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DISSOCIATION OF *TETRAHYMENA* 30 S DYNEIN INTO 14 S SUBUNIT BY SONICATION

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Summary

The sonication of 30 S dynein obtained from *Tetrahymena* cilia induced dissociation into 14-S subunits, some of the enzyme still remaining as intact 30 S dynein and partially dissociated dynein (21 S) in a minor amount. It was demonstrated that the enzymatic properties of the 14 S subunit are quite similar to those of 30 S dynein except for the Ca^{2+}:Mg^{2+} ratio. ATPase (EC 3.6.1.3) (ATP phosphohydrolase) activity of the 14 S subunit was steadily enhanced by increasing concentrations of Mg^{2+}. It was also activated by Ca^{2+} with an optimum at 6 mM but inhibited by a further increase in concentration. The Ca^{2+}:Mg^{2+} ratio at 1 mM was about 0.62. 0.6 M KCl stimulated ATPase activity of the 14 S subunit two-fold. The Mg^{2+}-ATPase had an optimum at pH 6.2 and revealed a high activity over pH 10. The Ca^{2+}-ATPase showed two optima at pH 6.2 and 9.5. The K_m for ATP was 10 μM. Only 10% of the 14 S subunit recombined with the outer fibers in the presence of Mg^{2+}. The 14 S subunit was shown to have the same mobility as that of 30 S dynein on sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Introduction

Dynein ATPase (EC 3.6.1.3) (ATP phosphohydrolase) forms the projections or so-called arms bound to the A-tubules of the outer doublets in cilia and flagella [1]. The induction of movement of glycerol- or Triton-treated cilia or flagella needs ATP [2—5]. Moreover, when the outer arms (a half of dynein) are removed, the reactivated spermatozoa beat at a frequency half that of the control [6]. In flagellar axonemes briefly digested with trypsin, sliding occurs between adjacent outer doublets, resulting in an increase in length by a factor of five [7,8]. These facts indicate that dynein plays a very important role in ciliary and flagellar motility.

Two forms of dynein are always extracted from *Tetrahymena* cilia, sedimenting at 30 S and 14 S [1]. Both dyneins differ in several enzymatic properties and behavior to the outer fibers [1,9,10]. Electron microscopic observations suggest that 30 S dynein is a linear connection of globular 14 S dynein particles of the same size [11]. Urea and sodium dodecylsulfate can dissociate 30 S dynein into a 14 S form although it has different properties to both 30-S and 14-S dyneins [12].

It is of interest to obtain the subunit(s) of 30 S dynein in a native state and characterize its properties thus comparing it with 30-S and 14-S dyneins. In this paper a new procedure for dissociating *Tetrahymena* 30 S dynein is presented, which produces a 14 S subunit much more native than that obtained by urea or sodium dodecyl sulfate treatment. Its properties are characterized and compared with 30-S and 14-S dyneins. Their electrophoretic properties are also described.

Materials and Methods

Tetrahymena pyriformis, strain W, was used throughout the present work. The isolation of cilia, extraction and separation of 30-S and 14-S dyneins, ATPase assay and sodium dodecyl sulfate-polyacrylamide gel electrophoresis were carried out as described elsewhere [12,16].

Dissociation of 30 S dynein

The 30 S dynein, fractionated in a medium containing 10 mM Tris · HCl (pH 8.2), 0.1 mM EDTA, 0.1 mM dithiothreitol and sucrose (about 15%), was sonicated for 3 min with cooling using a Toyo-Riko sonifier (model 2N-100) with a resonance frequency at the maximum power. The sonicated 30 S dynein fraction was dialyzed against a solution of 10 mM Tris · HCl (pH 8.2), 0.1 mM EDTA and 0.1 mM dithiothreitol to remove sucrose, followed by a sucrose density gradient centrifugation [12].

In some experiments, the sonication of the 30 S dynein fraction prepared in 10 mM Tris · HCl (pH 8.2) and 0.1 mM dithiothreitol was performed in the presence of 1 mM $MgCl_2$. Aggregates formed during the procedure were removed by centrifugation after dialysis, and the supernatant was subjected to centrifugation on a sucrose density gradient containing 1 mM $MgCl_2$ instead of 0.1 mM EDTA.

Recombination of dynein fraction with the outer fibers

The outer fiber fraction was first re-dialyzed against the extraction medium to remove remaining dynein. The dynein-free outer fibers were then mixed with 14 S or 30 S dynein or 14 S subunit in 25 mM Tris · HCl (pH 8.2) and 10 mM $MgCl_2$ and left standing overnight in an ice bath. After centrifugation, the supernatant was assayed for ATPase activity of dynein which was not bound to the outer fibers. The control experiment was performed without addition of the outer fiber fraction. In some cases, a reassembly buffer for polymerization of brain tubulin [13] was used with the modified medium (10 mM $MgSO_4$, 50 mM KCl, 1 mM ethylene glycol bis (2-aminoethyl)ether tetraacetic acid (EGTA), 4 M glycerol and 5 mM 2-(*N*-morpholino)ethane sulfonic

acid (MES)/KOH at pH 6.6) for the recombination test.

To obtain the species of dynein recombined with the outer fibers, the precipitate was dialyzed against the extraction medium to re-extract bound dynein and followed by centrifugation on a sucrose density gradient.

Protein determination

Protein concentration was determined by the method of Lowry et al. [14] with bovine serum albumin (from Sigma Chem. Co.) as a standard.

Results

Dissociation of 30 S dynein

Fig. 1B shows a typical sedimentation profile of the sonicated 30 S dynein fraction through a sucrose density gradient. Compared with the sedimentation pattern of the crude dynein fraction (Fig. 1A), the protein profile gave a main peak at 14 S having a gentle slope on the heavier side, a small peak at 30 S and a tiny shoulder around 4 S. The ATPase pattern had a main peak at 14 S just corresponding to the main peak of protein, a small 30 S peak and a broad peak around 21 S. This result indicated that more than half of 30 S dynein was dissociated into the 14 S subunit, some still remaining as intact 30 S dynein and small amounts of partially dissociated dynein. The specific activity at the 14 S peak was about 0.3 μmol P_i/mg protein per min. Here, it should be mentioned that the 14 S component obtained by sonication is designated as 14 S subunit in this paper to distinguish it from 14 S dynein which is originally extracted from the axonemes. The sonication treatment was revealed to be much milder for dissociating 30 S dynein into its subunit(s) than urea or sodium dodecyl sulfate treatment, in which the ATPase peak did not correspond to the protein peak and the enzymic activity was considerably reduced by the dissociating reagents [12].

When 30 S dynein fraction was sonicated in the presence of 1 mM $MgCl_2$, the protein solution became turbid. After centrifugation at 45 000 $\times$ *g* for 20 min, the supernatant fluid was applied on a sucrose density gradient (Fig. 1C). The 14 S subunit was separated even more sharply than in the case of sonication without $MgCl_2$. Any peaks of ATPase at 30 and 21 S could not be seen, with only a small ATPase activity being detected around an *S* value heavier than 30. Since loss of the enzyme activity was larger during sonication with $MgCl_2$ than sonication without $MgCl_2$, the preparation of the 14 S subunit was performed in the absence of $MgCl_2$ throughout the present experiments.

Intact 30 S dynein was always detected even when the sonication time was prolonged up to 5 min. When the 14 S subunit fraction was subjected to further sucrose density gradient centrifugation, no 30 S dynein was detected although a small peak of contaminant by 21 S dynein was shown. Therefore, an equilibrium between 30 S dynein and the 14 S subunit was not conceivable. Once dissociated, the 14 S subunit did not return to an oligomer.

The 14 S subunit fraction thus obtained was used in the following experiments.

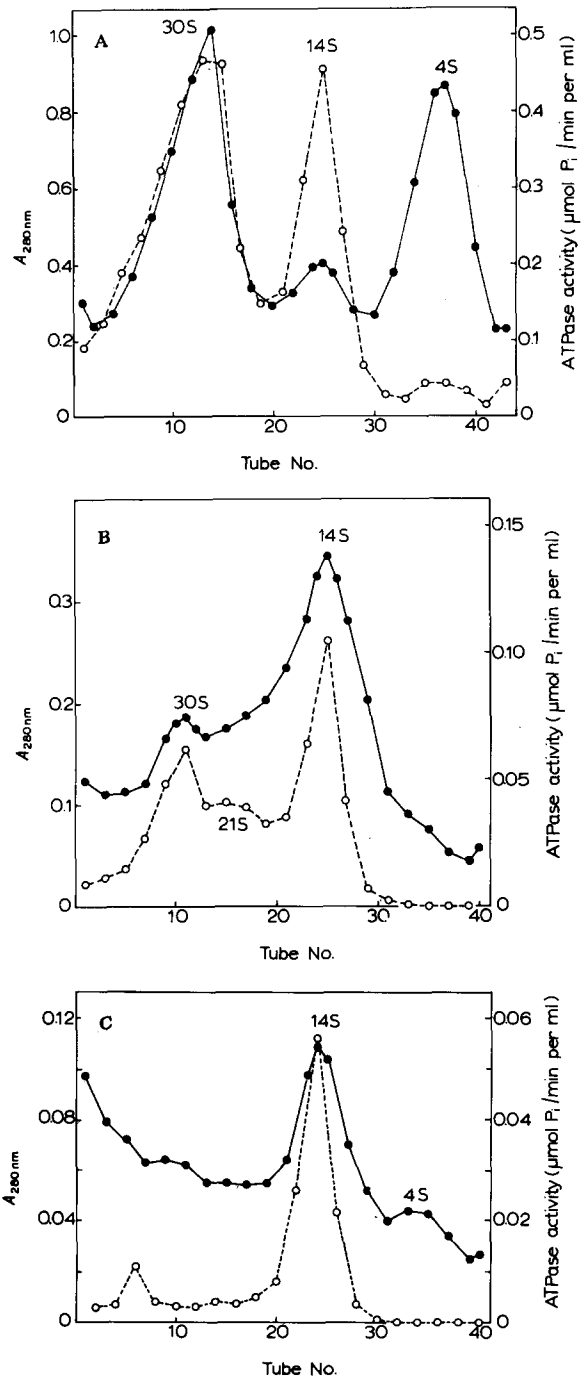

Fig. 1. Sucrose density gradient centrifugation of the crude dynein fraction (A) and the 30 S dynein fraction sonicated in the absence (B) or presence (C) of Mg^{2+}. The density gradient was made in 10 mM Tris · HCl (pH 8.2) containing 0.1 mM EDTA (A and B) or 1 mM $MgCl_2$ (C) and 0.1 mM dithiothreitol. 50 µl (A), 100 µl (B) and 200 µl (C) of each fraction were incubated in 1 ml of the standard assay medium [12] for 20 min (A), 60 min (B) and 40 min (C) at 25°C. ●——●, absorbance at 280 nm; ○- - - - -○, ATPase activity (µmol P_i/min per ml).

Enzymatic properties of the 14 S subunit

Several enzymatic properties of the 14 S subunit were compared with those of 30-S and 14-S dyneins to examine which type of dynein the 14 S subunit belongs to.

The 14 S subunit was also activated by both Mg^{2+} and Ca^{2+} divalent cations at a low concentration. The specific activity of the 14 S subunit in a standard assay system [12] was about 0.3 μmol P_i/mg protein per min, somewhat lower than that of 30 S dynein (0.45 μmol P_i/mg protein per min). The ratio of the activity with 1 mM $CaCl_2$ to that with 1 mM $MgCl_2$ (Ca:Mg ratio) was consistently about 0.62. This value was between that of 30 S dynein (0.75) and 14 S dynein (0.39) [9].

As to the effect of divalent cations, especially Mg^{2+}, towards the dynein ATPase activity, there is a striking difference between 30-S and 14-S dyneins [12]. A low concentration of Mg^{2+} activates both dyneins. A higher concentration of the divalent ion activates 30 S dynein, but inhibits 14 S dynein. The 14 S subunit was steadily activated by increasing Mg^{2+} concentration similar to 30 S dynein (Fig. 2). Although a high concentration of Ca^{2+} inhibits both 30-S and 14-S dyneins, a low concentration activates them; the optima are known to be 2—4 mM and 1 mM for 30-S and 14-S dyneins, respectively [12]. The optimum concentration for the 14 S subunit was around 6 mM, slightly higher than that of 30 S dynein (Fig. 2). Therefore, it was concluded that the 14 S subunit behaved exactly like 30 S dynein, towards divalent cations with the

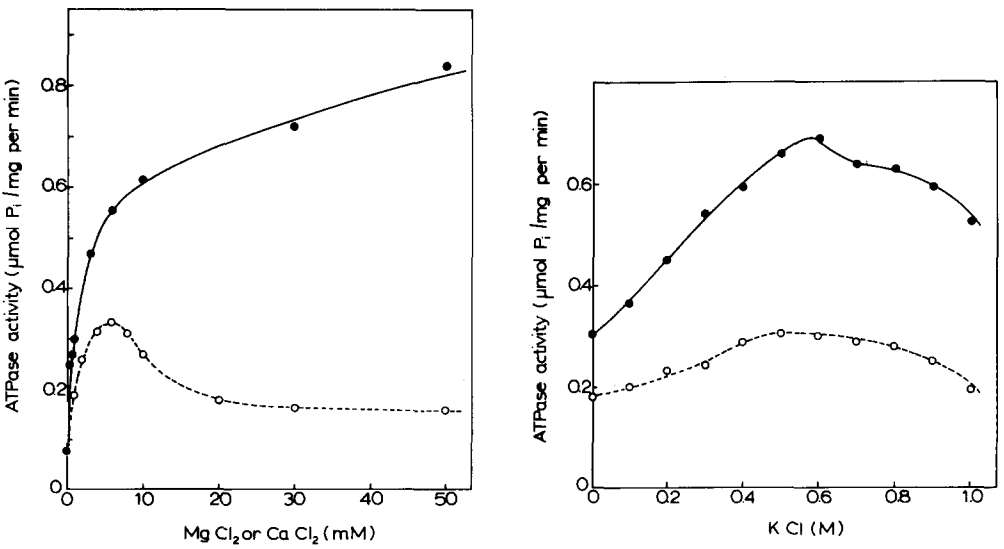

Fig. 2. Effects of divalent cations on ATPase activity of the 14 S subunit. The ATPase activity of the 14 S subunit (31.1 μg) was assayed in 1 ml of the standard assay medium except for varying concentrations of $MgCl_2$ or $CaCl_2$ as indicated. The reactions were performed at 25°C for 30 min. The specific activity was expressed as μmol P_i/mg protein per min. ●———●, Mg^{2+} activation; ○- - - - -○, Ca^{2+} activation.

Fig. 3. Effects of KCl concentration on ATPase activity of the 14 S subunit. The ATPase activity (31.1 μg of the enzyme) was assayed in 1 ml of the standard assay medium except for varying concentrations of KCl as indicated. The reactions were performed at 25°C for 60 min. ●———●, Mg^{2+} activation; ○- - - -○, Ca^{2+} activation.

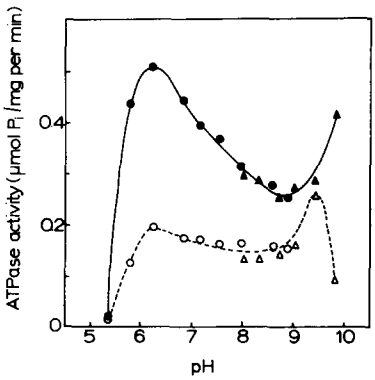

Fig. 4. Effects of pH on ATPase activity of the 14 S subunit. The assay medium consisted of 1 mM $MgCl_2$ or $CaCl_2$, 1 mM ATP, and 25 mM Tris/maleate/NaOH (pH 5.3—8.9) or 25 mM borate/NaOH (pH 8.0—9.9). 28.1 μg of the enzyme were used in each 1 ml of the assay media. The reactions were performed at 25°C for 60 min. ●———●, Mg^{2+}-ATPase in Tris/maleate/NaOH; ▲———▲, Mg^{2+}-ATPase in borate/NaOH; ○- - - - -○, Ca^{2+}-ATPase in Tris/maleate/NaOH; △- - - - -△, Ca^{2+}-ATPase in borate/NaOH.

exception that the Ca:Mg ratio of the 14 S subunit differed somewhat from that of 30 S dynein.

It has been known that the Mg^{2+}-ATPase activity of 30 S dynein is enhanced two fold by KCl at a concentration of 0.6 M, while 14 S dynein is inhibited at that concentration [9]. The activity of the 14 S subunit increased proportionally with KCl concentration until 0.6 M, with slight inhibition at KCl concentrations beyond 0.6 M (Fig. 3). A similar activation profile by KCl was obtained with the Ca^{2+}-ATPase activity. Therefore, the behavior of the 14 S subunit with KCl was in a good agreement with that of 30 S dynein.

The change in the activity of the 14 S subunit as a function of varying pH values is shown in Fig. 4. The Mg^{2+}-ATPase had an optimum at pH 6.2 and revealed a high activity over pH 10. The Ca^{2+}-ATPase of the 14 S subunit gave a similar pattern to that of the Mg^{2+}-ATPase with an alkaline optimum shifting to pH 9.5. This pH dependency resembled that of 30 S dynein, which is activated at an acidic pH (pH 6 by Gibbons [9], Shimizu and Kimura [15], Kaji (per-

TABLE I

RECOMBINATION OF DYEIN WITH THE OUTER FIBERS

Dyein fractions were mixed with the outer fiber suspension in the medium, A or B indicated below, and kept standing overnight at 0°C. The supernatant of the mixture was assayed for ATPase activity of dynein not binding to outer fibers. The amount of recombination was defined as $(T - S)/T \times 100$, where T and S represent total activity and activity remained in the supernatant, respectively. A. 25 mM Tris · HCl (pH 8.2), 10 mM $MgCl_2$; B. 5 mM MES/KOH (pH 6.6), 10 mM $MgSO_4$, 50 mM KCl, 1 mM EGTA, 4 M glycerol. Results are expressed in %.

Dynein	Medium A	Medium B
14 S subunit	10.0 ± 4.4	24.5 ± 3.4
30 S dynein	66.4 ± 1.8	81.9 ± 2.9
14 S dynein	10.0 ± 1.7	54.1 ± 2.3

sonal communication)) as well as at an alkaline pH (pH 8.5 by Gibbons [9], pH 10.5 by Shimizu and Kimura [15], a pH beyond 10 by Kaji (personal communication)). 14 S dynein, on the other hand, shows only one optimum around an alkaline pH (pH 9.0 by Gibbons [9]).

The K_m value for ATP was 10 μmol with respect to the 14 S subunit. Those for 30 S dynein and 14 S dynein are known to be 11—13 μmol and 33—35 μmol, respectively [9]. The value for the subunit also coincided with that of 30 S dynein.

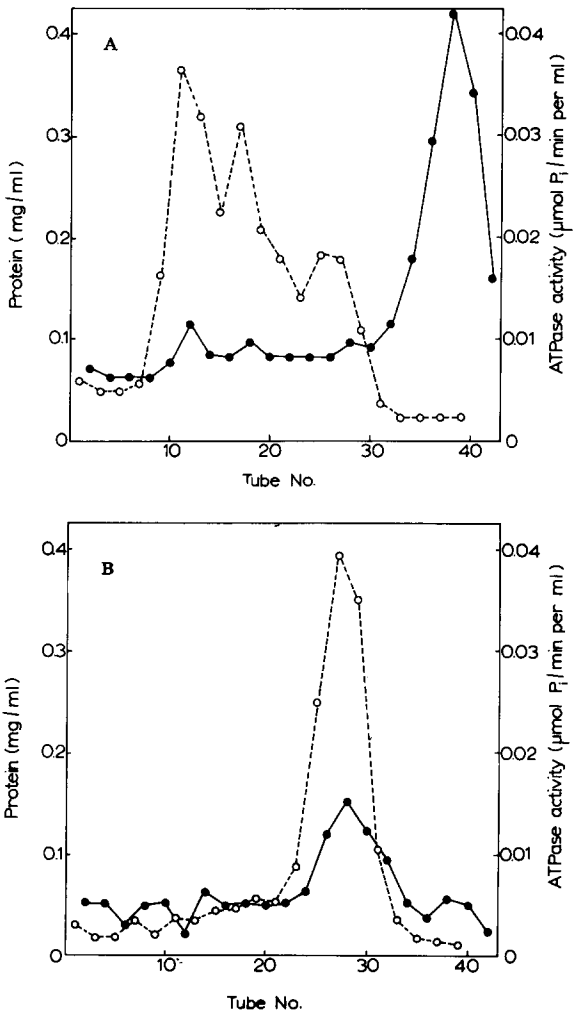

Fig. 5. Identification of dynein species recombined with the outer fibers by mixing with freshly sonicated 30 S dynein fraction. The sonicated 30 S dynein fraction was immediately mixed with the outer fiber suspension in 25 mM Tris · HCl (pH 8.2) containing 5 mM $MgCl_2$ and allowed to stand overnight. After centrifugation, the supernatant (B) and bound fraction (A) re-extracted from the pelleted outer fibers were subjected to sucrose density gradient centrifugation. 0.4 ml of each fraction was used for ATPase assay in the standard assay medium at 25°C for 60 min. ●———●, protein (mg/ml); ○- - - - -○, ATPase activity (μmol P_i/min per ml).

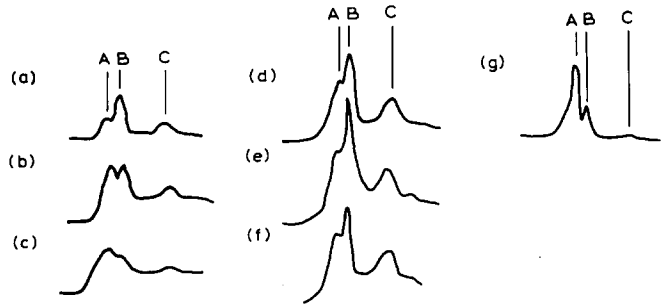

Fig. 6. Densitometric scan of the 14 S subunit, 30 S dynein and 14 S dynein electrophoresed on sodium dodecylsulfate-polyacrylamide gels. Only the bands corresponding to dynein are shown in the figure. Proteins migrated from left to right. A, B and C indicate A, B and C bands, respectively, according to Mabuchi and Shimizu [16]. (a) 14 S subunit, (b) mixture of 14 S subunit and 30 S dynein, (c) 30 S dynein, (d) 14 S subunit, (e) mixture of 14 S subunit and 14 S dynein, (f) 14 S dynein, (g) 30 S dynein fraction sonicated, immediately followed by precipitation with trichloroacetic acid.

Recombination with outer fibers

30 S dynein recombines with the outer fibers in the presence of Mg^{2+}; 14 S dynein has less ability [1]. First, the recombination experiments were performed in a medium containing 25 mM Tris · HCl (pH 8.2) and 10 mM $MgCl_2$. The results are summarized in Table I. Over 90% of freshly prepared 30 S dynein recombined with the outer fibers, nearly 70% for normal 30 S dynein preparation, only 10% for 14 S dynein, and also 10% as for the 14 S subunit. Two possibilities were considered: (1) the subunit form had little ability to recombine; (2) the dissociation procedure preserved ATPase activity of the subunit, but destroyed the recombination ability.

To examine the 2nd possibility, the same experiment was carried out in a buffer used for polymerization of brain tubulin [13], which favored the recombination of dynein with the outer fibers. The amount of 30 S dynein bound to the outer fibers increased from 66% to 80% and that of 14 S dynein recombined increased more than five-fold. However, the 14 S subunit recombined only two-fold (25%). The second of the above possibilities might be true. In addition, without addition of the outer fiber fraction, no forms of dynein were ever precipitated by centrifugation in either series of the experiment.

Assuming the possibility that the 14 S subunit lost its recombination ability during dialysis and centrifugation on a sucrose density gradient, sonicated 30 S dynein fraction was immediately mixed with the outer fiber suspension and the species of bound dynein was analyzed (see legend of Fig. 5). The results shown in Fig. 5 indicate that this possibility was ruled out, that is, the amount of the 14 S subunit recombined with the outer fibers did not significantly increase. It was clear that the remaining 30 S dynein and the partially dissociated 21 S dynein almost completely retained the ability to recombine with the outer fibers.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

It was reported by Mabuchi and Shimizu [16] that 14 S dynein has a polypeptide chain (B chain, molecular weight 520 000) slightly shorter than

that of 30 S dynein (A chain, molecular weight 560 000) when determined on sodium dodecyl sulfate-polyacrylamide gel electrophoresis. An alkylated 14 S subunit fraction was co-electrophoresed with 30-S and 14-S dyneins. The fraction showed the B chain as a major component that corresponds to that of 14 S dynein (Fig. 6). It was unlikely that when 30 S dynein was dissociated into the 14 S subunit by sonication, its chain length differed from that dissociated by sodium dodecylsulfate. The shortening of the chain length must have occurred during the sucrose density gradient centrifugation, probably by some contaminant protease. Therefore, immediately after sonication of the 30 S dynein fraction, proteins were precipitated by 5% trichloroacetic acid to prevent proteolysis, followed by reduction and alkylation prior to the electrophoresis. This sample showed A chain as a main band and B chain as a minor (Fig. 6).

Discussion

The previous work showed that the treatment of 30 S dynein with urea or sodium dodecyl sulfate causes its dissociation into the 14 S subunit, with modifications of the enzymatic properties different from both 30-S and 14-S dyneins [12]. The present sonication procedure considerably improves the isolation of the 14 S subunit in the following two points of view: (1) the ATPase peak and protein peak of the dissociated dynein coincide with each other at 14 S, (2) the 14 S subunit still preserves the enzymatic properties of its oligomer. Further improvements must be achieved in some respects: (1) the 14 S subunit loses most of its ability to recombine with the outer fiber, (2) the polypeptide chain length of the 14-S subunits shortens, (3) the present 14 S subunit cannot be reconstituted into 30 S dynein.

The sonicated 30 S dynein fraction contains 21 S dynein in addition to the 14 S subunit and non-dissociated 30 S dynein. The 21 S dynein fraction capable of recombining with the outer fibers is thought to be a partially dissociated product and seems to be equivalent to a 22—23 S ATPase appearing when the 30 S dynein fraction is treated with urea [12].

By some methods of extraction or in some biological sources dynein of a larger sedimenting coefficient corresponding to 30 S dynein of *Tetrahymena* cilia is extracted. Sperm flagella of sea urchins yield only 12 S dynein by usual extraction, but KCl can extract not only 12 S dynein but also 22 S dynein, presumably an oligomer, both having several different enzymatic properties (unpublished). When extracted with KCl from starfish sperm, only 22 S dynein is obtained, which is easily dissociated into 12 S under a low ionic strength (Mabuchi, personal communication). An ATPase of a larger size is also obtained from cilia of sea urchin embryos with KCl extraction (Kimura, personal communication). *Chlamydomonas* flagella have two types of dynein, namely 12 S and 18 S even when extracted under a low ionic strength [17]. Thus, the presence of oligomeric dynein appears to be universal in a variety of animal cells.

The enzymatic properties of the 14 S subunit obtained by sonication is principally the same as those of 30 S dynein. Conversely speaking, the oligomeric form does maintain enzymatic properties of the subunit: 30 S dynein is a simple summation of the subunit with respect to enzymatic properties. Com-

paring the 14 S subunit with 14 S dynein, there are quite different characteristics in their enzymatic properties, although the methods for their preparation are different. A preliminary analysis of enzymatic characteristics of a trypsin-digested 30 S dynein (12 S fragment) reveals that the fragment possesses enzymatic properties different from 14 S dynein. Although it is clear that the 14 S subunit is susceptible to proteolytic digestion and the shortening of the chain length happens to result in accordance with B chain of 14 S dynein, 14 S dynein could be an ATPase differing from 30 S dynein. It would not be surprising if more than one ATPase system is functioning within complex motile systems such as cilia or flagella as indicated by Warner and Satir [18] and that the spoke-heads also have a mechanochemical activity.

Acknowledgements

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