N-Ethylmaleimide Inhibits Ncd Motor Function by Modification of a Cysteine in the Stalk Domain†

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ABSTRACT: *N*-Ethylmaleimide (NEM), which reacts readily with exposed sulfhydryl groups, has been shown to inhibit the activity of the microtubule (MT) motors kinesin, Ncd, and dynein. Currently, the mechanism of inhibition is not known for any of these proteins. To investigate the mechanism by which NEM inhibits Ncd, the recombinant Ncd motor-stalk protein MC1 (modified claret 1) was treated with varying concentrations of NEM (0−10 mM) and cosedimentation and ATPase assays were used to assess the effects of modification on MC1 interactions with MTs. In the cosedimentation assay, treatment with ≤0.1 mM NEM enhanced MC1 binding to MTs in the presence of MgATP but had no effect on MC1 binding to MTs in the presence of MgAMP-PNP. In comparison, treatment with ≥0.5 mM NEM induced aggregation of MC1 and resulted in sedimentation of the motor in the absence of MTs. NEM modification had no effect on the basal ATPase rate but produced a decrease in the MT-stimulated ATPase rate. Labeling of MC1 with [^{3}H]NEM indicated that enhanced MT binding was associated with an average labeling of 1 Cys residue per MC1 polypeptide, while aggregation was associated with an average labeling of 2 Cys residues per MC1 polypeptide. Protein digestion, structural analysis, and mass spectrometry indicate that modification of Cys^{313} or Cys^{324} in the stalk domain is correlated with enhanced binding of MC1 to MTs. These results suggest that NEM enhances Ncd binding to MTs by disruption of neck and/or stalk function and demonstrate the importance of this region in motor function.

Microtubule (MT)[1] motor proteins are mechanochemical enzymes that hydrolyze ATP to generate the movement of various cellular components along MTs. Although many MT motor proteins have been identified (for reviews, see refs *1*−*3* and the kinesin homepage www.blocks.fhcrc.org/~kinesin), and the structures of some have been determined (*4*−*8*), the mechanism(s) by which motor proteins bind and "walk" along MTs is still not well understood. One approach to investigating these mechanisms is chemical modification, a powerful technique for probing functionally important amino acids. Cys residue modification, for example, has provided insight into structure−function relationships in the actin-dependent motor protein, myosin. In particular, *N*-ethylmaleimide (NEM) treatment of myosin has been shown to inhibit myosin's ATPase activity (*9*−*14*) and enhance myosin's binding to actin (*15*, *16*) and has led to the identification of two sulfhydryl groups, termed SH1 and SH2, that are involved in myosin's nucleotide binding and hydrolysis activities.

On the basis of the informative results obtained with myosin, several MT motor proteins have been treated with NEM to determine the functional consequences of Cys modification. As with myosin, NEM has been shown to inhibit the function of all the MT motor proteins that were tested, although different MT motors exhibit different levels of sensitivity to NEM and the mechanism by which NEM inhibits motor function is different for different MT motors. In terms of sensitivity to NEM, cytoplasmic dynein, axonemal dynein, and the kinesin-like protein Ncd (non-claret disjunctional) are inhibited by relatively low concentrations of NEM (10−100 μM) (*17*−*21*), while inhibition of kinesin requires much higher concentrations of NEM (1−5 mM) (*22*−*26*). In terms of the mechanism by which NEM inhibits the function of a given MT motor, differences between motors are most obvious in the in vitro motility assay, which tests a motor's ability to bind MTs and generate movement. As evaluated by this assay, NEM inhibits kinesin movement by interfering with the ability of kinesin to bind MTs (*23*, *24*, *26*), but inhibits Ncd (*20*) and cytoplasmic dynein (*21*) movement by enhancing the binding of these motors to MTs. NEM has also been reported to inhibit axonemal dynein motility, but the specific effect (suppressed vs enhanced binding) was not discussed (*19*). Surprisingly, and in contrast to the results observed in the motility assay, NEM treatment appears to enhance kinesin binding to MTs in the presence of MgATP in a cosedimentation assay (*25*, *26*). Since the effects of NEM on motors other than kinesin have not been examined in the cosedimentation assay, it is not known if this assay dependence is also exhibited by other MT motors. NEM has also been found to inhibit the MT-stimulated ATPase rate of kinesin (*25*) and cytoplasmic dynein (*18*, *27*),

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[1] Abbreviations: MC1, modified claret 1; MT, microtubule; Ncd, nonclaret disjunctional; NEM, *N*-ethylmaleimide.

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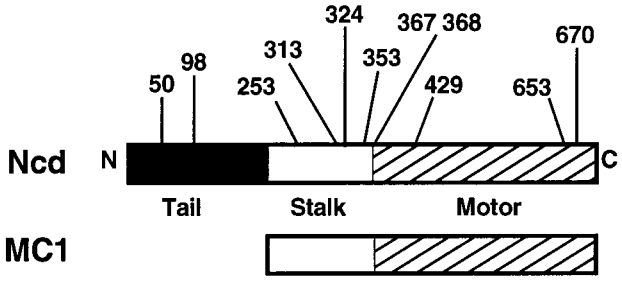

FIGURE 1: Schematic of the MC1 protein. MC1 is comprised of Ncd residues 209−700 and includes the entire stalk domain and motor domain. Nine of the eleven Cys residues of Ncd are present in MC1 and are referenced in the text by their position in the Ncd sequence (*32*). The delineations between the tail, stalk, and motor domains are based on sequence analysis (*29*, *32*), but structural analysis (*5*, *8*, *39*) places the stalk−motor border slightly closer to the N-terminus (349 vs 356), and therefore, Cys^{353} may be considered part of the motor domain (see the Discussion).

as well as the basal ATPase rate of cytoplasmic dynein (*18*). NEM effects on the ATPase activity of Ncd have not been reported, nor have the effects of NEM on the basal ATPase rates of motors other than cytoplasmic dynein.

Although by no means complete, the existing data regarding NEM inhibition of MT motors raise several questions. (i) Why does NEM treatment of the minus end-directed Ncd and dynein motors inhibit motility by enhancing binding to MTs, while treatment of the plus end-directed kinesin inhibits motility by reducing the level of binding to MTs? (ii) Do Ncd and dyneins, like kinesin, exhibit assay dependence in terms of NEM effects? (iii) For each motor, what Cys residue(s) is responsible for the inhibitory effects associated with NEM treatment?

To address some of these questions and gain further insight into Ncd motor activity, we treated the Ncd motor-stalk construct MC1 [modified claret 1 (*28*, *29*)] with a range of NEM concentrations (0−10 mM), and used cosedimentation and ATPase assays to determine the effects of modification on the Ncd motor. MC1 is comprised of Ncd residues 209−700 and includes nine of the eleven Cys residues present in the full-length protein (Figure 1). MC1 forms a homodimer and exhibits ATP-dependent MT binding, MT-stimulated ATPase activity, and one-dimensional diffusional movement of MTs (*28*, *29*). Cosedimentation experiments with NEM-treated MC1 demonstrated that low concentrations of NEM ($\leq$0.1 mM) enhanced the binding of MC1 to MTs in the presence of MgATP. However, higher concentrations of NEM ($\geq$0.5 mM) caused aggregation of the MC1 protein that interfered with characterization of the motor's activity. In comparison, NEM concentrations as high as 5 mM had no effect on the basal ATPase rate of MC1, while inhibiting the MT-stimulated ATPase activity of MC1 in a dose-dependent manner. Enhanced MT binding in the presence of MgATP correlated with the average labeling of $\approx$1 Cys residue per MC1 polypeptide, while pelleting in the absence of MTs correlated with the average labeling of $\approx$2 Cys residues per MC1 polypeptide. Formic acid digestion of MC1 indicated that the critical Cys residues associated with both enhanced binding and aggregation were located within Ncd residues 209−524. Mass spectrometry analysis of endoproteinase Lys-C and trypsin MC1 digests was consistent with this result and, in combination with analysis of the Ncd monomer and dimer structures (*5*, *8*), suggested that modification of Cys^{313} or Cys^{324} is associated with the enhanced binding to MTs. Given the location of these residues in the stalk region, modification of either Cys^{313} or Cys^{324} could result in enhanced MT binding via disruption of the normal head−head interactions needed for movement (*8*, *30*, *31*). Modification of both Cys^{313} and Cys^{324}, or perhaps one of these and either Cys^{253} or Cys^{367}, may be responsible for the aggregation observed at higher NEM concentrations.

MATERIALS AND METHODS

Protein Purification. The pET3/MC1 construct (*28*, *29*) was transformed into BL21(DE3) cells. Protein expression was induced by addition of 0.25 mM IPTG. After 4 h, cells were pelleted and washed in AB buffer [20 mM Pipes (pH 6.9), 1 mM $MgSO_4$, and 1 mM EGTA] with or without 5% sucrose (AB/sucrose), and the resulting pellets were stored at −70 °C. Frozen cells were resuspended in AB/sucrose or phosphate buffer [10 mM phosphate (pH 7.2), 0.1 M NaCl, 2 mM $MgCl_2$, and 1 mM EGTA] containing 5% sucrose (PB/sucrose) and lysed by addition of 0.2 mg/mL lysozyme and one freeze−thaw cycle. DNase I and $MgCl_2$ were added to final concentrations of 40 μg/mL and 10 mM, respectively, and after 30 min on ice, lysates were centrifuged at 20000*g* for 15 min at 4 °C. Low-speed supernatants were subsequently centrifuged at 100000*g* for 15 min at 4 °C, and the resulting high-speed supernatant was fractionated by S-Sepharose ion exchange chromatography. Bound MC1 protein was eluted with AB/sucrose or PB/sucrose containing 0.2 M NaCl. MgATP (0.1 mM) was present throughout the purification process, while PMSF (1 mM) and DTT (1 mM) were added to the lysis and column wash buffers but omitted from the elution buffer. The eluted protein was dialyzed against AB/sucrose containing 0.1 mM MgATP at 4 °C, then quick-frozen in liquid nitrogen, and stored at −70 °C. MC1 concentrations are reported as polypeptide (monomer) concentrations. Amino acids are numbered according to their position in the Ncd sequence (*32*).

Tubulin was purified as described previously, then quick-frozen in liquid nitrogen, and stored at −70 °C (*33*). Protein concentrations were determined with a Bradford assay (Bio-Rad) using bovine serum albumin (BSA) as the standard.

MC1 Modification and Functional Assays. NEM (Sigma) or biotin-NEM [*N*-(3-maleimidylpropionyl)biocytin, Molecular Probes] stock solutions were prepared fresh as 5× stock solutions in AB/sucrose. MC1 (final concentration of 7.5 μM) was treated with 0.05, 0.1, 0.5, 1, 5, and 10 mM NEM, yielding molar ratios of 6.7:1, 13:1, 67:1, 133:1, 667:1, and 1333:1, respectively. Modifications were performed at 22 °C for up to 30 min, and reactions were quenched by the addition of DTT (final concentration of 50 mM) and then the mixtures placed on ice for 10−15 min prior to use. Unmodified control samples were treated identically except that AB/sucrose was substituted for NEM.

To examine MT binding in a cosedimentation assay, unmodified or modified MC1 (final concentration of 2.5 μM) was mixed with paclitaxel-stabilized MTs (final tubulin concentration of 2.5 μM) in AB/sucrose containing either MgATP or MgAMPPNP (final concentration of 5 mM). Control reaction mixtures were prepared without MTs to evaluate nonspecific pelleting of MC1. After 30 min at 22 °C, samples were centrifuged at 100000*g*, and supernatant

and pellet fractions were analyzed via SDS−PAGE. Coomassie Blue-stained gels were quantified as % MC1 in supernatant versus pellet using a gel documentation system (Alpha Imager, Alpha Innotech Corp.).

Basal and MT-stimulated ATPase rates were determined by a malachite green phosphate assay (*34*). All reaction mixtures contained 0.1 μM motor, and MT-stimulated reaction mixtures contained GTP-depleted paclitaxel-stabilized MTs (final concentration of 2 μM). Reactions were initiated by adding 1 mM MgATP and proceeded for a total of 25−30 min with samples taken at 5 min intervals. MT-stimulated reactions were quenched by the addition of an equal volume of 0.5 M HCl and then centrifuged to pellet precipitated protein. Reaction samples were mixed with malachite green−molybdate color reagent at a 4:1 ratio (v/v) of protein sample to malachite dye reagent (*35*), and the A_{630} was measured after incubation for 10 min at 22 °C.

Identification and Characterization of Reactive Cys Residues. MC1 (7.5 μM) was treated with 0.05, 0.1, 0.5, 1, and 5 mM [^{3}H]NEM (either 2.6×10^{13} or 3.4×10^{13} cpm/mol). For each concentration, samples were taken at 5, 10, 20, and 30 min, and the reaction was quenched by adding DTT (final concentration of 100 mM). Samples were separated by SDS−PAGE and stained with Coomassie Blue, and MC1 bands were excised from the gel. Each gel slice was then solubilized with 1 mL of 30% H_2O_2 for 24 h at 60 °C and mixed with 9 mL of scintillation fluid, and the counts contained in each slice were determined.

For formic acid digestion, MC1 was modified with biotin-NEM as described above and modified protein samples (40 μg) were precipitated by a chloroform/methanol procedure (*36*). The dried protein pellets were resuspended in 20 μL of 88% formic acid (Fisher) and incubated for 24 h at 37 °C. Samples were then diluted with 480 μL of mQ water, concentrated [Microcon-3 or -10 concentrator (Amicon)], and neutralized by the addition of 2 volumes of 1 M Tris. Samples were separated by SDS−PAGE or Tricine−PAGE, and either stained with Coomassie Blue or subjected to Western blot analysis. Biotin-NEM-labeled fragments were detected by an anti-biotin antibody (Clone BN-34, Sigma) and an alkaline phosphatase-conjugated secondary antibody (Pierce). Blots were developed with Vistra ECF (Amersham) and visualized with a Molecular DynamicsStorm Imager.

To identify reactive Cys residues, samples were either digested with endoproteinase Lys-C and subjected to mass spectrometry analysis (Biomolecular Resources Center, University of Virginia, Charlottesville, VA) or digested with trypsin and fractionated by HPLC (Applied Biosystems 130A Separation System). Enzymatic digestions were performed at a 1:25 enzyme:protein ratio (w/w) for 24 h at 37 °C (*37*). For endoproteinase Lys-C digestions, MC1 was labeled with 1 mM biotin-NEM for 10 min which should label an average of two Cys per polypeptide (see Figure 5). For tryptic digestions, MC1 was labeled with 0.1 or 1 mM [^{3}H]NEM or NEM for 10 min which should label an average of 1 or 2 Cys residues per polypeptide, respectively (see Figure 5). Tryptic peptides were separated by reversed-phase chromatography on a C8 column using a gradient of 0 to 65% acetonitrile (0.1% TFA) over the course of 55 min. Fractions containing NEM-labeled peptides were identified by separating digested, [^{3}H]NEM-labeled MC1 samples (1 and 2 Cys residues per polypeptide) and using a liquid scintillation counter (Beckman model LS 6000SC) to identify fractions containing [^{3}H]. A_{214} profiles of [^{3}H]NEM-labeled samples and cold NEM-labeled samples were identical. For both 1 and 2 Cys residues per polypeptide digest, two fractions (in addition to the flow through) contained counts significantly (≥5-fold) above background; the first (5-fold greater than background) eluted at ≈10% acetonitrile and the second (8−12-fold greater than background) at ≈23% acetonitrile. Corresponding fractions from cold NEM-labeled MC1 were then analyzed by mass spectrometry (Macromolecular Resources, Colorado State University, Fort Collins, CO).

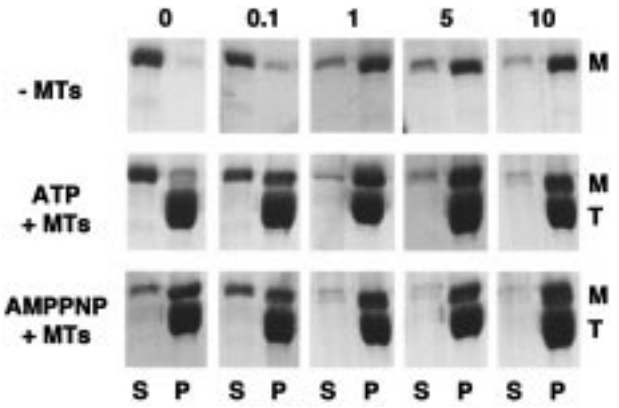

FIGURE 2: Effect of NEM on MC1 binding to MTs. MC1 (7.5 μM) was treated with the indicated concentrations of NEM (millimolar) for 30 min at 22 °C and then subjected to a cosedimentation assay as described in Materials and Methods. Supernatant (S) and pellet (P) fractions were separated by SDS−PAGE and stained with Coomassie Blue. The results for MC1 (2.5 μM) in the absence of MTs, in the presence of MTs (2.5 μM tubulin) and 5 mM MgATP, and in the presence of MTs (2.5 μM tubulin) and 5 mM MgAMP-PNP are shown in the top, middle, and bottom rows, respectively. The positions of MC1 (M) and tubulin (T) are denoted on the right.

RESULTS

Effects of NEM on the MT Binding and ATPase Activities of MC1. To characterize the effect of NEM on the MT binding properties of MC1, protein samples were treated with 0−10 mM NEM for 30 min and subjected to a MT cosedimentation assay in the presence of either 5 mM MgATP or MgAMP-PNP. Representative cosedimentation experiments are shown in Figure 2, and a quantitative summary (expressed as % MC1 in the pellet fraction) of these experiments is presented in Figure 3. The results for unmodified MC1 samples were consistent with previously reported cosedimentation experiments with MC1 (*29*); ≈25% of the unmodified MC1 was present in the pellet fraction in the absence of MTs or in the presence of MTs and MgATP, while ≈70% of the unmodified MC1 was found in the pellet fraction in the presence of MTs and MgAMP-PNP (Figures 2 and 3). Compared to unmodified MC1, treatment of MC1 with 0.05 or 0.1 mM NEM for 30 min caused a relatively small increase (to 30 and 36%, respectively) in the amount of MC1 that pelleted in the absence of MTs (Figure 3a), and in the presence of MTs and MgAMP-PNP [to 78 and 86%, respectively (Figure 3c)]. However, the same treatment conditions caused a significant ($P < 0.001$) increase (from 27 to 77 and 78%, respectively) in the amount of modified MC1 that pelleted in the presence of MTs and MgATP

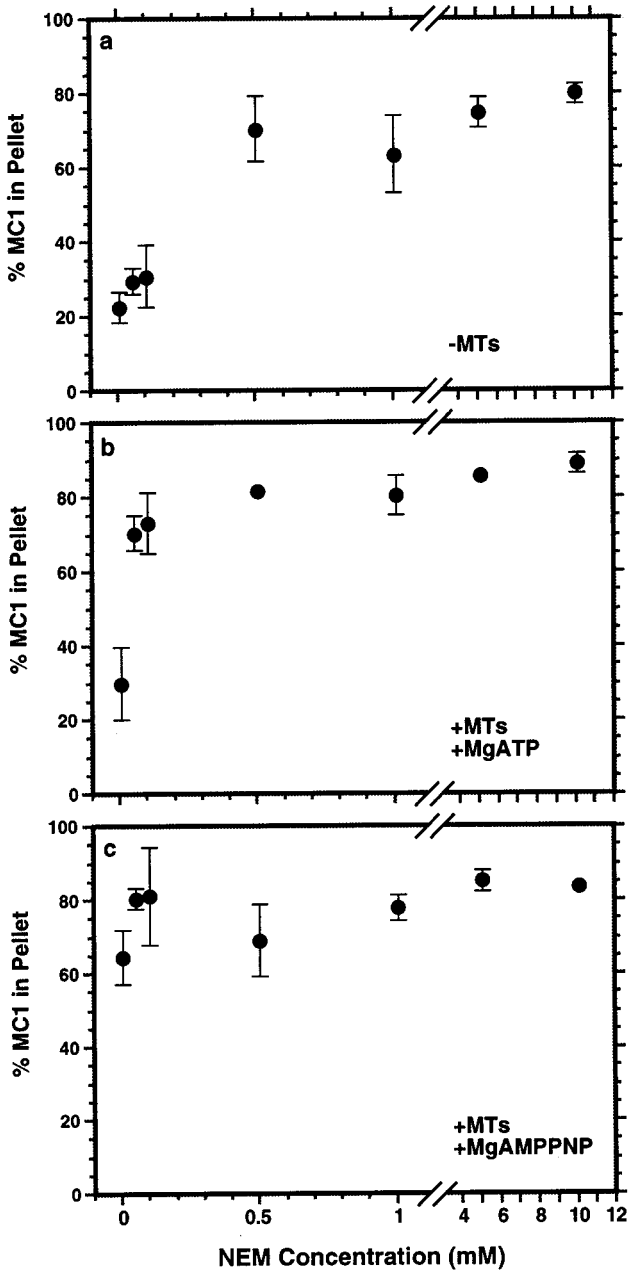

FIGURE 3: Quantitation of MC1 binding to MTs as a function of NEM concentration. MC1 was modified and subjected to MT cosedimentation as described in the legend of Figure 2. The average ± standard deviation of six experiments is shown for each NEM concentration. For each experiment, the amount of MC1 in the supernatant and pellet fractions was determined with a gel documentation system (Alpha Innotech). The percentages of MC1 found in the pellet fractions in the absence of MTs (a), in the presence of MTs and 5 mM MgATP (b), and in the presence of MTs and 5 mM MgAMP-PNP (c) are shown for each NEM concentration.

(Figure 3b). Thus, under these conditions, modified MC1 bound MTs in the presence of MgATP as effectively as in the presence of MgAMP-PNP. When the NEM concentration was increased to 0.5 mM, the modified MC1 was found almost completely (≈75% or greater) in the pellet fraction regardless of whether MTs were present, and similar results were also observed when MC1 was modified with 1, 5, or 10 mM NEM (Figures 2 and 3). A time course evaluation with selected NEM concentrations (0, 0.1, and 1 mM) showed that the NEM effects shown in Figure 3 were

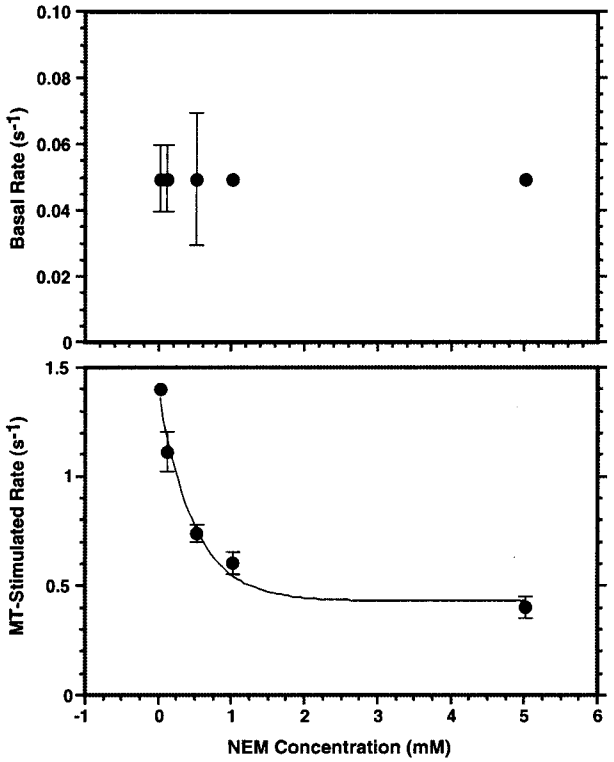

FIGURE 4: Effect of NEM on the basal and MT-stimulated ATPase activity of MC1. MC1 was treated with the indicated concentrations of NEM to analyze the effect of modification on the basal and MT-stimulated ATPase rates. The rate of phosphate release was determined using the malachite green assay as described in Materials and Methods. Averages and standard deviations for basal rates (top) are representative four to nine experiments and for MT-stimulated rates (bottom) three to six experiments.

generally obtained after treatment for 10−20 min (data not shown). The overall conclusion from these experiments was that treatment of MC1 with NEM produced two different effects depending on the NEM concentration that was used; lower NEM concentrations (≤0.1 mM) enhanced binding of the modified motor to MTs under conditions (MgATP) that normally release the majority of motor to the supernatant fraction, while higher NEM doses (≥0.5 mM) caused aggregation of the modified motor. Further, the aggregation made it difficult to evaluate the effect of the larger NEM doses on MT binding since pelleting was most likely due to aggregation and not binding to MTs.

To further characterize the effects of NEM on MC1 activity, we next determined the effect of NEM on the ability of MC1 to hydrolyze ATP. Modifications were performed as described above for the MT cosedimentation assay, and the effects of NEM on the basal and MT-stimulated ATPase rates of MC1 were measured (Figure 4). The results for unmodified MC1 were consistent with previously reported values (*29*); the basal rate of unmodified MC1 was 0.05 ± 0.02 s^{-1} ($n = 7$), and this increased ≈28-fold to 1.40 ± 0.21 s^{-1} ($n = 6$) upon addition of MTs. Treatment of MC1 with NEM concentrations as high as 5 mM had no effect on the motor's basal ATPase rate (Figure 4), but inhibited the MT-stimulated ATPase rate of MC1 in a dose-dependent manner (Figure 4). Compared to those of unmodified MC1, the MT-stimulated ATPase rates after treatment with 0.1, 0.5, 1, and 5 mM NEM were decreased by 21, 46, 57, and 70%, respectively.

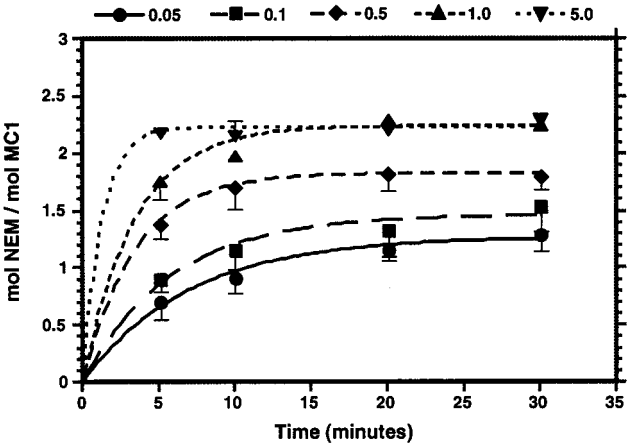

FIGURE 5: Time course of NEM labeling of MC1. MC1 (7.5 μM) was treated with 0.05, 0.1, 0.5, 1.0, and 5.0 mM [^{3}H]NEM (3.4 $\times$ 10^{13} cpm/mol) for the times indicated and then subjected to SDS−PAGE. MC1 bands were excised from the gel and processed as described in Materials and Methods. Each data point is the average of six experiments. Error bars (SEM) point up for 5.0 mM NEM data, and down for all others. An exponential curve was fit to each concentration.

Identification and Characterization of Reactive Cysteines. To correlate the observed effects on MC1's MT binding, solubility, and ATPase activity with modification of specific Cys residues, the number of NEM-modified residues per polypeptide was determined as a function of NEM concentration and time. MC1 was treated with 0.05−5 mM [^{3}H]-NEM, and samples were taken at 5, 10, 20, and 30 min (Figure 5). After treatment with 0.05 or 0.1 mM NEM for 10 min, an average of ≈1 Cys residue per MC1 polypeptide was modified, and this ratio increased to an average of 1.4 and 1.6 Cys per MC1 polypeptide after 30 min, respectively. In comparison, treatment of MC1 with 0.5 or 1 mM NEM for 10 min labeled an average of 1.7 and 2 Cys residues per MC1 polypeptide, respectively, while treatment for 20−30 min labeled an average of ≈2 Cys residues (1.9 and 2.2) per MC1 polypeptide. At the highest NEM concentration used in these experiments (5 mM), an average of 2.1 Cys residues were labeled after 5 min and this ratio increased slightly to an average of 2.3 Cys residues per MC1 polypeptide by 30 min. Thus, at the maximum NEM:MC1 polypeptide ratio that was used for modification reactions (>600:1), the maximum number of Cys residues labeled was ≈2 out of the 9 per polypeptide. Taken together, the MT binding data presented in Figures 2 and 3, and the labeling data presented in Figure 5, suggest that modification of one critical Cys residue is associated with enhanced MT binding and that modification of a second Cys is associated with aggregation of MC1.

MC1 labeled with biotin-NEM was used to facilitate identification of the specific modified Cys residues. In terms of MT binding in the cosedimentation assay, modification of MC1 with biotin-NEM was found to produce results identical to those observed for NEM, in terms of both enhanced MT binding at lower biotin-NEM concentrations and induced aggregation at higher biotin-NEM concentrations (compare Figure 6a with Figure 2). Note also the increase in the apparent molecular mass of MC1 after modification with biotin-NEM. Biotin-NEM-modified MC1 was initially subjected to digestion with formic acid, which preferentially cleaves peptide bonds between aspartic acid and proline.

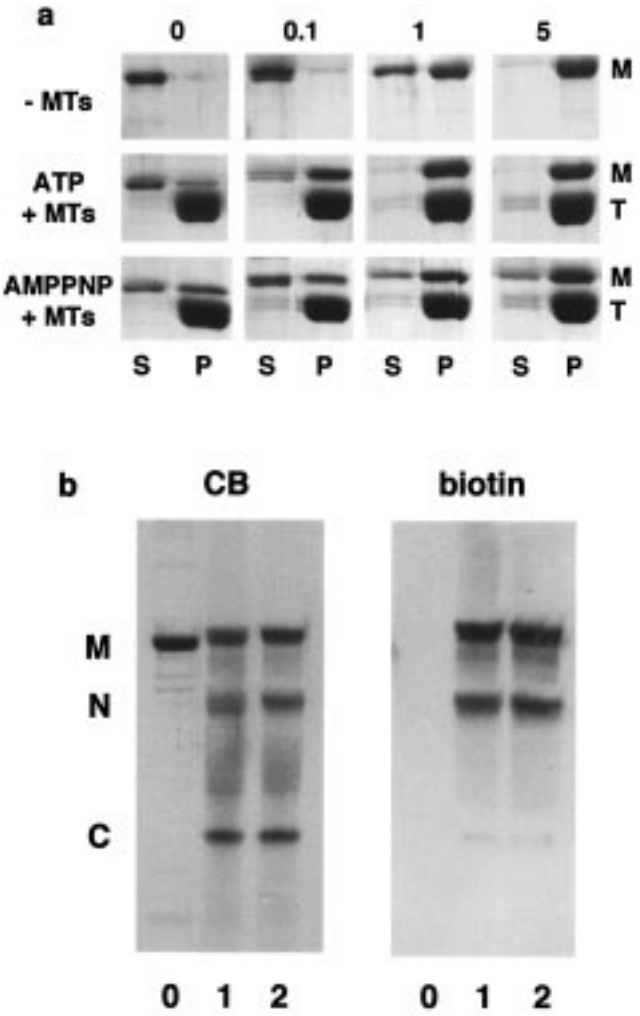

FIGURE 6: Biotin-NEM modification and formic acid cleavage of MC1. (a) MC1 (7.5 μM) was treated with the indicated concentrations of biotin-NEM (millimolar) for 30 min at 22 °C and then subjected to a cosedimentation assay as described in Materials and Methods. Supernatant (S) and pellet (P) fractions were separated by SDS−PAGE and stained with Coomassie Blue. The results for MC1 (2.5 μM) in the absence of MTs, in the presence of MTs (2.5 μM tubulin) and 5 mM MgATP, and in the presence of MTs (2.5 μM tubulin) and 5 mM MgAMP-PNP are shown in the top, middle, and bottom rows, respectively. The positions of MC1 (M) and tubulin (T) are denoted on the right. Note the increase in the apparent molecular mass of MC1 after modification with biotin-NEM. (b) MC1 was treated with 0.1 and 1.0 mM biotin-NEM to label an average of 1 (lane 1) and 2 Cys residues per polypeptide (lane 2), respectively. Undigested, unmodified MC1 is shown in lane 0. Biotin-NEM−MC1 samples were treated with 88% formic acid for 24 h at 37 °C. Formic acid cleaves between Asp^{524} and Pro^{525} in MC1, yielding two fragments: one N-terminal 36 kDa fragment (N) and one C-terminal 21 kDa fragment (C) from intact MC1 (M). Digested samples were either stained with Coomassie Blue (CB) or probed with an anti-biotin antibody after transfer to nitrocellulose membrane (biotin).

MC1 contains one such formic acid-sensitive site between Asp^{524} and Pro^{525}, and digestion should yield a 36 kDa fragment and a 21 kDa fragment. MC1 was treated with 0.1 or 1.0 mM biotin-NEM for 10 min; on the basis of the data obtained with [^{3}H]NEM (see Figure 5), these conditions would be expected to label an average of 1 and 2 Cys residues per polypeptide, respectively. After formic acid digestion, samples were separated by SDS−PAGE, and either stained with Coomassie Blue or transferred to nitrocellulose and probed with an antibody against biotin (Figure 6b).

Coomassie Blue staining showed two major products of the expected size in the biotin-NEM-modified formic acid digests. Qualitatively, modification was evident as a shift of the biotin-NEM-treated samples to a higher apparent molecular mass. A similar shift was also apparent for the upper but not lower fragment (data not shown). Of the two fragments, the larger fragment (and labeled, undigested MC1) was clearly recognized by the anti-biotin antibody, while labeling was barely detectable on the smaller fragment. This labeling pattern suggested that the critical Cys residues are located between Ncd residues 209 and 524, a region that includes 7 of the 9 total Cys residues (Figure 1).

To identify which of the 7 Cys residues were associated with enhanced MT binding and/or motor aggregation, MC1 samples were subjected to enzymatic digestion with either endoproteinase Lys-C or trypsin. For endoproteinase Lys-C digestion, MC1 was first treated with biotin-NEM under conditions (1 mM for 10 min) that should modify ≈2 Cys residues per polypeptide, and then digested as described in Materials and Methods. The resulting peptides were characterized by mass spectrometry, and peptides corresponding to 72% of the MC1 sequence were identified (data not shown). No peptides containing Cys residues modified by NEM could be identified. However, included among the identified peptides were 7 of the 9 MC1 Cys residues in the unmodified state. The 2 Cys residues not accounted for by this analysis were Cys^{313} and Cys^{324}, which are contained in the same peptide. Since endoproteinase Lys-C tends to produce long fragments with high charge states, unmodified and NEM-modified MC1 samples (with an average labeling of 1 or 2 Cys residues per polypeptide) were next digested with trypsin to create shorter peptides that might facilitate identification by mass spectrometry. Further, to enrich peptides with modified Cys residues, trypsin-digested MC1 samples were fractionated by reversed-phase HPLC as described in Materials and Methods. Two fractions were shown to contain NEM-modified peptides; one eluted with 10% acetonitrile and the other with 23% acetonitrile (data not shown). These fractions from unmodified, 1 Cys residue per polypeptide, and 2 Cys residues per polypeptide MC1 tryptic digests were analyzed by mass spectrometry and the results compared. For the fraction eluting with 10% acetonitrile, comparison of unmodified and modified samples did not reveal any species that were present in both modified samples and absent from the unmodified sample, or that could be identified as a MC1-derived peptide. However, for the fraction eluting with 23% acetonitrile, comparison of unmodified and modified MC1 mass results revealed the presence of two additional species with molecular masses of 2913 and 2929−2932 Da in both modified samples. These masses corresponded well to the predicted masses of MC1 peptide ^{313}CNEQQAAELETCKEQLFQSNMER^{335} in the nonoxidized (predicted mass of 2914 Da) and Met-oxidized forms (predicted mass of 2930 Da) assuming one of the Cys residues (Cys^{313} or Cys^{324}) was labeled with NEM. No additional peptides could be identified in the 2 Cys residues per polypeptide samples that were not present in the 1 Cys residue per polypeptide samples.

DISCUSSION

Cysteine modification previously has been shown to inhibit the function of several MT motors, but the specific mechanism by which such modification inhibits function is not well understood for any of these proteins. To investigate the mechanism by which NEM inhibits Ncd motor function, a recombinant protein (MC1) comprised of the stalk and motor domains of Ncd was treated with NEM and the effects of resulting modification were correlated with labeling of specific Cys residues.

On the basis of the results observed in the cosedimentation assay, NEM modification produced two different, concentration-dependent effects on the motor. Lower concentrations of NEM (≤0.1 mM) caused MC1 to bind more tightly to MTs in the presence of MgATP while having little effect on the amount of MC1 that pelleted in the absence of MTs or in the presence of MTs and MgAMP-PNP (Figures 2 and 3). These results are consistent with previous motility experiments which showed that NEM treatment of coverslip-bound Ncd induced tight binding to MTs even in the presence of MgATP (*20*). Enhanced binding to MTs correlated with an average labeling of 1 Cys residue per polypeptide (Figure 5). At this labeling ratio, the basal ATPase rate was identical to that of unmodified MC1, suggesting that this level of modification did not directly affect ATP binding, hydrolysis, or product release. However, this same treatment decreased the MT-stimulated ATPase rate by 21%. Given the results observed in the cosedimentation assay, this reduction is clearly not due to an inhibition of MT binding, and may instead result from a disruption in the "communication" either between the MT-binding and ATP-binding sites within the catalytic core of one of the MC1 motor domains or between the two motor domains of the dimer (see below).

In comparison to the enhanced binding observed for modification with ≤0.1 mM NEM, treatment of MC1 with NEM concentrations of ≥0.5 mM induced aggregation of the protein. This aggregation was evident in the cosedimentation assay as pelleting of modified MC1 in the absence of MTs (Figure 3a), and was correlated with an average labeling of 2 Cys residues per polypeptide (Figure 5). At this labeling ratio, the basal ATPase rate was identical to that of unmodified MC1, suggesting that any conformational changes in the MC1 structure associated with aggregation do not directly affect ATP binding, hydrolysis, or product release. In comparison, labeling of MC1 with 2 Cys residues per polypeptide decreased the MT-stimulated rate of MC1 by 42% compared to unmodified MC1, and as much as 70% if the average level of labeling increased to 2.3 Cys residues per polypeptide (the maximal labeling level observed). At these labeling ratios, it is difficult to distinguish whether inhibition is due to direct effects on motor activity or indirect effects due to aggregation (which may sterically inhibit interaction with MTs), although the data do suggest that some portion of the MC1 population is able to interact with MTs even at the highest labeling level. One final point concerning aggregation is that the maximum amount of protein that pelleted was similar to the amount of unmodified MC1 that bound MTs in the presence of MgAMP-PNP (Figure 3). This suggests that a small fraction of the MC1 population, perhaps due to misfolding following synthesis, is inactive in terms of MT binding and also cannot be induced to aggregate.

The results of the cosedimentation and ATPase experiments are consistent with a model in which modification of 1 critical Cys produces enhanced MT binding in the presence of MgATP and modification of a second, different Cys

residue induces MC1 aggregation. To obtain a low-resolution map of the location of reactive Cys residues, MC1 was modified with biotin-NEM under conditions expected to produce an average labeling of 1 or 2 Cys residues per polypeptide and then digested with formic acid (Figure 6b). Biotin was detected almost exclusively on the N-terminal 36 kDa fragment which contains Ncd residues 209−524, and this result indicates that the reactive Cys residues associated with both enhanced binding and aggregation are located between Ncd residues 209 and 524, and that neither Cys^{653} nor Cys^{670} is easily accessible or involved in enhanced binding or aggregation. Interestingly, a previous report found that a maleimide EPR spin probe predominantly labeled an Ncd monomeric motor domain protein (comprised of Ncd residues 335−700) on Cys^{670} with no apparent effect on MT binding or ATPase activity (*38*). The differences regarding the identity of the reactive Cys, as well as the effect of modification, are most likely due to the different proteins used in that study (*38*) and in the results reported here. One possibility is that Cys^{670} may be the most accessible and/or reactive Cys in the Ncd monomeric motor domain, but that the additional Cys residues in MC1 may be even more accessible and/or reactive. Alternatively, the presence of the stalk domain and the resulting dimeric structure of MC1 may somehow inhibit modification of Cys^{670} in this protein. In any event, our results demonstrate that for MC1, Cys^{653} and Cys^{670} are not reactive to NEM and therefore not involved in the inhibitory effects observed here for MC1 or previously for full-length Ncd (*20*).

Which of the 7 Cys residues present in the fragment of residues 209−524 are modified, and which are associated with the observed effects? Treatment of MC1 with excess [^{3}H]NEM produced a maximal labeling of ≈2 Cys residues of the 9 total and thus suggests that the other 7 Cys residues are relatively inaccessible. The formic acid digest results discussed above indicate that 2 of the 7 inaccessible MC1 Cys residues are Cys^{653} and Cys^{670}. Of the 7 Cys residues present in the fragment of residues 209−524, 4 are in the motor domain (Cys^{353}, Cys^{429}, Cys^{367}, and Cys^{368}) and 3 are in the stalk domain (Cys^{253}, Cys^{313}, and Cys^{324}). As noted by Naber et al. (*38*), analysis of the Ncd motor domain structure (*5*) indicates that Cys^{353} and Cys^{429} are buried and probably inaccessible to NEM. Cys^{367} and Cys^{368} are found in loop 1 which, in combination with loops 5 and 9, surrounds access to the ATP binding cleft (*5*). Structure analysis suggests that Cys^{368} may be relatively inaccessible given the orientation of the side chain, but it appears that Cys^{367} might be available for modification. Mass spectrometry of MC1 modified with biotin-NEM and subsequently digested with endoproteinase Lys-C identified peptides containing 7 of the 9 Cys residues in unmodified form, but did not reveal any peptides that contained biotin-NEM-modified Cys residues. A peptide containing both Cys^{313} and Cys^{324} was not identified, and although intriguing, the failure to identify this peptide is not conclusive evidence that the unaccounted for Cys residues are modified, and may be simply coincidental. However, when NEM-modified MC1 was digested with trypsin and fractions containing NEM-labeled peptides were analyzed by mass spectrometry, a peptide (^{313}CNEQQAAELETCKEQLFQSNMER^{335}) with the appropriate mass including 1 NEM-labeled Cys was identified from MC1 digests with an average labeling of either 1 or 2 Cys residues per polypeptide (no equivalent peptide was found in the same fraction from unmodified MC1 digests). This finding suggests that modification of either Cys^{313} or Cys^{324} is associated with the observed effects of NEM on MC1. Interestingly, this peptide contains an internal Lys residue which would be expected to be cleaved by trypsin. Perhaps modification of Cys^{324}, which immediately precedes the Lys residue, inhibits trypsin digestion at this site.

Taken together, the results from formic acid digestion, structural analysis, and mass spectrometry suggest that modification of Cys^{313} or Cys^{324} is coupled to enhanced MT binding. How could modification of a Cys residue in the stalk domain be responsible for enhanced MT binding of Ncd and MC1 in the presence of MgATP? One possibility is suggested by the recently published structure of an Ncd dimer similar to MC1 (*8*). In this structure, Cys^{313} and Cys^{324} are located in the stalk domain just below (N-terminal to) the neck region (Ncd residues 335−347) (*8*). The neck and adjacent stalk residues appear to interact with the Ncd catalytic core and have been implicated as being important in motor function and in directionality (*8*, *31*). Further, the neck and/or stalk region is pictured as a dynamic structure that changes conformation (*8*, *31*, *39*) and as such may allow accessibility to Cys residues in the adjacent area. Modification of one of these residues could disrupt either interaction of the catalytic core with the neck and/or stalk or the interaction between the neck and/or stalk of the two polypeptides. Such disruption may in turn interfere with interactions and/or cooperativity between the two motor domains (heads) of the dimeric motor (*6*, *8*, *31*, *39*−*44*), such that one head is unable to facilitate release of the other from the MT. This would explain the observed enhancement of MT binding in the presence of MgATP and may also act to decrease the MT-stimulated ATPase rate of the motor.

The proposed mechanism of NEM inhibition is not necessarily inconsistent with recent evidence indicating that Ncd is nonprocessive, since as pointed out by the authors, their results do not exclude interaction and/or cooperation between motor domains (*45*). Our proposed mechanism is also supported by two additional observations. First, the fact that NEM treatment has no effect on binding of modified MC1 (1 Cys residue per polypeptide) to MTs in the presence of MgAMP-PNP (Figure 3) suggests that the "normal" rigor attachment to MTs occurs with modified MC1, and therefore that NEM does not affect interactions between the MT-binding site and the ATP-binding site within a single motor domain. On the other hand, a mechanism that involves disruption of interactions between the two motor domains should have no impact on MgAMP-PNP-induced MT binding, and therefore accounts for both the MgAMP-PNP and MgATP MT binding results. The second supporting observation involves the effects of NEM on MC1 motility. As previously shown, MC1 does not move MTs unidirectionally in the motility assay but rather supports one-dimensional diffusional movement (*28*). Examination of the effect of NEM on this diffusional movement found no difference in the ability of unmodified or modified MC1 (1 Cys residue per polypeptide) to support this movement (K. K. Phelps and R. A. Walker, unpublished data). This suggests that NEM does not influence the weak MT interactions associated with diffusional movement (*28*, *46*), and is consistent with the mechanism proposed above since diffusional movement is

not likely to be inhibited by disruption of interactions and/or cooperativity between the two motor domains.

A final question is as follows. How does NEM induce MC1 aggregation? We were unable to identify a specific Cys that, when modified, caused aggregation. Formic acid digestion indicates that the critical Cys lies between Ncd residues 209 and 524. Using the same structural arguments presented above, Cys^{353} and Cys^{429} (and perhaps Cys^{368}) are buried and probably not accessible (although it is possible that modification of another Cys may subsequently expose one of these). This leaves Cys^{253}, either Cys^{313} or Cys^{324} (whichever is not associated with enhanced MT binding), and Cys^{367} as possible candidates. Since aggregation does not affect MC1's basal ATPase rate and does not completely eliminate the MT-stimulated ATPase rate, this suggests that a stalk Cys and not a motor Cys may be involved in MC1 aggregation. One possibility may be that Cys modification "opens up" the stalk domain in such a way that the α-helices form associations with other "opened" α-helices which in turn links multiple dimers together.

In conclusion, we have provided evidence that modification of Cys^{313} and/or Cys^{324} is associated with the inhibitory effects of NEM on Ncd (*20*). This finding underscores the importance of the stalk and/or neck region specifically for Ncd function and generally for other dimeric members of the kinesin superfamily.

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