Structure of the γ Heavy Chain of the Outer Arm Dynein from Chlamydomonas Flagella

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Abstract. We describe here the vanadate-dependent photocleavage of the γ heavy chain from the Chlamydomonas outer arm dynein and the pathways by which this molecule is degraded by endoproteases. UV irradiation in the presence of ATP, Mg²⁺, and vanadate cleaves the γ chain at a single site (termed V1) to yield fragments of Mₚ 235,000 and 180,000. Irradiation in the presence of vanadate and Mn²⁺ results in cleavage of the γ chain at two other sites (termed V2a and V2b) to yield fragment pairs of Mₚ 215,000/200,000 and 250,000/165,000. The mass of the intact chain is therefore estimated to be 415,000 D. We have located the major tryptic and staphylococcal protease cleavage sites in the γ chain, determined the origins of the resulting fragments, and identified the regions which contain the epitopes recognized by two different monoclonal antibodies. Both antibodies react with the smaller V1 fragment; the epitope recognized by antibody 25-8 is within 9,000–52,000 D of the original γ-chain terminus contained in that fragment, whereas that recognized by antibody 12γB is within 16,000 D of the V1 site. The data permit the construction of a linear map showing the structural organization of the polypeptide. The substructure of the γ chain is similar to that of the α and β chains of the outer arm dynein with regard to polarity as defined by the sites of vanadate-dependent photocleavage, and to that of the β chain with regard to a highly sensitive protease site located ~10,000 D from the original terminus contained in the smaller V1 fragment.

Dyneins are mechanochemical transducers which use the hydrolysis of ATP to generate the forces required for interdoublet microtubule sliding within the flagellar axoneme (Gibbons, 1965; Huang et al., 1979; Paschal et al., 1987a; Shimizu, 1975; Takahashi and Tonomura, 1978; for reviews see Gibbons, 1981, and Johnson et al., 1984); this interdoublet sliding leads directly to flagellar movement (Satir, 1968; Shingyogi et al., 1977). Dynein-like enzymes also have been purified from bovine brain (Pallini et al., 1982, 1983; Paschal et al., 1987b), echinoderm eggs (Pratt, 1980), and the nematode Caenorhabditis elegans (Lye et al., 1987), an organism that does not contain flagella. Dyneins exhibit ATPase activity (Pfister and Witman, 1984; Pfister et al., 1982; Piperno and Luck, 1979). Each subunit exhibits ATPase activity (Pfister and Witman, 1984; Pfister et al., 1984, 1985), and studies using photoaffinity analogs of ATP have demonstrated that each heavy chain contains a site of ATP binding (Pfister et al., 1984, 1985).

To further elucidate the molecular basis for the mechanochemical properties of the dynein ATPases, we have examined the substructure of the Chlamydomonas outer arm dynein heavy chains. Previously, we described the organization of the α and β heavy chains (Mₚ >400,000) associated with a number of intermediate and/or light chains (King et al., 1986; King and Witman, 1988a; Pfister et al., 1982; Piperno and Luck, 1979). Each subunit exhibits ATPase activity (Pfister and Witman, 1984; Pfister et al., 1984, 1985), and studies using photoaffinity analogs of ATP have demonstrated that each heavy chain contains a site of ATP binding (Pfister et al., 1984, 1985).

This is the third paper in a series describing the substructure of the Chlamydomonas dynein chains. Previous reports have detailed properties of the α and β chains (1987. J. Biol. Chem. 262:17596-17604; 1988. J. Biol. Chem. 263:9244-9255).
V2 site(s) of cleavage occur within the larger fragment produced by cleavage at the V1 site. The V1 and V2 sites are thought to correspond to portions of the polypeptide that interact with ATP; these sites are separated by 75,000 D in the β chain and by up to 100,000 D in the α chain, suggesting that each chain is highly folded to bring these distant sites together within its ATP hydrolytic domain.

More recently, we have used a series of monoclonal antibodies specific for the α and β heavy chains to probe partial proteolytic digests of the chains (King and Witman, 1988b). The results enabled us to locate a number of protease-sensitive sites within the chains, to identify the regions of each molecule from which specific fragments derived, and to determine which regions contain the epitopes recognized by our monoclonal antibodies. For the purposes of structural mapping, the data revealed that each molecule could be divided into three distinct regions: a large (Mr ∼300,000) central section which contains the sites of vanadate-dependent photocleavage, and two flanking regions of 70,000–90,000 D. One of the flanking regions of the β chain contained a distinct segment of 10,000 D that was preferentially removed by proteases; this segment was located at the original β chain terminus contained in the smaller V1 fragment.

In this report, we describe substructural features of the γ heavy chain from the Chlamydomonas outer arm dynein. This polypeptide contains a single V1 site and two V2 sites of photocleavage. From the sums of the masses of the fragments, the γ heavy chain is estimated to have a mass of 415,000 D. Examination of the immunoreactivity of fragments generated by endoproteolysis of the γ chain has enabled us to locate the regions of this molecule that contain the epitopes recognized by two different monoclonal antibodies, to identify a number of sites susceptible to proteolysis, and to determine the origins of the resulting fragments. The results reveal structural similarities between this chain and the α and β chains, and permit the construction of a linear map of γ chain substructure that will be very useful for locating other regions of structural and functional interest.

Materials and Methods

Purification of the γ Subunit of the Outer Arm Dynein

Flagella were isolated from Chlamydomonas reinhardtii strain 1132D by the dibucaine procedure and demembranated with NP-40 (Witman, 1986). The outer arm dynein was extracted from the flagellar axonemes by treatment with 0.6 M KCl and the γ subunit subsequently isolated by sucrose density gradient centrifugation. For some experiments, the γ subunit was further purified by hydroxylapatite column chromatography (King et al., 1986; Pfister et al., 1982).

Monoclonal Antibodies

Two monoclonal antibodies which react specifically with the γ heavy chain were used in this study. The generation and characterization of antibody 12γB has been described previously (King et al., 1985). Antibody 25–8 was kindly provided by Dr. Gianni Piperno (Rockefeller University, New York).

Vanadate-dependent Photocleavage

For cleavage at the V1 site, the purified γ subunit or the 0.6 M KCl extract was dialyzed into 30 mM Tris-HCl pH 7.5, 0.5 mM EDTA, 5 mM MgSO4, 1 mM phenylmethyl sulfon fluoride; ATP (A5394; Sigma Chemical Co., St. Louis, MO) or 8-N3ATP (A2392; Sigma Chemical Co.) and vanadate (No. 7260; Fluka Chemical Corp., Ronkonkoma, NY) were then added and the samples irradiated, on ice, at either 365 nm using a Spectronics EN-280L lamp (Spectronics Corp., Westbury, NY; power output 1,300 μWcm⁻² at 15 cm) or at 254 nm with a UVG-11 lamp (UltraViolet Products Inc., San Gabriel, CA; power output 580 μWcm⁻² at 15 cm). In the latter case, dithiothreitol (DO632; Sigma Chemical Co.) was also added to the sample as a free radical scavenger (final concentration 1 mM) to reduce the amount of nonspecific photolyis that occurs upon prolonged irradiation at 254 nm (Lee-Eiford et al., 1986; Gibbons et al., 1987; King and Witman, 1987).

For cleavage at the V2 sites, the isolated dynein was dialyzed into 10 mM Hepes pH 7.5; 1 mM MnCl₂ and the indicated concentration of vanadate was then added, and the samples cleared by irradiation at 365 nm (Gibbons and Gibbons, 1987; King and Witman, 1987).

Proteolytic Digestion

Digestion of the γ chain by Nα-tosyl-L-phenylalnine-chloro-methyl-ketone-treated trypsin (T8542; type XIII; Sigma Chemical Co.) was performed as described previously (King and Witman, 1988b). For cleavage with Staphylococcus aureus V8 protease, 10 μl of a 1 mg/ml stock solution of the protease (No. 399001; Miles Laboratories, Inc., Naperville, IL) was added per milliliter of γ subunit (∼50 μg). Aliquots were removed after various periods of time and prepared for electrophoresis by the addition of 5 × concentrated electrophoresis sample buffer, β-mercaptoethanol (final concentration 10% vol/vol), and boiling for 5 min. For certain experiments, the concentration of staphylococcal protease rather than the time of incubation was varied.

Gel Electrophoresis and Phophoryl Staining

Samples were separated in either 2–8 M urea, 3–5% acrylamide, or 0–2.4 M glycerol, 5–15% acrylamide gradient gels (King et al., 1986; Pfister et al., 1982). After electrophoresis, gels were either silver stained (Merril et al., 1981) or the separated proteins were electrotransferred to nitrocellulose sheets and immunostained (King et al., 1985, 1986; Otter et al., 1987).

Calibration of gels and determination of the relative molecular masses of γ chain fragments were as described previously (King and Witman, 1987, 1988).

Results

Photocleavage at the V1 site

When the γ subunit of the Chlamydomonas outer arm dynein was irradiated at 254 or 365 nm in the presence of 5 mM Mg²⁺, 50 μM vanadate and either 0, 10, or 100 μM ATP (or 8-N3ATP, a photoaffinity analog of ATP), the amount of γ heavy chain detectable in silver-stained gels decreased. Accompanying this decrease, two new peptide species of Mr 235,000 and 180,000 appeared (Fig. 1 a). Vanadate (but not nucleotide) was required for photocleavage at the V1 site; no specific cleavage products were obtained when vanadate was omitted from the sample. Immunoblot analysis, using monoclonal antibodies 12γB and 25–8 that react specifically with the γ heavy chain, revealed that both antibodies recognized the M, 180,000 fragment (Fig. 1 b). This result confirmed that the M, 180,000 fragment is derived from the γ chain, and indicated that the 12γB and 25–8 epitopes must be located in that portion of the molecule between the V1 site and the original γ chain terminus contained in the M, 180,000 fragment (Fig. 1 c).

The M, 235,000 fragment was recognized by neither anti-γ chain antibody (Fig. 1 b). However, this fragment appeared concomitantly with the M, 180,000 fragment and was always present in an equimolar ratio as estimated from the intensity of bands in Coomassie Blue-stained gels. As these samples did not contain significant amounts of other dynein heavy chains (Fig. 1 a), the M, 235,000 fragment must derive from the V1-cleaved γ chain as the complement to the M, 180,000 fragment (Fig. 1 c). Thus, the γ chain
contains a single V1 site at which cleavage may occur and has a mass of 415,000 D (235,000 + 180,000).

**Photocleavage at the V2 sites**

Irradiation of the γ heavy chain at 365 nm in the presence of 150 μM vanadate and 1 mM Mn²⁺ generated four discrete fragments (M, 250,000, 215,000, 200,000, and 165,000) that were detectable in silver-stained gels (Fig. 2 a). The M, 250,000 and 215,000 fragments contained the epitopes recognized by antibodies 12γB (Fig. 2 b) and 25–8 (see Fig. 3 b); the M, 200,000 and 165,000 fragments were not immunoreactive. Therefore, under these conditions the γ chain is cleaved at two sites (termed V2a and V2b) to yield fragment pairs of M, 215,000/200,000 and 250,000/165,000, respectively. As the larger fragment of each pair contained both epitopes, the V2a and V2b sites must occur within the larger (M, 235,000) V1 fragment, 35,000 and 70,000 D, respectively, from the V1 site (Fig. 2 c). Two additional minor fragments of M, 240,000 and 160,000 were also observed in the samples subjected to cleavage at the V2 sites (Fig. 2 a). As neither of these fragments was immunoreactive, it is unlikely that they derive from the γ chain; they probably represent photocleavage products of the small amount of inner arm dynein heavy chain present in these sucrose gradient-purified samples (Pfister et al., 1982).

The amount of the M, 250,000 fragment obtained by cleavage at the V2b site was not comparable to that of the complementary M, 165,000 fragment (Fig. 2 a). This indicates that after cleavage at the V2b site, γ chain molecules also may be cleaved at the V2a site, leading to the accumulation of the M, 165,000 and 215,000 fragments. The M, 200,000 fragment generated by cleavage at the V2a site did not appear to be converted to the M, 165,000 fragment. This suggests that cleavage of the γ chain at the V2a site precludes the subsequent cleavage of the same molecule at the V2b site.

It is also possible that, in a small proportion of γ chain molecules, a simultaneous double scission at both the V2a and V2b sites occurs.

The reactions leading to photocleavage at the V1 and V2 sites exhibited different dependencies on the vanadate concentration. The γ chain was cleaved at the V1 site by irradiation for 30 min at 365 nm in the presence of as little as 1 μM vanadate (Fig. 3 a). In contrast, no cleavage at the V2 sites was observed after irradiation for 30 min in the presence of up to 50 μM vanadate (Fig. 3 b). Some cleavage at the V2 sites did occur at 100 μM vanadate, but cleavage was much more rapid at higher levels (0.5–1 mM). These observations indicate that cleavage at the V2a and V2b sites occurs under similar conditions, and provides further evidence that the vanadate species required for photocleavage at the V2 sites are oligomeric (see Discussion).

**Tryptic Digestion of the γ Chain**

When the γ chain was digested with trypsin, the first cut occurred at a site (Tγ1) ~9,000 D from one terminus to generate an immunoreactive fragment of M, ~406,000 (γT406; see footnote 1 for nomenclature of fragments) that was recognized by antibodies 12γB and 25–8 (Fig. 4, a and b, lanes 2–7); estimation of the size of the M, 406,000 fragment is based on the analysis of tryptic fragments of the photocleaved chain described immediately below. To unambigu-

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1. Nomenclature of fragments and protease-sensitive sites follows the conventions described previously for fragments of, and cut sites within, the α and β chains (King and Witman, 1988b). For example, T1γ refers to a tryptic cut site within the γ chain. Cleavage at this site generates fragments of M, 363,000 and 43,000, which are designated γ1T363 and γ1T43. The fragments generated by staphylococcal protease (SP) and vanadate-dependent photocleavage are designated in a similar manner.
Figure 2. Cleavage of the γ chain at the V2 sites. (a) High molecular weight region of a silver-stained 2–8 M urea, 3–5% acrylamide gradient gel (SS). Lanes were loaded, from left to right, with 3 μg sucrose gradient-purified γ subunit that had been irradiated at 365 nm for 0, 15, 30, 45, 60, and 90 min in the presence of 150 μM vanadate and 1 mM Mn²⁺. The positions at which the γ chain and V2 fragments migrated are indicated at left. (b) Nitrocellulose replica of a similar gel loaded with samples identical to those shown in a and immunostained with antibody 12γB. Immunoreactive species are indicated at right. (c) Diagram illustrating the locations of the V2a and V2b cleavage sites within the γ chain, and the relationships between the various fragments. γ chain molecules cleaved at the V2b site subsequently may be cleaved at the V2a site. The numbers beside each fragment indicate its mass in daltons (× 10⁻³).

ously determine the location of this tryptic site, the γ chain was cleaved at the V1 site before proteolytic digestion. As demonstrated in the previous section, cleavage of the γ chain at the V1 site yielded a fragment of Mr 180,000 that was recognized by both anti-γ chain antibodies (Fig. 4, a and b, lane 1). Upon incubation of the photocleaved products with trypsin, the Mr 180,000 fragment was degraded and an immunoreactive peptide of Mr 171,000 obtained. Conversion of the Mr 180,000 fragment to the Mr 171,000 fragment occurred under the same conditions as conversion of the γ chain to the Mr 406,000 fragment, and therefore must also have resulted from cleavage at the Tγ site. Because the Mr 171,000 fragment was observed only in samples cleaved at the V1 site, it must contain one of the new termini generated by photocleavage. Therefore, the tryptic site that generates γ₄₀₆ and the Mr 171,000 fragment must be located ~9,000
Figure 3. Effect of vanadate concentration on photolysis at the V1 and V2 sites. Nitrocellulose replicas of 2–8 M urea, 3–5% acrylamide gradient gels probed with antibody 25–8. (a) Photolysis at the V1 site. Each lane was loaded with 4 lag sucrose gradient-purified 7 subunit that had been incubated with 50 ~atM ATP, 5 mM Mg2+ and, from left to right, 0, 1, 5, 10, 50, 100, 500, and 1,000 ~atM vanadate for 15 min before irradiation at 365 nm for 30 min. (b) Photolysis at the V2 sites. Lanes were loaded with 4 ug 7 subunit that had been incubated with 1 mM Mn2+ and the vanadate concentrations indicated in a for 15 min and then irradiated at 365 nm for 30 min. The 7 chain and immunoreactive photocleavage products are indicated.

D from the original terminus of the 7 chain contained in 7V1180 (Fig. 4 c). The larger (M, 235,000) V1 fragment was not degraded under these conditions (not shown).

Tryptic cleavage of 77430 yielded a fragment of Mr 43,000 that was recognized by antibody 25–8; the complementary fragment of Mr 363,000 retained the 12B epitope (not shown). In tryptic digests of photocleaved samples, cleavage of the M, 171,000 fragment at the same site yielded peptides of M, 128,000 and 43,000 recognized by 12YB and 25–8 respectively (Fig. 4, a and b, lanes 6–8). Therefore, as both epitopes are located within 7V1180, this cut site (T12) must occur 43,000 D from T11, and the epitope recognized by antibody 25–8 must be within 9,000–52,000 D of the 7 chain terminus contained in 7V1180 (Fig. 4 c). The 12YB epitope is contained within the 128,000-D region delimited by T12 and the V1 site (Fig. 4 c).

Both 7T23 and 7T350 were subsequently digested; the former to fragments of M, 41,000 and 39,000, the latter by cleavage at T13 to yield a fragment of M, 358,000 (not shown). In tryptic digests of the 7 chain photocleaved at the V1 site, the M, 128,000 fragment recognized by antibody 12YB was cleaved at T12 to yield an M, 123,000 fragment (Fig. 4 b, lanes 6–8). As the M, 123,000 fragment was observed only in samples that previously had been cleaved at the V1 site, it must retain the new terminus exposed by photocleavage at that site. Therefore, the T12 site must occur 123,000 D from the V1 site, ~5,000 D from T13. We have not been able to unambiguously locate the tryptic sites at which cleavage of 7T23 occurs to yield 7T23 and 7T39.

The 7T358 fragment was cleaved by trypsin at multiple sites to generate a series of immunoreactive fragments. Because the M, 123,000 fragment generated by tryptic digestion of the V1-cleaved 7 chain was not degraded under these conditions (Fig. 4 b, lanes 6–8), the sites at which trypsin cleaves the 7 chain to generate these fragments must be located within the larger (M, 235,000) V1 fragment. A major site (T6) is located 117,000 D from the original 7 chain terminus contained in 7V1180 (Fig. 7). Cleavage at this site releases a fragment of M, 241,000 which retains the 12YB epitope. Other major tryptic sites (T7, T8, and T9) are located 29,000, 18,000, and 6,000 D, respectively, from the V1 site; cleavage at these sites releases immunoreactive fragments of M, 152,000, 141,000, and 129,000, respectively (see Fig. 7).

Digestion of the 7 Chain by Staphylococcal Protease

Initial cleavage of the 7 chain by staphylococcal protease removed ~9,000 D from one terminus of the molecule (Fig. 5). After cleavage of the 7 chain at the V1 site, staphylococcal protease converted 7V1180 to a fragment of M, 171,000 (Fig. 6 a, b, c, lane 2). Therefore, the SP1 site must be located within 7V1180, ~9,000 D from the original 7 chain terminus contained in the latter fragment and very close to the T11 site (Fig. 6 d).

The truncated 7 chain molecules were then degraded at a second site (SP12) to yield a fragment of M, 251,000 which retained the 12YB epitope, but was not recognized by antibody 25–8 (Fig. 6 a, c–c, lanes 6 and 7). Consequently, SP12 must lie between the 12YB and 25–8 epitopes, 16,000 D from the V1 site (Fig. 6 d). The epitope 12YB must be contained in that portion of the chain between SP12 and the V1 site. This confirms the order of the epitopes deduced from analysis of the tryptic fragments. The localization of the 12YB epitope to within the 16,000-D region delimited by the SP12 and the V1 sites is confirmed by the existence of an endogenous 7 chain fragment of M, 170,000 which contains the 25–8 but not the 12YB epitope (see Fig. 4, a and b, lane I, and Fig. 6, b and c, lane I).

The complementary fragment (M, 155,000) that was generated from 7V1180 by cleavage at the SP12 site is highly susceptible to further proteolysis and yields a series of fragments (M, 110,000–39,000) recognized by antibody 25–8. However, as these fragments contain only one epitope and do not span the V1 site, it has not been possible to unambiguously determine their origin.

The regions of the 7 chain which correspond to the frag-
Figure 4. Tryptic digestion of the γ chain cleaved at the V1 site. Nitrocellulose replicas of a 0-2.4 M glycerol, 5-15% acrylamide gradient gel. Lanes were loaded, from left to right, with 4 µg γ subunit that had been cleaved at the V1 site by irradiation at 365 nm for 60 min in the presence of 100 µM ATP and 100 µM vanadate, and subsequently digested with 0, 5.5, 27.5, 55, 138, 275, 410, and 550 ng trypsin for 5 min. Replicas were probed with antibodies 25-8 (a) and 12γB (b). An endogenous fragment (Mr 170,000) and its tryptic product (Mr 141,000) that react with 25-8 but not 12γB are visible but not marked in a. (c) Diagram indicating the relationship between the V1 and tryptic fragments of the γ chain. The numbers beside each fragment indicate its mass in daltons ($\times 10^3$).

ments described above, the sections of the molecule which contain the epitopes recognized by antibodies 12γB and 25-8, and the locations of sites susceptible to proteolysis and vanadate-dependent photocleavage are shown diagrammatically in Fig. 7.

Discussion

The γ heavy chain from the Chlamydomonas outer arm dynein was cleaved at a single site (the V1 site) by UV irradiation at either 254 or 365 nm in the presence of vanadate, ATP, and Mg$^{2+}$. Two discrete fragments of $M_r$ 235,000 and 180,000 were obtained. The latter fragment was recognized by two monoclonal antibodies (12γB and 25-8), both of which react specifically with the γ chain. Therefore, the $M_r$ 180,000 fragment derived from the γ chain. The $M_r$ 235,000 fragment was not immunoreactive. However, because the γ chain was the only high molecular weight dynein polypep-
Figure 5. Truncation of the γ chain by staphylococcal protease. Nitrocellulose replica of a 2–8 M urea 3–5 % acrylamide gradient gel probed with antibody 25–8. Lanes were loaded with 4.5 μg sucrose gradient-purified γ subunit that had been digested with, from left to right, 0, 2.5, 12.5, 25, 62.5, 125, 187, and 250 ng staphylococcal protease for 5 min. The γ chain and Mγ 406,000 fragment are indicated.

tide present in significant amounts and as the Mγ 235,000 fragment was only observed in samples containing the Mγ 180,000 fragment, this nonimmunoreactive fragment must correspond to the complementary region of the γ chain. Ultraviolet irradiation also induced V1 photocleavage of the γ chain when ATP was omitted from the reaction mix, indicating that Mg2+-vanadate alone is sufficient for scission of the peptide backbone. A similar result was reported previously for the α heavy chain (but not the β chain) of Chlamydomonas outer arm dynein (King and Witman, 1987).

When the γ chain was irradiated at 365 nm in the presence of Mn2+ and vanadate, cleavage occurred at two discrete sites (termed V2a and V2b) different from the one observed in the presence of Mg2+ and vanadate. Fragment pairs of 215,000/200,000 and 250,000/165,000 were obtained; the larger fragment from each pair contained the 12γB and 25–8 epitopes. Therefore, the V2a and V2b sites are located within the larger (Mγ 235,000) V1 fragment, 35,000 and 70,000 D, respectively, from the V1 site.

The V1 and V2 photocleavage reactions do not exhibit the same dependence on vanadate concentration. Cleavage at the V1 site occurred at approximately the same rate in the presence of vanadate ranging from 1 μM to 1 mM. In contrast, photocleavage at the V2 sites was not observed in samples containing 0.1–100 μM vanadate. In the low micromolar range, vanadate exists mainly as monomeric H2VO4−; at 1 mM, cyclic trimeric (V3O8−) and/or tetrameric (V4O12−) species account for >85 % of the anions present (Chasteen, 1983). It has been suggested previously that the vanadate species required for photocleavage at the V2 sites might be oligomeric (Gibbons and Gibbons, 1987; King and Witman, 1987, 1988a; Tang and Gibbons, 1987). The observation that photocleavage of the γ chain at the V2 sites only occurs under conditions where these forms predominate further supports that hypothesis.

The V1 site of cleavage is thought to occur within the ATP

Figure 6. Staphylococcal protease digestion of the γ chain cleaved at the V1 site. The lanes in each panel were loaded with 2.2 μg γ subunit that had been cleaved at the V1 site by irradiation at 365 nm for 45 min in the presence of 50 μM ATP and 50 μM vanadate. After photocleavage, the samples were digested with 200 ng staphylococcal protease for, from left to right, 0, 30, 60, 90, 120, 180, and 240 min. For a, the samples were separated in a 2–8 M urea, 3–5 % acrylamide gradient gel, and subsequently silver stained. b and c show nitrocellulose replicas of the high molecular weight region of 0–2.4 M glycerol, 5–15 % acrylamide gradient gels probed with antibodies 25–8 (b) and 12γB (c). The γ chain and V1 fragments are marked at left; fragments generated by staphylococcal protease are indicated at right. An endogenous fragment of Mγ 170,000 can be seen in lane 1 of b. In each panel, all lanes are from a single gel; irrelevant lanes have been omitted. d illustrates the location of the sites sensitive to staphylococcal protease and the origin of the resulting fragments.
Figure 7. Linear map of the γ chain and properties of its proteolytic and photolytic fragments. Diagram illustrating the regions of the γ chain from which specific fragments were derived. Indicated on the map of the intact molecule at top are the trypsin- and staphylococcal protease-sensitive sites (small arrows labeled T and SP, respectively), the sites of vanadate-dependent photocleavage (large arrows labeled V1, V2a, and V2b), and the regions of the molecule which contain the epitopes recognized by antibodies 12γB and 25-8 (shading labeled B and 25-8). The protease or the vanadate-dependent cleavage procedure used to obtain a fragment, the relative molecular mass of the fragment, and its immunoreactivity are tabulated at right. The scale of the map is shown at the bottom of the figure; units are daltons (×10^3).

The present study indicates that the γ chain has a mass of 415,000 D. Because the γ subunit also contains three light chains (one of 18,000 D and two of 22,000 D) (King and Witman, 1988a), we calculate that this subunit has a mass of ~475,000 D. This value agrees extremely well with the mass of 460,000 ± 51,700 D determined for this complex by scanning transmission electron microscopy (Witman et al., 1987).
1983), and confirms that this complex can contain only a single copy of the γ heavy chain. Similarly, we calculated a mass of 1,240,000 D for the α-β dimer (King and Witman, 1987); the scanning transmission electron microscopy mass for this complex was 1,220,000 ± 117,000 D. Therefore, both methods of analysis indicate that the intact outer arm dynein from *Chlamydomonas* has a mass of ~1,700,000 D.

Previously, we demonstrated that the V2 sites of photobleaching within the α and β chains occur within the larger V1 fragment (King and Witman, 1987). The data presented here demonstrate that the chain exhibits the same inherent polarity. Similar results have also been obtained for the outer arm dynein heavy chains from sea urchin sperm flagella and *Tetrahymena* cilia (Gibbons and Gibbons, 1987; Tang and Gibbons, 1987), suggesting that the overall structure of these polypeptides has been conserved throughout evolution.

Because the COOH terminus of the β heavy chain is located within the smaller V1 fragment from that polypeptide (King and Witman, 1988a), we predict that the COOH terminus of the γ chain is located within the analogous (M, 180,000 fragment). The maps shown in Figs. 1, 2, 4, 6, and 7 have been oriented so as to reflect this prediction.

By examining the immunoreactivity of chain fragments generated by endoproteases, we have identified the regions of this molecule from which these fragments originally derived, and thus located the portions which contain the epitopes recognized by monoclonal antibodies 123'B and 25-8. Both epitopes are located within the M, 180,000 V1 fragment; that recognized by 123'B is within 16,000 D of the V1 site, whereas the 25-8 epitope is within 9,000–52,000 D of the original γ chain terminus.

The location of the site at which the γ chain is initially degraded by endoproteases is markedly similar to that of the first cleavage site within the β heavy chain (King and Witman, 1988b). Both molecules are cleaved at a site ~10,000 D from the original terminus contained in the smaller V1 fragment, suggesting that these portions of the β and γ chains are structurally and possibly functionally related. In contrast, the analogous region from the α chain does not contain a highly sensitive protease site ~10,000 D from the terminus.

The sites of vanadate-dependent photobleaching are located within the central regions of the α, β, and γ heavy chains, indicating that these regions contain the sites of ATP binding. We have recently proposed that the central regions of the α and β chains comprise the globular domains observed by scanning transmission electron microscopy (King and Witman, 1988a). Considering the substructural similarities between the dynein heavy chains noted above, it seems probable that the central portion of the γ chain (perhaps in association with one terminal region and/or one or more light chains) comprises the globular domain of the γ subunit that is observed by electron microscopy (Goodenough and Heuser, 1984). One (or both) terminal regions would then correspond to the stem of the complex.

The use of endoproteases and vanadate-dependent photobleaching has allowed for a detailed analysis of the substructure of the heavy chains from the *Chlamydomonas* outer arm dynein. The information provided by these studies will enable other regions of functional significance, such as the sites modified by photoaffinity analogs of ATP, the microtubule-binding sites, and sites of interaction with the dynein light chains, to be located within this very large molecule.

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