Direct Photoaffinity Labeling of Cysteine-295 of α-Tubulin by Guanosine 5′-Triphosphate Bound in the Nonexchangeable Site*

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The αβ-tubulin heterodimer has two high affinity guanosine 5′-triphosphate binding sites, so that purified tubulin usually contains two molecules of bound guanosine nucleotide. Half this nucleotide is freely exchangeable with exogenous guanine nucleotide, and its binding site has been readily localized to the β-subunit. The remaining nonexchangeable guanosine 5′-triphosphate can only be released from tubulin by denaturing the protein. We replaced the exchangeable site nucleotide of tubulin with 2′-deoxyguanosine 5′-diphosphate, exposed the resulting tubulin to ultraviolet light, degraded the protein, and isolated ribose-containing peptide derived from the nonexchangeable site. A large cyanogen bromide peptide was recovered, and its further degradation with endoproteinase Glu-C established that cysteine-295 of α-tubulin was the major reactive amino acid cross-linked to guanosine by ultraviolet irradiation.

EXPERIMENTAL PROCEDURES

Materials—Bovine brain tubulin was purified as before (10). The boronate matrix (Affi-gel 601) was from Bio-Rad, Texas Red hydrazide was from Pierce, EP-GC (sequencing grade) was from Promega, and 16% polyacrylamide gels, polyvinylidene difluoride, and nitrocellulose membranes were from Novex.

Preparation of dGDP-Tubulin—Tubulin (1, 800 mg) at 20 mg/ml in 1 M monosodium glutamate (pH 6.6), 2 mM dGTP, and 1 mM MgCl2 was incubated at 37 °C for 20 min. Polymer was harvested by centrifugation at 35,000 rpm for 20 min in a 37 °C rotor, and the polymer pellet was homogenized in 25 ml of 1 M glutamate on ice. Denatured protein was removed by centrifugation at 0 °C (20 min at 35,000 rpm), and another assembly/disassembly cycle was performed with the supernatant. Four cycles were performed, yielding 510 mg of dGDP-tubulin.

Photoaffinity Labeling—The reaction mixture contained dGDP-tubulin at 12 mg/ml in 0.2 M 4-morpholineethanesulfonate (pH 6.9) and 2 mM MgCl2-EGTA-dithiothreitol. About 2–4 ml of this mixture was spread in plastic weighing boats on ice and irradiated at 254 nm for 15 min (2750 μW/cm²). N-Ethylmaleimide (6 mM) was added to the mixture, which was left at 4 °C overnight. Protein was harvested by centrifugation at 15,000 rpm for 15 min. Residual protein was precipitated with 50% trichloroacetic acid and harvested by centrifugation. The combined pellets were dried by lyophilization.

Chemical and Enzymatic Digestion of Irradiated dGDP-Tubulin—The tubulin (2 mg/ml) was treated with alkaline phosphatase (5) for 3 h at 37 °C in 0.1 M Tri-HCl (pH 9) and then with 20 mg/ml CNBr in 70% formic acid for 24 h in the dark. CNBr was removed by repeated lyophilization and resuspension of the peptides in water. For EP-GC digestion, CNBr peptides were dissolved in 1% SDS, diluted 10-fold with 0.1 M phosphate buffer (pH 9), and treated at an enzyme/substrate ratio of 1:50 in 0.1 M phosphate buffer (pH 7.8) at 22 °C for 20 h.

Boronate Chromatography—Peptide mixtures were applied to Affi-gel 601 (5) (column, 1 × 15 cm; flow rate, 1 ml/min). CNBr peptides were dissolved in boiling 1 M Tris-HCl (pH 8.0), and the solution was diluted 10-fold with 50 mM glycine-NaOH buffer (pH 10). CNBr/EP-GC peptides were dissolved in boiling 1% SDS and diluted 10-fold with the 50 mM glycine buffer. After sample application, the column was washed with glycine buffer until elution of unbound peptides was complete.
Bound peptides were eluted with 0.1 M formic acid. Peptide-containing fractions were pooled and processed for SDS-PAGE.

RESULTS AND DISCUSSION

GDP-tubulin was induced to assemble with dGTP (9), with four assembly cycles performed. Nucleotide content of the resulting dGDP-tubulin is shown in Fig. 2C, in comparison with standards (Fig. 2A) and the nucleotide content of the original GDP-tubulin (Fig. 2B). Despite the multiple dGTP-driven assembly cycles, there was 5–10% residual GDP, presumably bound to the E site. This could have resulted from incomplete exchange, due to the lower affinity of dGTP for the E site (9), copolymerization of GDP-tubulin with dGTP-tubulin (11), and/or slow leaching of N site GTP into the medium from denatured tubulin.

Photolabeling conditions were studied with [8-14C]GDP-tubulin (12), and a 15-min exposure to UV light seemed optimum (Fig. 3). To follow putative labeling of the N site, we compared orcinol reactivity (detects ribose but not deoxyribose (13)) of dGDP-tubulin and GDP-tubulin following exposure to UV light and recovery of protein by gel filtration in 8 M urea. Both dGDP-tubulin and GDP-tubulin became orcinol-reactive following UV irradiation, and orcinol reactivity of protein did not occur without irradiation. The GDP-tubulin was as much as 3–4-fold more reactive than the dGDP-tubulin, indicating reduced efficiency of the covalent interaction of N site GTP relative to E site GDP. We attempted to quantitate the extent of the orcinol reaction, but the tubulin requirement was prohibitive. Moreover, tubulin quenched color obtained with ribose standards.

We proceeded to CNBr digestion of UV-exposed dGDP-tubulin. An additional experiment confirmed that we had a ribose-containing peptide. The entire digest was subjected to SDS-PAGE and transferred to nitrocellulose, and the membrane treated by a method designed to label glycoproteins (periodate...
oxidation and then reaction with Texas Red hydrazide). Although multiple bands were observed following staining of a duplicate sample with Coomassie Blue (not shown), there was a single prominent band following the periodate/hydrazide reaction (Fig. 4, gel A). This result required that the tubulin be exposed to UV light.

We proceeded to removal of ribose-containing peptides by chromatography of the CNBr digest of dGDP-tubulin on the boronate matrix. No peptide bound to the boronate matrix unless the tubulin had been UV irradiated. The bound peptide fraction was eluted with formic acid and subjected to SDS-PAGE. There were two peptide bands (Fig. 4, gel B), both of which were sequenced (Table I). The major b1 peptide yielded an amino acid sequence for 17 cycles consistent with the large CNBr peptide spanning residues 204–302 of α-tubulin (14), with the exception of Cys-213 (cysteine residues cannot be identified by automated Edman degradation). We therefore conclude that this peptide has been cross-linked to the N site GTP by UV irradiation. The minor b2 peptide yielded a sequence for 13 cycles (except for Cys-12) consistent with the CNBr peptide spanning residues 2–72 of β-tubulin (15). This peptide includes the Cys-12 residue that cross-links to the E site GDP in the dGDP-tubulin (Fig. 2C).

To further define the reactive N site amino acid in the α-tubulin, we employed several proteases. The best results were obtained with EP-GC, which cleaves at the carboxyl side of glutamate and, to a lesser extent, aspartate residues. A CNBr digest of dGDP-tubulin was further digested with this protease and applied to the boronate matrix. The acid eluate on SDS-PAGE yielded two peptide bands (Fig. 4, gel C), which were sequenced (Table I). The c1 peptide spanned residues 291–302 of α-tubulin, with the exception of Cys-295. The c2 peptide spanned residues 3–19 (except Cys-12) of β-tubulin, again including the E site Cys-12 residue (5).

### Table I

| Cycle | CNBr peptides | CNBr/EP-GC peptides |
|-------|---------------|---------------------|
|       | (Pro)         |                     |
| 1     | Val (α-204)   | Arg (β-2)           |
| 2     | Asp           | Glu                 |
| 3     | Asn           | Ile                 |
| 4     | Glu           | Val                 |
| 5     | Ala           | His                 |
| 6     | Ile           | His                 |
| 7     | Tyr           | Glu                 |
| 8     | Asp           | Ala                 |
| 9     | Ile           | Gly                 |
| 10    | X (α-213)     | Gln                 |
| 11    | Arg           | X (β-12)            |
| 12    | Arg           | Gly                 |
| 13    | Asn           | Asn                 |
| 14    | Leu           | Gly                 |
| 15    | Asp           | Ala                 |
| 16    | Ile           | Lys                 |
| 17    | Glu           |                     |

Because a modified amino acid residue would not be identified by sequential Edman degradation and because every expected amino acid except Cys-295 was identified in peptide c1, we conclude that it is α-tubulin Cys-295 that reacts covalently with the N site GTP during UV irradiation. Presumably the same photoreaction described by Shivanna et al. (5) that occurs between the E site GTP and β-Cys-12 occurs between the N site GTP and α-Cys-295. This mechanism included loss of the C-6 carbonyl from guanine, with the covalent bond formed between the S atom of cysteine and the C-5 atom of guanosine. We sought evidence for this by micro-HPLC coupled to mass spectrometry. The boronate-bound samples used to generate the b1, c1, and c2 peptides were examined by this technique, and each HPLC peak was subjected to high resolution mass spectrometry. Mass spectral peaks were obtained corresponding to peptides cross-linked to the guanosine fragment predicted by the mechanism of Shivanna et al. (5) for α-residues 204–302 and β-residues 4–22 (Table II).

Analysis of sequence homologies in proteins has been invaluable in predicting protein function and ligand binding sites, including nucleotide sites. Tubulin sequences, however, are sufficiently different from those of other GTP binding proteins to have been a theoretical challenge for precise identification of GTP binding sites (16, 17). Nonetheless, the extensive sequence homology between α- and β-tubulin, combined with localization of the E site to β-tubulin (5–8), led to the widespread assumption that the N site is on α-tubulin. Our studies confirm this prediction and represent the first successful attempt at defining tubulin amino acid residues near the N site GTP by cross-linking experiments. Based on the proposed mechanism of the photoinduced covalent bond between a cysteine residue and GTP (5), this reaction should occur between the S atom of α-Cys-295 and C-5 of the guanine moiety. It is particularly interesting that the homology between α- and β-tubulin in the region of α-Cys-295 is not extensive, and that there are no obvious sequence homologies between the α-tubulin peptide we have isolated and the β-tubulin E site peptides previously reported (5–8). This may reflect the profoundly different properties of nucleotide bound at the E and N sites.
at either the Protein Chemistry Facility, W. Alton Jones Cell Science Center, Lake Placid, NY (CNBr peptides) or the Protein and Carbohydrate chromatography. The peptide fractions bound to the matrix and eluted with formic acid were dried, and LC-MS was performed on the specimens.

Nogales et al. (18) presented a detailed model of the tubulin α-β-dimer based on electron crystallographic analysis of paclitaxel-stabilized sheets of antiparallel protofilaments induced by zinc. In this model each subunit had a bound guanine nucleotide in a Rossmann-type fold, confirming the prediction that the α-subunit contained the N site. There was little difference in the overall conformation of the two subunits. In the zinc sheets all GTP binding sites were shielded by the adjacent subunit, explaining the nonexchangeability of all polymer-bound nucleotide in both E (19) and N sites. Although specific αβ pairs in soluble dimers could not be identified, Nogales et al. (18) proposed that in the heterodimer the N site on α-tubulin remained shielded by β-tubulin, explaining its inaccessibility, whereas the E-site on β-tubulin would become exposed to the medium, explaining the rapid equilibration of bound with free nucleotide. This model agreed with observations indicating that the β-subunit was at the plus end of microtubules (20).

This “steric hindrance” explanation of N site properties, however, fails to explain the total nonexchangeability of nucleotide bound to α-tubulin, because the αβ-heterodimer readily dissociates into its subunits (21–26). Yet N site GTP remains totally nonexchangeable in cells (27) and through multiple cycles of assembly with radiolabeled GTP (9, 12) or GTP analogs (Ref. 28 and Fig. 2C). Moreover, no evidence for nucleotide exchange into the N site of dissociated tubulin monomer could be found when such exchange was specifically sought (26). Thus, the N site remains inaccessible in the dissociated α-subunit, as well as in α-tubulin bound to the β-subunit in heterodimer.

In terms of the current studies, in the model of Nogales et al. (18) Cys-295 of α-tubulin appears to be too distant from the N site GTP to account for the covalent interaction we have observed. Because the reaction occurs rapidly and only a single amino acid reacts with the GTP, it seems unlikely that the reaction is nonspecific or results from tubulin denaturation. Most likely there is a conformational change in soluble heterodimer relative to zinc polymer that brings Cys-295 close enough to the guanine residue for the photoreaction to occur. An alternative possibility, particularly in view of the apparently low efficiency of the α-Cys-295 reaction as compared with the β-Cys-12 reaction, is that the reactive residue is close to the guanine moiety only when the αβ-heterodimer dissociates. A substantial conformational change could occur in the α mono...