Effect of Spin-Labeled Maleimide on 14S and 30S Dyneins in Solution and on Demembranated Ciliary Axonemes†

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ABSTRACT: The effects of *N*-(1-oxyl-2,2,6,6-tetramethyl-4-piperidinyl)maleimide (SLM) on the pellet height response and ATPase activity of glycerinated Triton X-100 extracted cilia of *Tetrahymena pyriformis* have been studied. Preincubation of cilia with SLM caused complete inhibition of the pellet height response and an initial increase in ATPase activity followed upon longer exposure to SLM by inhibition of ATPase. The effect of SLM on extracted 30S dynein was the reverse of that for whole cilia: ATPase activity was increased when 30S dynein was added to a mixture of ATP and SLM and inhibited when the 30S dynein was preincubated with SLM. The activity of 14S dynein was only inhibited by SLM. Electron spin resonance spectra of ciliary axonemes that had reacted with SLM for various times showed that much of the covalently bound SLM was strongly immobilized even after 1 min of reaction, when ATPase activity increased twofold. The proportion of strongly immobilized label increased with longer times of reaction. Addition of ATP to SLM-labeled axonemes caused a small decrease in the height of the spectral peak corresponding to strongly immobilized label as compared with that of weakly immobilized label, indicating an increase in rotational freedom of some covalently bound label. The results suggest that ATP causes a conformation change affecting a sulfhydryl group(s) involved in the mechanochemical system. It was also shown that β,γ-methylene ATP (AMP-PCP) is an inhibitor of dynein ATPase. This analogue of ATP is not hydrolyzed by whole cilia or by the extracted dyneins and does not cause a pellet height response. With Mg^{2+} as divalent cation, AMP-PCP inhibits 30S dynein more than it inhibits 14S dynein; with Ca^{2+}, the inhibition of 30S dynein is reduced, and there is no inhibition of 14S dynein. Under conditions where AMP-PCP inhibited 30S dynein ATPase it was much less effective than ATP in protecting against the loss of ATPase activity by SLM. Although SLM inhibited Mg^{2+}-activated 14S and 30S dyneins in solution, it did not inhibit ciliary ATPase activity. These results support the view that at least 2 SH groups are involved in ciliary motility and that their reactivity to SH reagents depends on whether the dyneins are in situ or have been extracted.

Several reports that ciliary beating was inhibited by SH poisons led us to investigate the role of SH groups in ciliary motility. It was found (Blum and Hayes, 1974a,b) that the pellet height response of glycerinated *Tetrahymena* cilia could be inhibited by NEM[1] while the ATPase activity was slightly enhanced. NEM also prevented the rebinding of 30S dynein onto EDTA-extracted axonemes and, under certain conditions, caused an enhancement of the ATPase activity of 30S dynein, whereas the ATPase activity of 14S dynein was only inhibited. Blum and Hayes noted that the effects of NEM on whole cilia and on 30S dynein were similar in several ways to the effects of NEM on myosin, and this analogy was independently developed by Shimizu and Kimura (1974). From this work it appears that there are at least two and probably three SH groups that are involved in the mechanochemistry of 30S dynein. It is generally thought that the 30S dynein, which can rebind to EDTA-extracted axonemes in the presence of Mg^{2+} (Gibbons, 1965a,b) thereby restoring the arms on the A subfibers of the outer axonemal doublets, is the physiologically active mechanochemical ATPase of cilia, and that the 14S dynein is simply the monomeric subunit of the 30S dynein (Gibbons and Rowe, 1965). Recent evidence, however, indicates that the globular subunits of 30S dynein differ slightly in molecular weight from 14S dynein (Mabuchi and Shimizu, 1974) and these two ATPases also differ in a number of kinetic properties (Gibbons, 1966; Raff and Blum, 1969a; Blum, 1972; Shimizu and Kimura, 1974; Hoshino, 1974). Although there is general agreement that the arms attached to the A subfibers of the outer doublets act as cross bridges which attach to the B subfibers of adjacent doublets and generate the sliding motion of one doublet relative to its neighbor (Summers and Gibbons, 1973), it had been thought that the shear bearing links, which are necessary for the conversion of the sliding motion into that of bending (see, e.g., Blum and Lubliner, 1973) were passive links, perhaps localized in the radial link system originating on the A subfiber of each outer doublet and extending to contact the central pair of microtubules. Electron microscopic studies by Warner and Satir (1974), however, indicate that the radial link system appears to form shear-resistant links at the leading edge of a propagating bend and to break these links at the trailing edge of the bend. These observations plus some cytochemical observations cited by Warner and Satir (1974) lead them to propose that the radial link system contained an ATPase. In view of the evidence cited above, a possible candidate for such an ATPase is the 14S dynein.

To obtain further information on the role of SH groups in ciliary motility, it seemed worthwhile to use a spin-labeled analogue of NEM such as *N*-(1-oxyl-2,2,6,6-tetramethyl-4-piperidinyl)maleimide, which, under some circumstances reacts selectively with SH_2 of myosin and has been useful as

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[1] Abbreviations used: NEM, *N*-ethylmaleimide; SLM, *N*-(1-oxyl-2,2,6,6-tetramethyl-4-piperidinyl)maleimide; AMP-PCP, β,γ-methylene ATP; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; 1M 7.5 buffer, 20 mM imidazole buffer (pH 7.5).

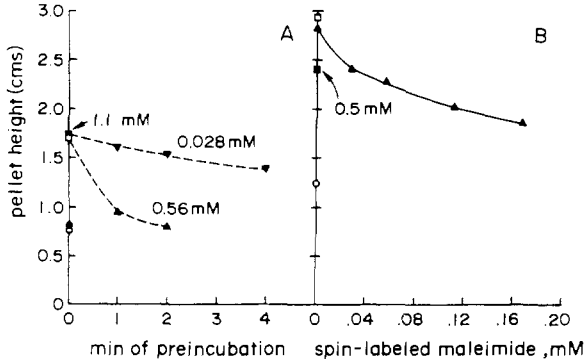

FIGURE 1: Effect of spin-labeled maleimide on the pellet height response of glycerinated cilia. Cilia were prepared as described in Materials and Methods. Circles show the height of the pellet obtained after 2.5 min centrifugation in the absence of ATP; (open circles) no SLM; (filled circle) (part A), 1.1 mM SLM. Square symbols show the height of the pellet obtained in the presence of ATP; (open square) no SLM; (filled square) 1.1 mM SLM (part A) or 0.5 mM SLM (part B). Triangular symbols show the pellet height when cilia were preincubated with the indicated concentrations of SLM for the times shown on the abscissa (part A) or when cilia were preincubated for 1 min at the concentrations of SLM shown on the abscissa (part B). All components of the reaction were in IM 7.5 buffer, and the experiments were done at room temperature. A different preparation of cilia was used for the experiments shown in part A as compared with those of part B. All pellet heights have been normalized to a height of 10.0 cm for the liquid in the hematocrit tube.

a probe for the study of conformational changes in the neighborhood of the active sites of myosin and heavy meromyosin (Stone and Prevost, 1973; Seidel et al., 1970; Seidel, 1972). Prior to any studies entailing the use of spin resonance techniques on labeled dyneins, it was necessary to investigate the effects of introducing SLM into the 14S and 30S dyneins and into glycerinated cilia. The changes observed suggested that SLM was a useful tool for studying the role of SH groups in ciliary motility independently of its potential value for spin resonance studies, and one which differed from NEM in several ways.

In addition to the studies on the effect of SLM on the ATPase activity of extracted 14S and 30S dyneins and of whole demembranated cilia, we also report here the results of electron paramagnetic resonance studies of SLM-labeled axonemes. A spectrum characteristic of both strongly immobilized and weakly immobilized nitroxide groups is obtained. The intensity of the peak corresponding to the strongly immobilized groups exceeds that of the weakly immobilized groups at each stage of reaction of the SLM with the cilia, i.e., during activation and during the subsequent inhibition of ATPase activity. Upon addition of ATP to the spin-labeled cilia, a small decrease in the ratio of strongly immobilized to weakly immobilized groups occurs, indicating a change in the environment of the spin-labeled SH groups.

Materials and Methods

Tetrahymena pyriformis, strain HSM, were grown and cilia were isolated by a modification of the glycerol method of Gibbons as described previously (Blum, 1973). Treatment of the cilia with 0.1% (w/v) Triton X-100 was as before (Blum and Hayes, 1974b). The Triton X-100 extracted axonemes were resuspended in 20 mM imidazole, pH 7.5, and dialyzed at 4 °C against a total of 2 l. of Tris-EDTA (1 mM Tris–0.1 mM EDTA, pH 8.2) containing 0.5 mM dithiothreitol, when specified. After at least 16 h of dialysis, the suspension was centrifuged for 30 min at 27 000*g* and the supernatant collected and recentrifuged for 15 min at 27 000*g* to remove any traces of axonemes. The crude dynein was then purified by sucrose density sedimentation as described elsewhere (Blum, 1973) except that, when specified, 0.5 mM dithiothreitol was added to the sucrose solutions.

The spin-labeled maleimide, *N*-(1-oxyl-2,2,6,6-tetramethyl-4-piperidinyl)maleimide, SLM, was synthesized by the method of Seidel et al. (1970) or purchased from Syva Associates (Palo Alto, Calif.) and stored in ice after being dissolved in buffer. Protein was assayed by the method of Lowry et al. (1951) or by the fluorescamine method of Udenfriend et al. (1972), using bovine serum albumin as the standard. Fluorescamine was prepared before each use and the standards were assayed under identical ionic conditions as the dynein. Orthophosphate was measured by a semimicromodification of the method of Taussky and Shorr (1953). All assays were run in duplicate; duplicates rarely differed by more than 5%. The pellet height response was measured as described before (Blum and Hayes, 1974a).

For studies of the electron paramagnetic resonance spectra, the Triton X-100 extracted cilia were washed twice with IM 7.5 buffer and resuspended in 2.9 ml of IM 7.5 buffer. Aliquots of 0.4 ml were added to 0.1 ml of 5 mM SLM and incubated at 25 °C for various times up to 12 min. The reaction was stopped by adding 0.1 ml of 10 mM dithiothreitol and the tubes were then placed in ice. All further processing was at 0 °C. Two samples of 0.05 ml were taken from each tube for ATPase assay, about 2 ml of IM 7.5 was added, and the mixture was centrifuged at 20 000*g* for 7 min. The supernatant was decanted and the washing repeated three more times to completely remove all traces of SLM that were not covalently attached to the axonemal pellet. The final pellet was resuspended in 0.3 ml of IM 7.5 buffer. About half of this was placed in a flat cell and its electron paramagnetic spectrum was measured. The effect of ATP was then ascertained by adding 0.05 ml of 12 mM ATP (in IM 7.5 buffer) to the remaining ~0.15 ml of cilia suspension and transferring this to a clean cell. Since it took several minutes to align the cell and adjust the spectrometer, the temperature had risen to room temperature by the time the first spectrum was recorded. Spectra were recorded in a Varian Model E9 spectrometer using a quartz flat cell with inside dimensions of 60 × 10 × 0.25 mm. All spectra reported here were taken at a power setting of 50 mW and a modulation amplitude of 2.5 G. At these settings none of the lines was noticeably broadened due to power saturation or to over-modulation. Spectra were recorded in triplicate at about 9540 MHz with a 4-min time scan and a recorder time constant of 1.0 s.

ATP, cysteine hydrochloride, β-mercaptoethanol, and dithiothreitol were purchased from Sigma; Triton X-100 was from Scientific Chemical Co.; AMP-PCP was from PL Biochemicals. All other chemicals were reagent grade.

Results

Effect of SLM on Glycerol-Extracted Triton X-100 Demembranated Cilia. When ATP is added to glycerinated *Tetrahymena* cilia in the presence of Mg^{2+} and the cilia are briefly centrifuged, the cilia swell and the height of the pellet is larger than that of a control pellet centrifuged at the same time in the absence of ATP. It has been shown that this response is a measure of some aspect of ciliary contractility closely related to beating (Raff and Blum, 1966, 1969b). If 0.56 mM SLM is preincubated with the cilia before the addition of ATP, the pellet height response is completely inhibited within 2 min (Figure 1A); at one-twentieth of this concentration, the inhibitory reaction is much slower. The loss of the

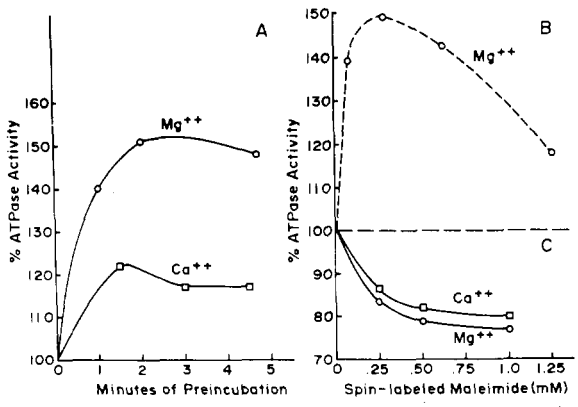

FIGURE 2: Effect of spin-labeled maleimide on the ATPase activity of glycerinated cilia. Cilia were prepared as described in Materials and Methods and used within 7 h after the Triton X-100 demembranation step. ATPase activity was assayed at 25 °C in duplicate; duplicate points fall within the size of each symbol. In part C, 0.2 ml of cilia was added to 0.8 ml of a mixture of ATP, SLM, and divalent cation in 20 mM imidazole buffer, pH 7.5, such that the final concentration of ATP was 1.0 mM, that of SLM was as shown on the abscissa, and that of Ca^{2+} or Mg^{2+} was 2.5 mM. The reaction was stopped after 10 min by the addition of 0.5 ml of 10% trichloroacetic acid and orthophosphate was assayed in an aliquot of the protein-free supernatant as described in Materials and Methods. For parts A and B of this figure, a similar procedure was followed except that the cilia were preincubated for the indicated times in 0.9 ml of imidazole buffer containing 2.8 mM divalent cation and 0.28 mM SLM (part A) and for 2 min at the concentrations shown for part B. After preincubation for the desired time, 0.1 ml of 10 mM ATP was added and the reaction allowed to proceed for 10 min. The data for parts A and C were obtained with one preparation of cilia (0.21 mg of protein per assay tube; 0.132 and 0.124 μmol min^{-1} mg^{-1} correspond to 100% ATPase activity in the presence of Mg^{2+} and Ca^{2+}, respectively). The data for part B of this graph are from a different preparation of cilia (0.13 mg of protein per assay tube; 0.22 μmol min^{-1} mg^{-1} corresponds to 100% ATPase activity).

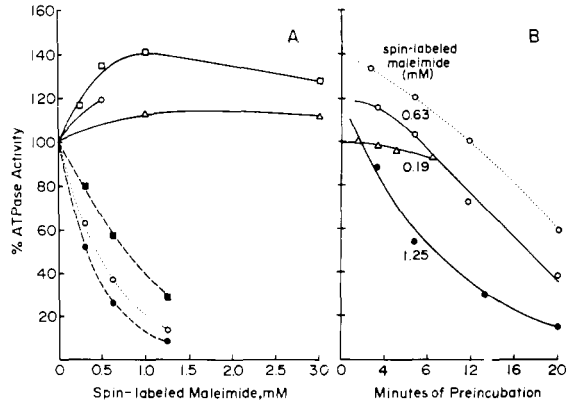

FIGURE 3: Effect of spin-labeled maleimide on the ATPase activity of 30S dynein. In the upper section of panel A (open symbols, solid lines) 30S dynein was added to a mixture of ATP and SLM in IM 7.5 buffer such that the final concentration of ATP was 1.0 mM and that of SLM as shown on the abscissa. The reaction was stopped after 20 min at 25 °C by the addition of 0.1 ml of 30% trichloroacetic acid. In the lower section of panel A and in panel B, the 30S dynein was preincubated with SLM in 0.8 ml total volume at the concentrations of SLM shown and then 0.2 ml of 5 mM ATP was added and hydrolysis allowed to proceed for 20 min. AMP-PCP, when present (O···O), was 0.31 mM during the preincubation period and 0.25 mM when ATP was present. Symbols have the following meanings: (circles) cilia preparation 112; (O—O, ●—●) assayed the same day as collected from the sucrose gradients, 100% ATPase = 0.83 μmol min^{-1} mg^{-1}; (O···O, ●- - -●) assayed 2 days later with 20 min preincubation, 100% ATPase = 1.10 and 0.64 μmol min^{-1} mg^{-1} in the presence and absence of AMP-PCP, respectively. (Triangles) Preparation 113, assayed 1 day after collection, 100% ATPase = 0.88 μmol min^{-1} mg^{-1}. (Squares) Cilia preparation 114, assayed 1 day after collection, 100% ATPase = 1.03 μmol min^{-1} mg^{-1}; (□—□) no preincubation; (■- - -■) 10 min preincubation.

pellet height response as a function of the concentration of SLM during a 1-min preincubation is shown in Figure 1B. If, however, the cilia are added to a mixture of SLM and ATP, one observes little or no effect even of high concentrations of SLM on the pellet height response. In the experiments of Figure 1A, the presence of ATP completely protected the pellet height response from the effects of 1.1 mM SLM during the ~4 min required for performance of the pellet height assay. In the experiment of Figure 1B, about 70% of the full pellet height response was obtained when 0.5 mM SLM was allowed to react in the presence of ATP for the time required for the assay, whereas less than 60% of full response was obtained if the cilia were preincubated with one-third less SLM in the absence of ATP. These two experiments represent the range of protection afforded by ATP against the inhibitory effects of high concentrations of SLM. SLM by itself has no effect on pellet height (Figure 1A).

The effect of SLM on the ATPase activity of glycerinated cilia also depends markedly on the presence of ATP (Fig. 2). If the SLM is added in the absence of ATP, then within 1 min there is an appreciable enhancement of the ATPase activity and, at 2 min, when the pellet height response is entirely inhibited (cf. Figure 1), a maximal enhancement of ATPase activity of about 1.5-fold is achieved. With further preincubation (Figure 2A) and at higher concentrations of SLM (Figure 2B), an inhibitory process becomes apparent, reducing the ATPase activity toward control level. If the cilia are added to a mixture of ATP and SLM, only the inhibitory process is observed (Figure 2C). Thus protection of the pellet height response by the presence of ATP (cf. Figure 1) corresponds to a slight inhibition of ciliary ATPase activity, while the inhibition of the pellet height response by exposure of the cilia to SLM in the absence of ATP corresponds to an enhancement of ciliary ATPase activity.

Because of evidence indicating the presence of a separate, Ca^{2+}-sensitive motile system which determines the direction of beating in both living and glycerinated models of *Paramecium* (Naitoh and Kaneko, 1973), it was of interest to inquire if either the enhancement or the inhibition of ATPase activity caused by SLM would be altered if the Mg^{2+} usually present in these studies was replaced by Ca^{2+}. The data in Figure 2 show that enhancement of ATPase activity occurs to a greater extent with Mg^{2+} than with Ca^{2+}, whereas the inhibition which occurs if ATP is present initially is about the same with either divalent cation. It should be noted that the ATPase activity of glycerinated cilia is practically unchanged by the substitution of 2.5 mM Ca^{2+} for 2.5 mM Mg^{2+}, so that the differences in enhancement which occur depending on which cation is present are not due to changes in the control activity.

Effect of SLM on ATPase Activity of 30S Dynein. When 30S dynein is added to a mixture of SLM plus ATP at 25 °C, one often observes an increase in ATPase activity with increasing concentration of SLM (Figure 3A). If the 30S dynein is preincubated with SLM in the absence of ATP, however, the ATPase activity is initially enhanced and then declines slowly with time; about 90% of the initial activity is lost upon exposure to 1 mM SLM for 20 min (Figure 3A). The time dependence of the enhancement depends on SLM concentration (Figure 3B). At 0.19 mM SLM, for example, no enhancement of ATPase activity was observed, but only a slow loss. At 0.63 mM the enhancement was already complete after 3 min of preincubation and activity then fell with further time of preincubation. At 1.25 mM SLM, the ATPase activity was

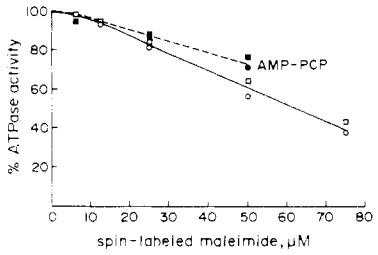

FIGURE 4: Effect of incubating spin-labeled maleimide with 30S dynein at 4 °C for 18 h. Dynein (30S) was incubated with SLM at the concentrations indicated on the abscissa at 4 °C in a total volume of 0.81 ml. AMP-PCP, when present, was 1.25 mM. After the incubation, the tubes were brought to 25 °C and 0.2 ml of 5 mM ATP in IM 7.5 buffer was added and the ATPase activity assayed. Results from two experiments are presented: (circles) cilia preparation 115, 0.013 mg of dynein per assay, 100% ATPase activity = 1.22 μmol min^{-1} mg^{-1}; (square symbols) preparation 114, 0.015 mg of 30S dynein per assay, 100% ATPase = 1.04 μmol min^{-1} mg^{-1}. For each preparation the ratio of activity measured in the absence of AMP-PCP to that in the presence of 1.0 mM AMP-PCP was 1.7.

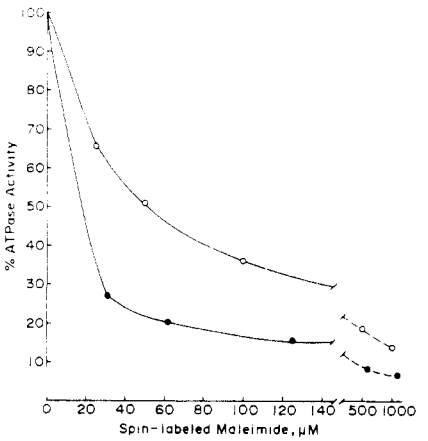

FIGURE 5: Inhibition of 14S dynein ATPase by spin-labeled maleimide at 25 °C. For the curves shown with open circles 0.2 ml of 14S dynein was added to 0.8 ml of a mixture of ATP and SLM such that the final concentration of ATP was 1.0 mM and that of SLM as indicated on the abscissa. For the curves shown with filled circles, the dynein was added to 0.6 ml of SLM at the concentrations shown on the abscissa and 0.2 ml of 5 mM ATP was added after 5 min. In both experiments, ATPase activity was assayed for 25 min in a buffer consisting of 20 mM imidazole, 2.5 mM total Mg^{2+}, 0.4 mM EDTA, pH 7.5. 100% ATPase activity corresponds to 0.68 μmol min^{-1} (mg of protein)$^{-1}$.

already inhibited after 3 min preincubation. Thus it appears that the enhancement of 30S activity occurs only in a narrow range of SLM concentrations and within the first few minutes of exposure to SLM in the absence of ATP. The enhancement is then rapidly obscured by an increasing inhibition. In the presence of ATP, however, the inhibitory process is markedly reduced, and one may observe enhancement even at very high SLM concentrations despite the fact that the SLM was present throughout the course of the ATPase assay, which in the experiments of Figure 3A was 20 min.

Shimizu and Kimura (1974) showed that, if low concentrations of NEM were incubated with 30S dynein overnight at 0 °C, the ATPase activity of the 30S dynein then behaved as did that of myosin similarly treated, i.e., a biphasic enhancement followed by a complete loss of ATPase activity. It was therefore of interest to incubate 30S dynein with low concentrations of SLM in the cold. Several such experiments were done at various concentration ranges of SLM. The data in Figure 4 show that even at concentrations as low as 5 μM there is no suggestion of an enhancement of ATPase activity; only inhibition is observed and, at higher concentrations (data not shown), one obtains complete loss of ATPase activity. Thus spin-labeled maleimide and *N*-ethylmaleimide differ in the specificity with which they react with SH groups at the active site at 25 vs. 0 or 4 °C.

Effect of SLM on ATPase Activity of 14S Dynein. Figure 5 shows that 14S dynein is much more sensitive to SLM at 25 °C than is 30S dynein (cf. Figure 3). Figure 5 also shows that, if the SLM is preincubated with 14S dynein in the absence of ATP, a much stronger inhibition of ATPase activity occurs than when the 14S dynein is added to a mixture of ATP and SLM. Both phenomena (i.e., inhibition at lower concentration of SH reagent than required for 30S dynein and protection against inhibition by ATP) are also observed when 14S dynein is reacted with NEM at 25 °C (unpublished data).

Inhibition of Dynein ATPase Activity by β,γ-Methylene ATP. Unlike myosin, the dyneins are highly selective with respect to the nucleoside triphosphates which they will hydrolyze (Gibbons, 1966; Raff and Blum, 1966, 1969a; Blum, 1972). None of the nucleotides so far examined have proved useful as inhibitors of the ATPase activity of either the 14S or 30S dyneins. Since substrate analogues are frequently of considerable value in clarifying the properties of enzymes, it was of interest to attempt to find an analogue of ATP which would inhibit dynein ATPase activity and/or of the pellet height response assay. Experiments with thio-ATP showed that, although this compound did inhibit 14S and 30S dynein ATPase activity, it was hydrolyzed at an appreciable rate and, furthermore, different batches did not give entirely reproducible results, perhaps because of the presence of impurities as revealed by thin-layer chromatography. AMP-PCP, which has proved useful in studies of myosin ATPase (Yount et al., 1971), was not hydrolyzed by 14S or 30S dynein, did not cause any change in the pellet height response, and was a fair inhibitor of dynein ATPase activity. Thin-layer chromatography by the procedure of Randerath and Randerath (1964) showed only a single spot, and this compound therefore appeared to be suitable for further use. The data in Figure 6 show that 1 mM AMP-PCP inhibits the Mg^{2+}-activated ATPase activity of 30S dynein by about 60% and that of 14S dynein by about 35% at an ATP concentration of 0.5 mM. If the divalent cation is Ca^{2+} instead of Mg^{2+}, there is a marked reduction in the effectiveness of AMP-PCP as an inhibitor of 30S dynein ATPase activity, and it no longer inhibits the 14S dynein at all. Since AMP-PCP discriminates between 14S and 30S dynein depending on whether Mg^{2+} or Ca^{2+} is the divalent cation, it appears to be useful for study of the functions of these two ATPases.

In an experiment in which the addition of 0.5 mM ATP to glycerinated cilia in IM 7.5 buffer caused an increase in pellet height from 1.2 to 2.9 cm, addition of 1 mM AMP-PCP together with the ATP yielded a pellet of about 2.5 cm height, with some cloudiness above the pellet, suggesting the beginning of dissolution of the cilia, as discussed elsewhere (Raff and Blum, 1966), but also clearly demonstrating no appreciable inhibition of the pellet height response under conditions where one would have expected considerable inhibition of the extracted 30S and 14S dyneins (cf. Figure 6). Experiments were therefore performed in which the ATPase activity of glycerinated cilia was measured in the presence and absence of AMP-PCP. Table I shows that at equimolar concentrations of ATP and AMP-PCP, where each of the extracted dyneins would have been strongly inhibited in the presence of Mg^{2+}, there was no inhibition of ciliary ATPase activity. Table I further shows, in agreement with the data in Figure 2, that

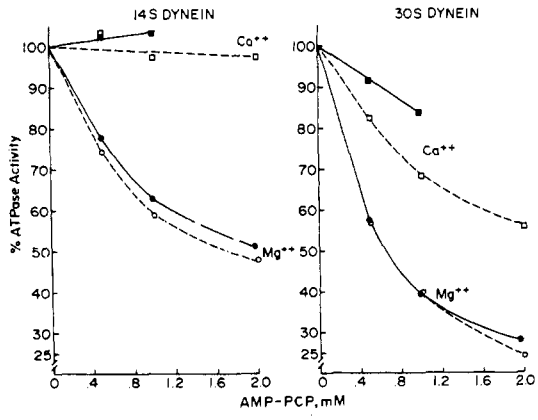

FIGURE 6: Effect of β,γ-methylene ATP on the ATPase activity of 14S and 30S dyneins. These dyneins were prepared as described in Materials and Methods, except that 0.5 mM dithiothreitol was included in the Tris-EDTA and the sucrose gradients had 0.5 mM dithiothreitol plus 0.1 mM EDTA. After collection from the gradients, the ATPase activities were measured in 20 mM imidazole buffer, pH 7.5, at 25 °C in the presence of 2.6 mM total Ca^{2+} or Mg^{2+} and ATP concentration of 0.5 mM, and at the concentration of AMP-PCP shown on the abscissa. The total concentration of EDTA during the incubation was 0.08 mM for the experiments shown by filled symbols and 0.03 mM for the experiments shown with open symbols. Symbols have the following meanings: (filled symbols) cilia preparation 109, 100% ATPase (in units of μmol min^{-1} mg^{-1}), 1.25 and 1.36 for the Ca^{2+}- and Mg^{2+}-activated 30S dynein, and 0.90 and 1.17 for the Ca^{2+}- and Mg^{2+}-activated 14S dynein; (open symbols) preparation 111, 100% ATPase, 0.52 and 0.55 for the Ca^{2+}- and Mg^{2+}-activated 30S dynein and 0.77 and 1.07 for the Ca^{2+}- and Mg^{2+}-activated 14S dynein, respectively.

TABLE I: Effect of β,γ Methylene ATP on ATPase Activity of Glycerinated Cilia in the Presence and Absence of Spin-Labeled Maleimide.[a]

Time of Preincubation in Absence of ATP (min)	AMP-PCP	SLM	ATPase Act. (μmol of P min^{-1} mg^{-1})	(%)
0	−	−	0.233	100
	+	−	0.241	103
0	−	+	0.191	82
	+	+	0.201	86
1.5	−	+	0.314	135
	+	+	0.277	97
3.0	−	+	0.331	142
	+	+	0.288	124

[a] Cilia (0.13 mg of protein in 0.8 ml of IM 7.5 buffer) were incubated in the presence (1.25 mM) or absence of AMP-PCP and of SLM (0.13 mM when present) for the times shown in the table, and then 0.2 ml of 5.0 mM ATP was added and hydrolysis allowed to proceed for 10 min before the reaction was stopped as described in the legend to Figure 2.

preincubation of cilia with SLM caused an increase of the ATPase activity, whereas, if the cilia were added to a mixture of ATP and SLM, a small inhibition of ATPase activity was observed. The presence of AMP-PCP did not alter the degree of inhibition, but reduced the enhancement of ATPase activity during the preincubation. Thus under conditions where the ATPase activity of the dyneins in situ was not inhibited by AMP-PCP this analogue nevertheless interfered with the ability of SML to cause enhancement of ATPase activity. It should be stressed, however, that the AMP-PCP merely reduced the degree of enhancement; if the SLM were reacted

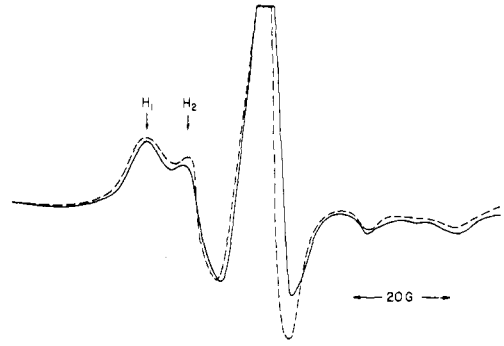

FIGURE 7: The electron paramagnetic resonance spectra of two suspensions of demembranated axonemes which have reacted with 1.0 mM spin-labeled maleimide for 7.0 min. All electron paramagnetic resonance spectra show the derivative of energy absorption (ordinate) as a function of field strength in gauss (G): (solid line) without ATP; (dashed line) after addition of ~3 mM ATP. The spectrum of the sample without ATP was multiplied by a constant and shifted slightly along the ordinate so that the baselines at the left and the height of the trough to the right of peak H_2 coincide with the corresponding portions of the spectrum of the sample with ATP. For further details, see legend to Figure 8.

with the cilia in the presence of ATP instead of AMP-PCP, only inhibition was observed.

Similar experiments were carried out with 30S dynein. Figure 3 shows that AMP-PCP, which inhibits the ATPase activity of extracted 30S dynein, had only a slight protective effect against loss of ATPase activity that occurs when 30S dynein is preincubated with SLM in the absence of ATP. When 1.25 mM AMP-PCP is present during overnight incubation of 30S dynein with SLM at 4 °C, the AMP-PCP scarcely affects the inhibition of ATPase activity (Figure 4). Since the analogue had little effect on the inhibition caused by incubation of SLM with 30S dynein at 4 °C, it was important to know whether the analogue itself inhibited 30S dynein ATPase at low temperatures. In an experiment at 0 °C in which the ATP concentration was 0.5 mM, 0.5, 1.0, and 2.0 mM AMP-PCP caused inhibitions of about 40, 65, and 75%, respectively. Thus at 0–4 °C, where AMP-PCP has little effect on the loss of ATPase activity caused by SLM, it is an effective inhibitor of ATPase activity.

Spin Label Studies of Demembranated Cilia. A concise introduction to the interpretation of spin label spectra has been given by Cooke and Morales (1969) and will not be repeated here.

Electron paramagnetic resonance spectra of spin-labeled ciliary axonemes are shown in Figure 7. These spectra are characterized by a higher intensity of the peak corresponding to the strongly immobilized label (H_1) than of that corresponding to the weakly immobilized label (H_2), but are otherwise similar to the spectra obtained with glycerol-extracted rabbit psoas fibers (Cooke and Morales, 1969). Figure 8 presents the results of two experiments on different preparations of cilia in which the cilia were preincubated with 1.0 mM SLM for various times and the reaction was terminated by addition of excess dithiothreitol, as described in Materials and Methods. As expected (see Figure 2), the ATPase activity rose about twofold within 1 min of preincubation and then declined with further time of preincubation; by 12 min a net inhibition of ATPase activity was obtained in both experiments. It can also be seen that the ratio H_1/H_2 increased smoothly from its value of 1.2 at 1 min (the shortest time studied) to over 1.5 after 12 min of preincubation. In the presence of 3–4 mM ATP, there was a small but definite reduction in the ratio H_1/H_2, corresponding to an increased degree of rotational freedom for label

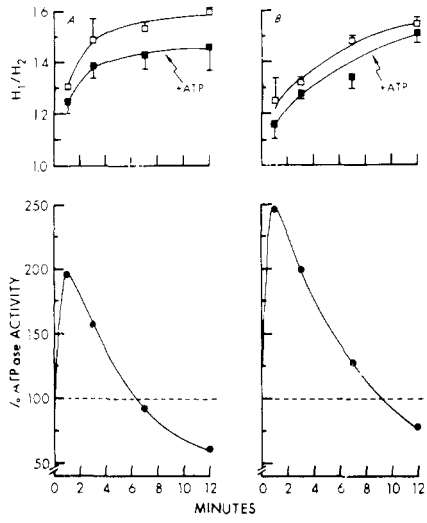

FIGURE 8: Effect of ATP on the electron paramagnetic resonance spectra of demembranated ciliary axonemes labeled with spin-labeled maleimide for the times shown on the abscissa. The lower curves show the effect of labeling with SLM on the ATPase activity. One hundred percent ATPase activity corresponds to 0.107 μmol min^{-1} mg^{-1} for experiment A and to 0.095 μmol min^{-1} mg^{-1} for experiment B. The upper curves show the ratio H_1/H_2 (see Figure 7) measured in the absence of ATP (□) and in the presence of ~3 mM ATP (■), as indicated. Error bars are shown only for ±1 standard deviation. For further details, see text.

that had been strongly immobilized in the absence of ATP. Since similar studies performed at lower ATP concentrations (data not shown) also indicated a decline in the ratio H_1/H_2, it is unlikely that the increase in H_1/H_2 results from the partial dissolution of dynein from the cilia (Raff and Blum, 1966), especially since there was no change in H_1/H_2 between the first spectrum (recorded about 3–5 min after ATP addition) and the third spectrum (recorded about 8 min later) by which time much of the ATP would have been hydrolyzed so that its concentration would surely have been less than 1 mM.

Discussion

Modification of whole cilia by spin-labeled maleimide causes a slight inhibition of ATPase activity if the reaction is carried out in the presence of ATP, but an appreciable enhancement of ATPase if the cilia are briefly preincubated with SLM prior to the addition of ATP. This is qualitatively similar to the effect of *N*-ethylmaleimide on whole ciliary ATPase (Blum and Hayes, 1974a). The two maleimide derivatives are also similar in their effect on the pellet height response; the presence of ATP affords almost complete protection against the loss of pellet height response which otherwise occurs very rapidly at much lower concentrations of either reagent. SLM and NEM differ, however, in their effects on extracted 30S dynein. The difference is most striking if the 30S dynein is reacted with low concentrations of either reagent at 0–4 °C; reaction with NEM leads to the biphasic enhancement–inhibition pattern typical of myosin (Shimizu and Kimura, 1974), whereas reaction with SLM leads only to inhibition. Thus in the absence of ATP, SLM appears to react more readily than NEM at 25 °C with the SH group(s) responsible for enhancement of ATPase activity, while at low temperatures the reverse appears to be true. Although NEM is thought to react exclusively with the SH_1 and SH_2 sites of myosin at the very low molar ratios of NEM to myosin which can be used, the reaction of either NEM or SLM with 30S dynein requires very high molar ratios of reagent to dynein. It is possible that SH groups (or, indeed, groups other than SH groups) elsewhere on the molecule react with either or both of these reagents at such high molar ratios. Shimizu and Kimura (1974) suggest that at least three SH groups are involved in the mechanochemistry of 30S dynein: one group that reacts at 10 μM NEM to yield complete loss of the ability of 30S dynein to rebind to EDTA-extracted axonemes; one group responsible for the enhancement of ATPase, reacting maximally at about 50 μM NEM; and one group responsible for the loss of catalytic activity at higher concentrations of NEM. The present data also support the view that more than two SH groups are involved since it took about 10 min of preincubation with 0.3 mM SLM at 25 °C to get even at 20% inhibition of 30S dynein ATPase (Figure 3A), whereas 1 min of preincubation with this concentration of SLM yielded almost maximal enhancement of ciliary ATPase (Figure 8) and a large inhibition of the pellet height response (Figure 1). Until experiments are performed to quantitate the number of groups that have reacted at various degrees of enhancement or inhibition for NEM and SLM, one cannot, however, be sure of the number of SH groups involved in the mechanochemistry of 30S dynein.

The effect of SLM on the mechanochemistry of cilia is of interest not only because of the differences between it and NEM and its potential use as a spin-labeling probe, but also because it further stresses the influence of the in situ environment on the properties of 30S dynein (Gibbons and Fronk, 1972; Hayashi and Higashi-Fujime, 1972; Blum, 1973). Incubation of whole cilia with SLM in the presence of ATP leads to a slight inhibition of ciliary ATPase (Figure 2), but incubation of extracted 30S dynein with ATP and SLM leads to an enhancement of ATPase (Figure 3). Since experiments with NEM have shown that SH groups on the EDTA-extracted axonemes are not involved in either the rebinding of native 30S dynein to the axonemes or in the enhancement of ATPase activity which occurs on rebinding (Blum and Hayes, 1974b), it appears that the interaction of 30S dynein with other proteins in situ modifies the rate at which some SH groups of 30S dynein react with SLM and/or the consequences of such reaction.

Further insight into the in situ environment of the SH groups that react with SLM may be gained from consideration of the electron paramagnetic resonance spectra of SLM-labeled axonemes. The first SH group(s) to react with SLM caused a doubling of ATPase activity (Figure 8). By this time (1 min), the ratio H_1/H_2 was about 1.2, indicating that much of the covalently bound SLM was strongly immobilized. This suggests but does not prove that the reaction of SLM with the SH group(s) responsible for enhancement of ATPase activity strongly immobilizes the bound SLM since if other SH groups also reacted in this brief interval and strongly immobilized the label, their contribution to the spectrum might obscure that of the SH group(s) involved in the enhancement of ATPase activity. With increasing time of reaction, there is a gradual inhibition of ATPase activity (Figure 8) and of pellet height response (Figure 1) accompanied by an increase in the ratio H_1/H_2 to values considerably larger than those observed in glycerinated psoas fibers (Cooke and Morales, 1969). It appears likely, therefore, that reaction of both the activating and the inhibiting SH groups with SLM leads to strong immobilization of the SLM. Support for the view that at least some of the SH groups involved in ATPase activity are in a microenvironment which strongly immobilizes any SLM that reacts with them comes from consideration of the effect of ATP on the spectrum. At every stage of the reaction, addition of ATP resulted in a small decrease in the ratio H_1/H_2 (Figure 8). This suggests that at least one of the SH groups involved in the en-

hancement or inhibition of ATPase activity in situ acquires greater rotational freedom in the presence of ATP. To our knowledge this is the first evidence suggesting that a localized conformation change occurs upon addition of ATP to axonemes. Further studies on SLM-labeled dyneins and on SLM-labeled axonemes in the presence of ADP and other nucleotides will be required before any detailed interpretation of these spectra is possible.

It is well known that both Mg^{2+} and Ca^{2+} are required for the contraction of striated muscle, with very low concentrations of Ca^{2+} serving to regulate ATPase activity and force generation. Reisler et al. (1974) have recently analyzed the differing roles of these two divalent cations in terms of their ability to form a bridge between the two SH groups at the active site of myosin and the ATP molecule. It has been known for some time (Gibbons, 1965b; Gibbons and Gibbons, 1972) that, unlike with muscle, Ca^{2+} is not required for the motility of cilia and flagella. Nevertheless, Ca^{2+} does appear to play some role in this system: it can replace Mg^{2+} at very low concentrations in the pellet height response assay (Raff and Blum, 1966); the presence of Ca^{2+} during extraction changes the nature of the beat observed following reactivation (Brokaw et al., 1974); Ca^{2+} plays a key role in the direction pointing response of cilia (Naitoh and Kaneko, 1973). In the present work we make two observations on the effect of Ca^{2+} vs. Mg^{2+} in the ciliary system. First, we find that cilia preincubated with SLM in the presence of Mg^{2+} and assayed for ATPase activity in the presence of Mg^{2+} display a greater degree of enhancement of the ATPase activity than cilia preincubated and assayed in the presence of Ca^{2+} (Figure 2A); whereas if the cilia are added to a mixture of SLM, ATP, and either divalent cation, there is little difference in the amount of inhibition observed over a wide range of SLM concentrations (Figure 3C). Shimizu and Kimura (1974) observed that cilia modified at 0 °C with NEM yielded similar biphasic curves when assayed in the presence of Ca^{2+} or Mg^{2+} except that less enhancement of the ATPase activity was observed with Ca^{2+}. Thus after modification of the SH group(s) responsible for enhancement of ATPase activity, hydrolysis of ATP proceeds more rapidly in the presence of Mg^{2+} than in the presence of Ca^{2+}. This is contrary to what is observed with myosin (see Reisler et al., 1974, for details) and indicates that the effects of SH group modification in dynein, while remarkably similar in many ways to that of myosin, are not identical.

A second difference in the effect of Ca^{2+} vs. Mg^{2+} appears when one considers the inhibitory effect of AMP-PCP on 14S dynein as compared with 30S dynein. Yount et al. (1971) observed that AMP-PCP was a mixed inhibitor of myosin ATPase when Mn^{2+} was the divalent cation, while it was a competitive inhibitor of heavy meromyosin ATPase when Ca^{2+} was the divalent cation, and stressed the importance of the metal ion on the nature of the inhibition. The effect of AMP-PCP on the extracted dynein also depends on the nature of the divalent cation; whereas AMP-PCP is a weaker inhibitor of 30S dynein in the presence of Ca^{2+} than Mg^{2+}, it does not inhibit 14S dynein at all if the Mg^{2+} is replaced by Ca^{2+}. Although AMP-PCP inhibited the activity of both Mg^{2+}-activated dyneins, it did not inhibit the Mg^{2+}-activated ATPase of cilia. This further emphasizes that there are some differences between dyneins in situ and after extraction. When SLM was preincubated with 30S dynein in the presence of AMP-PCP, there was hardly any change in the rate of loss of activity caused by the SLM (Figure 3). These experiments were done in the presence of Mg^{2+}, where appreciable inhibition of ATPase activity occurred. Thus AMP-PCP is clearly much less effective than ATP in protecting the SH group(s) responsible for the loss of catalytic activity. If one assumes that there is only one kind of site where both ATP and AMP-PCP can bind, then it appears that these two compounds may not bind to the site in the same way.

Acknowledgments

We are grateful to Dr. K. V. Rajagopalan for allowing us to use his Varian spectrometer.

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