Chlamydomonas α-Tubulin Is Posttranslationally Modified in the Flagella during Flagellar Assembly

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ABSTRACT The principal α-tubulin within *Chlamydomonas reinhardtii* flagellar axonemes differs from the major α-tubulin in the cell body. We show that these two isoelectric variants of α-tubulin are related to one another since posttranslational modification of the cell body precursor form converts it to the axonemal form. During flagellar assembly, precursor α-tubulin enters the flagella and is posttranslationally modified within the flagellar matrix fraction prior to or at the time of its addition to the growing axonemal microtubules. Experiments designed to identify the nature of this posttranslational modification have also been conducted. When flagella are induced to assemble in the absence of *de novo* protein synthesis, tritiated acetate can be used to posttranslationally label α-tubulin in vivo and, under these conditions, no other flagellar polypeptides exhibit detectable labeling.

Cilia and flagella[1] are regenerated following their detachment from many cell types (3, 7, 10, 14, 15, 25, 27, 28, 34–36) and this detachment results in the induction of the synthesis of tubulin and many other flagellar proteins (10, 14, 27, 35, 36). This synthesis requires transcription of flagellar protein mRNAs whose abundance rises dramatically following amputation of the flagella (10, 15, 17–19, 20, 31–33). Flagellar regeneration therefore allows the study of a model system that is inducible for both gene expression and organelle morphogenesis.

The unicellular green alga *Chlamydomonas reinhardtii* offers many special advantages for studies of flagellar regeneration. Large populations of cells can be easily deflagellated and will synchronously regenerate new flagella (14, 25, 28, 36). The detached flagella can be purified and fractionated to allow biochemical characterization (37). Moreover, numerous mutants affecting the structure and function of flagella exist which, coupled with biochemical and ultrastructural analysis, have allowed assignment of polypeptides to specific structural components of the *Chlamydomonas* flagellum (1, 11, 12, 22–25, 27, 37).

One interesting feature, which *Chlamydomonas* shares with some other flagellar regenerating systems (e.g., reference 3), is the existence of a flagellar protein precursor pool. In vegetatively growing *Chlamydomonas*, this precursor pool is sufficiently large to allow regeneration of half-length flagella in the absence of *de novo* protein synthesis (14, 28). This indicates that flagellar proteins can exist both assembled within flagella and as an unassembled pool within the cell body; deflagellation mobilizes these previously unassembled precursor proteins so that they assemble into flagella.

In this work, we were interested in determining whether the use of flagellar precursor proteins during flagellar assembly was accompanied by their modification. We concentrated our efforts on the major flagellar polypeptide α-tubulin because other work had indicated that the α-tubulin mRNA which is stimulated following flagellar amputation, when translated in vitro, encodes an α-tubulin which does not co-migrate with the major flagellar α-tubulin (14, 18, 33), suggesting that α-tubulin is posttranslationally modified. We will show that the mRNA encoded α-tubulin (α-1) is the form stored within the flagellar precursor pool. When the precursor pool is used during flagellar axonemal assembly, the α-1 tubulin is posttranslationally modified to α-3 tubulin within that ensemble of proteins that fills the space between axonemal elements and the flagellar membrane, the flagellar matrix. This posttranslational modification can be demonstrated by labeling with tritiated acetate in vivo, indicating that *Chlamydomonas* α-tubulin is modified by the addition of a carbon-containing moiety.

MATERIALS AND METHODS

Materials: [^{3}H]acetate (as $C^3H_3COO^-$ Na^+, 1.6 Ci/mM) was obtained from New England Nuclear (Boston, MA) and DNAse I, RNAse A, GTP, and cycloheximide from Sigma Chemical Co. (St. Louis, MO). Carrier ampholytes for isoelectric focusing (pH ranges 5–7 and 3–10) were obtained from Bio-Rad Laboratories (Richmond, CA) or Serva Biochemicals (Garden City Park, NY). Ultrapure urea for isoelectric focusing was purchased from either Schwarz-Mann (Spring Valley, NY) or BRL (Gaithersburg, MD) and was recrystalized before use. SDS for electrophoretic sample preparation was specially pure grade

[1] In this report, the word flagella will be used in describing phenomena in either flagella or cilia.

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(BDH Chemicals Ltd., Poole, England) while the SDS gel electrophoretic tank buffer contained technical grade SDS (J. T. Baker Chemical Co., Phillipsburg, NJ). All other chemicals were reagent grade or better.

Cell Culture and In Vivo Labeling: Wild type *C. reinhardtii*, strain 21gr vegetative cells, were used in all experiments. Conditions and media for cell growth have been described (14). In some cases (see below), cells were grown in the acetate-containing medium 2 (30) that was supplemented with five times the normal phosphate content. Cells were deflagellated by mechanical shear and flagellar lengths determined as described (28). Tritiated acetate was used in all in vivo labeling experiments. *Chlamydomonas* can use acetate as a carbon source for all carbon-containing molecules and acetate, therefore, can be used to measure amino acid incorporation into TCA-precipitable protein in vivo. When protein synthesis is inhibited, labeled acetate can only add to *Chlamydomonas* proteins by posttranslational modification (e.g., acetylation and methylation). Cells that were used for acetate labeling were grown in 1.5 l of medium 2 to a cell density of $1-2 \times 10^6$ cells/ml, concentrated by centrifugation, washed once in medium 2 without acetate, and resuspended in acetate-free medium at $1-1.5 \times 10^7$ cells/ml. These cells were acetate-starved for 30 min and then divided into two equal aliquots: one aliquot contained 10 μg/ml cycloheximide (experimental) while the other aliquot lacked this drug (control). 10 min later, each aliquot was in vivo labeled with 200 μCi/ml tritiated acetate for a period of 30 min. Both aliquots were then deflagellated in their respective media and allowed to regenerate new flagella for 40 min. At this point, samples for two-dimensional electrophoresis were prepared from cells, their flagella and flagellar fractions (see below). The time course of in vivo labeling of TCA-precipitable protein was monitored as described (14, 28).

Cell Fractionation: Cell bodies, flagella, axonemes, and flagellar membrane/matrix fractions were prepared as described (37, 39) except that cell bodies were pelleted through a solution containing 10 mM HEPES-KOH (pH 7.5), 5 mM $MgSO_4$, 1 mM dithiothreitol (DTT), and 25% sucrose (wt/vol). Resulting cell body pellets were frozen in liquid N_2 and stored at −80°C until use. Tubulin was prepared from frozen cell bodies and chromatographed on DEAE cellulose essentially as described (23). The difference was that the buffer used for cell disruption and column chromatography was 10 mM HEPES-KOH (pH 7.5), 1 mM $MgSO_4$, 1 mM DTT, 0.1 mM GTP, and 0.24 M sucrose. Following chromatography, the tubulin-containing fraction was dialyzed against 10 mM NH_4HCO_3, 0.1% β-mercaptoethanol, and lyophilized.

Sample Preparation and Two-dimensional Gel Analysis: Whole-cell samples were prepared for two-dimensional electrophoresis by a new method (May, G., and J. Rosenbaum, manuscript in preparation) modified from published techniques (15, 21, 27). Cells were pelleted and resuspended in the previously described buffer (15) then lysed by adding first 0.1 vol of a solution containing 3.3% SDS (wt/vol) and 10% 2-mercaptoethanol (vol/vol) and then 0.1 vol of nuclease solution (15). Following incubation of the cell lysate at 0°C for 15 min, cell walls and starch were removed by centrifugation for 10 min at 2,300 *g* (IEC PR-6 model centrifuge, 269 rotor, 3,200 rpm; Damon/IEC Div., Needham Heights, MA) and 9 vol of acetone was added to the resulting supernatant. After 1–2 h at 0°C, the resulting acetone precipitate was pelleted at 2,3000 *g* (as above).

Lyophilized cell body tubulin, pelleted axonemes and flagella, and acetone precipitates of cells or flagellar membrane/matrix fraction were resuspended directly in lysis buffer (21) and stored at −80°C until use. Two-dimensional electrophoresis and gel staining were performed as described (15, 18), except that isoelectric focusing was performed for 20 h at 400 V. Fluorography (13) was performed on stained gels with Autofluor® as recommended by the manufacturer (National Diagnostics, Somerville, NJ). Gels impregnated with this fluor were neither opaque nor appreciably destained and allowed accurate alignment of dried, stained gels with their respective fluorographs.

Protein Determination: Protein concentrations were determined by the method of Bradford (6) with chicken ovalbumin as a standard.

RESULTS

Deflagellation of *Chlamydomonas* stimulates the synthesis of many flagellar proteins, including both α- and β-tubulin and their corresponding mRNAs (reviewed in reference 33). This synthesis begins within 6–8 min and reaches a maximum, in the case of the tubulins, at ~50 min following deflagellation (14, 15, 20, 27, 32). It has recently been proposed that, during flagellar protein synthesis, α-tubulin is posttranslationally modified prior to its assembly into the flagella (15, 18, 33). Our purpose was to determine whether this modification occurs in the cell body or flagella, and to obtain information concerning the chemical nature of the modification. An initial report of these results has been presented (16).

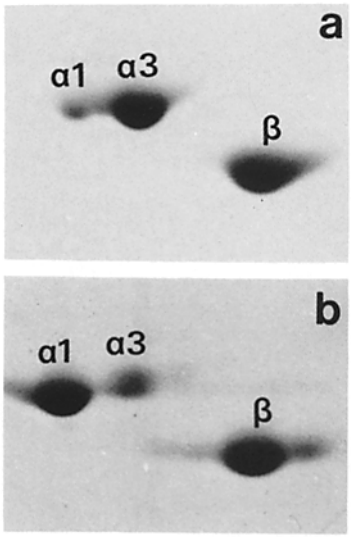

FIGURE 1 Two-dimensional (2-D) electrophoresis of *Chlamydomonas* flagellar (*a*) and cell body (*b*) tubulin. Both panels show Coomassie Blue-stained gels that were electrophoresed with 25 μg total protein; only the tubulin regions are shown.

Chlamydomonas contains a flagellar precursor pool within the cell body (14, 28), and we wished to know whether the tubulin in this pool differed from that found in the flagella.[2] In Fig. 1 *a*, it can be seen that the isolated flagella contain two α-tubulins, designated α-1 and α-3 and that α-3 is, by far, the principal α-tubulin. Conversely, if one examines the tubulins of the flagellar precursor pool (located in the cell body fraction which is obtained by removal of flagella from cells) one observes that the major α-tubulin is not α-3, as in the flagellum but, rather, α-1 (Fig. 1 *b*; see also references 15, 18, 33). This suggested that the α-tubulin in the precursor pool, probably for use in flagellar assembly, was stored as a precursor protein (α-1).

Previous studies have shown that when cells are deflagellated the α-tubulin mRNA that accumulates, when translated in vitro, encodes α-1 (15, 18, 32, 33). In in vivo experiments, when ^{35}S was used to label proteins synthesized during flagellar regeneration, the cell bodies contained principally labeled α-1 tubulin while the primary labeled α-tubulin in the flagellum was α-3 (15, 16, 18). Finally, pulse-chase labeling studies have shown that, during flagellar regeneration, cell body α-1 can be chased into flagellar α-3 tubulin (16, 18). These results suggest that the major flagellar α-tubulin (α-3) is synthesized as a precursor (α-1) which is posttranslationally modified.

Flagellar assembly involves addition of precursors to the distal tip of the flagellum as it elongates (2, 5, 9, 29, 38). We wanted to know where in the *Chlamydomonas* cell, cell body or flagella, α-tubulin posttranslational modification occurred and how it was related to flagellar assembly. Consequently, cells were deflagellated in the presence of colchicine in order to stimulate flagellar protein synthesis under conditions where flagellar assembly was blocked (14, 28). We found that when flagellar assembly was inhibited, only the precursor α-1 tubulin accumulated witin the cell body. This indicated that assembly of the flagellum was required for the posttranslational conversion of α-1 to α-3 tubulin (8, 16) (L'Hernault, S., and J. Rosenbaum, manuscript in preparation). This find-

[2] We have not noticed any 2-dimensional gel differences between the β-tubulin of the precursor pool and that within flagella. Consequently, we have limited our discussion to α-tubulin.

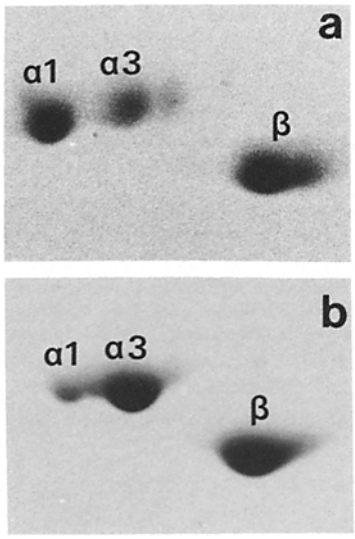

FIGURE 2 2-D gels of membrane/matrix (*a*) and axonemal (*b*) fractions. Both panels show Coomassie Blue-stained gels electrophoresed with 75 μg (*a*) or 25 μg (*b*) of total protein and only the tubulin regions are shown.

ing prompted us to ask whether the flagellar membrane/matrix fraction, which includes all nonionic detergent-soluble flagellar proteins, contained primarily the precursor (α-1) or the modified (α-3) form of α-tubulin. When flagella are fractionated into axonemal and membrane/matrix fractions, a small amount of tubulin is present in the membrane/matrix fraction and a large proportion of the α-tubulin in this fraction is in the α-1 precursor form (Fig. 2*a*).[3] By contrast, flagellar axonemal α-tubulin, as expected from earlier results (15, 18), is composed predominately of α-3 (Fig. 2*b*). These results indicate, therefore, that the precursor α-1 tubulin passes through the cell body and into the flagellar matrix where it is modified prior to or at the time of its addition to the distal tip of the axonemal microtubules (2, 5, 9, 29, 38).

The following experiments were performed to determine the nature of this α-tubulin posttranslational modification. Previously, it had been shown that vegetative *Chlamydomonas* will regenerate flagella of about half-length when cellular protein synthesis is completely inhibited with cycloheximide (14, 28). The α-tubulin in these half-length flagella was, as expected, predominately α-3 (as in Fig. 1 *a*; data not shown) which indicated that the enzyme(s) responsible for the posttranslational conversion of α-1 to α-3 in the flagellar matrix was fully active in the absence of protein synthesis. In principal, by choosing the appropriate radioactive precursor, one should be able to radioactively tag the posttranslational modification during flagellar assembly in the absence of protein synthesis. Several labeled compounds ($^{32}PO_4$, $^{35}SO_4$, [^{3}H]acetate) and protocols were assessed by this method, and the following method proved to be successful. Tritiated acetate was added to cycloheximide-treated cells 30 min prior to deflagellation (Fig. 3). The cells were then deflagellated in the continuous presence of both cycloheximide and tritiated acetate. Since there is no new protein synthesis, the cytoplasmic precursor pool (28) is the exclusive source of proteins for the half-length flagella which cycloheximide-treated cells can regenerate (Fig. 4). Flagellar axonemes from these half-length flagella were overloaded on two-dimensional gels so that many flagellar proteins in addition to the tubulins could be observed by Coomassie Blue staining (Fig. 5*a*). These gels were fluorographed and, of all the polypeptides visible in the stained gels (Fig. 5*a*), the only radioactivity was localized over α-3 tubulin (Fig. 5*b*); quadrupling the fluorographic exposure time did not reveal any additional labeled polypeptides (data not shown). These results demonstrate that α-3 tubulin is produced by post-translational modification and that this modification represents the addition of a carbon-containing moiety.

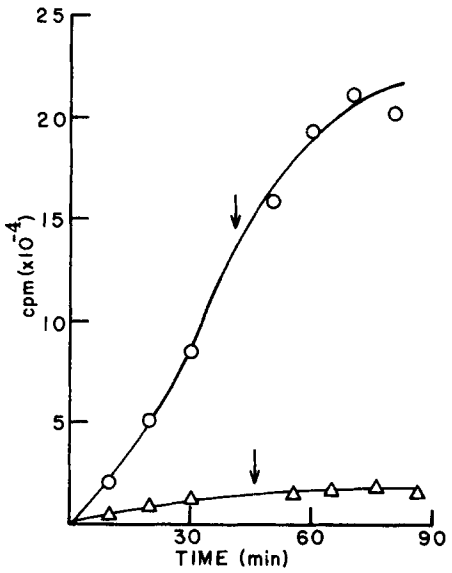

FIGURE 3 Incorporation of [^{3}H]acetate into *Chlamydomonas* TCA-precipitable protein. [^{3}H]acetate was added to *Chlamydomonas* in the presence (Δ) or absence (O) of cycloheximide (10 μg/ml). [^{3}H]-acetate was added at time zero to nondeflagellated cells which were subsequently deflagellated at the indicated times (arrows). Cells remained in their respective media throughout the time course of the experiment.

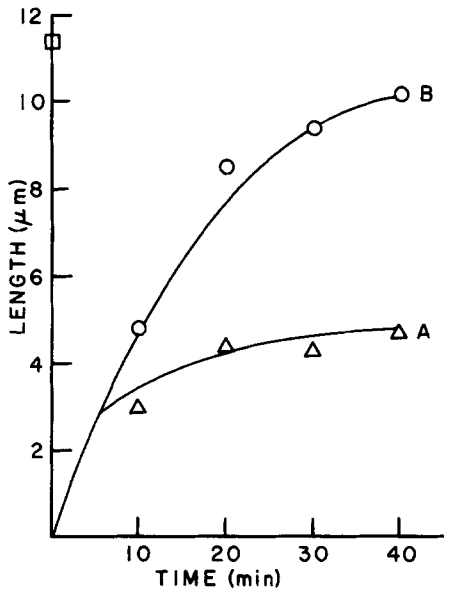

FIGURE 4 The effect of inhibition of protein synthesis on flagellar regeneration kinetics in *Chlamydomonas* vegetative cells during [^{3}H]acetate labeling. Curve *A*, regeneration in the presence of cyloheximide (10 μg/ml); curve *B*, regeneration in the absence of cycloheximide. The flagellar length prior to deflagellation is designated by the square (□) on the ordinate.

[3] Our laboratory has conducted an extensive experimental analysis of membranes isolated from regenerating and full-grown *Chlamydomonas* flagella by several different methods and has found little, if any, associated tubulin.

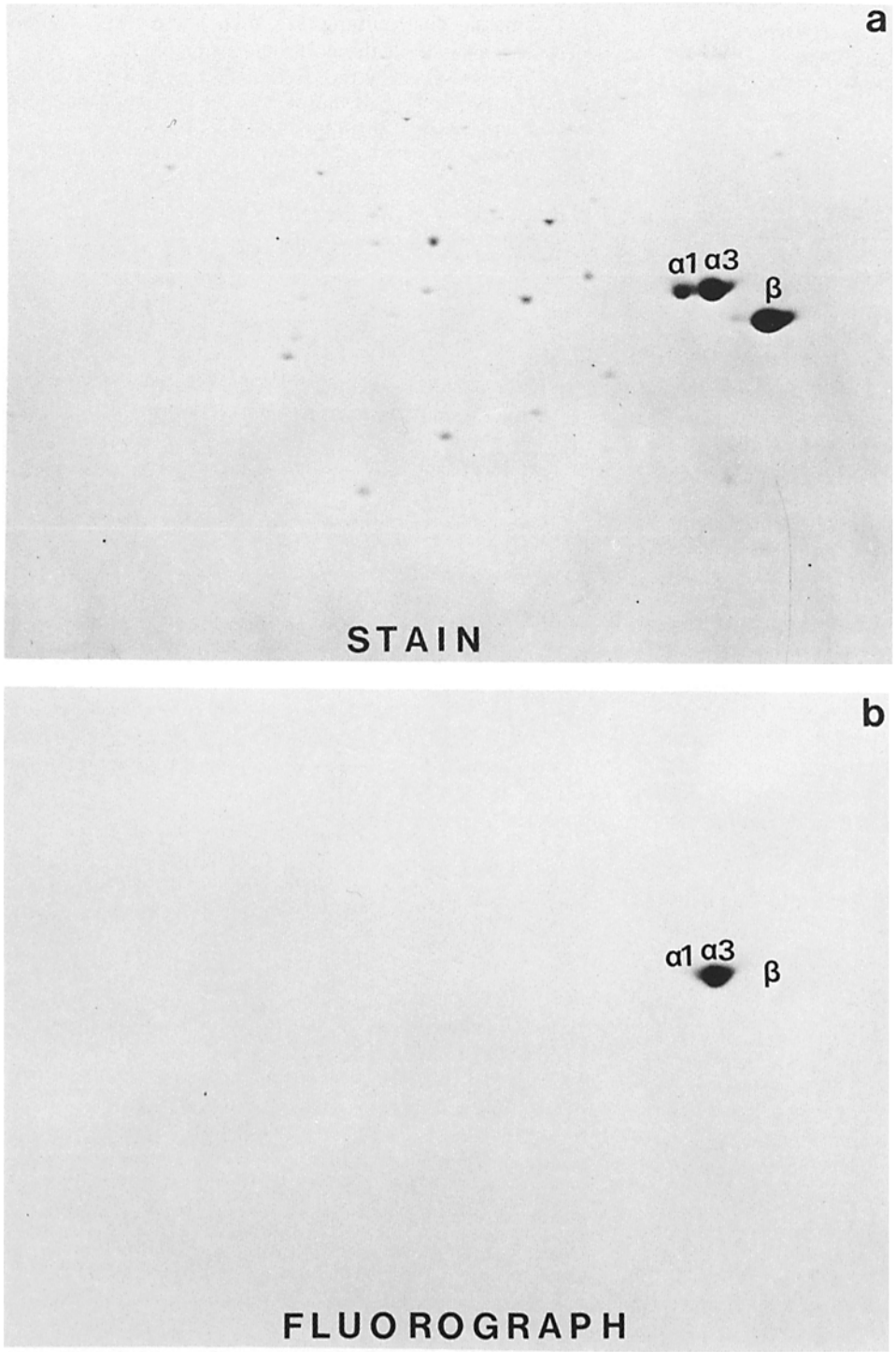

FIGURE 5 2-D electrophoresis of *Chlamydomonas* axonemes. Axonemes were prepared from flagella that had been labeled with [^{3}H]acetate in vivo (see Materials and Methods) in the presence of 10 μg/ml cycloheximide (i.e., posttranslational labeling). Both panels *a* and *b* are the same gel that was electrophoresed with ~35 μg of these axonemes containing 2×10^4 cpm. The Coomassie Blue-stained pattern of this gel appears in *a*, while the subsequently prepared fluorograph appears in *b*.

DISCUSSION

These results show that the principal axonemal α-tubulin in *Chlamydomonas* flagella (α-3) is synthesized as a precursor (α-1) which is subsequently modified. This posttranslational modification occurs within the flagellar matrix fraction during axonemal assembly and is probably related to the addition of tubulin dimers to the growing distal tip of the axoneme (2, 5, 9, 29, 38). This covalent posttranslational modification can be specifically labeled by adding tritiated acetate to cells that are assembling flagella in the absence of de novo protein synthesis. The modification is, therefore, most likely acetylation or, perhaps, the result of the addition of an acetate metabolite. It is not tyrosinolation which is a widespread α-tubulin carboxyl terminal posttranslational modification (e.g., references 4, 26) because *Chlamydomonas* α-1 and α-3 tubulins have different isoelectric points (15). We are currently determining the specific posttranslational modification by comparative amino and carboxyl terminal analysis, peptide mapping and amino acid analysis of *Chlamydomonas* α-1 and α-3 tubulins.

Our experiments also provide clear evidence for a discrete matrix compartment in the flagellum that is distinct from the microtubular axoneme. In the past, this compartment was an operational one, defined as the proteins removed from the flagellum by a certain concentration of a specific detergent

for a specific time and temperature (e.g., references 37, 39). Our studies now show that tubulin is consistently found in this compartment and that additional tubulin does not appear in the matrix fraction when detergent treatment of flagella is increased from one to 15 min (May, G., and J. Rosenbaum, manuscript in preparation). The discreteness of the matrix compartment is best indicated by the fact that it contains a large amount of precursor tubulin (α-1); extensive axonemal contamination of this compartment would have resulted in the presence of relatively large amounts of axonemal α-3 tubulin.

All axonemal proteins, including tubulin, must be transported through the flagellar matrix fraction during flagellar elongation, since the axoneme assembles at its distal tip (2, 5, 9, 29, 38). One potential level to regulate axonemal assembly, therefore, would be the posttranslational modification of axonemal precursors within the matrix fraction. In addition to the results reported here on α-tubulin modification, other axonemal polypeptides may be posttranslationally modified, for example, by phosphorylation (1, 12, 24). In one case, mutationally impaired ability to phosphorylate specific flagellar radial spoke polypeptides has been correlated with defects in spoke assembly (12). Recently, our laboratory has found that the phosphorylation of a specific class of flagellar polypeptides is stimulated during flagellar regeneration (May, G., and J. Rosenbaum, manuscript in preparation). Now that the matrix fraction can be delineated (based on α-1 tubulin content) it will be interesting to determine whether other axonemal precursor proteins within the matrix can be identified and whether other posttranslational modifications, such as phosphorylation, can be shown to occur within this fraction.

The posttranslational modification of α-tubulin reported here presumably occurs by an enzymatic mechanism. The activation of this enzyme(s) raises some intriguing questions of how its metabolic regulation is related to its cellular location. Our inhibitor studies indicate that posttranslational modification of α-tubulin requires flagellar assembly but does not require *de novo* protein synthesis. Therefore, cells which are deflagellated in the absence of *de novo* protein synthesis must have both the precursor α-tubulin (α-1) and the enzyme(s) that catalyzes the posttranslational modification together in the same cytoplasmic pool. However, the posttranslational modification appears to occur only within the flagella, and this indicates that the modification process is somehow activated during transport of the precursor α-tubulin (α-1) and its modifying enzyme(s) into the flagella.

Chlamydomonas flagella can be induced to resorb and this involves nonproteolytic disassembly of the axonemal polypeptides which can be re-used when the flagella are allowed to re-assemble (14). Recently, we determined that, upon flagellar resorption, posttranslationally modified axonemal tubulin (α-3) is changed back to its α-1 precursor (16). This supports the notion that posttranslational modification confers on α-tubulin some property that is associated with assembled axonemal microtubules but missing from unassembled tubulin. After the precise posttranslational modifying moiety has been identified, attempts will be made to inhibit this process to further study the role that posttranslational α-tubulin modification plays in flagellar assembly and function.

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