Biochimica et Biophysica Acta 481 (1977) 660—666

BBA 68078

STUDIES ON THE INITIAL PHASE OF DYNEIN ATPase ACTIVITY

KEN-ICHI NAKAMURA and ETSUKO MASUYAMA

Department of Living Science, Hiroshima Women's University Uzina, Hiroshima (Japan)

(Received August 24th, 1976)

Summary

Kinetic measurement of the reaction of dynein ATPase (ATP phosphohydrolase, EC 3.6.1.3) extracted from the gills of *Mytilus edulis* shows that in the presence of Mg^{2+} there is a very rapid initial liberation of P_i from the dynein-ATP system, followed by a slower liberation in the steady state. In view of following results, we have confirmed that this phenomenon is not due to the accumulation of end products, a fall in substrate concentration, nor to the presence of labile impurities in ATP but is due to the catalytic activity of dynein ATPase.

1. The replacement of native dynein by heat denatured dynein or other kinds of Mg^{2+}-ATPase could not produce such a burst phenomenon under the same condition.

2. Both the rate of initial burst and that of steady state were proportional to enzyme content over a wide range under our standard condition.

3. Initial burst was also observed under the constant ATP level by using a ATP generate system.

4. Preincubation of dynein with P_i prior to initiation of the reaction did not eliminate the initial burst.

Some properties of the initial rapid liberation of dynein ATPase were also examined. These are shown below.

5. The free ADP liberation did not show any initial burst though the P_i liberation did in the initial phase and the rate of free ADP liberation was almost equal to that of P_i liberation of the steady state.

6. Mg^{2+} was more effective than Ca^{2+} for the appearance of the initial burst, while the liberation of P_i in the steady state was activated more by Ca^{2+} than by Mg^{2+}. The addition of K^+ in the presence of Mg^{2+} resulted in a marked increase of P_i liberation in the steady state but not in the initial state.

7. The activation energy of the initial burst was 9.7 kcal, which is slightly smaller than that of myosin ATPase.

Introduction

In order to elucidate the mechanism underlying flagellar and ciliary movement at the molecular level, many workers have investigated the enzymatic properties of axonemal ATPase (ATP phosphohydrolase, EC 3.6.1.3) called dynein [1—10]. It is generally accepted that dynein generates motive force of ciliary and flagellar movement. Recently Summers and Gibbons [11] have suggested that the active sliding of the doublet microtubles is mediated through interactions with dynein, which is similar to myosin-actin interaction of muscle. However there is no direct evidence concerning the detailed mechanism by which dynein induces the sliding. An intensive investigation of the reaction mechanism of dynein ATPase activity should provide a clue to the elucidation of this mechanism.

During a study on the kinetic property of dynein ATPase derived from the gills of *Mytilus edulis*, we have observed that in the initial stage the rate of P_i liberation from the dynein-ATP system is about 2 times greater than the rate in the steady state. This phenomenon seems to correspond to the "initial burst" of ATPase activity of muscle myosin [12]. Tonomura et al. [13,14] have observed the initial rapid liberation of P_i (initial burst) from the myosin-ATP system and attributed the appearance of the burst to the formation of phosphoryl myosin, which is the key reaction in muscle contraction. Therefore a more intensive investigation of this phenomenon in the dynein-ATP system should shed some light on the mechanism which underlies motility of flagella and cilia. In the present study we have confirmed that the appearance of the rapid liberation of P_i from the dynein-ATP system is in the initial stage not due to artifact but to catalytic ATPase activity of dynein. In addition, several properties of the initial burst of dynein ATPase were compared with those of muscle myosin ATPase.

Materials and Methods

Pieces of separated gills of *Mytilus edulis* were homogenized in 10 volumes of 70% glycerol with a teflon homogenizer to detach the cilia. After removing the treated gills by low speed centrifugation, axonemes were collected by centrifugation at 20 000 × *g* for 15 min and then suspended in a small volume of Tris/EDTA solution (0.1 mM EDTA and 1 mM Tris · HCl, pH 8.2), and dialyzed overnight against the same buffer solution. The dialysate was centrifuged at 20 000 × *g* for 15 min and the resultant supernatant (Fraction I by Gibbons [1]) was used as a crude dynein sample. The dyneim thus obtained was stored at 4° C and used within two days after preparation.

Preparation of microsomal ATPase

Microsomal ATPase was prepared from a bovine liver according to the method of Hulsmans [15].

ATPase assay by measurement of P_i liberation

The standard assay medium consisted of 20 mM Tris · HCl (pH 7.4), 10 mM $MgCl_2$, 2 mM ATP and dynein (0.4—0.8 mg/ml). The reaction was performed at

27° C. After preincubation for 5 min the reaction was initiated by adding enzyme and terminated by adding 7% trichloacetic acid. The precipitate was removed by filtration and an aliquot of the filtrate was used for analysis of P_i content by the method described previously [16]. A zero blank was prepared by adding trichloroacetic acid prior to the initiation of the reaction, and then was subtracted from each sample.

ATPase assay by measurement of free ADP liberation

For measurement of liberation of free ADP, 2 mM phospho*enol*pyruvate and 0.04 mg/ml of pyruvatekinase were added to 0.1 M KCl, 10 mM $MgCl_2$ and 20 mM Tris · HCl (pH 7.4) solution containing 2 mM ATP. Under this condition phospho*enol*pyruvate was made to react quantitatively with the free ADP to produce pyruvate by the action of pyruvatekinase. The reaction was initiated by adding dynein and stopped by adding 2,4-dinitrophenylhydrazine. The amount of pyruvate produced was assayed by the method of Reynard et al. [17]. A zero time blank was prepared by adding 2,4-dinitrophenylhydrazine prior to the initiation of the reaction and was subtracted from each sample.

Protein determination

Protein concentration was determined by the method of Lowry et al. [18] with bovine serum albumin as a standard.

Reagents

The crystalline disodium salt of adenosine 5′-triphosphate (ATP), phospho-*enol*pyruvate and pyruvatekinase were obtained from Sigma Chemical Co. All other chemicals used in this experiment were of the best quality commercially available. Glass redistilled water was used throughout the experiment.

Results

The time-course of dynein ATPase activity in the presence of 10 mM $MgCl_2$ and 2 mM ATP is shown in Fig. 1. In the hydrolysis of ATP, the phosphate liberation was biphasic: an initial rate which was faster than the rate of the following steady state. The curve slope indicates that the transient change occurred 1 min after reaction was started, which was much later than that of myosin ATPase [12].

The initial burst may be due to an artifact of nonenzymatic hydrolysis of ATP. This possibility was examined by the use of heat-treated dynein and other kinds of Mg^{2+}-ATPase (microsomal ATPase). If the initial burst was not caused by properties of dynein ATPase but caused in part by nonenzymatical breakdown of ATP in the initial stage, these enzyme should also produce an initial burst under the same condition. As evident from Fig. 1, initial rapid liberation of P_i was not observed in either enzyme system. Hence we concluded that the initial burst was not due to the presence of labile impurities in the ATP but to the catalytic activity of dynein ATPase.

In order to confirm that substrate concentration was not rate limiting under our usual condition, various amounts of dynein were added to the standard assay mixture. The obtained result showed that both the rate of P_i liberation of

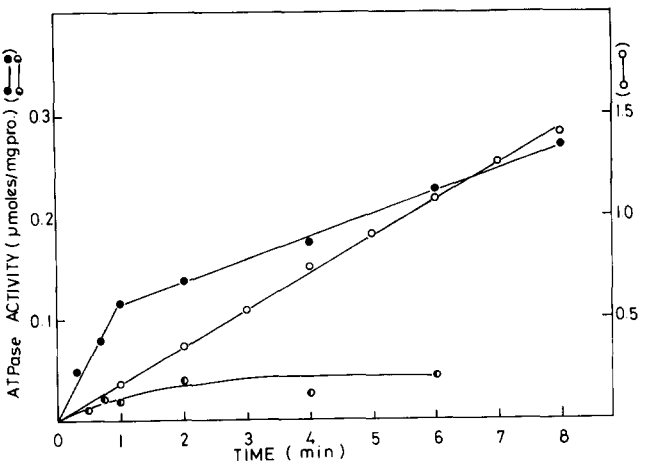

Fig. 1. Enzyme activity as a function of time. ATPase activity was measured in the presence of 20 mM Tris · HCl (pH 7.4), 10 mM $MgCl_2$, and 2 mM ATP at 27°C. The reaction was started by the addition of the following enzymes respectively. ●———●, native dynein; ◐———◐, heat-treated dynein (prepared by preincubation at 95°C for 10 min); ○———○, microsomal ATPase. A zero time blank was subtracted from each sample.

initial phase and that of steady state were proportional to enzyme content over a wide range (0.2—0.8 mg/ml). Furthermore the time course of P_i liberation was examined under a constant ATP level by using an ATP generate system. As shown in Curve A of Fig. 2, initial rapid liberation of P_i was also observed under this condition. These findings indicate that the transition from the initial burst to the lower steady state is not due to a fall in substrate concentration.

As for muscle myosin [13,14], kinetic studies on the initial burst have shown that the rate of P_i liberation and that of free ADP liberation in the steady state are almost equal but that initial burst is not observed in the free

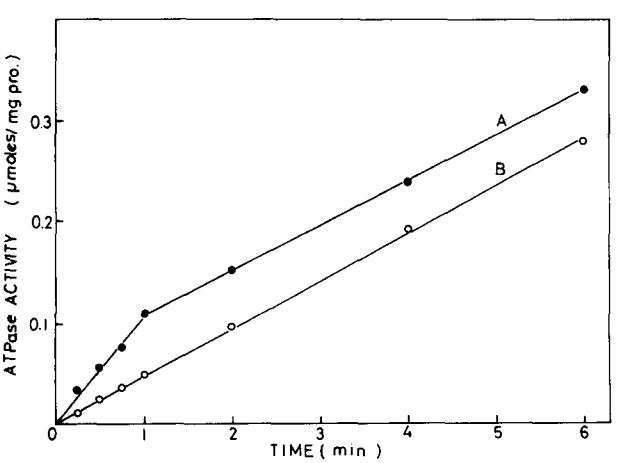

Fig. 2. Time course of P_i liberation and free ADP liberation from the dynein-ATP system coupled with ATP generate system. For details, see Materials and Methods. (A) ●———●, P_i liberation; (B) ○———○, free ADP liberation.

ADP liberation from the myosin-ATP system though it is in P_i liberation. The foregoing results have led to the conclusion that myosin · phosphate · ADP complex ($E^{P_i}_{ADP}$) is formed in the early stage of the reaction and the reactive complex is labile to acid, and that it must be decomposed after stopping the reaction by adding trichloroacetic acid. Consequently P_i bound to myosin appears in the supernatant and brings rise to the initial burst. In the reaction process of myosin-ATP system, the decomposition of the reactive complex is considered to be a rate determining step, hence the rate of liberation of free ADP is in accord with that of P_i in the steady state. In connection with myosin-ATP system, it seemed also desirable to measure the liberation of free ADP together with that of P_i in the dynein-ATP system. The liberation of free ADP was therefore measured by the produced pyruvates in the presence of pyruvate-kinase and phospho*enol*pyruvate. As indicated in Fig. 2, the result of the experiment shows that the free ADP liberation did not show any initial burst though the P_i liberation did during the initial phase of the reaction and the rate of free ADP liberation was almost equal to that of P_i liberation of the late steady state. This result is in accord with what was previously reported in the myosin-ATP system [13].

In order to investigate the general properties of a initial burst, effects of various cations on dynein ATPase were examined. As shown in Fig. 3 the replacement of Mg^{2+} by Ca^{2+} decreased the amount of initial burst of P_i liberation, while the liberation rate of P_i in the steady state (1—6 min) was slightly increased. In addition it was observed that the initial burstwas negligible when divalent metal was removed from the system. It has been well known that the addition of K^+ in the presence of Mg^{2+} results in further increase of dynein ATPase activity [5,8]. Our present results demonstrated that the augmentation by K^+ was effective only for the steady state but not for the initial phase. Therefore it may be concluded that Mg^{2+} is the ion most neccessary for the appearance of the initial burst.

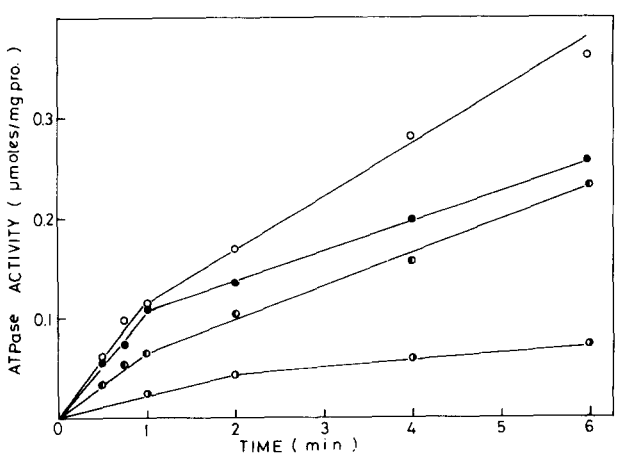

Fig. 3. Effect of cations on P_i liberation from the dynein-ATP system. ATPase activity was assayed in the absence of cations (●——●) or in the presence as indicated (●——●, 10 mM $MgCl_2$; ●——●, 10 mM $CaCl_2$; ○——○, 10 mM $MgCl_2$ + 0.2 M KCl). Other conditions were the same as Fig. 1.

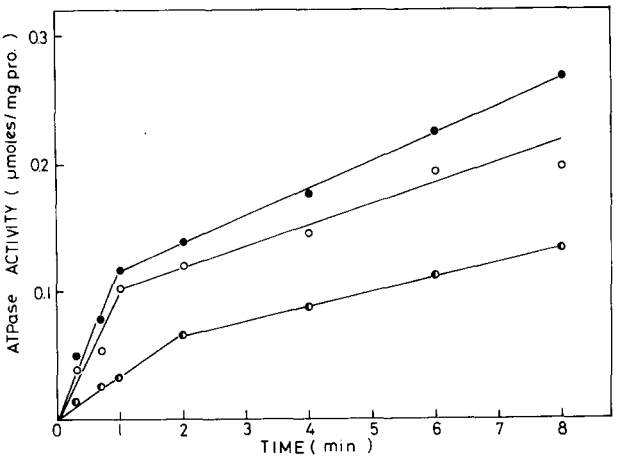

Fig. 4. Effect of temperature on P_i liberation from the dynein-ATP system. The reaction condition was the same as Fig. 1 except that the reaction was carried out at various temperatures as indicated below. ●———●, at 4° C; ○———○, at 16° C; ●———●, at 27° C. Other conditions were the same as Fig. 1.

Fig. 4 shows the time course of P_i liberation under the various incubation temperatures. Both the rate of the initial burst and that of the steady state were markedly low at 4° C and the transient time shifted from 1 to 2 min. The temperature coefficient of the initial burst was measured between 4 and 27° C and the activation energies were calculated to be 9.7 kcal. The obtained value was slightly smaller than that of myosin [12].

The transient process is not caused by product inhibition, since preincubation of dynein with 0.1 mM P_i prior to initiation of the reaction did not eliminate the transition at all and moreover the initial burst was observed also in the system coupled with ATP generate system in which ADP so produced should be converted into pyruvate instantly by the action of pyruvatekinase.

Discussion

In the preliminary experiment (Nakamura, K., in preparation), we have observed that some of the enzymatic properties of dynein extracted from the cilia of gills of *Mytilus edulis* were basically similar to those of dynein obtained from the cilia of protozoa or the flagella of spermatozoa. For example, this enzyme was soluble at low ionic strength, and ATPase was activated by Mg^{2+}, Ca^{2+} and Mn^{2+} and inhibited by Cd^{2+} and Hg^{2+}. Two optima appeared at pH 7.4 and 8.5. Addition of K^+ in the presence of Mg^{2+} resulted in doubling of ATPase activity, etc.. The kinetic measurement of the ATPase reaction of dynein extracted from *Mytilus edulis* shows that there is a rapid liberation of P_i at the initial stage followed by a slower liberation at the steady state. This burst phenomenon has been observed previously in myosin but not been reported in other dyneins. Whether or not such an initial burst can be seen in other dyneins is now under investigation.

As for the transition of the rate of P_i liberation by the dynein-ATP system, the following mechanism might be possible. The first is a two step mechanism,

namely (1) E + ATP → EADP + P_i and (2) EADP → E + ADP, and the second step is much slower than the first step. In such a case, P_i will be produced rapidly at the initial stage, while the rate of liberation of free ADP which is available for the kinase system will be almost equal to that of P_i liberation in the steady state. The second mechanism is formation of the reactive dynein · phosphate · ADP complex ($E^{P_i}_{ADP}$). The decomposition is a rate determining step in the reaction. As described before, this mechanism has already been adopted in the myosin-ATP system [13]. The third is a conformational change in the dynein from an initial active form to a second less active form. This possibility seems to be improbable, because the rate of free ADP liberation was constant during the reaction. Thus our investigation of the dynein-ATP system has elucidated some features of the initial phase of P_i liberation. Although it is difficult at present to clarify the precise mechanism of the initial burst, we expect that the burst phenomenon is involved in the fundamental mechanism of ciliary movement as is the case of muscle contraction. In order to elucidate the molecular mechanism of flagellar and ciliary movements more precisely, we attempt to investigate a stoichiometric relation between the amount of the initial burst of P_i liberation and dynein.

Acknowledgment

The authors wish to express their gratitude to professors Hideaki Shinkawa and Tetsukazu Okinaga for their suggestions and discussions, and also to Miss Yuki Hamada for her excellent technical assistance in some of this work.

References

1 Gibbons, I.R. (1963) Proc. Natl. Acad. Sci. U.S. 50, 1002–1010
2 Gibbons, I.R. and Rowe, A.J. (1965) Science 149, 424–426
3 Raff, E.C. and Blum, J.J. (1969) J. Biol. Chem. 244, 366–376
4 Stephens, R.E. and Levine, E.E. (1970) J. Cell Biol. 46, 416–421
5 Hayashi, M. and Higashi-Fujime, S. (1972) Biochemistry 11, 2977–2982
6 Ogawa, K. and Mohri, H. (1972) Biochim. Biophys. Acta 256, 142–155
7 Ogawa, K. (1973) Biochim. Biophys. Acta 293, 514–525
8 Hoshino, M. (1974) Biochim. Biophys. Acta 351, 142–154
9 Blum, J.J. and Hayes, A. (1974) Biochemistry 13, 4290–4298
10 Mohri, M. and Ogawa, K. (1974) in The Functional Anatomy of the Spermatozoon (Afzelius, B.A., ed.), pp. 161–167, Pergamon Press, Oxford
11 Summers, K.E. and Gibbons, I.R. (1971) Proc. Natl. Acad. Sci. U.S. 68, 3092–3096
12 Tonomura, Y. and Kitagawa, S. (1957) Biochim. Biophys. Acta 62, 15–21
13 Imamura, K., Tada, M. and Tonomura, Y. (1966) J. Biochem. 59, 280–289
14 Tonomura, Y. (1972) in Muscle Proteins, Muscle Contraction and Cation Transport, pp. 82, University of Tokyo Press, Tokyo
15 Hulsmans, H.A.M. (1961) Biochim. Biophys. Acta 54, 1–14
16 Nakamura, K-I. (1976) Bull. Hiroshima Women's Univ. 11, 11–15
17 Reynard, A.M., Hass, L.F., Jacobsen, D.D. and Boyer, P.D. (1961) J. Biol. Chem. 236, 2277–2283
18 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 240, 265–275