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

Stephen M. King and George B. Witman

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

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, Mg^{2+}, and vanadate cleaves the γ chain at a single site (termed V1) to yield fragments of M_r 235,000 and 180,000. Irradiation in the presence of vanadate and Mn^{2+} results in cleavage of the γ chain at two other sites (termed V2a and V2b) to yield fragment pairs of M_r 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 flagellated cells, strongly suggesting that dynein is involved in motile events occurring within cellular compartments other than the flagellum. Dyneins are thus of considerable interest as the probable mechanochemical transducers in a variety of microtubule-based motility systems.

The dyneins of the *Chlamydomonas* flagellum have been particularly useful for studying the molecular structure of these important enzymes (King and Witman, 1988a; Luck, 1984). The outer arm dynein from *Chlamydomonas* contains three subunits (α, β, and γ). The α and β subunits are purified together as a heterodimeric complex, whereas the

γ subunit becomes separated during extraction of the outer arm dynein from the axoneme and is obtained as a discrete particle. Each subunit consists of a single high molecular weight polypeptide (the α, β, and γ heavy chains; M_r >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).

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 (M_r 480,000 and 440,000, respectively) (King and Witman, 1987, 1988b). Both molecules contain a single site that is cleaved by UV irradiation in the presence of Mg^{2+}, ATP and vanadate; these are termed the V1 sites. The α chain also contains three other sites at which cleavage occurs as a result of UV irradiation in the presence of vanadate and Mn^{2+}, while the β chain contains one other site cleaved under these conditions; these are termed the V2 sites. Cleavage at the V2 sites requires higher vanadate concentrations than does photocleavage at the V1 sites and does not occur in the presence of Mg^{2+} or ATP. Both polypeptides exhibit the same polarity in that the

---

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 (M_r ~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 sulfonyl 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 Spectroline 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 (D0632; Sigma Chemical Co.) was also added to the sample as a free radical scavenger (final concentration 1 mM) to reduce the amount of nonspecific photolysis 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 were then added, and the samples cleaved by irradiation at 365 nm (Gibbons and Gibbons, 1987; King and Witman, 1987).

Proteolytic Digestion

Digestion of the γ chain by N^α tosyl-L-phenylalanine-chloro-methyl-ketone-treated trypsin (T8642; 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 (~150 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 Protein Blotting

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, 1988b).

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 Mg^{2+}, 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 M_r 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_r 180,000 fragment (Fig. 1 b). This result confirmed that the M_r 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_r 180,000 fragment (Fig. 1 c).

The M_r 235,000 fragment was recognized by neither anti-γ chain antibody (Fig. 1 b). However, this fragment appeared concomitantly with the M_r 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_r 235,000 fragment must derive from the V1-cleaved γ chain as the complement to the M_r 180,000 fragment (Fig. 1 c). Thus, the γ chain
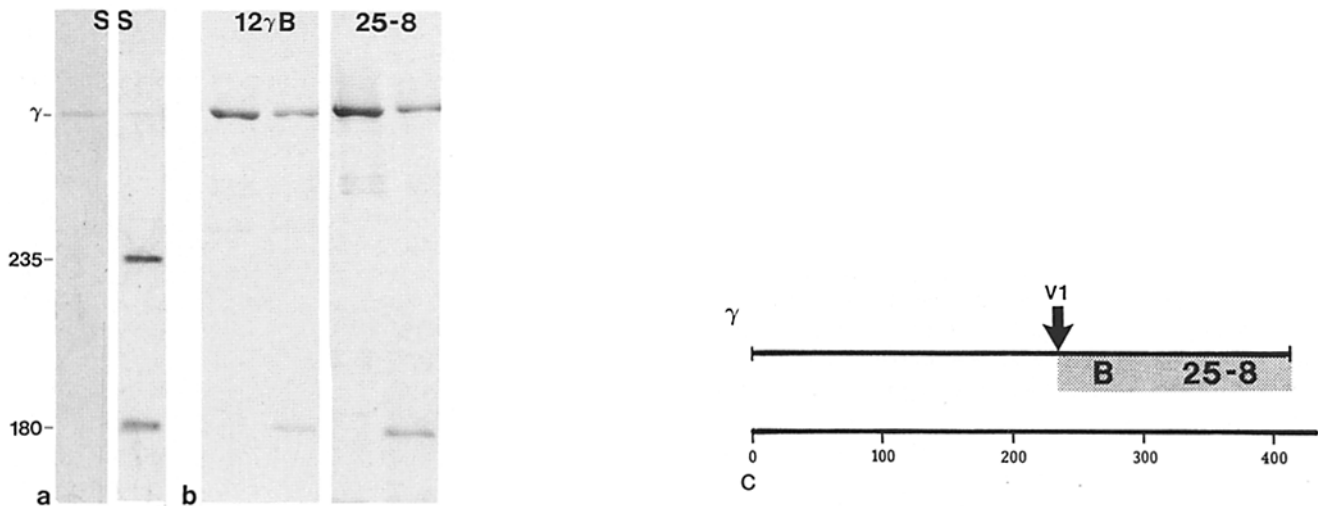
Figure 1. Cleavage of the γ chain at the V1 site. (*a*) Two lanes from a silver-stained 2-8 M urea, 3-5% acrylamide gradient gel. The lane at left was loaded with 2 μg hydroxylapatite-purified γ subunit, that at right was a similar sample that had been incubated with 50 μM ATP and 50 μM vanadate and irradiated at 365 nm for 40 min. The γ chain and V1 fragments are indicated. Note that in silver-stained gels, intermediate-sized fragments are often more intensely stained than an equimolar amount of the heavy chain from which they derived. (*b*) Nitrocellulose replicas of a 3-5% acrylamide gel probed with antibodies 12γB and 25-8. The first lane on each panel was loaded with 14.8 μg of a high salt extract of axonemes; the second lane with an identical sample that had been incubated with 100 μM vanadate, 100 μM 8-N₃ATP, and 1 mM DTT and irradiated at 254 nm for 45 min. (*c*) Map of the γ chain showing the location of the V1 site and the region which contains the epitopes recognized by antibodies 12γB and 25-8 (shading labeled *B* and *25-8*).

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_r 250,000, 215,000, 200,000, and 165,000) that were detectable in silver-stained gels (Fig. 2 *a*). The M_r 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_r 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_r 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_r 235,000) V1 fragment, 35,000 and 70,000 D, respectively, from the V1 site (Fig. 2 *c*). Two additional minor fragments of M_r 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_r 250,000 fragment obtained by cleavage at the V2b site was not comparable to that of the complementary M_r 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_r 165,000 and 215,000 fragments. The M_r 200,000 fragment generated by cleavage at the V2a site did not appear to be converted to the M_r 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 (T^γ₁) ∼9,000 D from one terminus to generate an immunoreactive fragment of M_r ∼406,000 (γ^T₄₀₆; 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_r 406,000 fragment is based on the analysis of tryptic fragments of the photocleaved chain described immediately below. To unambigu-

---

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, 1988*b*). For example, T^γ₂ refers to a tryptic cut site within the γ chain. Cleavage at this site generates fragments of M_r 363,000 and 43,000, which are designated γ^T₃₆₃ and γ^T₄₃. The fragments generated by staphylococcal protease (SP) and vanadate-dependent photocleavage are designated in a similar manner.
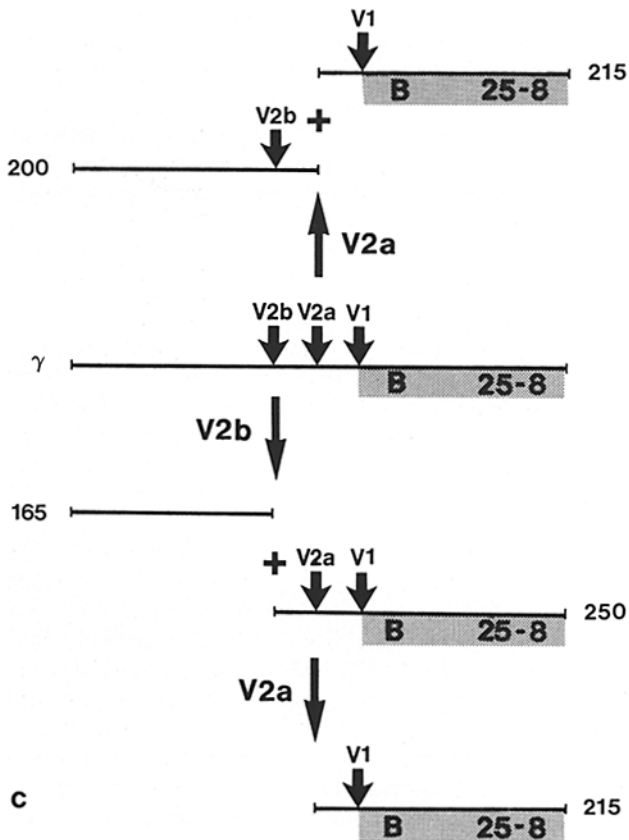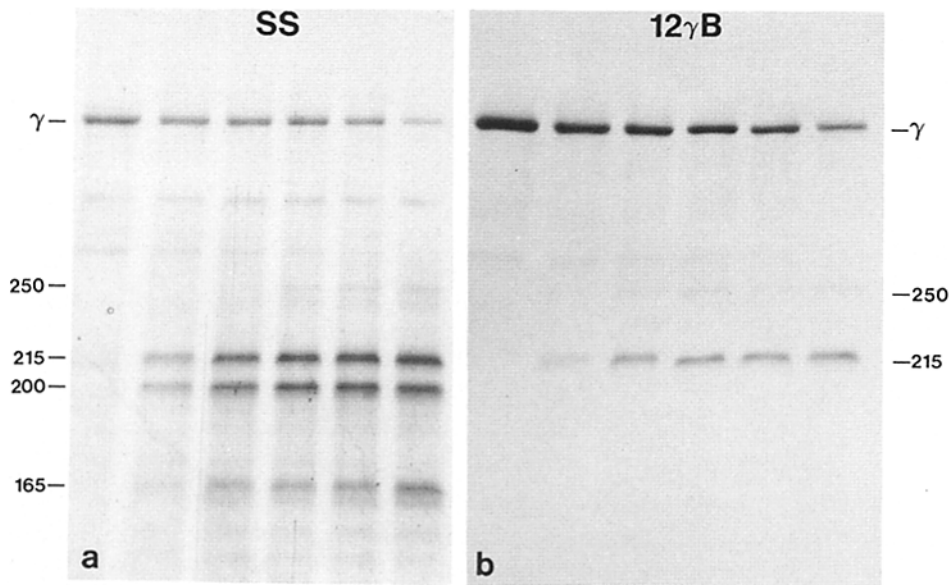
Figure 2. Cleavage of the γ chain at the V2 sites. (*a*) High molecular weight region of a silver-stained 2–8 M urea, 3–5% acrylamide gradient gel (*SS*). Lanes were loaded, from left to right, with 3 µg sucrose gradient–purified γ subunit that had been irradiated at 365 nm for 0, 15, 30, 45, 60, and 90 min in the presence of 150 µM vanadate and 1 mM Mn^{2+}. 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 ($\times$ 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 M_r 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 M_r 180,000 fragment was degraded and an immunoreactive peptide of M_r 171,000 obtained. Conversion

of the M_r 180,000 fragment to the M_r 171,000 fragment occurred under the same conditions as conversion of the γ chain to the M_r 406,000 fragment, and therefore must also have resulted from cleavage at the T^γ_1 site. Because the M_r 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 γ^T_{406} and the M_r 171,000 fragment must be located ∼9,000
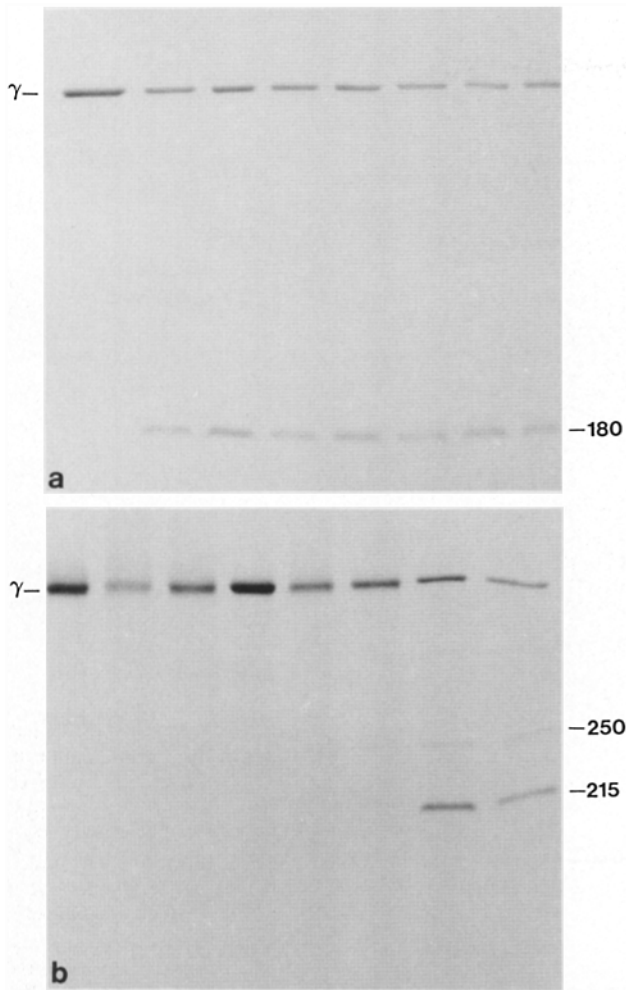
Figure 3. Effect of vanadate concentration on photolysis at the V1 and V2 sites. Nitrocellulose replicas of 2–8 M urea, 3–5% acrylamide gradient gels probed with antibody 25–8. (*a*) Photolysis at the V1 site. Each lane was loaded with 4 μg sucrose gradient-purified γ subunit that had been incubated with 50 μM ATP, 5 mM Mg^{2+} and, from left to right, 0, 1, 5, 10, 50, 100, 500, and 1,000 μM vanadate for 15 min before irradiation at 365 nm for 30 min. (*b*) Photolysis at the V2 sites. Lanes were loaded with 4 μg γ subunit that had been incubated with 1 mM Mn^{2+} and the vanadate concentrations indicated in *a* for 15 min and then irradiated at 365 nm for 30 min. The γ chain and immunoreactive photocleavage products are indicated.

D from the original terminus of the γ chain contained in γ^{VI}_{180} (Fig. 4 *c*). The larger (M_r 235,000) V1 fragment was not degraded under these conditions (not shown).

Tryptic cleavage of γ^T_{406} yielded a fragment of M_r 43,000 that was recognized by antibody 25–8; the complementary fragment of M_r 363,000 retained the 12γB epitope (not shown). In tryptic digests of photocleaved samples, cleavage of the M_r 171,000 fragment at the same site yielded peptides of M_r 128,000 and 43,000 recognized by 12γB and 25–8 respectively (Fig. 4, *a* and *b*, lanes *6–8*). Therefore, as both epitopes are located within γ^{VI}_{180}, this cut site (T$^\gamma_2$) must occur 43,000 D from T$^\gamma_1$, and the epitope recognized by antibody 25–8 must be within 9,000–52,000 D of the γ chain terminus contained in γ^{VI}_{180} (Fig. 4 *c*). The 12γB epitope is

contained within the 128,000-D region delimited by T$^\gamma_2$ and the V1 site (Fig. 4 *c*).

Both γ^T_{43} and γ^T_{363} were subsequently digested; the former to fragments of M_r 41,000 and 39,000, the latter by cleavage at T$^\gamma_3$ to yield a fragment of M_r 358,000 (not shown). In tryptic digests of the γ chain photocleaved at the V1 site, the M_r 128,000 fragment recognized by antibody 12γB was cleaved at T$^\gamma_3$ to yield an M_r 123,000 fragment (Fig. 4 *b*, lanes *6–8*). As the M_r 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 T$^\gamma_3$ site must occur 123,000 D from the V1 site, ∼5,000 D from T$^\gamma_2$. We have not been able to unambiguously locate the tryptic sites at which cleavage of γ^T_{43} occurs to yield γ^T_{41} and γ^T_{39}.

The γ^T_{358} fragment was cleaved by trypsin at multiple sites to generate a series of immunoreactive fragments. Because the M_r 123,000 fragment generated by tryptic digestion of the V1-cleaved γ chain was not degraded under these conditions (Fig. 4 *b*, lanes *6–8*), the sites at which trypsin cleaves the γ chain to generate these fragments must be located within the larger (M_r 235,000) V1 fragment. A major site (T$^\gamma_4$) is located 117,000 D from the original γ chain terminus contained in γ^{VI}_{235} (Fig. 7). Cleavage at this site releases a fragment of M_r 241,000 which retains the 12γB epitope. Other major tryptic sites (T$^\gamma_5$, T$^\gamma_6$, and T$^\gamma_7$) are located 29,000, 18,000, and 6,000 D, respectively, from the V1 site; cleavage at these sites releases immunoreactive fragments of M_r 152,000, 141,000, and 129,000, respectively (see Fig. 7).

Digestion of the γ Chain by Staphylococcal Protease

Initial cleavage of the γ chain by staphylococcal protease removed ∼9,000 D from one terminus of the molecule (Fig. 5). After cleavage of the γ chain at the V1 site, staphylococcal protease converted γ^{VI}_{180} to a fragment of M_r 171,000 (Fig. 6, *a–c*, lane *2*). Therefore, the SP$^\gamma_1$ site must be located within γ^{VI}_{180}, ∼9,000 D from the original γ chain terminus contained in the latter fragment and very close to the T$^\gamma_1$ site (Fig. 6 *d*).

The truncated γ chain molecules were then degraded at a second site (SP$^\gamma_2$) to yield a fragment of M_r 251,000 which retained the 12γB epitope, but was not recognized by antibody 25–8 (Fig. 6, *a–c*, lanes *6* and *7*). Consequently, SP$^\gamma_2$ must lie between the 12γB and 25–8 epitopes, 16,000 D from the V1 site (Fig. 6 *d*). The epitope 12γB must be contained in that portion of the chain between SP$^\gamma_2$ and the V1 site. This confirms the order of the epitopes deduced from analysis of the tryptic fragments. The localization of the 12γB epitope to within the 16,000-D region delimited by the SP$^\gamma_2$ and the V1 sites is confirmed by the existence of an endogenous γ chain fragment of M_r 170,000 which contains the 25–8 but not the 12γB epitope (see Fig. 4, *a* and *b*, lane *1*, and Fig. 6, *b* and *c*, lane *1*).

The complementary fragment (M_r 155,000) that was generated from γ^{SP}_{406} by cleavage at the SP$^\gamma_2$ site is highly susceptible to further proteolysis and yields a series of fragments (M_r 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 γ chain which correspond to the frag-
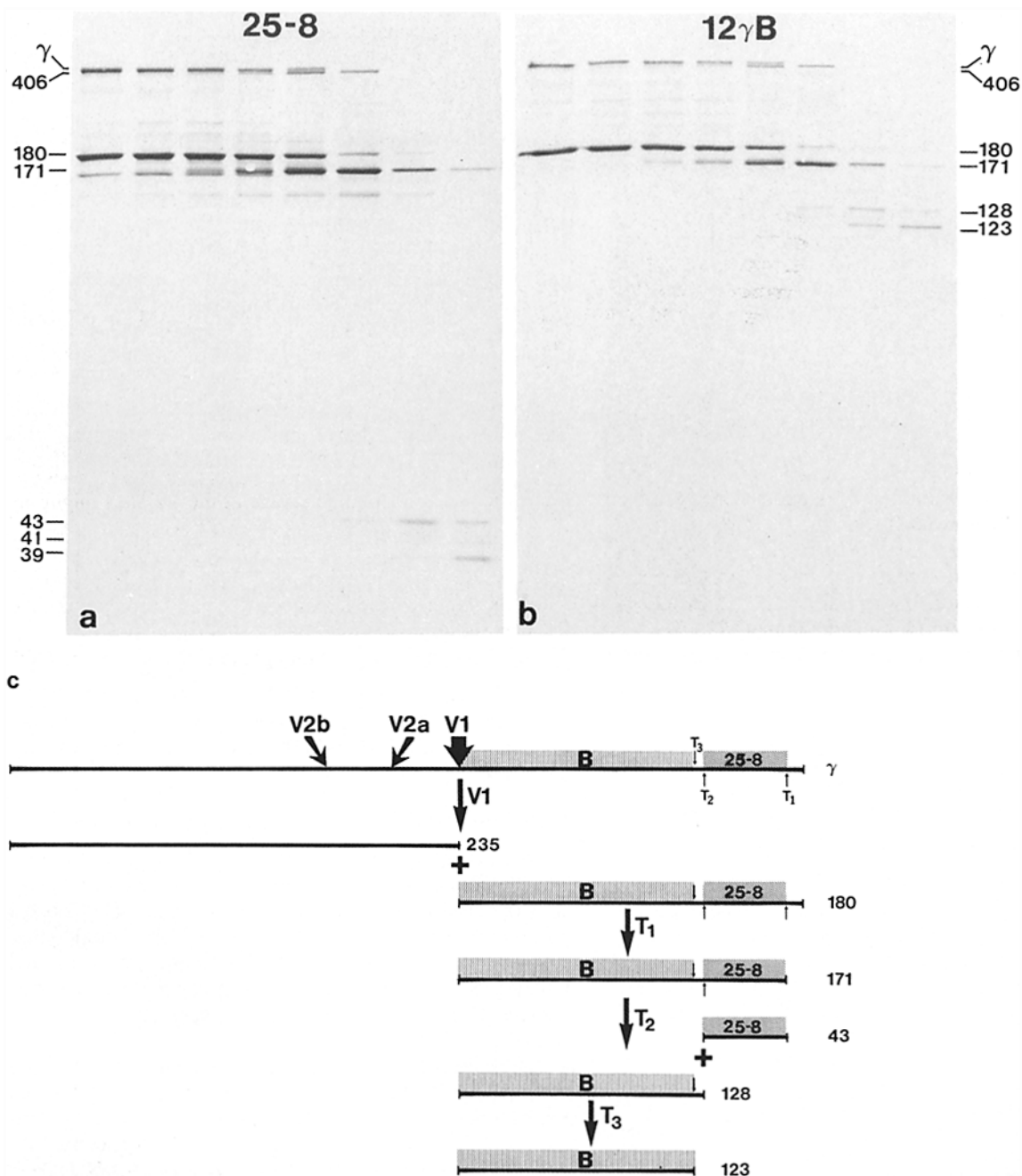
Figure 4. Tryptic digestion of the γ chain cleaved at the V1 site. Nitrocellulose replicas of a 0–2.4 M glycerol, 5–15% acrylamide gradient gel. Lanes were loaded, from left to right, with 4 μg γ subunit that had been cleaved at the V1 site by irradiation at 365 nm for 60 min in the presence of 100 μM 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 (M_r 170,000) and its tryptic product (M_r 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-
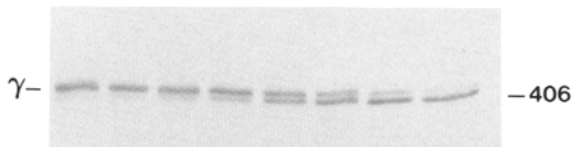
Figure 5. Truncation of the γ chain by staphylococcal protease. Nitrocellulose replica of a 2–8 M urea 3–5% acrylamide gradient gel probed with antibody 25-8. Lanes were loaded with 4.5 µg sucrose gradient–purified γ subunit that had been digested with, from left to right, 0, 2.5, 12.5, 25, 62.5, 125, 187, and 250 ng staphylococcal protease for 5 min. The γ chain and M_r 406,000 fragment are indicated.

tide present in significant amounts and as the M_r 235,000 fragment was only observed in samples containing the M_r 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 Mg^{2+}·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 Mn^{2+} and vanadate, cleavage occurred at two discrete sites (termed V2a and V2b) different from the one observed in the presence of Mg^{2+} 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 (M_r 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 <~100 µM vanadate. In the low micromolar range, vanadate exists mainly as monomeric $H_2VO_4^-$; at 1 mM, cyclic trimeric ($V_3O_9^{3-}$) and/or tetrameric ($V_4O_{12}^{4-}$) 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
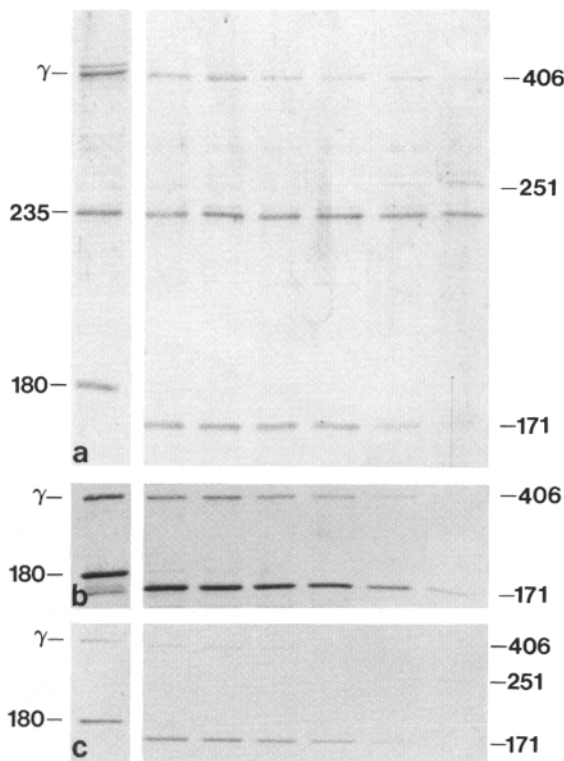
Figure 6. Staphylococcal protease digestion of the γ chain cleaved at the V1 site. The lanes in each panel were loaded with 2.2 µg γ subunit that had been cleaved at the V1 site by irradiation at 365 nm for 45 min in the presence of 50 µM ATP and 50 µM vanadate. After photocleavage, the samples were 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 M_r 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.
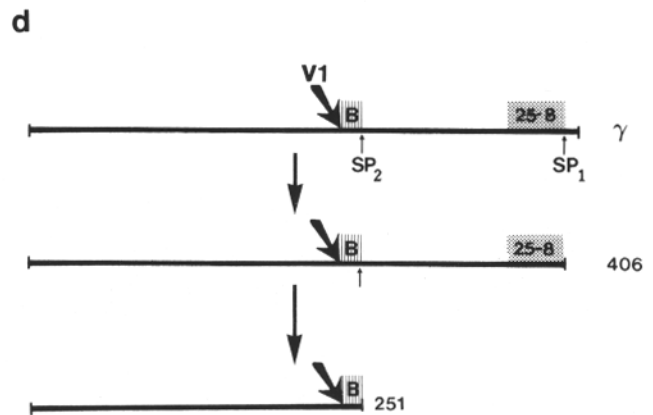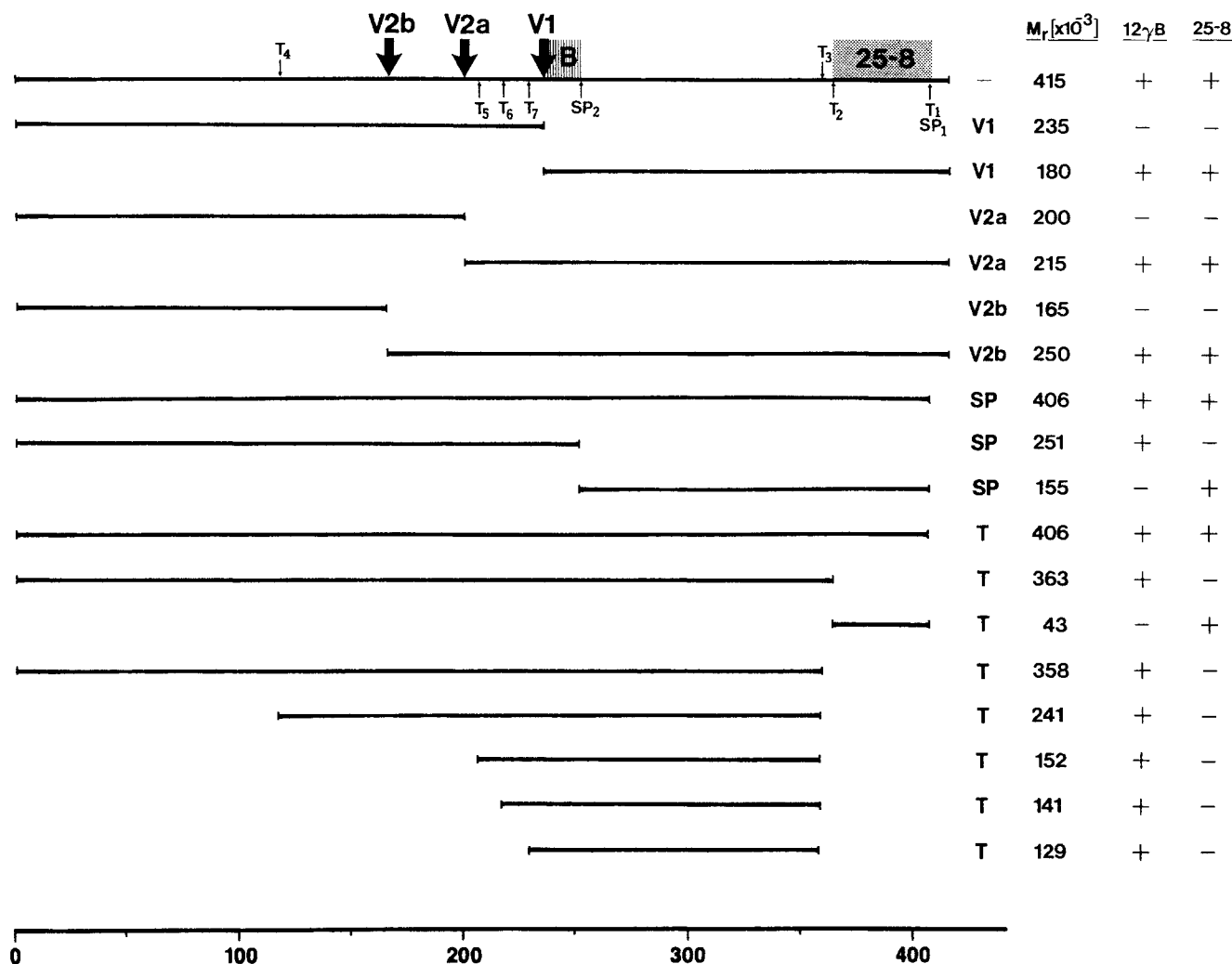
Figure 7. Linear map of the γ chain and properties of its proteolytic and photolytic fragments. Diagram illustrating the regions of the γ chain from which specific fragments were derived. Indicated on the map of the intact molecule at top are the trypsin- and staphylococcal protease-sensitive sites (*small 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}).

		$M_r [\times 10^3]$	12γB	25-8
	—	415	+	+
V1		235	−	−
V1		180	+	+
V2a		200	−	−
V2a		215	+	+
V2b		165	−	−
V2b		250	+	+
SP		406	+	+
SP		251	+	−
SP		155	−	+
T		406	+	+
T		363	+	−
T		43	−	+
T		358	+	−
T		241	+	−
T		152	+	−
T		141	+	−
T		129	+	−

hydrolytic domains at, or very close to, the region with which the γ-phosphate of ATP normally interacts (Gibbons et al., 1987; King and Witman, 1987). Recent studies using photoaffinity analogs of ATP suggest that the V2 sites also occur within the nucleotide-binding domains (King, S. M., and G. B. Witman, manuscript in preparation). Inasmuch as the vanadate-dependent cleavage sites within the α, β, and γ chains are separated by 35,000–100,000 D, the central regions of these molecules must be extensively folded so as to bring both the V1 and V2 sites within the hydrolytic domains.

Vanadate-dependent photocleavage probably occurs via an energy transfer from the vanadate chromophore to the side chains of specific amino acyl residues within the catalytic sites (Gibbons et al., 1987). The γ chain contains two sites at which photocleavage may occur. Three V2 sites have been identified within the α chain, whereas the β chain is cleaved

at only a single V2 site (King and Witman, 1987). This variation in the number of V2 sites suggests that there are significant differences in the primary and/or tertiary structures of the hydrolytic domains of these dynein polypeptides. Functional differences between these sites have been observed previously. The specific Mg^{2+}·ATPase activities of the purified subunits are not equivalent (Pfister and Witman, 1984; Pfister et al., 1982), and the subunits also differ in their ability to accept different metal–nucleotide complexes as substrates (King and Witman, 1988a).

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.,

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 photocleavage within the α and β chains occur within the larger V1 fragment (King and Witman, 1987). The data presented here demonstrate that the γ 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_r 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 γ 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 12γB and 25-8. Both epitopes are located within the M_r 180,000 V1 fragment; that recognized by 12γB 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 photocleavage 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 photocleavage 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.

References

Chasteen, N. D. 1983. The biochemistry of vanadium. *Struct. Bond.* 53: 107-138.

Gibbons, I. R. 1965. Chemical dissection of cilia. *Arch. Biol.* 76:317-352.

Gibbons, I. R. 1981. Cilia and flagella of eukaryotes. *J. Cell Biol.* 91(Suppl.): 107-124.

Gibbons, I. R., and B. H. Gibbons. 1987. Photocleavage of dynein heavy chains. *In* Perspectives in Biological Energy Transduction. Y. Mukohata, M. Nakao, S. Ebashi, M. F. Morales, and S. Fleischer, editors. Academic Press, Inc., Tokyo. 107-116.

Gibbons, I. R., A. Lee-Eiford, G. Mocz, C. A. Phillipson, W.-J. Tang, and B. H. Gibbons. 1987. Photosensitized cleavage of dynein heavy chains. Cleavage at the V1 site by irradiation in the presence of ATP and vanadate. *J. Biol. Chem.* 262:2780-2786.

Goodenough, U. W., and J. E. Heuser. 1984. Structural comparison of purified dynein proteins with *in situ* dynein arms. *J. Mol. Biol.* 180:1083-1118.

Huang, B., G. Piperno, and D. J. L. Luck. 1979. Paralyzed flagellar mutants of *Chlamydomonas reinhardtii* defective for axonemal doublet microtubule arms. *J. Biol. Chem.* 254:3091-3099.

Johnson, K. A., M. E. Porter, and T. Shimizu. 1984. Mechanism of force production for microtubule-dependent movements. *J. Cell Biol.* 99:132s-136s.

King, S. M., and G. B. Witman. 1987. Structure of the α and β heavy chains of the outer arm dynein from *Chlamydomonas* flagella. Masses of chains and sites of ultraviolet-induced, vandate-dependent cleavage. *J. Biol. Chem.* 262:17596-17604.

King, S. M., and G. B. Witman. 1988a. The molecular structure of *Chlamydomonas* outer arm dynein. *In* Cell Movement. Vol. 1. F. D. Warner, P. Satir, and I. R. Gibbons, editors. Alan R. Liss, Inc., New York. In press.

King, S. M., and G. B. Witman. 1988b. Structure of the α and β heavy chains of the outer arm dynein from *Chlamydomonas* flagella. Location of epitopes and protease-sensitive sites. *J. Biol. Chem.* 263:9244-9255.

King, S. M., T. Otter, and G. B. Witman. 1985. Characterization of monoclonal antibodies against *Chlamydomonas* flagellar dyneins by high resolution protein blotting. *Proc. Natl. Acad. Sci. USA.* 82:4717-4721.

King, S. M., T. Otter, and G. B. Witman. 1986. Purification and characterization of *Chlamydomonas* flagellar dyneins. *Methods Enzymol.* 134:291-306.

Lee-Eiford, A., R. A. Ow, and I. R. Gibbons. 1986. Specific cleavage of dynein heavy chains by ultraviolet irradiation in the presence of ATP and vanadate. *J. Biol. Chem.* 261:2337-2342.

Luck, D. J. L. 1984. Genetic and biochemical dissection of the eukaryotic flagellum. *J. Cell Biol.* 98:789-794.

Lye, R. J., M. E. Porter, J. M. Scholey, and J. R. McIntosh. 1987. Identification of a microtubule-based cytoplasmic motor in the nematode *C. elegans*. *Cell.* 51:309-318.

Merril, C. R., D. Goldman, S. A. Sedman, and M. H. Ebert. 1981. Ultrasensitive stain for proteins in polyacrylamide gels shows regional variation in cerebrospinal fluid protein. *Science (Wash. DC).* 211:1437-1438.

Otter, T., S. M. King, and G. B. Witman. 1987. A two-step procedure for efficient electrotransfer of both high molecular weight (>400,000) and low molecular weight (<20,000) proteins. *Anal. Biochem.* 162:370-377.

Pallini, V., M. Bugnoli, C. Mencarelli, and G. Scapigliati. 1982. Biochemical properties of ciliary, flagellar and cytoplasmic dyneins. *Symp. Soc. Exp. Biol.* 35:339-352.

Pallini, V., C. Mencarelli, L. Bracci, M. Contorni, P. Ruggiero, A. Tiezzi, and R. Manetti. 1983. Cytoplasmic nucleoside-triphosphatases similar to axonemal dynein occur widely in different cell types. *J. Submicrosc. Cytol.* 15:229-235.

Paschal, B. W., S. M. King, A. G. Moss, C. A. Collins, R. B. Vallee, and G. B. Witman. 1987a. Isolated flagellar outer arm dynein translocates brain microtubules *in vitro*. *Nature (Lond.).* 330:672-674.

Paschal, B. M., H. S. Shpetner, and R. B. Vallee. 1987b. MAP 1C is a microtubule-activated ATPase which translocates microtubules in vitro and has dynein-like properties. *J. Cell Biol.* 105:1273-1282.

Pfister, K. K., and G. B. Witman. 1984. Subfractionation of *Chlamydomonas* 18 S dynein into two discrete subunits containing ATPase activity. *J. Biol. Chem.* 259:12072-12080.

Pfister, K. K., R. B. Fay, and G. B. Witman. 1982. Purification and polypeptide composition of dynein ATPases from *Chlamydomonas flagella*. *Cell Motil.* 2:525-547.

Pfister, K. K., B. E. Haley, and G. B. Witman. 1984. The photoaffinity probe 8-azidoadenosine 5'-triphosphate selectively labels the heavy chain of *Chlamydomonas* 12 S dynein. *J. Biol. Chem.* 259:8499-8504.

Pfister, K. K., B. E. Haley, and G. B. Witman. 1985. Labeling of *Chlamydomonas* 18 S dynein polypeptides by 8-azidoadenosine 5'-triphosphate, a photoaffinity analog of ATP. *J. Biol. Chem.* 260:12844-12850.

Piperno, G., and D. J. Luck. 1979. Axonemal adenosine triphosphatases from flagella of *Chlamydomonas reinhardtii*. Purification of two dyneins. *J. Biol. Chem.* 254:3084–3090.

Pratt, M. M. 1980. Identification of a dynein ATPase in unfertilized sea urchin eggs. *Dev. Biol.* 74:364–378.

Satir, P. 1968. Studies on cilia. III. Further studies on the cilium tip and a 'sliding filament' model of ciliary motility. *J. Cell Biol.* 39:77–94.

Shimizu, T. 1975. Recombination of ciliary dynein of *Tetrahymena* with the outer fibers. *J. Biochem. (Tokyo).* 78:41–49.

Shingyogi, C., A. Murakami, and K. Takahashi. 1977. Local reactivation of Triton-extracted flagella by iontophoretic application of ATP. *Nature (Lond.).* 265:269–270.

Takahashi, M., and Y. Tonomura. 1978. Binding of 30 S dynein with the B-tubule of the outer doublet of axonemes from *Tetrahymena pyriformis* and adenosine triphosphate induced dissociation of the complex. *J. Biochem. (Tokyo).* 84:1339–1355.

Tang, W.-J. Y., and I. R. Gibbons. 1987. Photosensitized cleavage of dynein heavy chains. Cleavage at the V2 site by irradiation at 365 nm in the presence of oligovanadate. *J. Biol. Chem.* 262:17728–17734.

Witman, G. B. 1986. Isolation of *Chlamydomonas* flagella and flagella axonemes. *Methods Enzymol.* 134:280–290.

Witman, G. B., K. A. Johnson, K. K. Pfister, and J. S. Wall. 1983. Fine structure and molecular weight of the outer arm dyneins of *Chlamydomonas*. *J. Submicrosc. Cytol.* 15:193–197.