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

II. ANALYSIS BY PROTEOLYTIC CLEAVAGE*

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The structure of the 21 S latent activity dynein-1 (LAD-1) particle has been investigated by limited proteolytic cleavage with trypsin and with chymotrypsin. The A, and A, heavy polypeptide chains show different characteristic digestion patterns which remain essentially unchanged whether the chains are components of the 21 S LAD-1 particle or are in the form of separated fractions, although changes in their relative digestion rates upon separation suggest that the A, chain in the 21 S particle is partially protected from digestion by the presence of the A, chain and intermediate chains 2 and 3. The progressive digestion of the A chains and intermediate chains causes an eventual dissociation of the 21 S particle to smaller particles sedimenting in the range 10 to 14 S. Within this broad peak, the fragments from the A, chain peak in the 12 to 14 S region, while those from the A, chain peak in the 12 to 14 S region. Digestion of whole axonemes to a stage at which the A, chain is substantially digested but the A, chain remains mostly intact, enables a large amount of 21 S dynein-1 to be solubilized by 3 mM MgATP in 0.6 M NaCl-sensitive bond of the outer arm to the A-tubule is diminished substantially by the early stages of digestion of the A, chain.

In the preceding paper (1), we described the separation and partial characterization of polypeptide subunits from the 21 S form of dynein-1, LAD-1, from sperm flagella axonemes of the sea urchin *Tripneustes*. In the present study, we report the use of the technique of limited proteolytic digestion to probe further the structure and function of these subunits and of the intact 21 S dynein-1, as well as to investigate their interactions with axonemal outer doublet tubules. Initial application of this technique to the structure of dynein arms has been carried out by Ogawa (2) who showed that the ATPase site and the A-tubule binding site appear to be located on different parts of the arm, and by Takahashi and Tonomura (3) who have shown by electron microscopy that the arms appear to have a structural polarity, with a trypsin-sensitive A-tubule binding site at one end and an ATP-labile B-tubule binding site at the other. The more detailed results of the present study, describing the digestion of specified polypeptide chains, taken in conjunction with the results of the preceding paper, suggest a tentative model for the outer dynein arm and its interaction with the flagellar axoneme in sea urchin sperm.

**MATERIALS AND METHODS**

The preparation of LAD-1 and its subunits and the determination of ATPase activity and protein concentration were carried out as described in the preceding paper (1). The subunit fractions containing the A, and A, chains are referred to subsequently as the A, chain fraction and A,IC fraction, respectively. Flagellar axonemes from *Tripneustes* sperm were isolated in an isolation buffer containing 0.1 M NaCl, 4 mM MgSO4, 0.1 mM EDTA, 7 mM 2-mercaptoethanol, and 5 mM imidazole/HCl, pH 7.0, as described previously (4).

**Digestion with Trypsin and Chymotrypsin**—Crude LAD-1 in 0.6 M NaCl/Mg2+ solution (0.6 x NaCl, 4 mM MgSO4, 0.1 mM EDTA, 7 mM 2-mercaptoethanol, 1 mM dithiothreitol, 5 mM imidazole/HCl, pH 7.0) was digested with trypsin or chymotrypsin at room temperature (23-24℃). Digestion with trypsin was terminated by the addition of a 10-fold weight excess of soybean trypsin inhibitor; chymotryptic digestion was terminated by the addition of p-nitrophenylsulfonyl fluoride (5) to a final concentration of 5 mM from a stock solution of 0.1 M concentration in 2-isopropyl alcohol. All samples in any series of digestions were maintained at room temperature for the duration of the longest digestion regardless of their own digestion time because of the effects of room temperature on the latent and Triton-activated ATPase activities of LAD-1 (1). Tryptic and chymotryptic digestion of separated A, chain and A,IC fractions (after dialysis back into 0.6 M NaCl/Mg2+ solution) and of isolated flagellar axonemes were carried out essentially as described for LAD-1.

To facilitate the comparison of digests of different durations and protease/protein ratios, a parameter termed the digestion index is defined by the equation:

\[
\text{digestion index} = \frac{\text{protease concentration}}{\text{protein concentration}} \times \text{digestion time (min)} \times 10^6
\]

This value has proved a fairly reliable semiquantitative measure of the extent of digestion when the protease concentration is less than about 25 µg ml⁻¹. Beyond this concentration, autolysis of protease (6) makes the digestion index measurement less reliable. It should be emphasized that digestion indices used in measuring specific stages of digestion, especially the first appearance of new fragments, are approximate and tend to vary somewhat from one digest to another; for instance, Fig. 3 shows that A, chain fragments from LAD-1 do not appear until a digestion index of about 500, but, in Fig. 6b, small amounts of early A, fragments appear at a digestion index of only 240.

**Sequential Extraction of Digested Flagellar Axonemes**—The effects of digestion on the structure of flagellar axonemes were investigated by sequential extraction of the digested axonemes in buffers of different composition. To minimize the structural disruption caused by successive centrifugation and resuspension of pellets, this procedure was carried out at 0-4 °C as follows. After extraction in a given buffer, the digested axonemes were first centrifuged at 12,000 × g for 10 min and the supernatant was decanted and immediately further clarified by recentrifugation at 100,000 × g for 15 min. The 12,000 × g pellet was then gently resuspended in the next extraction medium and the sequence was repeated. A typical sequential extraction of digested axonemes is shown in Fig. 1. To maintain conditions as nearly equivalent as possible from one extraction to the next, pellets
Molecular Weights of Fragments—Polyacrylamide gel electrophoresis in the presence of Na dodecyl SO₄ was carried out as described in the preceding paper (1). The apparent molecular weights of polypeptide chains were measured by comparing their migration in such gels to that of standard polypeptides of known molecular weight including filamin, 250,000 (7); the myosin heavy chain, 200,000 (9); phosphorylase a, 92,500 (10); and dynein intermediate chains 1 to 3, 122,000, 90,000, and 74,000 (11). The standard polypeptides gave a reasonably linear dependence of log (molecular weight) on migration, and this line was extrapolated in order to estimate apparent molecular weights higher than 250,000. Care was taken to use only gels in which the polypeptides concerned had migrated more than one-quarter of the distance toward the bottom, because significant deviations from linearity tend to occur close to the top of the separating gel (9). On the basis of such an extrapolated calibration, the apparent molecular weights of the A₁, and A₁₀ chains were 330,000 and 320,000, respectively; these values are close to those reported previously using different gel buffer systems (12, 13). The various proteolytic fragments of the A₁, and A₁₀ chains will be identified by adding the apparent molecular weight (in thousands) as a superscript, together with the letter T or C, depending on whether the fragment is tryptic or chymotryptic. Thus, the tryptic fragment of the A₁₀ chain with an apparent molecular weight of 275,000 will be denoted A₁₀T₂₇₅.

Chemicals and Reagents—L-1-Tosylamido-2-phenylethyl chloromethyl ketone-treated trypsin (type THTFPC) and soybean trypsin inhibitor (type SI) were obtained from Worthington Biochemical Corp. Chymotrypsin (type I-S) and phenylmethylsulfonyl fluoride were obtained from Sigma Chemical Co. ATP was obtained from Boehringer Mannheim Biochemicals. Sources of all other chemicals and reagents were as reported in the preceding paper (1).

RESULTS
Effects of Digestion on LAD-1 and on Separated A Heavy Chain Fractions

Polypeptide Fragments—The progressive changes in the polypeptide composition of LAD-1 as a result of digestion with trypsin or chymotrypsin at various protease/substrate ratios are shown in Fig. 2. Initially, trypsin causes complete conversion of the A₁₀ chain to a single fragment migrating with apparent molecular weight of about 275,000, A₁₀T₂₇₅, at a digestion index of 500 to 1,500, and during this time, the A₁₀ chain does not appear to be greatly affected (Fig. 2). (The time taken to digest 50% of the A₁₀ chain is determinable with greater precision and occurs at a digestion index of about 100 to 150; see Table 1). During the formation of the A₁₀T₂₇₅ fragment, the band corresponding to A₁₀ chain becomes fuzzy and moves closer to that of the A₁₀ chain (this appearance is more obvious at protein loads lower than those shown in Fig. 2), which suggests that the formation of the A₁₀T₂₇₅ fragment is preceded by a less specific cleavage of small peptides from the A₁₀ chain.

![Flow diagram showing typical sequential extraction of digested axonemes.](http://www.jbc.org/)

**Fig. 1.** Flow diagram showing typical sequential extraction of digested axonemes. IB, isolation buffer: 0.6 M NaCl, 0.6 M NaCl/Mg⁺ solution. The wash supernatant stage was sometimes omitted.

**Fig. 2.** Polyacrylamide gel electrophoresis in the presence of Na dodecyl SO₄ showing digestion of crude LAD-1 by various concentrations of trypsin and chymotrypsin. T, trypsin; CT, chymotrypsin. Ratios above each group of five gel lanes represent the protease/LAD-1 mass concentration ratio for each group of digestions. The concentration of crude LAD-1 was 0.25 mg ml⁻¹ in all cases. Twelve and one-half μg of protein were loaded on each lane. Numbers above each lane are digestion index for that lane (see "Materials and Methods" for definition of digestion index). In all groups of digestions, the five digestion times were 0 min, 2 min, 5 min, 15 min, and 60 min. The scale to the right of the gel shows approximate apparent molecular weight. SC, sky chain; IC, intermediate chain; T, tubulin. Arrows indicate A₁₀T₂₇₅ fragment; diamonds (○) indicate A₁₀C₂₇₅ and A₁₀C₁₀₀ fragments (see text for details). The gel contained a 3 to 6% w/v gradient of acrylamide. In undigested samples, the band just below IC1 and that between IC3 and the tubulin doublet (T) correspond to polypeptides not forming part of the 21 S LAD-1 particle (see Fig. 1 of Ref. 1).
one or both ends of the \( A_\alpha \) chain. The absence of other high molecular weight fragments at this and earlier stages suggests that, after minor terminal peptide cleavage, the \( A_\alpha \) chain is converted directly to the \( A_{275\alpha} \) fragment, and that this fragment is then relatively resistant to further proteolysis. Conclusive evidence that the \( A_{275\alpha} \) fragment is derived from the \( A_\alpha \) chain is found in the formation of this fragment during the digestion of separated \( A_\alpha \) chain fraction by trypsin (Fig. 3a).

Digestion of LAD-1 beyond a digestion index of about 500 results in fragmentation of the \( A_\beta \) chain. At a digestion index of 6000, proteolysis of the \( A_\beta \) chain results in the appearance of at least four new bands between the remainder of the original \( A_\beta \) chain and the \( A_{275\beta} \) fragment (Fig. 2). These intermediate bands must correspond to multiple fragments of the \( A_\beta \) chains, since all of the \( A_\beta \) chains have been earlier converted to \( A_{275\beta} \). This conclusion is supported by digests of the separated \( A_{25/IC} \) fraction with trypsin, in which all those fragments identified as deriving from the \( A_\beta \) chain appear, but the \( A_{275\beta} \) fragment is absent (Fig. 3a).

Beyond a digestion index of about 6000, the origin of newly appearing bands in digests of LAD-1 is impossible to determine directly owing to the large number of bands generated by the proteolysis of the \( A_\beta \) chains. However, the origin of bands in digests of LAD-1 can be identified by comparison to the digestion patterns of the separated \( A_{25/IC} \) and \( A_\beta \) chain fractions (Fig. 3). At a digestion index of about \( 6 \times 10^4 \), two bands with apparent molecular weights of 200,000 and 115,000 appear (indicated by \( \diamond \) in Fig. 2) which may correspond to the two polypeptide components of molecular weights 190,000 and 135,000 described by Ogawa as comprising the tryptic fragment 1A from sperm flagellar dynein of the sea urchin, \textit{Anthocidaris crassispina} (14). Digestion of the separated \( A_{25/IC} \) and \( A_\beta \) chain fractions with trypsin indicates that the 115,000 molecular weight fragment is probably derived from the \( A_\beta \) chain (Fig. 3b). The origin of the 200,000 molecular weight fragment is not yet certain, but it may be derived from the \( A_\alpha \) chain (Fig. 3b). These fragments will be tentatively designated \( A_{115\beta} \) and \( A_{200\beta} \).

The action of chymotrypsin on LAD-1 in digests up to an index of 6000 is broadly similar to that of trypsin in as much as the \( A_\alpha \) chain is digested before the \( A_\beta \), and it gives rise to a major fragment, \( A_{275\alpha} \), that has the same apparent molecular weight as that of the \( A_{275\alpha} \) fragment occurring in tryptic digests (Fig. 2). However, trypsin and chymotrypsin follow different digestion sequences in the formation of the \( A_{275\alpha} \) fragment. Whereas tryptic digestion of LAD-1 or of the separated \( A_\alpha \) chain fraction gives rise directly to the \( A_{275\alpha} \) fragment, the corresponding chymotryptic digests pass through at least two intermediate fragments with molecular weights of about 325,000 and 285,000 before formation of the \( A_{275\alpha} \) fragment (Figs. 2 and 4). Chymotryptic digestion of the separated \( A_\alpha/IC \) fraction to an index of \( 10^4 \) or more gives rise to a fragment which co-electrophoreses with the \( A_{115\beta} \) fragment (data not shown).

Intermediate chain 1 is digested very rapidly by either trypsin or chymotrypsin (Fig. 2, Table I). Intermediate chains 2 and 3 are less susceptible to large scale proteolysis, although in the case of intermediate chain 2, chymotrypsin removes a very small portion by a digestion index of 200, as evidenced by the slight increase in mobility of this band at this time (Fig. 2).

Analysis of proteolysis of the light chains (11) is complicated because both the proteases used, as well as soy bean trypsin inhibitor, have molecular weights of 20,000 to 25,000 and migrate in the same region as the upper two light chains, 1 and 2. However, at trypsin/protein ratios of 1:2500 light chains 1 and 3 are attacked at about the same rate as intermediate

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**Table I**

<table>
<thead>
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<th>Chain</th>
<th>Axoneme</th>
<th>LAD-1</th>
<th>Isolated</th>
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<td>100-150</td>
<td>100-150</td>
<td>200</td>
</tr>
<tr>
<td>( A_\beta )</td>
<td>200-400</td>
<td>3000-5000</td>
<td>500</td>
</tr>
<tr>
<td>IC1</td>
<td>20-60</td>
<td>240-300</td>
<td></td>
</tr>
<tr>
<td>IC2</td>
<td>200-300</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IC3</td>
<td>750-1000</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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**Fig. 3.** Electrophoretic comparison between tryptic digests of isolated \( A_{25/IC} \) and \( A_\beta \) chains and tryptic digests of crude LAD-1. Numbers at side represent apparent molecular weights of fragments in thousands; numbers at bottom give digestion indices; a, \( A_\alpha \), 7.5 µg; LAD-1, 12.5 µg; \( A_\beta \), 7.5 µg; b, \( A_\alpha \), 12.5 µg; LAD-1, 29 µg; \( A_\beta \), 12.5 µg. Photographs are of midportions from 3 to 6% w/v polyacrylamide gradient gels (neither origin nor dye front is shown).

**Fig. 4.** Electrophoretic comparison between tryptic and chymotryptic digestion of separated \( A_\alpha \) chain. Numbers and symbols are as described in Fig. 3. Thirty-five µg of protein loaded on both lanes. T, trypsin; CT, chymotrypsin. Photograph is of midportion from 3 to 6% w/v polyacrylamide gradient gel.

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**TABLE I**

<table>
<thead>
<tr>
<th>Chain</th>
<th>Resistance</th>
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<td>IC1</td>
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</tr>
<tr>
<td>IC2</td>
<td>200-300</td>
</tr>
<tr>
<td>IC3</td>
<td>750-1000</td>
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</table>
chain 1, while light chains 2 and 4 are less susceptible to digestion (data not shown).

Sedimentation Behavior—The effect of digestion by trypsin on the physical integrity of the 21 S LAD-1 particle was investigated by zonal centrifugation on sucrose density gradients. Prior to digestion most of the A heavy chains and the intermediate chains sediment as a sharp single peak at about 21 S, while traces of these chains trail behind this peak and are augmented in the 12 to 14 S region by small amounts of the other heavy chains, forming a minor second peak (Fig. 5a) (11).

After digestion of the crude LAD-1 with trypsin to a digestion index of 240, the sucrose gradient sedimentation pattern is markedly altered (Fig. 5b). A peak of protein remains at about 21 S, but it now consists of the Aβ chain and the Aα subunit fragment. Intermediate chains 2 and 3 remain in the 21 S peak but intermediate chain 1 is absent. Much of the A heavy chain polypeptide has shifted from the 21 S peak to the 12 to 14 S peak of the gradient, and a significant amount of the Aβ and the Aα subunit fragment remains between the two peaks. However, whereas the two A polypeptides appear to co-sediment exactly in the 21 S peak, the peak of the Aβ chain in the 12 to 14 S region is displaced slightly toward the bottom of the gradient relative to that of the Aα subunit fragment. The bands migrating on the gel between the Aβ chain and the Aα subunit fragment in the 12 to 14 S peak are tryptic fragments of the Aα chain and those migrating just ahead of the Aα subunit fragment are fragments of both Aβ and Aα, caused by further tryptic digestion. These bands occur only in the 12 to 14 S peak.

After digestion to an index of about 1.2 x 10², there are no intact Aα and Aβ chains, and all polypeptide fragments within the apparent molecular weight range 100,000 to 300,000 sediment with a velocity of 12 to 14 S (Fig. 5c). Within this peak, there is a partial separation of two distinct groups of heavy chain fragments, with that including the Aα113T fragment sedimenting slightly faster than that containing the Aα290T fragment. Therefore, even after substantial proteolysis, the polypeptide fragments of the Aα and Aβ chains still constitute discrete particles that sediment at velocities close to those found after much less proteolysis (cf 12 to 14 S regions of Fig. 5, b and c).

ATPase Activity—Almost all of the ATPase activity in crude LAD-1 is associated with the 21 S LAD-1 particle (4, 11). Fig. 6 shows the effects of digestion with trypsin on the total amount of ATPase activity in preparations of LAD-1, both with and without treatment by 0.1% Triton X-100 subsequent to digestion and prior to assay. The untreated ATPase activity increases steadily with digestion, attaining a peak, at a digestion index of about 3 x 10², of more than 20 times the undigested activity. The Triton-treated ATPase activity of the digested LAD-1 remains almost constant with increasing digestion until it merges with the upward curve of the untreated ATPase activity at a digestion index of about 10,000. It attains a peak, approximately equal to that of the untreated ATPase activity, at a digestion index of 3 x 10². After further digestion, both the untreated and Triton-treated ATPase activities decrease sharply, falling to zero at a digestion index of about 10⁵. The increase in untreated ATPase activity accompanies the disappearance of the intact Aα chain, and at the peak of ATPase activity, the single most intense polypeptide band is that of the Aα113T fragment (Fig. 6), but we cannot yet say if there is a causal relationship between the fragmentation of these polypeptides and the changes in ATPase activity.

The ATPase activities of the separated Aα/IC and Aβ chain fractions in 0.6 M NaCl/MgCl₂ solution are also activated by tryptic digestion. The Aα/IC fraction is activated almost 5-fold over its initial ATPase activity at a digestion index of 1.2 x 10⁵, the onset of this activation corresponding with the appearance of the Aα113T fragment. At the same digestion index, the Aβ chain fraction is activated about 2-fold, but the activation does not appear to parallel the appearance of a
single identifiable fragment (data not shown).

The sedimentation distribution of ATPase activity is superimposed on the gels in Fig. 5. Most of the ATPase activity of the undigested LAD-1 co-sediments with the polypeptides of the 21 S peak (11). Treatment of the gradient fractions in this peak with Triton X-100 results in a 4-fold increase in ATPase activity. After digestion of LAD-1 with trypsin to an index of 240, the distribution of ATPase activity of untreated fractions is altered, with roughly equal amounts now being associated with the 21 S and 12 to 14 S peaks (Fig. 5b). Treatment of the fractions with Triton X-100 increases the ATPase activity of the material in the 21 S peak about 3-fold, but has no effect on the ATPase activity of the material in the 12 to 14 S peak. After digestion to an index of 1.2 X 10^3, all ATPase activity sediments with the 12 to 14 S peak (Fig. 5c). The peak ATPase activity of the untreated fractions is about 10 times greater than that of undigested 21 S LAD-1 and co-sediments with the A^15T fragment.

Effects of Digestion on Flagellar Axonemes

Polypeptide Fragments—Analysis of the digestion of whole axonemes is complicated by the presence of many polypeptides that do not appear in significant quantities in LAD-1. Furthermore, the absolute rates of digestion of individual polypeptides in LAD-1 and in the whole axoneme are not directly comparable because of the uncertain effect of the additional protease substrates available in the latter. However, the digestion of whole axonemes gives rise to essentially the same A and A chain fragments as were observed in digests of LAD-1, although the A chain in whole axonemes is digested by trypsin much more rapidly relative to the A chain than in LAD-1 (Table 1).

Although the digestion of the A chains with trypsin is similar in both crude LAD-1 and whole flagellar axonemes, there is a difference between the two in the initial stages of digestion of the A chain prior to and during formation of the A^275T fragment. In the latter, the initial event is the rapid and complete conversion of the A chain to a discrete fragment, A^275T, that migrates as a sharp band only slightly ahead of the original A chain (Fig. 8, arrowhead). This contrasts with the less specific terminal peptide cleavage of the A chain in LAD-1 which leads to the formation of a broad, fuzzy band (Fig. 2). The second stage of the digestion of the A chain in whole axonemes involves the formation of a second discrete fragment (Fig. 8, arrow), that co-migrates with the A^275T fragment formed by digestion of crude LAD-1 with trypsin.

Solubilization of High Molecular Weight Proteins by ATP and by 0.6 M NaCl—When suspended in isolation buffer containing 0.1 M NaCl, axonemes are stable (4, 15) and centrifugation at 100,000 X g for 15 min sediments almost all the protein. Suspension in 0.6 M NaCl/Mg^2+ solution, however, solubilizes a substantial fraction of the axonemal proteins, including much of the LAD-1 (4).

Digestion with trypsin markedly alters the solubility properties of certain axonemal components, particularly those of the dynein arms. Fig. 7 shows the supernatants from successive extractions of trypsin-digested axonemes. Digestion supernatants (see Fig. 5 for definition of terms) contain tubulin and lower molecular weight polypeptides in amounts that increase with the digestion index, but contain very little high molecular weight material migrating in the region of the dynein heavy chains. In contrast, supernatants resulting from centrifugation of the washed, digested axonemes in isolation buffer with 3 mM ATP added immediately prior to centrifugation contain relatively large quantities of the dynein heavy chains and related fragments, whereas the amount of tubulin at most equals that in the digestion supernatants. Subsequent extraction of the digested axonemes with 0.6 M NaCl/Mg^2+ solution shows a complementary decline in the solubilization of heavy chains and related fragments, presumably because of the lesser amount of heavy chain material remaining after extraction with ATP.

Higher resolution electrophoresis to determine what specific fractions of the heavy chain material in undigested and digested axonemes are released by ATP is shown in Fig. 8. Very
ATP there is a small but significant release of seven of the greater amounts of heavy chain material, among which are some of the A heavy chains and their fragments (Fig. 8). At a digestion index of 120, resuspension in the absence of ATP is impossible owing to the existence of many additional protease substrates (such as tubulin, spokes, and nexin links) in the latter possibility.

The next step in the tryptic digestion of the A, chain, leading to formation of the A, fragment, is common to digests of intact axonemes, solubilized LAD-1 and the isolated A, chain fraction. In all cases, the fragments formed co-electrophorese exactly as a single sharp band, suggesting that they represent almost identical polypeptides and that both this cut and the earlier cuts that form either the A, fragment or the “fuzz” of polypeptides in the region of A, are all made at the same end of the overall A, chain. If this were not so the A, fragment from both LAD-1 and isolated A, chain would also presumably be seen as a fuzzy band.

Digestion of the A, chain in both axonemes and LAD-1 by either chymotrypsin or elastase also gives rise to an electrophoretic band that co-migrates exactly with the A, fragment. The consistent production of this fragment by various proteases under different conditions suggests that the site at which this cleavage occurs represents an exposed hinge or joint between a relatively small terminal portion of the chain and a larger protease-resistant core. The A, fragment is relatively resistant to further proteolysis when it is part of the intact axoneme or the LAD-1 particle, but digestion of the separated A, chain fraction by trypsin shows that the stability of the A, core is reduced in the absence of the other LAD-1 polypeptide chains. This may indicate a conformational loosening of the A, core or the exposure of new cleavage sites by the separation of the A, chain from other LAD-1 polypeptides. The appearance in separated A, chain digests of fragments which do not appear in digests of LAD-1 supports the latter possibility.

The digestion patterns of the A, and A, chains are different in all the conditions so far examined, suggesting that the primary structures of the two chains are not closely homologous.

The polypeptide associations of the A chains can be elucidated in part by the changes in their resistance to digestion under various conditions. When the A, chain is incorporated into the LAD-1 particle, its resistance is 5- to 10-fold greater than that in the separated A, chains, indicating that its primary cleavage sites are substantially more accessible in the latter, either because they occur in what constitutes the interface area of the A, and A, chains in the intact LAD-1 particle or because of blocking by other polypeptides such as intermediate chains 2 and 3. The time course of the digestion of intermediate chains 2 and 3 appears to be consistent with the possibility that it is their digestion that leads to exposure of the primary cleavage sites of the A, chain during extended digestion of the LAD-1 particle. The A, chain, on the other hand, maintains approximately the same resistance to digestion both in the LAD-1 particle and as a separated fraction, suggesting that the accessibility of the A, sequence is little affected by the interaction of the A, chain with the other LAD-1 polypeptides. Direct comparison of the digestion indices of the A chains either as separated fractions or in the LAD-1 particle with those of the A chains in intact axonemes is impossible owing to the existence of many additional protease substrates (such as tubulin, spokes, and nexin links) in the latter. However, such comparisons may be obtained indirectly by comparing the A, resistance ratios under the various conditions. This ratio changes from about 1:2 for

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**DISCUSSION**

The rapidity and relatively simple pattern of the early stages of digestion of the A, chain enable certain conclusions to be drawn about the structure of this polypeptide. The formation of the discrete A, fragment during the digestion of axonemes, and the less specific cuts made in solubilized LAD-1 or A, chain suggest that the single hypersensitive proteolytic site on axonemally bound A, chain is submerged among less sensitive sites when the chain is detached from the axoneme.

![Figure 8](http://www.jbc.org/content/521/2/521/f8.large.jpg)

**Figure 8.** Extraction supernatants from digested axonemes in the presence and absence of ATP. Equal quantities (6 mg) of trypsin-digested axonemes were resuspended in 2 ml of isolation buffer either with or without 3 mM ATP. After centrifugation at 12,000 × g for 10 min, the supernatants were clarified by centrifugation at 100,000 × g for 15 min and 50 μl of each were electrophoresed. The numbers at the top of the figure refer to the digestion indices to which the axonemes were digested. Supernatants from extractions in the presence of ATP are signified by W (for “wash”); supernatants from extractions in the presence of ATP are signified by W + ATP. α indicates intact A, chain; β indicates intact A, chain. Arrowhead, A, fragment; Arrow, A, fragment; asterisk, see text. The photographs show the midportion of a 3 to 8% w/v polyacrylamide gradient gel.

The amounts of heavy chain polypeptide solubilized by isolation buffer in the absence of ATP, while in the presence of ATP there is a small but significant release of seven of the eight heavy chains present in intact axonemes, with only the C chain being absent (11). After digestion to an index of 8, only a small amount of the A chain material is released after resuspension in isolation buffer alone, but resuspension in the presence of 3 mM ATP causes the release of substantially greater amounts of heavy chain material, among which are some of the A heavy chains and their fragments (Fig. 8). At a digestion index of 120, resuspension in the absence of ATP causes the release of an increased quantity of the A, chain and the A, fragment, but still far less than that released in the presence of ATP. The heavy chain material released by ATP from axonemes at this digestion index is relatively resistant to further proteolysis when it is part of the intact axoneme or the LAD-1 particle, but digestion of the separated A, chain fraction by trypsin shows that the stability of the A, core is reduced in the absence of the other LAD-1 polypeptide chains. This may indicate a conformational loosening of the A, core or the exposure of new cleavage sites by the separation of the A, chain from other LAD-1 polypeptides. The appearance in separated A, chain digests of fragments which do not appear in digests of LAD-1 supports the latter possibility.

The digestion patterns of the A, and A, chains are different in all the conditions so far examined, suggesting that the primary structures of the two chains are not closely homologous.

The polypeptide associations of the A chains can be elucidated in part by the changes in their resistance to digestion under various conditions. When the A, chain is incorporated into the LAD-1 particle, its resistance is 5- to 10-fold greater than that in the separated A, chains, indicating that its primary cleavage sites are substantially more accessible in the latter, either because they occur in what constitutes the interface area of the A, and A, chains in the intact LAD-1 particle or because of blocking by other polypeptides such as intermediate chains 2 and 3. The time course of the digestion of intermediate chains 2 and 3 appears to be consistent with the possibility that it is their digestion that leads to exposure of the primary cleavage sites of the A, chain during extended digestion of the LAD-1 particle. The A, chain, on the other hand, maintains approximately the same resistance to digestion both in the LAD-1 particle and as a separated fraction, suggesting that the accessibility of the A, sequence is little affected by the interaction of the A, chain with the other LAD-1 polypeptides. Direct comparison of the digestion indices of the A chains either as separated fractions or in the LAD-1 particle with those of the A chains in intact axonemes is impossible owing to the existence of many additional protease substrates (such as tubulin, spokes, and nexin links) in the latter. However, such comparisons may be obtained indirectly by comparing the A, resistance ratios under the various conditions. This ratio changes from about 1:2 for

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2 W.-J. Y. Tang, unpublished results.
The presence of a sufficient concentration of MgATP$^{2-}$, the arms dissociate from the B-tubules but remain attached to the A-tubule of the adjacent outer doublet, while in the presence of a sufficient concentration of MgATP$^{2-}$, the arms dissociate from the B-tubules but remain attached to the A-tubule (3). Thus, the extraction of the major polypeptides of the outer dynein arm from digested flagellar axonemes of *Triposoma* in the presence of 3 mM ATP, 4 mM Mg$^{2+}$, and 0.1 M NaCl suggests that the ATP-labile interaction of the arms to the B-tubule is largely unaffected by such mild digestion. The efficiency of extraction in the presence of ATP depends on the degree of digestion, suggesting that the effect of digestion on the extractability of outer arms by ATP is cumulative and does not depend on cleavage at a single or relatively few sites. This is consistent with the outer arm possessing relatively extensive interactions with the A-tubule, and suggests the existence of an equilibrium between attachment and solubilization which is progressively shifted toward the latter as digestion proceeds. Takahashi and Tonomura (3), using the electron microscope, noted similar behavior in ciliary axonemes of *Tetrahymena*, where tryptic digestion appeared to reduce or abolish the interaction of 30 S dynein with the A-tubule, while preserving an ATP-labile interaction with the B-tubule. The partial blockage of the primary A,$^{275}$ cleavage site when the LAD-1 particle is bound to the axoneme, and the correlation between the digestion of the A$_r$ chain and the release of LAD-1 polypeptides from digested axonemes (Fig. 7) suggests that the A$_r$ chain may participate in the binding of the dynein arm to the A-tubule (Fig. 9). Data are insufficient to comment on the role of the intermediate chains in this interaction. The resistance of the A$_r$ chain to digestion at digestion indices which enable release of substantial quantities of LAD-1 polypeptides suggests that the A$_r$ chain is not directly involved in A-tubule binding. However, the ATP-labile blocking by the A$_r$/IC fraction of beat frequency restoration to dynein-depleted demembranated sperm flagella (1) suggests that the A$_r$ and/or intermediate chains participate in the binding of the dynein arm to the B-tubule (Fig. 9).

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