The Acetylation of Alpha-Tubulin and Its Relationship to the Assembly and Disassembly of Microtubules

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Abstract. A tight association between *Chlamydomonas* alpha-tubulin acetyltransferase (TAT) and flagellar axonemes, and the cytoplasmic localization of both tubulin deacetylase (TDA) and an inhibitor of tubulin acetylation have been demonstrated by the use of calf brain tubulin as substrate for these enzymes. A major axonemal TAT of 130 kD has been solubilized by high salt treatment, purified, and characterized. Using the *Chlamydomonas* TAT with brain tubulin as substrate, we have studied the effects of acetylation on the assembly and disassembly of microtubules in vitro. We also determined the relative rates of acetylation of

tubulin dimers and polymers. The acetylation does not significantly affect the temperature-dependent polymerization or depolymerization of tubulin in vitro. Furthermore, polymerization of tubulin is not a prerequisite for the acetylation, although the polymer is a better substrate for TAT than the dimer. The acetylation is sensitive to calcium ions which completely inhibit the acetylation of both dimers and polymers of tubulin. Acetylation of the dimer is not inhibited by colchicine; the effect of colchicine on acetylation of the polymer can be explained by its depolymerizing effect on the polymer.

ACETYLATION of several proteins is known to occur on the epsilon-amino group of their lysine residues (1, 2, 4). Although reversible acetylation has been studied most intensively with histones, in vivo and in vitro, the regulation of this modification and its physiological role still remain to be clarified. Recently we showed, by labeling in vivo with ^{3}H-acetate, that the acetylation and deacetylation of alpha-tubulin occur in *Chlamydomonas* during flagellar regeneration and resorption, respectively (3, 7–10). Acetylated alpha-tubulin was found almost exclusively in the flagellar apparatus, whereas cytoplasmic alpha-tubulin was found predominantly in the nonacetylated form (3, 8, 12). These results from labeling in vivo suggested that *Chlamydomonas* contains an alpha-tubulin acetyltransferase (TAT)[1] and a tubulin deacetylase (TDA) which carry out the reversible acetylation. Moreover, the results also indicated that there must be a mechanism by which acetylated alpha-tubulin accumulates principally in the flagellum.

Recently, we have demonstrated the presence of a TAT activity, using brain tubulin as a substrate, in flagella isolated from *Chlamydomonas* (5). We have purified and characterized the flagellar TAT to understand the role of alpha-tubulin acetylation in flagellar formation and/or function, and to determine the mechanism by which the acetylated alpha-tubulin is accumulated specifically in the flagellum. In this paper we show that (a) the TAT is highly specific for alpha-

tubulin, is tightly bound to the flagellar axoneme, and can be released from the axonemes by high salt treatment, (b) the enzyme will acetylate both dimers and polymers of tubulin, (c) calcium is a potent inhibitor of the acetylation of both dimers and polymers, (d) the acetylation does not affect either temperature-dependent assembly or disassembly of brain microtubules in vitro, and (e) a TDA and an inhibitor of tubulin acetylation can be found in the cytoplasm but not in the flagella.

Materials and Methods

Materials

[3]H-Acetyl CoA (1.54 Ci/mmol) was purchased from Amersham Corp. (Arlington Heights, IL); CoA and calf thymus histone IIs were from Sigma Chemical Co. (St. Louis, MO). All other chemicals were obtained from the same sources as described previously (5). Microtubule proteins (MTP) were prepared from calf brain by two cycles of polymerization/depolymerization as described previously (15). Microtubule-associated proteins (MAPs) and phosphocellulose (PC) tubulin were separated on a phosphocellulose column according to the procedure described previously (15). Flagella were detached from cell bodies of *Chlamydomonas reinhardtii* strain 21gr by dibucaine and isolated as described previously (19).

Solubilization and Isolation of Flagellar TAT

Flagella (200–250 mg of protein) suspended in 50 ml of HM buffer that contained 10 mM Hepes, pH 6.8, 1 mM MgSO$_4$, 1 mM dithiothreitol (DTT), 0.1 mM GTP, 0.5 mM phenylmethylsulfonyl fluoride, and 0.24 M sucrose were exposed to 0.2% Nonidet P-40 at 0°C for 15 min to lyse the flagellar membranes, and centrifuged at 20,000 g for 15 min. The pellet, containing the axonemes, was resuspended in 50 ml of HM buffer plus 0.66 M NaCl and kept on ice for 30 min. After centrifugation at 20,000 g for 15 min, the

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supernatant (NaCl extract) containing most of the TAT was dialyzed against TDSA buffer that contained 10 mM Tris-HCl, pH 7.5, 1 mM DTT, 15% sucrose, and 0.02% NaN$_3$. The NaCl extract was first fractionated on a hydroxylapatite column (bed vol 20 ml) equilibrated with TDN buffer that contained 10 mM Tris-HCl, pH 7.5, 1 mM DTT, and 0.1 M NaCl. Proteins bound to the column were eluted by a stepwise gradient of potassium phosphate (50, 100, and 400 mM) in TDN buffer. Most of the TAT activity was eluted with 50 mM phosphate. This TAT fraction (HA 50) was concentrated by dialysis against sucrose. The HA 50 was then fractionated on a Sephadex G-150 column (1.6 × 78 cm) equilibrated with TDN buffer. Most of the TAT activity was eluted at a position that corresponded to the elution of a globular protein of ~130 kD. This TAT fraction, called Sephadex 130, was further fractionated on a DEAE-cellulose column (bed vol 5 ml) equilibrated with TD buffer that contained 10 mM Tris-HCl, pH 7.5, and 1 mM DTT. Bound proteins were eluted by a stepwise gradient of NaCl (50, 100, and 400 mM). The major TAT activity (TAT-2) was eluted with 50 mM NaCl, whereas a minor TAT activity (TAT-1) was recovered in the flow-through fractions.

Preparation of Chlamydomonas Cell Body Extract

Deflagellated cells (cell bodies) were washed with 2 vol of HMDS buffer that contained 10 mM Hepes, pH 7.5, 5 mM MgSO$_4$, 1 mM DTT, and 4% sucrose. They were resuspended in 2 vol of HM buffer, disrupted with a French press at 500–600 kg/cm^2, and centrifuged at 20,000 g for 30 min. The slightly green supernatant, containing ~17 mg protein/ml, was used as the cell body extract.

Assay for TAT Activity

Calf brain MTP (25 μM) was incubated with various fractions at 37°C for 60 min in 100 μl of ADE buffer that contained 27–36 μM ^{3}H-acetyl CoA, 1 mM DTT, 0.8 mM EGTA, 40 mM Pipes, pH 6.9, 0.4 mM MgSO$_4$, 0.2 mM GTP, and 1.6 M glycerol. The TCA-insoluble radioactivity of ^{3}H-acetate incorporated into alpha-tubulin was measured by the filter paper assay described previously (5).

Assay for TAT Inhibitor and TDA Activities

The NaCl extract of flagella (0.7 mg protein/ml) was used as the source of TAT in the assays for the TAT inhibitor and for TDA. To detect the activity of TAT inhibitor, the cell extract to be tested was added, at 0 min, to a mixture of flagellar TAT and brain MTP in 100 μl of ADE buffer and incubated at 37°C for 60 min. For measurement of the TDA activity, brain MTP was preacetylated by TAT at 37°C in 100 μl of ADE buffer with ^{3}H-acetyl CoA at a reduced concentration (10 μM) to ensure that practically no radioactive acetyl CoA was left in the reaction mixture after incubating for 60 min. The cell body extract was then added to the reaction mixture which was incubated at 37°C for a further 60 min. Loss of TCA-insoluble radioactivity of ^{3}H-acetate was measured.

Assembly and Disassembly of Brain Microtubules Composed of Acetylated and Nonacetylated Tubulins

For the preparation of acetylated or nonacetylated (control) tubulin, brain MTP (3.7 mg/ml) was incubated with the NaCl extract of flagella (0.3 mg/ml) at 37°C for 4 h, in the presence of either 100 μM acetyl CoA (acetate donor) or CoA (acetylase inhibitor), in 1 ml of ADE buffer. Under these conditions, in the presence of acetyl CoA, microtubules were assembled and acetylated at a stoichiometry of 1.1 acetate per tubulin dimer (mol/mol). After incubation for 4 h, preparations of both acetylated and control microtubules were centrifuged at 100,000 g for 45 min. In both cases 75% of the

total tubulin was polymerized and recovered in the pellet. For depolymerization, the microtubules in each pellet were resuspended at a concentration of 1.4 mg/ml in 2 ml of cold PM buffer that contained 100 mM Pipes, pH 6.9 with KOH, 1 mM MgSO$_4$, 2 mM GTP, and 2 mM EGTA. The suspensions were held at 0°C for 30 min and then centrifuged at 100,000 g for 30 min. About 75% of the tubulin present in each pellet was depolymerized in both cases. The resultant supernatants, containing ~56% of the original tubulin dimers, were the starting materials for the kinetic analysis of polymerization and depolymerization. These supernatants (final concentration of tubulin, 0.8 mg/ml) were incubated at 37°C for 30 min for repolymerization, and the microtubules harvested by centrifugation at 100,000 g for 45 min. In the presence of 4 M glycerol, ~60% of the tubulin dimers in the supernatants assembled into microtubules independently of whether the tubulins were acetylated or nonacetylated. To estimate the amount of tubulin that polymerized and depolymerized at each step, each supernatant and pellet was subjected to SDS/urea (10%) PAGE and the stained gel was scanned at 550 nm.

Preparation of Microtubule Fragments, Measurement of Their Lengths, and Rate of Acetylation

For the preparation of relatively long (control) microtubules, brain microtubule proteins (containing 6 S tubulin and MAPs) and PC tubulin (6 S tubulin), each at 3.6 mg/ml in PM buffer, were mixed in a ratio of 1:1 and incubated at 37°C in the presence of 1 M glycerol. The polymerization reached a steady state within 30 min (A$_{350}$ 0.65). For the preparation of short fragments of microtubules, the microtubule preparation just described was passed vigorously through a 25-gauge needle, 5.0-cm long, 15 times. Aliquots of the control and the sheared preparations of microtubules were diluted five times with PM buffer plus 4 M glycerol, fixed with 2% glutaraldehyde, and negatively stained as described previously (13) for measurement of microtubule length by electron microscopy. To compare the rate of alpha-tubulin acetylation of the two preparations that contained either long or short microtubules, each preparation, at a final concentration of 2.0 mg/ml, was incubated at 37°C with the NaCl extract of the flagella as source of TAT, in the presence of 1 M glycerol in the standard TAT assay mixture for varying lengths of time. As the initial rate and not the extent of tubulin acetylation was to be measured, the amount of TAT added was limited to 1.4 units/ml (for definition of the unit, see Table I) so that the stoichiometry of the acetylation of tubulin after 80 min was still ~0.1 acetate per alpha-tubulin (mol/mol) in both cases. To quantify the acetylation of alpha-tubulin in each case, at each time point a sample of the reaction mixture was subjected to ultracentrifugation for 5 min in an airfuge at 30 psi. The microtubules in the pellet were dissolved in SDS PAGE sample buffer, boiled for 5 min, and subjected to SDS/urea PAGE to separate alpha-tubulin from beta-tubulin and MAPs. The gels were stained and each of the radioactive alpha-tubulin bands was cut out, dissolved by heating at 70°C for 7 h in 0.5 ml of 30% H$_2$O$_2$, and the radioactivity was measured by scintillation counting in 8.0 ml of Aquasol. In a parallel experiment, the protein content of each pellet was determined to calculate the specific radioactivity of the sedimented microtubules.

Results

Mg^{2+}-dependent Association of TAT with Flagellar Axonemes

In earlier experiments (5), whole flagella isolated from *Chlamydomonas* were shown to contain considerable TAT activity. Attempts were made to solubilize this flagellar TAT

Table I. Solubilization of TAT from Axonemes

Fraction	Total units*(% yield)	Total protein	Units/mg protein
		mg	
Whole flagella	90 (100)	50	1.8
Detergent extract	2 (3)	29	0.07
Axonemal pellet	88 (97)	21	4.2
NaCl extract	84 (92)	7	12.0
NaCl pellet	4 (5)	14	0.3

Flagella (8 mg protein/ml) were first extracted by 0.2% Nonidet P-40, and then the residual axonemal pellet was further extracted by 0.66 M NaCl.
* 1 unit is defined as the activity required to transfer 1 nmol of acetyl group to tubulin in 20 min at 37°C under standard TAT assay conditions.

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Table II. Mg²⁺-dependent Reassociation of TAT with Axonemes

Fraction	−Mg²⁺	+Mg²⁺
	%	%
Supernatant	94	44
Pellet	6	56

8.4 U of TAT (NaCl extract) and 1.0 mg of axonemes (NaCl pellet), the same aliquots, were incubated together at 0°C for 30 min in the presence or absence of 3 mM Mg²⁺, centrifuged at 20,000 g for 15 min, and the TAT activity in the supernatant and pellet was measured.
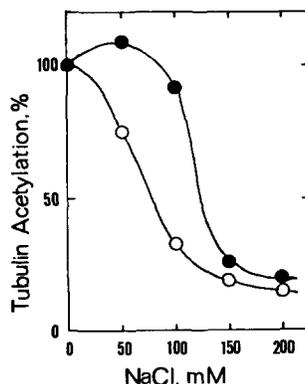
activity as shown in Table I. When whole flagella were extracted with 0.2% Nonidet P-40, most of the flagellar TAT activity remained associated with the sedimented axonemes. The majority of the axoneme-associated TAT activity could be released from the axonemes by treatment with 0.66 M NaCl in the presence of 0.66 mM Mg². However, in the presence of 5 mM Mg²⁺, the high salt treatment did not release the TAT activity from the axonemes, a result that suggests that Mg²⁺ is essential for the interaction of TAT with the axonemes. Once separated from the rest of the axonemes, the TAT activity remained soluble even after removal of NaCl. In the presence of 3 mM Mg²⁺, more than half of the TAT activity could be reassociated with axonemes within 30 min at 4°C (Table II). Unlike the interaction of TAT with the axonemes, which is Mg²⁺-dependent, the acetylation of brain alpha-tubulin by TAT did not require Mg²⁺ or any other divalent cations. Furthermore, NaCl at concentrations >0.1 M almost completely inhibited the activity of the axonemal TAT in both its free and bound forms (Fig. 1).
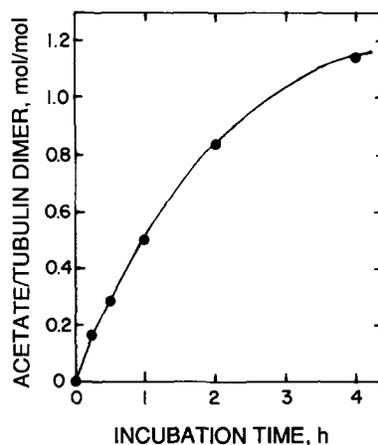
Stoichiometry of Alpha-Tubulin Acetylation

Using calf brain tubulin as substrate and the NaCl extract of flagellar TAT, we obtained a stoichiometry of alpha-tubulin acetylation of up to 1.1 mol acetate per mole of tubulin dimer after incubation for 4 h at 37°C (Fig. 2). The presence of 10-fold molar excess of acetyl CoA over the concentration of tubulin dimers did not change the final stoichiometry of the acetylation (data not shown).

The Effect of Ca²⁺ on Alpha-Tubulin Acetylation

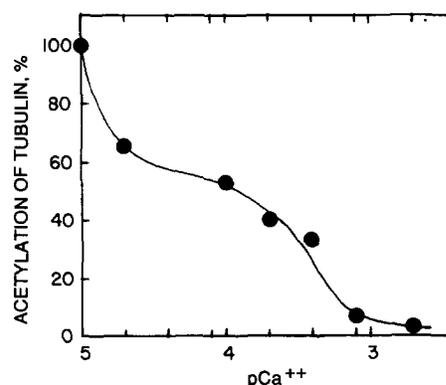Ca²⁺ (10⁻³ M) almost completely inhibited the acetylation of tubulin, and the curve of extent of inhibition versus Ca²⁺ concentration appeared to be biphasic (Fig. 3). The inhibition was not due to an effect of Ca²⁺ on assembly or disas-

Figure 2. Stoichiometry of tubulin acetylation. The time course of tubulin acetylation using in vitro-assembled brain MTP (25 μM) as a substrate was followed in the presence of 0.28 units of NaCl extract of flagellar axonemes as a TAT source.
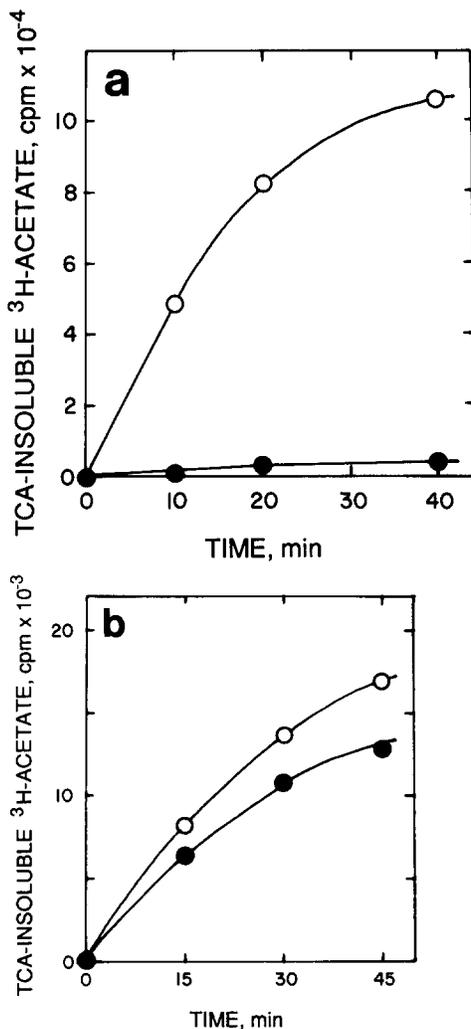

Figure 3. Effect of Ca²⁺ on tubulin acetylation. Acetylation of brain MTP (1.4 mg/ml) was measured for 60 min at various concentrations of Ca²⁺, as indicated, with 0.35 units of NaCl extract of flagellar axonemes.

sembly of microtubules, since these studies were also performed using PC tubulin dimers at concentrations below the critical concentration for assembly (Fig. 4 a). Moreover, since the PC tubulin used in these studies was free of calmodulin, MAPs, and tau proteins, the effect of calcium could not be due to the presence of any of these proteins. Instead, the effect of Ca²⁺ on tubulin acetylation must be due to its binding to TAT itself, tubulin dimer, or acetyl CoA. Since histone could serve as a substrate, albeit a poor one, for the flagellar TAT, we determined the effect of Ca²⁺ on the acetylation of histones by TAT. In Fig. 4 b it can be seen that Ca²⁺, which strongly inhibits the acetylation of brain tubulin by flagellar TAT (Fig. 4 a), has little effect on the acetylation of histones by TAT. Therefore, the major target of Ca²⁺ would appear to be neither the TAT itself nor acetyl CoA, but rather the tubulin dimer.

Two Forms of Flagellar TAT

When the NaCl extract of flagella was fractionated on DE-52 immediately after the removal of NaCl by dialysis, two peaks of TAT activity appeared: ∼60% of the TAT activity (TAT-2) was eluted with 50 mM NaCl and the rest of the TAT activity (TAT-1) was recovered in a flow-through fraction (Fig. 5 a).


Figure 1. Effect of salt on tubulin acetylation. Acetylation of brain MTP was measured for 60 min at various concentrations of NaCl, as indicated, with 0.14 units of axonemal bound (detergent-insoluble; ●) or free (0.66 M NaCl-soluble; ○) TAT preparations (for the definition of TAT unit, see Table I). 100% activity corresponds to 0.4 acetate per tubulin dimer (mol/mol) per hour.

Figure 4. Effect of Ca^{2+} on acetylation of tubulin dimers and histones. (*a*) The time course of the acetylation of PC tubulin (1.4 mg/ml) without MAPs was followed in the presence (●) and absence (○) of 1 mM Ca^{2+}. (*b*) The time course of the acetylation of histones (7.0 mg/ml) was followed in the presence (●) and absence (○) of 1 mM Ca^{2+}. In both cases 0.14 units of the same NaCl extract of flagellar axonemes were used as an acetyltransferase source. Note that the concentration of histone (a poor substrate) was five times higher than that of tubulin dimer and that there is a 10-fold difference in the ordinates (acetyltransferase activity) for tubulin compared with those for histone.

However, TAT-1 and TAT-2 could not be separated either by hydroxylapatite chromatography or by gel-filtration on Sephadex G-150. Both forms of TAT were eluted with 50 mM phosphate from hydroxylapatite and had a native molecular weight of ∼130 kD (Fig. 5 *b*).

Purification of TAT-2

Since no TAT activity could be detected in *Chlamydomonas* cell bodies (deflagellated cells), the flagella, representing only 0.3% of the total cellular proteins, were used as the starting material for TAT purification. By a combination of three different chromatographic procedures, TAT has been purified at least 110-fold from the 0.66 M NaCl extract of axonemes (for details, see Table III). SDS PAGE analysis indicated that the final TAT-2 fraction from DE-52 contained a
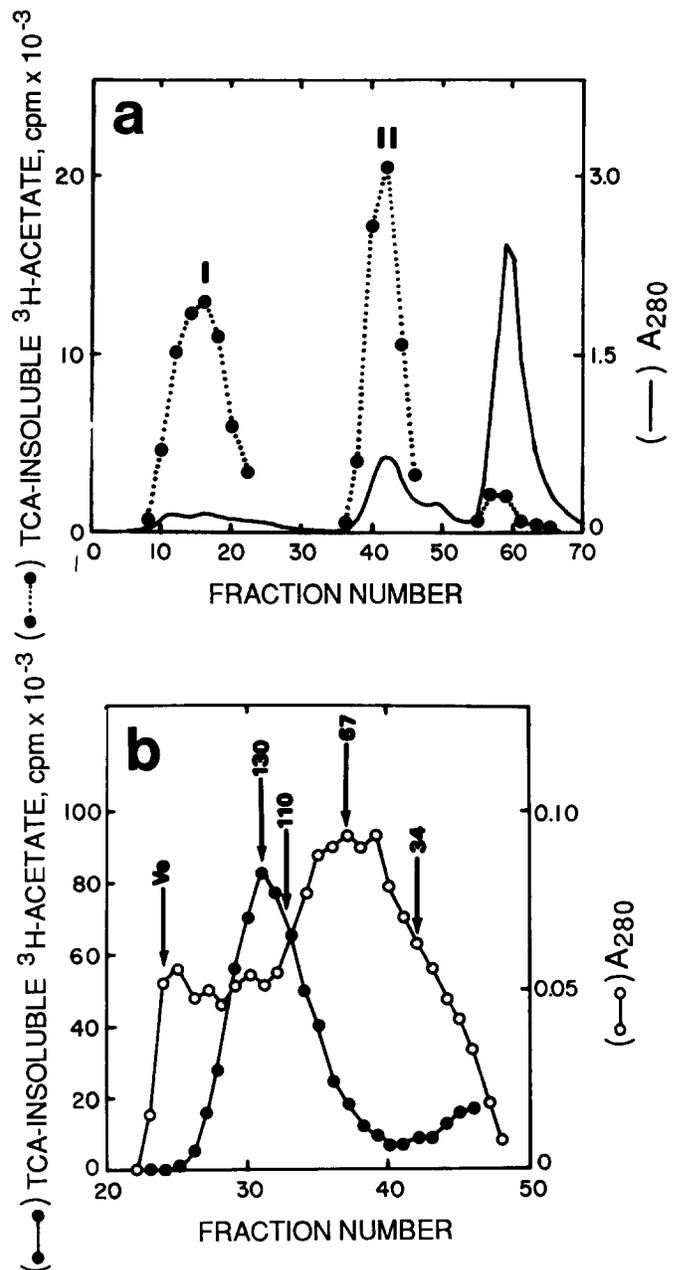
Figure 5. DEAE-cellulose chromatography (*a*) and gel-filtration on Sephadex G-150 (*b*) of the axonemal TATs. (*a*) 20 ml of NaCl extract of the flagellar axonemes after dialysis against TDSA buffer was applied to DE-52 (bed vol 20 ml) equilibrated with TD buffer and the bound proteins were eluted by a stepwise gradient of NaCl (50 and 400 mM). 10 μl of each fraction was assayed for TAT activity for 60 min under the standard conditions. Peaks I and II of TAT activity correspond to the flow-through and 50 mM NaCl eluate fractions, respectively. (*b*) 50 mM phosphate eluate from hydroxylapatite of NaCl extract of the flagella was further fractionated on Sephadex G-150. The TAT activity of each fraction was measured as described in Fig. 5 *a*. The arrows indicate molecular weights of standard proteins.

67-kD peptide as the major component and some smaller peptides as minor contaminants (data not shown).

K_m for Acetyl CoA

To determine the affinity of purified TAT-2 for its substrate,

Table III. Purification of Alpha-Tubulin Acetyltransferase

Fraction	Total units* (% yield)	Total Protein	Units/mg protein (fold)
		mg	
Whole flagella	288 (100)	226	1.2 (1)
NaCl extract	444 (150)	54	8 (7)
HA 50	144 (50)	1.5	96 (77)
Sephadex 130	60 (21)	0.4	150 (118)
DE OO (TAT-1)	6 (2)	—	— —
DE 50 (TAT-2)	14 (5)	<0.1	>140 (>113)

* 1 unit is defined as the activity required to transfer 1 nmol of acetyl groups to tubulin in 20 min at 37°C under standard TAT assay conditions.

acetyl CoA, the rate of tubulin acetylation was measured at various concentrations of acetyl CoA. As shown in Fig. 6, the K_m for acetyl CoA was ~2 μM.

CoA Is a Competitive Inhibitor of Tubulin Acetylation

It was previously shown that the acetylation of histones is inhibited by CoA, an end product of the acetylation (17). We found that the acetylation of alpha-tubulin is also inhibited by CoA competitively (Fig. 6). The K_i for CoA was ~8 μM.

Cytoplasmic Localization of TDA and an Inhibitor of Tubulin Acetylation

We initially found that flagella isolated from *Chlamydomonas* contained TAT activity (5). We also expected to find TAT activity in the cytoplasm because, after deflagellation in the presence of cycloheximide, a potent inhibitor of protein synthesis, each flagellum can regenerate to half of its original length by using alpha- and beta-tubulins in the cytoplasmic pool (14), and the alpha-tubulin from this pool is acetylated in the flagella (3, 8, 12). We tried to measure cytoplasmic TAT activity, using brain tubulin as a substrate, but precise quantification was hindered by the presence of an apparent TAT inhibitor as well as TDA.

The presence of a cytoplasmic TAT inhibitor and the activity of TDA were demonstrated by addition of cell body extract to a standard TAT assay mixture that contained flagella, brain MTP, and ^{3}H-acetyl CoA, as shown in Fig. 7. In the absence of cell body extract (open circles), acetylation of tubulin proceeded for up to 60 min until the stoichiometry
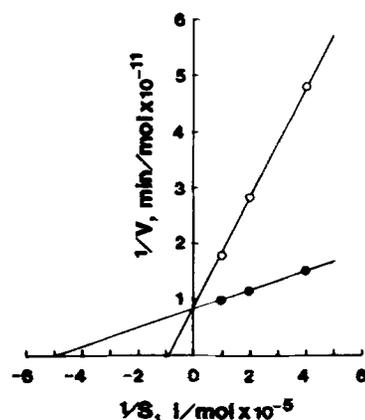
reached ~0.4 acetate per tubulin dimer (mol/mol), and then completely ceased because the supply of acetyl CoA was exhausted. When the cell body extract was added to the same reaction mixture after 60 min (closed circles), the radioactive acetate bound to tubulin was gradually released, indicating that the cell body extract contains, at least, a TDA. Under these conditions, however, only half of the acetylated alpha-tubulin was deacetylated after 60 min because the amount of extract added was limited.

When the same amount of cell body extract was added to the reaction mixture at 0 min (triangles) instead of 60 min, the acetylation of tubulin was almost completely inhibited. If it were the case that only a TDA was present in the extract, then this deacetylase could have reduced the extent of tubulin acetylation by less than half. Instead, the complete inhibition of acetylation by the added extract strongly suggests that a tubulin acetylation inhibitor (TAI), in addition to a TDA, is present in the cell body extract. In fact, when the cytoplasmic extract was fractionated on DE-52, TDA activity was recovered only in the flow-through fractions, whereas TAI activity was split into two fractions, which eluted with 50 mM and 400 mM NaCl, neither of which showed any TDA activity (data not shown). These results indicate that TAI and
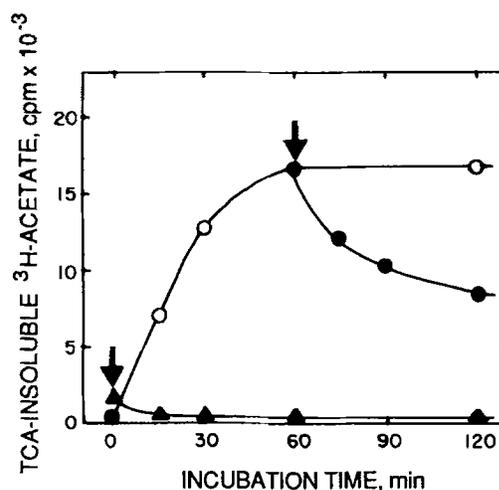
Figure 6. Determination of K_m and K_i for acetyl CoA and its competitive inhibitor CoA. The rate of tubulin acetylation by TAT-2 was measured at various concentrations of ^{3}H-acetyl CoA in the presence (○) or absence (●) of 24 μM CoA.

Figure 7. TAT inhibitor and TDA activities of *Chlamydomonas* cell body extract. (○) Brain MTP was acetylated by *Chlamydomonas* flagella over 60 min, at which point, the supply of ^{3}H-acetyl CoA was completely exhausted. (▲) To assay for cytoplasmic TAT inhibitor activity, *Chlamydomonas* cell body extract was added to the TAT assay mixture at 0 min (*arrow*). (●) To assay for cytoplasmic TDA activity, *Chlamydomonas* cell body extract was added to the TAT assay mixture at 60 min, as a second arrow indicates. For details, see the text.
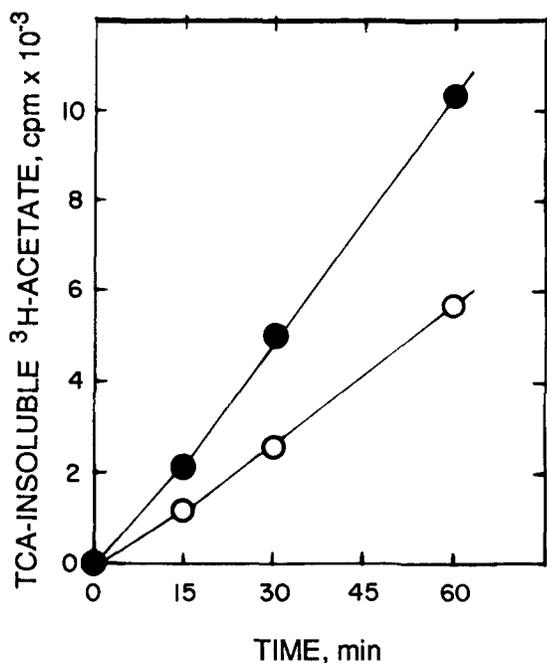
Figure 8. Acetylation of tubulin dimer and polymer. The time course of PC tubulin (1.4 mg/ml) acetylation, with 0.13 mg/ml MAPs (●) (tubulin polymers) or without MAPs (tubulin dimers) (○), was followed in the presence of 0.28 units of NaCl extract of the flagellar axonemes.

TDA activities in *Chlamydomonas* cell body are associated with distinct and different protein molecules. In addition, it should be noted that neither *Chlamydomonas* flagella nor brain microtubules contained any detectable TDA activity. No significant loss of radioactivity from acetylated alpha-tubulin was observed in the 60 min that followed the complete depletion of acetyl CoA from the reaction mixture, in the absence of the cell body extract (see open circles). Furthermore, no subfractions derived from the flagella showed any detectable TAI activity (data not shown).

Acetylation of Dimers and Polymers of Tubulin

To clarify whether the substrate for TAT is alpha-tubulin in either alpha/beta heterodimer, polymer (microtubule), or both, the acetylation of tubulin dimers was compared with that of microtubules. The acetylation of tubulin was carried out using PC tubulin (1.4 mg/ml) in the presence and absence of a fraction that contained MAPs. Under these conditions tubulin polymerized in the presence of MAPs but remained as dimers in their absence, as previously described (15). As shown in Fig. 8, both dimers and polymers served as substrate for solubilized axonemal TAT, although the polymer appeared to be a better substrate than the dimers.

The above results, which demonstrate that microtubules are a better substrate than tubulin dimers, could have been due to the presence of MAPs in the preparation of microtubules. MAPs could stimulate acetylation of tubulin either by promoting polymerization of tubulin or by activating the TAT. The second possibility has been excluded by the following experiments. Colchicine is known to inhibit polymerization of tubulin by its specific binding to tubulin and not to MAPs. We examined the effects of colchicine on acetylation of tubulin under conditions where the tubulin either remains
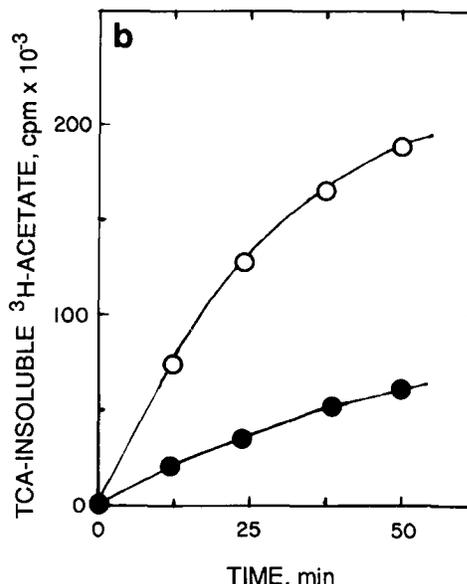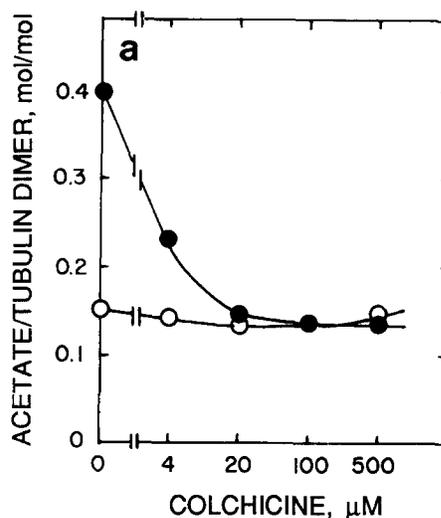

Figure 9(a). Effect of colchicine on acetylation of tubulin dimer and polymer. Acetylation of PC tubulin (1.4 mg/ml) by 0.14 units of NaCl extract of the flagellar axonemes was measured for 60 min at various concentrations of colchicine, as indicated, without (○) or with (●) MAPs (0.13 mg/ml). (b) Effect of taxol on acetylation of microtubules. Acetylation of PC tubulin (14 μM) with MAPs (0.13 mg/ml) by 0.14 units of NaCl extract of the flagellar axonemes was measured over 50 min, in the presence (●) or absence (○) of 20 μM taxol.

as dimer or is assembled into microtubules, i.e., with or without MAPs. Since the acetylation of tubulin dimers (14 μM PC tubulin without MAPs) was not significantly inhibited by colchicine at concentrations up to 0.5 mM (Fig. 9 *a*, open circles), and since colchicine cannot bind MAPs, the only possible way for colchicine to affect the rate of tubulin acetylation is by the disassembly of microtubules which have been formed in the presence of MAPs. Indeed, as shown in Fig. 9 *a* (closed circles), colchicine at concentrations as low as 1 μM significantly inhibited the acetylation of tubulin when microtubules (14 μM PC tubulin with MAPs) were used as a substrate. The inhibition never exceeded 70% even at a concentration as high as 0.5 mM, suggesting that the incomplete inhibition by colchicine of acety-

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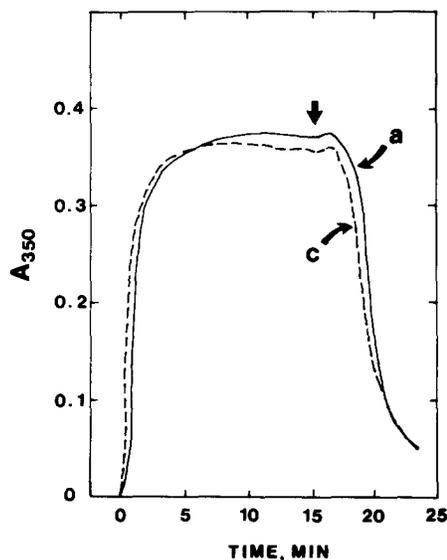
Figure 10. Time course of polymerization and depolymerization of the acetylated and nonacetylated (control) tubulins. The acetylated (*a*) and control (*c*) tubulin dimer preparations (0.8 mg/ml), obtained after one additional cycle of temperature-dependent polymerization/depolymerization as described under Materials and Methods, were incubated at 37°C in the presence of 0.3 M glycerol and 2 mM EGTA for in vitro polymerization. After 15 min, as indicated by the arrows, the temperature was shifted to 4°C to initiate depolymerization. The time courses of both polymerization and depolymerization were followed by monitoring the turbidity changes at 350 nm.

lation of microtubules is due to the colchicine-induced depolymerization of microtubules, the residual 30% being due to acetylation of the dimer form. Therefore, we believe that MAPs stimulate the acetylation by promoting polymerization of tubulin. Note that the binding of colchicine to tubulin dimers in the absence of MAPs does not affect the acetylation of tubulin (Fig. 9 *a*, open circles). By contrast, taxol, which inhibits depolymerization of microtubules by binding to any tubulin dimer along a microtubule, significantly reduced the rate of acetylation of the microtubules (see Fig. 9 *b*).

Effect of Alpha-Tubulin Acetylation on the Polymerization and Depolymerization of Microtubules

For a comparison of the extent and rate of polymerization and depolymerization of acetylated tubulin with those of nonacetylated tubulin, brain tubulin was incubated at 37°C for 4 h with TAT in the presence of either acetyl CoA (substrate) or CoA (control). Both acetylated and nonacetylated microtubules were centrifuged, and each pellet was solubilized in cold PM buffer and subjected to one more cycle of temperature-dependent depolymerization/polymerization. The amount of tubulin in each pellet and supernatant was quantitated by SDS/urea PAGE. As described under Materials and Methods, no significant difference in the extent of polymerization and depolymerization was observed between acetylated and nonacetylated tubulins.

The kinetics of the temperature-dependent polymerization and depolymerization of the acetylated and nonacetylated control tubulins were followed in the presence and absence of Ca²⁺. As shown in Fig. 10, in the absence of Ca²⁺, both the rate and extent of polymerization and depolymerization
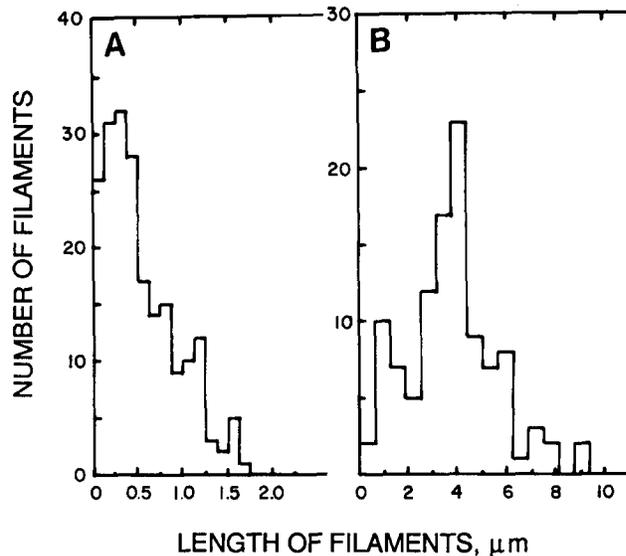

Figure 11. Length distribution of sheared and control microtubules. Calf brain microtubules were mechanically sheared, fixed with glutaraldehyde, and negatively stained as described under Materials and Methods. The length of the sheared microtubules was compared with the control microtubules by electron microscopy. (*A*) Sheared microtubules, the average length of ∿200 filaments was 0.6 μm. (*B*) Control microtubules, the average length of 100 filaments was 3.4 μm.

of the acetylated tubulin were almost indistinguishable from those of the control tubulin. This result was observed at concentrations of glycerol from 0.3 to 4.0 M. However, in the presence of 20 μM Ca²⁺, the nonacetylated tubulin polymerized significantly faster than the acetylated tubulin at concentrations of glycerol from 2 to 4 M (data not shown). These results indicate that, in the absence of Ca²⁺, under physiological conditions, acetylation does not significantly affect either the rate or the extent of polymerization or depolymerization of tubulin. In the presence of Ca²⁺, acetylation affects the polymerization of tubulin significantly only at high concentrations of glycerol, i.e., under rather unphysiological conditions.

Multiple Sites for Acetylation along a Microtubule

If the flagellar TAT preferentially acetylated alpha-tubulin at the ends of microtubules, the initial rate of tubulin acetylation should be proportional to the number of filament ends. Therefore, we determined whether the rate of tubulin acetylation was increased when the number of ends was increased by shearing, at a fixed concentration of microtubules. Tubulin (3.6 mg/ml) was polymerized at 37°C in the presence of 1 M glycerol, and a portion of the microtubules was sheared by passing it through a 25-gauge needle. As shown in Fig. 11, the average length of the unsheared microtubules was ∿3.4 μm, whereas the sheared microtubules were, on average, 0.6-μm long. The time course of acetylation of the sheared and control microtubules was followed at 37°C in the presence of flagellar TAT and ³H-acetyl CoA. Since, under the experimental conditions, ∿15% of the total tubulin remains as dimers, the acetylated microtubules in both microtubule preparations at each time point were collected by centrifugation in an airfuge and their radioactivity measured as described under Materials and Methods. As shown in Fig.
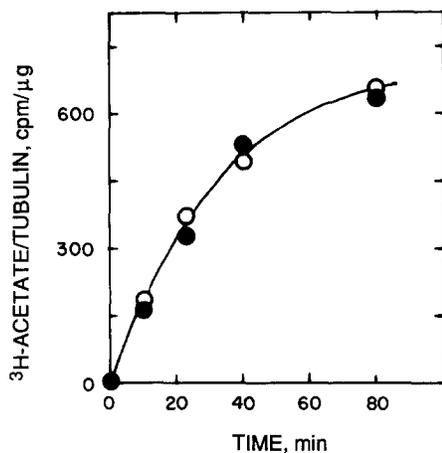
Figure 12. Acetylation of alpha-tubulin in preparations of control and sheared microtubules. The time course of tubulin acetylation of the control and sheared microtubules (see Fig. 11) was followed at 37°C in the presence of NaCl extract of the flagellar axonemes and ³H-acetyl CoA as described under Materials and Methods. After the indicated time of incubation, each sample was centrifuged in an airfuge, and the microtubules in each pellet were subjected to SDS/urea PAGE and the radioactivity in each alpha-tubulin band was measured as described under the Materials and Methods. ●, control microtubules; ○, sheared microtubules.

12, no significant difference in the rate of acetylation was detected in any time period, up to 80 min, between the sheared and control microtubules. These results indicate that the TAT can acetylate alpha-tubulin at any site throughout the length of the microtubule.

Discussion

In earlier reports we showed that *Chlamydomonas* alpha-tubulin is synthesized in the cytoplasm as a precursor, transferred up the shaft of the flagellum, and then acetylated at the flagellar tip assembly site (3, 7–10). More recently, we demonstrated the presence of an acetyltransferase activity in the flagella isolated from *Chlamydomonas* which was highly specific for alpha-tubulin in both *Chlamydomonas* and calf brain (5). The flagellar enzyme did not acetylate any other proteins including beta-tubulin, dynein, and MAPs present in preparations of either calf brain microtubules or *Chlamydomonas* flagella. In addition, histones were shown to be poor substrates for the flagellar enzyme, indicating that the flagellar acetyltransferase is different from any of the histone acetyltransferases that have been isolated. Now, using brain tubulin as a substrate, we have shown that alpha-tubulin acetyltransferase can be solubilized from the flagellar axoneme by treatment with a high concentration of NaCl. The enzyme has been partially purified and some of its characteristics determined. Based on the elution of the enzymatic activity from molecular sieve columns, the native molecular weight of the enzyme appears to be ∼130 kD. SDS PAGE of more highly purified enzyme fractions suggests that the enzyme is a homodimer of a 67-kD peptide. However, proof of the identity of the peptide responsible for the enzymatic activity will require an antibody that is specific for the peptide and can immunoprecipitate or inhibit the enzymatic activity. The acetylation of both tubulin dimers and microtubules (see

below) is strongly inhibited by calcium, and our results suggest that this inhibition is due to the binding of calcium to the tubulin dimer rather than to the enzyme itself. Colchicine does not inhibit the acetylation of tubulin dimers, and the partial inhibition of microtubule acetylation by colchicine can be explained by the fact that the microtubules are a somewhat better substrate for the acetyltransferase than the tubulin dimers, and colchicine causes depolymerization of the microtubules. We found that taxol also inhibits the acetylation of microtubules. The effect of taxol is probably due to its binding to a site near the acetylation site in alpha-tubulin rather than to the acetyltransferase because taxol has no effect on acetylation of histones by the same enzyme.

The physiological function of alpha-tubulin acetylation is unknown, although it has been suggested that it may be related to the stabilization of microtubules. The principal example of acetylated and stable microtubules is found in the flagellum, where both central and outer doublet microtubules are acetylated (12) and are more stable than the majority of cytoplasmic microtubules to both cold and colchicine treatments, although the central and outer doublet microtubules themselves have different stabilities (18).

If acetylation affects the stability of microtubules, it is not clear as yet how it achieves this stabilization. The results presented in this paper suggest that acetylation does not affect temperature-dependent tubulin dimer–dimer interactions because the kinetics of assembly and disassembly in vitro of brain microtubules are similar for both acetylated and nonacetylated tubulins. It seems more likely that, if acetylation is affecting the stability of microtubules, the stability is due to the binding of specific MAPs to the walls of acetylated microtubules. That the acetylated lysine residue is accessible on the walls of microtubules is indicated by the fact that intact brain microtubules can be acetylated and deacetylated along their entire lengths in vitro. If specific MAPs bind to the acetylated domains on the microtubules, one might also expect that the binding proteins would cover the acetylated lysines, thereby making them inaccessible. In preliminary experiments, we have been able to show that acetylated lysines on isolated axonemal microtubules are protected from deacetylation. Because one can acetylate intact brain microtubules in vitro, it may now be possible to use these acetylated microtubules to isolate specific binding proteins from extracts of flagella or cell bodies.

On the basis of the results presented in this paper, we suggest that one possible explanation for the localization of acetylated microtubules principally in the flagellar apparatus would be that the tubulin acetyltransferase is tightly associated with the microtubules in the flagellar apparatus, whereas both tubulin deacetylase and inhibitor(s) of tubulin acetylation appear to reside almost exclusively in the cytoplasm. The localizations of these enzymes could account, therefore, for the specific localizations of the acetylated microtubules.

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