Chymotryptic Digestion of *Tetrahymena* Ciliary Dynein. I. Pathway of the Degradation of 22S Dynein Heavy Chains

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Abstract. As shown in the preceding paper (Toyoshima, Y. Y., 1987, *J. Cell Biol.*, 105:887-895) three-headed *Tetrahymena* 22S dynein consists of three heavy chains (HCs) and is decomposed into two-headed (H) and one-headed (L) fragments by chymotryptic digestion. To accurately determine the presence of multiple ATPases and ultimately the location of various domains, it is necessary to determine the identity of each HC fragment relative to the original HCs in 22S dynein. The degradation pathway of each HC was determined by peptide mapping and immunoblotting.

The three HCs (Aα, Aβ, and Aγ) were immunologically different; although SDS-urea gel electrophoresis showed that Aα HC was apparently resistant to the digestion, actually three distinct HCs contributed to the same band alternately. H fragment was derived from Aγ and Aβ HCs, whereas L fragment originated from Aα HC. Since both fragments were associated with ATPase activity, these results directly demonstrate the presence of multiple ATPase sites in *Tetrahymena* 22S dynein.

From the recent electron microscopic studies, it has been established that *Tetrahymena* ciliary 22S dynein consists of three globular heads and thin stalks (2, 3, 12). SDS-urea gel electrophoresis showed that three heavy chains (HCs) are present in the 22S dynein preparation (12). To study the functions of these HCs and their relationships to the heads, we must decompose the molecule into functional units. As shown in the preceding paper, chymotryptic digestion splits the molecule into two portions, both associated with ATPase activity: one consisting of two globular heads linked by stalks (H fragment), and the other consisting of a single head (L fragment). This does not necessarily mean that 22S dynein has multiple ATPase sites, since the L fragment might come from the H fragment. To examine this possibility, we must determine the degradation pathway of the HCs.

In this work, I determined the pathway by peptide mapping and immunoblotting. The HC constituting L fragment originated from Aγ-HC and was not a digestion product of H fragment. Thus the presence of multiple ATPase sites is demonstrated directly. Further, these results indicate that the three HCs are different species and strongly suggest that one 22S dynein molecule consists of three distinct HCs, each of which would correspond to one head.

Materials and Methods

Chymotrypsin Digestion of *Tetrahymena* 22S Dynein

Chymotryptic digestion of *Tetrahymena* 22S dynein was performed as described in the preceding paper (12). Dr. Toyoshima's present address is Department of Cell Biology, Stanford University School of Medicine, Stanford, CA 94305.

1. Abbreviation used in this paper: HC, heavy chain.

PAGE and Peptide Mapping

SDS- and SDS-urea-PAGE in a discontinuous Tris-glycine buffer system (4) and in a continuous Tris-glycine buffer system (13), respectively, were done as described in reference 12. The two gel systems resolved individual components with different degree of resolution. For peptide mapping, specimens were treated with N-(7-dimethylamino-4-methylcoumarinyl) maleimide before electrophoresis on SDS-urea gels as described previously (13). HC bands were cut out as slices under a UV light and put into the sample slots in stacking gel of the Laemmli system (4). To compare the peptide maps accurately, two gel slices containing the HCs to be compared were put into the same slot. Concentration of α-chymotrypsin was adjusted with an SDS sample buffer and 0.2-10 μg of chymotrypsin was loaded onto the gel slices in the slots. Electrophoresis was done for 14 h at a constant current of 8 mA (1). Gels were stained with silver using a silver staining kit (Daiichi Chemicals, Tokyo, Japan).

Preparation of Antibodies

N-(7-dimethylamino-4-methyl-coumarinyl) maleimide-labeled 22S dynein and its chymotrypsin digest (at stage II as defined in the preceding paper) were subjected to electrophoresis on slab gels in an SDS-urea buffer system. Dynein HC bands were identified under UV light and cut out. The gel slices were homogenized with Teflon-glass homogenizer and then mixed with the same volume of complete Freund's adjuvant. About 100 μg of protein in 1 ml was injected into a rabbit and boosted 3 wk later with the same amount of protein in incomplete Freund's adjuvant. Animals were first bled 3 wk after the second injection, and were subsequently bled every 5-7 d. Preimmune serum was obtained from animals 1 wk before the first injection. Serum samples were initially screened in Ouchterlony immunodiffusion assay (6).

Immunoblotting

Immunoblotting was done essentially as described by Towbin et al. (II) after SDS-urea gel electrophoresis. A nitrocellulose sheet with a pore size of 0.1 μm (pH 7.9; Schleicher & Schnell, Inc., Keene, NH) and horseradish peroxidase-conjugated goat IgG raised against rabbit IgG (60 μg/ml antibody, Cappel Laboratories, Malvern, PA) were used. For coloring reaction, 4-chloro-l-naphthol (BioRad Laboratories, Richmond, CA) was used.

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Identification of Heavy-Chain Bands on SDS–Urea and SDS Gels

As described in the preceding paper (12), three HCs originally found in a 22S dynein preparation from Tetrahymena cilia were digested with chymotrypsin to produce three other major bands in the high molecular mass region on SDS–urea gels (see Fig. 3 b in reference 12). To determine the degradation pathway, peptide mapping and immunoblotting were used. For peptide mapping, it is advantageous to use a discontinuous buffer system for high resolution. Figs. 1 and 2 establish the relationships of HC bands between SDS–urea and the Laemmli systems. In Fig. 1, HCs were recovered from SDS–urea gels and were subjected to electrophoresis again in the Laemmli system. Band-1 and -2 HCs, and band-4 and -5 HCs, respectively, had apparently the same mobilities and were indistinguishable; whereas band-3 HC had different mobilities depending on the stage of digestion. That is, band-3 on SDS–urea gels actually corresponded to three distinct HCs termed band-3a, -3b, and -3c HCs as the ascending order of the mobility.

Fig. 2 presents SDS–urea (a) and the Laemmli (b) gels of the chymotrypsin digests of 22S dynein, showing the identification of HCs at three stages defined in reference 12. The transition of band-3 HC is clearly shown in Fig. 2 b; band-3a disappeared at stage I; band-3c appeared at stage I and was lost at stage II; band-3b emerged at stage II.

Peptide Mapping of the Fragments of Heavy Chains

In reference 12, I showed that chymotrypsin decomposed three-headed 22S dynein into two-headed (H fragment) and single-headed (L fragment) particles. The HCs contributing to these fragments were different depending on the digestion stage (Fig. 6 of reference 12). Considering the presence of three different HCs at band-3 at different stages, H fragment consisted of band-2 and -5 HCs; band-2, -3b, and -5 HCs; and band-3b, -5, and -6 HCs at stages I, II, and III, respectively; L fragment consisted of band-3c HC, band-3c and -4 HCs, and band-4 HC, at stages I, II, and III, respectively.

To inspect the pathway, peptide maps of HCs at stages I and II were compared in all possible combinations (Fig. 3). Underscores in Fig. 3 indicate the combinations in which the peptide map shown on the left was judged to contain all the bands found in that on the right. We must note that the density distribution over the bands may be significantly different between the two maps of HCs with the common origin depending on the digestion conditions.

From the comparisons of 22S dynein HCs, it is evident that band-1, -2, and -3 (i.e., Aa, Ab, and A0) HCs have different peptide maps. HCs at stages I and II were unambiguously related to the parent HCs at the previous stages. By comparing peptide maps of 22S dynein HCs and those at stage I, it is clear that band-2 HC remains unchanged, and that band-3c and -5 HCs come from band-1 and -3a HCs, respectively. Comparisons of the maps of 22S HCs and those at stage II show that the origins of band-3b, -4, and -5 HCs are band-2, -1, and -3a HCs, respectively. Peptide maps of HCs at stages I and II were compared to confirm these relationships.

Thus, the pathway is determined uniquely: band-1 HC degrades to band-4 HC via band-3c, band-2 HC goes to band-
3b HC, and band-3a HC changes to band-5 HC, as illustrated in Fig. 5 a.

**Immunoblotting of Heavy Chains and Their Chymotrypsin Digests**

Polyclonal antibodies were raised against band-2, -4 and -5 HCs at stage II and immunoblotting of 22S dynein and its chymotrypsin digests was performed (Fig. 4). Each of these three antibodies stained only one HC of 22S dynein, thus demonstrating the presence of three immunologically different HCs. It is obvious that band-4 was derived from band-1 (A\textsubscript{a}) HC, whereas band-5 originated from band-3a (A\textsubscript{v}) HC (Fig. 4 a).

Band-3 HCs at different stages were reacted with different antibodies, again showing that three HCs (namely, band-3a, -3b, and -3c HCs) had roughly the same mobilities on SDS-urea gels. Band-3 HC in intact 22S dynein reacted uniquely with anti-band-5 HC (Fig. 4 a, lane 5), indicating that band-5 HC was derived from band-3a (A\textsubscript{v}) HC. At stage II, band-3 HC was stained with both anti-band-2 HC and anti-band-4

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*Figure 3.* Peptide mapping of 22S dynein HCs and their chymotryptic fragments to determine the relationships of digestion products at different stages. Arabic and Roman numerals indicate HC bands and digestion stages, respectively, as shown in Fig. 2. Underscores mark the pairs in which all the bands in the right lane had their counterparts in the left lane.
HC antibodies (Fig. 4 b, lanes 2 and 4). Apparently band-3b and -3c HCs were contained in this particular preparation, since band-3b HC appeared to come from band-2 (A0) HC (Fig. 4 c, lane 2). At stage III anti-band-2 HC antibody stained mainly band-3b but also band-4. Actually, SDS–urea gels showed two barely separated bands at the location of band-4 (Fig. 2 a, lane III) and SDS gels showed a faint band below the dense one corresponding to band-4 and -5 HCs (Fig. 2 b, lane III). When H fragment at stage II was digested again with chymotrypsin, band-4 HC appeared (data not shown). Thus, band-4 at stage III may consist of two HCs derived from band-1 and from band-2 via band-3b HCs. At stage III, anti-band-5 antibody stained band-6 HC, indicating that band-6 HC was derived from band-5 HC.

These results are consistent with those obtained by peptide mapping (Fig. 3) and the degradation pathway shown in Fig. 5 a. Thus, if we assume that each 22S dynein molecule consists of three different HCs (hetero-trimer), and take the degradation pathway into consideration, HC composition of 22S dynein and its H and L fragments at different stages is determined as schematized in Fig. 5 b. Note that H fragments at stages II and III are mixtures of two-headed particles consisting of band-2 and -5 HCs and band-3b and -5 HCs (stage II), and those of band-3b and -5 HCs and band-3b and -6 HCs (stage III), respectively.

**Discussion**

**Does Tetrahymena 22S Dynein Consist of Three Different Heavy Chains?**

Peptide mapping of three HCs of 22S dynein suggested that these three HCs are different species (Fig. 3). Immunoblotting of 22S dynein clearly showed the presence of three immunologically different HCs (Fig. 4). Nishino and Watanabe (5) also reported that there were three immunologically different HCs in *Tetrahymena* dynein, although their SDS–urea gel showed only two HC bands (Fig. 2 in reference 5).

There was no direct evidence against the hypothesis that 22S dynein consists of three different HCs (1-2-3 composition). The possibility of a homo-trimer is excluded, since all dynein molecules in 22S dynein could be decomposed into H and L fragments. Another possibility that there are two populations with 1-2-2 and 1-3-3 compositions remains. In this case, H fragment should comprise homo-dimers of HCs 3b-3b and HCs 5-5. If this is the case, density gradient centrifugation or ion-exchange column chromatography could separate peak H into two peaks since band-2 and -3 HCs would be different in net charge, and band-3b and -5 HCs are different in mass. However, in DEAE–Sephacel chromatography (data not shown) and in density gradient centrifugation (Fig. 5 in reference 12), band-3b and -5 HCs were always present in roughly equal amounts and apparently moved as an entity. Thus, the hetero-trimer composition (1-2-3) of 22S dynein seems to be more plausible than the 1-2-2 and 1-3-3 compositions. Immunoadsorption with anti-band-2 HC and anti-band-5 HC antibodies should help resolve these possibilities.

**Multiple ATPase Sites on Tetrahymena 22S Dynein**

Shimizu and Johnson (9) showed the presence of three ATP-binding sites in *Tetrahymena* 22S dynein from the dissociation kinetics of dynein from microtubules. Pfister et al. (7, 8) demonstrated that all three HCs in *Chlamydomonas* 18S and 12S dynein have ATP-binding sites. Sea urchin 21S dynein has been shown to have two ATPases (10, 13).

As described in reference 12, both H and L peaks of digestion products of 22S dynein have ATPase activity. Band-4 HC, which constitutes the L peak, originates from band-1 (A0) HC; thus an ATPase site should reside on A0 HC. Similarly band-2 (A0) HC and/or band-3a (A1) HC, origin of band-3b and -5 HCs, respectively, should be associated with ATPase activity. Therefore, provided that 22S dynein consists of three different HCs, we can conclude that one 22S dynein molecule has at least two ATPase sites.

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