Structure of the $\alpha$ and $\beta$ Heavy Chains of the Outer Arm Dynein from *Chlamydomonas* Flagella

LOCATION OF EPITOPE S AND PROTEASE-SENSITIVE SITES*

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Stephen M. King and George B. Witman†

From the Cell Biology Group, Worcester Foundation for Experimental Biology, Shrewsbury, Massachusetts 01545

We describe here the pathways by which the $\alpha$ and $\beta$ heavy chains of the outer arm dynein from *Chlamydomonas* flagella are degraded by endoproteases. By probing the digestion products with monoclonal antibodies, we have located a number of protease-sensitive sites within both polypeptides and identified the regions of each molecule from which specific fragments are derived. These data also define the regions within each chain which contain the epitopes recognized by our monoclonal antibodies. The locations of the cleavage sites reveal both differences and similarities between the two molecules. The sites at which the $\beta$ chain is initially cleaved by elastase differ depending upon whether the particle is digested in situ or in solution, indicating that the $\beta$ chain undergoes a significant conformational change following extraction from the axoneme. Evidence was also obtained that heterogeneity exists among the purified $\alpha$ chain molecules. The possible arrangement of the different regions of the heavy chains within the $\alpha$-$\beta$ dimer is discussed.

NOMENCLATURE OF FRAGMENTS AND SITES OF CLEAVAGE

In this study, enzymatic cut sites are designated by a C, E, or T which refers to the protease used: chymotrypsin, elastase, or trypsin, respectively. This is followed by a superscript $\alpha$ or $\beta$ to indicate the chain digested and a subscripted number to denote the site (1, 2, 3 etc.). Individual fragments are designated by an $\alpha$ or $\beta$ to indicate

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* This is the second paper in a series describing the substructural organization of the *Chlamydomonas* dynein complex. The first (King, S. M., and Witman, G. B. (1987) J. Biol. Chem. 262, 17596-17604) reported the masses of the $\alpha$ and $\beta$ chains and the locations of sites of vanadate-dependent photolysis. This study was supported by Grants GM20626 and CA12708 awarded by the National Institutes of Health. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† To whom correspondence should be addressed.

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the chain from which they originated. A superscript letter indicates the protease used to obtain the fragment, and a subscripted number denotes the molecular weight (x 10^3), e.g. $\alpha_{167}$ refers to an $M_r$ 167,000 fragment of the $\alpha$ chain obtained by digestion with elastase. The mass values assigned to fragments of $M_r > \sim 100,000$ are not meant to imply that their masses have been determined to the nearest 1,000 daltons; rather these are nominal values such that the sum of the masses of the two fragments generated by a single cleavage equals the mass of the original fragment or polypeptide. However, in all cases the nominal value is close to or identical with the experimentally determined value.

The fragments obtained from the UV-induced, vanadate-dependent cleavage procedures described previously (King and Witman, 1987) are designated in a similar manner; e.g. $\alpha_{290}$ refers to the $M_r$ 290,000 fragment of the $\alpha$ chain generated by cleavage at its V1 site and $\beta_{260}$ to the $M_r$ 260,000 fragment of the $\beta$ chain obtained by cleavage at its V2 site.

MATERIALS AND METHODS

Purification of the $\alpha$-$\beta$ dimer from the outer dynein arm of the *Chlamydomonas* flagellum (King et al., 1986; Pfister et al., 1982; Witman, 1986), generation, and characterization of monoclonal antibodies which recognize the $\alpha$ (18aA and 18aB) and $\beta$ (18B and 18B'C) heavy chains (King et al., 1985, 1986). UV-induced cleavage at the V1 sites (King and Witman, 1987), and electrophoretic (King et al., 1985, 1986) procedures used to examine the properties of the intact molecules and their fragments were as described previously.

Proteolytic Cleavage—Purified $\alpha$-$\beta$ dimer or native axonemes were subjected to partial proteolytic cleavage with $\alpha$-chymotrypsin (Type VII (N'-tosyl-L-lysine chloromethyl ketone-treated), C-3142; Sigma), elastase (Type IV, E-0258; Sigma), or trypsin (Type I, T-8003 or Type XIII (N'-tosyl-L-phenylalanine chloromethyl ketone-treated), T-8642; Sigma). Fifty $\mu$l of solution containing the purified dynein (10-15 $\mu$g) or axonemes (20 $\mu$g) were placed in 500-$\mu$l micro test tubes (Bio-Rad Laboratories), 10 $\mu$l of protease-containing solution added, and the samples mixed by vortexing. The amount of protease added was varied between 0.5 ng and 10 $\mu$g. All digestions were performed in either 30 mM Tris-HCl, pH 7.5, 0.5 mM EDTA, 1 mM DTT, 25 mM KCl, or 30 mM Tris-HCl, pH 7.5, 0.5 mM EDTA, 5 mM MgSO$_4$ for 5 min at room temperature. Digestion of sucrose gradient-purified dynein occurred in the presence of ~15% (w/v) sucrose. Chymotryptic and elastase digests were stopped by the addition of 15 $\mu$l of 5 x-concentrated electrophoresis sample buffer, 5 $\mu$l of $\beta$-mercaptoethanol and immediate boiling for 5 min. Tryptic digests were halted either by the method described above or by the addition of a 10-fold excess (by mass) of soybean trypsin inhibitor (Type I-S, T-9003; Sigma) prior to preparation of the samples for electrophoresis.

The relative molecular masses of fragments of $M_r < \sim 100,000$ were determined using 0-2.4 M glycerol, 5-15% acrylamide gradient gels containing sodium dodecyl sulfate. Fragments of $M_r > \sim 100,000$ were analyzed in 2-8 M urea, 3-5% acrylamide gradient gels. The region of the gel in which fragments of $M_r > 205,000$ migrated was calibrated using $M_r$ values of 450,000, 440,000, 390,000, 290,000, and 205,000 for the $\alpha$ and $\beta$ chains, the fragments $\alpha_{290}$ and $\beta_{260}$, and myosin, respectively (King and Witman, 1987). Other $M_r$ standards were as detailed previously (King and Witman, 1987).

RESULTS

Partial proteolytic digestion of the $\alpha$-$\beta$ dimer isolated from the outer dynein arm of *Chlamydomonas* resulted in a highly complex pattern of fragments when analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Fig. 1, a and b). Several major tryptic fragments are pointed out in the figure. However, in order to positively identify the polypeptide from which a given fragment originated, and also to determine from where in that polypeptide the fragment was derived, it was essential to use monoclonal antibodies as markers for specific regions of each heavy chain.

The $\beta$ Chain

*Tryptic Digestion of the $\beta$ Chain*—A tryptic digest of the purified $\alpha$-$\beta$ dimer probed with monoclonal antibodies 18B and 18B'C is shown in Fig. 2, a and b. For the initial stages of the digest, the kinetics of appearance and disappearance of the immunoreactive fragments indicated that there were two concurrent pathways of cleavage of the $\beta$ chain that resulted in a fragment of $M_r$ 220,000 recognized by both antibodies (Fig. 2c).

In one pathway, cleavage first occurred at a site (Tf) approximately 10,000 daltons from one end, yielding a fragment of $M_r$ 430,000 that was reactive with monoclonal antibodies 18B and 18B'C (Fig. 2, a and b; lanes 2-5). Cleavage at or very near this site was observed in all proteolytic digestions of the isolated $\alpha$-$\beta$ dimer (see Figs. 3 and 5), indicating that this region of the molecule is particularly susceptible to en-
zymatic cleavage. This initial cleavage was followed by cleavage at a second site (Tf) to generate a fragment (β250) that was recognized by both 18βB and 18βC (Fig 2, a and b; lanes 3–5); the complementary fragment (M, 210,000) has been identified in silver-stained gels (see Fig. 1a).

In the alternative pathway, cleavage of the β chain occurred first at the Tf site, which in this case yielded an immunoreactive fragment of M, 230,000 (Fig. 2, a and b; lanes 2–4) together with the nonimmunoreactive M, 210,000 fragment noted above. Cleavage of β250 at Tf then yielded an M, 220,000 fragment which contained both epitopes and was identical to the β250 fragment obtained in the first pathway by cleavage of β250 at Tf. The fact that β250 was converted to β250 by a cut at Tf indicates that the latter cut site must occur on that end of the β chain which contains the epitopes recognized by 18βB and 18βC (i.e. within the M, 185,000 V1 fragment (King and Witman, 1987)). Therefore, Tf and Tf must be located 10,000 and 230,000 daltons, respectively, from that terminus (Fig. 2c).

The β250 fragment obtained by either pathway was then cleaved at Tf to yield a fragment of M, 175,000, which was recognized by 18βC but not 18βB (Fig. 2, a and b; lanes 4–6). Elastase digestion of the β chain indicates that 18βB is located closer to the β chain terminus contained in β250 than in 18βC (see below and Figs. 3 and 4); hence, Tf must be located 55,000 daltons from that terminus. The fragment complementary to β250 has not been identified.

Because the 18βB epitope was removed or destroyed by cleavage at Tf, it is likely that the epitope is at this site or within the 45,000-dalton segment removed by cleavage there; this is confirmed by the results obtained using other proteases (see below). Because 18βC is within β150 and β125 (King and Witman, 1987), it must be located between the V1 site and Tf (Fig. 2c).

The M, 175,000 fragment was further digested via fragments of M, 160,000 and 150,000 to a relatively stable fragment of M, 120,000 that retained the 18βC epitope (Fig. 2b; lanes 5–7). The locations of the cut sites that generate these fragments are uncertain. However, based on the further localization of the 18βC epitope (see section on elastase digestion), that terminus of β175, nearest the V1 site must be removed by one of the cleavages that generate the smaller fragments.

The nonimmunoreactive fragment β150 produced by cleavage of the intact β chain or β150 subsequently appeared to be cleaved to yield the M, 125,000 and 85,000 fragments indicated in Fig. 1, a and b, respectively (compare lanes 5–8 in Fig. 1a with lanes 4–7 in Fig. 1b). Because neither fragment was immunoreactive, this assignment derives solely from the kinetics of appearance of these fragments. Based on structural analogy with chymotryptic digests of the β chain (see below), we predict that this tryptic site (T) is located within β150.
85,000 daltons from the original terminus (Fig. 2c).

Elastase Digestion of the \( \boldsymbol{\beta} \) Chain—When the purified \( \alpha-\beta \) dimer was digested with elastase, the first cleavage within the \( \beta \) chain again removed approximately 10,000 daltons from one terminus (Fig. 3, a and b); analysis of elastase-digested V1 fragments indicates that this cut site (\( \beta_{85}^{E1} \)) is within \( \beta_{85}^{A} \) (not shown) and is therefore very near \( \beta_{85}^{E1} \). The \( M, 430,000 \) truncated \( \beta \) chain contained the epitopes recognized by antibodies 18\( \beta B \) and 18\( \beta C \). The next cleavage generated a fragment of \( M, 400,000 \) (previously designated "band 10"; Pfister and Witman, 1984; Pfister et al., 1982) that was recognized by 18\( \beta B \) but not by 18\( \beta C \) (Fig. 3, a and b; lanes 3–5). Because the epitope recognized by antibody 18\( \beta B \) is located within \( \beta_{85}^{A} \) (King and Witman, 1987), the \( \beta_{85}^{E1} \) cut site that removed or destroyed the 18\( \beta B \) epitope must be within the same V1 fragment, and the 18\( \beta B \) epitope must be located between \( \beta_{85}^{E1} \) and \( \beta_{85}^{E2} \) i.e. within approximately 40,000 daltons of the terminus (Fig. 3c). The \( \beta_{85}^{E1} \) fragment was then cleaved at \( \beta_{85}^{E1} \) to yield a nonimmunoreactive fragment of \( M, 345,000 \) that has been identified in silver-stained gels (not shown). Because the 18\( \beta C \) epitope is also located within the \( \beta_{85}^{E2} \) fragment, the \( \beta_{85}^{E2} \) site must occur approximately 55,000 daltons from \( \beta_{85}^{E1} \), and 18\( \beta C \) must be located between \( \beta_{85}^{E1} \) and \( \beta_{85}^{E2} \) i.e. between 40,000 and 95,000 daltons from the terminus (Fig. 3c).

When the \( \beta \) chain was digested in situ by treatment of isolated axonemes with elastase, cleavage did not occur at \( \beta_{85}^{E1} \) or \( \beta_{85}^{E2} \); instead the first cut (\( \beta_{85}^{E1} \)) generated fragments of \( M, 367,000 \) and 73,000 recognized by 18\( \beta B \) and 18\( \beta B \), respectively (Fig. 4, a and b; lanes 2–4). These results confirm that 18\( \beta B \) is located within 73,000 daltons of one terminus and place the 18\( \beta C \) epitope within the 22,000-dalton domain delimited by \( \beta_{85}^{E1} \) and \( \beta_{85}^{E2} \), i.e. 73,000–95,000 daltons from the original terminus contained in \( \beta_{85}^{E1} \) (Fig. 4c). \( \beta_{85}^{E7} \) was then cleaved to yield fragments of \( M, 124,000, 86,000, \) and 47,000 that were recognized by 18\( \beta C \) (Fig. 4b; lanes 3 and 4). The \( \beta_{85}^{E7} \), \( \beta_{85}^{E8} \), and \( \beta_{85}^{E9} \) fragments must retain that terminus of \( \beta_{85}^{E7} \) which contains the 18\( \beta C \) epitope; otherwise they would not be immunoreactive. Hence, the cleavage sites that generate these fragments (\( \beta_{85}^{E7}, \beta_{85}^{E8}, \) and \( \beta_{85}^{E9} \)) are located 197,000, 159,000, and 120,000 daltons, respectively, from the original terminus contained in \( \beta_{85}^{E1} \) (Fig. 4c). The fact that elastase digestion of the \( \beta \) chain proceeds by a different pathway in situ than within the isolated \( \alpha-\beta \) particle indicates that the conformation and/or accessibility of the \( \beta \) chain is quite different under the two conditions. Specifically, when the \( \beta \) chain is removed from the axoneme, the \( \beta_{85}^{E1} \) site becomes unavailable, and new elastase-sensitive sites \( \beta_{85}^{E7}, \beta_{85}^{E8}, \) and \( \beta_{85}^{E9} \) are exposed.

Chymotryptic Digestion of the \( \beta \) Chain—In chymotryptic digests of the \( \alpha-\beta \) dimer, four discrete immunoreactive fragments were observed (Fig. 5, a and b). The first cleavage of the \( \beta \) chain again generated a fragment of \( M, 430,000 \) (Fig. 5, a and b; lanes 2–6); presumably this cut site (\( \beta_{85}^{C2} \)) occurred close to \( \beta_{85}^{E7} \) and \( \beta_{85}^{E1} \). The next cleavage, at cut site \( \beta_{85}^{C2} \), yielded a fragment of \( M, 348,000 \) recognized by both antibodies (Fig. 5, a and b; lanes 4–6); the complementary fragment (\( M, 82,000 \)) has been identified in silver-stained gels (data not shown). Because the 18\( \beta B \) epitope is located within 73,000 daltons of the \( \beta \) chain terminus contained in \( \beta_{85}^{C1} \) (see above and Fig. 4), the \( \beta_{85}^{C2} \) site must be located at the opposite end of the molecule, 82,000 daltons from that terminus (Fig. 5c). The \( \beta_{85}^{C1} \) fragment was then degraded by cleavage at \( \beta_{85}^{C2} \) and \( \beta_{85}^{C1} \) to yield \( \beta_{85}^{C2} \) and \( \beta_{85}^{C1} \) (Fig. 5, a and b; lanes 6 and 7). Because these fragments were recognized by both antibodies and because the mass differences between fragments were greater than the maximal distance of the 18\( \beta B \) epitope from that terminus of the \( \beta \) chain contained in \( \beta_{85}^{C1} \), the chymotryptic sites \( \beta_{85}^{C1} \) and \( \beta_{85}^{C2} \) must occur 235,000 and 120,000 daltons, respectively, from that terminus. The \( \beta_{85}^{C1} \) site and \( \beta_{85}^{C1} \), which we have tentatively assigned to the same region, are the only protease-sensitive sites which we have identified in the 200,000-dalton portion of the chain extending from \( \beta_{85}^{C1} \) to the \( \beta \) chain terminus contained in \( \beta_{85}^{C1} \). The \( \beta_{85}^{C1} \) fragment was the smallest peptide obtained from the \( \beta \) chain that contained both the 18\( \beta B \) and 18\( \beta C \) epitopes; the existence of this fragment confirms that these epitopes are no further apart than 110,000 daltons.

A map of the \( \beta \) chain depicting the location of protease-sensitive sites, the origin of the fragments identified, the regions containing the epitopes recognized by 18\( \beta B \) and 18\( \beta C \), and the sites of V1 and V2 cleavage is shown in Fig. 6.

The \( \alpha \) Chain

Elastase Digestion of the \( \alpha \) Chain—The immunoreactivity of fragments of the \( \alpha \) chain obtained by digestion with elastase is shown in Fig. 7. The initial cleavage, at \( \beta_{85}^{E1} \), yielded a fragment of \( M, 392,000 \) that was recognized by monoclonal antibodies 18\( \alpha A \) and 18\( \alpha B \) (Fig. 7, a and b; lanes 1–4), and a smaller, nonimmunoreactive, fragment of \( M, 88,000 \) that has been identified in silver-stained gels (not shown). The larger fragment co-migrated with the major endogenous fragment that was previously designated "band 11" (Pfister and Witman, 1984; Pfister et al., 1982). The \( \alpha_{85}^{E5} \) fragment was subsequently cleaved, at \( \beta_{85}^{E6} \), to generate a fragment of \( M, 317,000 \) recognized by both antibodies (Fig. 7, a and b, lanes 2–7), and a nonimmunoreactive fragment of \( M, 75,000 \) (not shown).

To determine the origin of these fragments relative to the V1 fragments, the \( \alpha-\beta \) dimer was cleaved first at the V1 site and then subjected to elastase digestion (Fig. 8, a and b). UV-induced cleavage at the V1 site of the undigested chain yielded an \( M, 190,000 \) fragment (\( \alpha_{85}^{E5} \)) recognized by antibody 18\( \alpha A \) (Fig. 8a, lane 1), and an \( M, 290,000 \) fragment (\( \alpha_{85}^{E5} \)) recognized by 18\( \alpha B \) (Fig. 8b, lane 1); these results were identical to those

Fig. 4. Elastase digestion of the \( \beta \) chain in situ. Nitrocellulose replicas of 5–15% acrylamide gradient gels stained with antibodies 18\( \beta B \) (a) and 18\( \beta C \) (b). Lanes were loaded with 20 \( \mu g \) of axonemes that had been digested with 0, 0.1, 1.0, and 5.0 \( \mu g \) of elastase. The positions of the \( \beta \) chain and immunoreactive fragments are indicated. Endogenous fragments of the \( \beta \) chain are also detectable in (b).
**FIG. 5. Chymotryptic digestion of the β chain.** Nitrocellulose replicas of a 3–5% acrylamide gradient gel. For each panel, lanes were loaded, from left to right, with 4.7 μg of sucrose gradient-purified α–β dimer that had been digested with 0, 0.07, 0.15, 0.30, 0.45, 0.74, and 1.48 μg of α-chymotrypsin. a shows a replica probed with antibody 18βB; b is a similar blot incubated with 18βC.

**FIG. 7. Elastase digestion of the α chain.** Nitrocellulose replica of a 3–5% acrylamide gradient gel. Lanes on each panel were loaded, from left to right, with 5.3 μg of sucrose gradient-purified α–β dimer after digestion for 5 min with 0, 0.18, 0.35, 0.71, 1.06, 1.77, and 3.50 μg of elastase. a was probed with antibody 18αA and b with 18αB. The elastase-generated fragments recognized by antibodies 18αA and 18αB are indicated at left and right, respectively. A number of endogenous fragments of the α chain (and their elastase cleavage products) are visible in b because of the high sensitivity of the immunostain procedure using this antibody. The origin of the minor fragment (Mr, 105,000) visible in panel a lanes 6 and 7 is uncertain; a complementary fragment recognized by 18αB has not been identified.
obtained previously (King and Witman, 1987). Following elastase digestion, \( \alpha_{V1} \) was converted to an \( M, 202,000 \) fragment which was still recognized by \( 18\alpha B \) (Fig. 8b, lanes 5–8); this conversion resulted from cleavage of \( \alpha_{V1} \) at the \( E_F \) site (Fig. 8c). Therefore, this site is located within the \( M, 290,000 \) V1 fragment, 88,000 daltons from the original terminus of the \( \alpha \) chain. The \( M, 202,000 \) fragment migrated slightly behind a similar fragment produced by photocleavage of the endogenous band 11 at the V1 site, indicating that the \( E_F \) site is closer to the terminus of the \( \alpha \) chain than is the site of endogenous cleavage. Elastase digestion of the photocleaved \( \alpha \) chain also resulted in conversion of \( \alpha_{V1} \) to a fragment of \( M, 115,000 \) which retained the \( 18\alpha A \) epitope (Fig. 8a, lanes 5–8); this was due to cleavage at the \( E_F \) site (Fig. 8c). Thus, this site is located within the \( M, 190,000 \) V1 fragment, 75,000 daltons from the original terminus of the \( \alpha \) chain. The \( 18\alpha A \) epitope, previously shown to be within 190,000 daltons of this \( \alpha \) chain (King and Witman, 1987), must be located within the 115,000-dalton region between the site of V1 cleavage and \( E_F \).

Returning to the pathway by which the intact chain was degraded, digestion of \( \alpha_{95} \) gave rise to \( \alpha_{107} \) which contained both epitopes (Fig. 7, a and b; lanes 3–7) and a rapidly degraded nonimmunoreactive fragment (\( M, 150,000 \)) observed in silver-stained gels. Because the \( 18\alpha A \) epitope is within the 115,000-dalton region delimited by the V1 site and \( E_F \) (Fig. 8c), \( \alpha_{107} \) must also contain this region, and the \( E_F \) site must be located on the opposite side of the V1 site close to the V2a site (Fig. 7c). We previously determined that the epitope recognized by \( 18\alpha B \) was located between the V2a and V1 photocleavage sites (King and Witman, 1987); the fact that \( \alpha_{107} \) contains this epitope confirms its location in that region and further delimits it to the 52,000-dalton segment extending from \( E_F \) to the V1 site.

The \( \alpha_{167} \) fragment was subsequently cleaved at \( E_F \) to generate an \( M, 67,000 \) fragment recognized by \( 18\alpha A \) and a complementary fragment of \( M, 100,000 \) recognized by antibody \( 18\alpha B \) (Fig. 7, a and b; lanes 4–7). The latter fragment was highly sensitive to further degradation, eventually yielding an immunoreactive fragment of \( M, 15,000 \) (not shown). Because \( \alpha_{165} \) contains the \( 18\alpha B \) epitope and, therefore, the V1 site, \( E_F \) occurs within \( \alpha_{165} \), 48,000 daltons from the V1 site. Thus, the \( 18\alpha A \) epitope is within the 67,000-dalton region bounded by \( E_F \) and \( E_B \).

When the \( \alpha \) chain was degraded by elastase in situ, the initial stages of the cleavage pathway appeared similar to those described above for the particle in solution.

**Tryptic Digestion of the \( \alpha \) Chain**—Initial cleavage of the \( \alpha \) chain by trypsin generated fragments of \( M, 390,000 \) and \( 90,000 \) (Figs. 1, a and b, 9a, and 10, a and b; see also Fig. 3 of King and Witman, 1987). The former was recognized by \( 18\alpha A \) and \( 18\alpha B \) (see Fig. 4 of King and Witman, 1987); the latter was detected in silver-stained gels (Fig. 1b; lanes 2–4). This tryptic site (\( T_I \)) is located 90,000 daltons from the \( \alpha \) chain terminus contained in \( \alpha_{165} \) (Fig. 9b; see also Fig. 4 of King and Witman, 1987), and ~2,000 daltons from \( E_F \). This region of the molecule is highly sensitive to proteolysis and contains the preferred first cleavage site for all proteases that we have used. The \( \alpha_{165} \) fragment was subsequently degraded, apparently to a fragment of \( M, 80,000 \) (Figs. 1b; lanes 3–8).

Subsequent digestion of the \( \alpha_{165} \) fragment was complex and proceeded by multiple pathways. The majority of the \( \alpha_{165} \) fragments were cleaved at \( T_2 \) to generate a fragment of \( M, 277,000 \) that contained both epitopes (Fig. 9a; lanes 2–8); the complementary fragment of \( M, 113,000 \) is indicated in Fig. 1a. However, at slightly lower concentrations of trypsin, a small percentage of \( \alpha_{165} \) was cleaved at \( T_3 \) to yield a fragment of \( M, 317,000 \) (Fig. 9a; lanes 2–7). Minor fragments of \( M, 300,000, 290,000, \) and \( 280,000 \) were also observed in these samples (Fig. 9a; lanes 2–4).

To determine the relationship of these fragments, the \( \alpha \) chain was first cleaved at the V1 site and then subjected to trypsin digestion. As before, UV-induced cleavage at the V1 site in the absence of protease produced the fragments \( \alpha_{165} \) and \( \alpha_{165} \), recognized by \( 18\alpha A \) and \( 18\alpha B \), respectively (Fig. 10, a and b). Trypsin digestion of the V1-cleaved \( \alpha \) chain first yielded a major fragment of \( M, 200,000 \) that was recognized by \( 18\alpha B \) but not \( 18\alpha A \) (Fig. 10, a and b; lanes 2–5); this was derived from \( \alpha_{165} \) by cleavage at the highly susceptible site \( T_2 \). The \( M, 200,000 \) fragment was then cleaved at \( T_2 \) to yield
FIG. 9. Tryptic digestion of the α chain. a, nitrocellulose replica of the high molecular weight region of a 3–5% acrylamide gradient gel immunostained with antibody 18αB. Lanes were loaded, from left to right, with 5 μg of hydroxylapatite-purified α-β dimer that had been digested with 0, 3, 18, 36, 92, 183, 275, 367, 734, and 1830 ng of trypsin. The positions of the α chain and its tryptic products are indicated. A number of endogenous fragments are also visible.

an M, 87,000 fragment recognized by 18αB (Fig. 10b, lanes 5–8). Inasmuch as the 18αB epitope is located in α\text{50}_\text{50} within 52,000 daltons of the V1 cleavage site (see above), T\text{2}_\text{y} also must be located in α\text{50}_\text{50}, 87,000 daltons from the V1 site (Fig. 10c). Minor fragments of M, 110,000, 100,000, and 90,000 were also observed in the samples cleaved at the V1 site and then digested with trypsin; each of these fragments was recognized by 18αB (Fig. 10b; lanes 4–8). Because the 18αB epitope is within 52,000 daltons of the V1 site, these fragments were generated from either α\text{50}_\text{50} or the M, 200,000 fragment by cleavage at locations 110,000 (T\text{7}_\text{y}), 100,000 (T\text{2}_\text{y}) and 90,000 (T\text{2}_\text{y}) daltons from the V1 site. These sites are in the correct locations to produce the minor fragments of M, 300,000, 290,000, and 280,000 observed in samples not subjected to cleavage at the V1 site (see above). Similarly, the generation of an M, 117,000 fragment recognized by antibody 18αA indicates the presence of a trypsin-sensitive site 73,000 daltons from the terminus contained in α\text{10}_\text{10}. This probably corresponds to the site (T\text{7}_\text{y}) at which α\text{50}_\text{50} was cleaved to yield α\text{50}_\text{17}.

In the samples not subjected to V1 cleavage, α\text{77}_\text{T} was converted by cleavage at the T\text{7}_\text{y} site to an M, 204,000 fragment (Fig. 1a, lanes 5–8 and Fig. 9a, lanes 3–9) that retained both epitopes; the complementary fragment (M, 73,000) is indicated in Fig. 1b (lanes 5–9). The α\text{17}_\text{T} fragment was presumably degraded to the same fragment by cleavage at the T\text{7}_\text{y} site. The α\text{77}_\text{T} fragment was then cleaved at either T\text{7}_\text{y} to yield minor fragments of M, 107,000 and 97,000 recognized by 18αB and 18αA, respectively (not shown) or at T\text{3}_\text{y} to produce a fragment of M, 145,000 that included both epitopes (Fig. 1a, lane 7). Because 18αA and 18αB are on opposite sides of the V1 site, α\text{77}_\text{T} and α\text{77}_\text{T} must originate by cleavage at a site within α\text{50}_\text{50}, 20,000 daltons from the V1 site. α\text{77}_\text{T} could potentially contain either terminus of α\text{50}_\text{50}. However, a V1/trypsin fragment of M, 28,000 recognized by 18αB has been obtained (not shown); this fragment is consistent with a tryptic cut site 28,000 daltons from the V1 site. Such a tryptic site would be in the correct location to generate a 145,000-dalton fragment from α\text{50}_\text{50}. It is therefore probable that T\text{3}_\text{y} occurs within α\text{50}_\text{50} and that the 18αB epitope is within the 28,000-dalton region delimited by T\text{3}_\text{y} and the V1 site (Fig. 9b, and see below).

At higher trypsin concentrations, an additional immunoreactive fragment (M, 330,000) was obtained from α\text{50}_\text{50} by cleavage at T\text{3}_\text{y} (Fig. 9a, lanes 4–10; see also Fig. 1a, lane 7). This fragment originates from a residual population of α\text{50}_\text{50} refractory to cleavage at T\text{2}_\text{y} or T\text{3}_\text{y}. The location of T\text{3}_\text{y} was determined by digesting dynein photocleaved at the V1 site with high concentrations of trypsin. Under these conditions, an M, 130,000 fragment appeared which was not present at lower trypsin concentrations (Fig. 10a, lanes 7 and 8). This fragment was recognized by 18αA, indicating that it was generated from α\text{50}_\text{50} by cleavage at a site 60,000 daltons from the α chain terminus contained in α\text{50}_\text{50}. This must be the same site as that which generated α\text{50}_\text{50} from α\text{50}_\text{50}. In the samples not subjected to V1 photocleavage, a fragment of M, 158,000, recognized by both 18αA and 18αB, appeared following formation of α\text{50}_\text{50} (not shown); this M, 158,000 fragment presumably derives from α\text{50}_\text{50} by cleavage at the T\text{3}_\text{y} site.

Analysis of proteolytic fragments also suggests that cleavage at the V1 site causes significant conformational changes within the α chain. A fragment of M, 143,000 (recognized by 18αB) was generated by continued digestion of dynein that was cleaved at the V1 site (Fig. 10b, lanes 5–8). This fragment has no detectable counterpart in nonphotocleaved samples, indicating that after cleavage at the V1 site, a previously inaccessible tryptic site (T\text{3}_\text{y}) becomes available within α\text{50}_\text{50} at a location 143,000 daltons from the V1 site.

The locations of the trypsin- and elastase-sensitive sites, the epitopes recognized by 18αA and 18αB, the V1 and V2 cleavage sites, and the regions of the intact molecule which correspond to the fragments described above are shown diagrammatically in Fig. 11.

Chymotryptic digests of the α chain have also been analyzed. The results do not add significantly to an understanding of α chain structure and are not reported here.

DISCUSSION

Endoproteolytic digestion has been used to investigate the substructure of a number of high molecular weight polypeptides, e.g. myosin (Chen and Reisler, 1984; Hynes et al., 1987; Lawler et al., 1984), fibronectin (Castellani et al., 1986; Hayashi and Yamada, 1981; Sekiguchi et al., 1985), spectrin (Morrow et al., 1980; Speicher et al., 1980), thrombospondin (Galvin et al., 1985; Lawler et al., 1985), microtubule-associated protein 2 (Vallee and Borisy, 1977), and the outer arm dyneins from sea urchin sperm flagella (Bell and Gibbons, 1982; Ogawa, 1973; Ow et al., 1987) and Tetrahymena cilia (Clutter and Johnson, 1986; Takahashi and Tonomura, 1978; Toyoshima, 1987a, 1987b).

In this study we have used a number of proteases to obtain discrete overlapping fragments from the α and β heavy chains of the Chlamydomonas outer arm dynein. By probing these
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**FIG. 10. Tryptic digestion of photocleaved α chain.** Nitrocellulose replicas of 3–5% acrylamide gradient gels probed with antibodies 18αA (a) and 18αB (b). Lanes were loaded with 6 μg of sucrose gradient-purified α-β dimer that had been cleaved at the V1 site by irradiation at 365 nm for 45 min in the presence of 100 μM ATP and 100 μM vanadate prior to digestion with, from left to right, 0, 4, 20, 40, 100, 200, 300, and 400 ng of trypsin. Bands recognized by 18αA and 18αB are indicated at left and right, respectively. The presence of the M, 117,000 fragment in lane 1 of a is due to V1 cleavage of an endogenous fragment.

fragments with a series of monoclonal antibodies we have been able to determine which fragments contain the specific sequences recognized by our antibodies and the origin of these fragments. From these data we have constructed maps indicating the locations of the epitopes recognized by our antibodies and also the sites at which the polypeptides are cleaved to yield the identified fragments (Fig. 12). Both α chain epitopes are located in the central portion of the polypeptide; 18αA is located within a region 338,000–405,000 daltons from the end of the molecule characterized by the highly susceptible tryptic site Tγ, whereas the 18αB epitope is in a region 262,000–290,000 daltons from that same end. The two epitopes we have located within the β chain are more closely spaced. 18βA is within 10,000–40,000 daltons of the β chain terminus contained in the M, 185,000 V1 fragment (βγ); 18βC is 73,000–95,000 daltons from the same terminus.

Both trypsin and elastase first cleaved the α chain at a site located ~90,000 daltons from the original terminus contained in its M, 290,000 V1 fragment (αγ). This region is extremely sensitive to proteolytic attack and also contains the preferred site of cleavage for chymotrypsin. Both trypsin and elastase subsequently removed the other terminus of this polypeptide as a fragment of M, ~75,000. For the purposes of structural mapping, these data subdivide the α chain into three discrete regions: two terminal sections of 90,000 and 75,000 daltons and a large 317,000 dalton central region which contains the epitopes recognized by 18αA and 18αB and the V1, V2a, V2b, and V2c photocleavage sites. Within this central region, there are additional sites which are highly susceptible to proteolysis. Interestingly, a number of these sites are located very close to the V2 sites, suggesting that portions of this region of the polypeptide are exposed at the surface of the α-β dimer.

The αβ0 fragment produced by the initial tryptic cleavage of the α chain subsequently was degraded by cleavage at sites Tγ, Tδ, or Tη to yield fragments of M, 277,000, 317,000, or 330,000, respectively. However, the pattern of appearance of these fragments indicated that they did not result simply from degradation of a uniform population of αβ0 by random cleavage at one of these three sites. The pathway involving cleavage at Tη was kinetically favored and was observed at the lowest concentrations of trypsin, yet only a small number of the αβ0 fragments were degraded via this pathway; the majority of αβ0 was degraded by cleavage at Tδ at higher trypsin concentrations. The pathway involving cleavage at Tγ was observed at still higher trypsin concentrations, and appeared to involve a small subpopulation of αβ0 that was refractory to cleavage at Tδ or Tη. These results strongly suggest that there is heterogeneity within the α chain molecules of a given dynein preparation. There are several possible origins for such heterogeneity. The α chain is phosphorylated at multiple sites in vivo (King and Witman, 1986, 1988; Piperno and Luck, 1981), and α chain molecules having different levels of phosphorylation may have different sensitivities to tryptic cleavage at Tγ, Tδ, and Tη. Purified α-β dimers exhibit variable morphology when viewed by scanning transmission electron microscopy (Witman et al., 1983), and different parts of the α chain may be accessible to trypsin depending upon the conformation of the dynein particle. Heterogeneity could also result from some of the molecules being associated with tubulin. Finally, the observed heterogeneity could reflect the existence of multiple α chain variants generated from different genes or by alternative splicing of the primary transcript (Andreadis et al., 1987).

Digestion of the β chain proceeded via different pathways from those observed for the α chain. Treatment of the isolated α-β dimer with trypsin, elastase, or chymotrypsin always resulted in an initial cleavage of the β chain at closely spaced sites, Tγ, Eγ, or Cγ, located ~10,000 daltons from that terminus of the polypeptide contained in βγ. In the tryptic digests, some of the β chain molecules were first cleaved at an alternate site 220,000 daltons from Tη, but in this case the second cleavage occurred at Tδ. These results indicate that in the isolated dimer this terminal region of the β chain is easily accessible to proteases. In chymotryptic digests, an exposed site (Cη) was unambiguously identified 82,000 daltons from

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3 S. M. King and G. B. Witman, unpublished observation.
the opposite terminus of the molecule.

Interestingly, when the β chain was digested in situ by treatment of intact axonemes with elastase, cleavage did not occur at Eβ; instead the first cut occurred at a site (Eβ) 73,000 daltons from the terminus contained in β50. Because cleavage at the latter site was not observed in digests of the solubilized dynein complex, removal of the α-β dimer from the axonemal superstructure apparently causes a change in the conformation of the particle such that the Eβ site becomes protected and the Eβ site becomes exposed and preferentially cleaved. Within the axoneme, Eβ may be protected as a result of steric constraints or masking of this part of the chain by another protein. This terminal region of the β chain may be involved in binding of the dynein arm to the A or B tubule or it may be involved in the association of the α-β dimer with the γ subunit of the outer arm. All these associations are disrupted by the high ionic strength conditions used to extract dynein from the axoneme.

Based on the locations of its major cleavage sites, the linear map of the β chain may be divided into four readily distinguishable regions: 1) a region of ~10,000 daltons at the terminus contained in β50; 2) a neighboring segment of ~60,000 daltons which contains the 188B epitope and is preferentially removed by elastase digestion of the chain in situ; 3) a large central section of ~290,000 daltons which contains the 188C epitope, the V1 and V2 photocleavage sites, and centrally located sites highly susceptible to tryptic and chymotryptic digestion; and 4) a region of ~80,000 daltons at the terminus contained in β20.

A comparison of the major cleavage sites within the α and β chains reveals both differences and similarities between the two chains. The initial cleavage of the α chain occurs much more rapidly than that of the β chain (see also Fig. 10 in Pfister and Witman, 1984), and the α chain lacks a site analogous to the highly sensitive Tγ, E5, and Cγ sites of the β chain. Nevertheless, the two polypeptides appear to be similar in that both have a number of elastase- and trypsin-sensitive sites in a region 40,000–90,000 daltons from the original terminus contained within their smaller V1 fragments, both contain one or more highly sensitive protease sites located 82,000–90,000 daltons from the opposite terminus, and both exhibit a highly sensitive tryptic site (Tγ and Tγ) located between the sites of V1 and V2 (V2b within the α chain) photocleavage. These considerations, together with other differences and similarities noted previously (King and Witman, 1987), suggest that the two chains are related in their overall structures, but that they differ in important details.

Scanning transmission electron microscopy has shown that the native α-β dimer has a mass of ~1,220,000 daltons and is composed of two globular domains, ~12.7 nm in diameter, connected via a Y-shaped stem (Witman et al., 1983). Analysis of the individual heads indicates that each globular domain has a mass of 375,000 ± 51,000 daltons, which, as noted previously (King and Witman, 1987), is too small to include an entire heavy chain. One possible explanation is that one head contains the 317,000-dalton central region of the α chain. One or both of the terminal regions might then comprise at least part of the stem. The second head could, within the limits set by the standard deviations of the mass analysis, contain all but 20,000 daltons of the β chain. However, it is more likely that at least one terminal region again comprises part of the stem domain; if one intermediate or several light chains are associated with the head, then both terminal regions may constitute the stem. Confirmation of this hypothesis will require electron microscopic analysis of the proteolyzed particle. In this regard, Clutter and Johnson (1986) recently described a single-headed particle obtained from Tetrahymena 30 S dynein by limited proteolysis with elastase. The mass of this particle was approximately 300,000 daltons, and this fragment may well correspond to a region analogous to the 317,000-dalton central region of the Chlamydomonas α chain. Also, Toyoshima (1987a, 1987b) has demonstrated that removal, by chymotryptic digestion, of ~80,000 daltons from the largest dynein heavy chain of Tetrahymena results in a single-headed particle that apparently lacks a stem component; this particle is probably analogous to the chymotryptic equivalent of α300.

The information presented here provides a firm foundation for further studies concerning structure-function relationships within the outer dynein arm from Chlamydomonas.
First, it will be possible to locate discrete functional domains within these polypeptides by labeling a particular site and then examining the distribution of label among the identified fragments from each molecule. We have recently used this methodology to locate within the α and β chains the domains that are covalently modified by photoaffinity analogs of ATP (King and Witman, 1986, 1988; and Footnote 1). Second, these antibodies could be used to screen cDNA and/or genomic expression libraries to obtain cloned DNA sequences that contain the coding region for known portions of these very large proteins. Third, localization of these epitopes within both the purified dynein and the exosome by immunoelectron microscopy should allow for a more detailed understanding of dynein arm morphology and of how images of the purified particles relate to the structures observed in situ. Fourth, the generation of site-specific antibodies directed against defined regions of the heavy chains will permit detailed analysis of the function of discrete domains within the outer dynein arm. Fifth, outer arm function may now be dissected by locating the specific regions of dynein polypeptides that are deleted in mutant Chlamydomonas; for example, cells carrying the supr-1 mutation, which suppresses flagellar paralysis in radial spoke-deficient mutants, synthesize β chain molecules ~1,000 daltons smaller than those from wild-type cells (Huang et al., 1982). Finally, the contribution of each dynein heavy chain domain to the mechanochemical cycle may be assessed by determining the capacity of proteolyzed complexes to translocate microtubules in vitro (Paschal et al., 1987).

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Mapping Dynein Heavy Chains

FIGURE 1a: Elastase Digestion of the α Chain
Diagram illustrating the pathway by which the α chain was degraded by elastase, and the region of the resulting fragment. The lines labeled α and β indicate the regions which remain in the kinase digested by methods α and β, respectively.

FIGURE 1b: Trypsin Digestion of Phosphoeluted α Chain
Diagram illustrating the relationship between the intact α chain, the products of cleavage at the T1 site, and the fragment produced by trypsin digestion of the phosphoeluted α chain.

FIGURE 1c: Trypsin Digestion of the α Chain
Diagram illustrating the alternative pathways by which the α chain was degraded by trypsin and the origin and reactivity of the resulting fragment.

FIGURE 1d: Trypsin Digestion of Phosphoeluted α Chain
Diagram indicating the relationship between the intact α chain, the fragment generated by phosphoelution at the T1 site, and the products of trypsin digestion of the phosphoeluted α chain.

FIGURE 1e: Location of Antigens and Preparation of Proteolytic and Phosphoelution Fragments from the α Chain
This figure shows the regions of the α chain from which discrete proteolytic fragments are derived. The cut sites that generate these fragments are indicated on the top of the labeled doublet (by a small arrow) and a letter to indicate the process which inactivates the α chain (α or T2, respectively). The processes used to obtain a fragment, the relative molecular mass of the fragment, and its immunoactivity are denoted at right. Immunoreactivity is depicted by the presence or absence of a line and the density of the silver-stained gel. The regions which contain the epitopes recognized by antibodies αT and αK are indicated by shading labeled αT and αK, respectively. The size of each fragment is indicated by the size of the arrow. The regions from which the products of phosphoelution are derived are also shown. The scale is given at the bottom of the figure, which also indicates its scale (mm).