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Microtubule Assembly Is Dependent on a Cluster of Basic Residues in α-Tubulin†

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ABSTRACT: Previous studies have shown that tubulin, a major protein component of the microtubule, is rendered assembly incompetent when a highly reactive lysine residue (HRL) in the α polypeptide of tubulin *dimer* is reductively methylated [cf. Sherman, G., Rosenberry, T. L., & Sternlicht, H. (1983) *J. Biol. Chem. 258*, 2148-2156]. In this study we demonstrate that the HRL in bovine brain tubulin is Lys-394, a residue proximal in the α-tubulin sequence to the highly negatively charged carboxy-terminus region (residues 412-450) previously implicated in assembly. pH studies were undertaken to probe the local environment of Lys-394. These studies indicated that Lys-394 reactivity toward HCHO is sensitive to the titration of a pK_a 6.3 group presumed to be a histidine residue. This assignment is supported by our finding that histidine modification via diethyl pyrocarbonate strongly affects Lys-394 reactivity toward HCHO as well as microtubule assembly. We propose on the basis of secondary structure considerations and published sequence data for a variety of tubulins that Lys-394 is part of an evolutionarily conserved cluster of basic residues (effective charge: 2+ to 2.5+ at neutral pH) composed of Lys-394, His-393, and Arg-390, which is important for tubulin function and which renders Lys-394 reactive as a nucleophile.

Tubulin, the major protein of microtubules, is a heterodimer consisting of two polypeptide chains, α and β, each ~50 kdaltons (Luduena et al., 1977). Microtubule assembly is affected by divalent cations (Rosenfeld et al., 1976; Larsson et al.; Solomon, 1977) and basic proteins, i.e., microtubule-associated proteins (MAPs)[1] (Lee et al., 1978a,b; Scheele & Borisy, 1979). A number of these agents are thought to bind at the carboxy termini of tubulin, i.e., at residue positions ~410-451 in α polypeptide and ~400-446 in β polypeptide, which contain ~40% of all the glutamates and ~20% of all the aspartates in tubulin (Ponstingl et al., 1981; Krauhs et al., 1981; Valenzuela et al., 1981), and are presumed to affect assembly in part by modulating electrostatic interactions between carboxy termini (Maccioni et al., 1984; Serrano et al., 1984). Proteolytically cleaved tubulins that lack carboxy-

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[1] Abbreviations: PB, microtubule protein stabilizing buffer (pH 6.7) consisting of 0.1 M MES, 2 mM EGTA, 0.1 mM EDTA, 2 mM mercaptoethanol, and 0.5 mM $MgCl_2$; MES, 2-(*N*-morpholino)ethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*',*N*'-tetraacetic acid; EDTA, ethylenediaminetetraacetic acid; MTP, microtubule protein; MAPs, microtubule-associated proteins; HRL, highly reactive lysine; CNBr-HRL, CNBr fragment that contains HRL; PMSF, phenylmethanesulfonyl fluoride; DEP, diethyl pyrocarbonate; $(\alpha/\beta)_{cpm}$, cpm in radiomethylated α-tubulin divided by the corresponding cpm in radiomethylated β-tubulin (α and β subunits obtained from radiomethylated MTP or from radiomethylated PC tubulin electrophoresed on SDS polyacrylamide gels); PC tubulin, phosphocellulose-purified tubulin; SDS, sodium dodecyl sulfate; DTT, dithiothreitol; Tris, tris(hydroxymethyl)aminomethane; PAGE, polyacrylamide gel electrophoresis.

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termini regions show a marked decrease in their ability to interact with MAP-2 and undergo aberrant assembly characterized by open and twisted sheets (Maccioni et al., 1984; Serrano et al., 1984; Sackett et al., 1985). Although it is generally known that cations and MAPs are important regulators of microtubule assembly, there is much less appreciation of the fact that basic residues in tubulin also play essential roles in assembly. We have used reductive methylation to probe the role of lysine residues in assembly (Szasz et al., 1982; Sherman et al., 1983) and previously demonstrated the presence of an essential, highly reactive lysine (HRL) in the α polypeptide of tubulin. Our studies complemented related studies by Maccioni et al. (1981) and Mellado et al. (1982) on the role of arginyl and lysyl residues in microtubule assembly and Lee et al. (1976) on the role of histidines in assembly.

In this paper we describe our identification of the HRL in bovine neuronal tubulin as Lys-394, a residue proximal to the highly negatively charged carboxy-terminus region. We propose a model based on secondary structure predictions and electrostatic considerations that rationalizes the enhanced nucleophilicity of Lys-394. This model implicates a cluster of basic residues consisting of Lys-394, His-393, and Arg-390 as being involved in assembly. Lee et al. (1976) reported that chemical modification of histidine(s) with diethyl pyrocarbonate (DEP) abolishes microtubule assembly. Histidine-modified MTP resembles Lys-394-modified MTP; i.e., colchicine and GTP binding are largely retained in the modified proteins while only modified tubulin dimer, not modified microtubule polymer, is rendered assembly incompetent (Lee et al., 1976; Szasz et al., 1982). This similarity suggested that the histidine(s) previously implicated in microtubule assembly might be in the α polypeptide and in close proximity to Lys-394. We consequently reexamined the effects of DEP on tubulin. Our analysis indicated that Lys-394 reactivity toward HCHO as well as microtubule assembly is strongly affected by histidine modification and suggests that His-393 and/or His-406, two basic residues near the carboxy terminus, are likely to be the essential histidine(s) detected by Lee et al. (1976).

Materials and Methods

Protein Preparation. Microtubule protein (MTP) was isolated by repetitive cycles of assembly–disassembly from bovine brains following a procedure (Sternlicht & Ringel, 1979) modified from Gaskin et al. (1974). Twice-cycled preparations were used immediately or stored at −20 °C in PB + 5 M glycerol buffer for subsequent use within 2 weeks. MTP was ~85% tubulin and ~15% MAPs as determined by gel electrophoresis (Laemmli, 1970). Protein concentrations were established by the Lowry method (1951) with bovine serum albumin as standard.

Reductive Methylation. Protein samples were dual-isotope labeled by reductive methylation to facilitate identification of the HRL in CNBr digests of α-tubulin. MTP (~15 mg/mL, 5–10 mL) depleted of GTP was reacted with limiting concentrations of $H^{14}CHO$ (0.2–0.3 mM, 35 mCi/mmol, New England Nuclear) and $NaCNBH_3$ (1.2–1.8 mM) at 37 °C for 15 min as described previously (Szasz et al., 1982). The reaction was quenched by the addition of 50 mM glycine, and the radiolabeled protein was extensively dialyzed at 4 °C against 1 mM ammonium bicarbonate and 0.2 mM PMSF. The ^{14}C-radiolabeled protein was denatured in 6 M guanidine hydrochloride (Schwarz/Mann ultrapure, 50 °C for 30 min) and reacted with ^3HCHO and $NaCNBH_3$ (37 °C) with concentrations and specific activity of ^3HCHO chosen to give approximately equal ^3H and ^{14}C cpm. After 15 min, unlabeled HCHO (20 mM) and $NaCNBH_3$ (100 mM) were added to quench the ^3H-labeling reaction and to fully dimethylate all lysine ε-amino residues (60 min, 37 °C). The radiolabeled material was reduced with 25 mM DTT (30 min, 50 °C), carboxyamidomethylated with 100 mM iodoacetamide (30 min, 50 °C in the dark), and then extensively dialyzed against 1 mM ammonium bicarbonate and 0.2 mM PMSF. The dialyzed material was lyophilized and stored at −70 °C for subsequent isolation of α polypeptide.

Separation of α Polypeptide. Dual-isotope labeled α polypeptide was isolated by preparative slab gel electrophoresis following procedures modified from Stephens (1975). A total of 1.5 mL of 8–10 mg of methylated protein in layering solution (4.5% SDS, 7.5% glycerol, 15 mM Tris, 60 mM DTT, and 0.025% bromophenol blue) was evenly distributed across the top surface of a stacking gel and electrophoresed at 50 mA for 16–20 h through a 0.7 cm thick running gel [9% acrylamide, 0.24% bis(acrylamide)]. Protein bands were visualized by the method of Higgins and Dahmus (1979) with 4 M sodium acetate, and the α polypeptide band was cut from the gel, soaked in water for 30 min to reduce salt content, placed in a 25-mm dialysis bag (Spectrapor, 12-kdalton cutoff) partially filled with electrophoresis buffer (0.38 M glycine, 0.05 M Tris, 0.08% SDS, 1 mM MET), and electroeluted overnight in a horizontal electrophoresis chamber at 125 mA. Electroeluted samples were extensively dialyzed (24 h at room temperature and 3 days at 4 °C against 1 mM ammonium bicarbonate and 0.2 mM PMSF), lyophilized, and stored at −70 °C. Aliquots subjected to analytical gel electrophoresis indicated >95% of the total protein was α polypeptide.

Cyanogen Bromide Digestion. Lyophilized α polypeptide was dissolved to a final concentration of ~5 mg/mL in 70% formic acid with 10 mg/mL CNBr and incubated for 18–36 h at room temperature. The digestion reaction was quenched by the addition of 10 volumes of water, dried under vacuum, and characterized by polyacrylamide gel electrophoresis in cylindrical gels (5 × 120 mm) performed by a procedure modified from Swank and Munkres (1971) that used pyronin Y as the tracking dye (Sherman et al., 1983). In this gel system, the small CNBr peptide that contains the HRL (CNBr-HRL peptide) elutes at R_f 0.92 (Sherman et al., 1983). Amino acid analysis of the cyanogen bromide digests indicated >80% conversion of methionine to homoserine and homoserine lactone.

Isolation of CNBr-HRL Peptide. A total of 1.5–3 mg of dual-isotope labeled CNBr-digested α polypeptide dissolved in 1–3 mL of 70% formic acid was layered on a 1.3 × 90 cm Bio-Gel P-30 sizing column (Bio-Rad) and eluted with 25% acetic acid, 15% 2-propanol, 5 mM mercaptoethanol, and 0.2 mM PMSF at a flow rate of 2–3 mL/h. Fractions of 2 mL were collected. Fractions containing the CNBr-HRL peptide identified by their enhanced $^{14}C/^3H$ ratio (see Results) were pooled, dried under vacuum, and redissolved in 70% formic acid for further purification by ion exchange (SP-Sephadex) chromatography. A total of 0.1–0.2 mg of partially purified CNBr-HRL was applied to a 1 × 6 cm SP-Sephadex C-25 column (Pharmacia) equilibrated in 25% acetic acid and eluted with a linear gradient of 25% acetic acid (buffer A, 100 mL) and 25% acetic acid–5% pyridine redistilled from ninhydrin (buffer B, 100 mL). All buffers were prepared from HPLC grade (Fisher) water and solvents. Fractions of 1.5 mL were collected, and the fractions enriched in purified CNBr-HRL were pooled, dried under vacuum, and stored at −70 °C for subsequent sequence analysis.

Amino Acid Sequence Determination. A partial sequence of the CNBr-HRL peptide was obtained by manual Edman degradation following Black and Coon (1982). N-Terminal amino acid residues were sequentially converted to their phenylthiohydantoin (PTH) derivatives and analyzed by HPLC (Beckman Model 344 equipped with a Hewlett-Packard 3390 integrator) on an Ultrasphere ODS-PTH column (Beckman Δ242942) maintained at 50 °C. The complete sequence of the CNBr-HRL was determined by solid-phase Edman degradation [cf. Tong & Elzinga (1983)]. Radiolabel content at each residue position was estimated by counting an aliquot of the PTH-amino acid samples at each cycle.

HRL Reactivity as a Function of pH. The α subunit contains the HRL and at low concentrations of radiolabeled HCHO is preferentially methylated (Sherman et al., 1983). This preferential methylation is evident from a comparison of the ratio of incorporated cpm in α polypeptide relative to incorporated cpm in β polypeptide $[(\alpha/\beta)_{cpm}]$. At pH 6.7 where the HRL is approximately an order of magnitude more reactive than a bulk lysine, $(\alpha/\beta)_{cpm}$ is ~2 (Sherman et al., 1983). We used the pH dependence of the $(\alpha/\beta)_{cpm}$ values to estimate the pH dependence of the HRL reactivity.

MTP stock was diluted 1:1 with PB and clarified by centrifugation (70000*g*, 30 min, 4 °C). Microtubule assembly was induced by the addition of GTP to 1 mM and the warming of the solution to 37 °C for 45 min. Microtubules were collected by centrifugation, resuspended in PB + 2.5 M glycerol at the appropriate pH (pH 5.5–9.0), and depolymerized at 4 °C for 30 min. The resulting suspensions were clarified by centrifugation, and the pH of the supernatants was checked and, if necessary, adjusted by the addition of HCl or NaOH. At each pH, aliquots were dual-isotope labeled at low HCHO concentration (0.5 mM radiolabeled HCHO, 3 mM $NaCNBH_3$, 15 min, 37 °C). Samples were first labeled with $H^{14}CHO$ and then denatured with 6 M guanidine hydrochloride, and labeled with ^3HCHO. Incorporated cpm in α subunit relative to β subunit as a function of pH, $\alpha/\beta(pH)_{cpm}$, was determined by SDS–PAGE with a dual-isotope program (Beckman) to correct for channel leakage. Tritium $(\alpha/\beta)_{cpm}$ ratios had pH-independent values of ~1.1 while carbon-14 cpm ratios varied with pH (see Results). HRL reactivity as a function of pH relative to that of bulk lysines, $R_{HRL}(pH)$, was estimated from the carbon-14 $(\alpha/\beta)_{cpm}$ values with the semiempirical relationship

$$R_{HRL}(pH) \approx NR_{HRL}(pH\ 6.7)[\alpha/\beta(pH)_{cpm} - 1.1] \quad (1)$$

In deriving eq 1, we assumed that radiolabel incorporation into N-termini methionines of native tubulin can be neglected [cf. kinetic study by Sherman et al. (1983)] and that the excess radiolabel in α tubulin relative to β tubulin is mainly in the HRL. This excess radiolabel, expressed as the difference in $(\alpha/\beta)_{cpm}$ ratios between native and denatured protein (right-hand side of eq 1), was taken as a linear measure of the relative reactivity of the HRL. $R_{HRL}(pH\ 6.7)$ denotes the relative reactivity of the HRL at pH 6.7, and N denotes an empirical normalization constant of the order of 1 chosen to make the left-hand side of the equation equal to the right-hand side when the pH is 6.7. Parameter values used (Figure 4) were $N = 0.9$ and $R_{HRL}(pH\ 6.7) = 10$ (Sherman et al., 1983).

R_{HRL} values deduced from eq 1 were minimal at low pH and maximal at pH 7–8 (see Results) and were rationalized in terms of a two-state model (eq 2), which assumed that HRL reactivity is dependent on the protonation state of a titratable group (G). That is, the functional dependence of R_{HRL} on pH was expressed as a weighted average of contributions from protein state A where G is fully unprotonated and protein state B where G is fully protonated:

$$R_{HRL}(pH) = R_A F_A + R_B F_B \quad (2a)$$

F_A and F_B denote respectively the fraction of unprotonated and protonated G at the "pH" of interest ($F_A + F_B = 1$) and R_A and R_B the relative reactivity of the HRL when G is respectively fully unprotonated and fully protonated. With the Henderson–Hasselbalch equation (Goldstein et al., 1974), which relates F_A and F_B to pH and pK_a, eq 2a reduces to

$$R_{HRL}(pH) = (R_A - R_B)[10^{pH}/(10^{pH} + 10^{pK_a})] + R_B \quad (2b)$$

where the bracketed expression on the right-hand side of eq 2b represents F_A, the fraction of unprotonated G, expressed as a function of the pK_a of G and the pH of the solution. At low pH, eq 2b reduces to $R_{HRL} = R_B$. R_B was observed to be ~1 (see Results), and the R_A and pK_a values were estimated from a least-squares fit of eq 2b to the HRL reactivity data (Figure 4B).

Histidine Modification. MTP was reacted with DEP, giving *N*-(ethoxyformyl)histidine MTP as the major product (Lee et al., 1976; Melchior & Fahrney, 1970). Briefly, stock MTP diluted 1:1 with PB buffer was clarified by centrifugation at 4 °C and adjusted to a final concentration of 30–40 μM in PB (pH 6.5). Aliquots of 1.5 mL were reacted with DEP (0.2–2.5 mM) for 12 min at room temperature. The reaction was quenched with imidazole-4-acetic hydrochloride (10 mM final concentration), and samples were centrifuged twice through 10-mL Sephadex G-25 columns equilibrated in PB + 2.5 M glycerol (pH 6.8) or extensively dialyzed overnight against PB + 2.5 M glycerol buffer (4 °C) to remove unreacted imidazole and its DEP derivative. Eluted or dialyzed protein was used in methylation and assembly studies. In the latter studies, DEP-modified MTP was supplemented with 1 mM GTP to induce microtubule assembly at 37 °C. Assembly was monitored by following turbidity changes at 350 nm on a Gilford 2400-2 multichambered spectrophotometer. Inhibition by DEP relative to unmodified controls (see Results) is based on turbidity changes noted after 30 min of assembly. Previous studies (Berne, 1974) have shown that turbidity is proportionally related to the mass concentration of microtubules formed [0.20–0.23 A/mg of polymer at 350 nm (Sternlicht & Ringel, 1979)].

The number of modified histidine residues in MTP and phosphocellulose-purified tubulin and the rate of reaction of these proteins with DEP were estimated by difference spectroscopy from the 240-nm band (extinction coefficient of 3600 $cm^{-1}\ M^{-1}$) that is characteristic of carboxyethylated histidine residues (Melchior & Fahrney, 1970). Measurements were done on an Aminco DW-2C spectrophotometer with split (two-compartment) quartz cuvettes. MTP (9 μM) was placed in compartment 1 and an equal volume of PB buffer supplemented with DEP (0.4–10 mM) was placed in compartment 2 of the sample and reference cuvettes. Reaction was initiated in the sample by rapid, repetitive inversions of the sample cuvette, and difference spectra were recorded at 50-s intervals in the wavelength range 190–300 nm. Because DEP was in molar excess relative to histidine residues, acylation proceeded by pseudo-first-order kinetics.

In a number of experiments, NH_2OH was used to deacylate the carboxyethylated histidine residues and regenerate histidine (Melchior & Fahrney, 1970). DEP-modified MTP was brought to 0.1 M in NH_2OH and dialyzed against 0.1 M NH_2OH in PB + 2.5 M glycerol (pH 6.8) for 3–9 h at 4 °C. Deacylation was followed spectrophotometrically on the Aminco DW-2C by removing aliquots at various time periods and measuring the decrease in the *N*-(ethoxyformyl)histidine ab-

sorbance at 240 nm. At the end of the 3–9-h reaction period, hydroxylamine was removed by extensive dialysis against PB + 2.5 M glycerol (4 °C), and the samples were clarified.

RESULTS

Background. Reductive methylation of proteins by HCHO and $NaCNBH_3$ is specific for α- and ε-amino groups and can be carried out at physiological pH, yielding mono- and dimethylated amino derivatives (Jentoft & Dearborn, 1979). Despite the conservative nature of the modification, tubulin is rendered >50% assembly incompetent at 1 mM HCHO, where ca. five methyls out of a possible total of ca. 68 are introduced into the protein (Szasz et al., 1982). In contrast, colchicine and GTP binding are largely retained at low HCHO concentrations (Szasz et al., 1982). When native tubulin dimer is methylated with limiting concentrations of radiolabeled HCHO at pH 6.7, ~2-fold more radiolabel incorporates into α subunit than into β subunit. This preferential incorporation was shown to be the consequence of a HRL in the α subunit that is approximately an order of magnitude more reactive than normal lysine (Sherman et al., 1983). The reactive lysine was localized to a small CNBr fragment (R_f 0.92) on the basis of tube gel electrophoresis. Amino acid analyses of methylated tubulin indicated that loss of assembly competence correlates with the methylation of the HRL (Sherman et al., 1983).

Identification of HRL. A CNBr peptide fragment containing the HRL (CNBr-HRL peptide) was chromatographically isolated from a CNBr digest of α-tubulin by a procedure that took advantage of the enhanced reactivity of the HRL in native dimer relative to denatured protein. In this procedure, α-tubulin was isolated electrophoretically from dual-isotope labeled MTP ($H^{14}CHO$ in the native state and ^3HCHO in the denatured state) and CNBr-digested (Materials and Methods). Digests were chromatographed on a P-30 sizing column, and two peaks (I and II) showing enhanced $^{14}C/^3H$ cpm ratios indicative of preferential labeling in the native state were detected (Figure 1A). Peak I, which had the highest $^{14}C/^3H$ cpm ratio arose from a small CNBr peptide fragment and contained 21% of the total ^{14}C counts. This value agreed well with the expected value of 35 ± 10% for the peptide fragment containing the HRL on the basis of the excess radiolabel in α-tubulin relative to β-tubulin. Peak I was subjected to further purification on SP-Sephadex (Figure 1B). Amino acid composition analysis of pooled fractions 60–70 indicated a high alanine content (16 mol %), a high leucine/isoleucine ratio, and little proline, tyrosine, or valine while the amount of methionine conversion products, homoserine and homoserine lactone, corrected for loss during hydrolysis and expressed as percent methionine indicated a polypeptide with 17–20 residues (data not shown). One-dimensional peptide analysis of the purified SP-Sephadex fraction on SDS–urea gels (Swank & Munkres, 1970) revealed a single major peak at R_f 0.92 (Figure 1C) and confirmed the assignment of peak I to the CNBr-HRL peptide. Analyses of the minor peak II (Figure 1A), which indicated a composition similar to that of peak I, suggested that peak II peptide was an artifact of the separation,[2] or, possibly, a modified form of peak I peptide.[3]

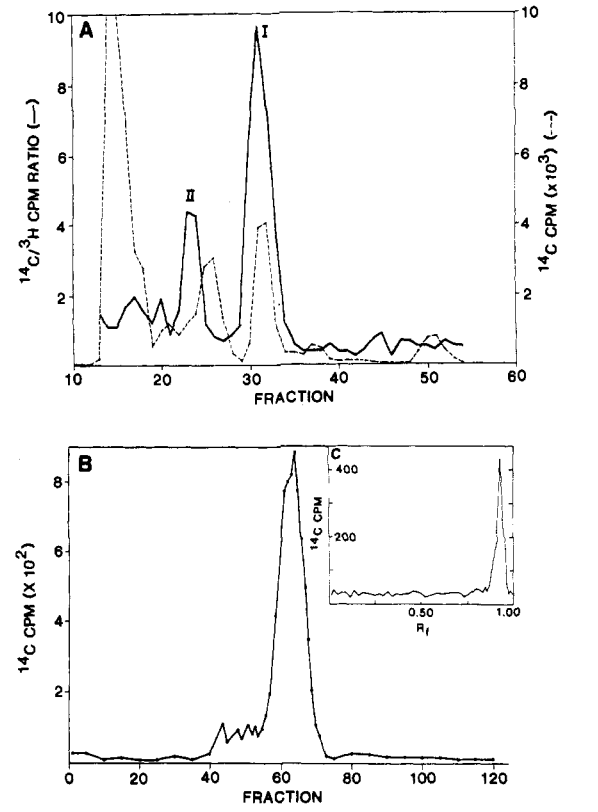

FIGURE 1: Isolation of the CNBr-HRL peptide. MTP was dual-isotope labeled with limiting concentrations of $H^{14}CHO$ in the native state and ^3HCHO in the denatured state. $(\alpha/\beta)_{cpm}$ values, which were 1.7 ± 0.1 (^{14}C label) and 1.2 ± 0.1 (^3H label), respectively, indicated preferential labeling of α-tubulin in the native state. Dual-isotope labeled α polypeptide was isolated electrophoretically and CNBr-digested (Materials and Methods). A total of 1.5 mg of the digest was dissolved in 1.5 mL of 70% formic acid and chromatographed on a P-30 column (A). Peptide elution was followed by monitoring radiolabel content, and fractions containing exceptionally reactive lysyl residues were identified from their enhanced $^{14}C/^3H$ ratios. Fractions 31–33 from peak I, which contained the largest radiolabel ratios, were combined, and an aliquot was removed for further purification on SP-Sephadex (B) (see Materials and Methods). An aliquot taken from the major peak in (B) (fractions 60–70) was electrophoresed on an SDS–urea tube gel (Swank & Munkres, 1971; Sherman et al., 1983), and the resulting gel was cut into 1-mm slices, eluted, and counted (C).

Comparison of the amino acid composition of peak I and its SP-Sephadex-purified fraction (Figure 1B) with the known compositions of CNBr peptides of α-tubulin (Ponstingl et al., 1981) suggested that peak I peptide could be CNBr peptide 378–398. Sequence analysis obtained by solid-phase Edman degradation of peak I peptide unequivocally established peak I peptide as CNBr peptide 378–398 (Figure 2; Table I). Analysis of radiolabel content in peak I peptide indicated that the major portion of the radiolabel was at position 17 (Figure 2C), corresponding to Lys-394. A smaller amount of radiolabel was also observed at position 3 and attributed to a lysine in a contaminant peptide (15–20% of total). This contaminant peptide was assigned to α CNBr fragment 399–413 on the basis of its N-terminal sequence, Tyr-Ala-Lys... (Table I), deduced from the minor PTH-amino acids detected in the first two sequencing cycles (cf. Figure 2A) and the fact that asparagine, the major amino acid at the third cycle (Figure 2B), does not incorporate radiolabel to any significant extent (Jentoft & Dearborn, 1979). A comparison of radiolabel content at positions 3 and 17 (Figure 2C) corrected for differences in residue recovery (Figure 2B) indicated that Lys-394 was 8–12-fold more reactive than the lysine in the contami-

[2] Small amounts of sulfonium salts of methionine are generated during carboxyamidomethylation. These modified methionines are known to be resistant to cyanogen bromide digestion (Tarchinsky, 1981).

[3] We considered the possibility that the peak II peptide, which ran larger than the peak I peptide (Figure 2), might be a posttranslational modification of the peak I peptide [the latter contains a signal sequence ...Asn-Thr-Thr-... for N-glycosylation at asparagine (cf. Table I)] but were unable to demonstrate the presence of hexosamines, sugars generally associated with N-glycosylation, in acid hydrolysates of peak II or peak I (unpublished work).

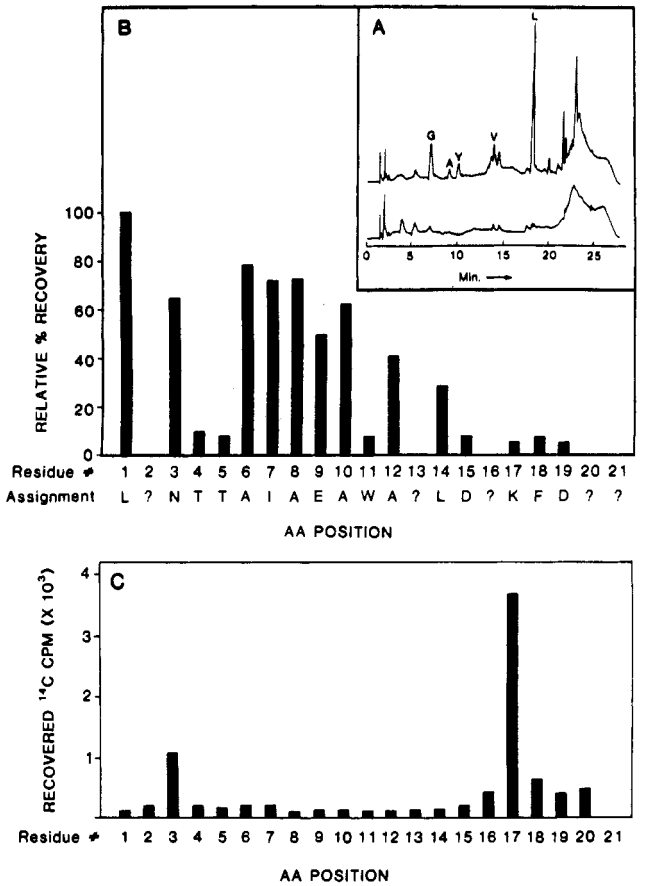

FIGURE 2: Amino acid sequence of the CNBr-HRL peptide. An aliquot of the CNBr-HRL peptide (peak I, Figure 1A) containing ca. 2 nmol of peptide was sequenced by solid-state Edman degradation and interpreted "blind" without reference to the published α-tubulin sequence [cf. Valenzuela et al. (1981)]. (A) HPLC chromatograms of the first cycle of phenylthiohydantoin amino acids (PTH-AA) (top) indicated the presence of ca. 1.4 nmol of leucine (L), which was assigned to the major peptide, and small amounts of other amino acid (G, A, Y, V) presumably derived from buffers and solutions used in the isolation of the CNBr-HRL and from a small contaminating peptide present in peak I (see Results). A control chromatogram of sequencing reagents taking through one degradation cycle indicated little amino acid contamination and no leucine (bottom). (B) Sequence assignment for the CNBR-HRL peptide and relative recovery of the PTH-AA derivatives. Standard single-letter amino acid code is used to represent the amino acids (see Table I). Residue 17 was assigned to dimethyllysine on the basis of its radiolabel content [see (C)] and was estimated to have been recovered at a level similar to that of adjacent residues 18 and 19. Positions 2, 16, 20, and 21 were ambiguous and could not be assigned. (C) Recovered ^{14}C cpm as function of residue position. Radiolabel at position 3 arises from a lysine in a contaminating peptide (see Results).

nating peptide. This estimated difference in reactivities is in excellent agreement with that deduced previously for HRL and "bulk" lysines from analyses of total mono- and dimethyllysine content in microtubule protein as a function of HCHO concentration (Sherman et al., 1983).

pH Studies of HRL Reactivity. The enhanced reactivity of Lys-394 in tubulin dimer indicates that this residue is in an altered environment relative to bulk lysines. Secondary structure predictions based on Chou and Fassman rules (Chou & Fassman, 1978; Valenzuela et al., 1981; Ponstingl et al., 1979) as well as our own predictions based on the Garnier algorithm (Blank et al., 1986) argue that Lys-394 is in an α-helical region. If true, then Lys-394 must be part of a positively charged cluster consisting of the basic residues Arg-390, His-393, and Lys-394 (Figure 3). Furthermore, the cluster is partly surrounded by a region rich in hydrophobic

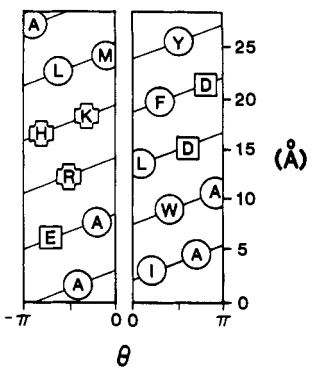

FIGURE 3: Lys-394 is in a cluster of positively charged residues. The 383–400-residue region in α-tubulin is presumed to be α-helical (Ponstingl et al., 1979; Blank et al., 1986). A cylindirical plot of this region is split at $\theta = 0$ to display a front ($-\pi < \theta < 0$) and back face ($0 < \theta < \pi$) [rise per residue in the axial direction is 1.5 Å; circumferential dimension (0 to π) is 7.2 Å]. Residue numbering proceeds from left to right along the diagonals starting with Ala-Ile-Ala at positions 383, 384, and 385 at the bottom and ending with Ala at position 400 at the top. Positively charged residues (enclosed in a "+"-shaped symbol) are clustered on the front face and are distant from the acidic residues (enclosed in a box) in the 383–400 region, which are dispersed primarily on the back face.

residues (Figure 3), which is consistent with Lys-394 not being fully accessible to solvent (see Discussion).

In an effort to further probe the local environment of Lys-394, we investigated the effects of pH on Lys-394 reactivity. We reasoned that electrostatic interactions between Lys-394 and its positively charged neighbor residues should lower the pK_a of Lys-394 (see Discussion) and attempted to estimate the Lys-394 pK_a from a plot of Lys-394 reactivity vs. pH. Exploratory studies indicated that the reactivity of the HRL is sensitive to pH since radiolabel incorporation into α-tubulin changes with pH while radiolabel incorporation into β-tubulin remains essentially constant. These studies also indicated that $(\alpha/\beta)_{cpm}$ values reversibly depend on pH and suggested the involvement of a titratable group.

MTP (10–20 μM) in PB + 2.5 M glycerol at pH 5.5–9 was dual-isotope labeled with 0.5 mM H^{14}CHO in the native state and 0.5 mM ^{3}HCHO in the denatured state, and $(\alpha/\beta)_{cpm}$ ratios were determined by SDS–PAGE (Materials and Methods). $(\alpha/\beta)_{cpm}$ ratios from denatured protein had values of $\sim$1.1 and were independent of pH while $(\alpha/\beta)_{cpm}$ ratios from native protein displayed a bell-shape dependence on pH (Figure 4A). The latter ratio had a value of $\sim$1 at pH 5.5, rose slowly between pH 5.5 and pH 7.0, reached a maximum of $\sim$2.6 between pH 7 and pH 8, and then fell rapidly to an apparent minimum close to 1 between pH 8.5 and pH 9.0 (Figure 4A). We have no simple explanation for this rapid drop and suspect that it results from a protein conformational change at high pH. Circular dichroism studies of calf brain tubulin, for example, indicate a major unfolding of tubulin between pH 9 and pH 10 (Lee et al., 1978). The reactivity of Lys-394 estimated from the $(\alpha/\beta)_{cpm}$ values (Materials and Methods, eq 1) is apparently comparable to that of bulk lysine at pH 5.5 but $\sim$15-fold larger than that of a bulk lysine at pH 7–8.5 (Figure 5). If one ignores the pH 9.0 data point, one can rationalize the reactivity vs. pH data in terms of a two-state model that assumes that the reactivity of the HRL is minimal when a titrable group is protonated and maximal when the group is unprotonated. The least-squares fit to the reactivity data based on this model (eq 2 under Materials and Methods) is shown as a solid curve in Figure 5, and the inflection point at pH 6.3 corresponds to the least-squares estimate for the pK_a of the titratable group. We interpreted this

Table I: Amino Acid Sequences of Peak I Peptides (Figure 1A)[a]

Major Peptide, M_r 2310 (~70% of Total)

	1	2	3	4	5	6	7	8	9	10
Fig. 2	Leu	?	Asn	Thr	Thr	Ala	Ile	Ala	Glu	Ala-
Ponstingl et al., 1981	Leu	Ser	Asn	Thr	Thr	Ala	Ile	Ala	Glu	Ala-

	11	12	13	14	15	16	17	18	19	20	21
Fig. 2	Trp	Ala	?	Leu	Asp	?	Lys*	Phe	Asp	?	?
Ponstingl et al., 1981	Trp	Ala	Arg	Leu	Asp	His	Lys	Phe	Asp	Leu-	Met[398]

Contaminating Peptide (~15–20% of Total)

	1	2	3
Fig. 2	Tyr	Ala	Lys**
Ponstingl et al., 1981	Tyr	Ala	Lys[401] . . . Gly - Met[413]

[a] An asterisk (*) indicates the highly reactive lysine (8–12-fold more reactive than Lys-401 in the contaminating peptide). Double asterisks (**) indicate that the sequence of the contaminating peptide could not be determined beyond position 3.

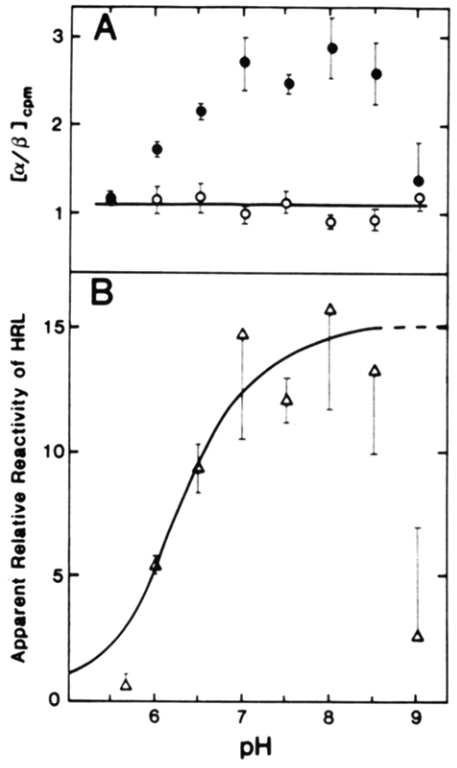

FIGURE 4: pH study of the reactivity of Lys-394. Native MTP was methylated (37 °C) at various pHs with $H^{14}CHO$, denatured, and radiomethylated with ^3HCHO. The extent to which each radiolabel incorporated into α- and β-tubulin was determined by SDS–PAGE. (A) $(\alpha/\beta)_{cpm}$ values of native protein (●) and denatured protein (○) as a function of pH. (B) Lys-394 reactivity relative to bulk lysine as a function of pH. Reactivities (△) were estimated from $(\alpha/\beta)_{cpm}$ values of native dimer in (A) (Materials and Methods and Results).

result as suggesting either that the ε-amine group of Lys-394 has a pK_a ~6.3, which is 3.5–4 pK units lower than that of bulk lysine, or that titration of another residue whose pK_a is normally in this range, e.g., histidine, affects Lys-394 reactivity.

Histidine Modification Affects HRL. To probe the relationship between histidyl residues and Lys-394 reactivity, we reacted tubulin with DEP, a reagent that converts histidine residues to the *N*-(ethoxyformyl) derivative (Melchior & Fahrney, 1970), and examined the effects of DEP modification

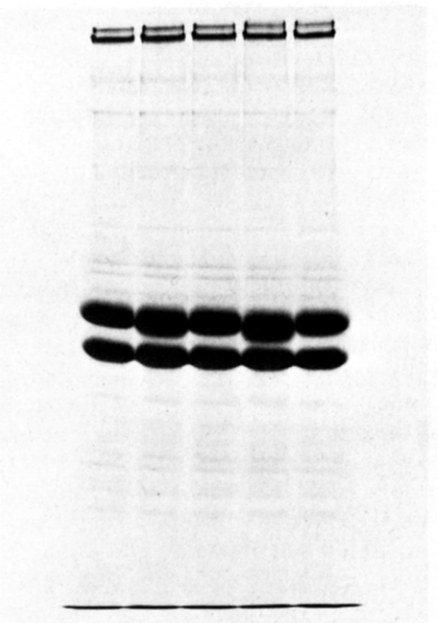

FIGURE 5: DEP *reversibly* affects electrophoretic mobility of α chain. A total of 100 μg of DEP-modified MTP was loaded per well and resolved by SDS–PAGE (9% acrylamide). (Lane 1) No DEP; (lane 2) 0.4 mM DEP; (lane 3) 0.4 mM DEP reversed with 0.1 M NH_2OH; (lane 4) 1.0 mM DEP; (lane 5) 1.0 mM DEP reversed with 0.1 M NH_2OH. Only the α band is visibly perturbed by DEP. At lower loading concentrations the α band is less distorted, and at 1.0 mM DEP one observes a small but distinct shift in α mobility toward lower molecular weight.

on tubulin methylation. Low molar ratios of DEP to total histidine ranging from ca. 0.3 to 3.5 were used to select for reactive histidine residues and to minimize formation of disubstituted histidine products, which have been implicated in DEP-induced denaturation (Miles, 1975). Our modification studies are modeled after an earlier study by Lee et al. (1976), which implicated two to three histidines in tubulin as essential for microtubule assembly.

In one series of experiments, 20–30 μM MTP in PB buffer (pH 6.5) was reacted with 0.2–1.0 mM DEP for 12 min at room temperature. Aliquots were removed for SDS–PAGE and HPLC analysis, and the remainder was used for reductive

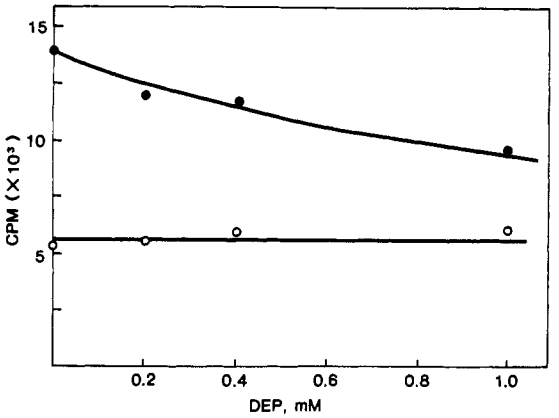

FIGURE 6: DEP inhibits radiomethylation of α subunit. MTP was reacted with DEP and then radiomethylated with 0.4 mM H^{14}CHO and 2.4 mM NaCNBH (15 min, room temperature). (●) α chain; (○) β chain.

methylation and for assembly studies at 37 °C. The latter two studies were done at pH 6.8. Difference spectroscopy based on the 240-nm absorbance of the ethoxyformyl derivative (Melchior & Fahrney, 1970) indicated that only 30–40% of the histidine residues in MTP are "available" for reaction with DEP. This "reactive" fraction reacts with DEP at a rate similar to that of histidine residues in denatured MTP and derives largely from tubulin since an almost identical fraction of reactive histidine residues is present in phosphocellulose-purified tubulin (data not shown). Noting that there are 23 histidines per tubulin dimer (Ponstingl et al., 1981; Krauhs et al., 1981), we estimated that ~4.4 and ~7.2 mol of the ethoxyformyl derivative, respectively, are introduced per mole of tubulin after 12 min of reaction with 0.4 and 1.0 mM DEP. Assembly studies confirmed Lee's et al. earlier study and indicated that 0.4 and 1.0 mM DEP causes respectively a ~45 and ~90% inhibition of assembly (data not shown). SDS–PAGE together with HPLC analysis demonstrated that both subunits of tubulin have reactive histidines that are modifiable at low DEP concentrations. DEP perturbs the electrophoretic mobility of α-tubulin and induces a preferentially broadening of the α band in SDS gels, which increases with increasing DEP (Figure 5). These DEP-induced changes are detectable at DEP concentrations as low as 0.2 mM and are apparently related to histidine residue modification since they are largely reversed with hydroxylamine, a reagent commonly used to deacylate (ethoxyformyl)histidine residues (Melchior & Fahrney, 1970; Means & Feeney, 1971) (Figure 5). On the other hand, when the subunits are separated by reverse-phase HPLC (Stephens, 1984), one observes that the β-tubulin peak preferentially broadens with increasing [DEP]. This broadening is detectable at 0.2 mM DEP and can be reversed with NH_2OH. There are 13 histidine residues in α-tubulin and 10 in β-tubulin (Valenzuela et al., 1981). If we assume that reactive histidine residues are distributed between subunits in approximately the same ratio as histidine content, we estimate that we generated 1.5–2 (ethoxyformyl)histidine derivatives in each subunit with 0.4 mM DEP and 3–4 ethoxyformyl derivatives with 1.0 mM DEP.

Although both subunits of tubulin are apparently modified by DEP, radiomethylation studies indicated that the subunits are affected differently. DEP causes a significant *decrease* in the amount of radiolabeled HCHO incorporated into α-tubulin under limiting conditions but causes only a small increase in radiolabel incorporation into β-tubulin (Figure 6). Analysis of CNBr-digested α-subunit demonstrated that radiomethylation of Lys-394 is specifically reduced by DEP. In

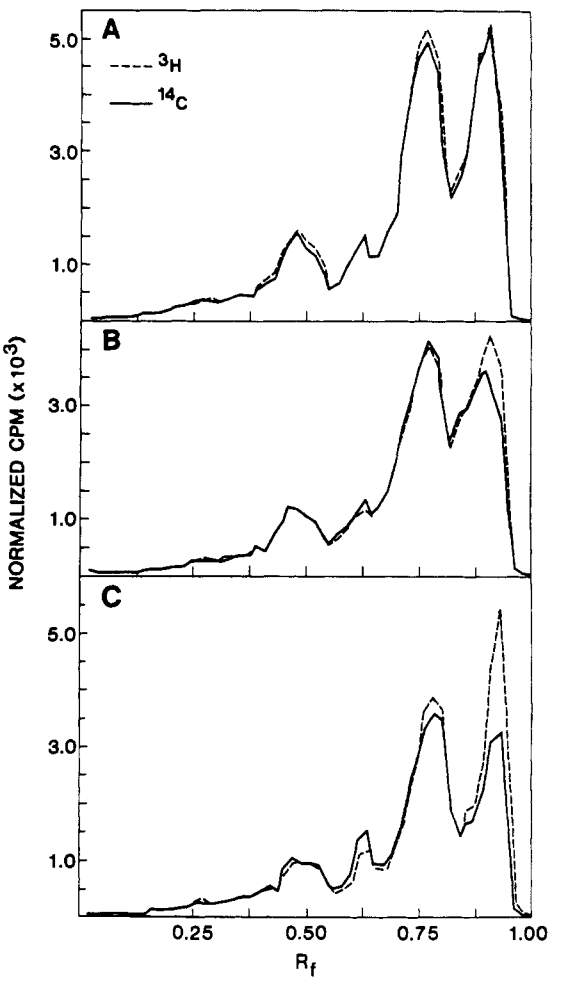

FIGURE 7: DEP modification alters the reactivity of the HRL. MTP samples (30 μM) carried through the modification sequence DEP modify → ^{14}C Methylate → hydroxylamine treat were mixed with corresponding samples from a preparation carried through the modification sequence ^{3}H methylate → DEP modify → hydroxylamine treat (see Results). Dual-radiolabeled α bands isolated from the mixtures by SDS–PAGE were CNBr-digested and analyzed on one-dimensional Swank and Munkres gels (Materials and Methods). (A) 0.0 mM DEP; (B) 0.4 mM DEP; (C) 1.0 mM DEP [(—) ^{14}C labeled; (--) ^{3}H labeled]. In this study samples were reacted with 0.1 M hydroxylamine to facilitate isolation of dual-labeled α polypeptide on SDS gels (cf. Figure 5), and controls (^{3}H-labeled samples) were exposed to DEP to eliminate possible artifactual differences on Swank and Munkres gels related to DEP-induced changes in electrophoretic mobility.

these studies, aliquots from a 30 μM MTP preparation were *first* reacted with 0.0, 0.4, or 1.0 mM DEP and then methylated with 0.5 mM H^{14}CHO. Following overnight dialysis against hydroxylamine, the ^{14}C-methylated samples were mixed with ^{3}H-methylated samples, which served as internal controls. The latter were derived from a common ^{3}H-labeled preparation obtained by *first* methylating with 0.5 mM ^{3}HCHO and then reacting radiolabeled aliquots with 0.0, 0.4, or 1.0 mM DEP. Dual-radiolabeled α- and β-tubulin isolated from the mixtures by preparative gel electrophoresis were counted, CNBr-digested, and subjected to one-dimensional peptide analysis (Figure 7). Measurements of total incorporated ^{14}C counts at 1.0 mM DEP relative to 0.0 mM DEP indicated a large decrease (34 ± 6%) in counts in α-tubulin and a small increase (<10%) in counts in β-tubulin, consistent with a specific loss in HRL reactivity with DEP modification. This interpretation was confirmed by peptide analyses. The R_f 0.92 peak in Figure 7 arises from the reactive Lys-394 residue, which is preferentially methylated at low [HCHO]

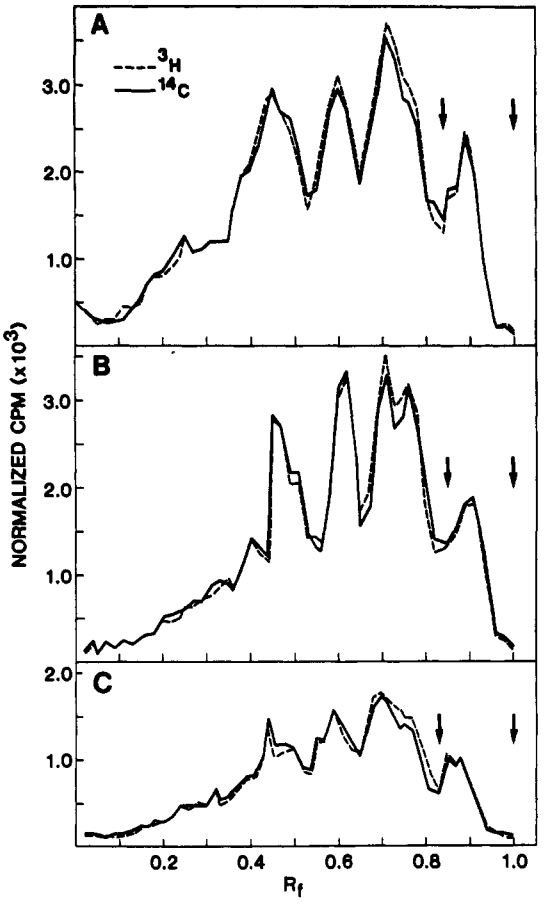

FIGURE 8: Altered reactivity of the HRL does not result from chemical modification of the HRL. MTP samples (30 μM) carried through the sequence DEP modify → denature → ^{14}C methylate → hydroxylamine treat were mixed with corresponding samples from a preparation that had been carried through the sequence denature → ^{3}H methylate → DEP modify → hydroxylamine treat (see Results). Dual-radiolabeled α polypeptide isolated from the mixtures by SDS–PAGE were CNBr-digested and analyzed on one-dimensional Swank and Munkres gels (Materials and Methods). As in Figure 7, samples were reacted with 0.1 M hydroxylamine to facilitate isolation of α polypeptide on SDS gels while controls (^{3}H-labeled samples) were exposed to DEP to eliminate possible artifactual differences on Swank and Munkres gels. (A) 0.0 mM DEP; (B) 1.0 mM DEP; (C) 2.5 mM DEP [(—) ^{14}C labeled; (--) ^{3}H labeled].

(cf. Figure 1). When methylation is done in the denatured state, this peak is significantly diminished (Sherman et al., 1983; also Figure 8). ^{14}C and ^{3}H radiolabel profiles were superimposable at 0.0 mM DEP (Figure 7A). However, at 0.4 and 1.0 mM DEP we observed respectively a ca. 12 and 40% decrease in the amount of radiolabel incorporated into the R_f 0.92 peak of ^{14}C-methylated samples relative to ^{3}H controls (Figure 7B,C). No other region displays a significant decrease in radiolabel content with DEP modification. The small decrease in ^{14}C counts relative to ^{3}H counts observed in the R_f 0.78 region (Figure 7C) may correspond to a change in the reactivity of other α-tubulin lysine(s) with DEP modification or more likely arises from an incompletely digested fragment containing the HRL [cf. Sherman et al. (1983)].

DEP principally modifies histidines but can also modify reactive amino residues in proteins (Melchior & Fahrney, 1970; Lundblad & Noyes, 1984). Our finding that Lys-394 reactivity as measured by radiolabel incorporation is affected by DEP (Figures 6 and 7) is consistent with either an *indirect* perturbation of reactivity via histidine modification or a *direct* perturbation by chemical modification of Lys-394 itself. Additional studies were undertaken to determine the extent to which Lys-394 could be methylated following reaction of tubulin with DEP and reactivity could be restored with hydroxylamine.

MTP in PB buffer (pH 6.5) was reacted in the native state with 0.0, 1.0 and 2.5 mM DEP and then denatured in 6 M guanidine hydrochloride, methylated with 2.5 mM H^{14}CHO (pH 6.8), and dialyzed overnight against hydroxylamine. Each ^{14}C-labeled sample was mixed with a corresponding ^{3}H-labeled sample prepared from guanidine hydrochloride denatured MTP that had been first methylated with 2.5 mM ^{3}HCHO and then reacted with DEP. Dual-radiolabeled α-tubulin polypeptides isolated from the mixtures by preparative gel electrophoresis were CNBr-digested and subjected to one-dimensional peptide analysis (Figure 8). We reasoned that if DEP did not acylate Lys-394 but perturbed Lys-394 reactivity indirectly, ^{14}C and ^{3}H profiles *should superimpose* at all DEP concentrations. On the other hand, if DEP acylates Lys-394, then N$^\epsilon$-acylated Lys-394 should be resistant to methylation and the ^{14}C profile in the R_f 0.92 region should show a *diminution* relative to that of the ^{3}H control. Correcting for contributions from one or two bulk lysine residues in the R_f 0.92 region, we would expect ^{14}C profiles of the [DEP] = 1 and 2.5 mM samples to show respectively a ca. 15–20 and 35–50% diminution of radiolabel content in the R_f 0.92 region relative to controls. We found no evidence for DEP acylation of Lys-394. A comparison of one-dimensional CNBr peptide maps (Figure 8) indicated that ^{14}C and ^{3}H profiles were *superimposable* at all DEP concentrations. Furthermore, the integrated area of the R_f 0.92 regions (areas between arrows in Figure 8) in all cases corresponded to ~12% of the total area, or to 2.3 ± 0.1 lysines out of a total of 19 α polypeptide lysines.

The above study (Figure 8) demonstrated that DEP did not react with Lys-394. Further studies done with NH_2OH indicated that histidine modification was mainly responsible for the loss of Lys-394 reactivity. NH_2OH at concentrations of 0.1–0.5 M readily deacylates carboxyethylated histidine residues (Melchior & Fahrney, 1970). We determined from spectrophotometric difference assays that *N*-(ethoxyformyl)-histidine residues in tubulin treated with 0.1 M NH_2OH have an apparent half-life at 4 °C of 1.5–2 h in PB + 2.5 M glycerol buffer (pH 6.7) (Materials and Methods). MTP (40 μM) in PB buffer was reacted with DEP (0.2–1.0 M) for 12 min at room temperature. Aliquots were reductively methylated with 0.5 mM H^{14}CHO. The remaining material was brought to 0.1 M NH_2OH, dialyzed for 3 or 9 h at 4 °C against PB + 2.5 M glycerol buffer supplemented with 0.1 M NH_2OH, and then dialyzed overnight against PB + 2.5 M glycerol and reductively methylated with 0.5 mM H^{14}CHO. $(\alpha/\beta)_{cpm}$ ratios for these DEP samples following 0-, 3-, and 9-h exposure to NH_2OH are given in Table II. We reasoned that full recovery of initial $(\alpha/\beta)_{cpm}$ ratios following reaction with hydroxylamine would be evidence that loss of Lys-394 reactivity toward HCHO results from a histidine-induced perturbation. On the other hand, if we did not recover initial $(\alpha/\beta)_{cpm}$ ratios with NH_2OH, this could be taken as evidence that other factors contributed to the loss of HRL reactivity. DEP caused a significant reduction in the $(\alpha/\beta)_{cpm}$ ratios and in the apparent HRL reactivity (Table II). A 9-h exposure to 0.1 M NH_2OH resulted in 100% restoration to unmodified $(\alpha/\beta)_{cpm}$ values in samples initially modified with [DEP] ≤ 0.4 M and ~70% restoration in samples initially modified with 1 mM DEP. Furthermore, the rate of recovery is consistent with deacylation of histidine residues (cf. 3- vs. 9-h exposure to NH_2OH, Table II). We conclude from these findings that histidine residue

Table II: Recovery of HRL following Reaction with NH_2OH[a]

DEP (mM)	$(\alpha/\beta)_{cpm}$ 0 h	$(\alpha/\beta)_{cpm}$ 3 h	$(\alpha/\beta)_{cpm}$ 9 h	app HRL reactivity as % of control[b] 0 h	app HRL reactivity as % of control[b] 3 h	app HRL reactivity as % of control[b] 9 h
0	1.90		1.80			
0.2	1.58	1.98	1.91	64	100	100
0.4	1.47	1.75	1.85	49	87	100
0.7	1.29	1.60	1.67	25	67	80
1.0	1.25	1.46	1.61	20	48	69

[a] At 0.1 M, 4 °C, with PB + 2.5 M glycerol (pH 6.7). [b] Estimated as $100\% \times [(\alpha/\beta)_{cpm} - 1.1]/[(\alpha/\beta)_{cpm,control} - 1.1]$, where $(\alpha/\beta)_{cpm,control}$ denotes $(\alpha/\beta)_{cpm}$ value (1.85 ± 0.06) of unmodified preparation.

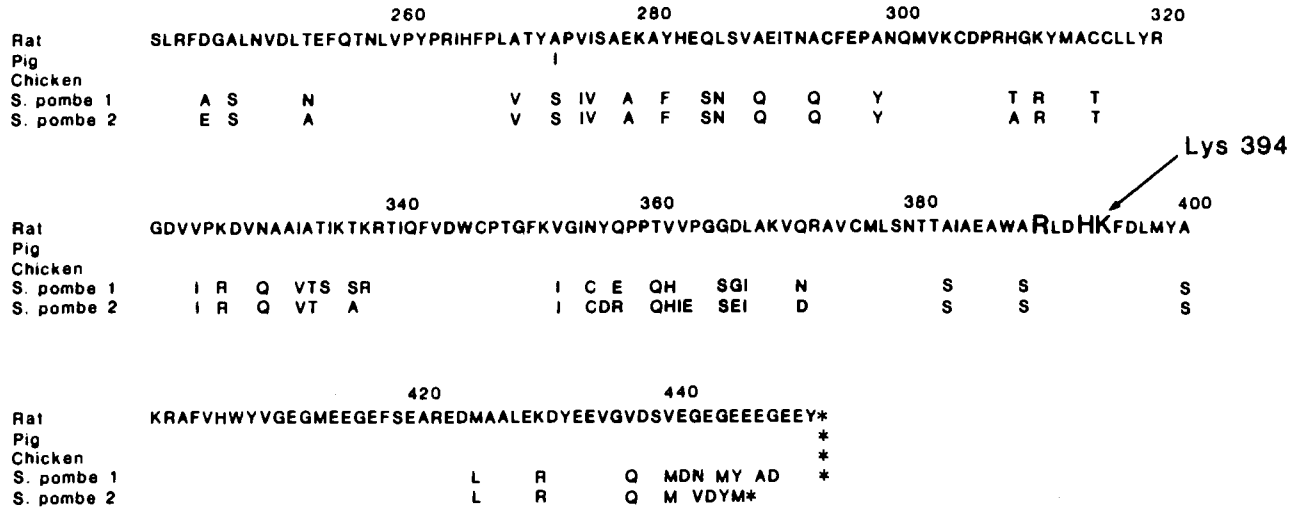

FIGURE 9: Positively charged cluster consisting of Lys-394, His-393, and Arg-390 is evolutionarily conserved. Amino acid sequences 241–450 from chicken (Valenzuela et al., 1981; Luduena & Woodward, 1973), pig (Ponstingl et al., 1981), rat (Lemischka et al., 1981, 1982), and yeast (Toda et al., 1984) α-tubulins are compared. Enlarged letters are used to indicate Arg-390, His-393, and Lys-394. Conserved sequences in the carboxy-terminus one-third segment of the molecule occur at positions 338–352, *390–398*, and 409–422. It is also worth noting that while the sequence for *Chlamydomonas* α-tubulin has not been published, preliminary data (Silflow & Youngblom, 1985; C. D. Silflow, private communication) indicate that the sequence of the Lys-394 region in *Chlamydomonas* α-tubulin is identical with the above and supports the concept of an evolutionarily conserved cluster of basic residues at positions 390, 393, and 394.

modification is mainly responsible for the loss of HRL reactivity although other factors [DEP-induced protein denaturation(?)] become important at high DEP concentrations ([DEP] ≥ 1.0 mM). If we make the assumption that (i) loss of HRL reactivity results from modification of histidine residues in the α chain and that (ii), as suggested by SDS–PAGE and HPLC analysis (see above), both subunits of tubulin have "reactive" histidine residues that are distributed between subunits in the *same* ratio as histidine content, we estimate that total loss of HRL reactivity correlates with the modification of three to four histidines in α-tubulin.

Discussion

In the above study we identified Lys-394 in α-tubulin (Figures 1 and 2, Table I) as the essential HRL previously implicated in assembly (Szasz et al., 1982; Sherman et al., 1983) and investigated the effects of pH change and histidine residue modification on the reactivity of the HRL (Figures 4–8). Identification of Lys-394 as the HRL rests on assembly studies and protein analysis of reductively methylated bovine brain MTP. However, Lys-394 and its nearest-neighbor residues are likely to constitute an essential region in *all* tubulins. Blank and Sternlicht,[4] for example, demonstrated that an essential HRL is present in α-tubulins from rat and chicken brain and from rat liver and chicken erythrocyte. In all instances the HRL was localized to a small CNBr fragment that coelectrophoresed on rod gels with the CNBr-HRL from bovine brain tubulin (R_f 0.92). In addition, while α-tubulins from evolutionarily diverse organisms show extensive amino acid sequence homology, only a limited number of internal sequences are *absolutely conserved*, presumably because these sequences are critical to tubulin function/structure. In the carboxy-terminus one-third segment of the molecule (residues 310–451), the totally conserved positions include Lys-394 and its positively charged nearest-neighbors, His-393 and Arg-390 (Figure 9).

Enhanced nucleophilicity observed for lysines in proteins is thought to involve a lowered pK_a induced by the local environment (Jentoft et al., 1981; Gerken et al., 1982]. A lowered pK_a would make a larger fraction of the unprotonated form of the reactive lysine available as a nucleophile at physiological pH. We propose on the basis of secondary structure analysis of α-tubulin that Lys-394, His-393, and Arg-390 form a cluster of positively charged residues (Figure 3). We suggest that positively charged neighboring residues, His-393 and Arg-390, electrostatically interact with Lys-394 to lower its pK_a. Furthermore, since the positively charged cluster appears to be in a region of low dielectric strength—the cluster is partly surrounded by a region rich in hydrophobic residues (Figure 3)—electrostatic repulsions between His-393, Arg-390, and Lys-394 should be enhanced. It is worthwhile noting that a similar configuration of hydrophobic and positively charged residues has been independently observed about reactive lysines in tropomysin by Hitchcock-Degregori et al. (1985). Tropomyosin is a coiled-coil structure consisting of two parallel α helices in register thought to be stabilized by electrostatic interactions between basic residues of one coiled helix with acid residues on the adjacent coiled helix (McLa-

[4] G. Blank and H. Sternlicht, submitted for publication.

chlan & Stewart, 1975). Hitchcock-Degregori et al. (1985) measured the acetylation reactivity of all lysyl residues in tropomyosin and attempted to classify lysine reactivities in terms of residue location. They intuitively expected that lysines on the outside of the coiled structure would be more reactive than internal lysines. This was not the case. One pair of highly reactive lysines, for example, occurred at a helical interface. This pair, Lys-149 and -152, together with His-153 *constituted a positively charged pocket surrounded by hydrophobic residues analogous to the positive pocket we believe exists in the Lys-394 region of α-tubulin.*

Studies undertaken to test the above concepts gave results consistent with the model. If Lys-394 is in a charged cluster (Figure 3), then modifications that disrupt the cluster should perturb Lys-394's reactivity as a nucleophile. We examined the effects of two potential cluster-disrupting modifications: pH titration and chemical modification of histidines (Figures 4 and 6–8). At physiological pH, His-393 is expected to be partially protonated. (Because this residue is part of the positively charged pocket, we would predict that its pK_a is reduced relative to that normal histidines.) Full protonation of this histidine, as at low pH, would increase electrostatic repulsion between the basic residues in the cluster, which potentially could destabilize the cluster. If destabilization occurred, the α-helical structure of the Lys-394 region would be perturbed and Lys-394 reactivity should be reduced. Alternatively, His-393 may function as a general acid–base catalyst in the methylation reaction and accelerate formation of the Schiff base intermediate (Jencks, 1969). Such capacity, which requires the histidine residue to be in the unprotonated form, would be abolished at low pH. We observed a ca. 15-fold decrease in the apparent reactivity of the HRL relative to bulk lysines as the pH decreased from 7.5 to 5.5, which was attributed to the protonation of the pK_a 6.3 group (Figure 4B). We do not believe that this decrease reflects a major conformational change of tubulin since no such conformational change has been detected by circular dichroism spectroscopy (Lee et al., 1978), nor do we believe that it reflects the titration of the ϵ-amino group of Lys-394. A pK_a value of 6.3 for the ϵ-amino group of Lys-394, while consistent with Lys-394 being a more effective nucleophile than bulk lysines at physiological pH, is exceptionally low. Jentoft et al. (1979), for example, report a pK_a of 9 for the highly reactive lysine in ribonuclease A. In addition, this low pK_a value does not agree with the calculated value of 8.5–9.0 for the pK_a of Lys-394 based on the proposed cluster geometry (Figure 3) and the point charge model of Mehler and Eichlele (1984) for estimating electrostatic effects in protein structure (M. Yaffe, unpublished work). Furthermore, preliminary ^{13}C NMR studies of ^{13}C-methylated MTP done at pH ≤ 8 suggest that dimethylated HRL has a $pK_a > 7.5$ (I. Ringel and H. Sternlicht, unpublished data). It is, however, reasonable to assign the 6.3 pK_a to a histidyl residue(s) since the apparent pK_a's of histidine residues are known to range from ~6 to 8 in proteins (Jardetsky & Roberts, 1981).

Assignment of the $pK_a = 6.3$ residue to a histidine(s) is supported by chemical modification studies that showed that carboxyethylation of a histidine residue(s) alters Lys-394 reactivity (Figures 6–8). We estimated that acylation of perhaps one and certainly no more than three to four histidine residues in α-tubulin affects Lys-394 reactivity when MTP is reacted with DEP. We do not know at present whether the loss of Lys-394 reactivity reflects a conformation change in the vicinity of Lys-394 induced by modification of histidine residues distant to Lys-394 or an alteration of a neighboring histidine residue involved in Lys-394 methylation. The carboxy-terminal one-third portion of the α chain is thought to fold into a separate domain distinct from the amino-terminus portion (Mandelkow et al., 1985). If so, it is reasonable to attribute loss of HRL reactivity to modification of His-393 and/or His-406, the only other histidine residue in the carboxy-terminal one-third portion of the α chain (Ponstingl et al., 1981). We observed that a fraction, 30–40% of the total tubulin histidine residues, are available for reaction with DEP (unpublished data). Similar fractions have been reported for a variety of native proteins and rationalized in terms of conformational restraints that limit the accessibility of histidyl residues to chemical reagents [cf. Lundblad & Noyes (1984)]. His-393, like Lys-394, is in a positively charged pocket and in the absence of steric constraints would be expected to react rapidly as a nucleophile. Kinetic analysis of the histidine modification reaction by difference spectroscopy failed to reveal a highly reactive histidine residue. All accessible histidine residues in tubulin appear to have reactivities similar to that of histidine residues in denatured protein (unpublished results). However, our analyses do not exclude the possibility that a moderately reactive histidine residue that is 2–4-fold more reactive than bulk histidines is present in α-tubulin and obscured by background residues.

The above discussion concerning the Lys-394 region raises a number of intriguing questions concerning the role of internal charge in microtubule assembly. Lys-394 and its neighboring residues in the positively charged cluster, His-393 and Arg-390, are proximal in the α-tubulin sequence to the highly charged carboxy-terminus region (residues 412–450) thought to play a critical role in microtubule assembly (Maccioni et al., 1984; Sackett et al., 1985). We suspect that the positively charged cluster electrostatically interacts with certain carboxylate residues in the highly negatively charged C-terminus to stabilize a conformation essential for microtubule assembly. This perception is supported by preliminary studies with EDC, a zero-length cross-linker specific for cross-linking carboxylate to amine residues, which indicates that the HRL is in close proximity to carboxylate residues (Blank et al., 1986). We expect that additional studies currently in progress, e.g., identification of the sites of histidine modification, circular dichroism analysis of synthetic peptides spanning the Lys-394 region, and cross-linking studies, will provide insights concerning the local environment of Lys-394 and how the Lys-394 region functions in microtubule assembly.

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Registry No. DEP, 95-92-1; HCHO, 50-00-0; L-Lys, 56-87-1; L-Arg, 74-79-3; L-His, 71-00-1.

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