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Regulation of Cytoplasmic Tubulin Carboxypeptidase Activity In Vitro by Cations and Sulfhydryl-Modifying Compounds

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Abstract α-tubulin subunits within microtubules (MTs) can be post-translationally detyrosinated by a tubulin-specific carboxypeptidase (TCP) activity to form biochemically distinct MTs. Attempts to characterize and purify TCP have suffered from the inability to detect low levels of activity and to distinguish TCP from other, competing enzyme activities. We recently developed an assay for TCP [Webster et al. (1992) Biochemistry 31:5849] that uses taxol-stabilized MTs as the substrate. In this study, we exploited the increased sensitivity and specificity of this new assay to explore the effects of various agents that might act to either stimulate or inhibit this enzyme in vitro. We tested a variety of both monovalent and divalent cations for their ability to affect TCP, and tested whether the cations were affecting the enzyme, the substrate, or both. We found that TCP displayed salt-sensitive binding to MTs, characteristic of other, more well characterized MT-associated proteins. While both calcium and magnesium stimulated TCP activity over a narrow concentration range (2–10 mM), they inhibited activity at higher concentrations. Other divalent cations tested, including zinc, copper, and cobalt, inhibited TCP at virtually all concentrations tested, but to different levels (zinc > copper > cobalt). Most of the zinc-induced TCP inhibition was attributed to the interference with the normal binding of TCP to MTs. In addition, we examined the involvement of free sulfhydryl groups (which are important for the activities of many types of enzymes) in TCP activity by the addition of sulfhydryl-modifying compounds during the assay, and found that their addition reduced TCP activity mainly (but not solely) by their action on the extract that contained the TCP. Finally, we tested the ability of DL-benzylsuccinic acid, a potent inhibitor of carboxypeptidase A, to inhibit TCP. While carboxypeptidase A has been found, in other studies, to be inhibited by micromolar concentrations, TCP was affected only at concentrations above 20 mM, adding another proof that carboxypeptidase A and TCP are distinct enzyme activities. © 1996 Wiley-Liss, Inc.

Key words: enzyme regulation, microtubule, post-translational modification, detyrosination, inhibitor, neuronal tissue

How do cellular microtubules (MTs), whose proportions of α-, β-tubulin heterodimers reflect the composition of the subunit pool, accomplish varied tasks that may be both temporally and spatially overlapping? One possibility is the post-translational detyrosination of α-tubulin, since it is both rapid and reversible, and occurs on MTs that have already assembled [Gundersen et al., 1987]. MTs modified in this way might then be recognized and bound by specific microtubule-associated proteins (MAPs), other cytoskeletal elements, or organelles.

Abbreviations used: CPA, pancreatic carboxypeptidase A; MAP, microtubule-associated protein; MT, microtubule; TCP, tubulin carboxypeptidase; Tyr, tyrosinated.

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Most α-tubulin gene products are subject to the reversible removal and subsequent reattachment of the C-terminal tyrosine residue [Arce and Barra, 1985; Raybin and Flavin, 1977], which is also dependent on the assembly status of the tubulin subunit. α-tubulin that has been incorporated within MTs is the primary substrate for tyrosine removal via the activity of tubulin carboxypeptidase (TCP) [Kumar and Flavin, 1981; Gundersen et al., 1987; Webster et al., 1992], while unpolymerized subunits are the main substrate for tyrosine re-addition via the activity of tubulin tyrosine ligase [Webster et al., 1987a; Wehland and Weber, 1987a]. This modification cycle is ubiquitous, having been observed in both invertebrate and vertebrate cells, including trypanosomes [Stieger et al., 1984], amphibians [Preston et al., 1981], sea urchins [Kobayashi and Flavin, 1981], and a variety of

mammalian cells [Gundersen and Bulinski, 1986], as well as during several developmental sequelae [Barra et al., 1980; Deanin et al., 1977; Gundersen et al., 1989; Warn et al., 1990; Webster et al., 1992].

In order to begin to understand the function of detyrosination, we sought to learn more about the detyrosinating enzyme TCP. Although the activity of this enzyme has been studied for almost 20 years [Argarana et al., 1978], little progress has been made toward understanding its role in cellular function. The purification and characterization of TCP have been hampered by the apparently low level of activity found in all tissues examined and by the inability to measure TCP activity reliably in those tissues [Flavin and Murofushi, 1984]. This inability was due to the insufficient sensitivity afforded by the standard assay, which uses radioactively labeled, unpolymerized tubulin dimers as the substrate, in a one-step procedure. In addition, the standard assay suffers from the inability to account for the relatively high level of endogenous substrate (Tyr tubulin) found in those tissues, which lowers the specific activity of the exogenously added ^{14}C- or ^{3}H-labeled substrate tubulin that is required to measure the enzyme activity [Argarana et al., 1980]. Finally, until recently there had been no demonstration of the specificity of the activity that was being measured, even when tissues (including brain tissue and cell homogenates) that undoubtedly contain many carboxypeptidase-like activities were used.

We overcame these obstacles by developing a new assay for TCP that is both more specific and more sensitive for TCP than was the old assay [Webster et al., 1992]. Three major improvements were made: the first was to use taxol-stabilized, ^{14}C-labeled MTs as the substrate for the reaction. The second was to divide the enzyme assay into several parts, in order to first bind all of the TCP in the sample to the MTs, then to separate the MT-bound TCP from other, "contaminating" enzyme activities by high-speed centrifugation, and finally to resuspend the TCP:MT complex in a suitable buffer for measuring the enzyme activity. The third improvement was to design a protocol that allowed us to measure the total amount of Tyr tubulin that was present in our TCP:MT complexes, in order to determine more accurately the specific activity of the substrate. Using this improved assay, we began an initial study of TCP activity in brain tissue, as well as in two cell lines that have been used extensively as models to study either neuronal or muscle differentiation [Webster et al., 1992]. In addition to documenting the increased sensitivity and accuracy of the assay, we also reported that, using the old assay, other carboxypeptidase-like activities (including lysosomal enzyme activities) were contributing to the total measured activity, while the new assay excluded such spurious activities.

Although substantial inroads were made in that study in characterizing TCP activity, several questions remained unanswered. One was, what molecules are capable of regulating TCP activity? A second question was, do the potential regulatory molecules exert their action mainly on the enzyme, on the substrate, on the binding of the enzyme to the substrate? To answer these questions, and as an initial step in the process of understanding the function of TCP and its possible regulation, we characterized TCP activity in vitro more fully by testing the effects of various cationic and sulfhydryl-modifying molecules, which were added at various stages of the assay. In this way, we discerned TCP inhibition that occurred by the modification of the substrate, and distinguished weaker effects (possibly due to the electrostatic inhibition of binding of TCP to its substrate) from stronger, more direct effects on the enzyme.

MATERIALS AND METHODS

Materials

^{14}C-tyrosine (NEC-289E, ~500 mCi/mmol) was obtained from New England Nuclear Research Products, Boston, MA. KCl, NaCl, $MgCl_2$, and $CaCl_2$ (all ACS grade) were purchased from Fisher Scientific, Pittsburgh, PA. Buffers were purchased from Research Organics, Inc., Cleveland, OH, and all other reagents were purchased from Sigma Chemical Co., St. Louis, MO. Taxol was a kind gift from the Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute, Bethesda, MD.

Brain Extract

^{14}C-labeled tubulin substrate. Adult bovine brains were obtained from the Excel Meat Packing Co., Plainview, TX. Preparation of brain extract and tubulin substrate were as described previously [Webster et al., 1992]. For the results reported here, the brains were homogenized in

50 mM MOPS, pH 7.0, 2 mM $MgCl_2$, 0.1 mM PMSF, and 5 mM DTT.

Polymeric TCP assay

The basic assay was performed as described [Webster et al., 1992]. TCP assays were performed using either MM buffer (50 mM MOPS, pH 7.0, plus 2 mM $MgCl_2$) or MHM buffer (25 mM MOPS, 25 mM HEPPS, pH 7.5, plus 5 mM $MgCl_2$). We used two buffer systems in this study, which gave identical levels of inhibition (relative to the untreated controls) for each agent tested. The only difference noted was a slightly higher specific activity for TCP using MHM buffer. The inhibitor of interest was usually added during the second incubation period (step 3 in Fig. 1). However, in some experiments the inhibitor was added at different stages of the assay, in order to test its effect on either the substrate itself or its ability to block the binding of TCP to the MT substrate. To test the effect of the inhibitor on the MT substrate, prepolymerized substrate (made by first allowing the tubulin dimers to self-assemble for 10 min at 37°C, followed by the addition of taxol to 20 μM and a further 10 min warm incubation) was incubated (at 37°C) with the inhibitor for 10 min. The MTs were then centrifuged at high speed (436,000 × *g* for 4 min). The supernatant from the centrifugation was subjected to TCA precipitation, after which the TCA pellet was solubilized with strong base and counted for radioactivity (to measure the level of substrate disassembly into tubulin dimers that was induced by the inhibitor). The substrate MT pellet (that had been treated with the inhibitor) was resuspended in MHM buffer and was then mixed with a fivefold dilution of brain extract containing 20 μM taxol. The extract:substrate mixture was analyzed as for a routine TCP assay, except that after the TCP:MT complex was pelleted (step 2 of the assay), the supernatant was TCA precipitated and analyzed for the level of tubulin dimers as before. When zinc was used as the inhibitor, occasionally the zinc:substrate mixture was chelated with EDTA (to 10 mM) in order to remove excess inhibitor before adding the substrate directly to the brain extract.

In other experiments, the extract was first incubated with the inhibitor and polymeric substrate together, and then centrifuged. The supernatant was treated in order to lower the level of inhibitor (by either dilution, chelation, or desalting) and was then incubated with new polymeric substrate, in order to determine the level of TCP that had not bound to the original substrate, but was still active.

Finally, in some experiments the brain extract was incubated with magnesium, zinc, or iodoacetic acid for 25 min at 37°C, desalted, and then mixed with substrate as for a routine TCP assay. From these experiments we sought to detect any direct effects of the inhibitor on the enzyme-containing extract, apart from any similar effects on the substrate.

RESULTS

The conventional assay that was used previously to measure TCP activity involved mixing the enzyme-containing material directly with unpolymerized, ^{3}H- or ^{14}C-labeled tubulin substrate, and then measuring the release of TCA-soluble material within a given time period (a "one-step" assay; Fig. 1a). In our assay (Fig. 1b), we incubated taxol-stabilized, ^{14}C-labeled MTs with enzyme-containing material (step 1), pelleted the enzyme:MT complex (step 2), and then resuspended that material in a suitable buffer and incubated it for 20–30 min at 37°C (step 3). In this way, we imposed more stringent requirements on the activity we measured; i.e., any ^{14}C-releasing activity first had to be able to bind to, then pellet with, MTs. In addition, we measured the amount of endogenous Tyr tubulin that pelleted with the added substrate MTs, in order to determine more accurately the specific activity of the substrate. We found earlier that these conditions separated tubulin carboxypeptidase activity from that of other, more nonspecific activities [Webster et al., 1992]. We routinely added potential activators or inhibitors of TCP to the resuspension buffer (i.e., at step 3), after the TCP-containing MTs had been pelleted.

We began this study with the realization that, because all previous work on this enzyme [with one exception: Webster et al., 1992] had been carried out using either unpolymerized dimers as substrate or microtubules that were at steady state with dimers [Arce and Barra, 1985; Lopez et al., 1990], the results of those studies probably reflected the pooled activities of several enzyme activities. For example, in a recent study [Webster et al., 1992] it was reported that much spurious "carboxypeptidase-like" activity was measured in extracts from brain tissue and from

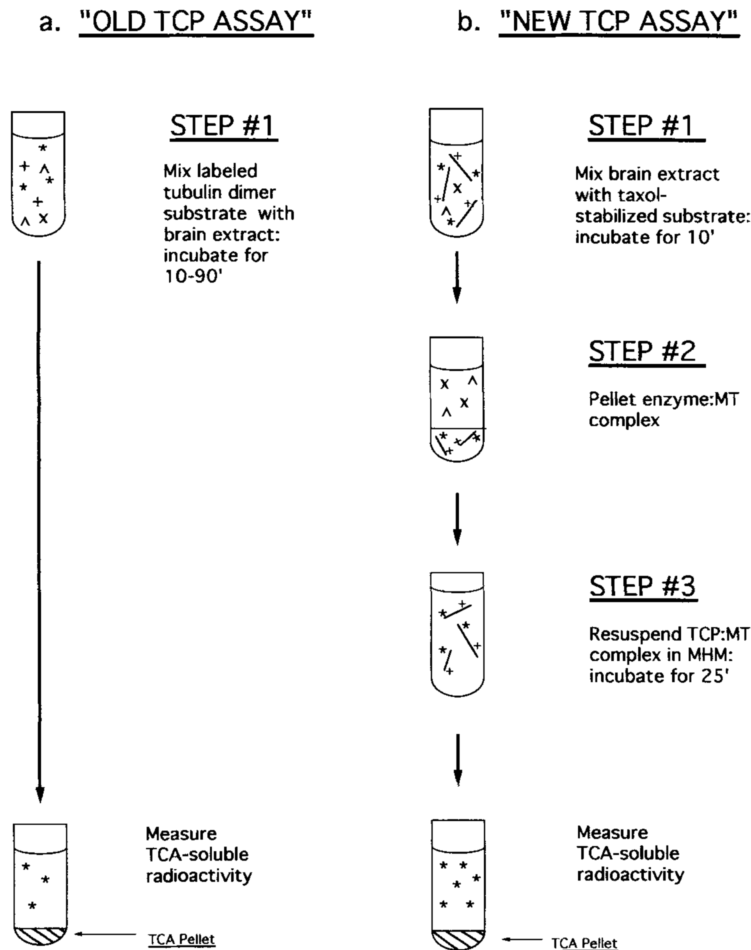

Fig. 1. The conventional, "old" TCP assay and the improved, "new" TCP assay developed by Webster et al. [1992] are compared in this cartoon. The "old" assay is depicted in **a**, wherein radioactively labeled (*), unpolymerized tubulin is mixed with enzyme-containing material ("+" = TCP, "Λ, x" = competing enzyme activities) for an established period of time, and is then precipitated with TCA. All TCA-soluble cpms are measured as TCP activity. The "new" assay is depicted in **b**, and reveals several intermediate steps. First, the enzyme-containing material is mixed with ^{14}C-labeled, taxol-stabilized MT substrate (step 1). Next, the TCP:MT complex is pelleted at high speed (step 2), and is then resuspended in a suitable buffer, which is essentially free of contaminating enzyme activities (step 3). Finally, this mixture is incubated for a set time interval before TCA precipitating it, as for the old assay. This procedure is both more sensitive and more specific than the protocol depicted in **a**.

cultured cell lines using the conventional assay (using unpolymerized tubulin as substrate), while an MT-enhanced activity (which was not affected by inhibitors of other carboxypeptidase activities) was measured using taxol-stabilized MTs. Although the tubulin carboxypeptidase activity was explored in a limited manner, the task remained to characterize its activity more fully, with respect to its possible regulation by cations and other domain-specific antagonists. Therefore, one goal was to establish the optimal parameters for activity in vitro using the new assay, as well as the activities of putative inhibitors and activators of TCP.

Differential Activation of TCP by a MOPS/HEPPS Buffer

Initially, we compared the level of TCP activity after the resuspension of the TCP:MT complex in various buffers (including PIPES, MES, and MOPS), over the pH range from 6.0 to 7.25, and found that MOPS buffer at pH 7.0 yielded the highest activity. Further, we documented a significant decrease in activity above pH 7.0, in agreement with the results reported by Argarana et al. [1978]. However, other work prompted us to extend that analysis in the present study. Although 50 mM MOPS yielded high

levels of TCP activity at pH 7.0 and pH 8.0 (Fig. 2a), the activity was dramatically lower at pH 7.5. This led us to explore TCP behavior in other buffers at these higher pH values, in order to test whether the effect was pH or buffer dependent. We used either 50 mM Tris (pK_a at 20°C of 8.3), 50 mM HEPPS (pK_a of 8.0), or a combination of 50 mM MOPS and 50 mM HEPPS. TCP responded quite differently according to the buffer type. The maximal values for TCP (~35% increase over MOPS, pH 7.0) were found using the MOPS/HEPPS combination at pH 7.5. Tris buffer was not suitable for measuring TCP activity, because the enzyme activity values were significantly lower using this buffer. TCP activity that was measured in HEPPS buffer alone, except for values at pH 7.5, was quite similar to that obtained using MOPS.

We reasoned that the increased activity values using the MOPS/HEPPS combination might be due merely to its increased ionic strength or buffering capacity. Therefore, we measured TCP activity in MOPS buffer alone (pH 7.0) at increasing buffer concentrations (Fig. 2b) in order to test that assumption. We found that (1) TCP activity did not increase with increasing buffer concentrations (over the range of 50–200 mM), (2) 50 mM MOPS represented the least concentrated buffer solution required for maximal activity (important for some chromatographic work), (3) TCP activity did not decrease when the concentrations of MOPS and HEPPS were each reduced to 25 mM (data not shown), and (4) the combination of MOPS and HEPPS, pH 7.5, overcame the diminution of activity that was observed in MOPS buffer alone.

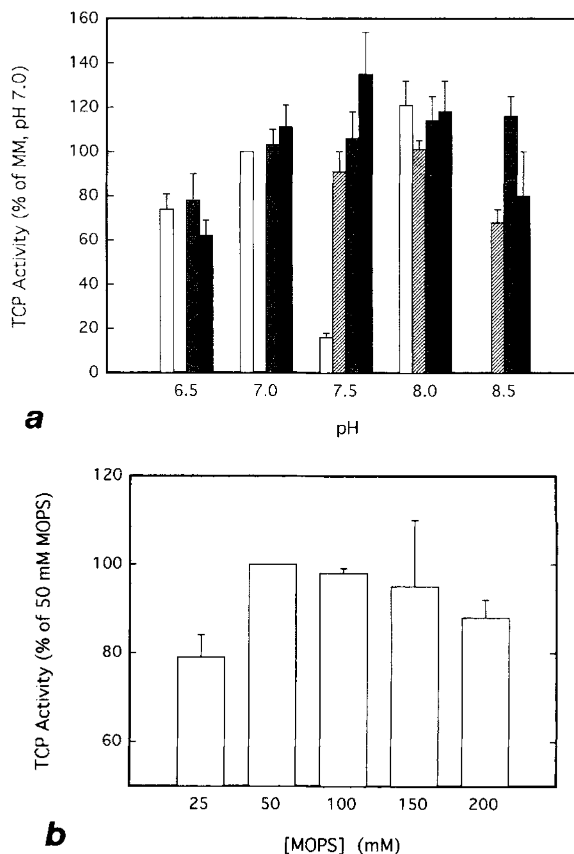

Fig. 2. TCP activity was measured under different buffer conditions. **a:** TCP activity was measured using the new assay, except that the TCP:MT complex was resuspended in either MOPS buffer alone (□), Tris buffer (▨), HEPPS buffer (▒), or a combination of MOPS and HEPPS (■), over the pH range from 6.5 to 8.5. All buffers contained 2 mM $MgCl_2$. Mean TCP activity values were calculated as the percent of activity in MOPS buffer alone, pH 7.0, ±SD. The MOPS/HEPPS combination, pH 7.5, yielded the highest values for TCP activity. **b:** TCP activity was measured in MOPS buffer alone (25–200 mM) to determine whether the increased buffering capacity alone was responsible for the higher values recorded for the MOPS/HEPPS combination in a. Mean TCP activity values were calculated as the percent of that found for 50 mM MOPS, ±SD, normally our "standard buffer" condition.

Monovalent Cations

We next sought to determine the strength of the binding of TCP to MTs by determining the activity of TCP on taxol-stabilized MTs in the presence of monovalent cations (at step 3 of our assay, shown in Fig. 1). TCP has been characterized as a MAP [Arce and Barra, 1983] and, indeed, we rely on its MT-binding properties to perform routine enzyme activity assays [Webster et al., 1992]. High-molecular-weight brain MAPs achieve half-maximal dissociation from taxol-stabilized MTs at ~150–200 mM monovalent salt concentrations (KCl or NaCl) [Vallee, 1982], indicating an ionic interaction of MAPs with the tubulin backbone of MTs. TCP activity declined at somewhat lower salt concentrations (half-maximal activity at ~100 mM KCl; Fig. 3), suggesting a weaker interaction with MTs. This result also suggested that other MAPs might normally outcompete TCP for MT binding sites when present at saturating levels (e.g., during normal brain preps). Indeed, we found that when we attempted to isolate large quantities of TCP with MTs, approximately half of the total activity remained in the MT-depleted supernatant (unpublished results).

Although the evidence suggested that TCP possesses properties that are characteristic of

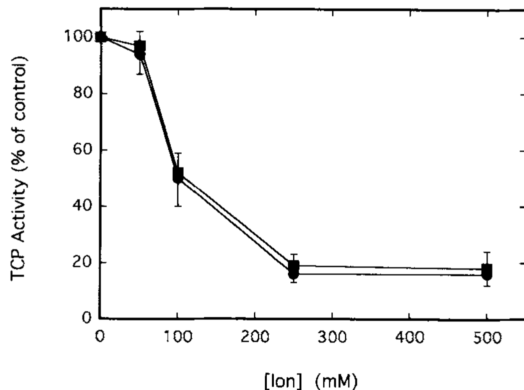

Fig. 3. TCP activity decreases with increasing monovalent cation concentrations. TCP activity from brain extract was determined using the "new" assay. Either KCl (■) or NaCl (●) was added during the second incubation period (step 3 in Fig. 1). Half-maximal inhibition of TCP activity was recorded at a monovalent cation concentration of ~100 mM (either KCl or NaCl), while maximal inhibition was observed at 250 mM salt. Values shown are the mean ± SD.

other MAPs, we considered that the observed inhibition of TCP by monovalent cations might arise by either of two mechanisms: (1) an inhibition of the binding of TCP to MTs, or (2) a direct inhibition of the enzyme. To evaluate these possibilities, we performed the following experiments. First, we added 250 mM KCl to the initial extract:substrate mixture (i.e., step 1), and then we measured the level of activity that was still able to pellet with MTs. We found that, under those conditions, only ~10% of the activity measured in untreated controls could be pelleted (Table I). Second, after mixing and pelleting the salt:extract:MT complex, we took the remaining supernatant and desalted it before adding it to new substrate. This material, now containing much lower levels of salt, contained much of the original TCP activity (data not shown). Third, we mixed undiluted extract (which would otherwise be diluted fivefold before mixing with substrate) plus salt together with twice the usual amount of substrate MTs (to prevent saturation of the substrate), pelleted the MTs, and then diluted the supernatant fivefold (to a KCl level that does not inhibit TCP activity; see Fig. 3) and reassayed the diluted mixture for TCP activity. Under these conditions, we were again able to recover much of the initial activity (data not shown). Therefore, the inhibition of TCP activity by either KCl or NaCl was the result of the inhibition of binding of TCP to MTs.

TABLE I. TCP Inhibition After Cation Addition to the Extract:Substrate Mixture*

Inhibitor	Conc'n (mM)	% Inhibition
Mg^{++}	25	64 ± 13
Mg^{++}	50	90 ± 1
Ca^{++}	50	88 ± 3
Zn^{++}	0.1	50 ± 22
K^{+}	250	91 ± 2

*Brain extract (diluted 1:5) was mixed together with ^{14}C-labeled, taxol-stabilized substrate and one of the cations listed above. After a 10 min incubation at 37°C, the mixture was sedimented as described in Materials and Methods. The MT pellet was resuspended in MHM buffer (with no added cation except for the usual 5 mM Mg^{2+}), and TCP activity was assayed. Values are displayed as the percent inhibition of untreated samples (n = 2–4).

Regulation by Magnesium and Calcium

Many enzymes are regulated, either positively or negatively, by divalent cations. We sought to determine whether TCP is similarly activated or inhibited, but in a manner essentially independent of the ability of the cations to depolymerize the MT substrate (made possible by the use of the taxol-stabilized substrate). Figure 4a shows that, over the narrow concentration range from ~2 to 10 mM, both calcium and magnesium stimulated TCP, but at higher concentrations (> 20 mM) acted as TCP inhibitors. In addition, the stimulation by calcium was not as great as with magnesium, and if calcium was added to MOPS buffer already containing 2 mM magnesium (which was our standard buffer for many of our experiments), no stimulation was observed. We suspected that the observed inhibition of TCP at higher cation concentrations was due to a weak interaction with either the enzyme or the substrate that interfered with the binding of TCP to MTs, because lower cation concentrations stimulated TCP activity and because TCP activity was inhibited when the extract and substrate were mixed with either 50 mM magnesium or calcium (i.e., step 1 of the assay shown in Fig. 1b; Table I). To test this possibility, we incubated brain extract with 50 mM magnesium for 25 min, desalted it, and then measured the TCP activity. Under these conditions we measured 100% of the level of TCP activity found in control samples. The results of these experiments strongly suggested that these divalent cations inhibited TCP activity by interfering with the binding of the enzyme to its substrate.

Further, the inhibition was not due to the depolymerization of the taxol-stabilized MTs (even at 50 mM concentration), because the amount of ^{14}C-labeled tubulin that was disassembled after a 25 min incubation with 50 mM calcium in buffer alone was minimal. In addition, the level of soluble substrate present after the second incubation (during which TCP activity is actually measured) was only mildly elevated (~7.5% of the total cpm for 50 mM calcium, 4% of the total cpm for magnesium, and 4% of the total cpm for the untreated controls) and did not account for the level of TCP inhibition observed (data not shown). In sum, the results indicated that (1) magnesium stimulated TCP activity, (2) calcium also stimulated TCP, albeit to lower levels than with magnesium, (3) either ion inhibited TCP at higher concentrations, probably by interfering with binding of the enzyme to its substrate, (4) the stimulatory activity of magnesium did not depend solely on its valence (since magnesium stimulated TCP more than did calcium), and (5) the inhibition of enzyme activity by these divalent cations was not explained by a reduced level of polymerized tubulin substrate.

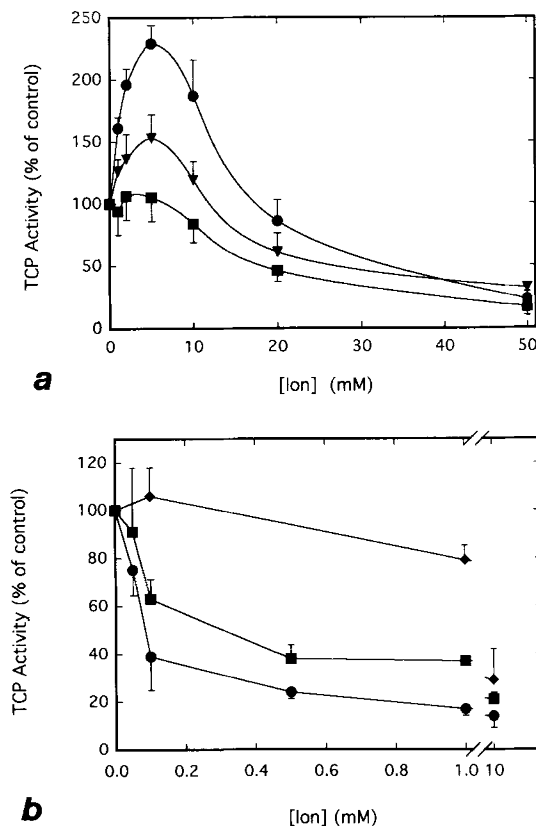

Fig. 4. Divalent cations can act as either stimulators or inhibitors of TCP activity. **a:** Over the concentration range of 2–10 mM, both Mg(II) (●) and Ca(II) that was added to MOPS buffer alone (▼) stimulated TCP activity, while the same Ca(II) concentrations added to MM buffer [containing 2 mM Mg(II)] (■) produced no stimulation. At higher concentrations of either cation (~20 mM+), TCP activity was inhibited to a maximal level of ~70–80%. **b:** Other divalent cations [Zn(II) (●), Cu(II) (■), or Co(II)] (◆) showed no stimulation of TCP activity at any concentration tested, but inhibited TCP at concentrations of 0.05–0.1 mM. Half-maximal inhibition occurred at ~0.075 mM [Zn(II)], 0.3 mM [Cu(II)], or 4 mM [Co(II)]. Values shown are the mean ± SD.

TCP Inhibition by Other Divalent Cations

In contrast to the results obtained by adding either calcium or magnesium, the addition of either zinc, copper, or cobalt inhibited TCP activity at virtually all of the concentrations tested (Fig. 4b). The inhibitory activity of zinc was the highest (half-maximal at ~75 μM), followed by copper (50% inhibition at ~0.3 mM), and then cobalt (50% inhibition at ~4 mM). We performed several types of experiments in an attempt to determine the site of inhibition by zinc. First, zinc was added directly to preformed, taxol-stabilized MT substrate, incubated for 10 min at 37°C, and then either pelleted and resuspended in MHM buffer or treated directly with EDTA (to 10 mM), in order to reduce the concentration of free zinc. This "pretreatment" of the substrate was followed by a routine TCP assay, in order to determine if replacement of the magnesium (that is normally bound with high affinity to the substrate MTs [Olmsted and Borisy, 1975] with zinc was generating part or all of the observed inhibition. Only mild inhibition of TCP was observed (~11%), and no (<5%) zinc-induced disassembly of the substrate (which does occur on MTs not stabilized by taxol) [Gaskin, 1981] was detected at any stage of the assay, indicating that TCP inhibition was not due to either the replacement of the (high-affinity-bound) magnesium with zinc on the MT lattice, or to the loss of substrate that was available for the binding of TCP. Therefore, any such substrate modification that might have been induced by zinc was not important for TCP binding or activity.

Second, zinc was added to the initial extract: substrate mixture (again without significant disassembly of the MT substrate; see Fig. 1) and, after pelleting the TCP:MT complex, TCP activity was measured. Approximately one-half of the activity found in untreated controls was measured in the treated samples (Table I). When the

extract supernatant (composed of the material that had *not* bound to the substrate) from the same tubes was treated with EDTA (to 10 mM) and was then added to new substrate, no TCP activity was detected. Therefore, the activity that was inhibited by zinc was not recovered in the supernatant, and was probably not due to the weak binding of the metal to the enzyme.

Finally, we preincubated the brain extract with 0.1 mM zinc for 25 min, desalted the mixture, and then performed a routine TCP assay. Under these conditions, TCP activity was not inhibited and the substrate did not disassemble, indicating that zinc did not bind tightly to the enzyme. It is somewhat puzzling why no TCP activity was recovered from the supernatant of the TCP:MT pellet after the EDTA treatment, whereas in other experiments no inhibition was observed when either the zinc-containing substrate or the enzyme material was treated with EDTA. However, the evidence supports the possibility that zinc interfered with the normal binding of TCP to the MTs, because inhibition was observed only when all three components were present together.

TCP Inhibition by Sulfhydryl-Reactive Compounds

It has been reported that 2 mM iodoacetate, a sulfhydryl-modifying (carboxymethylating) reagent, virtually abolishes TCP activity (again, using the conventional assay) [Hallak et al., 1977; Argarana et al., 1978]. Our results showed that, while inhibition was nearly maximal at 2 mM (Fig. 5a), significant TCP activity remained. The concentration of iodoacetic acid that was required to observe 50% inhibition was $\sim 5 \times 10^{-4}$ M, and the maximum inhibition achieved was ~80%. Likewise, TCP was also inhibited by n-ethylmaleimide (NEM), a reagent that modifies sulfhydryl groups on proteins by alkylation. The concentration at which half-maximal inhibition was observed ($\sim 2 \times 10^{-4}$ M) was similar to that of iodoacetic acid, as was the maximum level of inhibition (~70%; Fig. 5b). As for the experiments involving the divalent cations, the taxol-stabilized substrate was pre-incubated with 2 mM iodoacetate for 10 min at 37°C, and was then pelleted and resuspended in buffer plus taxol. This pretreated substrate was then used in a routine TCP assay of brain extract, in order to determine if the TCP inhibition was due solely to the modification of the substrate. We found that such pretreatment resulted in only moderate inhibition of TCP activity (~30% inhibition) and no significant substrate disassembly, indicating that the majority of the inhibition was due to the direct modification of the enzyme. When extract was incubated with 2 mM iodoacetic acid for 25 min at 37°C, desalted, and then assayed for TCP activity, virtually none was detected. Although we concluded that the major site of inhibition was on the enzyme-containing extract, with a lesser effect on the substrate, we could not determine whether these agents modified the MT-binding site or the active site on TCP. However, the sum of our results strongly suggested that the inhibition was achieved mainly by the modification of protein(s) (most likely TCP itself) present in the brain extract.

Effect of a Potent CPA Inhibitor on TCP Activity

Finally, we analyzed the inhibition of TCP by DL-benzylsuccinic acid, which is a potent inhibitor of carboxypeptidase A (CPA; its half-maximal inhibition is at ~1 μM [Byers and Wolfenden, 1972]; see Table II). We found that, in contrast to its strong ability to inhibit CPA, DL-benzylsuccinic acid inhibited TCP only at high concentrations (half-maximal at ~38 mM; Fig. 6), and imparted a maximal inhibition of ~85%. We compared the behavior of CPA toward the inhibitors studied in this report (Table II). Although the concentrations of some of the inhibitors that gave half-maximal inhibition are similar, there are also some notable differences, including the ~30-fold greater sensitivity of CPA to inhibition by zinc when measured using its optimal substrate, but at least a fourfold decrease in sensitivity (compared with TCP) when tubulin dimers were used, and the inhibition (~50%) of CPA by 2 mM magnesium, a concentration that *stimulates* TCP. Other differences have been noted previously [Webster et al., 1992]; namely, the inhibition of CPA by either EDTA or the potato inhibitor, both of which have no effect on TCP. Thus, it is clear that, although CPA can detyrosinate MTs in vitro [Webster et al., 1987a], it is an enzyme activity distinct from TCP.

DISCUSSION

The post-translational modification of Tyr MTs by detyrosination biochemically marks subsets of stable MTs. TCP activity modifies MTs by removing the C-terminal tyrosine from α-tubulin, and seems to show little, if any, activity

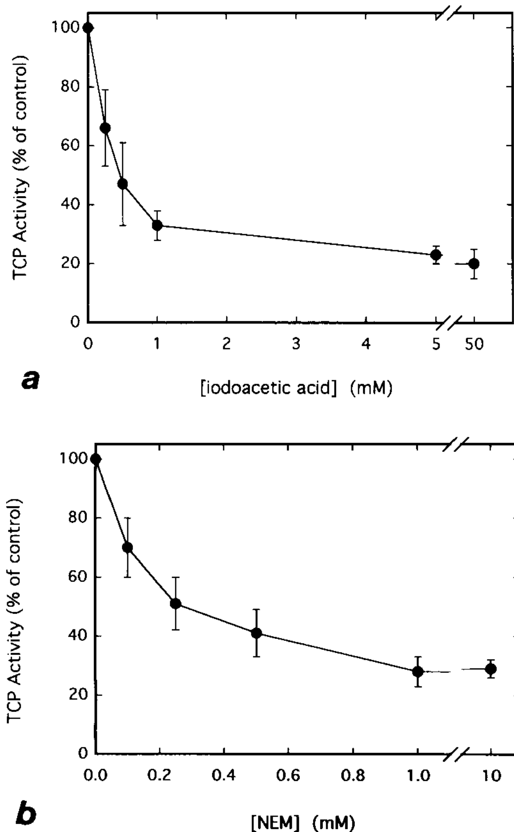

Fig. 5. TCP activity is inhibited by sulfhydryl-modifying compounds. Iodoacetic acid, when added to the second incubation buffer, inhibited TCP activity half-maximally at ~0.5 mM (**a**), and the maximum level of inhibition observed was ~80% (at ~3–5 mM). Likewise, n-ethylmaleimide (NEM) also inhibited TCP activity under these conditions (**b**), with half-maximal inhibition occurring at ~0.2 mM and a maximum inhibition of ~70% (at 1 mM concentration). Values shown are the mean ± SD.

toward unpolymerized tubulin dimers in vivo [Wehland and Weber, 1987b]. Since the rate of detyrosination by TCP is slow compared with the rate of turnover of most MTs [Webster et al., 1990], only MTs that have become stabilized (by an unknown mechanism) normally become appreciably detyrosinated (i.e., visible by immunofluorescence using specific antibodies) [Gundersen et al., 1987; Webster et al., 1987b]. A question that arises is how particular MTs (or MT subsets) can be involved in distinct cellular functions. One possibility is that some MTs become biochemically modified, and therefore specialized, to perform a particular task. Detyrosination (and therefore TCP activity) might be crucial within such a scenario.

We recently developed an improved assay for TCP in order to begin the characterization of TCP [Webster et al., 1992] and to aid in the eventual purification of the enzyme. To define the activity of this enzyme more fully, we now sought to determine whether TCP activity might be regulated in vitro by cations and other domain-modifying reagents. Although experiments that are similar to some of the ones described here were performed earlier [Argarana et al., 1978, 1980], they were accomplished using unpolymerized tubulin as substrate (see Fig. 1), which is not the preferred substrate for TCP, either in vitro [Kumar and Flavin, 1981] or in vivo [Gundersen et al., 1987; Wehland and Weber, 1987b]. Since other carboxypeptidase-like activities routinely contribute to the overall activity measured under those conditions [Webster et al., 1992], it was important to re-evaluate the existing data, as well as provide new information, using the improved assay.

We performed several different types of enzyme assays in order to evaluate the effect of the inhibitors on both the MT substrate and the enzyme. For our "standard" inhibitor assay, we incubated the brain extract with the taxol-stabilized substrate, pelleted the MTs, and then resuspended them in buffer containing the inhibitor and assayed TCP activity. We realized that, under these conditions, the inhibition might arise either by the modification of the substrate, the modification of the enzyme by binding at or near the active site, or by the inhibition (ionically or sterically) of the binding of TCP to the MTs. In an attempt to discern which of these possibilities was contributing to the observed inhibition, we added the inhibitor at different stages of the assay and compared the results with those obtained under "standard" conditions. Although there are some limitations to this approach (as discussed below), we were able to differentiate strong effects on the enzyme-containing material from effects that occurred on the substrate alone. We therefore applied this experimental rationale to the study of putative activators and inhibitors of TCP activity in vitro.

It is important to point out both the similarities and the differences between our results and the results of earlier studies [Argarana et al., 1978, 1980; Barra and Argarana, 1982]. The first difference is that in our study, the pH optimum for activity was 7.5, while in one of the earlier studies it was reported to be between 6.3 and 7.0 [Argarana et al., 1978]. The greatest activity we could measure at pH 6.5, using the

TABLE II. Carboxypeptidase A Inhibitors*

Inhibitor	Concn. (M)	% Inhib.	Substrate	Ref.
DL-benzylsuccinic acid	1.1×10^{-6}	50	Hippuryl-L-phenylalanine	*b*
Co(II)	1×10^{-2}	<10	Dns-Gly-Ala-Phe	*d*
Cu(II)	1×10^{-4}	<10	Dns-Gly-Ala-Phe	*d*
Zn(II)	2.6×10^{-6}	50	Dns-Gly-Ala-Phe	*d*
Mg(II)	2×10^{-3}	49	Tubulin dimers	*c*
Mg(II)	2×10^{-2}	78	Tubulin dimers	*c*
Zn(II)	1×10^{-4}	18	Tubulin dimers	*c*
EDTA	5×10^{-3}	73	Tubulin dimers	*c*
Iodoacetic acid	2×10^{-3}	40	Tubulin dimers	*c*

*The inhibition of CPA by various agents was measured spectrophotometrically (*b*) in 0.025 M Tris-HCl buffer, pH 7.5, containing 0.5 M NaCl; in 0.01 M sodium phosphate buffer, pH 7.0, with no added NaCl (*c*); or in 0.05 M HEPES buffer, pH 7.5, containing 0.5 M NaCl (*d*). *b*, Byers and Wolfenden [1972]. *c*, Argarana et al. [1980]. *d*, Larsen and Auld [1990].

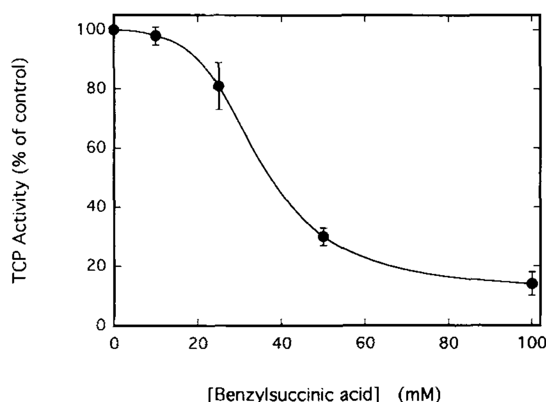

Fig. 6. TCP activity was measured in the presence of a potent inhibitor of pancreatic CPA, DL-benzylsuccinic acid. This agent was only a mild inhibitor of TCP, displaying half-maximal inhibition at ~38 mM and maximum inhibition at 60–100 mM. By comparison, CPA is inhibited half-maximally by DL-benzylsuccinic acid at ~1 μM (see Table II). TCP and CPA are distinct enzyme activities.

MOPS/HEPPS combination, was approximately 50% lower than at pH 7.5. Because we noted differences in TCP activity, measured at the same pH, that corresponded to the specific buffer type used and its concentration (e.g., between the 50 mM MOPS buffer and the MOPS/HEPPS combination at pH 7.5, and between 25 and 50 mM MOPS at pH 7.0), some of the difference reported in these studies might be attributed to the use of 7 mM sodium citrate–phosphate buffer in their experiments. However, their use of unpolymerized tubulin as substrate, which does not discriminate against related carboxypeptidase-like activities (especially at lower pH values) [Webster et al., 1992], suggests that the lower pH optimum that they reported reflected the optima of multiple enzyme activities.

MAPs were originally defined as those proteins that copurified with tubulin during protocols designed to purify microtubules [Borisy et al., 1975; Sloboda et al., 1975], and were found to stimulate the assembly of tubulin in vitro [Murphy and Borisy, 1975; Sloboda et al., 1976]. In addition, high-molecular-weight brain MAPs were further characterized with respect to their salt-sensitive dissociation from the tubulin backbone of taxol-stabilized MTs [Vallee, 1982]. Because TCP has also been characterized as a MAP (its activity cycled with MTs) [Arce and Barra, 1983], we sought to determine the strength of its binding to MTs. We found that TCP dissociates from MTs at a somewhat lower salt concentration than other, well characterized MAPs [Vallee, 1982], consonant with our previous observation that TCP partitioned approximately equally between the MT pellet and the supernatant during the bulk preparation of MTs (containing both tubulin and MAPs). Both sodium chloride and potassium chloride dissociated TCP from MTs at the same concentrations, and TCP activity was recovered from the salt-containing supernatants. Thus, we concluded that TCP is a relatively weak binding MAP, as measured by its dissociation from MTs in low salt concentrations.

We found that 5 mM calcium stimulated TCP. Earlier reports stated that 5 mM calcium inhibited TCP activity by ~40% [Arce et al., 1978; Kumar and Flavin, 1981]. It was also reported that, under those conditions, most of the microtubule protein (tubulin plus MAPs, but not taxol stabilized) used as the substrate had depolymerized, which probably accounted for the loss of activity that they observed. In this study, we found that even 50 mM calcium did not depoly-

merize the taxol-stabilized MTs, allowing us to measure TCP activity independently of any calcium-induced disassembly of the substrate. The level of stimulation by calcium was less than by magnesium, indicating that the latter was providing more than mere positive charge to the interaction between the enzyme and the substrate (i.e., more than a charge modification on the MTs). Further, when calcium was added to brain extract in MM buffer (containing 2 mM $MgCl_2$), the normal level of stimulation was abolished, suggesting that calcium opposed the effect of magnesium on TCP. Calcium cannot replace the high-affinity magnesium that is bound to tubulin [Buttlaire et al., 1980], but has been shown to antagonize the magnesium-induced stimulation of tubulin assembly [Olmsted and Borisy, 1975]. Calcium may exert effects on MTs by binding to the C-termini of tubulin subunits, since subtilisin-cleaved tubulin becomes resistant to Ca^{2+}-induced, but not cold- or dilution-induced, MT disassembly [Saoudi et al., 1995]. It is possible that the binding of calcium (or magnesium) to the C-terminus of α-tubulin interferes with TCP directly, but only at high concentrations (>10 mM). Our use of taxol-stabilized MTs in these assays allowed us to measure TCP inhibition at cation concentrations that would otherwise disassemble the substrate. Both divalent cations became inhibitory over a similar, higher concentration range, suggesting that a similar, possibly nonspecific effect on the enzyme reaction had occurred when either cation was present. We could not determine whether the effect was due to the binding of the cation(s) at a specific site or was due to a general charge modification of the MT substrate.

Zinc, copper, and cobalt also inhibited TCP, in general agreement with earlier reports [Argarana et al., 1978, 1980]. However, the extent of inhibition was somewhat greater in this study [~40% inhibition by 0.1 mM copper in this study vs. ~15% in Argarana et al., 1978; ~60% inhibition by 0.1 mM zinc in this study vs. ~5–10% in Argarana et al., 1978, 1980]. The differences may be ascribed either to the increased sensitivity of the assay used in this study, or to the aforementioned contributions of other enzyme activities that are usually measured when tubulin dimers are used as the substrate.

Both zinc and cobalt can readily substitute for the tightly bound magnesium on tubulin [Buttlaire et al., 1980], although cobalt may bind to an additional site [Himes et al., 1982]. However, these ions inhibited TCP activity to different levels and at disparate concentrations, suggesting a different mode of involvement in TCP inhibition. Further, preincubation of the MT substrate with either of these cations did not decrease TCP activity, suggesting that substrate modification did not contribute to the inhibition. In other studies, we found that the activity from tubulin-depleted, partially purified TCP preparations could be removed after passing the enzyme-containing material over a metal chelation column that had been "loaded" with either zinc or copper (unpublished results), but much of the activity could not be recovered under routine elution conditions (including "stripping" the column with EDTA). Although the reason for the loss of activity was not determined, and might have been due to the progressive denaturation of the enzyme, one possibility is that the metal cation bound TCP to another protein (remnant levels of tubulin?) and rendered it inactive.

Although copper has not been shown to replace the tightly bound magnesium on tubulin, it has been shown to inhibit tubulin polymerization in vitro [Wallin et al., 1977]. Further, zinc, copper, and cobalt reduce the number of free sulfhydryl groups on tubulin, which may be responsible for their effects on tubulin polymerization. The sum of our results suggests that these divalent metal cations inhibit TCP activity (or its release) by interfering with the binding of TCP to MTs in a manner that requires the presence of the enzyme, the substrate, and the cation together. Inhibition by a direct, weak interaction with the enzyme (possibly by blocking free sulfhydryl groups that are essential for activity) is not favored, since pretreated and then desalted extract displayed normal levels of TCP activity.

We also investigated the requirement of free sulfhydryl groups for TCP activity by measuring the inhibitory capacity of two well known sulfhydryl-modifying agents, iodoacetic acid and n-ethylmaleimide. These compounds inhibited TCP over similar concentration ranges (0.1–5 mM), implying that free sulfhydryl groups are required either to bind TCP to MTs or directly for their activity. Again, when preincubated with substrate MTs only, neither of these two reagents promoted significant MT depolymerization and produced only a moderate decrease in

TCP activity, suggesting that MT modification did not contribute significantly to the inhibition of TCP. Brain extract that was treated directly with iodoacetic acid (before the addition of substrate MTs) lost all TCP activity in an irreversible manner, strengthening the probability that these agents directly affected the enzyme. In other work, we found that partially purified TCP bound to thiol-reactive chromatographic matrices (unpublished results). Under those conditions, the TCP activity was quantitatively eluted with DTT, demonstrating the presence of a free sulfhydryl group(s) within the enzyme [Krieger et al., 1976]. However, it is possible that another protein(s) that is essential for enzyme:substrate interaction was being affected instead of (or along with) TCP. Although there is no evidence for the requirement of accessory proteins for TCP activity, the answer to this question will await the analysis of the purified enzyme.

Finally, we measured the effects of a potent inhibitor of CPA, DL-benzylsuccinic acid, on TCP activity when added during the second incubation. In an earlier study [Webster et al., 1992], we described conditions that inhibited CPA activity (the presence of EDTA, carboxypeptidase inhibitor from potato, 8-hydroxyquinoline sulfonic acid, or DTT) but did not affect TCP. Here we add further evidence that TCP is an enzyme activity distinct from CPA, since the concentration of DL-benzylsuccinic acid that is required for half-maximal inhibition of TCP is over 10^4-fold higher than is required for CPA. CPA-like activities from mast cells [Goldstein et al., 1989; Reynolds et al., 1989] and from epidermal cell preparations [Kikuchi et al., 1989] have been partially characterized, but they also are distinct from TCP. Thus, it is unlikely that TCP merely represents a ubiquitous, cytoplasmic form of CPA.

We recorded two results where the site of inhibition could not be determined. When zinc was added directly to the extract, only ~50% of the control activity was measured. However, the supernatant that remained after pelleting the MT substrate contained no measurable activity. Therefore, we cannot be certain whether the remaining 50% of the control activity represented TCP that had bound zinc and was still bound to the substrate, or TCP that had bound zinc but had been released from the MTs. It is also possible that, under these conditions, TCP became progressively denatured, accounting for the inability to recover the remaining activity. Likewise, when we incubated extract with iodoacetic acid, desalted the material, and then assayed it for TCP activity, we could not discern if the enzyme had bound to MTs. The answers to these questions will require specific probes (e.g., antibodies) for TCP. However, the sum of the results from this study allowed us to make some worthwhile observations concerning TCP.

In summary, we have exploited a recently improved assay for TCP and have investigated the regulation of TCP activity in vitro by cations and sulfhydryl-modifying compounds. We have also begun to explore the mechanism by which these compounds affect TCP activity; by affecting the enzyme directly, by interfering with its binding to the MT substrate, or by modifying the substrate alone. We conclude that the divalent cations exert their inhibition mainly by interfering with the binding of the enzyme to the substrate, and only to a lesser degree (if at all) by the direct binding to the enzyme. Iodoacetic acid inhibited activity both by inactivating TCP directly and, to a lesser degree, by modifying the taxol-stabilized MTs. These results are likely to be important for our understanding of TCP activity in vivo, and have already aided us in devising a useful protocol for purifying TCP to near homogeneity. The characterization of purified TCP, and the preparation of specific, antagonistic reagents against it, will be useful for more detailed studies of its role in MT function in vivo, both in established cell lines and during the differentiation of cells during development [Larsen and Auld, 1991].

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