ heavy chain and the intermediate chains 1, 2, 3, sedimenting at about 10 S, and the other, containing predominantly aggregates of the Aα heavy chain sedimenting over the range 12 to 30 S. Zonal centrifugation of the Aα/intermediate chain (Aα/IC) fraction after dialysis back into 0.6 M NaCl/MgCl2 solution, shows that the Aβ heavy chain and intermediate chain 1 now co-sediment as a distinct complex, forming a peak at about 14 S, whereas intermediate chains 2 and 3 constitute a separate complex sedimenting in two peaks of 10 S and 15 to 17 S. The Aα chain fraction, after dialysis back into 0.6 M NaCl/MgCl2 solution, sediments at about 12 S. Although neither the Aα/IC fraction nor the Aα chain fraction appears able to reform a 21 S particle alone, dialysis of the pooled Aα and Aα/IC fractions back into 0.6 M NaCl/MgCl2 solution causes a partial re-formation of a 21 S particle which contains the Aα and Aβ chains and intermediate chains 1, 2, and 3 in approximately the same proportions as found in the original LAD-1 particle. Neither the Aα fraction, the Aα/IC fraction, nor a mixture of the two appears able to restore the beat frequency of outer arm-depleted sperm flagella. However, a 1:1 mixture of the Aα chain and Aα/IC fractions, and (less effectively) the Aα/IC fraction alone, appear to block the manifestation of frequency restoration of subsequently added LAD-1 in a way that is reversed gradually over 2 to 8 min in the presence of ATP. The different enzymatic properties of the ATPases in the Aα and Aα/IC fractions suggest that the outer arms of sea urchin sperm flagella each contain two distinct dynein ATPases.

The normal oscillatory beating of cilia and flagella is widely thought to be the result of coordinated sliding movements between the doublet microtubules of the axoneme that lead to the formation of bends at the proximal end of the flagellum and their propagation to the tip (1–3). A variety of evidence indicates that this relative sliding of the doublet tubules is largely the result of a shear stress produced by the dynein arms through a mechanochemical cycle involving changes in angular orientation of the arms and their cyclic detachment and reattachment to the B-tubule concomitant with the binding and hydrolysis of molecules of ATP (4–7).

Although the overall action of the dynein arms in the generation of shear stress between doublet tubules seems clear, the details of the mechanochemical cycle are largely speculative, partly because so little is known about the functional substructure within the dynein arms. For some time, it has been evident from electron microscopy that the outer and inner arms have distinct structures, with the former being longer and more complex (8, 9). More recent studies have attributed to the outer arm a variety of structures, including one resembling a bent hammer (9, 10) and one in the shape of a Y (11, 12), but all seem to agree that both the inner and outer arms are to some extent hooked in appearance when viewed in axonemal cross-sections. Warner et al. (13) have shown by the negative staining technique that the dynein arms of Tetrahymena cilia appear, in side view, to be composed of three or four uniform, globular subunits, although the relationship of these subunits to the hooked profile in cross-sections is at present uncertain.

Recent evidence from high resolution Na dodecyl SO4-polyacrylamide gel electrophoresis has shown that the complex electron microscopic structure of the dynein arms is paralleled by a correspondingly complex polypeptide composition (14). For instance, an 18 S form of dynein which is solubilized from Chlamydomonas flagellar axonemes by high salt concentrations has been found by Piperno and Luck (15) to contain up to 13 different polypeptides with molecular weights between 330,000 and less than 15,000. Treatment of sea urchin sperm flagellar axonemes with an 0.6 M NaCl extraction medium solubilizes a 21 S form of dynein 1, LAD-1, that is thought to represent most or all of the outer arm on the doublet tubules of the axoneme (16). Purification of LAD-1 by sucrose density gradient sedimentation and subsequent electrophoresis in the presence of Na dodecyl SO4 has revealed that the 21 S particle contains at least nine different polypeptides: two heavy chains, designated Aα and Aβ, with apparent molecular weights of 330,000 and 320,000, three intermediate chains with apparent molecular weights of 122,000, 90,000, and 76,000, and at least four light chains with apparent molecular weights between 24,000 and 14,000 (17). So far, however, there is relatively little data on the stoichiometry of these subunits (16, 17) and virtually none on their substructural arrangement within the outer arm.

A more complete understanding of the role of the dynein arms in flagellar movement will require a detailed analysis of their structural and functional organization derived from a comprehensive characterization of their component parts. In

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1 The abbreviations used are: LAD-1, latent activity dynein-1; IC, intermediate chain; EGTA, ethylene glycol bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid; IC2/IC3, intermediate chain 2/intermediate chain 3 complex.
Polypeptide Subfractions of Dynein-1

Preparation of LAD-1 and its Subunits—Sperm were obtained from the sea urchin *Tripneustes gratilla* by the injection of 0.5 M KCl into the body cavity. Flagellar axonemes were isolated from the sperm as previously reported (16) or by a modified sucrose procedure that avoids the use of Triton X-100 (18). Crude LAD-1 was extracted by suspending the axonemes, usually at 3 mg of axonomal protein/ml, in 0.6 M NaCl/Mg2+ solution (0.6 M NaCl, 4 mM MgSO4, 0.1 mM EDTA, 1 mM dithiothreitol, 7 mM 2-mercaptoethanol, 5 mM imidazole/HCl, pH 7.0) at 4°C for 10 min as previously reported (16). Whenever necessary, the enzyme was concentrated by ultrafiltration under N2 with an Amicon UM20 membrane.

The 21 S LAD-1 was dissociated into subunits by dialysis against a low ionic strength solution containing 5 mM imidazole/HCl, pH 7.0, 0.5 mM EDTA, and 14 mM 2-mercaptoethanol (IEM buffer) for 24 h at 4°C with at least two changes of 100 volumes each of the IEM buffer. Table I shows typical recoveries of protein and ATPase activity at various stages.

Zonal centrifugation was carried out on 11-ml to 20% w/v sucrose density gradients prepared in an appropriate buffer. After sedimentation in an SW 41 rotor (Beckman Instruments, Spinco Division) at 35,000 rpm for 15 h at 4°C, each gradient was separated into about 20 fractions of equal volume by carefully lowering a glass capillary densest fraction first, with a peristaltic pump. The fractions are collected by stopping the pump when a given amount of liquid has been collected, the stopcock opened, and the liquid allowed to flow. The fractions were then concentrated under N2 using an Amicon UM20 membrane.

**Table I**

| Stage | Volume [mg] | Total protein [mg] | Total ATPase activity [units] | Specific activity [µmol P_Blue R-250/mg/min] |
|-------|------------|--------------------|-------------------------------|---------------------------------------------|
| 1     | 185        | 0.15               | 28 (100%)                     | 7.4 (100%)                                  |
| 2     | 2          | 0.25               | 23 (82%)                      | 4.4 (60%)                                   |
| 3     | 3          | 0.21               | 19 (68%)                      | 13.5 (182%)                                 |

Results and Reagents—Imidazole was obtained from Sigma and recrystallized twice from 80% v/v ethanol containing 1 mM EDTA before use. Tris(hydroxymethyl)aminomethane was obtained from Sigma and, for the purpose of ATPase assays and sperm reactivation, was recrystallized first from 1 mM EDTA and then from 80% v/v methanol before use. For electrophoresis, Tris was used without recrystallization. Pyruvate kinase, lactate dehydrogenase, bovine serum albumin, Coomassie Brilliant Blue R-250, and "Na lauryl SO," were all purchased from Boehringer Mannheim Biochemicals. Acrylamide, N,N'-methylenebisacrylamide and ammonium peroxodisulfate were from Eastman Kodak Co. All other chemicals and reagents were of analytical grade. Distilled, deionized water was used throughout.

**Results**

Improvements in Na dodecyl SO4 polyacrylamide gel electrophoresis technique have made it possible to study in greater detail the axonomal proteins of sperm flagella of the sea urchin *Tripneustes gratilla*. The inset in Fig. 1 shows the high molecular weight polypeptide composition of the whole axonemes.
the presence of Na dodecyl SO₄. The sample, 1.0 mg of LAD-1 (0.29 pmol of P₆₈ μmol) was loaded on a 5 to 30% sucrose density gradient prepared in 0.6 M NaCl/Mg²⁺ solution, centrifuged as described, and then separated into 20 fractions. Fifty μl of each fraction were heated to 100 °C for 5 min with 1% (w/v) Na dodecyl SO₄, 0.15 M 2-mercaptoethanol, and then electrophoresed on a separate track of the 3 to 8% polyacrylamide slab gel in the presence of 0.1% Na dodecyl SO₄. Other portions of each gradient fraction were used for assay of the latent and Triton-activated ATPase activity; recovery of neme: In addition to the previously identified C, A, and Aβ heavy chains (17), there are three polypeptides in whole axonemes that migrate more slowly than the C chain and these will be named sky chains 1, 2, and 3. Upon extraction of axonemes with 0.6 M NaCl/Mg²⁺ solution, sky chains 2 and 3 are solubilized, along with the A, and the Aβ chains and some of the C chain material, while sky chain 1 remains bound to the axoneme. When this 0.6 M NaCl/Mg²⁺ extract (crude LAD-1) is subsequently sedimented through a 5 to 20% sucrose density gradient made up in the same buffer, the major protein peak sediments at approximately 21 S (16). Electrophoretic analysis of the fractions from such a sucrose density gradient (Fig. 1) shows that the 21 S particle contains two species of heavy chain, A, and Aβ; three species of intermediate chain, numbered 1, 2, and 3, and at least four distinct light chains (17). Both the latent ATPase activity and that activated by treatment with Triton X-100 (16) parallel the distribution of the 21 S LAD-1 polypeptides (Fig. 1).

Behavior of LAD-1 at Low Salt Concentration—Preliminary experiments showed that the sedimentation coefficient of LAD-1 increased when the salt concentration was lowered, to about 25 S in 0.1 M NaCl and to 30 to 40 S in 10 mM NaCl. Partial dissociation of the LAD-1 particle occurred at this lower salt concentration, with peak of the Aβ chain sedimenting more slowly than that of the A, chain.

Dialysis of crude LAD-1 against the low salt IEM buffer causes a complete dissociation of the 21 S particle, and fractions containing the separated A, and Aβ heavy chains can then be obtained by sedimentation through a sucrose density gradient made up in the same solution. Under these conditions, the Aβ chain and the three intermediate chains appear to sediment approximately together as a fairly compact peak at a position corresponding to a sedimentation coefficient of 8 to 10 S (Fig. 2). However, examination of many preparations indicates that the distribution of intermediate chains 2 and 3 is reproducibly broader than that of the intermediate chain 1 which parallels closely that of the Aβ chain. The A, chain is largely aggregated and sediments over a broad region of the gradient, corresponding to about 12 to 30 S. The peak of ATPase activity usually coincides with the peak of the A, and the three intermediate chains, and there appears to be little ATPase activity associated with the fractions containing the A, chains under these conditions. Combining samples from appropriate regions of such gradients yields two fractions: one (comprising fractions 12 to 19) designated the A, chain fraction contains the A, chain, as well as some sky chain and C chain; while the second (comprising fractions 7 and 8), designated the Aβ/IC fraction, contains the Aβ chain and intermediate chains 1, 2, and 3, along with small amounts of polypeptides not derived from the 21 S LAD-1 particle.

Re-formation of the 21 S Particle—Experiments have been performed to determine whether the 21 S LAD-1 particles that have been dissociated by dialysis at low ionic strength are able to re-form if the salt concentration is increased. When either the A, or the Aβ/IC fraction is dialyzed separately back into 0.6 M NaCl/Mg²⁺ solution, essentially no re-formation of the 21 S particle occurs. Density gradient centrifugation of the redialyzed A, chain fraction shows a more compact peak (Fig. 3a) than was seen in low salt solution (Fig. 2), with an approximate sedimentation coefficient of 10 to 12 S. ATPase activity is now present and parallels the distribution of the A, chain. The corresponding redialyzed Aβ/IC fraction sediments at 10 to 14 S, with the distribution of intermediate chain 1 closely paralleling that of the Aβ chain in fractions 8 to 11 (Fig. 3b), while intermediate chains 2 and 3 are distributed more broadly across the gradient from fractions 5 to 13, forming two peaks on either side of the Aβ peak, the slower at approximately 9 to 10 S and the faster at about 15 to 17 S. The distribution of ATPase activity parallels approximately that of the Aβ chain and intermediate chain 1.

When pooled fractions containing the separated A, and Aβ chains are combined and dialyzed against 0.6 M NaCl/Mg²⁺ solution for 24 h, their distribution after density gradient sedimentation (Fig. 3c) shows partial re-formation of a 21 S peak (fractions 12, 13) that contains approximately equal amounts of the A, and the Aβ chains. The remaining Aβ chain
and some A<sub>c</sub> chain sediment in the 10 to 14 S region (fractions 8 to 10). The proportion of re-formed 21 S particles, determined by the relative electrophoretic band intensities of the A<sub>c</sub> chain in the 10 to 14 S and the 21 S peaks, has varied between preparations, with more than 50% re-formation occurring in the best cases. Assays of ATPase activity reveal two peaks, one in the region of the 10 to 14 S peak and a second at about 21 S. The material in the re-formed 21 S peak usually shows a higher degree of Triton-activation (2- to 3-fold) than that in the 10 to 14 S peak (less than 2-fold). The distribution of the intermediate chains in such recombined preparations is shown more clearly in the more heavily loaded gel shown in Fig. 3d. Intermediate chain 1 shows two peaks, one of which co-sediments with the A<sub>c</sub> chain in the 10 to 14 S peak, while the second co-sediments with the two A chains in the 21 S peak. The distribution of intermediate chains 2 and 3 also shows two peaks, one co-sedimenting with the re-formed 21 S peak, the other sedimenting at about 7 to 10 S.

**Sedimentation Properties of A<sub>d</sub>/IC Fraction**—Analytical ultracentrifugation of the A<sub>d</sub>/IC fraction in IEM buffer after dialysis to remove sucrose shows a symmetrical peak with a sedimentation coefficient (s<sub>20,w</sub>) of 9.3 ± 0.5 S (Table II). Small amounts of contaminant material form a "skirt" at the leading and the trailing edges of the main peak. The A<sub>d</sub>/IC fraction that has been dialyzed back into the 0.6 M NaCl/Mg<sup>2+</sup> solution prior to sedimentation usually shows a less symmetrical distribution, with a main peak with a s<sub>20,w</sub> of 14.2 ± 0.3 S and a fairly substantial trailing shoulder.

Analytical ultracentrifugation of the pooled A<sub>c</sub> fraction has so far been unsatisfactory because of persistent aggregation of the A<sub>c</sub> chain.

**Enzymatic Properties**—In order to minimize the aggregation of the A<sub>c</sub> chain, the separated A<sub>c</sub> or A<sub>d</sub>/IC fractions were usually dialyzed back into 0.6 M NaCl/Mg<sup>2+</sup> solution prior to study of their enzymatic properties. Double reciprocal plots of ATPase activity against ATP concentration for intact LAD-1 have given a K<sub>m</sub> of 1.3 ± 0.6 μM (Table II). In some preparations, a second kinetic component with a higher apparent K<sub>m</sub> of 12 to 24 μM was observed; this is probably attributable to contamination with a small percentage of activated dynein-1, for treatment of LAD-1 with Triton X-100 causes an increase in the K<sub>m</sub> to 45 ± 9 μM along with an approximately 10-fold increase in V<sub>max</sub>.

The specific MgATPase activity of the A<sub>c</sub> chain fraction depended on the presence or absence of MgSO<sub>4</sub> during the dialysis back into 0.6 M NaCl buffer. After dialysis into 0.6 M NaCl/Mg<sup>2+</sup> solution, an average specific MgATPase activity of 0.24 ± 0.12 μmol of P<sub>i</sub> mg<sup>-1</sup> min<sup>-1</sup> was obtained, whereas after dialysis in the absence of MgSO<sub>4</sub>, the activity averaged only 0.06 ± 0.01 μmol P<sub>i</sub> mg<sup>-1</sup> min<sup>-1</sup> (Table I). These data suggest that the ATPase activity of the A<sub>c</sub> chain fraction is labile in the absence of Mg<sup>2+</sup>. The specific MgATPase activity of the A<sub>d</sub>/IC fraction, on the other hand, was independent of the presence or absence of MgSO<sub>4</sub> during dialysis, and an average value of 0.7 ± 0.3 μmol P<sub>i</sub> mg<sup>-1</sup> min<sup>-1</sup> was found in both cases. The ATPase activities of both the A<sub>c</sub> fraction (dialyzed into 0.6 M NaCl/Mg<sup>2+</sup> solution), and the A<sub>d</sub>/IC fraction increased 1.5- to 2-fold upon treatment with Triton X-100 prior to the ATPase assay (Fig. 3, a and b).

The divalent cation dependence and the effect of NaCl concentration on the ATPase activities of the separated A<sub>c</sub> and A<sub>d</sub>/IC fractions are qualitatively similar to those on the ATPase activity of LAD-1 (Fig. 4). In most cases, the ATPase activity in the presence of either Mg<sup>2+</sup> or Ca<sup>2+</sup> is approximately the same, and the activity increases with increasing concentrations of NaCl up to about 1 M. This increase in the Mg<sup>2+</sup>-activated activity is less pronounced when either the A<sub>d</sub>/IC fraction or LAD-1 have been treated with 0.1% Triton X-100 prior to the assay. The activity of the A<sub>c</sub> fraction is affected less by increasing concentrations of NaCl, and treatment of the A<sub>d</sub> fractions with Triton X-100 showed little effect under these conditions.

The dependence of the Ca<sup>2+</sup>-activated ATPase activity of LAD-1 and the A<sub>c</sub> and A<sub>d</sub>/IC fractions upon NaCl concentration is generally similar to that of the MgATPase, except that treatment of LAD-1 with Triton X-100 tends to decrease the Ca<sup>2+</sup>-activated ATPase activity observed at high concentrations of NaCl.

**Effect of Triton X-100 on the Sedimentation of LAD-1**—Exposure of LAD-1 in 0.6 M NaCl/Mg<sup>2+</sup> solution to 0.1% w/v Triton X-100 for 10 min at room temperature (23 °C), with subsequent sedimentation at 4 °C in sucrose gradients made up in the same solvent causes substantial changes in the sedimentation pattern of ATPase activity, as described earlier by Gibbons and Fronk (16). Electrophoretic analysis of such gradients (Fig. 5a) shows that most of the LAD-1 polypeptides now sediment in the 10 to 14 S region. The A<sub>c</sub> and A<sub>d</sub> heavy chains sediment as two partially resolved peaks, with the A<sub>c</sub> chain occurring primarily in fractions 5 to 8 and the A<sub>d</sub> chain in fractions 7 to 10, corresponding to approximately 8 to 12 S and 10 to 14 S, respectively. Intermediate chain 1 co-sediments with the A<sub>c</sub> chain. Intermediate chains 2 and 3 peak in

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**FIG. 2.** Same as for Fig. 1, for a sample of LAD-1 (1.2 mg; 0.75 amol of P<sub>i</sub> min<sup>-1</sup>) that had been dialyzed against IEM solution for 24 h and subsequently sedimented through a 5 to 20% sucrose density gradient prepared in IEM solution. Fifty μl of each fraction were loaded on this slab gel. The latent ATPase activities of the individual fractions are shown superimposed on gel. Recovery of latent ATPase was 0.73 μmol of P<sub>i</sub> min<sup>-1</sup> (81%). Fractions 12 to 19 from this gradient were combined as the A<sub>c</sub> fraction and fractions 7 and 8 were combined as the A<sub>d</sub>/IC fraction.
Polypeptide Subfractions of Dynein-1

Fig. 3. Same as Fig. 1, but showing the electrophoretic pattern after resedimentation on a sucrose density gradient in 0.6 M NaCl/Mg\(^{2+}\) solution of (a) the A, chain fraction (0.05 mg sample), (b) the A,\%/IC fraction (0.40 mg sample), and (c and d) (0.15 mg and 0.40 mg, respectively) the pooled A, and A,\%/IC fractions. These A chain fractions were obtained from a density gradient in IEM solution as shown in Fig. 2, and were dialyzed back into 0.6 M NaCl/Mg\(^{2+}\) solution prior to loading on the second density gradient. The A,\%/IC fraction (b) was contaminated with a small amount of A, chain which sediments as an (A,\%/A,) complex peaking in fractions 12 and 13. d is the same type of sample as c, but shows a different preparation of pooled A, and A,\%/IC fractions. In this experiment, a smaller proportion of the A heavy chains reassembled into a 21 S particle (as compared with c), but the higher protein load enables the distribution of the intermediate chains to be seen more clearly. Latent ATPase activities of 0.04 μmol of P\(_i\) min\(^{-1}\) and 0.14 μmol of P\(_i\) min\(^{-1}\) were recovered from gradients c and d, respectively. These activities correspond respectively to 100% and 135% recovery relative to an equal weight of fresh LAD-1 (0.15 mg for gradient c, 0.40 mg for gradient d). High ATPase recoveries are caused by the incomplete reconstitution of low latent activity 21 S particles. Only the gel lanes corresponding to fractions 2 to 16 are shown in these figures. One hundred fifty μl of each sample were loaded in a and c, and ATPase activity are as in Fig. 1.

TABLE II

Properties of the A, fraction, the A,\%/IC fraction and LAD-1

The A, and A,\%/IC fractions, obtained by dialysis of LAD-1 against IEM solution and density gradient separation in the same medium, were dialyzed back into 0.6 M NaCl/Mg\(^{2+}\) solution for 24 h before ATPase activities were measured. The numbers in parentheses represent the number of experiments performed for that type of sample.

| LAD-1 | A, fraction | A,\%/IC fraction |
|-------|-------------|------------------|
| Latent | Triton-treated | Latent | Triton-treated |
| \(K_m\) (μM) | 1.3 ± 0.6 (5) | 45 ± 9 (6) | 7.3 ± 0.4 (2) | 2.8 ± 0.4 (3) |
| Specific activity (μmol P\(_i\) mg\(^{-1}\) min\(^{-1}\)) | 0.26 ± 0.04 (19) | 2.7 ± 0.4 (19) | 0.06 ± 001 (-Mg\(^{2+}\)) (4) | 0.7 ± 0.3 (6) |
| \(s_{0.1}\) (S) | 21.4 ± 0.6 (3) | 12.3 (1) | 9.3 ± 0.5 (IEM) (2) | 14.2 ± 0.3 (0.6 M NaCl/Mg\(^{2+}\)) (2) |

\(\ast\) Value for latent enzyme was measured using sucrose gradient-purified material.
\(\ast\) Value for Triton-treated enzyme used unpurified material and was not extrapolated to zero concentration.
\(\ast\) Material showed major 14 (S) peak, minor 12 S peak.
during assay. The A, and the Ap/IC fraction, after separation by phosphate was determined by the Fiske-SubbaRow procedure. Triton either 2 mM MgSO4 to the that the specific activity scale 

X-100 at 23 

X-100 treatment was done by incubating the sample with 0.1% Triton ananol, all dialyzed against 0.6 M NaCl, density gradient sedimentation in IEM solution and the LAD-I were and the Ap/IC fraction, as a function of the ATPase activity is in fraction 7 where both the A, and the A, fraction were electrophoresed. Visual comparison of band intensities suggests that the specific activity of fractions containing predominantly the A, chain is comparable to those containing predominantly the A, and intermediate chains under these conditions, which stands in contrast to the much lower ATPase activity associated with the A, chain after low salt dialysis (Fig. 2).

If Triton-treated LAD-1 is sedimented into a sucrose density gradient prepared in 0.6 M NaCl/Mg2+ solution containing no Triton X-100, a partial re-formation of a 21 S peak containing the A, and A, heavy chains and intermediate chains 1, 2, and 3 occurs (Fig. 5b). The efficiency of the reformation of the 21 S particle, judged in terms of the amount of A, and A, chains sedimenting at a velocity of 21 S, appears to be greater than 50%.

**Test of Functional Activity of A Chain Fractions using Outer Arm-depleted Axonemes—Assessment of the functional capability of LAD-1 obtained from axonemes isolated from the new sucrose procedure to restore the beat frequency of outer arm-depleted sperm (18), showed that most preparations raised the average beat frequency from about 15 Hz to 24 Hz, in agreement with our earlier report using LAD-1 from axonemes prepared by the Triton procedure (16). However, some preparations of LAD-1 made by the new procedure raised the beat frequency to 28 to 29 Hz, which almost equals that of standard reactivated sperm.

The pooled A, fractions and the pooled Ap/IC fractions from sucrose density gradients, either still in the IEM sucrose solution or dialyzed back into 0.6 M NaCl/Mg2+ solution, were tested for their effect on the beat frequency of outer arm-depleted sperm. Incubation of sperm samples with the A, fraction, the Ap/IC fraction, or with a 1:1 mixture of these fractions, had no effect on the beat frequency when the sperm were subsequently reactivated with 1 mM ATP.

Preincubation of outer arm-depleted sperm with a 1:1 mixture of the separated A, and Ap/IC fractions that had been dialyzed back into 0.6 M NaCl/Mg2+ solution (designated A,-Ap/IC mixture) resulted in a substantial inhibition of the frequency increase obtained upon incubation with intact LAD-1. The beat frequency of such sperm observed immediately after addition of ATP (~15 Hz) was nearly the same as that of the original outer arm-depleted sperm to which no LAD-1 has been added. However, this inhibition was partially reversed in the presence of ATP; their beat frequency rose quite rapidly with time after addition of the ATP, until by 6 to 8 min, it had increased to about 22 Hz. A similar result was observed when outer arm-depleted sperm were preincubated with the Ap/IC fraction before incubation with intact LAD-1, but in this case the increase in beat frequency following the addition of ATP occurred more rapidly so that the beat frequencies were as high as 17 to 18 Hz when initially measured and rose to 22 Hz within 2 min. Preincubation with the A, fraction alone, on the other hand, appeared to have no effect upon the restoration of beat frequency by subsequently added LAD-1. Samples of A,-Ap/IC mixture that had been heated at 50 °C for 5 min before assay no longer blocked the restoration of beat frequency by LAD-1. For reasons that are as yet unexplained, the restoration of beat frequency by LAD-1 added after preincubation with the A,-Ap/IC mixture or the Ap/IC fraction proceeds only to a value of ~ 22 Hz, significantly lower than the value of ~ 25 Hz obtained with LAD-1 alone under otherwise identical conditions.

**DISCUSSION**

The nature of the interactions between the two species of heavy chain, A, and A, in the 21 S LAD-1 particle has been partially elucidated from their changes in sedimentation be-
behavior as the composition of the medium is changed. In the 0.6 M NaCl/Mg²⁺ extraction solution which maintains the functional capability of LAD-1 and constitutes "standard conditions" for its physicochemical investigation, the two heavy chains co-sediment in apparently equimolar quantities as a compact peak at 21 S (16, 17). In the low ionic strength IEM solution, however, the Aα and Aβ chains are dissociated, suggesting that the interactions between them are predominantly hydrophobic (26). This is supported by the similar dissociation resulting from addition of Triton X-100 to the 21 S LAD-1 particle in 0.6 M NaCl/Mg²⁺ solution, for such nonionic detergents mediate the exposure of hydrophobic surfaces to the external medium (27).

Conversely, the aggregation of the Aα chain at low ionic strength, and the dispersion of these aggregates upon restoring to 0.6 M NaCl/Mg²⁺ solution (Figs. 2 and 3a), suggest that the Aα polypeptide chain contains one or more regions of ionic character that tend to associate in the absence of solution ions, but that are dissociated by ionic competition in sufficiently high concentrations of salt. This hypothesis is supported by the observation that at pH 8, even at low ionic strength, the Aα chains are much less aggregated than at pH 7, presumably because they have a greater net negative charge at the higher pH (data not shown). The location of this ionic region on the Aα chain is unknown, but since the outer arms are solubilized by 0.6 M NaCl, it may be the same region as that involved in the salt-sensitive binding of the 21 S LAD-1 particle to the A tubule of doublet to form the outer dynein arm (28). If this is the case, then in the absence of A-tubule binding sites, the Aα chains might well interact with each other nonspecifically, which would explain the heterogeneous aggregates of the solubilized Aα chain at low ionic strength (Fig. 2).

The isolated Aα/IC fraction in IEM buffer sediments in the analytical ultracentrifuge with a sedimentation coefficient of 9.3 S, while its sedimentation coefficient increases to about 14.3 S after it is dialyzed back into 0.6 M NaCl/Mg²⁺ solution. While it cannot be completely excluded that this change in sedimentation coefficient is caused by a secondary charge effect at the lower salt concentration (29), it seems more likely to be a result of either a major conformational change in the Aα chain, or a monomer-dimer transition. Sucrose density gradient centrifugation of the Aα/IC fraction indicates that the sedimentation of intermediate chain 1 closely parallels that of the Aα chain under all conditions examined, suggesting that the Aα chain and intermediate chain 1 occur as a complex (Aα/IC1) that is maintained by relatively strong interactions over a wide range of salt concentration. Intermediate chains 2 and 3 have likewise been found to co-sediment under all conditions examined, suggesting that they also occur as a relatively stable complex (IC2/IC3).

The Aα chain fraction displays no definite peak in either sucrose density gradient or analytical centrifugation at low ionic strength at pH 7.0. In 0.6 M NaCl/Mg²⁺ solution, it still displays no definite peak in the analytical ultracentrifuge, although when sedimented in sucrose density gradients, most of the protein in the Aα chain fraction sediments as a peak with a sedimentation coefficient of about 10 to 12 S. The only other condition in which the separated Aα chains sedimented as an apparently compact peak without signs of aggregation was when LAD-1 in 0.6 M NaCl/Mg²⁺ solution was treated with 0.1% Triton X-100 and sedimented in a sucrose density gradient prepared in the same solvent (Fig. 5), and under these conditions the Aα chains also sedimented at 11 S.

The reconstituted 21 S particles obtained by mixing the separated Aα and Aβ/IC fractions and dialyzing against 0.6 M NaCl/Mg²⁺ solution contain the A chains and intermediate chains in approximately the same proportions as occur in the original 21 S particle. The fact that no 21 S particle is formed by either the Aα or the Aα/IC fraction alone provides the first direct evidence that LAD-1 is a single species of particle containing both the Aα and Aβ chains in equal quantity, rather than a mixture of fortuitously co-sedimenting particles containing the Aα chain and Aβ chain separately. The apparent lack of interaction between the IC2/IC3 complex and either of the Aα chain or the Aα/IC1 complex separately suggests that the IC2/IC3 complex becomes incorporated into the 21 S particle within the interface region between the Aα and Aβ chains, and that its affinity for the separated Aα chain and Aα/IC1 complex is relatively low. It is notable that when the Aα/IC fraction is dialyzed alone back into 0.6 M NaCl/Mg²⁺ solution, the IC2/IC3 complex appears to aggregate with itself to give a peak of 15 to 17 S rather than associating with the Aα/IC1 complex (Fig. 3b).

The distribution of ATPase activity upon centrifugation of preparations of LAD-1 dissociated with Triton X-100 in 0.6 M NaCl/Mg²⁺ solution suggests that there are approximately equal amounts of ATPase activity associated with the Aα chain fractions (Fig. 5), and appears in conflict with the distribution of ATPase activity in centrifuged preparations of LAD-1 dissociated by dialysis against IEM solution, which show ATPase activity associated with the Aα/IC fraction but little or no ATPase activity in the Aα chain fraction. A possible explanation for this discrepancy is provided by the results showing that the dialysis-separated Aα chain fraction regains a substantial amount of ATPase activity when it is dialyzed against 0.6 M NaCl/Mg²⁺ solution, whereas its activity after dialysis against an 0.6 M NaCl solution lacking Mg²⁺ remains minimal (Table I). The simplest interpretation of the data is that the fractions containing the Aα, and the Aβ chains initially have comparable amounts of ATPase activity, but that the activity of the Aα chain fraction is labile in the absence of Mg²⁺ while that of the Aα chain fraction is not. A subsequent dialysis of the separated Aα chain fraction against 0.6 M NaCl/ Mg²⁺ then presumably enables a partial recovery of its ATPase activity. This interpretation is consistent with the results showing that the 21 S particles formed by recombination of the Aα and Aα/IC fractions in 0.6 M NaCl/Mg²⁺ solution regain some but not all of the original properties of LAD-1, including a somewhat greater degree of ATPase latency than is present in the Aα and Aα/IC fractions dialyzed separately against 0.6 M NaCl/Mg²⁺ solution. However, it has not yet been possible to obtain reconstituted 21 S particles with a full ATPase latency of approximately 10-fold, and possibly for this reason they have lacked the functional capability to restore the beat frequency of outer arm-depleted sperm flagella.

In intact axonemes in the absence of ATP, the outer arms are thought to form rigor cross-bridges between the doublet tubules, with one end of each arm being attached via an 0.6 M NaCl-sensitive binding to the A-tubule of a doublet and the other end of the arm attached via an ATP-sensitive binding to the B-tubule of the adjacent doublet (30). The fact that the arm-depleted flagella with the Aα/IC mixture are capable of blocking the restoration of beat frequency by subsequently added intact LAD-1 suggests that the reconstituted 21 S particles, although nonfunctional themselves, are nevertheless capable of binding to the A-tubule and/or B-tubule sites with an affinity comparable to that of the intact functional LAD-1 particles. Since this inhibition of the beat frequency increase is largely relieved within 6 to 8 min after addition of ATP, it seems likely that the strongest interaction of the nonfunctional reconstituted 21 S particles
occurs with ATP-sensitive sites on the B-tubule and that their affinity for the salt-sensitive site on the A-tubule is weak compared to that of native outer arms.

The similar inhibition of the beat frequency increase by preincubation of outer arm-depleted flagella with the separated A,IC fraction, together with rapid relief of this inhibition by ATP, suggests that the 14 S particles in this fraction also became attached to the doublet tubules by ATP-sensitive bonds, and block either the binding or the function of subsequently added intact LAD-1 particles. The failure of the A, fraction to show a similar blocking effect may be associated with its reduced ATPase activity after dialysis and sedimentation of the fractions by a single ATPase. Thus it appears that, as in the case of Chlamydomonas flagella, where genetic and biochemical evidence indicate the presence in the outer arms of two distinct ATPases consisting of nonoverlapping sets of polypeptides (15), the outer arms of the sea urchin sperm flagella likewise contain two distinct ATPases. The different enzymatic properties of the A,- and A,,-associated ATPases suggest that, rather than being equivalent to the two apparently identical ATPase-containing heads in myosin cross-bridges of muscle, the two ATPases in the flagellar outer arm serve functionally distinct purposes in motility. It is possible, for instance, that the two ATPases may control, at least partially, the direction and waveform of flagellar beating by causing tubule sliding in opposite directions, although in trypsin-treated axonemes disintegration by sliding occurs in one direction only (31). Alternatively, one of the ATPases may be the main power producer in a single direction of tubule sliding interaction while the second ATPase adds power to this interaction under specific conditions where it is required. In either case, it might be expected that the regulatory systems controlling the activity of the two ATPases would be distinct in their actions.

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