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A Comparison of the Effects of Gentle Heating, Acetone, and the Sulfhydryl Reagent Bis (4-Fluoro-3-Nitrophenyl) Sulfone on the ATPase Activity and Pellet Height Response of Tetrahymena Cilia

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Incubation of glycerol-extracted, Triton X-100 demembranated Tetrahymena cilia with 2–10 vol % acetone caused an enhancement of ATPase activity by 2- to 3-fold, depending on concentration and time of incubation. Axonemal ATPase activity was also increased upon incubation with bis (4-fluoro-3-nitrophenyl) sulfone (FNS). Acetone and FNS enhanced the activity of solubilized 30S dynein, but slightly inhibited that of 14S dynein. Heating at 38°C, incubation with FNS, and incubation with acetone activated axonemal ATPase to the same extent. Subsequent studies of 1) the effect of time of preincubation with a spin-labeled maleimide (SLM) at 25°C as a function of pH on the ATPase activity, 2) the concentration dependence of the inhibition of ATPase activity by N-ethylmaleimide or SLM, 3) the ratio of ATPase activity assayed at 25°C to that assayed at 0°C, and 4) the ratio of ATPase activity at pH 8.6 to that at pH 6.9 did not reveal any difference in the properties of the axonemal ATPase after near maximal enhancement by the heat, acetone, or FNS treatments. It was concluded that enhancement of ATPase activity by gentle heat treatment, by incubation with acetone (or other organic solvents), or by FNS results from a conformation change of 30S dynein.

The effect of acetone and of FNS on the pellet height response (a measure of the increase in height of the pellet of cilia precipitated by brief centrifugation in the presence of ATP as compared to the absence of ATP) was also determined. Enhancement of ATPase by these reagents did not lead to a decrease in pellet height response. This observation, in conjunction with other data, indicates that there are at least 3 states of the cross-bridge cycle of dynein arms in cilia.

Key words: cilia, dynein, conformation change, sulfhydryl groups, ATPase activity

INTRODUCTION

The motility of cilia and flagella is based on a sliding filament mechanism (1, 2). Sliding is believed to result from the cyclic attachment and detachment of dynein arms projecting from the A subfibers of each doublet to the B subfibers of the adjacent doublets

Abbreviations used: SLM – N-(1-oxyl-2,2,6,6-tetramethyl-4-piperidinyl) maleimide (spin labeled maleimide); NEM – N-ethylmaleimide; PPDM – N,N'-phenylene-dimaleimide; FNS – bis (4-fluoro-3-nitrophenyl) sulfone; EGTA – [ethylenebis (oxyethylenenitrilo)] tetraacetic acid; DTT – dithiothreitol; PCMB – p-chloromercuribenzoate.

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(3). Warner and Satir (4) have suggested that the radial links also undergo a cyclic attachment to and detachment from projections on the central sheath, and that this system is responsible for the conversion of interdoublet sliding into local bending. Although there is at present almost no knowledge of any of the details of the mechanochemical cycle of the dynein arms, this cyclic process clearly requires that there be a conformation change in at least one step of the cycle.

Blum and Hayes (5, 6) reported that treatment of 30S dynein with NEM or by gentle heating increased its ATPase activity but reduced its ability to recombine with EDTA-extracted ciliary axonemes. Gibbons et al. (7) have recently found that 2 kinds of dynein can be extracted from sea urchin sperm tails. These forms, called dyneins 1 and 2, differ only slightly in molecular weight and in those kinetic properties so far examined. It was found that the latent ATPase activity of dynein 1 could be enhanced 5- to 10-fold by treatment with low concentrations of p-chloromercurisulfonate, and by heating at 40°C for 10 min (8). As expected from the pellet height response studies of Blum and Hayes (6) on 30S dynein from Tetrahymena cilia, the p-chloromercurisulfonate-treated or heat-treated dynein 1 from sperm tails had no frequency restoring capability (7, 8). Gibbons et al. (8) also found that treatment of sperm dynein 1 with 1% Triton caused an enhancement of ATPase activity and a loss of frequency restoring capability. They suggested that soluble dynein can exist in 2 conformational forms, only one of which has physiological activity. That the similarity between the effects of heat-treatment and of treatment with an SH reagent may be due to a conformational change of the dynein is also indicated by a comparison of some properties of 30S dynein extracted from heat-treated cilia with 30S dynein extracted from unheated cilia (9). After heat treatment, the ATPase activity of the dynein in situ differed from that in unheated cilia as shown by an increased sensitivity to a lower temperature of assay (0°C) and by a loss of the activation normally observed upon reaction with NEM or PPDM. Upon extraction of the heat-treated cilia with Tris-EDTA, there was a large loss of ATPase activity so that the heat-treated cilia yielded a crude dynein fraction with a lower specific activity than that obtained from unheated controls despite the fact that the ATPase activity of the heat-treated cilia was higher than that of the controls.

Direct evidence for a conformational change in situ comes from a study of the effect of ATP on cilia that had been reacted with SLM, a spin-labeled analogue of NEM. It was found (10) that the addition of ATP changed the spin resonance spectrum in a manner indicating an increase in rotational freedom of the covalently attached label.

From these and earlier studies (11), it appeared that the reaction of monovalent SH reagents proceeded in at least 2 stages. The first SH group(s) to react caused an enhancement of the ATPase activity, presumably due to a conformation change, while the second SH group(s) to react caused an inhibition of ATPase activity and a loss of functional capacity. When it was observed that PPDM, a divalent SH reagent, caused an enhancement of 30S dynein ATPase at very low concentrations (9), it appeared possible that this reagent might be a useful tool for studying the functional roles of the SH groups of dynein. During the course of these studies it was found that acetone, used to dissolve the PPDM, caused an enhancement of the ATPase activity of cilia. Further studies showed that acetone could also cause an enhancement of the ATPase activity of extracted 30S dynein, and it appeared likely that heat-treatment and exposure to acetone or SH reagents were causing similar conformational changes in the dynein. The differences observed by Blum and Hayes (9) in their study on the ATPase properties of dynein ATPase in situ and after extraction following heat treatment of cilia, however, suggested that the conformational

states in situ might not be identical to those of the solubilized dynein. There are obviously many approaches that could be used to further analyze this complex system. In the present paper, we began by investigating the effects of FNS on axonemal ATPase. This divalent sulfhydryl reagent, which has proved useful in studies on the role of SH groups in the ATPase activity of myosin (E. Reisler, personal communication) was found to cause only an enhancement of the ATPase activity of 30S dynein and of axonemal ATPase. It was then possible to compare the properties of axonemal ATPase in situ after activation of its latent ATPase activity by heat-treatment, incubation with acetone, or incubation with FNS, and evidence is presented which suggests that these 3 different treatments produce a similar conformation change. We have also studied the effects of these treatments on the pellet height response, which serves as a convenient assay of some aspects of the motile system (12, 13). These studies show that enhancement of ATPase activity by acetone or FNS is not necessarily accompanied by a loss of pellet height response, and lead to the conclusion that there are at least 3 states of the dynein cross-bridges.

MATERIALS AND METHODS

Preparation of Cilia and Dynein

Tetrahymena pyriformis, strain HSM, were grown and harvested as described elsewhere (14). Before the continuous-flow centrifuge was stopped, 500 ml of ice-cold solution A (25 mM Tris, 25 mM sodium acetate, adjusted to pH 7.5 at room temperature with ice) was poured through the centrifuge. The centrifuge was then stopped, and the cells resuspended in about 600 ml of solution A. The suspension was then centrifuged at ~ 200 × g for 3 min, the supernatant decanted by suction, and the washed cells resuspended in 100–200 ml of solution A and 5 volumes of solution B [25 mM Tris, 25 mM sodium acetate, 0.1% (wt/vol) Na_2 EDTA, and 15% (wt/vol) glycerol, pH 7.5] was then added. The cell suspension, in a 2-liter Erlenmeyer flask, was then swirled vigorously by hand while being kept at 0°C. After 4 min of swirling, 1 ml of 3 M $CaCl_2$ was added per 120 ml, and the suspension again swirled vigorously for 4 min at 0°C, during which time deciliation occurred. The suspension was then centrifuged at 2,500 × g for 10 min. The pellets were washed with approximately 100 ml of the same proportion of solutions A, B, and $CaCl_2$, and the supernatant recentrifuged to remove all remaining cells. The cell-free supernatant was then filtered through a 13.5 cm diameter Pyrex filter (type C, 40–60 μm pore size) at 4°C. The filtrate was centrifuged at 23,500 × g for 20 min and the pellet of cilia resuspended in ~ 60 ml of IM 8.3 buffer (20 mM imidazole, 2.5 mM $MgSO_4$, pH 8.3) and centrifuged at 27,000 × g for 20 min. After a second resuspension in ~ 60 ml IM 8.3 buffer and centrifugation, the washed pellet of cilia was resuspended in 4 ml of IM 8.3 buffer plus 40 ml of 60% glycerol in IM 8.3 (6 volumes of glycerol:4 volumes IM 8.3) and stored at −20°C. This method is a very slight modification of the procedure given to us by Dr. Robert Conner (personal communication), and we have found it to yield cilia of higher yield and greater purity than the method we have hitherto used. When desired, cilia in the glycerol storage medium were diluted with 5 volumes of IM 7.5 buffer (20 mM imidazole, 2.5 mM $MgSO_4$, pH 7.5), centrifuged for 15 min at 27,000 × g, and the pellet resuspended in 20 ml of 0.1% (vol/vol) Triton X-100 in IM 7.5 buffer. The cilia were stirred at 0°C for 5 min by a magnetic stirrer and collected by centrifugation at 12,000 × g for 10 min. The demembranated axonemes were washed twice (12,000 × g for 10 min each time) with about 30 ml IM 7.5 buffer and resuspended in whatever buffer was

desired for the experiments to be performed (see below). Axonemes were always prepared (from storage) just before use.

Partially purified dynein preparations were made by dialyzing demembranated cilia against 1 mM Tris, 0.1 mM EDTA, pH 8.2, and resolving the 14S and 30S fractions by centrifugation on sucrose density gradients as described in detail elsewhere (14).

Measurement of ATPase Activity

Assay of ATPase activity was performed by adding 0.1 or 0.2 ml of cilia (in 16.7 mM Tris, 16.7 mM imidazole, 2.5 mM Mg^{2+}, 0.13 mM EDTA, pH 7.5) to a mixture of 0.3 ml of IMT buffer (50 mM Tris, 50 mM imidazole, 7.5 mM Mg^{2+}, and 0.4 mM EGTA), pH 6.9–8.6 as specified, plus SH reagent (and DTT when desired) in water such that the final volume was 0.9 ml during the preincubation at 25°C. At the appropriate time 0.1 ml of 10 mM ATP (dissolved in H_2O with pH adjusted to ~7) was added and the reaction allowed to proceed until terminated by the addition of trichloroacetic acid [0.1 ml of 30% (wt/vol) for dynein ATPase assays, 0.5 ml of 10% (wt/vol) for assays of demembranated cilia] and ATPase activity determined as described elsewhere (14). Thus the ionic conditions during assay of the ATPase activity ranged from 15 mM Tris, 17 mM imidazole, 2.50 mM Mg^{2+}, 0.01 mM EDTA, 0.12 mM EGTA when 0.1 ml of cilia or dynein were used to 15 mM Tris, 15 mM imidazole, 2.75 mM Mg^{2+}, 0.12 mM EGTA, 0.02 mM EDTA when 0.2 ml of cilia or dynein were used, and 1.11-fold higher for each component during preincubation.

In experiments on demembranated cilia or on 14S dynein the results of paired assays rarely differed by more than 5%, whether ATP was initially present or whether the enzyme was preincubated for up to 1 hr. With 30S dynein, however, we frequently obtained highly variable data if ATP was not initially present. The variability could be considerably reduced and sometimes eliminated if siliconized test tubes were used, and could be completely eliminated if brand new disposable test tubes were used. In all cases when variability occurred, one observed only an enhancement of activity compared to the expected values. Most of the experiments on 30S dynein reported in this paper were done with new disposable test tubes. For those few done with siliconized test tubes, the errors were less than ± 10%.

Protein Determination

Protein was determined by the method of Lowry et al. (15) using bovine serum albumin as a standard.

Pellet Height Assays

Pellet height assays were performed in Wintrobe hematocrit tubes according to our standard procedure (12); the pellet heights reported have been corrected to a meniscus height of 10.0 cm.

Preparation of Reagents

SLM and NEM were dissolved in water or in IMT buffer, pH 6.9–8.6 as specified below, diluted with 2 volumes of H_2O. The diluted IMT buffer will be referred to as buffer D. PPDM and FNS were dissolved in 10 ml of acetone and diluted further with acetone and water as desired. All solutions of SH reagents were made immediately before use.

Reagents

Acetone (99 mol % pure) was purchased from Fisher Scientific Co.; NEM and FNS from Sigma; DTT from Bachem Feinchemikalien A.G., Switzerland; PPDM from Aldrich Chemical Co.; ATP from Pabst. All other chemicals were of the highest purity commercially available.

RESULTS

Effect of Acetone on ATPase Activity of Demembranated Ciliary Axonemes

Figure 1 shows the results of an experiment in which various amounts of acetone were preincubated with cilia at pH 7.5 for times up to 1 hr and then the ATPase activity assayed after an 11.7 min incubation with ATP. It will be noted that even in the absence of acetone there is a "spontaneous" but slow enhancement of ATPase activity. Incubation

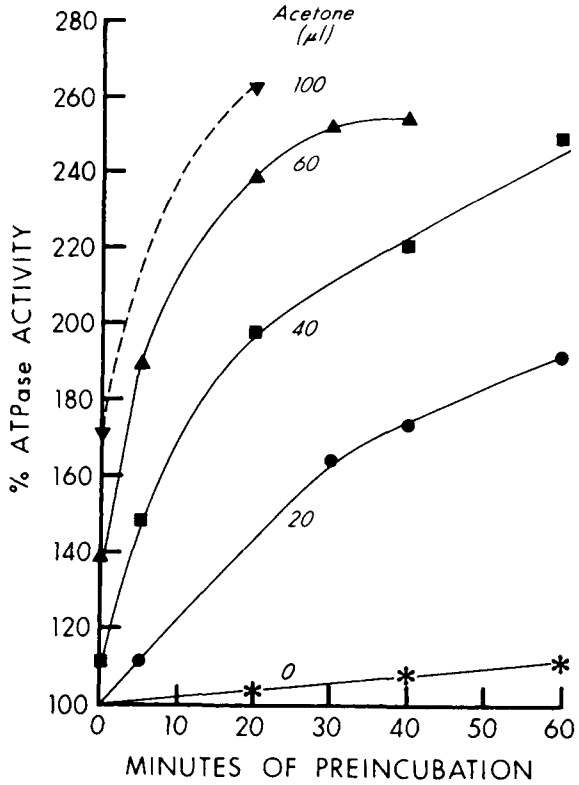

Fig. 1. Enhancement by acetone of the ATPase activity of demembranated cilia. Cilia (0.15 mg protein) were incubated at 25°C with the indicated amounts of acetone in pH 7.5 buffer for the times shown on the abscissa. At the times shown, 0.1 ml of 10 mM ATP was added (bringing the total volume to 1.0 ml). ATP hydrolysis was allowed to proceed for the next 12 min, and the reaction terminated by addition of 0.5 ml 10% (wt/vol) trichloroacetic acid, and the phosphate released was measured as described in Materials and Methods. One-hundred percent ATPase activity corresponds to 126 nmol/min·mg protein.

with 20 μl acetone per 0.9 ml increased the rate of enhancement so that the ATPase activity had almost doubled within an hour. It is also seen that this curve passes through the origin, showing that the presence of ATP during the assay interval completely prevented the enhancement of ATPase activity by this concentration of acetone. With increasing concentrations of acetone, the rate of enhancement of ATPase activity increased. It will also be noted that the ATPase activities observed at zero time of preincubation (i.e., ATP and acetone present together for 11.7 min) increase with increasing acetone concentration, indicating that the ATP becomes less and less effective in preventing the enhancement of ATPase activity as the acetone concentration increases.

In studies to be reported elsewhere we have found that the activating and inhibiting effects of NEM, SLM, FNS, PPDM, and carboxypyridine disulfide on axonemes and on 30S and 14S dyneins are sensitive to pH in the range 6.9–8.6. Since some of these reagents were dissolved in acetone, it was important to ascertain the effects of acetone on cilia as a function of pH. Figure 2A shows that the enhancing effect of 20 μl of acetone preincubated with cilia for up to 30 min decreased slightly with increasing pH in the range 6.9–8.6. In other experiments, however, there was a slight increase in the rate of enhancement with increasing pH. Thus acetone controls were always included in each experiment. It is again evident that ATP prevented the enhancing effect of 20 μl acetone since if the acetone had been effective during the 11.7 min duration of the ATPase essay, the zero time point activities should have been close to the values obtained after 5 min preincubation. Figure 2B shows the effect of preincubating cilia with various concentrations of acetone for 20 min at pH 6.9 and at pH 8.6, the 2 extreme values of pH used in the subsequent

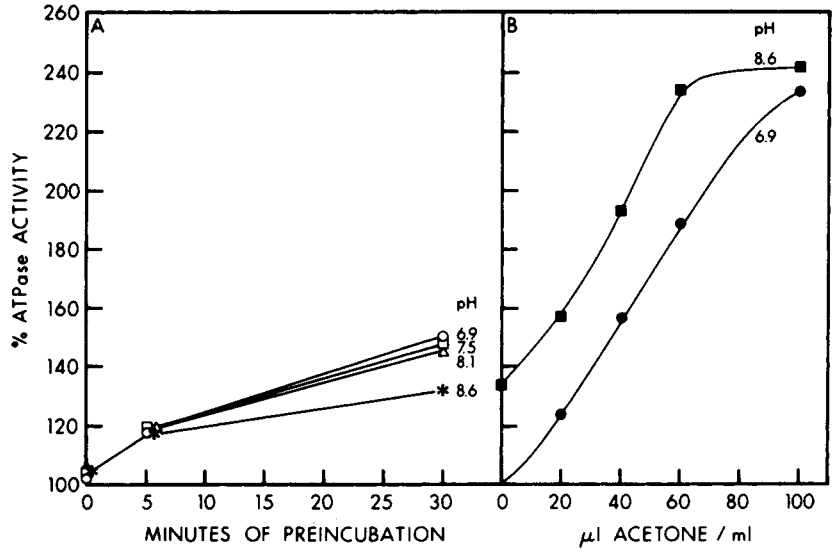

Fig. 2. Effect of pH on the enhancement of ATPase activity of cilia by acetone. In panel A, cilia (0.168 mg protein) were preincubated with 20 μl acetone in a total volume of 0.9 ml in buffers at the pH values shown. In panel B the cilia (0.136 mg) were preincubated for 20 min with the amounts of acetone shown on the abscissa. ATPase activity was then determined 12 min after the addition of 0.1 ml ATP. One-hundred percent ATPase activity in the absence of acetone corresponds to 123, 129, 140, and 157 nmol/min·mg at pH 6.9, 7.5, 8.1, and 8.6, respectively, for the cilia in panel A, and to 187 and 247 nmol/min·mg at pH 6.9 and 8.6, respectively, for the cilia in panel B.

studies with SH reagents. It can be seen that up to about 6 vol % of acetone there was little difference in the enhancing effect at these 2 pH values. At higher concentrations of acetone, the increase in ATPase activity continues at pH 6.9 but appears to level off at pH 8.6, perhaps due to a slow denaturation process as indicated by a decline in ATPase values at longer times (data not shown).

Cilia were also incubated with acetone for 18 hr at 0°. The cilia were then centrifuged at 0°C, resuspended in buffer D, and assayed at 25°C. It was found (data not shown) that there was only a small enhancement of ATPase activity even at concentrations of acetone up to 8 vol %.

Similar experiments were also performed with 14S and 30S dyneins. Table I shows the results of one such experiment. It can be seen that preincubation of 30S dynein with up to 4 vol % acetone caused an enhancement of up to 2.4-fold of the ATPase activity, with less enhancement being obtained at 6 vol %. The presence of ATP almost completely prevented the enhancement of activity even for 6 vol % acetone. Acetone only inhibits 14S dynein ATPase. There was little effect of pH in the range 6.9–8.6 on the rate of inhibition of 14S dynein ATPase by acetone (data not shown). As with 30S dynein, the presence of ATP largely prevented the effect of acetone even at 6 vol % acetone (Table I). There was very little effect of up to 4 vol % acetone on the ATPase activity of 30S or 14S dynein if the dynein was preincubated for 18 hr at 0°C and then assayed at 25°C in the presence of ATP (data not shown).

A few experiments were also performed with other organic solvents. The ATPase activity of cilia was increased 2-fold by 10 min preincubation with 6 vol % ethanol. The ATPase activity of 30S dynein was also enhanced by 4 vol % 1-propranol, 2-propranol, 1-butanol, and isobutanol, while that of 14S dynein was only inhibited by these solvents.

Effect of FNS on the ATPase Activity of Demembranated Cilia

In studies to be reported in detail elsewhere (Blum and Hayes, manuscript in preparation) we have found that FNS activates the ATPase activity of 30S dynein and of axonemes at 25°C, the degree of activation increasing with increasing pH in the range 6.9–8.6. However, 14S dynein ATPase activity was slightly inhibited. Figure 3 shows the results of an experiment in which demembranated axonemes were incubated with FNS or PPDM at 0°C for 18 hr and then warmed to 25°C and assayed for ATPase activity. It can be seen that whereas PPDM yields a biphasic response, such as observed earlier for NEM

TABLE I. Effect of Acetone on the ATPase Activity of 30S and 14S Dynein

Time of preincubation (min)	Acetone (μl)	% ATPase activity	
		30S Dynein	14S Dynein
15	0	(100)	(100)
15	10	100	–
15	20	107	–
15	40	242	74
15	60	173	65
0	60	109	92

30S dynein and 14S dynein in buffer at pH 7.5 and in a total volume of 0.9 ml were preincubated with acetone as shown in the table at 25°C and then 0.1 ml of 10 mM ATP was added and the ATPase activity assayed after a 15 min incubation with the ATP.

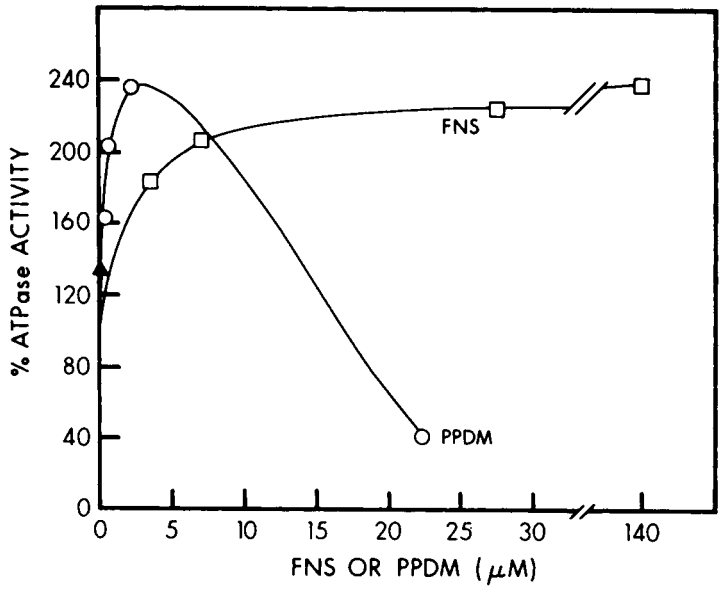

Fig. 3. Effect of FNS and PPDM on the ATPase activity of demembranated cilia. Cilia were incubated with the indicated concentrations of FNS or PPDM for 18 hr at 0°C in 0.9 ml of Buffer D (pH 7.5) containing 0.02 ml acetone. The cilia were then briefly warmed to 25°C and 0.1 ml of 10 mM ATP was added and the ATPase activity assayed after 11 min as described in Methods. One-hundred percent ATPase activity corresponds to 130 nmol/min·mg. Each tube contained 176 μg ciliary protein. The datum shown by a filled triangle (▲) is the activity of a control sample incubated for 18 hr at 0°C with 0.02 ml acetone but no sulfhydryl reagent.

(16) and SLM (10), FNS appears to react only with the SH group(s) responsible for enhancement of the ATPase activity. Of particular interest is the observation that the same maximal degree of enhancement of ATPase activity (~ 240%) was caused by each reagent. Since comparable increases in ATPase activity are caused by acetone (see Fig. 1) and by heating at ~ 38°C it was of interest to inquire whether the same state of ATPase in situ was achieved when enhancement occurred by these 3 quite different treatments.

Comparison on the ATPase Activities of Demembranated Cicia After Near-Maximal Enhancement by Heat, Acetone, or FNS

Figure 4 shows the results of an experiment in which a preparation of cilia was divided into 3 portions. One portion was treated with 7.8 vol % acetone at 25°C for 30 min and then chilled to 0°C. The second portion was heated at 38.2°C for 25 min and then chilled. The third portion was kept at 0°C and 55.6 μM FNS was added. Each sample was incubated at 0°C for ~18 hr and then washed once by adding ice-cold buffer D, pH 7.5, and centrifuging for 10 min at 12,000 × g. The washed pellets were resuspended in this buffer and assayed for ATPase activity during preincubation with SLM at 4 pH values. It can be seen that treatment with acetone, heat, or FNS enhanced the ATPase activity (assayed at pH 7.5) by about 260, 320, and 265%, respectively. (Earlier experiments had shown that these treatments yielded about the same final degree of activation, but variations from experiment to experiment make it difficult to achieve the maximal enhancement in any given experiment.) When the washed cilia were preincubated with SLM at 25° C for varying times, it was found that there were no appreciable differences in the rate of change of ATPase activity. In the acetone-treated and FNS-treated cilia, where

less than maximal enhancement of ATPase activity had been achieved, a slight residual capacity for enhancement at short times of exposure and at low pH (i.e., pH 6.9 and 7.5) remained, but then the time course of inhibition at each pH was as expected from results obtained with untreated cilia (data not shown).

In a similar experiment (Fig. 5) where about a 220% enhancement of ATPase activity was achieved by each of the 3 treatments, we studied the concentration dependence of the ATPase activity during a 10 min preincubation at several concentrations of SLM (at pH 8.6) and of NEM (at pH 8.1). The results of this experiment show that there was little difference in the concentration dependence of the inhibitory process between any of the 3 differently treated samples. The data in Figs. 4 and 5 therefore indicate that the properties of the SH group(s) responsible for the inhibition of catalytic activity are closely similar in axonemes in which near maximal enhancement of the ATPase activity has been achieved by 3 quite different treatments. It should also be noted that preincubation with NEM did not lead to a net inhibition of activity as compared to the original activity prior to the heat, acetone, or FNS treatments, whereas preincubation with SLM caused a large net inhibition. Similar results have been obtained during preincubation of control cilia with NEM and SLM.

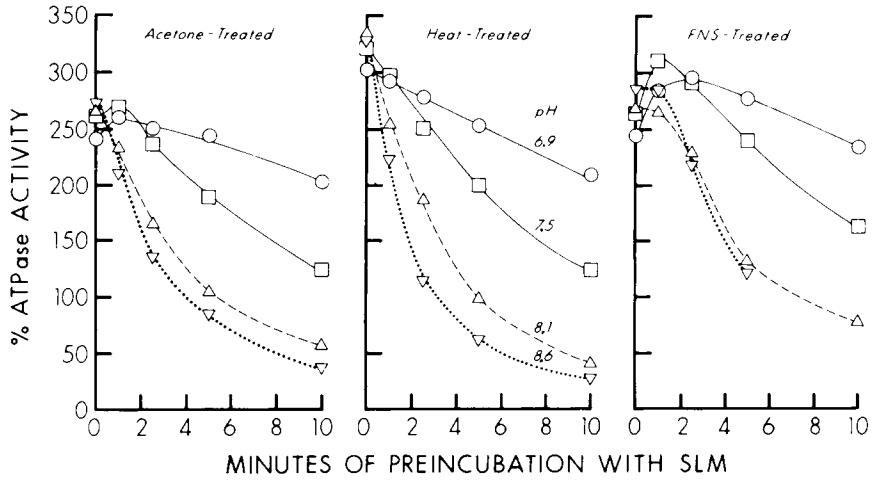

Fig. 4. pH dependence of effect of SLM on ciliary ATPase activity following treatment of cilia with acetone, heat, or FNS. Demembranated axonemes were prepared as described in Materials and Methods and resuspended in buffer at pH 7.5. Three samples of 3.0 ml (7.57 mg protein) were treated as follows: sample 1, 0.32 ml buffer and 0.28 ml of acetone were added and the mixture incubated at 25°C for 30 min and then placed in ice; sample 2, 0.6 ml buffer added and the mixture heated at 38.2°C for 25 min and then placed in ice; sample 3, 0.52 ml buffer plus 0.08 ml of 2.5 mM FNS in acetone, at 0°C. Aliquots were taken for ATPase assay at 25°C and the remainder of each sample (3.4 ml) was kept at 0°C for about 18 hr, and then 7 ml of pH 7.5 buffer were added and the tubes centrifuged for 10 min at 12,000 × g. The supernatants were decanted and the pellets resuspended in 4.0 ml of buffer and samples taken for assay of protein content and ATPase activity at 25°C. One-hundred percent activity (untreated cilia) corresponds to 114 nmol/min·mg. For ATPase assay, aliquots of 0.10 ml of the treated cilia were added to 0.7 ml of buffers at the indicated pH values (○——○, pH 6.9; □——□, pH 7.5; △---△, pH 8.1; ▽...▽, pH 8.6) containing SLM and incubated for the times shown on the abscissa. The concentration of SLM during the preincubation was 625 μM. The reaction was terminated by addition of 0.1 ml 10 mM DTT and ATPase activity was then assayed 12 min after adding 0.1 ml of 10 mM ATP. The amount of protein used in each assay was, respectively: acetone-treated, 0.135 mg; heat-treated, 0.132 mg; FNS-treated, 0.145 mg.

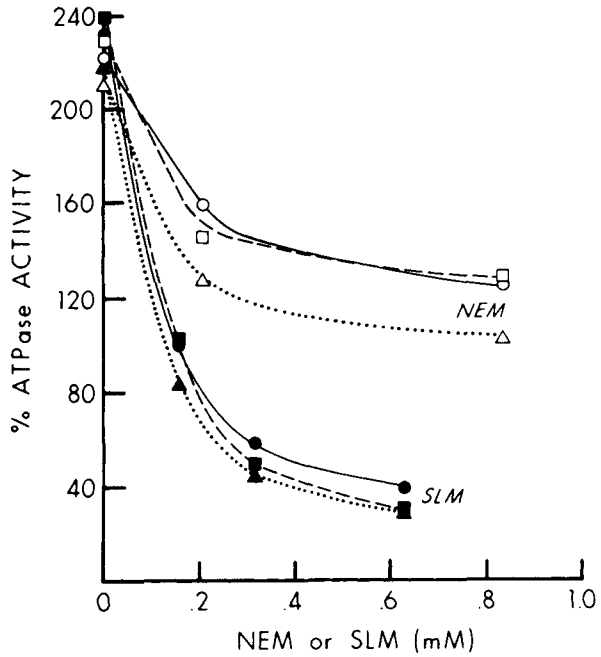

Fig. 5. Effect of pretreatment of cilia with FNS, acetone, or heat on subsequent concentration dependence of inhibition of ATPase by NEM or SLM. Demembranated axonemes were treated with FNS, acetone, or heated at 38.4° as described in the legend to Fig. 4. After about 18 hr at 0°C the cilia were washed and resuspended in buffer and preincubated for 10 min at 25°C in pH 8.6 buffer for the experiments with SLM or in pH 8.1 buffer for the experiments with NEM. The volume during preincubation was 0.9 ml and the concentrations of NEM and SLM were as shown on the abscissa. At the end of the 10 min preincubation 0.1 ml of ATP was added and the ATPase activity assayed after 15 min. Each tube contained 0.0622 mg protein. One-hundred percent activity for the untreated cilia was 147 nmol/min•mg.

Further evidence that there was no large difference in the properties of the axonemal ATPase after these different treatments was obtained by comparing the ratio of ATPase activity at pH 8.6 to that at pH 6.9 and the ratio of activity at 25°C to that at 0°C. In the 2 experiments shown in Figs. 4 and 5 plus one other, the ratios of ATPase activity at pH 8.6:pH 6.9 ranged between 1.06 and 1.43, with no significant difference related to type of pretreatment. The ratios of ATPase activity at 25°C to that at 0°C ranged between 6.2 and 8.8, again with no significant differences between the 3 pretreatments. Thus neither the pH dependence of the SLM effect, the concentration dependence of SLM or NEM, the ratio of activity at pH 8.6 to that at pH 6.9, nor the ratio of activity at 25°C to that at 0°C appear to differ in cilia that have been activated by treatment with heat, acetone, or FNS.

Effect of Acetone and FNS on the Pellet Height Response

When Tetrahymena cilia are exposed to ATP and briefly centrifuged at low speed, the height of the pellet obtained is greater than that of control cilia centrifuged identically but in the absence of ATP (12). It has been shown elsewhere that the pellet height response is a measure of some aspect of normal beating (13). In all our previous studies, whether

with heat treatment (6, 9, 14), NEM (5, 6), SLM (10), or PCMB (5), an inhibition of the pellet height response was observed which appeared to be correlated with either an enhancement of ATPase activity or, in the case of PCMB, with an inhibition of this activity, although in the latter case measurements of the ATPase activity and of the pellet height response were not performed on the same samples of cilia. In view of the apparent selectivity of acetone and FNS for the enhancement process, it was of interest to examine this question more closely. Cilia were therefore exposed to 13 vol % acetone at 25°C or to 160 μM FNS (and 2 vol % acetone) at 0°C for 18 hr, and then washed and assayed for ATPase activity and pellet height response. It was found that the acetone and FNS treatments had enhanced the ATPase activity 141% and 178%, respectively, but the pellet height response was not changed compared to that of untreated controls. A similar experiment with 2.1 μM PPDM yielded a 165% increase in ATPase activity and no change in pellet height response. These results clearly demonstrate that enhancement of ciliary ATPase activity is not necessarily accompanied by an inhibition of the pellet height response.

DISCUSSION

Raff and Blum (12) first reported that gentle heating of glycerol-extracted Tetrahymena cilia caused a slight increase in ATPase activity and a loss in pellet height response. Later Blum and Hayes (5) found that the ATPase activity of ciliary axonemes and of solubilized 30S dynein was enhanced approximately 2-fold by preincubation with high concentrations of NEM, and noted that this constituted a surprising analogy to the effect of NEM on myosin. It was then shown that treatment of 30S dynein with high concentrations of NEM at 25°C impaired the ability of the dynein to recombine with EDTA-extracted axonemes (6). Shimizu and Kimura (16), working independently of Blum and Hayes, reported that if the reaction of NEM with 30S dynein was carried out at 0°C, the typical biphasic curve characteristic of myosin was obtained, and at much lower concentrations of NEM. Shimizu and Kimura (16) found that preincubation of axonemes with 10 μM NEM at 0° largely prevented the recombination of the 30S dynein with EDTA-extracted axonemes. Since the loss of recombining ability occurred prior to full activation of the ATPase activity, they suggested that at least 3 SH groups were involved in the functioning of dynein; reaction of the most sensitive group with NEM prevented binding, reaction of the next most sensitive group caused enhancement of the ATPase activity, and reaction of the least sensitive group caused an inhibition of catalytic activity. The present finding that acetone (and several other organic solvents) cause an enhancement of the ATPase activity of 30S dynein and of axonemes is reminiscent of the effects of organic solvents on the ATPase activity of myosin (Refs. 17–19 and references therein). In the case of myosin, where much more extensive studies have been performed, it appears certain that the effects of the organic solvents cannot be due to small changes in bulk dielectric constant but rather are indications of a change in protein conformation which, according to Stone and Prevost (19) enhances ATPase activity and interferes with an ATP-induced conformation change. Since gentle heating, exposure to SH reagents, to acetone and other solvents, and to Triton (8) would appear to have very little in common, a similar deduction appears virtually certain for both Tetrahymena and sea urchin sperm tail dynein. The observation that 30S dynein but not dynein in situ may be activated by contact with the surface of well-washed test tubes indicates that conformational changes

may be induced more easily in solubilized dynein than in dynein in situ. This is not surprising in view of several reports showing a number of differences between free and bound dynein (14, 20, 21). Whereas previous studies have simply shown that the ATPase properties differed, the present observations indicated that dynein in situ is held in a more stable configuration, in agreement with the conclusions derived from observations on the loss of activity of 30S dynein upon extraction from heat-treated cilia (9).

The finding that low concentrations of FNS enhance the ATPase activity of 30S dynein ATPase and of axonemal ATPase is not surprising in view of earlier experiments with NEM, SLM, and PPDM, where similar magnitudes of enhancement have been obtained. However, FNS appears to be more selective than any of the other SH reagents we have so far examined, in that for no combination of concentrations (up to 140 μM) or times (up to 18 hr at 0°C and 30 min at 25°C) have we observed any inhibitory effect. Likewise, PPDM, also a divalent SH reagent but with a span of 12–14 Å as compared to 10–12 Å for FNS, can yield the same enhancement as FNS but can cause net inhibition at high concentrations (e.g., 20 μM). It is not known whether this apparent selectivity of FNS for the SH group(s) responsible for enhancement of ATPase activity is related to the potentially divalent nature of this reagent, since the present experiments do not indicate whether FNS (or PPDM) is acting as a monovalent or a divalent reagent when it reacts with axonemes or with extracted 30S dynein. Its apparent selectivity for the activating SH group(s) under the conditions of these experiments, however, makes it useful for comparing the properties of the in situ ATPase after near maximal enhancement by FNS, acetone, or heat treatment. Our results showed no appreciable differences in the properties of the enhanced ATPase with respect to 4 criteria: dependence of SLM effect with time of preincubation; concentration dependence of inhibition by SLM or NEM after 10 min preincubation; ratio of ATPase activity at pH 8.6 to that at pH 6.9; ratio of ATPase activity at 25°C to that at 0°C. This does not, of course, prove that an identical conformation change is caused by each of the 3 treatments, since small differences in these criteria have been ignored and, also, it is possible that larger differences may be revealed by use of some other criteria. Nevertheless it is clear that to a first approximation these 3 treatments have caused a similar conformation change and, furthermore, that this conformation change does not destroy all mechanochemical function, as indicated by an unimpaired pellet height response.

Shimizu and Kimura (16) showed that the first SH group(s) to react with NEM at 0°C interfered with the rebinding of 30S dynein to EDTA-extracted axonemes. Presumably this SH group is located at or near that portion of the 30S dynein arm which interacts with the dynein binding site on the A subfiber of each doublet. The next SH group to react causes an enhancement of 30S dynein ATPase. The present experiments show that the enhancement of ATPase activity in situ, whether caused by acetone, PPDM, or FNS, does not interfere with the pellet height response. We have shown elsewhere (12) that gentle heating inhibits the pellet height response, but this does not necessarily mean that heat-treatment affects dynein in situ differently than does treatment with acetone or SH reagents, since heat treatment (but not treatment with NEM) strongly reduces the capacity of EDTA-extracted axonemes to bind 30S dynein (6). It is not yet known whether the enhancement of axonemal ATPase activity by acetone or PPDM or FNS is accompanied by a loss of the ability of cilia to beat. If one assumes, as indicated by all available evidence (13), that the pellet height response assay measures some aspect of beating which is related to the mechanochemical cycle of the dynein arms, then one must postulate at least 3 states for the cross-bridge cycle in cilia. First, a relaxed state, in which the arms

do not contact the sites on the adjacent B subfibers. Second, the rigor state, induced by dilution of the ATP to 10–15 μM (22). In this state the dynein arms bridge the distance from the A subfiber to the adjacent B subfiber. Third, a stage in which the ciliary ATPase is enhanced, but the pellet height reponse is not interfered with. Since the pellet height response occurs at a much higher ATP concentration than the maximum permissible for the rigor state, it cannot be the same as the rigor state. Possible cross-bridge cycles in cilia have been considered by several workers and it seems likely that at least 4 states are required (e.g., see diagram in Ref. 23), but this is the first evidence for the existence of a state other than the relaxed and the rigor states.

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