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Effects of Sulfhydryl Reagents on the ATPase Activity of Solubilized 14S and 30S Dyneins and on Whole Ciliary Axonemes as a Function of pH

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The effects of five sulfhydryl (SH) reagents – N-ethylmaleimide (NEM), a spin-labeled maleimide (SLM), N-N′-phenylenedimaleimide (PPDM), bis(4-fluoro-3-nitrophenyl)sulfone (FNS), and carboxypyridine disulfide (CPDS) – on glycerol-treated, Triton X-100-demembranated ciliary axonemes of Tetrahymena, on the 30S and 14S dyneins extracted from such axonemes, and on the residual ATPase activity remaining associated with axonemes that have been extracted twice with Tris-EDTA have been examined as a function of pH in the range 6.9–8.6.

Preincubation of axonemes and of solubilized 30S dynein with low concentrations of each of the five SH reagents, at 0°C and at 25°C, caused enhancement of the latent ATPase activity. PPDM was the most effective reagent, causing half-maximal enhancement (after 18 h at 0°C) at ~ 0.5 μM, corresponding to 0.19 moles/10^5 g axonemal protein. The rate constants, k_a, for the enhancement reaction at 0°C depended on whether the 30S dynein was in situ or solubilized; the ratio k_a (in situ) /k_a(solubilized) was > 1 for NEM, ~ 1 for PPDM, and < 1 for FNS. For each SH reagent except CPDS, k_a (at 0°C) increased markedly with increasing pH in the range pH 6.9–8.6; for CPDS k_a increased only about fourfold.

At long times of preincubation and high concentrations of NEM, SLM, PPDM, and CPDS, the enhancement of ATPase activity was followed by a loss of activity. The values of k_L, the rate constants for loss of ATPase activity from the peak enhanced level, were much lower than the corresponding values for k_a, and increased with increasing pH. With SLM and PPDM, inhibition continued until the ATPase activity was almost completely inhibited. With NEM, however, the initial rate of loss from the peak enhanced value decreased as the ATPase activity returned toward the control (unmodified) level, and further inhibition was very slow. The differences in degree of inhibition obtained with SLM as compared to NEM suggest that there are at least two classes of inhibitory SH groups on 30S dynein.

Abbreviations: SLM, N-(1-oxyl-2, 2,6,6-tetramethyl-4-piperidinyl) maleimide (spin-labeled maleimide); NEM, N-ethylmaleimide; CPDS, carboxypyridine disulfide (6,6′-dithiodinicotinic acid); PPDM, N,N′-p-phenylenedimaleimide; FNS, bis(4-fluoro-3-nitrophenyl)sulfone; EGTA, [ethylenebis(oxyethylenenitrilo)]-tetraacetic acid; DTT, dithiothreitol; PCMPS, p-chloromercuriphenylsulfonate.

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The ATPase activity of 14S dynein was only inhibited by preincubation with NEM, SLM, PPDM, and, to a lesser extent, CPDS; k_L increased with increasing pH. Preincubation of 14S dynein with FNS yielded conflicting results when the reaction was "stopped" by adding dithiothreitol. When 14S dynein was preincubated at 0° C with FNS and the ATPase activity was then assayed at 25°C, a biphasic pattern of enhancement followed by inhibition was obtained.

The residual ATPase activity of twice-extracted axomenes was relatively insensitive to each of the SH reagents studied; an initial rapid loss of some 20–40% of the ATPase activity occurred, followed by a very slow further loss of activity. Increasing the pH increased this slow rate of inhibition. The residual ATPase activity of unmodified twice-extracted axonemes decreased slightly with increasing pH, in contrast to the slight increase observed with increasing pH for the ATPase activity of axonemes and of solubilized 30S and 14S dyneins.

The presence of ATP during preincubation of axonemes with PPDM at 0°C prevented the enhancement of ATPase activity; only a slow loss of ATPase activity was observed. This rate of loss of ATPase activity was slower than the rate of loss observed (after peak enhancement of activity was reached) when PPDM reacted with axonemes in the absence of ATP. In these properties the SH groups of 30S dynein responsible for the enhancement of latent ATPase activity and for the inhibition of ATPase activity do not resemble the SH_1 and SH_2 groups of myosin, respectively, since the presence of ATP increases the rates of reaction of SH_1 and SH_2 of myosin with SH reagents.

Key words: cilia, 14S dynein, 30S dynein, sulfhydryl groups, pH, ATPase activity

It has been known for many years that sulfhydryl reagents can inhibit the motility of cilia and flagella [1]. Ogawa and Mohri [2] first observed that low concentrations of some heavy-metal ions and SH reagents could slightly enhance the ATPase activity of solubilized dynein obtained from sea urchin sperm flagella. Blum and Hayes [1] then showed that high concentrations of NEM caused a slight enhancement of the ATPase activity of cilia of Tetrahymena and a doubling of that of crude dynein. They also found that preincubation of the cilia with high concentrations of NEM inhibited the pellet height response of these cilia, the inhibition being considerably less if ATP was initially present. Shimizu and Kimura [3] showed that incubation of 30S dynein from Tetrahymena cilia with low concentrations of NEM at 0°C for about 18 h caused a biphasic enhancement followed by inhibition of the ATPase activity, whereas the ATPase activity of 14S dynein was only inhibited. From these studies it became apparent that myosin and 30S dynein not only participated in sliding filament-based motile systems but also that their ATPase sites behaved similarly with respect to interactions with some sulfhydryl reagents [1–3].

Reaction of 30S dynein with NEM not only affects the ATPase activity but also prevents recombination of the 30S dynein onto extracted axonemes [3, 4]. The concentration dependence of the loss of recombining ability, the enhancement of the ATPase activity, and the inhibition of ATPase activity suggests that there are at least three types of SH groups present in 30S dynein – the most reactive to NEM being those responsible for inhibition of the ability to rebind to EDTA-extracted axonemes; the next most reactive, called SH_1, being those responsible for enhancement of ATPase activity; and the least reactive, called SH_2, being those responsible for loss of catalytic activity [3]. Since exposure of EDTA-extracted axonemes to NEM did not prevent the rebinding of native 30S dynein, it seems unlikely that any SH groups on the proteins of the EDTA-extracted

axonemes play an important role in allowing the 30S dynein to rebind and express its ATPase activity [4]. SH groups on axonemal proteins do, however, influence wave shape [5] and pellet height response [1, 6].

Although NEM is a useful reagent for studying the role of SH groups in enzymatic processes, much can be learned by using other sulfhydryl reagents. Thus SLM, a spin-labeled analogue of NEM that has been used to study conformation changes in actomyosin systems, affects the ATPase activity and pellet height response of cilia in a fashion similar (but not identical – see below) to NEM [6, 7]. Studies of the effect of ATP on the electron spin resonance spectra of SLM-labeled axonemes showed an increase in rotational freedom of some of the covalently bound label, thus providing direct evidence that ATP caused a conformational change in axonemes. Studies with PPDM, a bivalent analogue of NEM with a span of 12–14 Å that has been used to map the role of SH groups at the ATPase site of myosin [8, 9], showed that this reagent could enhance the activity of ciliary axonemes and of 30S dynein at much lower concentrations than were required when either NEM or SLM were used [10], suggesting that PPDM might be particularly useful for estimating the number of SH groups involved in the enhancement of ATPase activity.

The ATPase activity of Tetrahymena cilia can also be enhanced by exposure to acetone, by gentle heating, and by incubation with FNS [6], a divalent sulfhydryl reagent with a span of 7–10 Å that is not a maleimide derivative. Although that study was primarily aimed at providing information concerning the conformation change in dynein in situ, it was noted that FNS did not appear to cause inhibition of the ATPase activity of either the intact axonemes or of 30S dynein. It was observed in our experiments with SLM [7] and with FNS [6] that the rate of enhancement of ATPase activity increased markedly with increasing pH in the range 6.9–8.6, whereas the ATPase activity was only slightly increased by increasing the pH in this range. In view of the potential further utility of sulfhydryl reagents with differing selectivities towards the SH groups involved in the ATPase activity and mechanochemical functioning of dynein, we undertook a study of the effect of NEM, SLM, PPDM, FNS, and CPDS (a divalent reagent with a span of about 2–3 Å that has proved useful for analysis of the role of vicinal dithiols in oxidative phosphorylation [11, 12]) on the ATPase activity of demembranated axonemes, 30S dynein, 14S dynein, and the residual ATPase remaining in the axonemes after two extractions with Tris-EDTA [13].

MATERIALS AND METHODS

Demembranated axonemes of Tetrahymena pyriformis, strain HSM, were prepared as described elsewhere [6]. Partially purified dynein preparations were made by dialyzing demembranated cilia against 1 mM Tris, 0.1 mM EDTA, pH 8.2, at 0–4°C, and resolving the 14S and 30S fractions by centrifugation on sucrose density gradients [10]. Twice-extracted axonemes were prepared by dialyzing the pellet obtained from the first extraction for another 24 h with Tris-EDTA.

Measurement of ATPase Activity

Assay of ATPase activity was usually performed by adding 0.1 ml of axonemes (in 8.33 mM Tris, 8.33 mM imidazole, 12.5 mM Mg^{2+}, 0.67 mM EGTA, pH 7.5) or of dyneins (in the same buffer plus sucrose) to a mixture of 0.3 ml IMT buffer (50 mM Tris, 50 mM imidazole, 7.5 mM Mg^{2+}, 0.4 mM EGTA, pH 6.9–8.6 as specified) plus SH reagent in H_2O such that the final volume was 0.9 ml for preincubations at 25°C or 0.8 ml for preincubations at 0°C. For preincubations at 25°C the preincubation was ended by adding

0.1 ml of 10 mM ATP (dissolved in H_2O, pH adjusted to ~ 7). Slight deviations from these conditions will be specified in the figure legends. The ATPase assay was terminated after an appropriate time at 25°C (generally 10–20 min) by adding trichloroacetic acid [0.1 ml of of 30% (w/v) for dynein ATPase assays, 0.5 ml of 10% (w/v) for axonemal ATPase assays] and the orthophosphate released was determined as described elsewhere [13]. For preincubations at 0°C the preincubation was ended by adding 0.1 ml of 10 mM DTT, also at 0°C. The tubes were kept on ice for at most a few more minutes, and then 0.1 ml of ATP was added and the tube immediately placed in a 25°C bath for ATPase assay. The ionic conditions during ATPase assay were 15 mM Tris, 17 mM imidazole, 2.50 mM Mg^{2+}, 0.12 mM EGTA, and 1.11-fold higher during preincubations at 25°C and 1.25-fold higher during preincubations at 0°C. All assays were performed in duplicate; paired assays rarely differed by more than 5%. Unless otherwise specified, 1 unit of ATPase activity is 1 nmole/min·mg protein.

Preparation of Reagents

SLM and NEM were dissolved in water. CPDS was dissolved in a smaller volume of IMT buffer (pH 8.6) and diluted with H_2O, and the pH was then adjusted to ~ 7.5. This solution was then further diluted with water, so that the contribution of ions from the buffer was negligible. PPDM and FNS were dissolved in acetone and diluted with acetone as desired. When these reagents were used, they were always used as 0.02 ml (the volumes during preincubation remaining at 0.8 ml and 0.9 ml for preincubations performed at 0°C and 25°C, respectively). Control tubes received 0.02 ml of acetone. All solutions of SH reagents (and of DTT) were made fresh daily and kept at 0°C.

Protein Determination

The protein content of axonemes and of pellet II (axonemes that had been twice extracted with Tris-EDTA) was determined by the method of Lowry et al [14], using bovine serum albumin as standard. Because the protein content of 30S dynein and 14S dynein solutions is very low, protein concentrations for the early experiments (in which dyneins were preincubated at 25°C) were not performed, and those data are reported as activity units per milliliter of dynein solution. For most of these experiments, however, protein content of the dynein solutions was determined by a modification of the Coomassie Blue dye binding procedure developed by Bradford [15] and by Sedmak and Grossberg [16]. Coomassie Blue reagent was purchased from Bio-Rad Laboratories, Richmond, California. Their suggested microassay procedure uses 0.2 ml of concentrated reagent plus 0.8 ml of sample. The blank in such a mixture was very high, so that concentrations below ~ 4 μg/ml could not be determined with precision. We found that diluting the Bio-Rad reagent 1:1 with 0.3 N perchloric acid and using 0.2 ml of the diluted reagent plus 0.8 ml of sample markedly reduced the blank and improved the sensitivity and accuracy so that protein concentrations as low as 2 μg/ml could be reliably assayed. The final procedure adopted was to add 0.05–0.10 ml of dynein to water so that the final volume was 0.8 ml. Then 0.2 ml of the 1:1 diluted dye reagent was added and 5 min later the absorbances at 465 and 595 nm were measured. Although these absorbances changed slowly with time, the ratio A_{595}/A_{465} was independent of time and was linear with protein concentration (using bovine serum albumin as standard) at least up to 10 μg protein per assay.

Reagents

The source of all reagents used is as earlier reported [6].

Effect of SH Reagents on the ATPase Activity of Demembranated Cilia at 25°C

Preliminary studies of the effect of NEM and SLM at different pH values suggested that pH studies might yield insight into the properties of the SH groups responsible for the activation and inhibition processes. Figure 1 shows that preincubation of cilia at 25°C with 625 μM NEM at pH 6.9 caused about a 1.4-fold enhancement of ATPase within 2–3 min followed by a very slow decline in ATPase activity. At pH 7.5 the increase in activity occurs somewhat faster, and the activity declines more rapidly than at pH 6.9. At pH 8.1 the enhancement appeared similar to that at pH 7.5, but inhibition sets in somewhat earlier and proceeds more rapidly. At pH 8.6 one observes either no activation or, as in this experiment, a slight activation followed by a rapid inhibition, which then proceeds very slowly so that ATPase activity remains near control values. It should be noted that the effect of pH on ciliary ATPase in the absence of NEM is in general to increase the ATPase activity up to about 1.3-fold at pH 8.6 (Fig 1).

Figure 1 also presents data on the pH dependence of the enhancement of ATPase activity for three divalent SH reagents which differ in hydrophobic properties, in number of charged groups, and in the distance between the two reactive centers. In the experiments with FNS and PPDM these reagents were dissolved in acetone and 0.02 ml was used per assay (final volume, 1 ml). The solid symbols show the enhancement of ATPase activity at each pH value during preincubation with acetone alone for 30 min (the maximum time of preincubation with FNS or PPDM), as reported in detail elsewhere [6]. Preincubation with 69 μM FNS caused an enhancement of ATPase above that due to preincubation with acetone alone, and no indication of an inhibitory effect was observed. PPDM behaved similarly to FNS in that the rate of enhancement of ATPase activity increased with increasing pH, but at high concentrations of PPDM (eg, 37μM as in Fig 1), strong inhibition was obtained. The enhancing effect of CPDS was also larger at pH 8.6 than at pH 6.9 (Fig 1).

Effects of SH Reagents on 30S and 14S Dyneins at 25°C

Studies similar to those presented above on axonemes are shown in Figure 2 for both 14S and 30S dynein. At the concentrations of acetone used (in the experiments with PPDM and FNS) there was practically no effect of the acetone on the ATPase activities of either dynein. With 14S dynein for each of the five SH reagents examined only inhibition was observed, and in each case the rate of loss of ATPase was faster at pH 8.6 than at pH 6.9. PPDM, NEM, SLM, and CPDS affected 14S dynein at concentrations comparable to those effective for studies on axonemes, but FNS had very little effect on 14S dynein even at rather high concentrations.

The effect of SLM on 30S dynein ATPase is similar to its effect on axonemal ATPase (cf Fig 1 with Fig 2). This is not so with NEM. In whole axonemes, as shown in Figure 1, enhancement was followed by partial inhibition within 2 min of preincubation. With 30S dynein, only enhancement was observed in the time period examined, the degree of enhancement increasing markedly with increasing pH.

Effects of Sulfhydryl Reagents on the ATPase Activity of Demembranated Axonemes at 0°C

In the experiments so far described, cilia and dyneins were preincubated with SH reagents for relatively short times (up to 30 min) at 25°C and then assayed for ATPase activity, and most of these experiments were done at a single concentration of reagent. To obtain a more quantitative description of the effects of these reagents on ciliary ATPase, a series of experiments was undertaken in which demembranated axonemes, solubilized

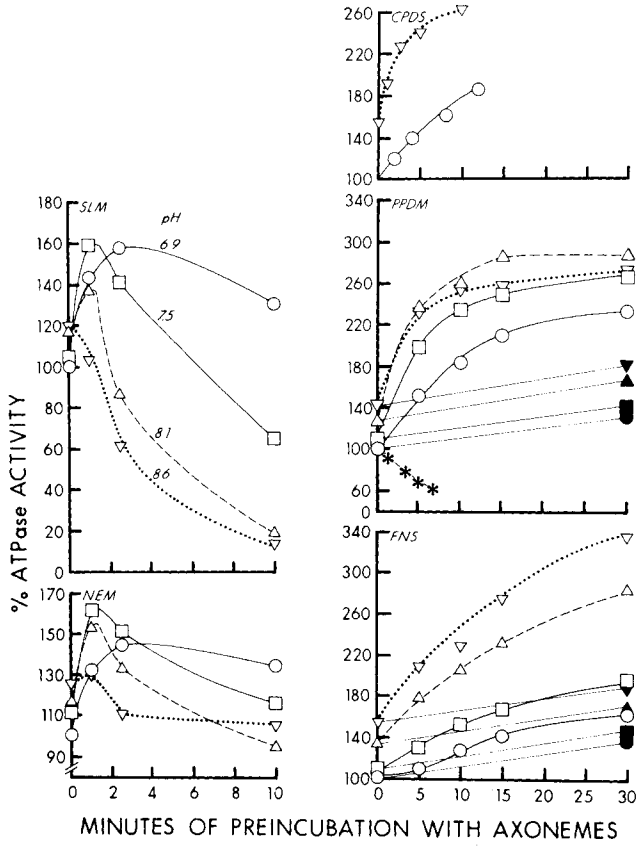

Fig 1. Effect of SH reagents on the ATPase activity of demembranated axonemes as a function of pH at 25°C. Cilia were preincubated with SH reagents at 25° for the times indicated on the abscissa in buffers of pH 6.9 (○—○), 7.5 (□—□), 8.1 (△- - -△), and 8.6 (▽• • •▽) as described in Materials and Methods. In the experiments with SLM and NEM, the preincubation volume was 0.8 ml, and the reaction was terminated by the addition of 0.1 ml 10 mM DTT, followed by 0.1 ml 10 mM ATP. For FNS, CPDS, and PPDM the preincubation volume was 0.9 ml and preincubation was terminated by addition of the ATP. In each experiment, ATP hydrolysis was allowed to proceed for 12 min before addition of trichloracetic acid and determination of phosphate released. The concentration of SH reagent during preincubation, the amount of ciliary protein per assay, and the ATPase activity at pH 6.9 (= 100% in each experiment) were, respectively: 625 μM NEM, 0.114 mg protein, 200 nmoles/min•mg; 625 μM SLM, 0.126 mg protein, 143 nmoles/min•mg; 69.4 μM FNS, 0.208 mg protein, 115 nmoles/min•mg; 2.22 μM PPDM, 0.208 mg protein, 123 nmoles/min•mg; 11.1 μM CPDS, 0.103 mg protein, 121 nmoles/min•mg. Controls for the experiments with FNS and PPDM contained 0.02 ml acetone. The effect of preincubation of the axonemes for 30 min with 0.02 ml acetone is shown for these two experiments by lines drawn to the solid symbols (●, pH 6.9; ■, pH 7.5; ▲, pH 8.1; ▼, pH 8.6). In the experiment with PPDM, * - - - * shows data from the same preparation of cilia but with 37.0 μM PPDM during preincubation at pH 8.1.

30S and 14S dynein, and axonemes that had been extracted twice with Tris-EDTA to remove all extractable dynein were preincubated at 0°C with SH reagents for varying times and concentrations.

Part A of Table I shows the effect of five SH reagents on the rate of enhancement of axonemal ATPase. To clarify the meaning of the numbers presented in Table I, we shall

describe the experiments for NEM in some detail. At each of the pH values shown, axonemes were preincubated with NEM at concentrations ranging from 25 to 250 μM and times ranging from 0.3 to 10 min, such that initial slopes of the rise in activity (units/min) could be obtained. These slopes were then plotted against the concentration of NEM for each pH value. For NEM the enhancement reaction was proportional to NEM concentration up to about 800 μM at pH 6.9 and 100 μM at pH 8.6. The best line was drawn through the points; the slopes of these lines, in units/min·μM, are reported in Table I. Since

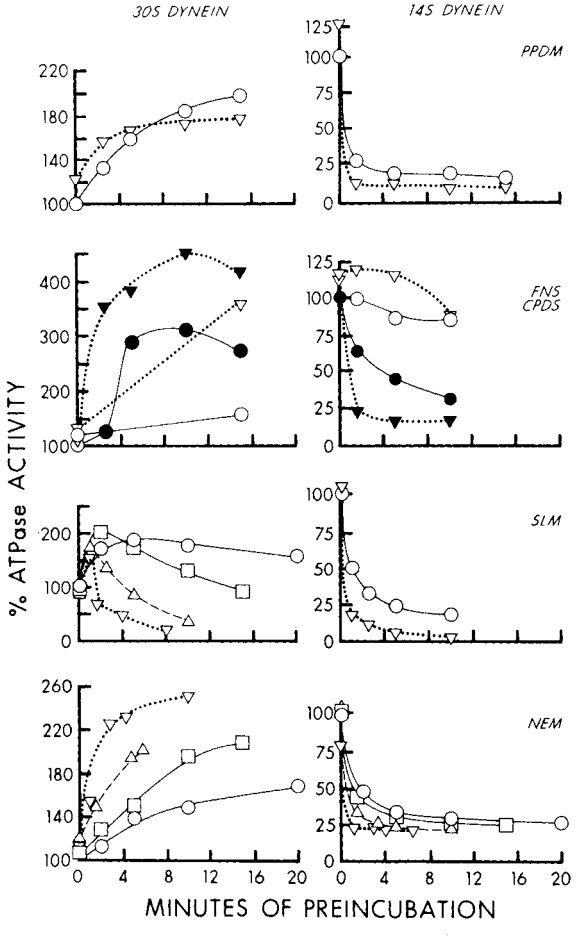

Fig 2. Effect of SH reagents on the ATPase activity of 30S and 14S dyneins as a function of pH at 25°C. Dyneins were preincubated with the indicated SH reagents at 25° for the times shown on the abscissa in buffers of pH 6.9 (○—○), 7.5 (□—□), 8.1 (△- - -△), and 8.6 (▽• • •▽). Addition of DTT and of ATP was as described in the legend to Figure 1. The duration of ATPase assay ranged from 15 to 20 min. For each experiment 100% ATPase activity is taken at pH 6.9. The concentration of SH reagents in the experiments with 30S and 14S dyneins, respectively, were: NEM, 625 μM, 625 μM; SLM, 625 μM, 312 μM; FNS, 62.5 μM, 139 μM; PPDM, 11.1 μM, 11.1 μM; CPDS, 111 μM, 27.7 μM. In the experiments with FNS, 0.02 ml and 0.05 ml acetone were used for the 30S and 14S dynein studies, respectively, and in those with PPDM, 0.01 ml was used. These quantities of acetone caused only a slight change in ATPase activity under these conditions. In the panels for FNS and CPDS, open symbols are for FNS (○—○, pH 6.9; ▽• • •▽ pH 8.6) and closed symbols for CPDS (●—●, pH 6.9; ▼• • •▼, pH 8.6).

TABLE I. Effect of pH on the Rates of Enhancement and of Subsequent Inhibition of ATPase Activity of Demembranated Axonemes by SH Reagents (units/min·μM)

pH	SH Reagents				
	NEM	SLM	PPDM	FNS	CPDS[a]
	A. Enhancement				
6.9	0.005	0.04	0.77	$0.07 \cdot 10^{-3}$	0.01
7.5	0.24	0.31	5.6	$0.34 \cdot 10^{-3}$	0.02
8.1	0.67	0.72	16.0	$1.7 \cdot 10^{-3}$	0.03
8.6	2.2	1.8	28.0	$7.2 \cdot 10^{-3}$	0.04
	B. Subsequent inhibition				
6.9	$0.30 \cdot 10^{-3}$	$0.64 \cdot 10^{-3}$	0.015	b	b
7.5	$2.3 \cdot 10^{-3}$	$4.8 \cdot 10^{-3}$	0.11		
8.1	$9.2 \cdot 10^{-3}$	$7.8 \cdot 10^{-3}$	0.22		
8.6	$16.0 \cdot 10^{-3}$	$27.0 \cdot 10^{-3}$	0.45		

[a]The data for CPDS are semiquantitative at best.
[b]Too slow for reliable assay; see text.
Axonemes were preincubated at 0° and the pH values shown for times suitable for obtaining several points during the enhancement phase (part A) and, in separate experiments, during the subsequent inhibition after the peak of ATPase activity had been obtained (part B). The range of concentrations used for the enhancement studies in part A were: NEM and SLM, 25–250 μM; PPDM, 1.25–12.5 μM; FNS, 78 and 156 μM only; CPDS 156 and 313 μM only. The range of concentrations used for the inhibition studies in part B were: NEM and SLM, 125–1000 μM; PPDM, 31.2 and 62.5 μM only.

different cilia preparations frequently had to be used to get enough different concentrations and times even for one reagent, the data in Table I should not be regarded as more accurate than within a factor of two. Thus the rate constants for activation (which will be referred to as k_a) for SLM and NEM (Table I, part A) are not different, to within the error inherent in this data. It is clear that for both of these monovalent maleimides, k_a increases in rough proportion to the increase in hydroxyl ion concentration. As expected from the observations made at 25°C (see Fig 1), where PPDM caused enhancement at lower concentrations than did NEM or SLM, k_a for PPDM was about 20 times higher than for SLM or NEM at each pH.

FNS reacts very slowly with the SH group(s) causing enhancement – about 500 times slower than NEM or SLM – but its rate of reaction also increases with increasing pH in rough proportion to the increase in hydroxyl ion concentration.

The data collected for CPDS were semiquantitative at best. Within the limits of reliability of these data, it was clear that CPDS enhances axonemal ATPase at pH 6.9 at a rate comparable to SLM and NEM (Table I). The increase in k_a with pH, however, appeared to be much smaller for CPDS than for any of the other four reagents studied.

As shown in Figure 1 for axonemes at 25°C, inhibition of ATPase activity follows the initial rise. For SLM and PPDM at 0°C, this inhibition continues until most of the activity is lost, and there is a sufficiently long interval when the ATPase declines more or less linearly to get a fair estimate of the rate of decline just after peak activity is reached. With NEM, however, the activity declines only towards the original control value, and estimation of the initial rate of of decline is somewhat subjective. Given the uncertainty in the estimates of the rate of loss of ATPase from the peak level (which we shall refer to as k_L), the data in part B of Table I indicate no appreciable difference between the k_L

for NEM and SLM, but these k_L values are about 100 times lower than the k_a values for these compounds. Similarly, the k_L for PPDM is about 50 times lower than the k_a for PPDM. For each of these maleimide derivatives, k_L increased about 50-fold with increasing pH from 6.9 to 8.6, so that the rate of loss of activity from the peak value also increases roughly in proportion to hydroxyl ion concentration.

A number of experiments were performed at high concentrations of CPDS (~ 1 mM) and FNS (~ 0.2 mM) in an attempt to measure k_L for these compounds at 0°C. It was found, however, that such experiments required many hours for appreciable losses of activity to occur, and no reliable estimates of k_L were obtained.

The data presented for PPDM in part B of Table I cover a limited range of PPDM concentrations (31–62 μM). We initially intended to investigate a much higher range of PPDM concentrations, but it was found that as the concentration of PPDM was increased above 65 μM, the rate of loss of activity *decreased*. This was observed in several axonemal preparations and with 30S dynein as well. It was also noted that at very high concentrations of FNS (> 300 μM) the rate of enhancement of ATPase activity decreased with increasing concentration. We have not investigated these phenomena further.

Given this information concerning k_a and k_L at 0°C, it was of interest to perform overnight titrations with various concentrations of each reagent. It can be seen (Fig 3) that NEM caused an enhancement of ATPase activity that was already maximal at 11 μM (5.6 moles/10^5 g axonemal protein). This is comparable to the amount reported by Shimizu and Kimura [3] to be required for maximal activation of 30S dynein (50 μM; 23 moles/10^5 g), assuming that dynein comprises 10% of axonemal protein [17]. With

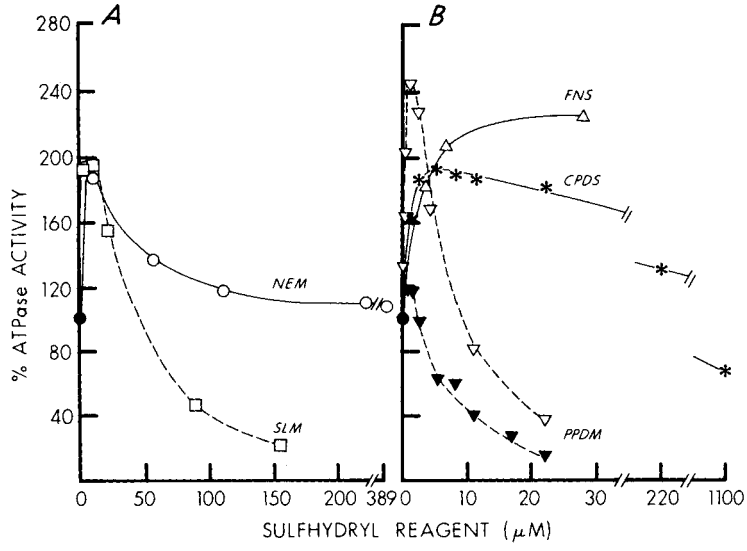

Fig 3. Effect of SH reagents on ATPase activity of demembranated axonemes at 0°C. Cilia were pre-incubated at 0° in 0.9 ml buffer at pH 7.5 for about 18 h. The tubes were then placed at 25° for about 3 min and 0.1 ml of 10 mM ATP was added and hydrolysis was allowed to proceed for 10 min. One experiment is also shown in which 30S dynein was incubated at 0° for 18 h with PPDM and then assayed at 25° as for cilia (▼- - -▼). For the experiments with cilia, 100% ATPase activity and the amount of protein per assay were, respectively: NEM, SLM, FNS, 130 nmoles/min•mg, 0.176 mg protein; CPDS 152 nmoles/min•mg, 0.156 mg protein; PPDM 88.4 nmoles/min•mg, 0.238 mg protein. For experiments with 30S dynein, its ATPase activity was 170 nmoles/min·ml.

PPDM, half-maximal activation was obtained at about 0.5 μM PPDM, corresponding in two experiments with different cilia preparations to 0.18 and 0.19 moles/10^5 g axonemal protein. If the only protein that reacted was 30S dynein, then up to 1.9 SH groups per 10^5 g dynein were responsible for the half-maximal activation, or, assuming a molecular weight of $\sim 7 \cdot 10^5$ [18], about 13 SH groups per mole of dynein in situ. To the extent that SH groups on other axonemal proteins had reacted [3, 5, 6], the number is correspondingly lower.

Figure 3 also shows the results of an experiment in which 30S dynein was incubated with PPDM for 18 h at 0°C. The ability of PPDM to cause enhancement was greatly reduced in the solubilized 30S dynein as compared to dynein in situ, although there was little if any change in the concentration dependence of ATPase inhibition. We have reported elsewhere [7] that incubation of 30S dynein with SLM at 0°C for 18 h caused only an inhibition of ATPase activity. This is quite different from the biphasic effect seen with axonemes at 0°C in Figure 3. Thus dynein in situ is more susceptible to the enhancing effects of low concentrations of PPDM and of SLM than is solubilized 30S dynein.

Incubation of axonemes with FNS for 18 h at 0°C caused only an enhancement of ATPase, even at concentrations up to 140 μM (data not shown), but full activation required much higher concentrations than required for SLM, NEM, or PPDM. CPDS also enhanced the ATPase activity of axonemes at 0°C maximally at fairly low concentrations (~ 5 μM), and at high concentrations caused a slow reduction in activity.

Effect of Sulfhydryl Reagents on the ATPase Activity of 30S Dynein at 0°C

Experiments similar to those just described for axonemes were also performed on 30S and 14S dyneins. Part A of Table II shows the rates of enhancement, k_a, for NEM, PPDM, and FNS at each of the pH values studied. For NEM, k_a increased about 20-fold between pH 6.9 and 8.6, which may not be significantly less of an increase than observed for the k_a of NEM on axonemes. The k_a values for PPDM increased with pH roughly in proportion to the increase in OH^- concentration, but k_a for FNS increased only fourfold with increasing pH (Table II, part A). For CPDS not enough data were obtained to yield values for k_a. The rate of enhancement at 0.625 mM CPDS increased only threefold with increasing pH.

Table II, part B, shows the rates of loss of activity of 30S dynein from its maximally enhanced value at one or two concentrations of NEM, SLM, and PPDM. As with demembranated axonemes, NEM did not cause a complete loss of activity at the times (< 30 h) and concentrations (< 0.8 mM) tested. In the experiments shown, 30S dynein was preincubated with 100 or 250 μM NEM at 0°C for about 17 h, and the rate of loss of ATPase activity was measured during the next 5 h. There was a slow loss of activity during this time and the rate of loss increased with increasing pH. This does not contradict the results of Shimizu and Kimura [3], since in an overnight incubation with 0.25 mM NEM they also obtained about the control levels of ATPase activity. Concentrations as high as 10^{-2} M were required to completely inhibit the activity of 30S dynein. Thus the inhibitory phase of the 30S dynein curve they obtained was probably not a true titration curve.

Table II, part B, shows that SLM caused a much faster loss of activity after the peak enhancement had been obtained than did NEM, and, as with whole axonemes, SLM caused an almost complete inhibition.

PPDM also caused a complete inhibition of 30S dynein activity after the peak had been achieved, and was several times more potent than SLM. As with whole axonemes, neither FNS nor CPDS caused an appreciable loss of the fully enhanced activity of 30S dynein at the times and concentrations used.

TABLE II. Effect of pH on the Rates of Enhancement and Subsequent Inhibition of the ATPase Activity of 30S Dynein by SH Reagents

pH	NEM (units/min·μM)	PPDM (units/min·μM)	FNS (units/min·μM)	CPDS (units/min at 625 μM)
		A. Enhancement		
6.9	$7.8 \cdot 10^{-3}$	22	0.08	57
7.5	$18 \cdot 10^{-3}$	55	0.08	76
8.1	$70 \cdot 10^{-3}$	150	0.14	96
8.6	$140 \cdot 10^{-3}$	244	0.32	136

	B. Subsequent inhibition					
	NEM, units/min at		SLM, units/min at		PPDM, units/min at	
pH	100 μM	250 μM	250 μM	500 μM	62.5 μM	
6.9			0.58	2.3	1.1	1.1
7.5	0.14	0.17	4.8	7.2	22	16
8.1			23	21	87	41
8.6	0.65	0.58	38	68	103	85

Experiments were performed as described in the legend to Table I except that solubilized 30S dynein was used instead of axonemes. In part A the data for FNS, NEM, and PPDM are given as in Table 1, i.e. in units ATPase activity/min·μM. The concentration ranges studied were 0 to 78 μM for FNS, 0 to 500 μM for NEM, and 0 to 1.25 μM for PPDM. For CPDS in part A and for all the studies in part B the rate of enhancement or the rate of loss of ATPase activity is given in units ATPase activity/min at the specified concentration of SH reagent. The rates of loss of 30S dynein ATPase with FNS and CPDS were too slow for reliable assay. Rates of loss of ATPase activity at 62.5μM PPDM are shown for two preparations of 30S dynein to give an indication of variability.

It was of interest to compare the rates of enhancement of the ATPase activity of solubilized 30S dynein with those of 30S dynein in situ (ie, whole axonemes). Since 30S dynein comprises about 10% of axonemal protein [17], the k_a values in Table I, part A, were multiplied by 10, as shown in Table III. These are then the k_a values that would have been computed for the axonemes on a per mg of 30S dynein basis. Table III shows that 30S dynein in situ reacted between 6–100 times faster with NEM than did soluble 30S dynein. The rate at which enhancement occurred with PPDM was roughly equal for 30S dynein in situ and the solubilized 30S dynein, while the rate at which enhancement occurred was between 5–140 times faster for solubilized 30S dynein than for dynein in situ with FNS.

A similar computation was made for the rates of loss of activity of 30S dynein in situ as compared to solubilized 30S dynein. The rates of loss were compared at 250 μM NEM, 250 μM SLM, and 62.5 μM PPDM. For NEM, the rate of loss of ATPase activity for dynein in situ was 30–70 times faster than the rate of loss observed for the solubilized 30S dynein, while for SLM and PPDM the rates of loss of the solubilized 30S dynein were comparable to those of the dynein in situ (data not shown).

Effect of Sulfhydryl Reagents on the ATPase Activity of 14S Dynein at 0°C

NEM, SLM, and PPDM only inhibit the ATPase activity of 14S dynein, and in each case the inhibition increased about 20 times as the pH increases from 6.9 to 8.6 (Table IV). The rates of inhibition caused by NEM and by SLM were comparable, but PPDM was about five times more potent in causing inhibition than was NEM. CPDS caused a variable inhibition of activity (data not shown).

TABLE III. Comparison of Rates of Enhancement of ATPase Activity of 30S Dynein in situ With Solubilized 30S Dynein

pH	Reagent	k_a (units/min·μM) 30S dynein in situ	k_a (units/min·μM) Soluble 30S dynein	k_a (in situ) / k_a(solubilized)
	NEM	0.05	0.0078	6.0
6.9	PPDM	7.7	22.0	0.4
	FNS	0.0007	0.08	0.007
	NEM	2.4	0.018	130.0
7.5	PPDM	56.0	55.0	1.0
	FNS	0.0034	0.08	0.04
	NEM	6.7	0.070	100.0
8.1	PPDM	160.0	150.0	1.0
	FNS	0.017	0.14	0.1
	NEM	22.0	0.14	160.0
8.6	PPDM	280.0	244.0	1.0
	FNS	0.072	0.32	0.2

The values for k_a for 30S dynein in situ were computed by multiplying the values given in Table I, part A, for whole axonemes by 10, on the assumption that 30S dynein comprises about 10% of axonemal protein. The values for k_a for solubilized 30S dynein are taken from Table 2, part A.

TABLE IV. Effect of pH on Rate of Inhibition of 14S Dynein ATPase by SH Reagents

pH	NEM (units/min·μM)	SLM (units/min) at 62.5 μM	SLM (units/min) at 125 μM	PPDM (units/min·μM)
6.9	0.60	44	74	4.4
7.5	3.4	124	300	16
8.1	10.0	140		48
8.6	25.0	448	1890	91

Experiments were performed as described in the legend to Table I except that 14S dynein was used instead of axonemes. The concentration range used for the studies with NEM was 0–125 μM and for PPDM, 0 to 5 μM.

Our initial experiments with FNS yielded highly puzzling results. Under very similar conditions, results as different as no effect and a large enhancement of activity were obtained. It was found that the results obtained depended on the time between addition of DTT to "stop" the reaction and the addition of ATP. It appeared likely, therefore, that a disulfide exchange reaction was occurring. To avoid this complexity, a series of experiments were performed in which 14S dynein was preincubated with FNS at 0°C for various times and then ATP was added and the ATPase activity assayed at 25°C as usual. The results of such an experiment at two concentrations of FNS are shown in Figure 4. It can be seen that there is about a 1.5-fold increase in the activity of 14S dynein during the first 20 min of preincubation and the activity then drops below the original level. This biphasic response pattern was scarcely affected by changing the FNS concentration from 39 to 78 μM. Since 30S dynein activity was increased by exposure to FNS, we considered

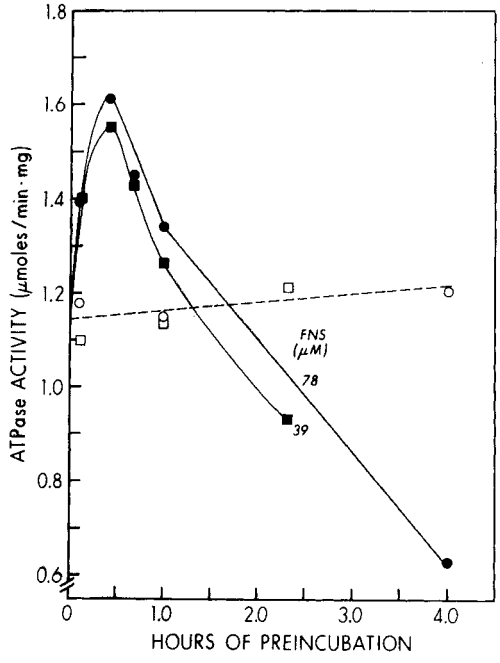

Fig 4. Effect of FNS on the ATPase activity of 14S dynein at 0°C. 14S dynein was preincubated at 0° and pH 8.6 for the times shown on the abscissa with 39 μM (■—■) or 78 μM (●—●) FNS. Controls were preincubated with 0.02 ml acetone (○---○, □---□) in a total volume of 0.9 ml. At the indicated times 0.1 ml of 10 mM ATP was added and the tubes transferred to 25° for ATPase assay (20 min). Each tube contained 7.1 μg protein.

the possibility that the increase in activity was due only to the effect of FNS on some contaminating 30S dynein, while the fall in activity was due to the effect of FNS on 14S dynein. This was unlikely for several reasons. First, examination of the pattern of ATPase activity in the gradient from which the 14S fractions were taken and pooled indicated that the maximum possible contamination of 14S fractions with 30S dynein was less than 10%, and this would not account for the 1.5-fold rise in activity found. Second, all the experiments with 30S dynein and FNS had been done with DTT addition prior to the ATP, and no indication of any fast exchange reaction had been found. Third, studies with SLM on the 14S and 30S fractions from this gradient showed that concentrations which would cause a large activation of the 30S dynein would cause, in the same time, only the expected large inhibition of the 14S dynein.

Effect of ATP and AMP-PCP on the Enhancement and Subsequent Inhibition of Axonemal ATPase Activity by PPDM at 0°C

In an earlier study [1], we showed that the presence of ATP protected against the inhibition of the pellet height swelling response of axonemes incubated with p-chloromercuribenzoate and with NEM. It was later observed that the presence of ATP partially prevented the inhibition of 30S dynein ATPase activity at 25°C by high concentrations of SLM so that enhancement could be observed even at the high SLM concentration [7]. Shimizu and Kimura [19] reported that ATP prevented enhancement of 30S dynein

ATPase activity by NEM. More recently, Shimizu et al [20] showed that ATP protects 30S dynein from the enhancing effect of PCMPS. Since PPDM appeared to be much more selective for the SH groups involved in causing enhancement of 30S dynein (both in situ and solubilized) than NEM (see Tables I and II, and Fig 3), it was of interest to ascertain whether the presence of ATP would protect the SH group(s) responsible for enhancement by PPDM. Panel A of Figure 5 shows the results of an experiment in which 1.25 μM PPDM was preincubated with axonemes at pH 7.5 in the presence and absence of ATP. Clearly, ATP prevented the enhancement of ATPase activity, but permitted an inhibition to occur. Panel B of Figure 5 shows the results of a similar experiment conducted on the same batch of axonemes at pH 8.6 and with a 100-fold higher concentration of PPDM. In the absence of ATP there was the expected very rapid enhancement of ATPase, followed by a slower inhibition. (As mentioned earlier, at such high concentrations of PPDM, the rate of the loss phase is very slow.) If ATP was present during the preincubation, only a slow inhibition of the ATPase was observed. It should be noted that the initial rate of loss of ATPase activity in panel B of Figure 5 was slower than the rate of loss in panel A, despite the 100-fold increase in PPDM concentration and the increase in pH. Thus the presence of ATP during preincubation with PPDM not only prevents the enhancement of axonemal ATPase but also reduces the rate of loss of ATPase activity below that of the abnormally slow rate observed in the presence of high PPDM concentrations.

Effect of Sulfhydryl Reagents on the Residual ATPase of Twice-Extracted Axonemes at 0°C

We have shown elsewhere [13] that after two extractions with Tris-EDTA up to 40% of the original ATPase activity remains in the pellet; we refer to this as the residual ATPase. Reaction of twice-extracted axonemes with very high concentrations of NEM at

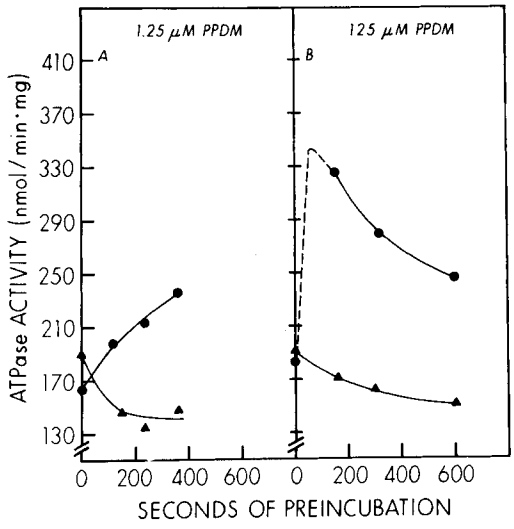

Fig 5. Effect of ATP on the enhancement of axonemal ATPase by PPDM at 0°C. Axonemes were preincubated at 0° with 1.25 μM PPDM at pH 7.5 (panel A) or 125 μM PPDM at pH 8.6 (panel B) for the times shown on the abscissa. Control tubes had 0.02 ml acetone. ●—●, no ATP present during preincubation; ▲—▲, 0.63 mM ATP present during preincubation. At the indicated times 0.1 ml of 10 mM DTT was added to each tube and then 0.1 ml of 10 mM ATP was added and the tubes were transferred to 25° for ATPase assay as described in Materials and Methods. The amounts of axonemal protein per assay were 68.4 and 68.4 μg in the experiments of panels A and B, respectively.

pH 7.5 and 25°C caused a rapid loss of about 40% of the residual ATPase activity, followed by a very slow further decline [1]. In view of our results on the effects of SH reagents on unextracted axonemes and on 14S and 30S dyneins, it was of interest to examine the effects of these reagents on the residual ATPase as a function of pH at 0°C (Table V). In each case we found an initial rapid decrease of 20–40% of the initial residual ATPase activity, followed by a slow further loss of activity. With increasing pH there was a tendency towards a slightly faster loss of residual ATPase activity, but the effect of pH was very much less than with either unextracted axonemes or with 30S or 14S dyneins. It is also evident that the residual ATPase activity decreases slightly with increasing pH (Table V), whereas the ATPase activity of unextracted axonemes and of 30S and 14S dyneins increases slightly with pH in this range.

DISCUSSION

Latency Properties of Axonemal ATPase and of Solubilized 30S Dynein ATPase

Although both myosin and dynein are involved in the generation of motility via a sliding filament mechanism, differences in molecular weight, substrate specificity, amino acid composition, and ability to interact with actin [21] do not support any evolutionary relation between these two proteins. The discovery that the 30S dynein of Tetrahymena and dynein 1 of sea urchin sperm flagella have a latent ATPase activity that can be activated by low concentrations of SH reagents, by heating, or by acetone or Triton X-100 [1, 3, 6, 22, 23] suggests, however, some similarity between the ATPase sites of the two different mechanochemical ATPases, as does the report that both myosin and 30S dynein catalyze an exchange of oxygen from water into the orthophosphate released upon hydrolysis of

TABLE V. Effect of SH Reagents on Twice-Extracted Axonemes at 0°C

Reagent	Concentration (μM)	pH	ATPase activity (nmoles/min·mg) at T = 0h	0.33 h	1h	2.5 h	3 h
NEM	250	6.9	76			64	
		7.5	69			56	
		8.1	67			46	
		8.6	65			43	
SLM	250	6.9	80				65
		8.6	63				36
PPDM	63	8.1	55	39			
		8.6	53	36			
FNS	78	6.9	70		69		
		8.6	56		42		
FNS	78	7.5	84				81
		8.6	77				53

Twice-extracted axonemes were prepared as described in Methods and stored at −20°C as described in Methods. They were then resuspended in buffer as described in Methods for axonemes, and incubated with the SH reagents for the times shown in the Table. The reactions were stopped by addition of 0.1 ml DTT and then ATP was added and the ATPase activity assayed at 25°C. ATPase activities listed in the column T = 0 were controls that had been preincubated at 0°C without SH reagents.

ATP [24]. Reaction of a single SH group (SH_1) at the active site of myosin causes enhancement of its ATPase activity (see references in Takamori, Kato, and Sekine [25]), but it is not yet known how many SH groups are involved in the enhancement of 30S dynein ATPase. Shimizu, Kaji, and Kimura [20] estimate that 2.7–5.4 moles/10^5 g are required for maximal activation of 30S dynein by PCMPS, corresponding to 19–38 moles/mole dynein if the molecular weight is taken as $7 \cdot 10^5$ daltons. Titration of whole axonemes with the more selective reagent PPDM yields a value of about 0.19 mole PPDM per 10^5 g axonemal protein for half-maximal enhancement of the latent ATPase activity. If the only groups to react were on the 30S dynein, this would correspond to about 13 SH groups per mole of dynein in situ. It is almost certain, however, that SH groups of other axonemal proteins react before the SH group(s) of the 30S dynein. Thus Cosson and Gibbons [5] report that brief treatment of sea urchin sperm with low concentrations of NEM or PPDM reduces wave amplitude without affecting the latent ATPase or frequency-restoring ability of the dynein. Titration studies with PPDM on solubilized 30S dynein will be required to obtain more realistic upper limits of the number of SH groups of type SH_1.

In the pH range investigated, k_a for solubilized 30S dynein and for axonemes increased markedly with pH for NEM, SLM, PPDM, and FNS (Tables I and II and Figs 1 and 2), though pH in this range had little effect on ATPase activity per se. This suggests that k_a increases as the SH_1 group(s) becomes more ionized. Similar behavior has been reported for the reaction of SH_1 of myosin with several analogues of NEM [25], but an increase in reactivity of SH groups towards SH reagents with increasing pH seems to be a general property of SH groups [26]. Similarity in behavior with respect to pH does not therefore imply any specific resemblance between SH_1 of myosin and SH_1 of 30S dynein.

Of particular interest are the differences in k_a values between 30S dynein in situ and after solubilization. Table III shows that k_a (in situ)/k_a (solubilized) was > 1 for NEM, ~ 1 for PPDM, and < 1 for FNS. This is consistent with earlier observations on changes in properties of 30S dynein ATPase upon extraction after heat treatment of axonemes [10]. It is likely that the difference is not due to the presence in the axoneme of a residue which simply impedes access to SH_1, since if this were the case, one would expect to find a decrease in k_a for the axonemes relative to that for the solubilized 30S dynein for each of the three SH reagents. Instead it appears that the conformation change which occurs in situ [7] alters the local environment in a more complicated way, causing k_a for NEM to increase and that for FNS to decrease relative to their respective k_a values with solubilized 30S dynein.

Blum and Hayes [4] observed that if axonemes were added to a mixture of NEM and ATP only a slow inhibition was observed, instead of the enhancement of ATPase activity which occurs during preincubation with NEM alone. This suggested that ATP prevented the reaction with NEM of those SH groups responsible for enhancement of the ATPase activity. Similar results were obtained for solubilized 30S dynein by Shimizu and co-workers, who found that the presence of ATP during preincubation protected against the enhancing effects of NEM [19] and PCMPS [20]. The present results show that ATP completely protected 30S dynein in situ against the enhancing effects of both low and higher concentrations of PPDM. It is instructive to compare these results with similar studies on myosin. When myosin in which SH_2 had been reacted with NEM was exposed to PPDM in the presence and in the absence of ATP [27], the Ca^{2+}-ATPase activity decreased more rapidly in the presence of ATP, indicating that the presence of ATP enhanced the reactivity of SH_1 of myosin. Takamori, Kato, and Sekine [25] studied the effect of NEM on myosin at pH 6.0, where SH_2 does not react in the absence of nucleotide and also found that the reactivity of SH_1 was strongly accelerated on addition of ATP or

ADP. Thus while SH_1 of myosin and SH_1 of 30S dynein resemble each other in that reaction with an SH reagent increases with increasing pH and enhances the ATPase activity, they are by no means identical. The presence of ATP accelerates the rate of reaction of SH_1 of myosin with NEM and PPDM but reduces the rate of reaction of the SH_1 group(s) of 30S dynein (solubilized or in situ) with NEM or PPDM. In view of this disparity, it would be erroneous to assume that SH_1 of dynein is entirely analogous to that of myosin.

Inhibition of Axonemal ATPase and of Solubilized 30S Dynein ATPase

Following enhancement of the latent ATPase activity by NEM and other maleimides, inhibition is observed. For myosin, it is known that the inhibition is caused by reaction of a single SH group, termed SH_2, although other groups, collectively termed SH_3, also react at sufficiently high NEM concentrations [28]. It is not known how many SH groups are responsible for the inhibitory phase of 30S dynein ATPase, but, following Shimizu and Kimura [3], we shall refer to the SH group(s) responsible for the inhibition as SH_2. Yamaguchi and Sekine [27] reported that SH_2 of myosin reacts more rapidly with NEM as the pH is increased from 6.3 to 7.9, and Reisler et al [8] reported a similar increase with pH for PPDM. The data in Tables I and II show that SH_2 of 30S dynein (solubilized or in situ) also reacts more rapidly with increasing pH for all the SH reagents studied and, as implied by the fact that the titration curve is biphasic, SH_2 reacts much more slowly than SH_1. For 30S dynein in situ, k_L for SLM, NEM, and PPDM is about 50–100 times smaller than k_a. For myosin, the ratio of the reaction rate of NEM with SH_1 to that with SH_2 is about 700:1 at pH 6.0 [25]. Since k_a for FNS (and, at least in the higher pH values, for CPDS) was much lower than the k_a for PPDM, NEM, and SLM, it is not surprising that the k_L for FNS (and CPDS) was too small to be measured.

After SH_1 of solubilized 30S dynein has reacted with SLM, the ATPase activity decreases towards zero as SH_2 reacts. This is not the case for NEM, where SH_2 of solubilized 30S dynein reacts very slowly indeed (Table II). Similarly, SLM reacts with 30S dynein in situ to cause almost complete inhibition (Fig 1 and Table I; see also Blum and Hayes [6]), while NEM causes only a relatively rapid initial decrease of ATPase activity from the peak towards the original unenhanced level. Reaction of axonemes with 250 μM NEM at 0°C and pH 8.6 caused a loss of about 4 units of ATPase per minute, compared with about 0.6 units/min for solubilized 30S dynein (cf Tables I and II). Thus even without taking into account the fact that 30S dynein comprises only about 10% of axonemal protein, it can be seen that SH_2 of 30S dynein in situ reacts more rapidly with NEM than does SH_2 in solubilized 30S dynein. The marked difference in rates of loss of ATPase activity from its peak enhanced level between NEM and SLM suggests that there are at least two classes of SH_2 groups in 30S dynein. The more sensitive of the two appears to react relatively rapidly with SLM and NEM, causing a loss of the enhanced activity. Then, at higher concentrations or longer times, SLM reacts with the second class of inhibitory groups, causing an almost complete loss of ATPase activity, while NEM reacts only very slowly.

That SH_2 of 30S dynein was not comparable to SH_2 of myosin first became apparent with the finding [10] that low concentrations of PPDM caused only an activation of the ATPase activity, in contrast to the modification of *both* SH_1 and SH_2 of myosin [8]. Further evidence indicating that SH_2 of 30S dynein was not similar to SH_2 of myosin came from the observation that neither ADP nor ATP enhanced the rate of reaction of dynein SH_2 with NEM [19], whereas it is well established that ATP (and, even more strongly, ADP) increase the rate of reaction of SH_2 of myosin with NEM [9, 25] and

PPDM [8]. Shimizu and Kimura [19] reported that the presence of ATP partially protected against the loss of ATPase activity of 30S dynein caused by high concentrations of NEM, but this depends in part on the nature of the SH reagent, since with PCMPS, ATP caused a small increase in the rate of loss of activity [20]. Our results show that very high concentrations of PPDM (125 μM) caused a decrease in the rate of loss of axonemal ATPase activity, compared to the rates of loss observed at lower concentrations of PPDM (eg, 50 μM). The reasons for this unusual behavior are at present unknown. The essential point for present purposes, however, is that the presence of ATP during incubation with high concentrations of PPDM not only prevented the enhancement of axonemal ATPase, but also reduced the rate of loss of ATPase activity (Fig 5). This is the opposite of the effects of NEM and PPDM on SH_2 of myosin. Since both SH_1 and SH_2 of dynein differ from SH_1 and SH_2 of myosin with respect to the effects of ATP on their reactivity with NEM and PPDM, it is clear that the SH groups of dynein are not closely analogous to those of myosin.

Inhibition and Enhancement of 14S Dynein ATPase Activity

Blum et al [7] showed that 14S dynein was inhibited by SLM and by NEM, and that ATP partially protects against the inhibitory effects of these reagents. Shimizu, Kaji, and Kimura [20] reported that 14S dynein was inhibited by PCMPS. The present results show that NEM, SLM, and PPDM all inhibit 14S dynein ATPase, the inhibition increasing at least 20-fold with increasing pH in the range 6.9–8.6 (Table IV). As expected from the results with 30S dynein, PPDM was effective at lower concentrations than either SLM or PPDM. In view of the seeming absence of any latent ATPase activity in 14S dynein, it was of interest to find that FNS, which reacted so slowly with the inhibitory groups of 30S dynein as to be virtually specific for the SH_1 type group(s), caused a biphasic response in 14S dynein at 0°C (Fig 4), though this was not observed at 25°C (Fig 2). A fuller understanding of the significance of the finding of a latent ATPase activity in 14S dynein must await further information on the structural organization of the proteins in the inner and outer forms of cilia, but the capacity of 14S dynein to undergo enhancement of its ATPase activity increases the likelihood that 14S dynein plays a role in ciliary mechanochemistry, either as a component of the larger 30S complex or as an independent entity.

Properties of the Residual ATPase Activity

The ATPase activity remaining in axonemes that have been twice-extracted with Tris-EDTA is almost equally active with Ca^{2+} or Mg^{2+} as the divalent cation, is insensitive to ethacrinic acid and to variations in KCl or NaCl concentrations in the range 0–0.12 M, and is relatively stable to heating at 56°C [13]. It was earlier reported that brief preincubation of twice-extracted axonemes with even very high concentrations of NEM at pH 7.5 caused the loss of only about one-third of the residual ATPase activity, though high concentrations of PCMB caused the loss of about 60% of the activity [1]. In view of the present findings that reactivity of both SH_1- and SH_2-type groups of 30S and 14S dynein increased markedly with increasing pH for several SH reagents, and that the reagents used here had differing rates of reaction with SH_1 and SH_2 depending on whether 30S dynein was in situ or solubilized, it was desirable to examine the effects of the SH reagents used in this study on twice-extracted axonemes. Our results (Table V) confirmed the earlier findings with NEM and showed that even at preincubation NEM caused only a partial inhibition of the ATPase activity and that the effectiveness of NEM increased only slightly with

increasing pH. SLM resembled NEM, while PPDM seemed to be somewhat more effective. FNS had little effect in 1 h and even a 3-h preincubation had no effect at pH 7.5. These results show that the effects of the various SH reagents used in this study on whole axonemal ATPase activity are almost entirely due to effects on the 30S dynein "in situ," thus validating use of this term to describe the results obtained from experiments with unextracted axonemes. In addition to demonstrating the insensitivity of the residual ATPase to the SH reagents employed in this study, our results also show that the pH dependence of the residual ATPase is opposite that of 30S dynein (either solubilized or in situ) and to 14S dynein. Thus in its insensitivity to SH reagents and its pH dependency the residual ATPase does not resemble dynein.

ACKNOWLEDGMENTS

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