THE INITIAL BURST OF PHOSPHATE LIBERATION BY 30 S DYNEIN ATPase FROM *TETRAHYMENA* CILIA

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Received 18 September 1979

1. Introduction

The sliding filament theory has been postulated for ciliary or flagellar motion of eukaryotic cells [1] and evidence supporting this theory has accumulated [2–7]. Several similarities of dynein, the ciliary or flagellar ATPase, to myosin have been reported with respect to their enzymatic characteristics [8–10], although there exist significant differences between the two proteins [8,10]. Dissociation of dynein bound to the B-tubule of the outer fiber has been demonstrated to occur upon addition of ATP [11]. This phenomenon seems to be analogous to the ATP-induced dissociation of actomyosin [12]. Therefore, cyclic attachment and detachment of dynein, to and from the B-tubule of the adjacent outer fiber, appears to generate the motive force for mutual sliding of the outer fibers during ciliary or flagellar motion, being accompanied with hydrolysis of ATP, in a process analogous to muscle contraction [13].

For the understanding of ciliary or flagellar motion, it is requisite to compare the elements of the motion with those involved in muscle contraction. Myosins are known to exhibit an initial (or early) burst of phosphate liberation upon addition of the substrate [14–17]. The rate of phosphate liberation by axonemal ATPase of gill cilia has been shown as higher in the early than in the steady state phase [18]. However, this phenomenon does not seem to indicate the initial burst of phosphate liberation because of the non-stoichiometric size and the extremely slow rate of phosphate liberation during the presteady state phase.

More recent observation using a crude KCl-extract of the axonemes indicated an initial burst of phosphate release with reasonable stoichiometry [19]. This result suggested a similarity between the reaction intermediate of the dynein ATPase and that of the myosin ATPase.

Here we confirm the existence of the burst phenomenon, using 30 S dynein of *Tetrahymena* cilia, which was extracted from the cilia with a buffer of low ionic strength and purified through sucrose density gradient centrifugation. The effects of inhibitors of dynein ATPase on the initial burst of phosphate release are also investigated.

2. Materials and methods

The preparative method for 30 S dynein from the cilia of *Tetrahymena pyriformis* strain W was described in [10]. [γ-^{32}P]ATP was synthesized by the method in [20]. All other chemicals of the highest purity were as in [21].

The concentration of vanadate (Na_3VO_4) solution was determined with a Hitachi 170-70 Zeeman Effect Atomic Absorption Spectrophotometer. SDS–polyacrylamide gel electrophoresis (3% gels) and the scanning of gels were carried out as in [17]. The concentration of protein was determined by the method in [22] using bovine serum albumin as a standard.

The ATPase assay to determine the app. K_m of 30 S dynein for ATP was carried out at 25°C in an assay solution consisting of 30 mM 3-(*N*-morpholino)-propanesulfonic acid (MOPS)–NaOH (pH 7.0), 3 mM $MgCl_2$ and varying concentrations of [γ-^{32}P]ATP. The reaction was stopped by the addition of 5% PCA (final concn.) containing 0.2 mM P_i. An aliquot of the supernatant, after charcoal treatment, was transferred into a vial and the radioactivity was measured by Cherenkov counting. If necessary, the charcoal treatment was repeated. The steady state rate was calculated from the time course of linear phosphate liberation.

The ATPase assay used for other determinations is described in the legends to the figures.

3. Results and discussion

Figure 1 shows the densitometric scan of SDS–polyacrylamide gel electrophoresis of 30 S dynein. One major band and several minor bands were detected, as reported [23]. The major band, designated as band A, has 560 000 chain mol. wt [23]. Since further purification of 30 S dynein by hydroxylapatite column chromatography revealed a band A with only trace amounts of other components [24], one can regard band A as main component of 30 S dynein molecule. Band A of the present 30 S dynein preparation accounted for ~80% of the total protein, as estimated from fig.1. We did not intend to purify it further because the hydroxylapatite-purified 30 S dynein had an app. K_m of 0.05 mM for ATP [24], which was much higher than that of the original 30 S dynein fraction [11].

30 S dynein was shown to have a low K_m value for ATP (~1 μM) as reported [11]. The ATPase activity was somewhat higher at a higher ATP concentration (0.03 mM). This suggested the presence of a second K_m [11] and might explain the higher K_m value (0.01 mM) reported [8,10].

The early phase of the phosphate release is shown in fig.2. The steady state rate of phosphate liberation was 0.07–0.09 μmol . mg protein^{-1} . min^{-1} and agreed well with that given in fig.3. Extraporation of the plot to zero time did not meet the origin but crossed the ordinate above it. The least square treatment of the data revealed a crossing point of 1.7 mol/

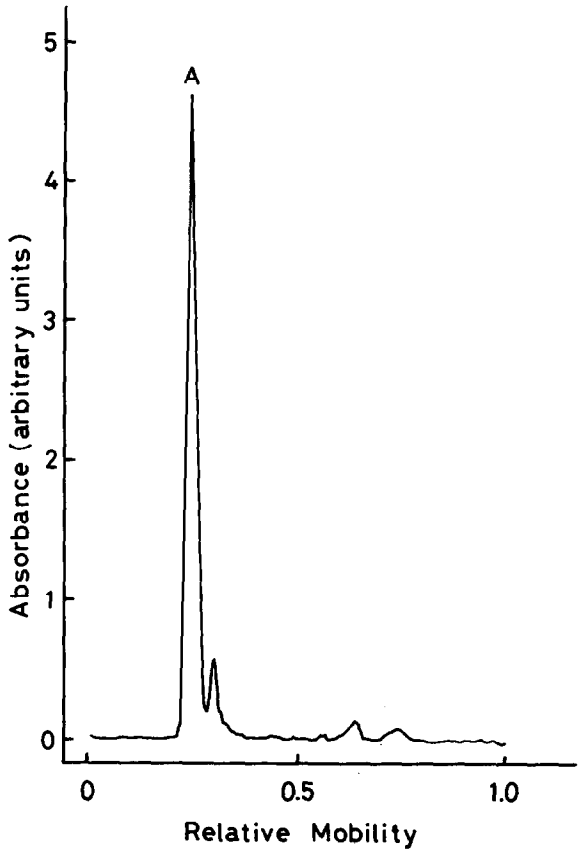

Fig.1. Densitometric scan of SDS–polyacrylamide gel electrophoresis of 30 S dynein. The gel was stained with Coomassie brilliant blue and scanned at 550 nm with a Shimadsu CS900 Chromatoscanner. (A) in the figure indicates band A.

10^6 g protein. In our method, the experimental error in the determination of the incubation time did not exceed 0.2 s. Therefore, the existence of initial burst of phosphate liberation by 30 S dynein ATPase may be taken for granted. The size of the burst was in the range of 1.2–1.9 mol inorganic phosphate/10^6 g protein, depending on the enzyme preparation. Taking the purity of band A into consideration, the size of the burst was determined to be 1.5–2.4 mol phosphate/10^6 g band A, a value slightly smaller than that estimated in [19] (1 mol phosphate/3.5 X 10^5 g dynein).

The present results strongly suggest that the main intermediate of 30 S dynein ATPase action resembles that of the myosin ATPase, M** · ADP · P_i [25], and

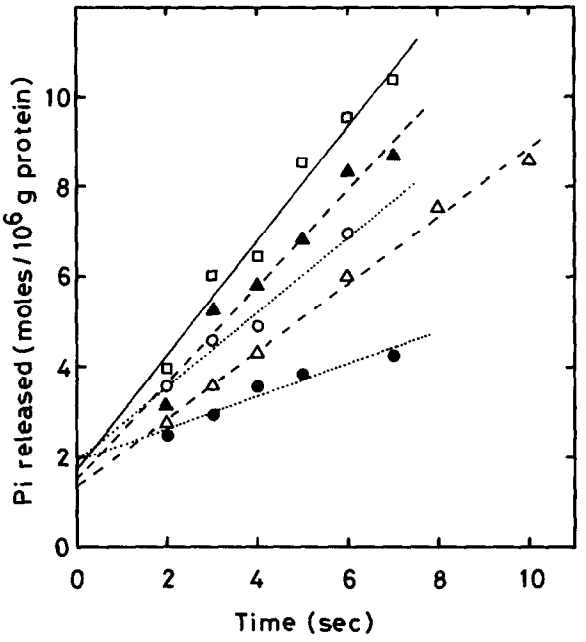

Fig.2. Early phase of phosphate liberation by 30 S dynein ATPase upon addition of ATP. 0.2 ml of the enzyme solution consisting of 30 mM MOPS–NaOH (pH 7.0), 3 mM $MgCl_2$ and appropriate concentration of 30 S dynein were stirred with a stirring bar in a water bath (5°C) and then mixed with 0.075 ml of the ATP solution consisting of 30 mM MOPS–NaOH (pH 7.0), 3 mM $MgCl_2$ and appropriate concentration of [γ-^{32}P]ATP. The final concentrations of the enzyme and ATP were 276–305 μg/ml and 12.3–13.6 μM, respectively. After various intervals indicated, 0.075 ml of 50% PCA–0.2 mM P_i was mixed to stop the reaction. The amount of released P_i was determined by the method in section 2. When the effect of AMPPNP or vanadate was investigated, the ligand was included in both the enzyme and ATP solution at the same concentration, prior to the mixing. (–□–) No addition; (- -▲- -) with 0.15 mM AMPPNP; (- -△- -) with 0.30 mM AMPPNP; (· ·○· ·) with 5×10^{-6} M orthovanadate; (· ·●· ·) with 5×10^{-5} M orthovanadate.

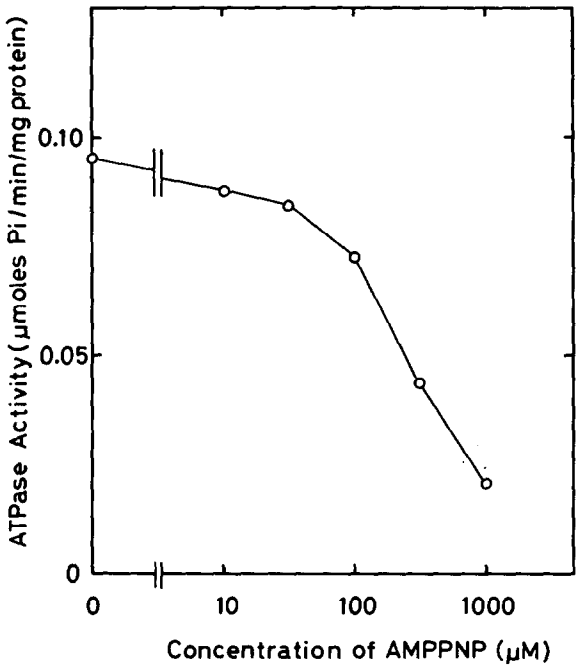

Fig.3. Effect of AMPPNP on 30 S dynein ATPase activity. The reaction mixture consisted of 30 mM MOPS–NaOH (pH 7.0), 3 mM $MgCl_2$, 12 μM ATP, 9.6 μg 30 S dynein/ml and various concentration of AMPPNP as indicated. The reaction was started by adding ATP and was terminated with addition of equal volume of 10% PCA containing 0.2 mM P_i after 4 min incubation at 5°C. The amount of P_i released was determined as in section 2.

will be designated as D · (ADP) · P_i; an intermediate containing P_i and possibly ADP, the P_i–enzyme bond being acid labile. The asterisks of M** mean an altered conformation of myosin. Such an alteration was, however, not detected in the dynein molecule [26] so that the asterisks were omitted.

Next, we investigated the effect of adenylylimidodiphosphate (AMPPNP) on the early phase of the action of dynein ATPase. We have reported the inhibitory effect of AMPPNP on the steady state rate elsewhere [21]. However, a reinvestigation was undertaken because for the determination of AMPPNP inhibition at steady state the condition for the ATPase assay had to be identical to that used for the burst study. Under the present conditions, the half-maximal inhibitory concentration of AMPPNP was ~0.3 mM (fig.3).

Figure 2 shows that in the presence of 0.15 mM or 0.30 mM AMPPNP the size of the initial burst was also reduced to some extent. Since preincubation of the enzyme with AMPPNP (fig.3) would repress the burst almost completely if the catalytic site of 30 S dynein and AMPPNP were in a state of slow equilibrium, the present results suggest that the association of AMPPNP with 30 S dynein represents a rapid equilibrium process.

Vanadate has recently been shown to be a potent

inhibitor of dynein ATPase [27,28]. The size of the burst of phosphate release was not decreased in the presence of various concentrations of orthovanadate (5×10^{-7} to 5×10^{-5} M); in some cases the size of the burst was even slightly increased, whereas the steady state rate of P_i liberation was reduced (fig.2). Vanadate was shown to inhibit dynein ATPase not in a competitive but possibly in a non-competitive manner [27,28]: that is, vanadate would bind only to the enzyme–substrate complex (intermediate). Our present results suggest that vanadate inhibits the breakdown of the D · (ADP) · P_i complex. The intermediate, D · (ADP) · P_i, might be the major one in the ATPase mechanism, being at equilibrium with other intermediates. The slight enlargement of the size of the burst would be due to the accumulation of D · (ADP) · P_i in the reaction process through the inhibitory action of vanadate.

In conclusion, 30 S dynein ATPase was shown to exhibit an initial burst of phosphate liberation, which supports the similarity of 30 S dynein and myosin. However, precise determination of the burst size and of other kinetic properties remain to be made after purifying 30 S dynein in the native state to understand the molecular feature of dynein.

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