

## Modification of the Microtubule-Binding and ATPase Activities of Kinesin by *N*-Ethylmaleimide (NEM) Suggests a Role for Sulfhydryls in Fast Axonal Transport<sup>†</sup>

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Received March 20, 1989; Revised Manuscript Received July 15, 1989

**ABSTRACT:** *N*-Ethylmaleimide, an agent which alkylates free sulfhydryls in proteins, has been used to probe the role of sulfhydryls in kinesin, a motor protein for the movement of membrane-bounded organelles in fast axonal transport. When squid axoplasm is perfused with concentrations of NEM higher than 0.5 mM, organelle movements in both the anterograde and retrograde directions cease, and the vesicles remain attached to microtubules. Incubation of highly purified bovine brain kinesin with similar concentrations of NEM modifies the enzyme's microtubule-stimulated ATPase activity and promotes the binding of kinesin to microtubules in the presence of ATP. These results suggest that alkylation of sulfhydryls on kinesin alters the conformation of the protein in a manner that profoundly affects its interactions with ATP and microtubules. The NEM-sensitive sulfhydryls, therefore, may provide a valuable tool for the dissection of functional domains of the kinesin molecule and for understanding the mechanochemical cycle of this enzyme.

The microtubule (Mt)-stimulated ATPase kinesin has been proposed to be a motor protein responsible for the movement of membrane-bounded organelles in fast axonal transport in neurons (Brady, 1985; Vale et al., 1985a). Recent biochemical, immunological, and ultrastructural studies on highly purified kinesin from bovine brain have supported this proposition and provided a detailed description of its molecular architecture (Bloom et al., 1988; Kuznetsov et al., 1988; Hirokawa et al., 1989; Pfister et al., 1989; Wagner et al., 1989). Kinesin is a highly elongated heterotetramer, consisting of two 124 000-dalton heavy-chain polypeptides and two 64 000-dalton light chains (Bloom et al., 1988; Kuznetsov et al., 1988; Wagner et al., 1989). On one end of kinesin are two globular domains which contain those portions of the heavy chains that interact with both Mt and ATP (Gilbert & Sloboda, 1986; Penningroth et al., 1987; Bloom et al., 1988; Hirokawa et al., 1989; Kuznetsov et al., 1989; Yang et al., 1989; Scholey et al., 1989). The opposite end of kinesin, which has a fanlike shape, contains the light chains, and is believed to interact with membrane-bounded organelles (Hirokawa et al., 1989). Monoclonal antibodies to both the heavy and light chains show that kinesin is located on membrane vesicles in a variety of cell types including primary rat neuronal and glial cells and squid axoplasm (Pfister et al., 1989; Brady et al., 1989).

From this basic understanding of the structure and function of kinesin, dissection of the functional domains of the protein can be extended by focusing on the sulfhydryl groups of kinesin. *N*-Ethylmaleimide (NEM) alkylates sulfhydryl groups and has been used to identify specific sulfhydryls associated with the functional sites of a wide variety of proteins, including the lac repressor, *S*-adenosylmethionine synthetase, and myosin (Sekine & Keilley, 1964; Brown & Matthews, 1979; Markham & Satishchandran, 1988). NEM alkylation of myosin, for

example, has identified two sulfhydryl groups with different reactivities which are important for mechanochemical functions (Sekine & Keilley, 1964; Yamaguchi & Sekine, 1966; Silverman et al., 1972; Wells & Yount, 1979; Burke & Knight, 1980). Alkylation of the most reactive sulfhydryl makes the binding of the myosin S1 fragment to F-actin insensitive to release by ATP and blocks the normal activation of myosin  $Mg^{2+}$ -ATPase by actin. Alkylation of this sulfhydryl also modifies two nonphysiological ATPase activities of myosin, inhibiting the  $K^{+}$ /EDTA-ATPase activity and stimulating the  $Ca^{2+}$ -ATPase. When both sulfhydryls are alkylated, the  $Ca^{2+}$ -ATPase activity is inhibited. The fact that NEM-modified heavy meromyosin (NEM-HMM) will bind to, but not release from, actin filaments *in vivo* has made NEM-HMM a useful intracellular probe of myosin's role in a wide range of cellular systems (Meeusen et al., 1980; Brady et al., 1985; Porrello et al., 1983; Cande, 1986).

The role of sulfhydryls in organelle transport was determined by analyzing the effect of NEM on the bidirectional transport of organelles in squid axoplasm and comparing the effect on fast axonal transport with *in vitro* characterization of two underlying molecular phenomena: the Mt-stimulated ATPase of kinesin and the binding of kinesin to Mts in the presence of ATP. The squid giant axon provides an especially useful model for study of organelle movements in fast axonal transport. The cytoplasmic contents of the axon can be extruded intact from the plasma membrane, creating a cylinder of axoplasm in which both the anterograde and retrograde directions of fast axonal transport can be observed by video-enhanced light microscopy for hours (Brady et al., 1982, 1985). Since the plasma membrane and other permeability barriers have been removed, molecules that do not cross the plasma membrane can be introduced at defined concentrations by perfusion into the native axoplasm. For example, nucleotides, regulatory proteins, antibodies to axoplasmic proteins, and isolated organelles have been perfused into axoplasm (Brady et al., 1982, 1984, 1985, 1989; Gilbert & Sloboda, 1984; Lasek & Brady, 1985; Schroer et al., 1985; Johnston et al., 1987). These studies provided biochemical and pharmacological

<sup>†</sup> This work was supported by NIH Grant NS23868 (S.T.B. and G.S.B.), NIH Postdoctoral Fellowship Award GM10143 (K.K.P.), Grant DMB-8701164 from the National Science Foundation Biological Instrumentation Program (G.S.B. and S.T.B.), and Welch Foundation Grant I-1077 (G.S.B. and S.T.B.).

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analyses of the mechanochemistry of membranous organelle transport along Mts.

When kinesin and microtubules are mixed and centrifuged in the presence of ATP, kinesin is found primarily in the supernatant, not the Mt pellet (Brady, 1985; Vale et al., 1985a). In the presence of ATP, the interaction of kinesin with Mts is transient, and ATP promotes release of kinesin from Mts. However, in the presence of 5'-adenylyl imidodiphosphate (AMPPNP), a nonhydrolyzable ATP analogue, most of the kinesin pellets with Mts (Brady, 1985; Vale et al., 1985a). AMPPNP, therefore, stabilizes the association between kinesin and Mts. This nucleotide-sensitive binding to Mts is an important part of the mechanochemical cycle of the ATPase and has become a diagnostic property for the identification of kinesin in a wide range of species and cell types (Brady, 1985; Scholey et al., 1985; Vale et al., 1985a; Amos, 1987; Murofushi et al., 1988; Saxton et al., 1988). Nucleotide-sensitive binding of kinesin to Mts is associated with the hydrolysis of ATP by the enzyme, and kinesin ATPase activity is substantially activated by Mts (Brady, 1985; Kuznetsov & Gelfand, 1986; Wagner et al., 1989).

Comparison of the properties of fast transport in axoplasm with the *in vitro* properties of purified kinesin allows us to better understand the roles played by kinesin in the molecular mechanisms of fast axonal transport. Perfusion of squid axoplasm with NEM stops the movement of organelles in both the anterograde and retrograde directions, causing the organelles to remain attached to the Mts. These effects of NEM in axoplasm suggested that nucleotide-sensitive binding of kinesin to Mts and possibly the Mt-stimulated ATPase activity of kinesin had been altered by alkylation of kinesin with NEM. As predicted, treatment of highly purified bovine brain kinesin with comparable concentrations of NEM did stabilize the binding of kinesin to Mts in the presence of ATP, and modified the Mt-stimulated ATPase activity of kinesin. The effects of NEM indicate that one or more sulfhydryls in kinesin are involved in the mechanochemical function of this motor protein for fast axonal transport.

#### EXPERIMENTAL PROCEDURES

**Squid Axoplasm Model.** Isolated axoplasm was prepared by extrusion from the axon as described previously (Brady et al., 1982, 1985) and perfused with half-strength buffer X (175 mM potassium aspartate, 65 mM taurine, 35 mM betaine, 25 mM glycine, 6.5 mM MgCl<sub>2</sub>, 5 mM EGTA, 1.5 mM CaCl<sub>2</sub>, 0.5 mM glucose, 1 mM ATP, and 10 mM HEPES, adjusted to pH 7.2 with KOH) that was supplemented with various concentrations of NEM ranging from 0.1 to 10 mM. Transport of organelles in both anterograde and retrograde directions was analyzed by using AVEC-DIC microscopy (Allen et al., 1981; Brady et al., 1982, 1985; Allen & Allen, 1983). Preparations were followed for at least 30 min, and a number of regions in both the interior and the periphery of the axoplasm were examined. For tabular presentation of the data, movement was scored by using the following system: +, normal or near-normal movement;  $\pm$ , partial reduction in the number of moving particles; -, complete inhibition of organelle transport.

**ATPase and Microtubule-Binding Assays.** Kinesin and tubulin used for *in vitro* assays were prepared as described previously (Wagner et al., 1989). Two different buffer systems were utilized: IME buffer (15 mM imidazole, 2 mM MgCl<sub>2</sub>, and 1 mM EGTA, pH to 7.0 with HCl) or buffer X2 (150 mM potassium aspartate, 10 mM HEPES, 1 mM EGTA, and 1 mM MgCl<sub>2</sub>, pH to 7.2 with KOH). For binding assays, kinesin (0.16–0.22 mg/mL), taxol-stabilized Mts made from

purified tubulin (1 mg/mL), or kinesin plus Mts were mixed in either IME buffer or buffer X2 with concentrations of NEM ranging from 0.1 to 5 mM for 30 min at room temperature. At the end of the incubation time, dithiothreitol (DTT) was added to a final concentration of 20 mM to quench the unreacted NEM. NEM-treated samples of kinesin or Mts were mixed with taxol-stabilized Mts or kinesin, respectively. Those mixtures, as well as samples of kinesin plus Mts that had been treated with NEM together, were supplemented with 10 mM ATP (final concentration), incubated at 37 °C for 15 min, and pelleted through a 30% sucrose cushion in IME or X2 plus ATP at 100000g (54000 rpm) for 30 min in a TLA 100.2 rotor in a Beckman TL-100 ultracentrifuge. Supernatants and pellets were mixed with SDS sample buffer, and equivalent volumes were analyzed by SDS gel electrophoresis. Quantitative densitometry of SDS-polyacrylamide gels was performed as described previously (Bloom et al., 1988). Similar results were obtained regardless of whether IME or X2 buffer was used. For studies on the effect of NEM on ATPase activity, kinesin in IME buffer was incubated with the indicated concentrations of NEM at room temperature for 15 min. The reaction was stopped by the addition of DTT again to a final concentration of 20 mM. The ATPase assay was performed as described by Wagner et al. (1989).

**Incorporation of [<sup>3</sup>H]NEM into Kinesin.** Highly purified kinesin (51  $\mu$ g) was reacted with 50  $\mu$ Ci of [<sup>3</sup>H]NEM (41.3 Ci/mmol) for 15 min, precipitated with trichloroacetic acid (TCA), and analyzed by SDS-polyacrylamide gel electrophoresis as described above. Labeled polypeptides were visualized by fluorography. To quantify the incorporation of [<sup>3</sup>H]NEM into kinesin, Coomassie blue stained bands were excised from the gels, solubilized with H<sub>2</sub>O<sub>2</sub>, and analyzed by scintillation counting (Black & Lasek, 1980).

#### RESULTS

Axoplasm that has been extruded from the membrane sheath continues to support bidirectional organelle transport, with properties essentially unchanged from those observed in the intact axon (Allen et al., 1982; Brady et al., 1982, 1985). The rate of anterograde organelle movement (the direction away from the nucleus, toward the synaptic terminal) is approximately 1.8  $\mu$ m/s, while that of retrograde transport (the direction toward the nucleus) is approximately 1.1  $\mu$ m/s. When axoplasm was perfused with buffers containing ATP, organelle transport was maintained. However, following perfusion with the buffer, some organelles detached from Mts, and both Mts and vesicles underwent independent thermal (Brownian) movement. This movement was readily distinguishable from organelle transport along Mts (Allen et al., 1982, 1983; Brady et al., 1982, 1983, 1985). Perfusion of axoplasm with buffers containing 1 mM NEM rapidly inhibited the translocation of endogenous organelles moving in both the anterograde and retrograde directions [Figure 1, and compare with moving organelles in Figures 1 and 2 of Brady et al. (1982); see also Brady et al. (1983, 1984, 1985) and Allen et al. (1983, 1985)]. To determine if different concentrations of NEM would allow us to discriminate between inhibitory effects on anterograde versus retrograde axonal transport, isolated axoplasm preparations were perfused with concentrations of NEM between 0.1 and 10 mM. There was no measurable difference between the effect of NEM on anterograde versus retrograde transport at any concentration of NEM used (Table I). NEM at concentrations as low as 0.5 mM stopped the movement of most organelles throughout the axoplasm. However, the velocity of those organelles which continued to move was comparable to those moving in un-

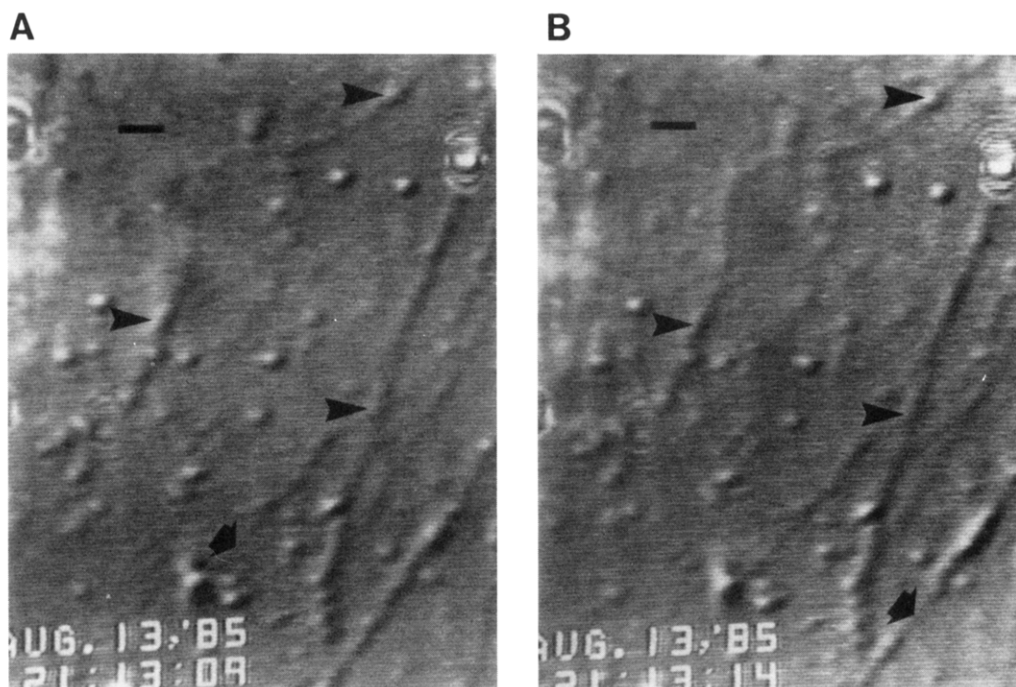


FIGURE 1: Inhibition of organelle movement by 1 mM NEM. Two frames of a video record, taken 5 s apart, of a region near the periphery of an axoplasm preparation illustrate the absence of organelle translocation following exposure of axoplasm to buffer containing 1 mM NEM. At this concentration, there was no preferential effect on either anterograde or retrograde movements. The observed Mts and organelles are not attached to the coverslip and are therefore moving about in the buffer. The arrowheads indicate several examples of different sized stationary organelles apparently attached to Mts, and the arrows point to relatively bare segments of Mts. Bars represent 1 micrometer. Compare with moving axoplasm [see Figures 1 and 2 of Brady et al. (1982)].

Table I: Comparison of the Effects of NEM on Purified Kinesin and Fast Axonal Transport

	organelle transport in squid axoplasm		properties of bovine kinesin	
	anterograde transport	retrograde transport	% kinesin bound to MTs	ATPase (% of control)
0 NEM	+	+	15	100
0.1 mM NEM	+	+	31	101
0.5 mM NEM	±	±	38	108
1.0 mM NEM	—	—	49	85
2.5 mM NEM	—	—	63	57
5.0 mM NEM	—	—	84	32
10.0 mM NEM	—	—	ND	21

treated axoplasm. All concentrations of NEM which inhibited motility also resulted in the decoration of Mts with membranous organelles (Figure 1).

Although isolated axoplasm is composed of hundreds of different polypeptides, many of which have reactive sulfhydryls, NEM inhibition of bidirectional fast axonal transport suggested that one or more components of the mechanochemical machinery for organelle transport could be perturbed by NEM. In preliminary experiments, it was found that treatment of isolated synaptic vesicles with 10 mM NEM did not inhibit the ability of those vesicles to move when reintroduced into axoplasm (Brady & Schroer, 1985; Brady, 1987). These experiments indicated that alkylation of an integral membrane component of the vesicle was not likely to be responsible for the effects of NEM on fast axonal transport. Therefore, we examined the possibility that either Mts or kinesin was sensitive to NEM.

To determine whether NEM affected kinesin, a motor protein for organelle transport, we determined the effects of NEM on kinesin Mt-stimulated ATPase activity. NEM alkylation of kinesin was found to affect the Mt-stimulated ATPase in a concentration-dependent manner. Figure 2 shows

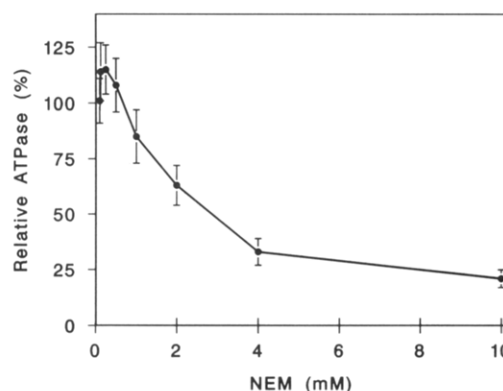


FIGURE 2: Mt-stimulated ATPase activity of kinesin is inhibited when kinesin is modified by NEM. Highly purified kinesin was reacted with various NEM concentrations ranging from 0.1 to 10 mM. The points on the graph are averages ( $\pm$ SEM) of experiments done on three kinesin preparations ( $n = 6$ ). The Mt-stimulated ATPase activity of untreated, control samples of kinesin was set equal to 100%, and under these incubation conditions, the Mt-stimulated ATPase of kinesin was inhibited 50% by 2.5 mM NEM.

that concentrations of NEM greater than 0.5 mM inhibited the Mt-stimulated ATPase activity, with half-maximal inhibition occurring at approximately 2.5 mM NEM. We also observed a small (15%) but reproducible increase in the Mt-stimulated ATPase activity when kinesin was reacted with concentrations of NEM of 0.1–0.5 mM (Figure 2). As would be expected for an agent which covalently modifies proteins, preliminary kinetic experiments indicate that inhibition of the ATPase activity by NEM is of the noncompetitive type.

Inhibition of the Mt-stimulated ATPase activity by NEM might explain the inhibition of organelle movement observed in the axoplasm. However, the dose-response curves for the inhibition of kinesin ATPase activity and axoplasmic organelle transport did not correlate exactly (Table I). Since treatment of the axoplasm with NEM resulted in organelles remaining

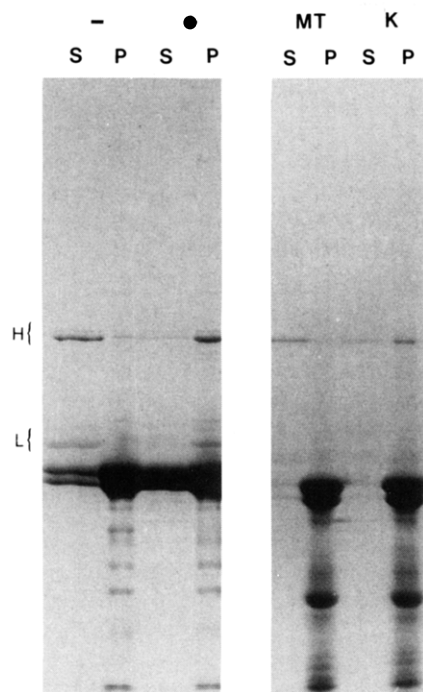


FIGURE 3: NEM modification of kinesin, but not Mts, stabilizes the binding of kinesin to Mts in the presence of ATP. Supernatant (S) and pellet (P) fractions, obtained as described below, were analyzed by SDS-PAGE. (Left panel) Kinesin and taxol-stabilized Mts were mixed with [(+) pair of lanes] or without [(-) pair of lanes] 2 mM NEM. Following a 15-min incubation, ATP (10 mM final concentration) was added and the mixture centrifuged. In the absence of NEM [(-) lanes], most of the kinesin remained in the supernatant (S). In contrast, when NEM was present [(+) lanes], most of the kinesin, both heavy (H) and light (L) chains, pelleted with the Mts (P). Very little kinesin remained in the supernate (S). (Right panel) Purified kinesin (K lanes) or taxol-stabilized Mts (Mt lanes) were incubated separately with 2 mM NEM; then the reaction was quenched with DTT. Unmodified Mts or kinesin was added to the NEM-treated kinesin or Mts, respectively. ATP (10 mM final concentration) was added to the reaction mixtures, and samples were centrifuged as above. When Mts were reacted with NEM alone (MT lanes), most of the kinesin remained in the supernatant fraction (S). However, when kinesin alone was exposed to NEM (K lanes), most of the kinesin pelleted despite the presence of ATP. Therefore, it is the modification of the kinesin by NEM, not the modification of Mts, that promotes kinesin binding to the Mts. The polypeptides with a greater electrophoretic mobility than tubulin are apparent breakdown products of the tubulin, which were present in some tubulin preparations but did not affect the results.

attached to Mts, NEM might have modified the interaction of kinesin with Mts in a manner distinct from its effect on the ATPase. The effects of NEM on the association of the kinesin with Mts were determined by using a previously described kinesin-Mt binding assay (Brady, 1987; Bloom et al., 1988).

When purified kinesin and taxol-stabilized Mts were incubated with ATP and centrifuged, both the heavy and light chains of the enzyme were found predominantly in the supernatant fraction (Figure 3). If comparable mixtures of kinesin and Mts were incubated with 2 mM NEM prior to incubation and centrifugation in the presence of ATP, most of the kinesin was found to sediment (Figure 3). In this *in vitro* assay, the only proteins present that might serve as a site for modification by NEM were kinesin and tubulin. Therefore, NEM alkylation of either tubulin or kinesin stabilized the binding of the enzyme to Mts even in the presence of ATP.

To determine which protein was the relevant target of NEM, kinesin and Mts were incubated separately with NEM. After unreacted NEM was quenched with dithiothreitol (DTT), the NEM-treated kinesin and Mts were mixed with ATP and their

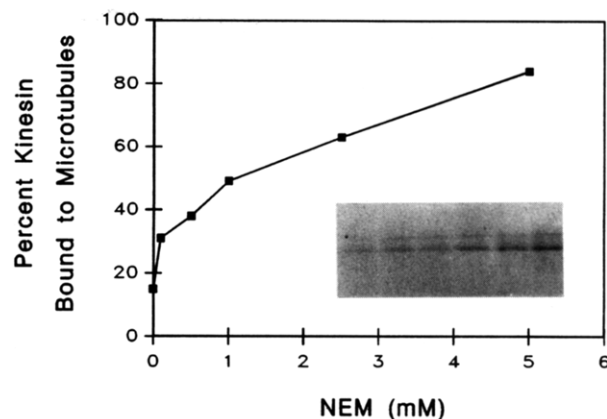


FIGURE 4: Amount of kinesin which pellets with Mts in the presence of ATP increases as kinesin is reacted with increasing concentrations of NEM. Kinesin alone was reacted with a range of NEM concentrations from 0 to 5 mM and then mixed with unmodified Mts and ATP (10 mM). Samples were then centrifuged and analyzed by SDS gel electrophoresis. Quantitative densitometry of SDS-polyacrylamide gels was then used to measure the amount of sedimentable kinesin at each concentration of NEM. The amount of sedimentable kinesin was graphed as a percent of the total kinesin in the supernate and pellet. The inset shows the portion of the gel which was used for the densitometry. While multiple isoforms of the kinesin heavy chain can be resolved by SDS-PAGE, the quantitation here was done only with the major form of the heavy chain (lower band) as detectability of the minor form (upper band) was not in the linear range of protein concentration for the lanes with the lower NEM concentrations. From left to right, the lanes are 0, 0.1, 0.5, 1.0, 2.5, and 5 mM NEM.

unmodified binding partner (either Mts or kinesin, respectively). Finally, the protein mixtures were centrifuged after a brief incubation period with ATP to allow binding to occur. Figure 3 shows that when Mts alone were reacted with NEM, most of the kinesin remained in the supernatant in the presence of ATP. This result is comparable to that which was observed with untreated proteins. However, when only the kinesin was modified by NEM, both the heavy and light chains cosedimented with the Mts in the presence of ATP. The stabilization of kinesin binding to Mts was similar to that observed when both components were treated with NEM. Kinesin modified by NEM did not sediment in the absence of Mts (data not shown). Together, these observations indicate that NEM modification of kinesin, but not of tubulin, was responsible for stabilizing the binding of the enzyme to Mts in the presence of ATP.

The effect of NEM on kinesin binding was analyzed quantitatively. Kinesin modified by various concentrations of NEM was mixed with purified tubulin and ATP, and the solutions were centrifuged. The levels of kinesin in the pellets were measured by quantitative densitometry of SDS-polyacrylamide gels. The amount of kinesin bound to Mts in the presence of ATP was dependent on the concentration of NEM used (Figure 4). Concentrations of NEM as low as 0.1 mM increased the amount of kinesin bound to Mts. Following treatment with 5 mM NEM, 85% of the kinesin sedimented in the presence of ATP. This level is almost 6-fold higher than comparable samples which had not been exposed to the alkylating reagent.

One or both of the subunit polypeptides of kinesin might be subject to alkylation by NEM. In order to determine which were modified by NEM, kinesin treated with an approximately 10-fold molar excess of [ $^3$ H]NEM was analyzed by SDS-PAGE fluorography (Figure 5). Multiple isoforms of both the heavy and the light chains (Pfister et al., 1989; Wagner et al., 1989) incorporated [ $^3$ H]NEM, indicating that sulfhydryls on both sets of polypeptides of kinesin are susceptible

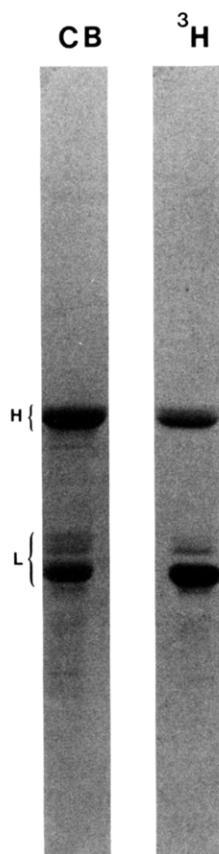


FIGURE 5: NEM modifies both the heavy and light chains of kinesin. The heavy (H) and light (L) chains of kinesin were labeled with [ $^3\text{H}$ ]NEM, indicating that both contain sulfhydryls sensitive to NEM. CB is the Coomassie blue stained gel, and  $^3\text{H}$  is the fluorograph of the gel.

Table II: Incorporation of [ $^3\text{H}$ ]NEM into Kinesin Heavy and Light Chains

polypeptide	mol of [ $^3\text{H}$ ]NEM/mol of polypeptide
heavy chain (124 K)	0.77
light chain (64 K)	2.41

to modification by NEM. The light chains, however, incorporated more NEM than the heavy chains under these conditions. The molar ratio of NEM to kinesin subunit polypeptide was 0.8 for the heavy chains and 2.4 for the light chains (Table II). These results indicate that the native kinesin molecule has at least six sites which react with NEM, a minimum of one for each of the heavy chains and two for each of the light chains.

#### DISCUSSION

NEM, which alkylates free sulfhydryls in proteins, has proven to be an effective tool for modifying both organelle movement in fast axonal transport and the *in vitro* properties of a purified motor protein for organelle transport, kinesin. When perfused into isolated squid axoplasm, NEM inhibited both anterograde and retrograde organelle transport, and many of the vesicles remained firmly attached to Mts. In reconstituted, *in vitro* assay systems using purified bovine brain tubulin and kinesin, we have shown that NEM alkylates several sites on both the heavy and light chains of kinesin, thereby altering the Mt-stimulated ATPase of kinesin and stabilizing the binding of kinesin to Mts in the presence of ATP. The *in vitro* effects of NEM on kinesin activity are consistent with the inhibition by NEM of fast transport in the axoplasm.

The amount of kinesin which pelleted with Mts was dependent on the concentration of NEM used, suggesting that modification of one or more sulfhydryls in kinesin alters the conformation of the enzyme and promotes a stable interaction with Mts. Furthermore, since NEM-treated enzyme was bound to Mts even in the presence of ATP, we conclude that alkylation by NEM inhibits the release of kinesin from Mts by ATP. This suggests that NEM is modifying sulfhydryls which are important in the mechanochemical cycle of kinesin. The globular domains of the heavy chains of kinesin are known to contain both the ATP and Mt binding sites (Bloom et al., 1988; Hirokawa et al., 1989; Scholey et al., 1989; Yang et al., 1989). As a result, it is tempting to assume that a sulfhydryl site on the heavy chain near to or part of one of these functional sites is responsible for the change in kinesin-binding properties. However, a modification of the light chains, or even another portion of the heavy chain, could also change the enzymatic properties of kinesin. The regulation of the various myosins by modification of either the heavy or the light chain provides a precedent for such hypotheses [reviewed by Kuznicki (1986) and Warrick and Spudich (1987)]. The role of the light chains is currently unknown, although they are presumed to be involved in binding of kinesin to membrane-bounded organelles (Hirokawa et al., 1989).

When kinesin was reacted with relatively low concentrations of NEM (less than 0.5 mM), we routinely detected a small increase in the Mt-stimulated ATPase activity of kinesin. At higher concentrations of NEM (greater than 0.5 mM), there is a concentration-dependent inhibition of the Mt-stimulated ATPase. When kinesin was incubated with NEM for longer periods of time (greater than 60 min), the inhibition of the Mt-stimulated ATPase approached 100%. NEM had little effect on the basal ATPase of kinesin (data not shown). Therefore, a sulfhydryl group which can be alkylated by NEM affects some aspect of the cycle of Mt-stimulated ATP hydrolysis. Further analysis of the modification(s) in kinesin activity caused by NEM should provide insight into the mechanochemical cycle of kinesins, the role of the light chains in the enzyme, or even the regulation of kinesin by a portion of the molecule distant from the functional sites.

While the significance of these two effects of NEM on the ATPase activity is currently under investigation, comparison with NEM modification of myosin (Sekine & Kielley, 1964) suggests the possibility that more than one site on kinesin is being modified and that the sites have slightly different sensitivities to NEM. Recently, the binding of certain antibodies to the heavy chains of sea urchin kinesin was found to increase Mt-stimulated ATPase activity (Ingold et al., 1988), and proteolytic digestion of bovine brain kinesin can produce a fragment with substantially increased ATPase activity (Kuznestov et al., 1989). The increase in the Mt-stimulated ATPase activity of kinesin following alkylation with NEM could be related to the changes in kinesin activity induced by proteolysis or antibody binding.

The incorporation of [ $^3\text{H}$ ]NEM into kinesin indicates that the native kinesin molecule ( $M_r$  379 000) has at least six sulfhydryl sites subject to alkylation by NEM. A minimum of one site is associated with each of the two heavy chains, and no less than two sites are present on each of the two light chains. These numbers are in agreement with those obtained in preliminary experiments using other sulfhydryl reagents. The effects of NEM on kinesin are complex, causing an increase in the binding of kinesin to Mts, as well as both stimulatory and inhibitory modifications of the Mt-stimulated ATPase activity. Alkylation of the heavy chain, the light



chain, or both polypeptides may be responsible for the effects of NEM on kinesin. Further investigation will be required to determine if these various perturbations of kinesin activity can be obtained separately, or if the NEM modification which alters the ATPase activity is the same as that which causes the increase in Mt binding. Regardless of the final number or the location of the important NEM-sensitive sites, understanding the role of those sites alkylated by NEM will enhance our understanding of the mechanism of ATP hydrolysis by kinesin, and the mechanochemical cycle of organelle transport along Mts in fast axonal transport.

The utility of the squid axoplasm as an experimental paradigm of organelle transport has been confirmed in this study. Just as perfusion of the axoplasm model with AMPPNP led to the isolation of kinesin (Lasek & Brady, 1985; Brady 1985; Vale et al., 1985a), the inhibition by NEM of both anterograde and retrograde organelle movement has led to the discovery that NEM-sensitive sulfhydryls in the kinesin molecule affect kinesin binding to Mts and the Mt-stimulated ATPase activity. The complexity of the axoplasm, with a protein concentration of 24 mg/mL, makes it likely that there are many proteins with sulfhydryls capable of reacting with NEM. Nevertheless, the effects of NEM on fast axonal transport in squid axoplasm are similar to the effects of NEM on the binding of bovine brain kinesin to Mts in vitro with respect to both nature of inhibition and concentrations at which the inhibition occurs. As a result, the targets for NEM in isolated axoplasm which inhibit organelle transport in the axoplasm are likely to include kinesin.

The correlation between membrane-bounded organelle movements in axoplasm perfused with NEM and kinesin-induced microtubule gliding activities is not as high. Kinesin attached to glass coverslips promotes the movement of microtubules in one direction, equivalent to anterograde vesicle transport (Vale et al., 1985a). Previous reports on kinesin from sea urchin, squid, and bovine brain indicate that a concentration of 3–5 mM NEM is needed to achieve detectable inhibition of Mt gliding (Vale et al., 1985a,b; Porter et al., 1987). Porter et al. (1987) reported not only that Mts failed to move on coverslips coated with sea urchin kinesin treated with 5 mM NEM but also that the Mts actually remained in solution rather than attaching to the coverslip. In contrast, however, we find that NEM-modified kinesin binds to Mts even in the presence of ATP. While these differing results could simply reflect species differences of the kinesins, it is more likely that they reflect the properties of the assay system used. For example, NEM appears to inhibit ATP-sensitivity release of kinesin bound to Mts, but may not promote binding of free kinesin to Mts like AMPPNP does. When the effects of NEM alkylation of free sulfhydryls on the mechanochemical properties of kinesin are measured, the ATPase and Mt binding assays can be more easily quantitated and have greater sensitivity than the gliding assay.

On the basis of Mt gliding assays, it has been proposed that kinesin and cytoplasmic dynein are the anterograde and retrograde organelle transport motors, respectively (Vale et al., 1985b; Paschal & Vallee, 1987; Gibbons, 1988). Since we have shown that NEM inhibits bidirectional organelle movements in axoplasm, we were interested in comparing our data on the effects of NEM on kinesin in vitro with reports on bovine brain cytoplasmic dynein (MAP 1C). Treatment of cytoplasmic dynein with 0.1 mM NEM completely blocks Mt gliding, while 1 mM NEM is needed to inhibit 66% of the ATPase activity (Paschal et al., 1987; Shpetner et al., 1988). Therefore, the effects of NEM on kinesin and cytoplasmic

dynein are similar enough that this alkylating reagent cannot be used to distinguish the role of the two Mt-based motor proteins, kinesin and dynein, in organelle transport in axoplasm.

In conclusion, concentrations of NEM of 0.5 mM or greater inhibit bidirectional organelle transport when perfused into squid axoplasm. When highly purified bovine brain kinesin is reacted with equivalent concentrations of NEM, the amount of the enzyme bound to Mts in the presence of ATP is increased, and kinesin Mt-stimulated ATPase activity is altered in a dose-dependent manner. These results suggest that alkylation of sulfhydryls on kinesin induces a conformational change in the molecule which inhibits the release of kinesin from Mts by ATP. Further studies on the sulfhydryls of kinesin should provide valuable insights into the mechanochemical cycle of this motor protein.

#### ACKNOWLEDGMENTS

We thank Dr. Matthew Suffness of the National Cancer Institute for supplying us with taxol and David L. Stenoien, Heather Schiff, and Martha Stokely for providing technical assistance. The studies on squid axoplasm were performed at the Marine Biological Laboratory, Woods Hole, MA.

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## Theory for the Observed Isotope Effects from Enzymatic Systems That Form Multiple Products via Branched Reaction Pathways: Cytochrome P-450<sup>†</sup>

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Received April 24, 1989; Revised Manuscript Received June 30, 1989

**ABSTRACT:** By use of cytochrome P-450 as the prototype, kinetic descriptions are derived for the observed isotope effects for several models of enzymatic systems which are capable of generating multiple products from single substrates. The models include rapid and slow equilibria between enzyme-substrate orientations as well as multiple simultaneous and multiple sequential isotope effects. When an equilibrium is established between enzyme-substrate complexes that are responsible for the oxidation of different positions of the substrate, the kinetics can be represented by competing pathways from the same intermediate. When direct interchange between the complexes does not occur, the alternate pathway mimics the presence of a competitive inhibitor in the substrate solution. In general, the presence of alternate pathways in competition with the isotopically sensitive step will tend to unmask the intrinsic isotope effect.

One of the important techniques used for unraveling the mechanism of a chemical reaction, particularly in determining transition-state structure, is the measurement of the isotope effect (most commonly deuterium) associated with the reaction. Indeed, the technique has been successfully employed by chemists for more than half a century to study the mech-

anisms of many common homogeneous reactions. Its application to biochemical systems, however, has not been as straightforward because the interpretation of the observed isotope effect for an enzymatically catalyzed reaction is invariably complicated by the multistep nature of such reactions. Fortunately, in approximately the last decade the kinetics involved and the interpretation of the observed isotope effects in enzymatically mediated reactions have been elucidated and described (Northrop, 1978, 1981; Cleland, 1982).

In general, the experimental design used for isotope effect experiments can be divided into three categories: noncompetitive intermolecular, competitive intermolecular, and in-

<sup>†</sup>Supported in part by the National Institutes of Health (Grant GM 36922) and in part by a PRAT fellowship (K.R.K.) from the National Institutes of General Medical Sciences.

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