The Local Electrostatic Environment Determines Cysteine Reactivity of Tubulin*

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Of the 20 cysteines of rat brain tubulin, some react rapidly with sulfhydryl reagents, and some react slowly. The fast reacting cysteines cannot be distinguished with [14C]iodoacetamide, N-[14C]ethylmaleimide, or IAE-DANS (15-(((2-iodoacetyl)amino)ethyl)amino) naphthalene-1-sulfonic acid), since modification to mole ratios ≪1 cysteine/dimer always leads to labeling of 6–7 cysteine residues. These have been identified as Cys-305α, Cys-315α, Cys-316α, Cys-347α, Cys-376α, Cys-241β, and Cys-356β by mass spectroscopy and sequencing. This lack of specificity can be ascribed to reagents that are too reactive; only with the relatively inactive chloroacetamide could we identify Cys-347α as the most reactive cysteine of tubulin. Using the 3.5-Å electron diffraction structure, it could be shown that the reactive cysteines were within 6.5 Å of positively charged arginines and lysines or the positive edges of aromatic rings, presumably promoting dissociation of the thiol to the thiolate anion. By the same reasoning the inactivity of a number of less reactive cysteines could be ascribed to inhibition of modification by negatively charged local environments, even with some surface-exposed cysteines. We conclude that the local electrostatic environment of cysteine is an important, although not necessarily the only, determinant of its reactivity.

The 20 SH groups of the tubulin dimer have long led to speculation as to their function. Requirements for a few of the SH groups have been identified. Thus, Cys-12β is near the binding site of the exchangeable GTP of β-tubulin (1), and a C12β mutation is lethal in haploid yeast, whereas a C12Aβ mutation is survivable (2). Cys-241β and Cys-356β are near or are part of the binding site for colchicine and other agents (3–5). What the precise role of the involvement of these cysteines may be is for the most part not clear. Some of the SH groups of tubulin form thioesters with palmitic acid both in vivo and in vitro; these may be responsible for membrane localization of tubulin (6–9). One of these has been located as Cys-376α (10). Except for this palmitoylation site, no specific functions have been identified for the 12 SH groups of α-tubulin, and the order of reactivities of the SH groups has not been definitively established. It has been repeatedly demonstrated (11) that reaction of an equivalent of 1 or 2 SH groups with the usual alkylating agents abolished polymerization competence, but their location in the sequence has not been unambiguously determined. Loss of colchicine binding requires modification of additional SH groups by these nonspecific SH reagents. For this reason we have approached the reactivity of tubulin SH groups in a more general sense, comparing the effects of thioether, disulfide, and thioester formation as well as their location in α-tubulin and β-tubulin.

Protein sulfhydryl groups can be involved in numerous reactions such as oxidation, disulfide interchange, thioether, and thioester formation. For the purpose of this discussion, we shall exclude oxidation. Although free radical reactions of SH groups are known, the remaining reactions all involve the thiolate anion as the reactive species, whereas the thiol group has very much lower reactivity (12). Cysteine reactivity toward various sulfhydryl reagents is regulated by a number of factors including first, exposure to the solvent, and second, dissociation of the thiol to the thiolate anion. RS– is a strong nucleophile (stronger than RO–) normally leading to S N2 reactions. Ionization is suppressed by neighboring acidic groups and enhanced by basic amino acids (12). Although the great preponderance of SH groups involved catalytically in enzyme reactions have low pKₐ values for dissociation to the thiolate anion, less is known about pKₐ values of SH groups of cysteines not directly involved in catalysis. The tubulin cysteine pKₐ values are not known. In general it is assumed and has been shown in certain cases that these approach the “normal” SH pKₐ values near pH 8.5–9.0.

Third, cysteine reactivity is regulated by the reactivity of the SH reagent. For disulfide interchange, the pKₐ isaryl-SH ≪ alkyl-SH, making the former more reactive. Thus, DTNB² is highly reactive with respect to rate and extent of reaction with native tubulin. Factors outlined fourth and sixth (in the next paragraphs) also contribute to this high reactivity of DTNB. It must be remembered that many of the SH reagents can also react with undissociated thiol albeit at a much lower rate. This must be kept in mind when ascribing low pKₐ values for SH groups from reactivity with a thiol reagent.

Fourth, cysteine reactivity is regulated by charge compatibility between the reagent and the cysteine environment, e.g. iodoacetate versus iodoacetamide or DTNB versus 2,2'-dipyridyl disulfide (13). 2,2'-Dipyridyl disulfide yields significant reactivity with cysteine at pH 2. Because the tubulin pKₐ values are not known, we tested cysteine (assuming a pKₐ ~ 8.5) reactivity at pH 2.0 and found a brisk reaction with this reagent.

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1 Because of a difference in alignment in the electron diffraction structure of β-tubulin, Cys-241 corresponds to Cys-239, and Cys-356 corresponds to Cys-354 in the linear sequence.

2 The abbreviations used are: DTNB, 5,5'-dithiobis(2-nitrobenzoic acid) or Ellman’s reagent; IAEDANS, 5-(((2-iodoacetyl)amino)ethyl)amino)naphthalene-1-sulfonic acid (IAEDANS is the fluorescent moiety that is bound to protein and lacks the iodine); NEM, N-ethylmaleimide; HPLC, high performance liquid chromatography; TPCK, 1-tosylamido-2-phenyl chloromethyl ketone; trypsin-TPCK, TPCK-treated trypsin; CAPS, 3-cyclohexylaminol-1-propanesulfonic acid; Mes, 4-morpholineethanesulfonic acid; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; MALDI-TOF, matrix-assisted laser desorption/ionization-time of flight.
gent. Presumably, this indicates that the weakly nucleophilic, undissociated thiol was one of the reactants.

Fifth, cysteine reactivity is regulated by the stability of the bonds formed (in decreasing order), thioether > disulfide > thioester. Most studies on tubulin SH groups have used thioether formation with iodoacetate or iodoacetamide or their derivatives or maleimides. The former react relatively slowly with native protein and with a rather limited number of cysteines (11). Sixth, cysteine reactivity is regulated by the nature of the leaving group of the sulfhydryl reagent, e.g. a thiolate, as found in DTNB, is a good leaving group as are other negatively charged species.

The great difficulty in analyzing very hydrophobic peptides produced by palmitoylation led us to take advantage of the greater stability and hydrophobicity of the thioether bond for subsequent manipulations such as the analysis of tryptic peptides. In the present study we have focused on the comparative reactivities for thioether formation of the SH groups of tubulin.

In a subsequent study we shall compare this with disulfide and thioester bond formation, the effect of the loss of the fast or slow reacting cysteines, and the effect of the size of the substituents on the ability of tubulin to polymerize and to react with ligands.

**EXPERIMENTAL PROCEDURES**

**Materials—**N-[ethyl-1-14C]Ethylmaleimide (50 μCi/mmol) in n-pentane, [1-14C]Iodoacetamide (50 μCi/mmol) in ethanol, and [carboxyl-14C]Chloroacetic acid (55 μCi/mmol) were purchased from American Radiolabeled Chemicals (St. Louis, MO). N-Ethylmaleimide and iodoacetamide were from Sigma, and 1,5-IAEDANS-[5-[(2-iodoacetylamino)ethyl]amino]naphthalene-1-sulfonic acid was from Molecular Probes. Syn-Monobromobimane (Molecular Probes) and Thioglo 1 (Calbiochem) were used from their acetone stock solutions. Trypsin-TPCK was obtained from Worthington. All other reagents were the highest grade available from Sigma unless otherwise noted. Pure (>99%) rat brain tubulin was prepared as described (14). All experiments were performed with buffer (0.3 M Mes, pH 6.9, 1.0 mM EGTA, and 1.0 mM MgCl2) in the dark, and tubulin concentration was 30 μM in all experiments. Stock solutions of the sulfhydryl reagents were prepared fresh in Mes assembly buffer (0.1 M Mes, 1.0 mM EGTA, 1.0 mM MgCl2, pH 6.9).

The specific activities of 14C-labeled reagents were adjusted with unlabeled compounds whenever necessary. The reverse phase HPLC columns were obtained from Phenomenex.

**Sulphydryl Modifications with 14C-Iodoacetamide, N-[14C]Ethylmaleimide, 14C-Chloroacetic Acid, and 1,5-IAEDANS—**Two types of experiments were performed, 1) a time course of tubulin sulphydryl modification at 37 °C at low (1:2) and high (50:1) molar ratios of reagent to tubulin and 2) an 8-h incubation at 4 °C. The tubulin samples were air dried overnight and exposed to phosphorimaging plates (BAS-IP MS 2340) for 10–20 days at room temperature. Then the phosphorimagng plates were scanned in an FLA3000G image analyzer (Fuji-film™ & Imaging, Fuji Medical Systems). The IAEDANS-modified tubulin samples were subjected to electrophoresis in 10% polyacrylamide gels (3.0 mm thick). The fluorescent gel bands were imaged using Molecular Dynamics' Fuji-Film LAS-3000 Advance Fluorescence and Visible Imaging Software. The gel was excited at 302 nm to see the emission at 490 nm.

**Trypsin Digestion—**The modified tubulin samples (0.5–1.0 mg) were digested with trypsin-TPCK (1:20 weight ratio) at 37 °C for 15–24 h in 0.1% Triton buffer, pH 8.5, with 5 mM CaCl2. The trypsin digests of modified tubulin were subjected to electrophoresis in 10% or 12% Tris-Tricine Novex precast gels.

**Peptide Separation Using C18 Reverse Phase HPLC—**To obtain a higher yield of labeled peptides, we used a preparative (250 × 10 mm, pore size 300 Å, particle size 10 μm) C18 reverse phase column. The tryptic digestion was applied to the column, and the column was eluted with a linear gradient of 5% methanol plus 95% water, 5 mM ammonium acetate for 20 min; 5% methanol plus 95% water, 5 mM ammonium acetate to 50% methanol plus 50% water, 5 mM ammonium acetate to 95% methanol plus 5% water, 5 mM ammonium acetate over a period of 150 min; 50% methanol plus 50% water, 5 mM ammonium acetate to 95% methanol plus 5% water, 5 mM ammonium acetate over a period of 45 min; 95% methanol plus 5% water, 5 mM ammonium acetate for 40 min. This gradient gave us good reproducibility and recovery of radioactivity (greater than 80%). A C12 reverse phase column (50 × 4.6 mm, pore size 90 Å, particle size 4 μm) was used for further purification of peptides. The absorbance at 214 nm was monitored; the peaks were collected manually and counted.

**Mass Spectra and Sequencing—**The radioactive and the fluorescent peaks were concentrated on a Speedvac instrument and sent for matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) and N-terminal sequence analysis to the Macromolecular Structure Facility, Michigan State University. The masses of the peptides were calculated from the Protein and Peptide Software developed by Dr. Lewis Pannell, NIH (sx102a.niddk.nih.gov/peptide).

**Structure Generation Using RASMOL—**RASMOL (19) was used to visualize and generate tubulin structure (Protein Data Bank code 1JFF).

**RESULTS**

**Kinetics—**It has been proposed that several SH groups of tubulin may form disulfide bonds (20), but in the hands of most investigators the 20 SH groups (12 in α- and 8 in β-tubulin) are readily shown to be reduced, and the rat brain tubulin used here consistently yields >19 SH groups by titration with excess DTNB in the absence of any denaturing agent. Moreover, the electron diffraction structure shows no disulfide bonds (21). The time courses of thioether formation with iodoacetamide, IAEDANS, or the maleimides were compared in an excess of reagent (reagent to tubulin = 50:1 or 2.5:1 per SH group at pH 6.9 and 37 °C (Fig. 1A). Both S2,2 displacing reagents, iodoacetamide and IAEDANS, showed a slow progressive increase in the number of cysteines reacting over a period of 3 h. Of considerable interest is the finding that the bulky IAEDANS reacted at the same rate as iodoacetamide. Another reagent in this class is syn-n-monomobromobimane (16), whose progress curve is shown in Supplemental Figure S8000 Advanced Fluorescence, Chemiluminescence, emission λ = 480 nm, quantum yield 0.2–0.3, as the reagent itself is negligibly fluorescent. The initial rate of reaction is not much faster than iodoacetamide, and the extent of reaction is comparable. As has been reported for small thiols (16), the reaction is pH-sensitive and is a linear function of the OH− concentration (over the pH range available due to the pI of the protein).
high mole ratio of reagent to tubulin. Tubulin (30 μM in 334 μl) samples were incubated with the sulfhydryl reagents succinyl-monobromobimane, 6:1 (•), [14C]NEM, 50:1 (○), [14C]iodoacetamide, 50:1 (□), 1,5-IAEDANS, 50:1 (▲), Thioglo 1, 40:1 (△) at 37 °C in the dark. A, low mole ratio of reagent to tubulin. Tubulin (30 μM in 334 μl) samples were incubated with [14C]NEM, 1:2 (○), and [14C]iodoacetamide, 1:2 (•) at 37 °C in the dark. C, tubulin (30 μM in 334 μl) samples were incubated with varying concentrations, 1.5, 1.2, 1.1, 1.0, 1.0, 1.0, 1.0, and 1.0, of [14C]iodoacetamide (○), 1,5-IAEDANS (●), and [14C]NEM (▲) at 4 °C in the dark for 8 h. At regular time intervals, the reaction was stopped by adding β-mercaptoethanol and processed as in “Experimental Procedures.”

Fig. 1. The reaction of sulfhydryl reagents with tubulin. A, high mole ratio of reagent to tubulin. Tubulin (30 μM in 334 μl) samples were incubated with the sulfhydryl reagents succinyl-monobromobimane, 6:1 (•), [14C]NEM, 50:1 (○), [14C]iodoacetamide, 50:1 (□), 1,5-IAEDANS, 50:1 (▲), Thioglo 1, 40:1 (△) at 37 °C in the dark. B, low mole ratio of reagent to tubulin. Tubulin (30 μM in 334 μl) samples were incubated with [14C]NEM, 1:2 (○), and [14C]iodoacetamide, 1:2 (•) at 37 °C in the dark. C, tubulin (30 μM in 334 μl) samples were incubated with varying concentrations, 1.5, 1.2, 1.1, 1.0, 1.0, 1.0, 1.0, 1.0, of [14C]iodoacetamide (○), 1,5-IAEDANS (●), and [14C]NEM (▲) at 4 °C in the dark for 8 h. At regular time intervals, the reaction was stopped by adding β-mercaptoethanol and processed as in “Experimental Procedures.”

(data not shown). As might be expected, monochlorobimane reacts much more slowly than its bromo congener (data not shown). Unfortunately, monobromobimane is subject to photolysis leading to fluorescent products; hence, minimal light exposure and careful blank corrections are critical at all time points. Because separation of excess reagent is required, we have not further pursued this labeling procedure. Nevertheless, all three reagents react with ~9 of the 20 SH residues in 3 h (Fig. 1A).

Thioether formation with maleimides occurs by nucleophilic addition to a double bond rather than by nucleophilic displacement. As shown in Fig. 1A (top curves), this is a much faster and more extensive reaction. Thus, at 15 min and 37 °C, virtually all of the cysteines reached the plateau value seen at 2 h. 3–4 SH groups did not react over the 2-h time period. Interaction with a fluorescent maleimide analogue, Thioglo 1 (17), occurs at an equally rapid rate under these conditions, and again, ~3–4 cysteines failed to react in the time allowed. It is of interest that here, too, a bulky fluorophore in no way impeded access to 16–17 cysteines of tubulin nor does the fluorophore significantly accelerate interaction, as has been noted for long chain alkylnialcimides on proteins but not small thiols (22). Thus, although reaction conditions are not identical, it is apparent that thioether formation by nucleophilic displacement is substantially slower than by the addition to double bonds. This difference in rates has been observed previously for small thiols; second order rate constants differ by between 1 and 2 orders of magnitude (23–25).

There are clearly fast and slow reacting SH groups in tubulin; the latter become fast upon denaturation with urea such that the full increase in fluorescence occurs virtually instantaneously (data not shown). With progressively increasing urea concentrations, two reaction classes (initial slopes) are observed, a relatively slow rate up to ~1.5 μmol urea and a faster rate with urea >1.5 μmol.

Because the main objective of the present study is to discover the location of the most reactive cysteines of tubulin, it is necessary to devise conditions for attaining limited stoichiometries by using low mole ratios of reagent to tubulin, low temperatures, or low pH. To minimize reactions with less reactive cysteines, we used mole ratios of 1:2 at 37 °C at pH 6.9 for varying times. Under these conditions the available iodoacetamide was not used up at 3 h (Fig. 1B), and, although there was very low incorporation at early time intervals (e.g. 0.09 SH/dimer), these amounts proved to be useful for further analysis as discussed below.

It is well known that tubulin decays at 37 °C (26, 27). Therefore, to minimize any contribution from denaturation to the accessibility or reactivity of tubulin cysteines, experiments were carried out at 4 °C with 30 μM tubulin at mole ratios of reagent/tubulin of 1:5 to 10:1 at pH 6.9 for prolonged periods. As shown in Fig. 1C, the 8-h incorporation rose gradually as a function of the mole ratio for both iodoacetamide and IAEDANS but never exceeded 4 SH/dimer. These samples also served as samples for tryptic peptide analysis. About twice as much substitution occurred with NEM.

Distribution of Label between α- and β-Tubulin—In preliminary experiments to compare the distribution of label between the two tubulin monomers, we used 0.06–0.6 mol of [14C]NEM/dimer, 0.05–0.3 mol of [14C]iodoacetamide/dimer, or 0.5–4.0 mol of IAEDANS/dimer. 10% sodium dodecyl sulfate polyacrylamide gels were used to separate α- and β-tubulin followed by transfer to polyvinylidene difluoride membranes, phosphorimaging (Figs. 2, A and B), or fluorescence analysis (Fig. 2C).

Under various incubation conditions using different temperatures, times, or mole ratios, no conditions could be found that led to unique labeling of only one monomer at the expense of the other. Even with mole ratios as low as 0.06 (Fig. 2A) of label/dimer both monomers were labeled. Identical results were obtained with [14C]NEM (Fig. 2B) or with IAEDANS (Fig. 2C). Although the literature deals almost exclusively with modification of SH groups on β-tubulin, the α-tubulin SH groups appear to react at least as vigorously, and in the case of IAEDANS, α-tubulin labeling exceeds that of β-tubulin. This suggests possibly fast, but partial, reactions on several cysteine residues whose reactivity cannot be distinguished by these reagents; moreover, it is clear that incorporation of 1 eq of any of these three sulfhydryl reagents cannot be unambiguously interpreted in terms of a single cysteine residue.

Low Resolution Mapping of the Most Reactive Tubulin Cysteines—The modified tubulin, digested with trypsin-TPCK for 24 h at 37 °C, was separated on a 16% Tris-Tricine gel. 12 Cysteines of 20 are present in larger (~2.4 kDa) tryptic peptides that could be identified in 16% Tris-Tricine gels. The following 4 α-tubulin tryptic peptides contain 7 cysteines: 1) residues 3–40 (3.8 kDa), including Cys-4, Cys-20, and Cys-25; 2) residues 125–156 (3.2 kDa), including Cys-129; 3) residues...
and 5.6, respectively. To obtain a higher yield of labeled peptides, tubulin (30 μM) samples were incubated with [14C]NEM (15 μM, 56 dpm/pmol) at 37 °C. The moles incorporated were 0.06 (lane 1, 15 min), 0.09 (lane 2, 30 min), 0.18 (lane 3, 60 min), 0.2 (lane 4, 90 min), 0.24 (lane 5, 120 min), 0.28 (lane 6, 150 min), and 0.33 (lane 7, 180 min). 8 μg of protein was loaded on each lane. B, tubulin (30 μM) samples were incubated with [14C]NEM (15 μM, 133 dpm/pmol) at 37 °C and processed as above. 30 μg of protein was loaded on each lane. The moles incorporated were 0.45 (lane 1, 15 min) and 0.45 (lane 2, 30 min). Also, tubulin (30 μM) samples were incubated with varying [14C]NEM concentrations at 4 °C for 5 h. The moles incorporated were 0.06 (lane 3, 1:10), 0.12 (lane 4, 1:5), 0.35 (lane 5, 1:2), 0.65 (lane 6, 3:1), and 2.4 (lane 7, 5:1). C, tubulin (30 μM) was incubated with 1.5-IAEDANS with varying concentrations at 4 °C for 8 h. 30 μg of protein was loaded on each gel: 3.0 mm and 10 lane). The moles incorporated were 0.02 (lane 5, 150 min), 0.65 (lane 3, 60 min), 0.2 (lane 2, 30 min), and 8.0 (lane 1). The samples were processed according to the procedure described under “Experimental Procedures.” The iodacetamide to tubulin ratio was 1:2 in the former case and 50:1 in the latter, and the moles of 14C incorporated were 0.16 and 5.6, respectively. To obtain a higher yield of labeled peptides, we digested the whole tubulin rather than separating and purifying α- and β-tubulins and 2) used a preparative (250 mm × 10 mm) C18 column. The formation of soluble peptide aggregates impeded the progress of the experiment, so a high concentration of about 4–6 μg guanidine-HCl was used for sample preparations. The common acetonitrile gradient did not give reproducible results with good recovery of radioactivity. We used methanol instead of acetonitrile for the fractionation of labeled tubulin digest. This gradient yielded good reproducibility and recovery of radioactivity (>80%).

Fig. 3A shows the HPLC chromatogram of a digest of tubulin modified with [14C]iodoacetamide to a mole ratio of 0.16/dimer. We identified five peaks, labeled as 1*, 2*, 3*, 4*, and 5*, with significant radioactivity (Fig. 3A). The inset in Fig. 3A shows the radioactivity of the peaks. Even though the mole ratio of 14C bound per tubulin dimer was 0.16, we observed 5 radioactive peaks, later identified as 3 coming from α-tubulin and 2 from β-tubulin (see below). Fig. 3B shows the HPLC chromatogram of [14C]iodoacetamide-reacted tubulin with a mole ratio of 5.6 per dimer. Again the radioactivity was localized on the same five peaks as at low mole ratios. Thus, there are at least five fast-reacting cysteines present in tubulin, and all of them react with iodoacetamide even at substoichiometric labeling.

Similar results were obtained when tubulin was labeled with [14C]NEM. Fig. 4A shows chromatograms of tubulin modified at a mole ratio of 14C to dimer of 0.45. Under these conditions seven labeled peaks could be identified (a) despite a total labeling stoichiometry of <1 NEM/dimer. This greater number of modified cysteines is expected because of the greater reactivity of NEM. When a much higher mole ratio of 5.1 NEM/dimer was used, only 1 additional labeled peptide was obtained as shown in Fig. 4B. In addition to the peaks labeled with iodoacetamide above, several of these peaks could not be identified on the basis of their masses, and these were not pursued further.

Our failure to identify a single most reactive cysteine using either iodoacetamide or N-ethylmaleimide suggested the use of a less potent reagent such as chloroacetaamide. When [14C]chloroacetamide (1.5 mM with 30 μM tubulin for 3 h at 37 °C) was used for kinetic analysis, the moles of 14C incorporated per dimer were only 0.2 as compared with 8 when iodoacetamide was used under the same conditions. When 1/10 as much chloroacetamide was used (Fig. 5), 0.02 mol of 14C were incorporated per dimer. Analysis of the tryptic peptides revealed a single radioactive peak corresponding to Cys-347α (that was also labeled by the other reagents as shown above) (Fig. 5, inset). It is clear that by this approach the one most reactive cysteine could be selected from the other fast-reacting cysteine residues.

**Masses and Sequences of Peptides Bearing the Most Reactive Cysteine Residues**—The tryptic peptides obtained from IAEDANS-, iodoacetamide-, and NEM-modified tubulin after HPLC separation (Table I, actual mass) are compared with their calculated masses (Table I, calculated mass). These values are in good agreement. Subsequent N-terminal sequencing revealed that each peptide had one or two unidentified residues in the cysteine position of the primary sequence. This accounted for the expected mass of the particular modification of the peptide. Table IA lists the four IAEDANS-modified peptides containing five cysteines, Cys-305α, Cys-315α-Cys-316α, Cys-347α, and Cys-356β. For reasons we don’t understand at present, iodoacetamide modified five peptides (see also Fig. 3, A and B), but one (peak 1) could not be identified. The modified cysteines are Cys-305α (peak 2), Cys-347α (peak 4), Cys-241γ (peak 5), and Cys-356β (peak 3). Seven peptides were labeled by NEM (Fig. 4, A and B) as shown in Table IC: Cys-305α (peak 2), Cys-315α-Cys-316α (peak 3), Cys-347α (peaks 6 and 7), Cys-37βa (peak 5), Cys-241γ (peak 8), and Cys-356β (peak 4). Again we could not identify peak 1. Note that during trypsin digestion at pH 8.5, the NEM group may undergo hydrolysis with a mass...
increase of 18 (H$_2$O), leading to 2 entries in column 2, Table 1C. In sum, these data show that the most reactive β-tubulin cysteines are Cys-241 (239) and Cys-356 (354), as has been found by others (see "Discussion"). α-Tubulin has five reactive cysteines: Cys-305, Cys-315, Cys-316, Cys-347, and Cys-376.

**DISCUSSION**

A major factor determining the reactivity of cysteines is their dissociation to the thiolate anion. Increased thiol dissociation can be promoted by one or more interactions in which the excess electron density of the thiolate product is stabilized by interactions with positive charge. Any full or partial positive charges in the neighborhood (<6.5 Å) will tend to stabilize the thiolate anion and, thus, lower its pK$_a$ and increase its reactivity. Short range effects resulting from hydrogen bonds and from main chain carbonyl carbon atoms may well contribute to thiol reactivity but cannot be usefully analyzed from the 3.5 Å structure currently available. They have not been considered in the present study. The other positive charges can derive from the following.

Coulombic Stabilization of the Thiolate Anions by Positively Charged Amino Acid Side Chains of His, Lys, and Arg at Distances Not to Exceed 6.5 Å—Snyder et al. (28) have shown

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**FIG. 3. Localization of most reactive cysteines with iodoacetamide.** Tubulin (30 μg) was incubated with $[^{14}$C]iodoacetamide (15 μM, 56 dpm/pmol or 1.5 mM, 2.44 dpm/pmol) for 60 min at 37 °C. Samples were processed as described under “Experimental Procedures.” Absorbance at 214 nm was monitored, and intensity is depicted in mV; peaks were collected, and their radioactivity was measured (insets). A, chromatogram of $[^{14}$C]iodoacetamide modified tubulin with a mole ratio of 0.16. B, chromatogram of $[^{14}$C]iodoacetamide modified tubulin at a mole ratio of 5.6.
with cyanogen bromide-generated peptides of small proteins that reaction rates of cysteine with DTNB vary over many orders of magnitude as a function of the charge of the nearest neighbor amino acid side chains in the primary sequence. These effects are markedly reduced at high ionic strength, attesting to the electrostatic nature of the activation of the thiols by the local environment. The dielectric environment, thus, has a considerable effect on the extent of these interactions. Several studies with small peptides confirm this effect with S-palmitoylation (29, 30). However, the distance between the positive charge and the thiolate anion is indeterminate in these peptides.

Cysteine-Aromatic Interactions (31)—The π electron cloud on the faces of the aromatic ring interact strongly with cations. With the large number of aromatic residues in tubulin, it is not surprising that there are six readily identified cation-π interactions. These are Phe-343/Lys-336, Tyr-399/Arg-402, Phe-92/Arg-79β, Phe-395/Arg-422, and Tyr-346/Lys-401α. The obverse of this electron distribution implies that ring edges are relatively positively charged.

**Fig. 4. Localization of most reactive cysteines with [14C]NEM.** Tubulin (30 μM) was incubated with [14C]NEM (15 μM, 120 dpm/pmol) for 15 min at 37 °C or 1.5 mM [14C]NEM (2.47 dpm/pmol) for 60 min at 37 °C. At the end of the incubation period, the samples were processed according to the procedure described under “Experimental Procedures.” Absorbance at 214 nm was monitored, peaks were collected, and their radioactivity was measured (insets). A, chromatogram of [14C]NEM modified tubulin with a molar ratio of 0.45. B, chromatogram of [14C]NEM modified tubulin with a molar ratio of 5.1.
capable of interacting with anions, although these interactions may not be as strong as the cation-π interaction. It has been shown that many cysteines of proteins are close enough to aromatic rings to interact in this way. In model helical peptides, interaction between phenylalanine and cysteine in the proper orientation (i, i+4) contributes significantly to helix stability (34). Although the dielectric constants of protein interiors are difficult to ascertain, the electrical potential is a reciprocal function of the dielectric constant; hence, the electrostatic effects may be enhanced by low local dielectric

![Fig. 5. Localization of most reactive cysteines with [14C]chloroacetamide. Tubulin (30 μM) was incubated with [14C]chloroacetamide (150 μM, 125 dpm/pmol) for 180 min at 37 °C. Samples were processed as described under Experimental Procedures."

**TABLE I**

MALDI-TOF and N-terminal sequencing data of tubulin cysteine peptides modified by 1,5-IAEDANS A, [14C]iodoacetamide B and [14C]NEM C

The weight values are given for [M + H]²⁺ ions. The weight values, [M+H]²⁺ of the unmodified peptides were 490.6 (305–308α), 1136.4 (312–320α), 1528.8 and 1542.8 (340–352α), 1809.1 (374–390α), 2653.1 (217–241β), and 972.2 (351–359β). The peptide mixtures obtained from the trypsin-TPCK digestion of modified tubulin were fractionated by reverse phase HPLC using a C18 preparative column. The peaks with significant radioactivity from [14C]NEM- and [14C]iodoacetamide-modified tubulin or with fluorescence from 1,5-IAEDANS-modified tubulin were collected and analyzed by MALDI-TOF, then by N-terminal (5 or 6 residues) sequencing.

| Peptide sequence | Calculated weight | Obtained weight | N-terminal sequencing |
|------------------|------------------|----------------|----------------------|
| (A) Molecular mass of AEDANS (307 Da) | 796.55 | 796.65 | XDPR (X = Cys-305α) |
| 305–308α | 1442.4 | 1441.52 | YMAXXI (X = Cys-315α) |
| 312–320α | 1748.4⁣ | 1748.11 | (X = Cys-316α) |
| 340–352α | 1834.8⁣ | 1836.25 | SIQFV (contains Cys-347α⁴) |
| 351–359β | 1278.15 | 1278.36 | TIQFV (contains Cys-347α⁴) |
| (B) Molecular mass of CH₂CONH₂ = 58 Da | 547.55 | 546.7 ± 0.1 | XDPR (X = Cys-305α) |
| 305–308α | 1585.8⁣ | 1599.8⁣ | TDQFV (contains Cys-347α⁴) |
| 340–352α | 1848.8⁣ | 1848.05 | SIQFV (contains Cys-347α⁴) |
| 351–359β | 1278.15 | 1278.36 | TIQFV (contains Cys-347α⁴) |
| (C) Molecular mass of [14C]NEM = 127 Da (1) and [14C]NEM + H₂O = 145 Da (2)⁶ | 614.6 (1) | 612.6 | XDPR (X = Cys-305α) |
| 305–308α | 632.6 (2) | 629.8 | YMAXXI (X = Cys-315α) |
| 312–320α | 1260.1 (1) | 1260.1 (2) | (X = Cys-316α) |
| 340–352α | 1654.8 (1) | 1654.8 (2) | 1671.6 ± 1.5 | TQVFV (contains Cys-347α⁴) |
| 374–390α | 1935.12 (1) | 1935.12 (2) | AVXNL (X = Cys-376α) |
| 217–241β | 2779.05 (1) | 2779.05 (2) | TQVFV (contains Cys-347α⁴) |
| 351–359β | 1096.15 (1) | 1096.15 (2) | TAVXD (X = Cys-354β⁶) |

⁣1-cysteine-modified peptide.
⁣2-cysteine-modified peptide.
⁣Weight differences due to different isotypes.
⁣See footnote 1.
⁣For NEM, the calculated masses were given for peptides with unhydrolyzed and hydrolyzed NEM moiety.

![Fig. 5 inset](Image 264x455 to 554x728)
The N Terminus of an α-Helix Dipole Is Positively Charged and May Thus Stabilize a Nearby Thiolate (36)—From model α helices, Kortemme and Creighton (37) measure a decrease in thiol pK$_a$ of 1.6 pH units. In selected cases two or even three helices may stabilize the same thiolate (36).

As mentioned above, it was not possible with these three reagents under these conditions to identify a unique cysteine residue. Five peptides that contained 6 of the 20 cysteines were the most easily labeled. They were Cys-315 plus Cys-316, Cys-347 and Cys-376 from α-tubulin, and Cys-241 and Cys-356 from β-tubulin (Fig. 6). The numbering follows that of the primary sequence.

Cysteine residue Cys-376, although nearly buried, appears to be activated by Arg-320 and Tyr-272 (127°) (Fig. 6A). The sulfur to ring-edge angle is listed only in the legend but not the figures (in parentheses). Whether or not prior substitution of another SH group promotes exposure of Cys-376 remains to be determined. Note that it was this residue in platelet tubulin that was the substrate for palmitoylation (10), but is not the most reactive toward reagents forming thioethers in rat brain tubulin. It is of interest that His residues are only rarely involved; Cys-20α has a His residue at 4.9 Å; helix dipoles appear not to participate in the activation of this group of cysteines.

The most extensively labeled thiol belonged to Cys-347α, which contained more than half the incorporated label (Fig. 6B). Factors that may have contributed to its reactivity are probable exposure to the solvent in the free dimer, the presence of Lys-336 at 6.1 Å, and the presence of two positively charged aromatic ring edges from Phe-343 and Trp-346, both at favorable angles, 146° and 127°, respectively. It is not known whether this relationship, present in the polymer, remains the same in the free dimer. Moreover, Cys-347α is the most exposed of the reactive cysteines.

The α peptide, containing two SH groups, Cys-315α and Cys-316α, was also labeled by the sulfhydryl reagents (Fig. 6C). The two SH groups are separated by 9 Å (data not shown), too far for ready disulfide formation. A remarkable feature of the Cys-315α SH environment is a cluster of four aromatic residues, Tyr-312 (132°, B8), Phe-296 (156°, B9), Phe-343 (143°) (loop between H10 and B9), and Phe-351 (88°, B9). D, environment of Cys-241 and Cys-356β. Both Cys-241 (H7) and Cys-356β (B9) share a positive group, Arg-320 (B8), and an aromatic residue, Phe-241 (T7 loop). The angle between the Cys-241 sulfur to the plane of Phe-244 is 160° and between the Cys-356 sulfur to the plane of Phe-244 is 141°.

**FIG. 6. Tubulin cysteines in positive surroundings.** Structures were generated using RASMOL. The tubulin dimer (accession number 1JFF) was displayed as a ribbon diagram (gray), and specific residues and/or specific regions were highlighted in different colors. The following colors were used: magenta, loops; cyan, β-sheets; yellow, α-helix. Cys, Arg, Lys, Asp, Glu, Phe, Tyr, Trp, and His residues are displayed as wire-frame models. When a Cys residue belongs to a (i) α-helix, it is displayed in yellow wire-frame, (ii) β-sheet, it is displayed in cyan wire-frame, and (iii) loop, it is displayed in magenta wire-frame; so also with other residues. Sulfur atoms, side chain nitrogen atoms of Arg and Lys, and side chain oxygen atoms of Asp and Glu are displayed as space-filling atomic models according to the Corey, Pauling, and Kendrew (CPK) color scheme: S, dark yellow; N, sky blue; and O, red. The angles between sulfur and the nearest ring carbon are listed in parentheses in the legend below. Helix (H), β sheet (B), and loop (T) locations are in parentheses in the legend below (21). A, environment of Cys-376α (in B10). Cys-376α has a positive neighbor, Arg-320 (B8), and an aromatic residue, Tyr-272 (127°, B7), at a distance < 6.5 Å. B, environment of Cys-347α (loop between H10 and B9). Cys-347α has a positive neighbor, Lys-336 (H10), and two aromatic residues, Phe-343 (146°) and Trp-346 (127°) (loop between H10 and B9), at a distance < 6.5 Å. C, environment of Cys-315α-Cys-316α (B8). Cys-316α has a positive neighbor, Lys-352 (B9), and an aromatic residue, Phe-255 (92°, H8). Cys-315α is surrounded by four aromatic residues, Tyr-312 (123°, B8), Phe-296 (156°, B9), Phe-343 (143°) (loop between H10 and B9), and Phe-351 (88°, B9). D, environment of Cys-241 and Cys-356β. Both Cys-241 (H7) and Cys-356 (B9) share a positive group, Arg-320 (B8), and an aromatic residue, Phe-241 (T7 loop). The angle between the Cys-241 sulfur to the plane of Phe-244 is 160° and between the Cys-356 sulfur to the plane of Phe-244 is 141°.
reactive SH of the two because Lys-352\(\alpha\) is at 6.3 Å and Phe-155 subtends an angle of 92°.

Two β-tubulin SH groups reacting at low reagent/tubulin ratios were Cys-241β and Cys-356β. These groups can be cross-linked by bifunctional reagents (11) and, although far apart in the primary sequence, both are activated by the same arginine (Arg-320β); hence, we represented them together in Fig. 6D. In addition, these residues are activated by Phe-244β, whose ring edge is positioned at favorable reaction angles, 160° to Cys-241 and 141° to Cys-356. Although Cys-241β is nearer to a formal positive charge than any other SH group of tubulin, it is not the most reactive. This may possibly be due to the fact that access to the reagent is constrained by the presence of Cys-241 in helix 7 and Arg-320 in a β-sheet rather than a loop. Note that these residues are also close to the colchicine-binding site, which may explain the inhibitory effect of site occupancy on cross-linking by bifunctional reagents (11). In this respect it is interesting that certain natural product antimicrotubule agents, quinones (38–40) and benzophenanthridines (41), inhibit microtubule assembly through interaction with SH groups.

The inactivity of a number of the cysteine thiol groups is also amenable to electrostatic analysis. All six remaining cysteines of β-tubulin find themselves in the vicinity of one or more carboxylate groups that are expected to suppress dissociation of the thiols (Fig. 7). Cys-129β and Cys-131β, which are surface-accessible, nevertheless do not react with the SH reagents under our conditions; Cys-129β is de-activated by Glu-3β, whereas Cys-131β is surrounded by three carboxylates that appear to be enough to overcome any activating effects of Arg-164β (Fig. 7A). Cys-129β and Cys-131β are negatively charged by a nearby phosphate as well as being blocked by GDP (Fig. 7). In the present study we have used two uncharged alkylating reagents and IAEDANS with a sulfonate group, and we have a measure of the distance between charge and SH, permitting a better estimate of some activating factors. The fact the IAEDANS is at least as active as iodoacetamide suggests that either its negative charge is far away from the interacting surface or that the anilino nitrogen mitigates the negative charge effect. It also suggests that bulky groups, as in IAEDANS, monobromobimane, or Thioglo 1, have ready access to non-surface cysteines. Under these conditions the common

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**Fig. 7. Tubulin cysteines in negative surroundings.** Color notations as in Fig. 6. A, environments of Cys-129β and Cys-131β (loop between H3 and B4). Cys-129β has a negative neighbor Glu-3 (N terminus). Cys-131β has one positive neighbor, Arg-164 (loop between H4 and B5), and three negative neighbors, Asp-130β (loop between H3 and B4), Asp-98α (T3 loop), and Glu-97α (T3 loop). B, environments of Cys-203β (T6 loop) and Cys-305β (loop between H9 and H9’). Cys-203β has one negative neighbor, Asp-205 (T6 loop) and two aromatic residues, Phe-267(131°, B7) and Phe-388(143°, H11). Cys-305β has two negative neighbors, Asp-205 (T6 loop) and Asp-306. C, environment of Cys-129α (loop between H3 and B4). Cys-129α has a negative neighbor, Glu-3 (N terminus).
reagents used were too active to permit distinctions between the most reactive cysteines. Thus, a unique most active thiolate could be identified only by use of the poorly active chloroacetamide. This residue was Cys-347a, the most reactive of all tubulin thiols studied. Several other reagents have identified Cys-241β as the only reactive thiol; these are, however, site-directed by binding in the area of the colchicine binding site (4, 5, 42).

Considerable difficulty exists in the interpretation of the solvent accessibility of the cysteine residues of tubulin. Using DTNB, many investigators have shown that all 20 Cys residues react. Of these, ~5 reacted very rapidly, whereas the remaining 15 were more slowly reacting. Roychowdhury et al. (43) interpret this in terms of 5 surface-exposed cysteines and 15 residues “buried,” as calculated according to Fraszskiewicz and Braun (44). The high activity of DTNB precludes rate differentiation within these groups. They also showed only minor circular dichroism changes from DTNB modification, reportedly excluding opening up of buried residues by prior modification of fast reacting residues (43). The results obtained in the present study as well as various results in previous reports, mostly on β-tubulin, are not consistent with that interpretation. Four of the five fast reacting cysteines are buried according to this study as well as various results in previous reports, mostly on α-tubulin.

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