Proteolytic Analysis of Domain Structure in the β Heavy Chain of Dynein from Sea Urchin Sperm Flagella†

Gabor Mocz,* Jarrett Farias, and I. R. Gibbons
Pacific Biomedical Research Center, University of Hawaii, Honolulu, Hawaii 96822
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ABSTRACT: The stability of different regions of the β heavy chain of dynein has been investigated by examining the perturbing effects of methanol, temperature, salt, and nucleotide on the pattern of tryptic digestion. In standard low-salt medium, tryptic proteolysis cleaves the β heavy chain into three principal polypeptides of 130, 215, and 110 kDa, with the 215-kDa central peptide containing the ATP binding site as well as the vanadate and iron photocleavage sites (Mocz, G., Tang, W.-J. Y., & Gibbons, I. R. (1988) *J. Cell Biol. 106*, 1607–1614). The 130-kDa peptide is the most stable, and its susceptibility to trypsin appears unaffected by methanol concentrations up to 25% or temperatures up to 45 °C, although a 5-kDa region at one end is lost in the presence of salt (>20 mM NaCl). The 215-kDa tryptic peptide contains two regions of different stability: its 123-kDa portion adjoining the 130-kDa peptide is destabilized by mild heat (37 °C) or by 25% methanol and becomes digested away to leave the more stable region of 92 kDa that is located toward the 110-kDa peptide and retains the V1 photocleavage site and most of the ATP binding site. The 110-kDa peptide is the least stable and at 37 °C, or in the presence of low concentrations of methanol or salt, is rapidly digested to small peptides. The presence of ATP during digestion of the β heavy chain retards the formation of the 130- and 215-kDa peptides and also protects the 215-kDa peptide from further digestion at 37 °C. In the combined presence of ATP and methanol, the cleavage site between the 215- and 110-kDa peptides becomes almost completely protected. Measurements of tryptophanyl fluorescence polarization confirm that the conformational state of the β chain is altered in the presence of either methanol or bound nucleotide. The distinct differences in stability of the major 130-, 215-, and 110-kDa tryptic peptides suggest that they each correspond to a tertiary structural domain of the intact β chain, with the 215-kDa peptide being composed of a flexible 123-kDa domain and a more stable 92-kDa domain. The latter may interact with the stable 130-kDa domain to form a stable core in the tertiary structure of the heavy chain. The binding of ATP in the central domain influences the susceptibility to trypsin of the linkages to both domains of the β chain.

Dynein ATPase is one of the most widely studied microtubule-associated energy-transducing enzymes. It constitutes the outer and inner arms on the doublet tubules of sperm flagellar axonemes, where it generates the sliding between doublets that underlies flagellar beating (Gibbons, 1981, 1988). Dynein has also been implicated in cytoplasmic motile functions, including chromosomal movement, retrograde organelle and axonal transport, the endocytic pathway, and the organization of the Golgi apparatus (Vallee & Shpetner, 1990).

In all cell types, dynein has the same basic structure and is composed of two or three distinct heavy chains of ~475 kDa, three intermediate chains (IC)[1] of 70–125 kDa, and at least four light chains of 15–25 kDa. Examination of solubilized dyneins by electron microscopy has shown that the molecule consists of two or three globular heads attached by slender stems to a common base, with each of the head/stem units corresponding to one of the distinct heavy-chain polypeptide subunits (Johnson & Wall, 1983; Witman et al., 1983; Sale et al., 1985). Similar examination of the dynein arms in situ on the axonemes has shown that their form is influenced by the presence or absence of ATP during preparation for microscopy. In the absence of ATP, the outer arms appear to consist of two apposed globular heads with visible stems, whereas in the presence of ATP the heads are more tightly associated and appear as a single globular unit (Goodenough & Heuser, 1984, 1985). More recently, an ATP-dependent conformational change has also been detected in soluble dynein by limited proteolysis (Inaba & Mohri, 1989, 1990). These structural differences, which depend on occupancy of the nucleotide binding site, may be related to the different transient states that the arms pass through during their normal ATP-driven cross-bridging cycle.

The separated β heavy chain of sea urchin sperm dynein is of particular interest because it retains the ability to interact with microtubules and to translocate them in an ATP-dependent manner in vitro (Sale & Fox, 1988; Vale et al., 1989). Study of the functional substructure of this β heavy chain by limited proteolysis has shown that the digested β chain is separable into fragments A and B by density gradient centrifugation (Ogawa & Mohri, 1975; Ow et al., 1987). On the basis of their different sedimentation rates, it was suggested that these fragments may correspond to the head and stem regions of the intact β chain. Fragment A is composed principally of 215- and 130-kDa peptides,[2] and it retains the ATPase function but has lost the ability to rebind to microtubules. Fragment B is composed of a 110-kDa peptide with no ATPase activity. The use of a set of four monoclonal antibodies in conjunction with specific site-directed photolysis

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* Address correspondence to this author at Kewalo Laboratory, 41 Ahui St., Honolulu, HI 96813.

[1] Abbreviations: HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; IC, intermediate chain.

[2] The molecular masses adopted for the β heavy chain and its major tryptic peptides in this paper are the values obtained by summation of the smallest photolytic and proteolytic peptides [see Discussion in Mocz et al. (1988)]. The values for tryptic peptides larger than 130 kDa are somewhat higher than those used for the same peptides in the earlier paper of Ow et al. (1987).

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has made it possible to map the tryptic peptides onto the linear structure of the β chain (Mocz et al., 1988; King & Witman, 1988). Epitopes 1 and 2 are located in the 130- and 215-kDa polypeptides of fragment A, respectively. Epitope 3b is located in the 110-kDa polypeptide of fragment B, while epitope 3a is located near one end of the 132-kDa precursor of this 110-kDa polypeptide and becomes lost in the cleavage of this precursor to form the 110-kDa polypeptide. The ATP binding site has been localized in the midregion of the β chain, where it appears to span a multiply folded region of ~100 kDa near the middle of the 215-kDa tryptic peptide of fragment A. Little information is yet available about the secondary and tertiary structure of intact dynein or of the separated α and β heavy chains. However, circular dichroic study of the intact heavy chains and of fragments A and B has not revealed any extensive α-helical regions similar to those present in the other motor proteins, myosin and kinesin (Mocz & Gibbons, 1990a).

In order to examine whether the major 130-, 215-, and 110-kDa tryptic peptides of fragments A and B constitute distinct structural domains of the intact β heavy chain, we have used additional limited proteolysis and changes in polarization of tryptophanyl fluorescence to probe the conformational stability of these peptides to methanol, salt, temperature, and ATP-induced perturbations. Our data suggest that the three major tryptic peptides correspond to structural domains of the intact β chain, with the 215-kDa domain being composed of a 123- and a 92-kDa subdomain.

Materials and Methods

Outer arm dynein with latent ATPase activity was extracted from sperm of the sea urchin *Tripneustes gratilla* as described previously (Bell et al., 1982). The separated α and β heavy chain fractions were obtained by dialysis of the dynein against 0.5 mM EDTA/5 mM HEPES/NaOH buffer, pH 7.0, followed by sucrose gradient centrifugation in the same medium with the addition of 50 μg/mL aprotinin (Bell et al., 1982). However, since preliminary work showed that, under all conditions used, tryptic digestion of the β chain in the separated β/IC fraction of dynein proceeded in the same manner as that of the β chain in unpurified dynein, most work was performed with unpurified dynein. All samples were dialyzed into low-salt medium containing 5 mM triethanolamine hydrochloride, pH 7.0, 0.5 mM EDTA, and 7 mM 2-mercaptoethanol before digestion.

Limited proteolysis was performed by adding trypsin (TRTPCK, Cooper) at a trypsin:protein ratio of 1:15 (w/w) to the low-salt medium containing 0.25–1.25 mg/mL protein and other components as required for particular experiments. The digestion was allowed to proceed for 60 min at 23 °C or 20 min at 37 °C and then was stopped by adding a 10-fold weight excess of soybean trypsin inhibitor (Cooper), followed by addition of sodium dodecyl sulfate and mercaptoethanol to final concentrations of 1% and incubation in a boiling water bath for 5 min. The possible effects of the addition of ATP on the activity of trypsin itself were examined by measuring the hydrolysis rate of benzoylarginine-*p*-nitroanilide (Erlanger et al., 1961) and were found to be negligible. In addition, the tryptic hydrolysis rate of this synthetic substrate in the high-salt medium used was compared with the rate in the low-salt medium and was found to be about 16% faster in 0.45 M acetate medium, probably because of its slightly higher pH. Effects of methanol and temperature on the kinetic parameters of tryptic hydrolysis have been described (Maurel et al., 1975).

Incubation of dynein with methanol was performed by adding samples in low-salt medium to cold methanol and allowing them to stand on ice for 15 min followed by 15 min at room temperature before digestion. In experiments in which ATP was added to the digestion medium, the proteins were incubated with ATP for 5 min prior to addition of methanol.

Photolytic cleavage of dynein heavy chains was performed by irradiating the samples with near-ultraviolet light (365 nm) in 0.45 M acetate medium containing vanadate or iron(III) gluconate as described previously (Gibbons et al., 1987; Mocz & Gibbons, 1990b).

Steady-state fluorescence polarization and emission spectra were recorded with an SLM-8000C spectrofluorometer (SLM Industries, Urbana, IL) equipped with an external refrigerated bath operating at 20 °C. Fluorescence excitation was performed at 295 nm. Emission spectra were recorded with a 4-nm band-pass for both the excitation and emission monochrometers. Spectra were recorded and integrated over the wavelength range 300–460 nm. For polarization measurements, the emission was observed through a barrier filter with a 300-nm cutoff. The changes in amplitude of fluorescence polarization are presented as percentages relative to the polarization amplitude of the unperturbed β/IC complex. For these experiments, the samples were diluted to have an absorbance of 0.1 at 280 nm.

Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate was performed on 6.5% gels by the method of Dreyfuss et al. (1984), followed by staining with Coomassie Blue R-250. Protein bands in the gels were electroblotted onto a poly(vinylidene difluoride) membrane (Immobilon, Millipore Corp.) and stained with monoclonal antibodies directed toward known regions of the β heavy chain of dynein as described by Mocz et al. (1988). Protein concentrations were determined spectrophotometrically by an absolute method based upon the difference in absorbance at 235 and 280 nm (Whitaker & Granum, 1980).

Results

Methanol-Induced Structural Changes. Limited proteolysis was performed in standard low-salt medium containing various concentrations of methanol up to 25%. Figure 1 shows that there are marked differences in the tryptic susceptibility of the various fragments. The 110-kDa peptide is the most sensitive and mostly becomes degraded to small peptides at methanol concentrations greater than 15%. The 215-kDa peptide of dynein also becomes more susceptible to trypsin, and antibody staining of the resultant digest (Figure 2) shows that one region of this peptide is now present as a new, more stable peptide of 92 kDa while the remainder is presumably lost as small peptides. The 130-kDa peptide, which bears epitope 1, is the most stable and its susceptibility to trypsin appears unaffected by up to 25% methanol.

Ethanol and 1-propanol affect the digestibility of dynein in a similar way, although somewhat lower concentrations are required for the same effect, presumably because of the greater hydrophobicity of these solvents. 1-Butanol did not show preferential destabilization of any of the peptides, and they were simultaneously lost in the course of digestion. Larger amphiphilic molecules, such as 0.5% Triton X-100, appeared to have no significant effect on the digestion pattern (data not shown).

Although methanol affects the tryptic susceptibility of some regions of the β heavy chain, concentrations up to 18–20% appeared to have no significant effect on the vanadate- and iron-mediated photocleavage reactions (data not shown). This indicates that the structure of the catalytic site itself is little affected, in agreement with earlier observations that these concentrations of methanol have little effect on the Triton-activated ATPase activity of dynein (Evans & Gibbons, 1986).

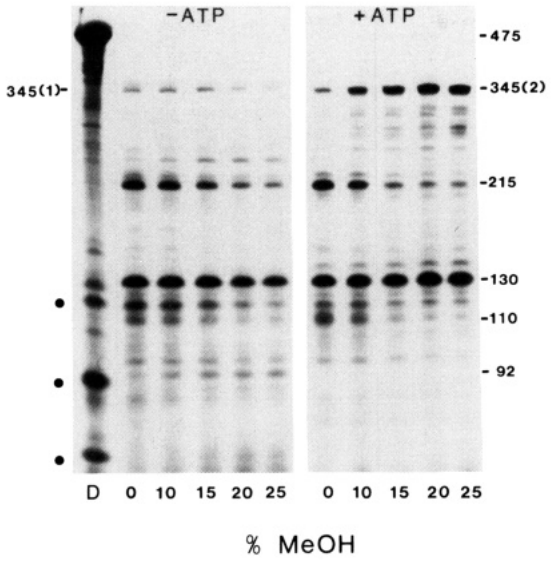

FIGURE 1: Effect of methanol concentration on the tryptic digestion of dynein. The electrophoresis gel shows dynein (0.6 mg/mL) digested for 60 min at 23 °C in low-salt medium containing the indicated concentrations of methanol at a trypsin:protein ratio of 1:15 (w/w). The leftmost lane shows undigested dynein. Tryptic digestion was performed in the absence or presence of 1.5 mM ATP as indicated. The numbers represent approximate molecular masses (in kilodaltons) of the principal cleavage peptides. Dots indicate the intermediate chains. A total of 30 μg of protein was loaded on each lane.

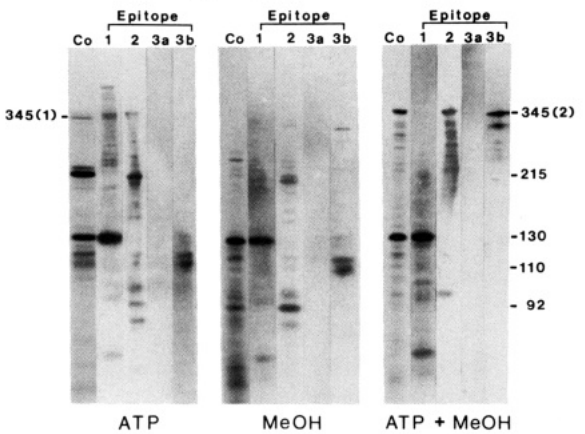

FIGURE 2: Distribution of epitopes in the dynein β chain after digestion in medium containing methanol and/or ATP. Immunoblots of electrophoresis gels of dynein are shown that had been digested for 60 min at 23 °C in low-salt medium containing added 1.5 mM ATP (left), 20% methanol (middle), or 1.5 mM ATP plus 20% methanol (right). Strips of each immunoblot were reacted with monoclonal antibodies against epitopes E1, E2, E3a and E3b on the β heavy chain (clones 6-31-24, C-241-2, 4-69-14, and C-26-5, respectively (Piperno, 1984; Mocz et al., 1988)). The strips labeled Co were stained with Coomassie Blue to show total protein. The peptide 345(1) contains epitopes 1 and 2, whereas peptide 345(2) contains epitopes 2 and 3b. In the sample digested in methanol, the higher sensitivity of the antibody staining shows up epitope 3b in a residual amount of 110-kDa peptide that is scarcely visible in the sample stained with Coomassie Blue. A total of 10 μg of protein was loaded on each lane.

ATP-Induced Structural Changes in the Presence of Methanol. Comparison of the peptide patterns in parallel digests performed in the absence and presence of 0.1–5 mM ATP indicated that concentrations of ATP of 2 mM or more moderately retard formation of the 130- and 215-kDa peptides by slowing cleavage of the 345-kDa precursor described previously (Ow et al., 1987), especially in the early phase of digestion.

In the joint presence of ATP and methanol, there is a remarkable change in the pattern as tryptic proteolysis leads to formation of two major peptides of 130 and 345 kDa with an

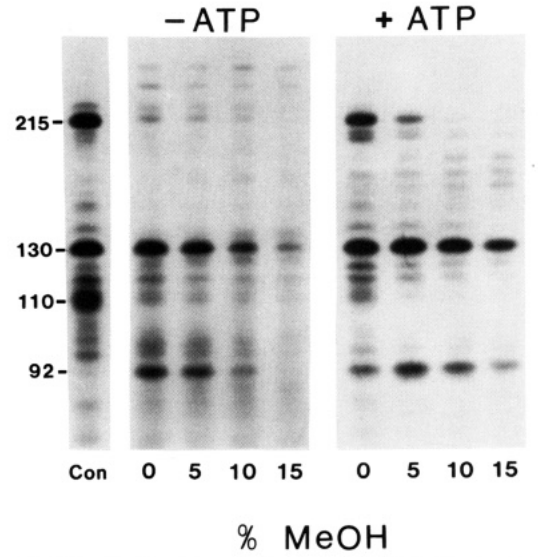

FIGURE 3: Tryptic digestion of dynein at 37 °C in the presence of methanol. Electrophoresis gels show the peptide pattern after digestion as in Figure 1 except that the medium contained up to 15% methanol and the reaction proceeded for 20 min at 37 °C. The leftmost lane shows a control pattern after a 60-min digestion at 23 °C in the absence of ATP and methanol.

apparent lack of the 110-kDa peptide (Figure 1). Although this 345-kDa peptide has a mass similar to that of the intermediate peptide mentioned above, the kinetics of its formation suggest that it has a different origin. Antibody staining of the peptides formed in the presence of ATP and methanol show that this 345-kDa peptide contains epitope 2 and epitope 3b while neither epitope 1 nor epitope 3a is present (Figure 2). Thus, it appears that this 345-kDa peptide, denoted 345(2), is composed of the central 215-kDa region and the 110-kDa region (fragment B), whereas the 130-kDa peptide represents the other end of the heavy chain. Epitope 3a is therefore located close to the terminus of the 110-kDa fragment. The T1 tryptic site is almost completely blocked by ATP in the presence of methanol.

The ~345-kDa digestion intermediate present in the samples digested in the presence of ATP without methanol, denoted 345(1), contains epitope 1 and epitope 2 (Figure 2), indicating that it represents a precursor of fragment A in which the presence of ATP retarded cleavage at the T2 tryptic site.

Tryptic Digestion of Dynein at 37 °C. The stability of dynein to temperature-induced perturbation has been followed over the range 0–45 °C. At temperatures above 30 °C, the 215-kDa fragment exhibits a markedly increased susceptibility to tryptic attack. At 37 °C, this peptide becomes almost wholly converted to a stable 92-kDa peptide (Figure 3). The 130-kDa fragment remains intact after digestion at 37 °C, whereas the 110-kDa peptide has an intermediate stability. The presence of ATP during digestion at 37 °C largely protects the 215-kDa peptide.

If the protein is first incubated for 30 min at 37 °C and subsequently cooled and digested at room temperature, the peptide digestion pattern obtained closely resembles that obtained when the digestion is itself performed at 37 °C, indicating that the temperature-induced structural change in the 215-kDa region is not readily reversible.

Although the ATPase activity of the β heavy chain is retained upon formation of the 215- and 130-kDa peptides by digestion at room temperature (Ogawa & Mohri, 1975; Ow et al., 1987), we have found that the ATPase activity diminishes to less than 1% of its former value upon digestion for 20 min at 37 °C, which converts the 215-kDa peptide to 92 kDa.

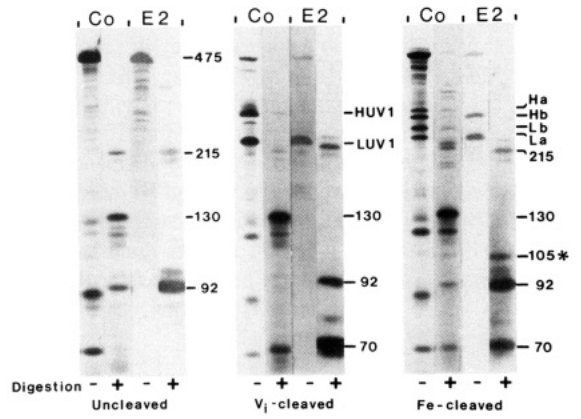

FIGURE 4: Epitope mapping of temperature-induced proteolytic digestion sites in dynein β heavy chain. Dynein was first subjected to photocleavage in 0.45 M acetate medium by irradiation at 365 nm, either for 30 min with 100 μM vanadate and 200 μM ATP or for 3 min with 350 μM iron(III) gluconate and 700 μM ATP, respectively. The unirradiated and irradiated samples were then dialyzed against standard low-salt medium and digested at a trypsin:protein ratio of 1:15 (w/w) for 20 min at 37 °C. Immunoblots of electrophoresis gels showing tryptic digests of unirradiated (left), vanadate-cleaved (middle), and iron-cleaved (right) dynein with control samples not digested with trypsin were reacted with the monoclonal antibody against epitope E2 on the β heavy chain (clone C-241-2). The lanes labeled Co were stained with Coomassie Blue to show total protein. The asterisk indicates the 105-kDa peptide present only in the iron-cleaved sample. HUV1 and LUV1 represent peptides formed by cleavage at the V1 photocleavage site. Ha and La represent peptides formed by cleavage at the Fe–a photocleavage site; Hb and Lb represent peptides formed by cleavage at the Fe–b photocleavage site. The antibody-stained 215-kDa peptide in the iron(III)-cleaved sample is derived from tryptic digestion of residual nonphotocleaved heavy chain.

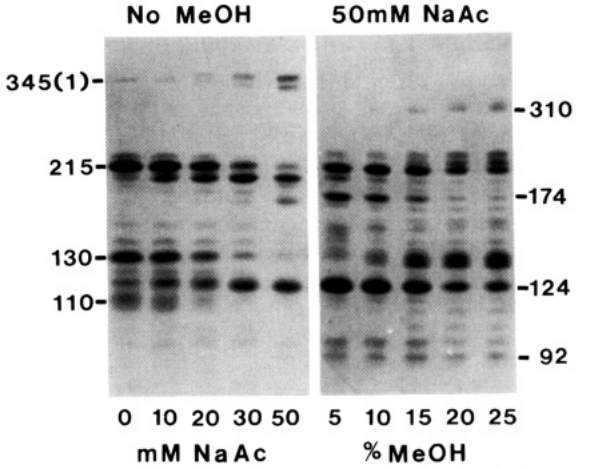

FIGURE 5: Reversal of salt-induced changes in the tryptic digestion pattern of dynein by methanol. The left five lanes of the electrophoresis gel show dynein (0.6 mg/mL) digested at a trypsin:protein ratio of 1:15 (w/w) for 60 min at 23 °C in low-salt medium containing the indicated concentrations of sodium acetate, in the absence of methanol. The right five lanes show similar samples digested in 50 mM sodium acetate and the indicated concentrations of methanol. The 310-kDa peptide is an intermediate in the digestion pathway under these conditions, and its formation is not discussed in detail.

When the digestion at 37 °C is performed in the presence of 5% methanol, the 130- and 92-kDa peptides appear relatively stable (Figure 3). At higher concentrations of methanol, the stability of these peptides decreases approximately in parallel so that little of either remains after digestion in 15% methanol. The presence of 1.5 mM ATP partially protects both the 130- and the 92-kDa peptides during digestion, which is especially apparent in the samples digested in 10–15% methanol. Sucrose gradient centrifugation of a dynein sample that had been digested at 37 °C showed that the stable 130- and 92-kDa peptides cosediment as a single peak at ~10.5 S, indicating that they interact strongly with each other in the tertiary folding of the β chain.

Localization of the 92-kDa Peptide. In order to determine the location of the heat-stable 92-kDa core peptide within the 215-kDa peptide from which it is formed, dynein that had been photocleaved at the V1 site by irradiation in the presence of ATP and vanadate, or that had been photocleaved at the Fe–a and Fe–b sites by irradiation in the presence of iron(III) gluconate ATP (Mocz & Gibbons, 1990b), was digested with trypsin for 20 min at 37 °C. In the sample digested after photocleavage at the V1 site, epitope 2 was present principally in a new peptide of 70 kDa, with some remaining in the residual nonphotocleaved 92-kDa peptide (Figure 4). In digests of dynein that had been photocleaved at the Fe–a and Fe–b sites, epitope 2 is present on a third peptide of 105 kDa as well as on the 70- and 92-kDa peptides on which it appeared in samples that had been cleaved with vanadate (Figure 4). Since the V1/Fe–a and Fe–b cleavage sites are located ~70 and 90–100 kDa, respectively, from the 215–110-kDa junction and photocleavage at the Fe–b site gives rise to a larger peptide of 105 kDa, the heat-stable 92-kDa peptide must be located at the junction of the 215-kDa fragment with the 110-kDa peptide. The 70-kDa peptide spans from the 215–110-kDa junction to the V1/Fe–a site, while the 105-kDa peptide spans from this junction to the Fe–b site (Figure 7).

Nucleotide Specificity. The action of ATP in modifying the trypsin digestion pattern of the β chain appears to be moderately specific. Other purine nucleotides such as ADP, GTP, GDP, ITP, and IDP had similar but much less pronounced effects. Pyrimidine nucleotides, such as CTP, CDP, UTP, and UDP, have little or no effect, nor have AMP–PNP and AMP–PCP, suggesting that these compounds do not bind to dynein under these conditions (data not shown).

Effect of Ionic Conditions. The digestion pattern of the β chain is sensitive to the presence of salt. Increasing the ionic strength of the digestion medium slightly by adding as little as 50 mM sodium acetate produces characteristic changes in the pattern of tryptic fragmentation (Figure 5). The 130-kDa low-salt peptide is completely replaced by a somewhat smaller 124-kDa peptide. The 215-kDa low-salt peptide is less stable and becomes digested rapidly to several smaller peptides in the range 160–180 kDa. The 110-kDa low-salt peptide appears to be degraded with little selectivity and is not seen when the sodium acetate concentration exceeds 20 mM. Essentially the same changes are seen with 50 mM NaCl, NH_4Cl or ammonium acetate or with as little as 1 mM magnesium acetate (data not shown).

Further increasing the ionic strength of the digestion medium to 0.5 M causes no additional changes in the fragmentation pattern, although the rate of proteolysis becomes generally slower and several moderately stable intermediates in the range 300–445 kDa are visible in the early phase of digestion. The presence of ATP during digestion retards the cleavage of the 345(1)-kDa intermediate to a significantly greater extent than that under low-salt medium (data not shown).

The digestion pattern is also sensitive to pH. Increasing the pH of digestion to 8.5 or higher favors the formation of the 124-kDa peptide, especially when ATP is included in the digestion medium. However, the degradation of the 215-kDa peptide is only slightly affected by higher pH (data not shown). These results indicate that the region of difference between the 130- and 124-kDa peptides is especially sensitive to such

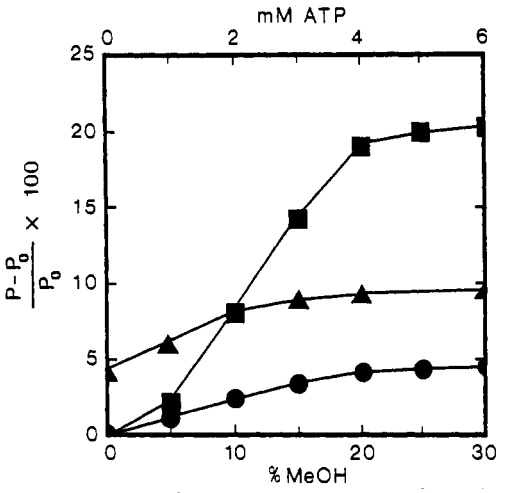

FIGURE 6: Dependence of the tryptophanyl fluorescence polarization of β/IC fraction on methanol and ATP concentration. The polarization data are plotted on the y axis as percentage change relative to the absolute polarization of the sample in standard low-salt medium (P_0). The dynein was in low-salt medium with additions as indicated: ■, methanol (bottom scale); ●, ATP (top scale); ▲, 5 mM ATP plus methanol (bottom scale). The absolute polarization of β/IC fraction in standard low-salt medium was 0.175 ± 0.016 (n = 6). Excitation wavelength: 295 nm. Temperature: 20 °C.

environmental factors as ionic strength and pH.

The effects of 50 mM sodium acetate on the digestion pattern can be partly reversed by including 15% methanol in the medium (Figure 5). At methanol concentrations exceeding 15%, the predominant products are the 130- and 215-kDa peptides despite the presence of salt.

Immunostaining of dynein and β/IC samples digested in the presence of salt has shown that the prominent 124-kDa peptide is not recognized by the antibody specific for epitope 1, which does recognize the somewhat longer 130-kDa parent peptide. Therefore, epitope 1 must lie close to one end of the 130-kDa peptide.

Structural Changes Monitored by Fluorescence. In order to confirm the changes in the structure of the β heavy chain suggested by the changes in digestion pattern, we examined tryptophanyl fluorescence emission and polarization of the β/IC complex under the same conditions as those used for the proteolysis. The fluorescence intensity of β/IC was enhanced about 20% by the presence of 20% methanol, whereas addition of NaCl up to 0.5 M had no significant effect (data not shown).

The changes in polarization of tryptophanyl fluorescence of the isolated β chain/IC complex in the presence of different concentrations of methanol and ATP are shown in Figure 6. Increasing concentrations of methanol result in a progressive enhancement of polarization, amounting to about 20% at methanol concentrations of 20% or above. Increasing concentrations of ATP alone result in a much smaller enhancement, amounting to about 5% with 5 mM ATP. Assays in the combined presence of methanol and 5 mM ATP show that the amount of polarization enhancement is substantially reduced compared to that with methanol alone, with 25% methanol giving an increase of only about 5% over that observed with 5 mM ATP alone. Similar results with a slightly larger amplitude of change were obtained with intact dynein that had been purified by sucrose density gradient centrifugation by the method of Bell et al. (1982).

These changes in fluorescence polarization and emission spectra indicate that the orientation of certain of the tryptophanyl residues and their interaction with the hydrophobic core of the dynein are altered by the presence of ATP or methanol.

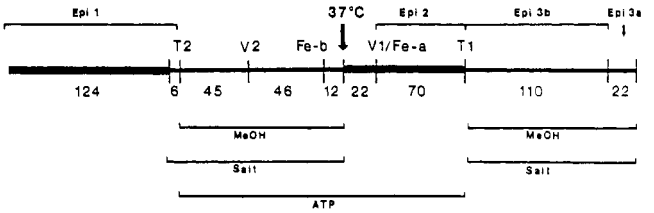

FIGURE 7: Linear map of stability regions in β heavy chain. The diagram shows the relative position of the various proteolytic and photolytic cleavage sites. The numbers represent approximate molecular masses (in kilodaltons) of the peptide regions spanning between the cleavage points. The site of temperature-sensitive proteolytic cleavage is indicated by 37 °C. The bold lines represent the most stable regions of the heavy chain. The regions predominantly affected by methanol, salt, and ATP are also indicated.

The magnitudes of the changes suggest that the conformational transitions associated with these and ligand-induced perturbations are localized to a relatively small portion of the molecule. The decreased magnitude of the polarization change induced by methanol in the presence of ATP supports the proteolytic evidence that ATP partially protects the dynein against the structural changes induced by methanol.

Discussion

We have shown that addition of methanol and salts or an increase in temperature changes the stability of the various regions of the dynein β chain to proteolytic cleavage. The results obtained are summarized in Figure 7 where the various stability regions are shown in relation to the principal tryptic (T1 and T2) and photolytic (V1, V2, Fe–a and Fe–b) cleavage sites (Mocz et al., 1988; Mocz & Gibbons, 1990b).

The effects of methanol can be attributed to increased hydrophobicity of the medium. Methanol/water mixtures more closely approximate the dielectric constant of protein interiors and therefore may be expected to induce conformational changes that bring hydrophobic areas to the protein surface where they are more susceptible to proteolytic cleavage. This is seen in the enhanced cleavage of the 110- and 215-kDa peptides.

It would be anticipated that the addition of salt or an increase in temperature would weaken hydrogen bonds while strengthening hydrophobic interactions to produce effects on the structure of dynein opposite from those of methanol (Biringer & Fink, 1988). In agreement with this expectation, we have found that an increase in salt concentration or in temperature affects approximately the same regions of the molecule. Moreover, the salt-induced changes can be reversed with methanol, although the thermal changes appear to be irreversible. The results suggest that the regions affected by salt and temperature have a moderately flexible conformation, while the unaffected regions possess a more limited conformational response to change in the chemical environment.

The early occurrence of tryptic cleavage at the site on the β heavy chain that produces the 345(1)-kDa peptide, in both the absence and presence of salt and methanol (Figure 1 and Ow et al., 1987), indicates that this site is a region of general protease sensitivity and lends support to the suggestion made previously (Ow et al., 1987) that the 345(1) peptide corresponds to the globular head. The complementary peptide of 110 kDa found in low-salt digests is postulated to correspond to the tail of the "tadpole" but is not preserved intact in the presence of salts and methanol or at elevated temperatures.

The salt concentration of the digestion medium modifies the accessibility to trypsin of a region near the middle of the β chain. In the near absence of salt, the 345(1)-kDa intermediate is rapidly cleaved to form complementary 215- and

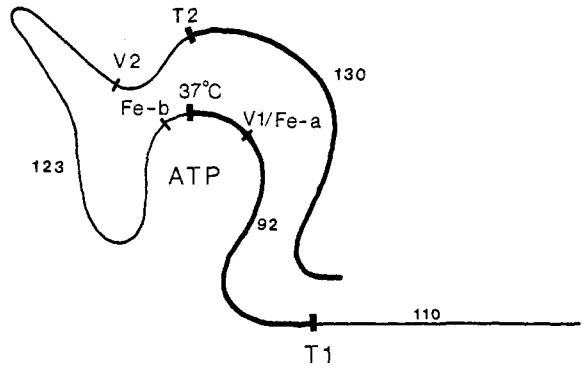

FIGURE 8: Structural model of β heavy chain. The highly schematic model is based on the conformational response to various structural perturbations as revealed by limited proteolysis in low-salt medium. The various segments correspond to those shown in Figure 7 but with fewer details indicated. The 130-kDa domain is proposed to interact with the 92-kDa domain of the 215-kDa peptide to form a stable folding unit. The remaining 123-kDa region of the 215-kDa peptide and the 110-kDa domain (Fragment B) represent salt-sensitive sections of molecule and may correspond to the regions of interaction with the B and A microtubules, respectively.

130-kDa peptides by scission at a site that appears to be completely inaccessible in media of higher salt concentration, whereas in 50 mM acetate with no ATP, this intermediate is cleaved at multiple sites to form peptides in the 160–180-kDa size range, a 124-kDa peptide, and small peptides. However, the fact that a similar pair of peptides is formed in both low- and high-salt digestion conditions shows that the 345(1)-kDa precursor of fragment A is organized into two major protease-resistant regions.

The binding of ATP to soluble dynein particularly affects the region of the 215-kDa peptide close to its junction with the 130-kDa peptide as is seen in its increased stability to tryptic digestion at 37 °C, even in the presence of methanol. Furthermore, ATP appears to block cleavage at the T1 site in the presence of methanol and to slow cleavage at the T2 site under both low- and high-salt conditions. This implies that ATP affects the structure of regions distant from its binding site, which from photocleavage data is believed to be close to the middle of the β heavy chain (Gibbons et al., 1987; Mocz & Gibbons, 1990b). These findings can be reconciled by postulating either that the tertiary folding of the molecule brings the ATP binding site in the midregion of the chain physically close to the T1 and T2 cleavage sites or that ATP binding at the catalytic site produces structural changes some distance away. Our results are in general agreement with those of Inaba and Mohri (1989, 1990), who recently suggested on the basis of their proteolytic experiments that there are two fundamental conformation states of dynein depending on the presence of ATP. The effect of ATP in reducing the rate of tryptic cleavage may be related to the structural change of the dynein arms observed in electron microscopic samples of axonemes prepared in the presence and absence of ATP (Goodenough & Heuser, 1984, 1985), although the two globular components of axonemal dynein are reported to become less tightly associated in the presence of ATP, and such a change might be expected to increase, rather than to decrease, the accessibility of the sites to trypsin. The changes in the polarization of tryptophanyl fluorescence provide further evidence that soluble outer arm dynein undergoes a structural change in the presence of ATP. This structural change may include either an increase in dimensional asymmetry reflecting movements of regions of the heavy chain toward the ATP binding site or a decrease in segmental flexibility of the various regions of the heavy chain upon ATP binding.

Within the limitations of the proteolytic approach, our data lead us to a view of dynein in which the β chain is organized into three or four regions of different stability that each correspond to a distinct structural domain in the tertiary structure of the native heavy chain. It is possible that the three major tryptic peptides may each correspond to a structural domain, for the 130- and 110-kDa peptides are the most and least stable regions of the β chain, respectively. However, the fact that the 215-kDa central peptide is comprised of a relatively stable region close to its junction with the 110-kDa peptide and a lower stability region close to its junction with the 130-kDa peptide suggests that it may be more valid to consider these regions as distinct domains, at least in terms of their susceptibility to solvent- and temperature-induced perturbations. The fact that the most stable 130- and 92-kDa domains are lost approximately in parallel upon digestion in increasing concentrations of methanol at 37 °C suggests that they either have a similar secondary structure or that they mutually interact in a manner that makes them dependent on each other for their stability. Figure 8 presents a schematic diagram illustrating one possible arrangement of the different stability domains of the β chain. The fact that the presence of ATP appears to have its greatest effect on the stability of the flexible 123-kDa domain of the 215-kDa peptide rather than on the more stable 92-kDa domain where most of the ATP binding site is believed to be located (Mocz et al., 1988) suggests that ATP binding may occur at the interface between these two domains with the 123-kDa domain being the portion of the β chain that is involved in the ATP-dependent cross-bridges to the B tubules that are responsible for motile activity. Correspondingly, the 110-kDa domain (fragment B) may participate in the interaction between the α and β heavy chains of a given dynein arm as well as in the fixed attachment of the dynein arm structure to the A tubules. Future work using intramolecular cross-linking and immunoelectron microscopy may help to elucidate the details of this or similar models of dynein heavy chains.

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Synthesis and Characterization of a Heterobifunctional Mercurial Cross-Linking Agent: Incorporation into Cobratoxin and Interaction with the Nicotinic Acetylcholine Receptor†

Eric R. Wohlfeil‡,§ and Richard A. Hudson*,‖

Department of Medicinal and Biological Chemistry, College of Pharmacy, and Department of Chemistry, College of Arts and Sciences, University of Toledo, Toledo, Ohio 43606, and Department of Biochemistry, Wayne State University School of Medicine, Detroit, Michigan 48201

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ABSTRACT: The heterobifunctional organomercurial reagents 3-(acetoxymercurio)- and 3-(chloromercurio)-5-nitrosalicylaldehyde were prepared, characterized in model studies, and used to probe the interaction between cobratoxin, purified from the venom of the Thailand cobra (*Naja naja siamensis*), and the affinity-purified nicotinic acetylcholine receptor (AcChR) from *Torpedo californica* electroplax. These reagents may also be useful in introducing chemically well-defined heavy metal atoms into proteins containing no reactive thiols. Model reagent adducts were prepared in situ by reductive amination with *N*-butylamine and N^{α}-acetyllysine-*N*-methylamide. The nitrophenolic pK_as of the amine adducts were similar to those of the aldehyde reagents though reduced by 1.3–1.5 units when compared with the hydroxylmethyl reduction product. Reaction of either mercuriosalicylaldehyde with cobratoxin led to a single major modification product incorporating 1 mol of the reagent into cobratoxin at Lys 23. The Lys 23 modified toxin had a reduced binding affinity for the AcChR over that of the native toxin (K_d 2.75 nM cf. 0.3 nM). Reduction of the purified AcChR with 1 mM dithiothreitol (DTT) followed by removal of excess thiol led to cross-linking reactions with the Lys 23 modified cobratoxin to both the α and β subunits of the AcChR complex. Reaction of DTT-treated AcChR with *N*-ethylmaleimide (NEM) blocked cross-linking, while treatment of the initially cross-linked toxin–AcChR complex with mercaptoethanol leads to reversal of cross-linking. These observations strongly support cross-linking mediated by the formation of a mercury–sulfur bond and further lend support the identity of the respective interacting sites in AcChR and toxin.

Multifunctional reagents have been extensively employed over the past two decades to probe the chemical reactivity of biopolymer surfaces, to study the origins of protein–protein interactions, and, in particular, to examine intrasite relationships at enzyme active centers. Here, we report the synthesis, characterization, and demonstration of the utility of 3-(acetoxymercurio)- and 3-(chloromercurio)-5-nitrosalicylaldehyde. These reagents contain a nitrophenolic group that can be used to follow protein modification by either the aldehyde or mercurial reactivities and to report on the microenvironment at the sites of modification (Hille & Koshland, 1967). Also, proteins or protein complexes thus modified may provide useful heavy metal derivatives for X-ray analysis of protein structure [cf. Edwards et al. (1974)].

These reagents may be employed in multiple though restricted ways [a preliminary report is given in Wohlfeil et al. (1985)]. The aldehyde may be used to reductively alkylate lysine or N-terminal amino groups. Intramolecular cross-

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‡ Department of Biochemistry, Wayne State University School of Medicine.

§ Present address: Department of Anesthesiology, Sinai Hospital, Detroit, MI 48235.

‖ Department of Medicinal and Biological Chemistry, College of Pharmacy, and Department of Chemistry, College of Arts and Sciences, University of Toledo.

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