Structure of the \( \alpha \) and \( \beta \) Heavy Chains of the Outer Arm Dynein from Chlamydomonas Flagella

MASSES OF CHAINS AND SITES OF ULTRAVIOLET-INDUCED VANADATE-DEPENDENT CLEAVAGE*

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We report here on the UV-induced vanadate-dependent cleavage of the \( \alpha \) and \( \beta \) heavy chains of the outer arm dynein from Chlamydomonas flagella. Both polypeptides are cleaved at a single site (termed the V1 site) by UV irradiation in the presence of\( \text{Mg}^{2+} \), ATP, and vanadate. The \( \alpha \) chain yields fragments of \( M \), 290,000 and 190,000. Fragments of \( M \), 255,000 and 185,000 are obtained from the \( \beta \) chain. Ultraviolet irradiation of the \( \alpha \) and \( \beta \) chains in the presence of vanadate and \( \text{Mg}^{2+} \) (but no nucleotide) induces cleavage of both molecules at sites (termed the V2 sites) distinct from the V1 sites. The single V2 site within the \( \beta \) chain is located 75,000 daltons from the site of V1 cleavage within the \( M \), 255,000 V1 fragment. The \( \alpha \) chain contains three distinct sites of V2 cleavage; all are located within the \( M \), 290,000 V1 fragment, 60,000, 90,000, and 100,000 daltons from the site of V1 cleavage. From these studies, we estimate the masses of the \( \alpha \) and \( \beta \) heavy chains to be 480,000 and 440,000 daltons, respectively.

Dyneins are multimeric ATPases which function as mechanochemical force transducers during ciliary and flagellar beating (Gibbons, 1965; Gibbons and Gibbons, 1973, 1979; Huang et al., 1979; Mabuchi et al., 1976; Ogawa et al., 1977; Shimizu, 1975; for review see Johnson et al., 1984). Within the flagellar axoneme, these enzymes form discrete structures termed arms which project from the A-tubule of the outer doublet microtubules. During a cycle of ATP binding and hydrolysis, the dynein arms undergo conformational changes including a transient interaction with the B-tubule of the adjacent doublet that result in the sliding movement of each doublet microtubule with respect to its neighbor (Avolio et al., 1984; Goodenough and Heuser, 1984; Sale et al., 1985; Sale and Satir, 1977; Satir, 1968; Satir et al., 1981; Summers and Gibbons, 1971; Takahashi and Tomonura, 1978; Tsukita et al., 1983; Warner, 1978; Warner and Mitchell, 1978; Witman and Minervini, 1982). This sliding leads to axonemal bending (Satir, 1968; Shinglyogi et al., 1977).

In the biflagellate green alga Chlamydomonas, the outer dynein arm is composed of three discrete subunits. During purification the \( \alpha \) and \( \beta \) subunits remain associated and are isolated as a separate particle (Pfister and Witman, 1984; Pfister et al., 1982; Piperno and Luck, 1979; Watanabe and Flavin, 1976). Each subunit contains one very high molecular weight polypeptide, the \( \alpha \), \( \beta \), and \( \gamma \) heavy chains (\( M \), > 400,000), each of which has a site of ATP hydrolysis (Pfister et al., 1984, 1985). In addition, the \( \alpha \) subunit contains a single light chain of \( M \), 16,000; the \( \gamma \) subunit contains two light chains (\( M \), 18,000 and 22,000); and the \( \beta \) subunit contains two intermediate chains (\( M \), 78,000 and 69,000) and seven distinct light chains (\( M \), 8,000–20,000). The heavy chains from the outer dynein arm are immunologically distinct from each other and also from the intermediate chains of the \( \beta \) subunit (King et al., 1985; Mitchell and Rosenbaum, 1986).

Our goal has been to identify and locate sites of structural and functional interest within the heavy chains of the Chlamydomonas outer arm dynein and to examine the relationships between the various components of these very large protein complexes (King and Witman, 1985, 1986). Recently, Lee-Eiford et al. (1986) described a procedure that resulted in a single site-specific cleavage of each heavy chain from sea urchin sperm flagella dynein, yielding fragments of \( M \), 228,000 and \( M \), 200,000. To achieve this, the dynein preparation was first incubated with ATP and vanadate to form an ADP-vanadate complex, which acts as a dead-end kinetic block within the catalytic sites of these enzymes (Shimizu and Johnson, 1983). Prolonged ultraviolet irradiation of the dynein-ADP-vanadate complex then resulted in cleavage of the heavy chains through a reaction mechanism that is not yet understood. This reaction showed an absolute requirement for the presence of both the vanadate anion and the nucleotide, suggesting that the sites of cleavage (designated the V1 sites) occurred within the ATP-binding domains of these molecules (Gibbons et al., 1987; Lee-Eiford et al., 1986).

A second site of vanadate-dependent cleavage has also been found within each of the sea urchin sperm dynein heavy chains (Gibbons and Gibbons, 1987). Cleavage at these sites (designated V2), located approximately 100,000 daltons from the V1 sites, was obtained by UV irradiation of dynein in the presence of vanadate and \( \text{Mn}^{2+} \) and was completely inhibited by the addition of ATP or dithiothreitol. It is at present preparation, the complex composed of the \( \alpha \) and \( \beta \) subunits from the Chlamydomonas outer arm, which was previously termed "18 S dynein," is now referred to as the \( \alpha-\beta \) dimer. Similarly, the third subunit of the outer arm (previously 12 S dynein) is referred to as the \( \gamma \) subunit.

The molecular weights of the individual polypeptides of the \( \alpha-\beta \) dimer are 480,000, 440,000, 78,000, 69,000, 29,000, 19,000, 16,000, 14,000, 14,000, 11,000, 8,000, and 8,000. Quantitative densitometry of Coomassie Blue-stained gels indicates that all polypeptides are present in 1:1 molar ratios except for the two \( M \), 8,000 light chains, both of which appear to be present at a stoichiometry of five copies/\( \alpha-\beta \) dimer (K. K. Pfister and G. B. Witman, unpublished results).
Vanadate-dependent Cleavage of Chlamydomonas Dynein

In this report we describe the vanadate-dependent cleavage products of the α and β heavy chains from the Chlamydomonas outer arm dynein. Both polypeptides are cleaved at a single site (V1) in the presence of vanadate and ATP. The β chain also contains a single V1 site, whereas there are three discrete sites within the α chain at which V2 cleavage may occur. By using monoclonal antibodies specific for each polypeptide, we have been able to unambiguously locate the sites at which cleavage occurs within each molecule. Finally, examination of the complementary fragments has allowed us to accurately determine the M₄ of these very large proteins. These results will be useful for locating other regions of structural and functional significance, such as sites of ATP binding (Pfister et al., 1985) and phosphorylation (King and Witman, 1986; Piperno and Luck, 1981), within these polypeptides.

MATERIALS AND METHODS

Purification of Outer Arm Dynein—Flagella were isolated from Chlamydomonas reinhardtii strain 1132D (mating type +/+) by the dibucaine procedure (Pfister et al., 1982; Witman, 1986), demembranated with Nonidet P-40, and the resulting axonemes extracted with 0.6 M KCl. The α-β dimer was then separated from the γ subunit by sucrose density gradient centrifugation. For some experiments the dimer was further purified by hydroxyapatite column chromatography (King et al., 1986; Pfister et al., 1982).

Monoclonal Antibodies—The generation and characterization of monoclonal antibodies which specifically recognize the high molecular weight components of the Chlamydomonas outer arm dynein have been described previously (King et al., 1986). Two antibodies which recognize different epitopes within the α chain are designated 18αA and 18αB; two that react exclusively with different regions of the β chain are designated 18βB and 18βC. Supernatants from hybridoma cultures were used directly as the source of primary antibody in the immunostaining procedure described below.

UV-induced Vanadate-dependent Cleavage at the V1 Site—The purified α-β dimer was dialyzed against 1500 volumes of 30 mM Tris-HCl, pH 7.5, 0.5 mM EDTA, 5 mM MgSO₄, 74 mM NaCl, 1 mM phenylmethylsulfonyl fluoride (TEM buffer) for 6-18 h. Twenty-microliter aliquots containing ~5 μg of protein were placed in 500-μl micro test tubes (Bio-Rad) to each of which was added 10 μl of a 400 μM stock solution of NaVO₄·H₂O (no. 7260, Fluka Chemical Corp., Ronkonkoma, NY) in TEM and 10 μl of either 40 or 400 μM 8-azidoadenosine 5′-triphosphate (A-2392, Sigma), or adenosine 5′-triphosphate (A-5594, Sigma). Samples were mixed by vortexing and incubated at room temperature for 15 min. To examine the effects of vanadate or ATP alone, the appropriate volume of TEM buffer was added in place of the anion or nucleotide. After incubation, 5 μl of 10X dithiothreitol (D 0632, Sigma) was added as a free radical scavenger, and the tubes were then placed on ice and irradiated for 20-90 min with UV light at 254 nm (UVG-11 lamp, Spectroline EN-280L lamp, Spectrumons Corp., Westbury, NY; power output, 1300 microwatts/cm² at a distance of 15 cm) or 365 nm (Spectronline EN-280L lamp, Spectronetics Corp., Westbury, NY; power output, 1300 microwatts/cm² at 15 cm), with either lamp placed directly on top of the tubes. After 5 × concentrated electrophoresis sample buffer and 5 μl of β-mercaptoethanol were added, and the samples were boiled for 5 min prior to gel electrophoresis.

UV-induced Vanadate-dependent Cleavage at the V2 Sites—The α-β dimer, purified by sucrose density gradient centrifugation, was dialyzed overnight against 500 volumes of 10 mM HEPES, pH 7.5. One hundred microliter aliquots were placed in micro test tubes (Bio-Rad) and 1 mM NaVO₄·H₂O and 10 mM MnCl₂·4H₂O were added to final concentrations of 150 μM and 1 mM, respectively. The samples were mixed by vortexing, incubated at room temperature for 15 min, and then placed on ice and irradiated for 0-6 h at 365 nm. Samples were prepared for gel electrophoresis as described above.

Propynyl Digestion—Proteolytic digestion was initiated by adding 60 μl of 0-50 μg/ml trypsin (T 8003, Sigma) in TEM to 300-μl aliquots of the purified dynein (~40 μg of protein). After vortexing, samples were incubated at room temperature for 5 min. The proteolytic digestion was stopped by the addition of a 10-fold excess (by mass) soybean trypsin inhibitor (T 9003, Sigma). Following addition of vanadate to 100 μM and a 15-min incubation, dithiothreitol was added to 1 mM and the samples irradiated for 45 min at 254 nm prior to preparation for electrophoresis.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis and Protein Blotting—Dynein polypeptides and the fragments generated by vanadate-dependent cleavage and trypsin digestion were separated on either 0-2.4 M glycerol, 5-15% acrylamide, or 2-8 M urea, 3-5% acrylamide gradient gels as described previously (King et al., 1986; Pfister et al., 1982). The latter formulation was required to obtain adequate resolution of polypeptides of M₄ > 200,000, whereas the former was used to examine smaller molecules and fragments. After electrophoresis, polyacrylamide gels were silver-stained by the procedure of Merrill et al. (1981). Alternatively, the separated polypeptides were electrophoretically transferred to nitrocellulose sheets (BA-83; Schleicher and Schuell). The buffer and power conditions necessary for the efficient elution of very high molecular weight species from polyacrylamide gels and for their subsequent retention on the nitrocellulose sheets have been described previously in considerable detail (King et al., 1985, 1986; Otter et al., 1987).

RESULTS

UV-induced Cleavage in the Presence of Vanadate, ATP, and Mg²⁺ (the V1 Sites)—When purified α-β dimer in TEM was incubated with 10 or 100 μM ATP or 8-N³ATP (a hydrolyzable photoaffinity analog of ATP) and 100 μM vanadate and then subjected to a prolonged (20 min) UV irradiation at 254 nm, the amounts of α and β heavy chains detectable in silver-stained gels decreased. Concomitantly, four discrete fragments (M₄ 290,000, 255,000, 190,000, and 185,000) were formed (Fig. 1, lanes 6-13). Estimation of the masses of these fragments is described below. When samples in TEM were incubated and then irradiated in the presence of 100 μM vanadate without nucleotide, only the M₄ 290,000 and 190,000 fragments were obtained (Fig. 1, lane 3). Incubation of the dynein preparation with either 0, 10, or 100 μM 8-N³ATP (or ATP), but no vanadate, followed by irradiation for 60 min resulted in no detectable cleavage products from either polypeptide (Fig. 1, lanes 2, 4, and 5, respectively). However, the amounts of α and β heavy chains remaining in these samples were greatly reduced, probably as a result of random UV-induced breakage of the polypeptides. This conclusion is supported by the observation that increasing concentrations of 8-N³ATP had an increasingly protective effect on the proteins (Fig. 1, lane 5), presumably due to screening of the proteins from UV irradiation. Nonspecific degradation was also greatly reduced by irradiation at 365 nm, which induced cleavage in the presence of vanadate and ATP (data not shown). Cleavage of both polypeptides was dependent upon the nature of the divalent cation present in the reaction mix. Cleavage in the presence of nucleotide occurred when the divalent cation in the buffer was 5 mM Mg²⁺, Ca²⁺, or Zn²⁺; however, when Mn²⁺ was substituted for Mg²⁺ no specific fragments were obtained from either heavy chain (data not shown).

Both the M₄ 78,000 and 69,000 intermediate chains are labeled by photoaffinity analogs of ATP, suggesting that these...
polypeptides also contain sites of ATP binding (Pfister et al., 1985). However, no detectable cleavage products were obtained from either intermediate chain following UV irradiation of the α-β dimer in the presence of Mg²⁺, ATP, and vanadate (results not shown).

**Origin of the V1 Fragments**—In order to unambiguously identify the polypeptide from which each V1 fragment was derived, the immunoreactivity of the fragments was determined (Fig. 2). This analysis made use of four monoclonal antibodies, the specificities of which have been described previously (King et al., 1985, 1986). Two antibodies (18aA and 18aB), which react exclusively with the α heavy chain, recognized the fragments generated by UV cleavage in the presence of vanadate alone. Antibody 18aA reacted with the M₁, 190,000 fragment, whereas 18aB bound specifically to the larger M₁, 290,000 fragment. Neither antibody recognized any of the intermediate fragments, thus the M₁, 190,000 and M₁, 290,000 fragments are complementary portions of the α heavy chain. Antibody 18aB also detected a minor fragment (M₁, 200,000) in the irradiated but not the control sample; this fragment is derived from the α chain to which 18aB bound specifically (Fig. 2 and Table 1). Therefore, this fragment is derived from the α chain and was observed in samples which contained the M₁, 185,000 fragment and was always present in amounts approximately equimolar to the M₁, 185,000 fragment as judged by the intensity of the bands in silver-stained gels. This strongly suggests that the M₁, 255,000 fragment represents that portion of the β chain complementary to the M₁, 185,000 immunoreactive fragment.

**Tryptic Digestion and Mass of the Dynein Heavy Chains**—Two of the fragments obtained by V1 cleavage of the α and β chains migrate, in acrylamide gradient gels, within the linear range defined by well characterized molecular weight standards, including myosin (M₉, 205,000). The relative molecular masses of these fragments, therefore, can be estimated directly from the molecular weight standards to be M₁, 190,000, for that derived from the α chain, and M₁, 185,000, for that derived from the β chain.

The complementarity of the fragments extends more slowly than myosin. In order to obtain an accurate estimate of the molecular mass of the larger of these fragments, i.e. that derived from the α chain and recognized by antibody 18aB, the α-β dimer was digested with increasing concentrations of trypsin (Figs. 3 and 4) and then subjected to the V1 cleavage procedure (Fig. 4). At the trypsin concentrations used, very little or no digestion of the β chain was observed in either silver-stained gels (Fig. 3) or on nitrocellulose blots probed with antibodies against this polypeptide (data not shown). However, trypsin treatment did result in a single cleavage of the α chain to yield the very large fragment previously designated band 11 (Pfister and Witman, 1984; Pfister et al., 1982) and a complementary smaller peptide of M₁, 90,000 (Fig. 3). The band 11 fragment, which was recognized by both 18aA and 18aB (Fig. 4), was not observed in the samples which contained the M₁, 185,000 fragment.
Thus, the intact 90,000 fragment of the larger V1 cleavage fragment at a site 90,000 daltons from the intact fragment from the control dynein preparation (a high salt extract of axonemes, dialyzed into TEM buffer); the second lane, the same preparation after incubation with 100 μM 8-NT, ATP and 100 μM vanadate and irradiation for 45 min. The panel at far left was probed with antibody 18aA, and the adjacent panel with 18aB. The immunopositive bands migrating ahead of the α heavy chain in the controls are endogenous proteolytic fragments of that chain. The right-hand panels were incubated with antibodies 18βB and 18βC. The α and β heavy chains, band 11, and UV-induced fragments migrating at M, 290,000, 190,000, and 185,000 are indicated.

4, upper panels), was then subjected to V1 cleavage, and the resulting fragments identified by immunoblot analysis (Fig. 4, lower panels). Antibody 18αA recognized a fragment of M, 190,000; this fragment is identical, in mass and immunoreactivity, to that obtained by cleavage of the intact α chain. Antibody 18αB recognized the M, 290,000 fragment obtained by cleavage of the α chain and a fragment of M, 200,000 seen only in those samples which initially contained band 11 and, therefore, derived from that band. The tryptic site that generates band 11 and the M, 90,000 fragment thus occurs within the larger V1 cleavage fragment at a site 90,000 daltons from one terminus of the original molecule. These results indicate that band 11 must have a mass of 390,000 daltons (190,000 + 200,000); the largest fragment obtained by V1 cleavage of the α chain must have a mass of 290,000 daltons (200,000 + 90,000); and the intact α chain must have a mass of 480,000 daltons (190,000 + 200,000 + 90,000).

By using the M, 290,000 fragment of the α chain as an additional molecular weight standard, the mass of the largest fragment from the β chain was estimated at 255,000 daltons. Thus, the β chain has a mass of 440,000 daltons (255,000 + 185,000).

The locations of the V1 cleavage sites within both the α and β chains, the site of tryptic cleavage within the α chain that generates band 11, and the regions of both molecules that contain the epitopes recognized by our monoclonal antibodies are depicted, diagrammatically, in Fig. 5.

![Diagram](image_url)

**Fig. 2. Immunoreactivity of the V1 cleavage fragments.** Each panel shows a nitrocellulose replica of two lanes from a 3-5% acrylamide gradient gel. The left-hand lane of each panel shows the control dynein preparation (a high salt extract of axonemes, dialyzed into TEM buffer); the second lane, the same preparation after incubation with 100 μM 8-NT, ATP and 100 μM vanadate and irradiation for 45 min. The panel at far left was probed with antibody 18αA, and the adjacent panel with 18αB. The immunopositive bands migrating ahead of the α heavy chain in the controls are endogenous proteolytic fragments of that chain. The right-hand panels were incubated with antibodies 18βB and 18βC. The α and β heavy chains, band 11, and UV-induced fragments migrating at M, 290,000, 190,000, and 185,000 are indicated.

![Graph](image_url)

**Fig. 3. Tryptic digestion of the α chain generates band 11 and a fragment of M, 90,000.** The upper panel shows the high molecular weight region of a silver-stained 3-5% acrylamide gradient gel. Lanes were loaded, from left to right, with 6 μg of the α-β dimer after digestion with 0, 2.5, 5, 25, 37.5, 50, and 100 ng of trypsin. The lower panel shows the intermediate molecular weight range (from M, 50,000–110,000) of a silver-stained 5-15% acrylamide gradient gel loaded with the same gel samples. The α and β heavy chains (α, β), the two complementary fragments of the α chain ("11", 90) and the two intermediate chains (78, 69) of the β subunit are indicated.

UV-induced Cleavage in the Presence of Vanadate and Mn²⁺ (the V2 Sites)—When the α-β dimer was incubated with 1 mM Mn²⁺ and 150 μM vanadate and then subjected to long wavelength UV irradiation, cleavage of both heavy chains was observed (Figs. 6 and 7). The amount of α chain present in the samples decreased rapidly, and this polypeptide was undetectable in samples irradiated for longer than 3.5 h. Concomitant with the decrease in the amount of α chain, six discrete fragments (M, 290,000, 280,000, 250,000, 230,000, 200,000, and 190,000) appeared. The M, 290,000, 280,000, and 250,000 fragments were recognized by both 18αB (Fig. 7) and 18αA (data not shown), whereas the remaining fragments were not immunoreactive. Because the mass of the α chain is 480,000 daltons, the complementary pairs of fragments must be 250,000 and 230,000, 280,000 and 200,000, and 290,000 and
FIG. 4. V1 cleavage of the band 11 tryptic fragment from the α chain.
Each panel shows a nitrocellulose replica of a 3–5% acrylamide gel. In the upper panels, lanes were loaded with 4.2 μg of α-β dimer that had been digested with, from left to right, 0, 6, 15, 31, 63, and 126 ng of trypsin to generate band 11 ("11"). The lower panels were loaded with the same samples after incubation with 100 μM vanadate and 100 μM 8-NADP for 15 min and UV irradiation at 254 nm for 45 min. The panels at left were immunostained with antibody 18αA, those at right with 18αB. The M, 290,000 (290) and 200,000 (200) fragments are derived from the α heavy chain (α) and band 11, respectively, and are complementary to the M, 190,000 (190) fragment.

190,000; the sites of cleavage which generated these pairs are referred to as V2a, V2b, and V2c, respectively (see Table I and Fig. 8). Inasmuch as the larger fragment from each pair was recognized by both 18αA and 18αB, the sites of cleavage must all occur within the M, 290,000 V1 fragment, 230,000, 200,000, and 190,000 daltons from the original terminus of the molecule (Fig. 8). When the time of irradiation was increased, a decrease in the amounts of the M, 290,000, 280,000, 230,000, and 200,000 fragments was observed, with a corresponding increase in the intensity of the bands which migrated at M, 250,000 and 190,000. This indicates that, within a given molecule, V2 cleavage occurred at more than one site with the result that the smallest fragments containing the original terminus of the α chain accumulated with time. This predicts that additional fragments of M, 40,000, 30,000, and 10,000 should also exist. An M, 40,000 fragment apparently derived from cleavage of the α chain at both the V2a and V2c sites has been observed in silver-stained gels, but because this fragment is not immunoreactive it has not been possible to unambiguously determine its origin.

The β chain was more resistant to V2 cleavage; detectable amounts of this polypeptide were observed in samples irradiated for as long as 5 h. However, after extensive irradiation, V2 cleavage of the β chain did occur at a single site yielding fragments of M, 260,000 and 180,000 (Fig. 6). The former fragment contained the epitopes recognized by 18βC (Fig. 7) and 18βB (data not shown). Thus, the single site of V2 cleavage within the β chain must occur within the M, 255,000 V1 fragment, 180,000 daltons from the terminus of the original molecule (Fig. 8).

Fig. 5. Location of V1 cleavage sites within the α and β chains. This diagram shows the positions of the V1 cleavage sites within the α and β chains (large arrows). The small arrow indicates the site at which trypsin cleaves the α chain to yield fragments of M, 90,000 (90) and 390,000 (band 11). The regions of these polypeptides that contain the epitopes recognized by each antibody are indicated by shading. Within the α chain 18αA (A) recognizes the M, 190,000 V1 fragment (190); 18αB (B) reacts with that region of the molecule between the V1 and tryptic sites. Both anti-β chain antibodies, 18βB (B) and 18βC (C), recognize the M, 185,000 V1 fragment (185). The scale of the maps is shown at the bottom of the figure; units are daltons (× 10^4).
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FIG. 6. UV-induced vanadate-dependent cleavage of the α and β chains at the V2 sites. This figure shows the high molecular weight region of a silver-stained 3–5% acrylamide gradient gel. Lanes were loaded, from left to right, with 4.1 µg of hydroxyapatite-purified α-β dimer irradiated at 365 nm for 0, 2, 5, 10, 20, 30, 45, 60, 90, 120, 150, 180, 210, 240, 300, and 360 min, respectively, in the presence of 150 µM vanadate and 1 mM Mn2+. The positions of the α and β heavy chains and the eight major fragments (M, 290,000, 280,000, 260,000, 250,000, 230,000, 200,000, 190,000, and 180,000) are indicated.

FIG. 7. Immunoreactivity of the V2 cleavage fragments. Nitrocellulose replica of a 3–5% acrylamide gradient gel that was loaded with the same samples of hydroxyapatite-purified α-β dimer (irradiated for various lengths of time in the presence of Mn2+ and vanadate) as shown in Fig. 6. The blot was first probed with antibody 18βC and then with 18αA; the combined result of the two probes is shown.

DISCUSSION

Ultraviolet irradiation of the α-β dimer from the Chlamydomonas outer dynein arm, after incubation with Mg2+, ATP, and vanadate, resulted in the specific cleavage of both the α and β heavy chains. The α chain yielded two fragments. The larger fragment (M, 290,000) was recognized by antibody 18αA, whereas the smaller fragment (M, 190,000) was recognized by antibody 18αA. The β chain was cleaved to fragments of M, 255,000 and 185,000. The latter fragment contained the epitopes recognized by both 18βB and 18βC. Irradiation of the α-β dimer after incubation with Mg2+ and vanadate but no nucleotide resulted in the cleavage of the α chain alone. Again fragments of M, 290,000 and 190,000, recognized by 18αB and 18αA, respectively, were obtained. Thus, by the criteria of mass and immunoreactivity of the fragments, V1 cleavage of the α chain in the presence of either vanadate and ATP or vanadate alone occurred at the same site. Substitution of Ca2+ or Zn2+ for Mg2+ also supported V1 cleavage of the α and β chains. However, when Mg2+ was replaced with Mn2+, cleavage did not occur in the presence of nucleotide, even though MnATP2− is a suitable substrate for dynein-mediated ATP hydrolysis (Ogawa and Mohri, 1972; King and Witman, 1987).

When the α-β dimer was irradiated at 365 nm in the presence of vanadate and Mn2+ but no nucleotide, both heavy chains were cleaved at sites distinct from the V1 sites. The β chain was cleaved to yield fragments of M, 260,000 and 180,000. The M, 260,000 fragment was recognized by antibodies 18βB and 18βC; therefore, the site of V2 cleavage occurred with the M, 255,000 V1 fragment at a point 75,000 daltons from the V1 site. V2 cleavage of the α chain occurred at three discrete sites (designated V2a, V2b, and V2c). Cleavage at the V2a site resulted in fragments of M, 250,000 and 230,000. The larger fragment was recognized by both anti-α chain antibodies; therefore, this site of cleavage occurred within the M, 290,000 V1 fragment, 100,000 daltons from the V1 site. V2 cleavage of the α chain at the V2b and V2c sites yielded fragments of M, 280,000 and 200,000, and 290,000 and 190,000, respectively. Again, the larger fragments were immunoreactive, indicating that these sites of cleavage also occurred within the M, 290,000 V1 fragment (90,000 and 100,000 daltons from the
FIG. 8. Location of the V2 cleavage sites within the α and β chains. This figure diagrams the regions of the α and β chains which correspond to the fragments observed following UV irradiation of dynein in the presence of vanadate and Mn²⁺. The sites at which V2 cleavage occurred to generate a particular set of fragments are indicated by the large arrows labeled V2a, V2b, and V2c. Shaded boxes indicate the regions containing epitopes recognized by antibodies 18αA, 18αB, 18βB, and 18νC. The scale is indicated at the bottom of the figure; units are daltons (× 10⁻²).

| Polypeptide | Cleavage site | Fragments | Immunoactivity |
|-------------|---------------|-----------|----------------|
| α           | V1 (290)      | 18αB      |                |
|             | V2a (230)     | 18αA 18αB |                |
| α           | V2b (200)     | 18αA 18αB |                |
| α           | V2c (290)     | 18αA 18αB |                |
| β           | V1 (190)      | 18βB 18νC |                |
| β           | V2 (180)      | 18βB 18νC |                |

V1 site, respectively. As the time of UV irradiation was increased, the intensity of the bands migrating at M, 290,000, 280,000, 230,000, and 200,000 decreased with a concomitant increase in the intensity of those migrating at M, 250,000 and 190,000. This indicates that cleavage of the α chain at one V2 site does not preclude cleavage of that same polypeptide at a second or a third site.

The sites of V1 cleavage are presumed to occur within the ATP hydrolytic domains of the dynein heavy chains (Lee-Eiford et al., 1986; Gibbons et al., 1987). This conclusion is based on the observation that vanadate-sensitized photocleavage of sea urchin sperm dynein at the V1 site occurs only in the presence of ATP, ADP, or other substrates, which probably help stabilize the vanadate anion at the γ-phosphate binding site (Gibbons et al., 1987). Our finding that V1 cleavage of the α chain of *Chlamydomonas* outer arm dynein can occur in the absence of nucleotide indicates that, at least in *Chlamydomonas*, Mg²⁺-vanadate alone is capable of binding to the hydrolytic site. The specific mechanism of vanadate-sensitized cleavage is not clear. However, the vanadate anion may rapidly esterify the hydroxyl group of tyrosine (Tracey and Gresser, 1986), and the oxovanadium (IV) cation (VO⁴⁺) may complex with the imidazole ring of histidine (Mulks et al., 1982). These observations have led to the proposal that such aminoacyl residues within the catalytic sites might be essential for vanadate-dependent cleavage (Gibbons et al., 1987).

The locations of the V2 sites are less certain. Under the conditions of pH and vanadate concentration used here to obtain V2 cleavage, the vanadate anion probably exists in both monomeric (H₂VO₄⁻) and polymeric forms (Cotton and Wilkinson, 1972). The latter include trimeric (V₃O₁₀⁻⁻) (Ingri and Brito, 1968; Howarth and Richards, 1986; Wells and Bagshaw, 1984) and tetrameric (V₄O₁₃⁻⁻) (Simon and Jahr, 1964; Tytko and Mehmke, 1983) species, both of which are thought to be cyclic structures (for review of the formation and structure of isopolyvanadates in solution, see Pope and Dale, 1968). It is possible that paramagnetic Mn²⁺ (but not diamagnetic Mn²⁺) coordinates with trimeric or tetrameric vanadate to form a complex which binds to part(s) of the hydrolytic domain (such as the sites normally occupied by the adenine and/or ribose sugar rings or by the α and β phosphates of ATP) not available in the presence of nucleotide. The interaction of trimeric or tetrameric vanadate with three separate aminoacyl residues within the α chain would then explain the observed presence of three sites of V2 cleavage in this polypeptide. In this case, the fact that only a single V2 site was observed within the β chain would suggest that there are differences in the primary structure of the hydrolytic domains from these two molecules. Assuming that there is only one hydrolytic domain/heavy chain, each chain would have to be extensively folded to bring its vanadate-dependent cleavage sites, which are separated by 60,000–100,000 daltons, within the hydrolytic domain.

Alternatively, the V2 sites may define one or more vanadate-binding domains separate from the ATP hydrolytic sites.
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Such sites could represent either catalytic or noncatalytic nucleotide or phosphate-binding domains. Experiments are currently underway to investigate these possibilities.

By determining the masses of complementary fragments obtained by tryptic and V1 cleavage of the α chain we were able to estimate the mass of the intact chain. Low concentrations of trypsin cleaved the α chain at a specific site to yield two discrete fragments: one of M, 90,000 that was not immunoreactive and a large complementary fragment (previously referred to as band 11 (Pfister et al., 1982; Pfister and Witman, 1984)) that was recognized by both 18aA and 18aB. Cleavage of band 11 at the V1 site resulted in a fragment of M, 190,000, identical to that obtained by V1 cleavage of the α chain, and a second fragment of M, 200,000 that was recognized by 18aB. Thus, the larger V1 fragment from the α chain must contain the tryptic site that is cleaved to yield band 11; this site is located 90,000 daltons from one terminus of the molecule and 200,000 daltons from the site of V1 cleavage. These results indicate that the larger V1 fragment from the α chain has a mass of 290,000 daltons. From the sums of the masses of their fragments, the sizes of the intact α and β heavy chains are estimated at 480,000 and 440,000 daltons, respectively.

These values for the intact chains are considerably larger than previous estimates based on electrophoretic studies where it was necessary to extrapolate from markers of known mass (Pfister et al., 1982; Piperno and Luck, 1979). However, the new values for the masses of the α and β chains agree extremely well with the value obtained for the native mass of the α-β dimer by scanning transmission electron microscopy (Witman et al., 1983). From the STEM analysis the mass of the dimer was found to be 1,220,000 daltons with a standard deviation from the mean of 117,000 daltons. Using the new values for the α and β heavy chains, the sum of the masses of the individual polypeptide chains of the α-β dimer is 1,240,000 daltons.

The STEM study also demonstrated that the α-β dimer is composed of two globular domains interconnected via a Y-shaped stalk. Each globular unit had a mass of approximately 375,000 daltons. Therefore, each head is too small to accommodate an entire heavy chain; both the α and β chains must contribute mass (perhaps as much as 100,000 daltons in the case of the α chain) to the stalk components of the dynein complex.

The α and β chains share a defined polarity; in both polypeptides the site(s) of V2 cleavage occurs within the larger V1 fragment (Fig. 8). Therefore, we predict that both molecules exhibit the same orientation with respect to their NH₂ and COOH termini. The maps shown in Figs. 5 and 8 have been drawn so as to reflect this prediction.

The studies reported here have highlighted a number of differences between the heavy chains of the α-β dimer. First, the α chain may be cleaved at the V1 site following incubation with Mg²⁺-vanadate alone, whereas the β chain also requires the presence of ATP (or ADP). This suggests that Mg²⁺-vanadate resides in the catalytic site of the α chain sufficiently long even in the absence of nucleotide to allow cleavage to occur, whereas a Mg²⁺-ADP-vanadate complex is required to immobilize the anion within the active site of the β chain. Second, three distinct sites of V2 cleavage have been identified within the α chain, but only one was observed within the β chain. Third, the mass of the α chain is approximately 40,000 daltons greater than that of the β chain. Other differences between the Chlamydomonas dynein α and β heavy chains and their active sites have been noted previously.

Pfister and Witman (1984) found the Mg²⁺-ATPase activity of the purified α subunit to be 0.6 μmol of phosphate released/min/mg, whereas the activity of the β subunit was approximately 10-fold higher (7.9 μmol of phosphate released/min/mg). King et al. (1985) used monoclonal antibodies to demonstrate that the α and β heavy chains are immunologically distinct from each other. Also, the α chain is phosphorylated in vivo whereas the β chain is not (King and Witman, 1986; Piperno and Luck, 1981). The large number of differences between these two chains suggests that they have distinctly different roles in the production and/or regulation of shearing forces during flagellar movement.

The vanadate-dependent cleavage procedures have also been applied to the outer arm dynein from sea urchin sperm flagella (Lee-Eiford et al., 1986; Gibbons and Gibbons, 1987; Gibbons et al., 1987). Both heavy chains, although separable by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, give rise to V1 fragments which migrate at M, 228,000 and 200,000. V2 fragment pairs of M, 170,000 and 260,000, and 175,000 and 255,000 are obtained from the α and β chains, respectively; the V2 sites are located within the larger of the V1 fragments. The relative positions of the V1 and V2 sites in the dynein heavy chains are thus similar in Chlamydomonas and sea urchin sperm.

The V1 and V2 cleavage procedures will prove to be extremely useful in mapping the locations of other domains within the α and β heavy chains, because 1) they have allowed accurate estimates of the masses of these very large polypeptides and 2) they mark specific sites within these molecules, with respect to which other sites of interest may be mapped.

We have utilized the sites of V1 and V2 cleavage, in conjunction with a number of endoproteases, to further define the regions which contain the epitopes recognized by our antibodies, to locate protease-sensitive sites, and to define the domains covalently modified by photoaffinity analogs of ATP (King and Witman, 1986, 1987).

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