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Effect of *N*-Ethylmaleimide and of Heat Treatment on the Binding of Dynein to Ethylenediaminetetraacetic Acid Extracted Axonemes†

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ABSTRACT: Cilia from *Tetrahymena pyriformis* were extracted with Tris-EDTA to yield crude dynein which for some experiments was further purified by centrifugation on sucrose density gradients to yield 30S dynein. Dynein was incubated with EDTA extracted axonemes for 30 min at 25° and the axonemes precipitated by centrifugation. The amount of dynein bound to the axonemes was computed from the loss of ATPase activity in the supernate and from the gain of ATPase activity in the pellet. In three-fourths of the experiments more activity appeared in the pellet than disappeared from the supernatant, indicating an enhancement of the ATPase activity of dynein upon binding to EDTA extracted axonemes. If dynein were reacted with *N*-ethylmaleimide (NEM) or heated at about 40–46° before incubation with native EDTA extracted axonemes, the dynein ATPase activity increased but its ability to bind to the axonemes and to display an enhancement of its ATPase activity on binding were reduced. If EDTA extracted axonemes were reacted with NEM prior to incubation with native dynein, there was little effect on their ability to bind the dynein, but if the axonemes were heated prior to the incubation with native dynein, the ability of the axonemes to bind the native dynein and to enhance the ATPase activity of the bound dynein was reduced. Thus an SH group(s) on the dynein but not on the EDTA extracted axonemes is required for binding of the dynein to the axonemes and for enhancement of the ATPase activity upon binding, whereas both sets of binding sites are very sensitive to thermal denaturation.

It is now established (Summers and Gibbons, 1971) that the ability of cilia and flagella to generate bending waves is based upon a sliding filament system. The paired arms projecting from the A subfibers of each outer doublet are thought to act as cross bridges that contact the B subfiber of the adjacent doublet and generate the sliding motion which, in the presence of shear resistant linkages (Brokaw, 1966; Lubliner and Blum, 1971), will lead to bending. Gibbons (1965a) showed that extraction of *Tetrahymena* cilia by Tris-EDTA led to the appearance of two forms of the ATPase dynein in solution and the disappearance of the arms. His finding that the 30S dynein could be rebound to the extracted axonemes with the reappearance of the arms and that ATP caused a light-scattering change of the axonemes only if 30S dynein had been added indicated that the 30S form was the mechanochemically effective ATPase of cilia and that it was a major component of the arms (Gibbons, 1965b). Since the arms are not the same shape other

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proteins may also be present in association with the dynein (see Linck, 1973). Ogawa and Mohri (1972) reported that a purified preparation of dynein obtained from sea urchin sperm flagella could rebind to EDTA extracted axonemes in the presence of Ca^{2+} or Mg^{2+}. This work was extended by Ogawa (1973) who showed that brief trypsinization of dynein produced a fragment, called fragment A, which could not recombine with EDTA extracted axonemes but whose ATPase activity was enhanced by the addition of a microtubule fraction. Otokawa (1972) also noted that the ATPase activity of 30S dynein from *Tetrahymena* was enhanced upon the addition of a microtubule protein fraction prepared from the cilia. It is apparent that further information on the binding of dynein to EDTA extracted axonemes will be essential for an understanding of cross bridge action and the generation of sliding motion in cilia and flagella.

We have noted earlier (Raff and Blum, 1966) that brief heating of glycerinated *Tetrahymena* cilia resulted in a slight enhancement of the ATPase activity and a complete loss of the swelling response of these cilia to ATP. This effect of heating was tentatively interpreted as an uncoupling. Similarly, when glycerinated *Tetrahymena* cilia were preincubated with *N*-ethylmaleimide (NEM)[1] in the absence of ATP, a slight increase in ATPase activity but a complete loss of the swelling response occurred (Blum and Hayes, 1974). It was therefore of interest to determine whether the sensitivity to heating and to NEM was a property of the dynein, of the EDTA extracted axonemes, or of both. In this paper crude dynein and purified dynein were treated with NEM or heated at various temperatures and then mixed with native EDTA treated axonemes and the effect of the NEM or heat treatment on ATPase activity and on the amount of rebinding was measured. Similar experiments were done in which the EDTA treated axonemes were exposed to NEM or heated and then allowed to interact with native dynein. Our results show that when dynein binds to EDTA extracted axonemes an enhancement of its ATPase activity frequently occurs. Pretreatment of the dynein with NEM causes a slight increase in its ATPase activity but a loss of the ability to bind to EDTA extracted axonemes, whereas treatment of EDTA extracted axonemes with NEM did not appreciably alter the ability of these axonemes to bind dynein. Both the dynein and the EDTA extracted axonemes are very sensitive to heating. These results help clarify the mechanism by which NEM prevents the swelling response of glycerinated cilia and point to a significant role for an SH group(s) in the mechanochemically effective interaction between dynein and EDTA extracted axonemes. The results also provide an explanation for the uncoupling action of heating at 40° on the swelling response of *Tetrahymena* cilia.

Materials and Methods

Tetrahymena pyriformis, strain HSM, were grown axenically at 26° in stirred aerated culture vessels in a medium consisting of 1% Difco proteose peptone, 0.05% Difco liver extract, 0.1% glucose, and about 0.5 ml of Dow Corning Antifoam FG-10 per liter, all in 0.02 M KH_2PO_4 adjusted to pH 6.5 with NaOH. The cells were chilled and harvested by continuous flow centrifugation (1500*g*) and the cilia isolated by a modification of the glycerol method of Gibbons as described previously (Blum, 1973). Conditions for the storage of cilia, for protein determination, and for the preparation of 14S and 30S dyneins on sucrose density gradients are as specified earlier. When a preparation was to be used, it was taken from storage at −20°, diluted with four volumes of ice-cold IM buffer (20 mM imidazole–2.5 mM $MgCl_2$ (pH 7.5)), and centrifuged at 27,000*g* for 30 min at 0°. The pellet was resuspended in 20 ml of IM buffer plus 20 ml of 0.2% Triton X-100 and stirred with a magnetic stirrer at 0° for 5 min. The suspension was then centrifuged for 5 min at 12,000*g;* the pellet was resuspended in 40 ml of IM buffer and recentrifuged for 5 min at 12,000*g*. The Triton X-100 extracted axonemes were resuspended in 20 ml of 20 mM imidazole (pH 7.5) and dialyzed against a total of 2 l. of Tris-EDTA (1 mM Tris, 0.1 mM EDTA (pH 8.2)) for at least 16 hr at about 4°. After dialysis the suspension was centrifuged for 30 min at 27,000*g*. The supernatant was collected and recentrifuged for 15 min at 27,000*g* to remove any traces of axonemes, and will be referred to as supernatant I or crude dynein. The axonemal pellet was resuspended in 40 ml of Tris-EDTA, stirred at 0° for 1 hr, and then centrifuged for 15 min at 27,000*g*. The twice extracted axonemal pellet was resuspended in 40 ml of IM buffer, washed by centrifugation for 15 min at 27,000*g*, and resuspended in about 10 ml of IM buffer. We shall refer to this suspension of demembranated axonemes from which all the extractable dynein has been removed as pellet II or as EDTA extracted axonemes.

Binding experiments were performed in a total volume of 2 ml of IM buffer and each point was run in duplicate. One pair of tubes contained dynein (crude dynein or 30S dynein obtained from the crude dynein by sedimentation on a sucrose gradient). Another pair contained pellet II. A third pair contained both dynein and pellet II. The tubes were incubated at 25° for 30 min to allow the binding to occur, and then centrifuged for 10 min at 27,000*g* at 0°. Each tube was then gently mixed and the centrifugation repeated. The supernatant from each tube was then very slowly decanted by suction, and the pellet was resuspended in 2.0 ml of IM buffer. From each supernatant and each pellet, 0.8 ml was taken for ATPase assay, which was performed by adding 0.2 ml of 5 mM ATP (in IM buffer with pH adjusted to 7.5) and allowing the reaction to proceed at 25° for 10–20 min, as desired. The reaction was terminated by adding 0.5 ml of 10% trichloroacetic acid. The tubes were then centrifuged in a clinical centrifuge for 5 min and 1 ml of the clear fluid was assayed for orthophosphate by a semimicro modification of the method of Taussky and Shorr (1953).

For experiments in which *N*-ethylmaleimide (NEM) was used, both it and cysteine-HCl were weighed out immediately before use and kept ice cold until needed, usually less than 2 hr after weighing. Each compound was dissolved in IM buffer and the pH adjusted to 7.5.

Protein was assayed by the method of Lowry *et al.* (1951), with bovine serum albumin as the standard.

NEM, ATP, and cysteine-HCl were purchased from Sigma; Triton X-100 was from Scientific Chemical Co. All other chemicals were reagent grade.

Results

Previous experiments (Ogawa, 1973; Hayashi and Higashi-Fujime, 1972) on the binding of dynein to EDTA extracted axonemes have been performed by mixing the dynein with the axonemes and dialyzing the suspension overnight or longer against a suitable buffer. This method has

[1] Abbreviations used are: NEM, *N*-ethylmaleimide; IM buffer, 20 mM imidazole–2.5 mM $MgCl_2$ (pH 7.5).

the advantage of removing EDTA from the crude dynein, of removing sucrose from the purified 30S dynein, and, presumably, of allowing ample time for equilibrium to be approached. A possible disadvantage is that changes which could alter the binding properties but not the total ATPase activity might occur. We have chosen instead to use a 30-min incubation at 25° and to have EDTA or sucrose present during the binding process. Several experiments showed that more dynein bound during an overnight incubation in the cold than during a 30-min incubation at 25°, but the difference was not great. Hayashi and Higashi-Fujime (1972) state that under their conditions association of crude dynein and the EDTA extracted axonemes took place within a few minutes and that further incubation did not increase the association. The presence of EDTA should have no effect since even in experiments in which 0.6 ml of crude dynein was used the EDTA concentration during the binding process is less than 0.03 mM while the Mg^{2+} concentration was over 2 mM. Furthermore, the EDTA concentration was kept constant in every experiment, and control experiments failed to show any effect of EDTA at these concentrations on either binding or on the subsequent ATPase activity measurements. Similarly, although there was no reproducible effect of sucrose on either the binding or the ATPase activity of either 14S or 30S dynein, we routinely adjusted the sucrose concentration to the same level in all samples of any given experiment.

In the experiment shown in Table I various amounts of 30S dynein were incubated with 0.33 mg of EDTA extracted axonemes for 30 min and the supernatants and precipitates obtained after centrifugation of these tubes were assayed for ATPase activity. In principle, there are two ways for computing the per cent binding. First, one can compute the binding from the amount of activity that disappears from the supernatant as a result of incubation with the pellet. If one defines SD, SM, and SA as the ATPase activity measured in the supernatants of the tubes containing dynein alone, mixed dynein plus EDTA extracted axonemes, and EDTA-extracted axonemes alone, respectively, then

$$\% \text{ binding (method S)} = \frac{SD - (SM - SA)}{SD} \times 100 \quad (1)$$

In general SA is very small, since very little ATPase activity remains in solution when the twice extracted axonemes are precipitated by centrifugation, and this computation assumes that any ATPase activity which "leaks" from the axonemes (SA) stays in the supernatant even if dynein is added, *i.e.*, it is assumed that there is nothing in the dynein which would cause SA to rebind to the axonemes. In fact, we have made computations of all our data on the other assumption as well (namely that the addition of dynein does cause SA to rebind to pellet II) and although in some experiments the quantitative conclusions would be changed, there would be no change in any of the qualitative conclusions. Throughout this paper, therefore, it is assumed that the addition of either crude or purified 30S dynein to a suspension of EDTA treated axonemes does not cause the small amount of ATPase activity which stays in solution if the EDTA treated axonemes themselves are centrifuged to rebind to the axonemes.

The binding should also be computable by measuring the increase in activity of the pellet. If one defines PM as the ATPase activity in the precipitate formed from tubes containing a mixture of EDTA extracted axonemes and dynein and PP as the activity in the precipitate formed from tubes containing only the EDTA extracted axonemes, then

$$\% \text{ binding (method P)} = \frac{PM - PP}{SD} \times 100 \quad (2)$$

If there is no change in the activity of 30S dynein when it rebinds to the axonemes, the two methods should give the same results. The data in Table I show that this is not the case; there is an increase in activity of the dynein when it binds onto the axonemes. A similar experiment was done in which the binding of 30S dynein to EDTA extracted axonemes was followed as a function of time after the preparation of the 30S dynein. When the 30S dynein was used within 1 day after collecting the fractions from the sucrose

TABLE I: Binding of 30S Dynein to EDTA Extracted Axonemes.[a]

30S Dynein (ml)	Pellet II (ml)	ATPase Activity (nmoles/min) in Supernatant	ATPase Activity (nmoles/min) in Precipitate	% Binding Computed from Loss of Activity from supernatant (Method S) (%)	% Binding Computed from Gain of Activity in precipitate (Method P) (%)
0.1	0	4.68	0.0		
0.2	0	10.5	0.0		
0.4	0	23.0	0.0		
0.6	0	29.6	0.1		
0	0.2	1.25	36.5		
0.1	0.2	4.37	40.3	33	81
0.2	0.2				
0.4	0.2	16.3	47.1	34	46
0.6	0.2	23.6	51.7	24	51

[a] Cilia (preparation 74) were treated with Triton X-100 and extracted with Tris-EDTA to yield crude dynein and EDTA extracted axonemes (pellet II) as described in the Materials and Methods section. 30S dynein was prepared from the crude dynein by sedimentation in a sucrose density gradient. Each tube contained 30S dynein and pellet II (1.66 mg of protein/ml) in the volumes shown, plus enough sucrose in IM buffer to make the total volume 2.0 ml and the final sucrose concentration about 6% (w/v). After incubating for 30 min at 25° each tube was centrifuged, the supernatant decanted and saved, and the precipitate resuspended in 2.0 ml of IM buffer. The ATPase of each fraction was then determined as described in the Materials and Methods section, and is reported in units of nmoles/min for the entire 2-ml sample that was originally incubated. Since there was 37.8 nmol/min of total ATPase activity by 0.2 ml of pellet alone, the ATPase activity of the EDTA extracted axonemes was 37.8/0.2 (1.66) = 114 nmol min^{-1} mg^{-1}, and the ATPase activity of this preparation of 30S dynein was 50 nmol min^{-1} ml^{-1} of pooled 30S dynein from the gradient. Each incubation was done in duplicate but only the average value is shown. The maximum difference in ATPase activity between the two members of any pair was 10%, *i.e.*, the actual values are within ±5% of the mean value shown.

density gradient, about 40% of the dynein bound to the axonemes if the computation was made by eq 1 but about 80% of the dynein would have bound if the computation was made by eq 2. Thus binding of freshly isolated 30S dynein to freshly extracted axonemes resulted in approximately a doubling of the bound ATPase. After about 1 week of storage, much of the binding ability of the 30S dynein was lost, but the dynein that did bind was still activated upon binding. In another experiment we found that 30S dynein that had been stored in the refrigerator for 5 days was no longer capable of binding to either freshly prepared EDTA extracted axonemes or to axonemes that had been stored in 50% glycerol at −20° for 6 weeks. The crude dynein from which the 30S dynein had been obtained, however, was still capable of binding to both fresh and 6-week old axonemes even after 6 days in the refrigerator. Twice as much of the crude dynein bound to the fresh axonemes as to the stored ones, but in both cases the activity of that dynein which did bind was activated about twofold. Thus purified 30S dynein deteriorates more rapidly than crude dynein, while the binding capacity of EDTA extracted axonemes for dynein is stable in glycerol at −20° for at least a few weeks. In all the experiments which follow, 30S dynein was used within 2 days of the sucrose gradient run, crude dynein was used within 5 days of the Tris-EDTA extraction, and axonemes were used within 8 days.

Twenty-two cilia preparations were examined during the course of this study, and from these 50 control binding assays were done (*i.e.*, assays in which neither the dynein nor the EDTA extracted axonemes were treated with NEM or by heating). In 38 of the 50 control assays the per cent binding computed by method P (eq 2) was 52 ± 13 while the per cent binding computed by method S (eq 1) was 37 ± 13. Because of the large differences in per cent binding as well as in the degree of enhancement on binding in the various experiments, the standard deviations attached to these means are large. It is clear, however, that in these 38 assays there was an appreciable enhancement of the activity of the bound dynein. In 11 of the assays the two methods of computation gave essentially the same results, and in one assay the per cent binding computed by method S was higher than that computed by method P. Thus enhancement of the activity of crude dynein and binding was only observed in about three-fourths of the assays performed. We do not understand the factors responsible for this variability. Most of the experiments reported here were performed with preparations that showed appreciable enhancement on binding. It must be emphasized, however, that the effects of NEM and of heating on the binding of crude dynein to EDTA extracted axonemes and on the change in its ATPase activity (in the absence of axonemes) occurred in all preparations whether or not the enhancement of activity on binding was demonstrable. Eight control assays were done with 30S dynein obtained from sucrose density gradients. In two of these, no ATPase activity disappeared from the supernatant even though a considerable increase in the ATPase activity of the pellets occurred after the 30-min incubation, *i.e.*, the system behaved as if enhancement of ATPase had occurred in both the 30S dynein that bound to the EDTA-extracted axonemes and in the 30S dynein that remained unbound. In the remaining six assays, the per cent binding computed by method S was 45 ± 23 while the per cent binding computed by method P was 80 ± 50. As with the crude dynein, the variability is large but with 30S dynein enhancement always seems to occur.

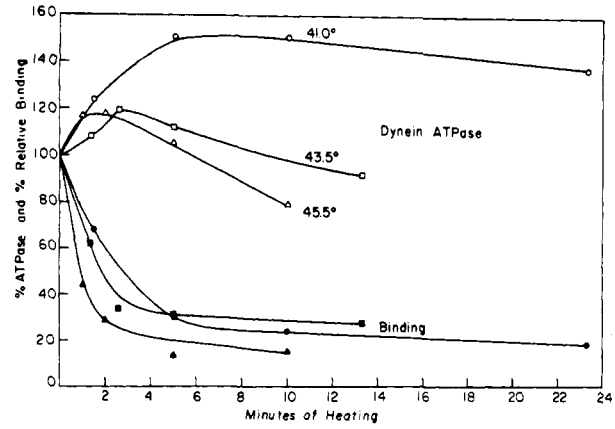

FIGURE 1: Effect of heating on the ATPase activity and the ability of dynein to rebind to EDTA extracted axonemes. Cilia preparation 66 was extracted with EDTA to yeild a preparation of crude dynein (0.88 mg of protein/ml) and of EDTA extracted axonemes as described in the Materials and Methods section. On the first day after preparation of the crude dynein, 1-ml aliquots were placed in a bath at 41.0° for the times shown on the abscissa and then placed in ice. When the last tube was finished, all the tubes were brought to 25° and allowed to react with 0.54 mg of EDTA extracted axonemes for 30 min and assayed for per cent binding as described in the section on Methods. The ordinate represents the per cent ATPase activity and the per cent binding relative to that of unheated dynein; 100% ATPase activity corresponds to 589 nmol min^{-1} mg of crude $dynein^{-1}$, and 100% relative binding corresponds to 80% binding as computed by method P. On the next day the same experiment was repeated at 45.5°, with 0.50 mg of EDTA extracted axonemes; 100% ATPase activity corresponds to 740 nmol min^{-1} mg of crude $dynein^{-1}$ and 100% relative binding corresponds to 65% binding as computed by method P. On the third day, the same experiment was repeated at 43.5°, with 0.57 mg of EDTA extracted axonemes; 100% ATPase activity corresponds to 710 nmol min^{-1} mg of $dynein^{-1}$, and 100% relative binding corresponds to 62% binding computed by method P.

To facilitate comparison of the effects of NEM or heat treatment on ATPase activity with binding ability, it is convenient to define the relative binding as the per cent binding computed by either eq 1 or eq 2 divided by the per cent binding computed the same way for the control (untreated) dynein or axonemes.

Effect of Heating on the Rebinding of Dynein to EDTA Extracted Axonemes. It has been reported that heating glycerinated *Tetrahymena* cilia at 40° for 11 min caused a slight increase in ATPase activity and a complete loss of the swelling response (Raff and Blum, 1966). When 14S and 30S dynein were heated at 47° or higher, ATPase activity was lost with increasing time of heating (Figure 3 of Blum, 1973), and the same pattern was observed when crude dynein was heated at temperatures of 47° or above (Figure 2 of Blum, 1973). Heating at 45°, furthermore, did not cause any increase in the ATPase activity of whole cilia (Blum, 1973). The data in Figure 1 show that when crude dynein is heated at 41° there is a 1.5-fold enhancement of ATPase activity which declines only slowly up to 23 min. At 43.5° the enhancement is smaller, occurs earlier, and is replaced by a net loss of activity within 9 min. At 45.5° the peak enhancement occurs still earlier and is replaced by net inhibition within 6 min. For each of these three experiments, the binding of the dynein to unheated axonemes was also measured. The solid lines in Figure 1 were computed according to method P (eq 2), *i.e.*, from the amount of activity appearing on the pellet. It can be seen that early in the heating, whether at 41 or 45.5°, when the ATPase activity of the dynein was enhanced, the ability of the dynein to rebind

to EDTA extracted axonemes was markedly inhibited. Further heating, which increased the thermal denaturation of the ATPase activity, caused only a small further loss of binding ability. A similar conclusion is reached if the computation is made from the amount of ATPase activity lost from the supernatant, *i.e.*, by method S (eq 1), but no conclusions could be drawn as to whether the heat treatment preferentially reduced the enhancement of dynein ATPase activity on binding. In any case, it is clear that brief heating of crude dynein at 41–45° enhances the ATPase activity while destroying the ability to bind to EDTA extracted axonemes.

Similar experiments were performed in which EDTA extracted axonemes were heated for various times and then the binding of native dynein to the heat-treated axonemes was assayed. In one such experiment (Table II) the ATPase activity of the unheated EDTA extracted axonemes was 13.6 units, of which 12.5 units were pelleted by centrifugation (column B) while 1.1 units were "soluble." With increasing time at 42.2°, this unextracted ATPase activity was partially inactivated as was the "soluble" ATPase. To each tube 67.2 units of fresh crude dynein were added (column D) and, after allowing binding to occur at 25° for 30 min, the mixture was centrifuged, yielding a supernatant and a pellet, the ATPase activities of which are shown in columns E and F, respectively. If there were no changes in total activity upon mixing, then the sum of columns B + C + D should equal the sum of columns E + F. For the unheated axonemes, these sums are 80.8 and 93.1 units, respectively, indicating the appearance of 12.3 units of ATPase activity, as shown in column I, although computed there by a different procedure. The theoretical units that were bound to the axonemes are then computed in column G on the assumption that the "soluble" ATPase of the axonemes did not rebind to the axonemal pellet. Given this theoretical amount of ATPase activity which disappeared from the supernatant and therefore should have been bound to the pellet, one computes in column H the expected ATPase activity of the pellet, which should be compared with the measured ATPase of the pellet given in column F. Column I shows that when this preparation of crude dynein bound to native EDTA extracted axonemes, its ATPase activity was enhanced. After 1.5 min at 42.2° there was little loss of binding and no loss of this "extra" ATPase activity (see column G). After 5 min, less enhancement of activity was observed although about 75% of the dynein that bound to the unheated axonemes was bound to the heated axonemes. Further heating caused no further loss of binding but no enhancement of activity was obtained. After 25 min at 42.2°, when the amount of dynein bound to the axonemes had only been reduced from 24.1 to 19.7 units, there was an almost complete loss of the enhancement of activity observed when the dynein bound to the native EDTA extracted axonemes. At higher temperatures (up to 55°) similar results have been obtained, *i.e.*, a rapid loss of ability of axonemes to cause enhancement of the ATPase of the dynein which binds and a slower loss in the per cent of dynein that can bind.

Effect of Cysteine on the Binding of Dynein to EDTA Extracted Axonemes. We have recently shown (Blum and Hayes, 1974) that incubation of NEM with crude dynein activates the ATPase activity while preincubation of glycerol-extracted cilia with low concentrations of NEM in the absence of ATP causes a loss of the swelling response which normally occurs when ATP is added to control cilia. It was therefore of interest to examine the effect of treating dynein with NEM on the ability of the dynein to bind to EDTA extracted axonemes and, conversely, to examine the ability of untreated dynein to bind to axonemes that had been treated with NEM. Fresh cysteine-HCl in excess of the NEM was used to render any unreacted NEM inoperative. Since cysteine can itself be looked upon as a sulfhydryl reagent, it was necessary to check its effects on the binding of dynein to axonemes even though in earlier experiments (Blum and Hayes, 1974), where cysteine was used for short times, we had found no effect of cysteine. Cysteine had no effect on the ATPase activity of EDTA extracted axonemes, but, as shown in Figure 2 for one of two such experiments, cysteine caused about a 25% decrease in the ATPase activity of crude dynein under the conditions of a standard binding assay as described in the section on Methods. The relative per cent binding, computed according to eq 1, decreased essentially in parallel with the inhibition of ATPase activity. Thus the data presented in the lower graph of Figure 2 indi-

TABLE II: Loss of Enhancement of Dynein ATPase Activity on Binding to Heated EDTA Extracted Axonemes.[a]

A	B	C		E	F		H	I
				After 30 min at 25°				
Time of Heating of Axonemes (min)	Axonemal ATPase (precipitable) (nmoles min^{-1} ml^{-1})	Axonemal ATPase (non-precipitable) (nmoles min^{-1} ml^{-1})	D Dynein ATPase (nmoles min^{-1} ml^{-1})	Supernatant ATPase (nmoles min^{-1} ml^{-1})	Pellet ATPase (nmoles min^{-1} ml^{-1})	G Theoretical Units Bound [D − E + C] (nmoles min^{-1} ml^{-1})	Theoretical Activity of Pellet [B + G] (nmoles min^{-1} ml^{-1})	"Extra" Activity of Pellet [F − H] (nmoles min^{-1} ml^{-1})
0	12.5	1.1	67.2	44.2	48.9	24.1	36.6	12.3
1.5	11.0	0.9	67.2	48.4	45.2	19.7	30.7	14.5
12.0	6.7	0.5	67.2	50.4	31.5	17.3	24.0	7.5
25.0	4.8	0.3	67.2	47.7	26.3	19.7	24.6	1.7

[a] EDTA extracted axonemes (preparation 84, 1.15 mg of protein/ml) in IM 7.5 buffer were heated at 42.2° for the times indicated and then chilled. Aliquots of 0.2 ml were then added to tubes containing either 1.6 ml of buffer plus 0.2 ml of Tris-EDTA or to 1.6 ml of buffer plus 0.2 ml of crude dynein (preparation 84, 0.99 mg of protein/ml) and incubated at 25° for 30 min. All tubes were prepared in duplicate but only the average value is shown in the table. The maximum difference of any measurement from the average was 10% and generally less than 5%. After the 30-min incubation the tubes were centrifuged as described in the Materials and Methods section and samples of 0.8 ml were taken for ATPase assay.

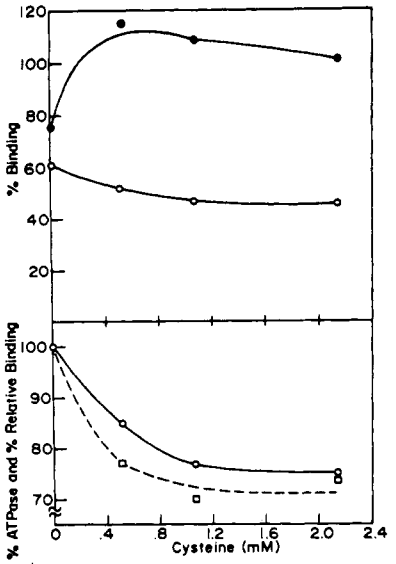

FIGURE 2: Effect of cysteine on the ATPase activity of dynein and its binding to EDTA treated axonemes. Crude dynein (0.184 mg/ml) and EDTA extracted axonemes (0.874 mg/ml) were prepared from cilia preparation 72 as described in the Materials and Methods section. Aliquots of 1 ml of dynein were mixed with 1.33 ml of IM buffer containing freshly dissolved cysteine such that the final concentrations of cysteine were 0, 2.68, 5.36, and 10.7 mM. Aliquots of 0.4 ml of each of these solutions were then added to duplicate tubes containing 1.6 ml of IM buffer or 1.4 ml of IM buffer plus 0.2 ml of the EDTA extracted axonemes, and the mixture was incubated for 30 min at 25°. The concentration of cysteine in each pair of tubes during the incubation is shown on the abscissa. After the 30-min incubation the samples were centrifuged and the ATPase activity of the supernatant and of the precipitate was determined for each tube. The points shown are the average values of each pair of measurements. The individual points differed by less than ±5% from the average values plotted. The lower part of this graph shows the ATPase activity (100% = 674 nmol min^{-1} mg of crude dynein $protein^{-1}$) and the relative percent binding (100% relative binding = 61.0% binding as computed by method S, eq 1). ATPase activity (□- - -□); relative % binding (O—O). The upper part of the figure shows the amount bound computed according to method S (eq 1) (O—O), and computed according to method P (eq 2) (●—●).

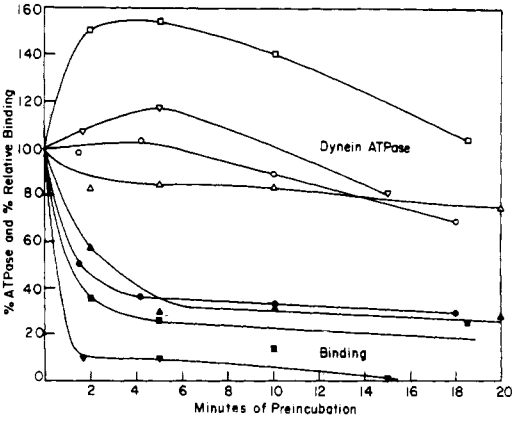

FIGURE 3: Effect of preincubation of dynein for various times with NEM on the ATPase activity and ability to bind to EDTA extracted axonemes. Four experiments are shown, each represented by the same symbol. Filled symbols are the relative per cent binding, computed by method P (eq 2). Open symbols show the per cent ATPase activity. The experiments are arbitrarily numbered as follows: (O, ●) expt I; (□, ■) expt II; (△, ▲) expt III; (▽, ▼) expt IV. Each experiment was done by taking a preparation of crude dynein, reacting it with NEM at 25° for the time shown on the abscissa, and stopping the reaction with cysteine. Controls received only the cysteine. The treated dynein was then incubated for 30 min by itself or in the presence of EDTA-extracted axonemes and the ATPase activity of the supernatant and precipitate was measured. The per cent binding was computed by method P (eq 2) and is shown as the relative per cent binding, *i.e.*, with the control value set at 100% in each experiment. The concentrations of NEM and dynein during the preincubation periods were: expt I, 6.25 mM and 0.56 mg/ml; expt II, 6.25 mM and 0.50 mg/ml; expt III, 3.13 mM NEM and 0.37 mg/ml; expt IV, 6.25 mM and 0.26 mg/ml. In each experiment 1 ml of the above mixture was added to 0.8 ml of freshly prepared 25 mM cysteine to stop the reaction and in each experiment 0.4 ml of the NEM-treated dynein was added to 1.6 ml of IM buffer or to 1.6 ml of IM buffer containing the following amounts of EDTA extracted axonemes: expt I, 0.44 mg of proteins; expt II, 0.30 mg; expt III, 0.36 mg; expt IV, 0.46 mg. The NEM-treated and control crude dyneins were incubated with the axonemes for 30 min and the supernatants and precipitate obtained by centrifugation were assayed for ATPase activity as described in the section on Methods. The crude dynein and EDTA extracted axonemes used in experiments II and IV were from cilia preparations 68 and 58, respectively. Experiments I and III were done on cilia preparation 69, experiment I immediately after the dialysis against Tris-EDTA and experiment III 2 days later.

cate that cysteine caused about 25% decrease in the ATPase activity of crude dynein and a comparable decrease in its capacity to rebind to EDTA extracted axonemes. The data in the upper part of Figure 2 show the reversibility of the cysteine effect. When the per cent binding computation is made according to eq 1 (*i.e.*, from measurements of supernatant activities), one observes a small decrease in per cent binding with increasing cysteine, as already shown in the lower part of this figure on a relative basis. If the computation is made according to eq 2, the enhancement of activity on binding appears to increase with increasing cysteine. This increase in apparent enhancement of activity results primarily from the decrease in supernatant activity, which enters into the denominator of eq 2, whereas the pellet activities, which enter into the numerator of eq 2, are measured in the absence of cysteine. Thus the small inhibition of dynein ATPase activity and of its binding to EDTA extracted axonemes is reversed upon removing the cysteine.

Effect of NEM on the Binding of Dynein to EDTA Extracted Axonemes. One series of experiments was performed in which a particular concentration of NEM was preincubated with dynein for various times and the reaction terminated by adding an excess of freshly prepared cysteine. The treated dynein was then incubated for 30 min with EDTA extracted axonemes and the ATPase activity and the binding of the dynein to the axonemes were measured as described in the Materials and Methods section. If NEM had no effect on the ATPase activity, one would expect about a 25% decrease in the ATPase activity of the dynein in those tubes which did not have any axonemes added, because of the effect of cysteine as described in the previous section (*cf.* Figure 2). Instead, one generally finds either a slight inhibition of activity or an activation of the ATPase, as previously described (Blum and Hayes, 1974). The four experiments shown in Figure 3 were chosen to display the range of results obtained. The effect of preincubation with NEM on the ability of the dynein to bind to EDTA extracted axonemes is also shown in Figure 3. It is clear that at short times (up to 3 min) when there is either very little inhibition of ATPase or else an appreciable activation of the ATPase of the dynein by NEM, there is a marked inhibition of the ability of the dynein to bind to the axonemes. With increasing time of preincubation with NEM, the dynein ATPase activity decreases (becoming less activated or more inhibited), and there is a further slight decline in the ability of the dynein to bind to axonemes. Because the inhibition of binding is large, it is difficult to tell whether the per cent binding computed by method P (eq 2) remained larger than the per cent binding computed by method S (eq 1), but in the

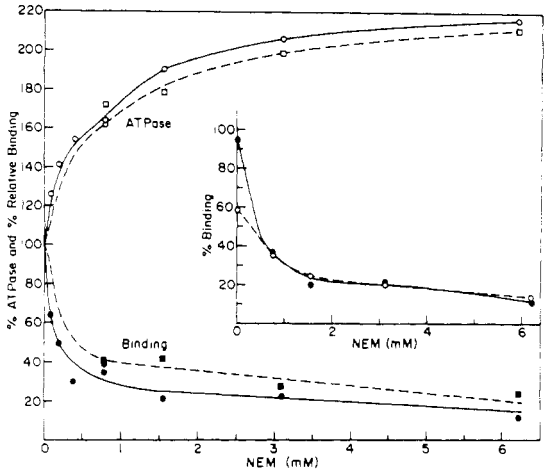

FIGURE 4: Effect of preincubation of dynein with various concentrations of NEM and the ATPase activity and ability to bind to EDTA extracted axonemes. Crude dynein and EDTA extracted axonemes were prepared from cilia preparation 70 as described in the Materials and Methods section; 1 ml of dynein (0.37 mg of protein) was incubated at 25° with 0.33 ml of NEM for 100 sec (□, ■) or 200 sec (○, ●) at the final concentrations shown on the abscissa. The reaction was stopped by adding 1 ml of 25 mM cysteine. Samples of 0.4 ml of control dynein (to which only the cysteine had been added) and of NEM-treated dynein were then added to 1.6 ml of IM buffer alone or of IM buffer containing EDTA extracted axonemes (0.35 mg of protein). The tubes were incubated for 30 min and then centrifuged and the ATPase activity of the supernatant and pellets were assayed as described in the section on Methods. The experiment in which the cilia were preincubated for 200 sec was done immediately after the extraction against Tris-EDTA; the 100-sec preincubation experiment was done 1 day later. The ordinate of the main part of the graph shows the per cent ATPase activity (100% ATPase corresponds to 480 nmol min^{-1} mg^{-1}) and the relative per cent binding computed by method P (eq 2). The amount bound is shown in the inset graph as computed by method P (●—●) and by method S (○- - -○) for the experiment in which the dynein was preincubated with NEM for 200 sec.

best experiment of the four shown it was clear that there was little if any enhancement of the ATPase activity of the bound dynein. Two of the experiments shown in Figure 3 were done on the same preparation of crude dynein and EDTA extracted axonemes. Experiment I (○, ● in Figure 3) was done immediately after the dialysis against Tris-EDTA, while experiment II (△, ▲ in Figure 3) was done 2 days later. It can be seen that brief incubation with NEM caused no inhibition of the fresh dynein but a small amount of inhibition in the dynein that had been stored in the refrigerator for 2 days. The ability of the fresh dynein to rebind to fresh EDTA extracted axonemes did not, however, differ from the ability of the 2-day old dynein to bind to 2-day old axonemes.

The experiments shown in Figure 3 were done by incubating crude dynein with high concentrations of NEM for various times. Experiments were also done in which the dynein was incubated with various concentrations of NEM for fixed times, with results shown in Figure 4. When increasing concentrations of NEM were incubated with dynein for 100 or 200 sec there was a more than twofold increase in the ATPase activity and a sharp loss of the ability of the dynein to bind to EDTA extracted axonemes. There was little difference in the results obtained from 100 sec *vs.* 200 sec preincubations. The inset to Figure 4 compares the amount of binding computed by method S with the amount of binding computed by method P. In the control experiments (dynein exposed to cysteine but not to NEM) there was an activation of the ATPase of the dynein when it bound to the axonemes, as noted in the discussion of Figure 2. Exposure of the dynein for 200 sec to 0.78 mM NEM reduced the amount bound to about 37% on either computation, *i.e.*, 37% of the dynein ATPase bound to the axonemes but there was no enhancement of the activity as a consequence of binding. The same results were obtained for the experiment done at 100-sec preincubation but are not plotted in the inset to Figure 4 since the points fall close to those shown. Thus NEM enhances the ATPase activity of crude dynein, interferes with its ability to bind to EDTA treated axonemes, and prevents the activation of its ATPase activity.

An experiment was also done in which the effect of NEM on the ATPase and binding ability of purified 30S dynein was measured. Preincubation of this preparation of 30S dynein with low concentrations of NEM caused almost a 40% enhancement of the ATPase activity which declined to 10% at higher NEM. As with crude dynein, there was a loss of binding ability when computed by method S, so that in the range of NEM concentration utilized binding dropped from about 70% to about 35% as computed from the changes in supernatant activity. The control dynein was very strongly activated on binding, its activity increasing over twofold. Treatment of the 30S dynein with NEM caused a marked reduction in this activation, so that 30S dynein which has been preincubated with 6.3 mM NEM bound to EDTA extracted axonemes to almost the same extent whether the data were computed by eq 1 or eq 2.

The preceding experiments with NEM were done by reacting NEM with dynein and then, after destroying all the unreacted NEM with cysteine, testing the ability of the dynein to bind to untreated EDTA extracted axonemes. Similar experiments were done in which EDTA extracted axonemes were preincubated with NEM, the unreacted NEM then destroyed by addition of cysteine, and untreated dynein allowed to bind onto the NEM-treated axonemes. We have earlier reported that even after two extractions with Tris-EDTA a significant amount of ATPase activity remains in the axonemes (Blum, 1973). It is not yet clear whether this represents unextractable dynein, a contaminating ATPase, or an ATPase other than dynein which is an integral part of the cilium. It was therefore necessary to measure the effect of NEM on this axonemal ATPase as well as on the capacity of the axonemes to bind untreated dynein. Figure 5 shows that preincubation of EDTA extracted axonemes with NEM caused about a 40% loss of axonemal ATPase within 2 min, after which there was little further loss of activity. In two experiments the binding of dynein to NEM-treated axonemes was increased by about 15% and in one experiment it was decreased by up to 20%. Thus to a first approximation one can say that treatment of EDTA-extracted axonemes with NEM causes a loss of almost half the axonemal ATPase but no appreciable change in the ability of the axonemes to bind dynein. Although not shown in Figure 5, the binding was computed by both method S (eq 1) and by method P (eq 2). NEM treatment of axonemes did not appreciably alter their ability to activate the ATPase activity of the dynein which bound onto the axonemes.

Interaction of Dynein with 4S "Matrix" Protein. When glycerinated *Tetrahymena* cilia are extracted with Tris-EDTA and the crude dynein is subjected to sucrose density gradient sedimentation, a large peak of 30S dynein, a small peak of 14S dynein, and a peak of matrix protein (mostly microtubular protein) sedimenting at 4S are obtained (Gibbons, 1965a; Raff and Blum, 1969). Otokawa (1972)

showed that the addition of 4S protein to 30S dynein caused a small but definite increase in the ATPase activity of 30S dynein. We have confirmed that observation, although we get only a small (~10–20%) enhancement of the ATPase activity. It was also found that 4S protein did not alter the activity of 14S protein.

Discussion

When 30S dynein rebinds to EDTA extracted axonemes in the presence of Mg^{2+} the arms are restored (Gibbons, 1965a) and the ability to respond to the addition of ATP by a change of turbidity is also restored (Gibbons, 1965b; Raff and Blum, 1969). This suggests that 30S dynein is a major component of the arms and is the mechanochemically effective ATPase. Although binding of the dynein restores the light-scattering response to ATP, the ability of ATP to cause swelling of a ciliary pellet is not restored (Raff and Blum, 1969). Thus the rebinding of 30S dynein to extracted axonemes restores only part of the complete system necessary for cyclic bending. For the sliding filament mechanisms to operate the arms must act as bridges between the A subfiber of one doublet and the B subfiber of the doublet adjacent to it (see *e.g.*, Summers and Gibbons, 1973). The failure of the rebound dynein to restore even the swelling response may indicate that other proteins are required (see, *e.g.*, Linck, 1973; Kincaid *et al.*, 1973), or that the dynein does not reposition itself as it was *in vivo*. It is well established that extracted dynein differs in several properties from unextracted dynein (Brokaw and Benedict, 1971; Gibbons and Fronk, 1972; Hayashi and Higashi-Fujime, 1972; Blum, 1973), but differences between extracted dynein and dynein after rebinding to EDTA-extracted axonemes are less well established.

Hayashi and Higashi-Fujime (1972) found that after overnight binding at 4° the rebound dynein had properties similar to those when it was *in situ* in the unextracted axonemes, *i.e.*, differing from those of the extracted dynein. Ogawa and Mohri (1972) found that the activity lost from the supernatant after a 2-day dialysis was recovered quantitatively in the axonemes, suggesting that no change had occurred in the activity of the dynein on rebinding. Ogawa (1973) treated sea urchin dynein with trypsin and found that the fragment containing the ATPase activity could not recombine with EDTA extracted axonemes but was activated by the addition of tubulin. In the present experiments, rebinding was allowed to occur for 30 min at 25°. This has the advantage of preventing the loss of ATPase by denaturation but, on the other hand, does not allow equilibrium to be attained. The finding that in three-fourths of the experiments there was an appreciable enhancement of the ATPase activity on binding is of interest since it demonstrates that a change in properties such as is observed when dynein is extracted from axonemes into solution can also occur when the extracted dynein repositions itself in the axonemal matrix. In one respect, at least, these changes are not reciprocal. When glycerinated *Tetrahymena* cilia are extracted with Tris-EDTA there is an increase in total ATPase activity (Blum, 1973). When crude dynein or 30S dynein from a sucrose density gradient rebinds to the axonemes, there also is an increase in total ATPase activity. If the dynein reentered the axonemal matrix in its original position and configuration, one might have expected to find a decrease in ATPase activity rather than an enhancement. The reason why enhancement was observed only three-fourths of the time is not known. The finding that there may be two forms

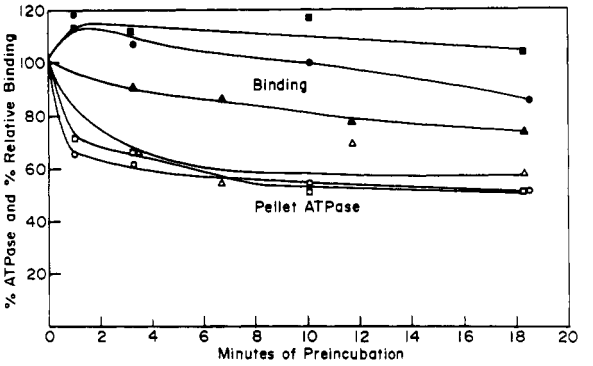

FIGURE 5: Effect of preincubation of EDTA extracted axonemes with NEM on axonemal ATPase and on the subsequent binding of dynein. EDTA extracted axonemes and crude dynein were prepared from three different cilia preparations: (○, ●) experiment I, cilia preparation 69; (□, ■) experiment II, cilia preparation 68; (△, ▲) experiment III, cilia preparation 59. Note that dynein and axonemes used in expt I and II are the same as the dynein and axonemes used in expt I and II, respectively, of Figure 5. Each experiment was done by taking a preparation of EDTA extracted axonemes, reacting it with NEM at 25° for the time shown on the abscissa, and stopping the reaction with cysteine. Controls received only the cysteine. The NEM-treated axonemes were then incubated for 30 min alone or with added untreated crude dynein and after centrifugation the ATPase activity of the supernatant and the precipitate was measured as described in the section on Methods. The per cent binding was computed by method P (eq 2) and is shown as the relative per cent binding, *i.e.*, with the control value set at 100% for each experiment. The concentrations of NEM and axonemal protein during the preincubation periods were: expt I, 6.25 mM and 1.82 mg/ml; expt II, 6.25 mM and 1.16 mg/ml, expt III, 3.13 mM and 0.886 mg/ml. In each experiment 0.1 ml of the above mixture was added to 0.8 ml of 25 mM cysteine to destroy any unreacted NEM and in each experiment 0.4 ml of the NEM-treated axonemes was added to 1.6 ml of IM buffer containing the following amounts of crude dynein: expt I, 0.099 mg; expt II, 0.134 mg; expt III, 0.135 mg; 100% ATPase corresponds to 152 nmol min^{-1} mg^{-1} for expt II and 85.3 nmol min^{-1} mg^{-1} for expt III; 100% relative binding corresponds to 32.1% binding as computed by method P (eq 2) for expt I, to 6.30% for expt II, and to 36.3% for expt III.

of dynein which differ in extractability as well as another protein which is associated with the dyneins (Linck, 1973; Kincaid *et al.*, 1973) suggests possible avenues for further exploration of the conditions required for enhancement of the ATPase activity to be observable all the time.

It should be noted that the increase in ATPase activity of 30S but not 14S dynein upon interaction with 4S protein (mostly tubulin) (Otokawa, 1972, and present data) is smaller than the degree of enhancement observed in many of our experiments on dynein binding to EDTA extracted axonemes. Whether this is because the binding to tubulin dimers is not as effective as is binding to polymerized tubulin or because of other proteins associated with the doublets *in situ* is not known, but it is clear that at least part of the ability to cause enhancement of dynein ATPase resides in the soluble protein(s) of the 4S fraction.

It is well established that flagellar motility is inhibited by SH poisons (Hoffman-Berling, 1955; Child, 1959; Karaev and Aivazyan, 1966). The swelling response of glycerinated *Tetrahymena* cilia is strongly inhibited by NEM if the cilia are preincubated with NEM in the absence of ATP (Blum and Hayes, 1974). It was therefore of interest to inquire whether the sensitivity to NEM resides largely on the dynein or on the EDTA extracted axonemes. We have earlier reported that incubation of NEM with crude dynein caused a large increase in ATPase activity, whereas partially purified 30S dynein obtained from a sucrose density gradient exhibited only a small increase in ATPase activity (Blum

and Hayes, 1974). The present results show that in both cases the increase in ATPase activity is associated with a sharp decline in the ability of the dynein to rebind to EDTA extracted axonemes and its ability to cause enhancement of the dynein ATPase activity. Incubation of EDTA extracted axonemes with NEM, however, caused little change in ability of native dynein to bind to the axonemes. The inhibition of the unextracted axonemal ATPase contrasts with the activation of extracted dynein, but does not contribute to deciding whether the unextracted axonemal ATPase is a separate ATPase characteristic of the cilia, a contaminating ATPase, or unextracted dynein which is affected differently by NEM than is extracted dynein. For present purposes, it does not matter which of these alternatives is correct, since the reaction of the EDTA-extracted axonemes with NEM did not appreciably alter the binding of native dynein to axonemes, whereas NEM markedly reduced the ability of dynein to bind to native EDTA extracted axonemes. It is reasonable to assume, therefore, that the loss of motility of cilia and flagella upon exposure to SH reagents and the loss of the pellet height response of glycerinated *Tetrahymena* cilia is caused primarily by reaction of an SH group(s) on the dynein which "uncouples" the ATPase and prevents it from effective participation in the cross bridge mechanochemical cycle.

Many studies (Gray, 1923; Holwill and Silvester, 1965; Sleigh, 1962; Holwill, 1970) have shown that both cilia and flagella are unusually sensitive to temperature; beat frequency increases with temperature up to about 30–40°, depending on the species, but at higher temperatures irreversible thermal damage occurs. Earlier work (Blum, 1973) had shown that dynein ATPase was very sensitive to thermal denaturation at temperatures above 47°. The present data show that dynein is remarkably sensitive to temperature. In the range from about 41 to 46° brief heating increases the ATPase activity. This activation then changes to inhibition with further time at temperature, the inhibition becoming dominant more rapidly at the higher temperatures. Concomitant with the increase in ATPase activity there is a loss of the ability of the dynein to bind to EDTA extracted axonemes and of the enhancement of its activity on binding. These changes, which can be considered as a thermally induced uncoupling, might of themselves be sufficient to account for the above noted sensitivity of ciliary beating to thermal denaturation. In contrast to NEM, which appears to affect the dynein but not the EDTA extracted axonemes, however, the axonemal binding sites are also highly sensitive to thermal denaturation. Heating the EDTA extracted axonemes at 42°, for example, rapidly destroyed the ability of the axonemes to enhance the ATPase activity of dynein and then reduced the amount of dynein that was bound. Thus both the site(s) on dynein and the sites on the axonemes which are required for the binding and for the enhancement of ATPase activity are very thermolabile. The loss in pellet height response of *Tetrahymena* cilia by heating at 40° for 11 min (Raff and Blum, 1966) thus probably results from the thermal denaturation of both sets of binding sites.

Since the binding of dynein to EDTA-extracted axonemes restores the arms, it seems clear that dynein rebinds onto the A subfibers. There is at present no information concerning the interaction between the opposite ends of the arms and the B subfibers even in native flagella or cilia. The inability of the reconstituted axonemes to exhibit the swelling response suggests that this interaction is not adequately restored. It is not known whether the enhancement of the ATPase activity on binding reflects interaction between the arms and the sites on the B subfibers or between the arms and the A subfibers, and much further work will be required to achieve an understanding of the cross bridge cycle in ciliary motility. The present work shows that sites on both the arms and the EDTA extracted axonemes are very sensitive to thermal denaturation and that an SH group(s) on the dynein is involved in both the ATPase activity and the binding of the dynein to the axonemes, and thus provides some potentially useful tools for further investigation.

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