**Direct Photoaffinity Labeling of Cysteine 211 or a Nearby Amino Acid Residue of β-Tubulin by Guanosine 5’-Diphosphate Bound in the Exchangeable Site**

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Tubulin with [8-14C]GDP bound in the exchangeable site was exposed to ultraviolet light, and radiolabel was cross-linked to two peptide regions of the β-subunit. Following enrichment for peptides cross-linked to guanosine by boronate chromatography, we confirmed that the cysteine 12 residue was the major site of cross-linking. However, significant radiolabel was also incorporated into a peptide containing amino acid residues 206 through 224. Although every amino acid in this peptide except cysteine 211 was identified by sequential Edman degradation, implying that this was the amino acid residue cross-linked to guanosine, radiolabel at C-8 was usually lost during peptide processing (probably during chromatography at pH 10). Consequently, the radiolabeled amino acid could not be unambiguously identified.

The α-β-tubulin heterodimer contains two tightly bound molecules of guanosine nucleotide (1, 2), one bound to each subunit (3). Because the nucleotide on β-tubulin is readily replaced with exogenous nucleotide, including radiolabeled compounds, its binding site has been called the exchangeable site (E site)1 (1, 4–7). GTP bound in the E site and its hydrolysis is generally necessary for microtubule assembly (8–10). The α-tubulin nucleotide is always in the form of GTP, and it probably derives from the tissue of origin of the tubulin because it has never been replaced with exogenous nucleotide (11, 12). The GTP bound to α-tubulin is thus described as being in the nonexchangeable site (N site).

To determine which amino acid(s) of β-tubulin were near the E site, Shivanna et al. (7) employed boronate column chromatography to isolate tryptic peptides enriched for guanosine after direct photoaffinity labeling of tubulin bearing E site [3H]GTP. Shivanna et al. (7) found most of the radiolabel retained by the boronate matrix had reacted with Cys-12 of β-tubulin. Earlier, Little and Ludueña (13) had demonstrated that tubulin depleted of E site nucleotide was susceptible to cross-link formation between Cys-12 and either Cys-201 or Cys-211 by N,N’-ethylenebis(succinimide). The second amino acid involved in the cross-link was not precisely determined. Formation of this dicysteine cross-link was inhibited by guanine nucleotides and by antimitotic drugs that themselves inhibit nucleotide exchange at the E site (14).

We recently applied the boronate methodology to isolate a peptide of α-tubulin cross-linked to the N site GTP by direct photoaffinity labeling by replacing E site GDP with dGDP (15). Following exposure of this tubulin to UV light, E site peptide(s) were not retained by the boronate matrix, and the predominant retained peptide was derived from α-tubulin. Sequence analysis indicated that the modified amino acid was α-Cys-295.

Although we had no radiolabel to assist in optimization of the photoreaction and peptide isolation, we had an ample supply of [8-14C]GDP-tubulin (16) and used this material to define conditions for the photoreaction and optimal use of the boronate matrix. Unlike Shivanna et al. (7), who used high performance liquid chromatography to purify further peptides retained by the boronate matrix before sequencing, we used SDS-PAGE and blot transfer methodologies for this purpose, and we usually observed several radiolabeled bands derived from [8-14C]GDP-tubulin. The most prominent yielded a sequence consistent with a guanosine-β-Cys-12 cross-link, as described by Shivanna et al. (7). This paper describes studies that demonstrate that a second reactive amino acid is probably β-Cys-211.

**EXPERIMENTAL PROCEDURES**

**Materials—**Preparation of [8-14C]GDP-tubulin (16) and of GDP-tubulin (17) from bovine brain were described previously. Affi-Gel 601 was from Bio-Rad, polyacrylamide gels and PVDF membranes from Novex, sequencing grade EP-GC from Roche Molecular Biochemicals, and CNBr, N-ethylmaleimide, and alkaline phosphatase from Sigma.

**Direct Photoaffinity Labeling—**As before (15), a 2-ml mixture containing [8-14C]GDP-tubulin at 12 mg/ml, 0.2 ml of 4-morpholineethanesulfonate (pH 6.9, NaOH), and 2 mM each of MgCl2, EGTA, and dithiothreitol was placed in a weighing boat on ice and irradiated at 254 nm for 15 min at 2750 microwatts/cm2. Tubulin was partially precipitated with N-ethylmaleimide (2.5 mM, at 4°C overnight) and harvested by centrifugation. Residual protein was precipitated with 50% trichloroacetic acid and collected by centrifugation. The combined pellets were lyophilized and treated with alkaline phosphatase for 3 h at 37°C in 0.1 M Tris-HCl (pH 9).

CNBr and EP-GC Digestion— Irradiated [8-14C]GDP-tubulin was treated with 20 mg/ml CNBr in 70% formic acid for 24 h in the dark at 37°C. CNBr and formic acid were removed by repeated lyophilization. For EP-GC digestion, irradiated [8-14C]GDP-tubulin was treated at an
enzyme to substrate ratio of 1:50–100 in 0.1 M phosphate buffer (pH 7.8), and the peptide mixture was lyophilized.

**RESULTS**

Following exposure to UV light, [8-14C]GDP-tubulin was digested with either CNBr or EP-GC. The lyophilized peptide mixtures were applied to boronate matrix columns in glycine buffer at pH 10, and bound peptides were eluted with formic acid. A typical experiment with EP-GC peptides is shown in Fig. 1, and the pattern obtained with CNBr peptides was similar (data not shown). About 25% of the recovered radiolabel was in the unbound fractions and 75% in the bound fractions. Without UV irradiation no radiolabel was retained by the boronate column. SDS-PAGE, with subsequent electrophoretic transfer of the peptides to PVDF membrane, and peptide sequencing were performed as before (15). Autoradiograms were prepared with Kodak Biomax MR film (24–48 h exposures).

The main results of our experiments are summarized in Table I. The CNBr digestion generated three prominent peptides, but the relative amounts varied in different gel patterns (Fig. 2A, II, Coomassie Blue stained, and III, autoradiogram). We cannot explain the faster mobility of the bound radiolabeled peptides, but this did not interfere either with resolution or subsequent sequencing.

The stained gel II (Fig. 2A) showed three major bands and one minor band. All except the upper band 1 were radiolabeled (Fig. 2A, III). Note that the specific activity of the upper radiolabeled band 2 is substantially lower than that of the lower radiolabeled bands 3a and 3b. Band 1 yielded a sequence through 19 cycles of Edman degradation (Table I) consistent with the CNBr peptide α204–302. Note that α-Cys-213 was not positively identified because cysteine residues are destroyed during the Edman procedure. This is the same peptide we obtained previously from dGDP-tubulin (15) following UV irradiation, CNBr digestion, and boronate chromatography, and we therefore identified it as being derived from the N site.

The upper radiolabeled band 2 yielded a sequence through 16 cycles of Edman degradation consistent with the CNBr β165–233 peptide (Table I), and it thus corresponds to the upper radiolabeled band in gel I.

Bands 3a and 3b both yielded sequences through 12 cycles of Edman degradation consistent with the CNBr β2–73 peptide (Table I), and these bands thus correspond to the lower radiolabeled bands in gel I.

To more precisely define the positions of the radiolabel in β-tubulin, we turned to enzymatic digestion to obtain smaller peptides. EP-GC yielded the best results (studies also performed with endoproteinase Lys-C and trypsin). It should be noted that EP-GC will cleave after aspartate as well as glutamate residues.

Initially we obtained puzzling results. Fig. 2B presents a Coomassie Blue pattern (I) together with its autoradiogram (II) of the peptide fraction bound to and eluted from the boronate column. The most heavily stained band in the electroblot was not radiolabeled. Nevertheless, it yielded the sequence of the EP-GC β206–224 peptide, which is a sequence within that of the radiolabeled CNBr peptide represented by band 2 of the gels shown in Fig. 2A. The peptide should have not been retained by the boronate matrix unless it possessed the ribose cis-diol, but not necessarily the radiolabel at the C-8 position in guanine. The heavily radiolabeled bands yielded a sequence consistent with the EP-GC β4–22 peptide, which would contain the known target Cys-12.

We continued to investigate the peptides generated by EP-GC digestion. We noted that before SDS-PAGE the bromphenol blue tracking dye added to the peptide sample had a green color. Small amounts of NaOH added to the sample restored the blue color of the dye. Sometimes this resulted in an additional radiolabeled band, and the most dramatic example of this change is shown in Fig. 2C (Coomassie Blue pattern, I, compared with the autoradiogram, II). The Coomassie Blue stained sample showed two minor bands (1a and 1b) and three major bands (2, 3a, and 3b), with only the latter being radiolabeled.

The nonradiolabeled bands, 1a and 1b, yielded sequences for 16 and 12 cycles, respectively, of sequential Edman degradation consistent with the EP-GC peptide α291–306 (Table I), the same peptide identified earlier from dGDP-tubulin (15) that led us to propose α-Cys-295 as the reactive amino acid at the N site (note that α-Cys-305 is not included in the large CNBr peptide derived from α-tubulin, which should terminate at α-Met-302).

Radiolabeled band 2 yielded a sequence for 19 degradation cycles consistent with the EP-GC peptide β206–224 (Table I). The mobility of this band does not differ substantially from the analogous band shown in Fig. 2B. Because all residues were positively identified except Cys-211, this suggests that the
The radiolabeled amino acid is in fact Cys-211. If the modification had occurred at a different residue, that amino acid should have either been unidentifiable or misidentified following the Edman procedure (but see “Discussion”). The microsequencing technique does not permit specific localization of the radiolabel in the peptides purified by SDS-PAGE, because specific activity is too low.

Radiolabeled bands 3a and 3b yielded sequences for 17 degradation cycles consistent with the EP-GC peptide $b_{4–22}$ (lacking only Trp-21, which would have been destroyed during the Edman procedure, and the terminal Glu-22). These bands, also, have similar mobility to the analogous bands shown in Fig. 2B. Only Cys-12 was not identified in this sequence, consistent with its bearing the radiolabeled guanosine fragment shown directly by Shivanna et al. (7). Note that there is much less difference in the specific activities of the band 2 and band 3 EP-GC peptides than in the analogous CNBr peptides (Fig. 2A).

**TABLE I**

| Cycle | CNBr peptides | EP-GC peptides |
|-------|---------------|----------------|
|       | 1 2 3a/b      | 1a 2 3a/b      |
| 1     | Arg           | Ile            |
| 2     | Asp           | Ala            |
| 3     | Asn           | Thr            |
| 4     | Glu           | Ala            |
| 5     | Ala           | Val            |
| 6     | Ile           | Val            |
| 7     | Tyr           | Pro            |
| 8     | Asp           | Ser            |
| 9     | Ile           | Gly            |
| 10    | X (Cys-213)   | Lys            |
| 11    | Arg           | Val            |
| 12    | Arg           | Ser            |
| 13    | Asn           | Asp            |
| 14    | Leu           | Thr            |
| 15    | Asp           | Val            |
| 16    | Ile           | Val            |
| 17    | Glu           | Met            |
| 18    | Arg           | Lys            |
| 19    | Pro           | Thr            |

Radiolabeled bands 3a and 3b yielded sequences for 17 degradation cycles consistent with the EP-GC peptide $b_{4–22}$ (lacking only Trp-21, which would have been destroyed during the Edman procedure, and the terminal Glu-22). These bands, also, have similar mobility to the analogous bands shown in Fig. 2B. Only Cys-12 was not identified in this sequence, consistent with its bearing the radiolabeled guanosine fragment shown directly by Shivanna et al. (7). Note that there is much less difference in the specific activities of the band 2 and band 3 EP-GC peptides than in the analogous CNBr peptides (Fig. 2A).

**Fig. 2.** SDS-PAGE patterns obtained with peptides derived from UV irradiated [8-14C]GDP-tubulin. A, peptides obtained by CNBr digestion; B and C, peptides obtained by EP-GC digestion. Panel A, lane I shows the autoradiogram of the PVDF electrophlet obtained following electrophoresis of an aliquot of the entire CNBr digest of irradiated [8-14C]GDP-tubulin; lanes II and III show, respectively, the Coomassie Blue stained electrophlet and its autoradiogram of the peptide fraction bound to the boronate matrix (formic acid eluate). The band numbers are described in the text. Panel B, lanes I and II show, respectively, the Coomassie Blue stained electrophlet and its autoradiogram of the peptide fraction bound to the boronate matrix (formic acid eluate). Sample pH not adjusted before electrophoresis. Panel C, lanes I and II show, respectively, the Coomassie Blue stained electrophlet and its autoradiogram of the peptide fraction bound to the boronate matrix (formic acid eluate). The band numbers are described in the text. Sample pH adjusted with NaOH as described in the text before SDS-PAGE.

**Fig. 3.** Effect of dialysis against 0.05 M glycine-NaOH (pH 10) of a CNBr digest of irradiated [8-14C]GDP-tubulin. Lane I, autoradiogram of undialyzed CNBr digest; lane II, autoradiogram of the digest after 12 h of dialysis at room temperature.
Our finding of a second peptide cross-linked to radiolabeled E site nucleotide by direct photoaffinity labeling differs from the result of Shivanna et al. (7), although in our studies β-Cys-12-containing peptides were always more heavily labeled than those containing β-Cys-211 (see "Discussion"). Besides differences in reaction conditions, our tubulin contained [8-14C]GDP and theirs contained [3H]GTP in the E site. It was thus possible that the difference in results was due to a subtle conformational change in the tubulin as a consequence of the change in nucleotide. However, using CNBr digestion followed by SDS-PAGE as our assay, we could find no condition that caused significant reduction in the upper radiolabeled band (Fig. 2A, gel I).

The sole remaining possibility was that the lability of the link from the nucleotide to the Cys-211 peptide caused substantial loss of radiolabel at some point during sample processing by Shivanna et al. (7). The experiment presented in Fig. 3 demonstrates that this is a reasonable explanation, and, in addition, explains the erratic retention of radiolabel we observed. A CNBr digest was prepared and dialyzed against 0.05 M glycine-NaOH (pH 10), the application buffer for the boronate column. SDS-PAGE was performed with the original digest and with samples that had been dialyzed for different times. The radiolabel initially present in the β165–233 peptide (gel I) was reduced after 2 h and nearly completely gone after 12 h (gel II) of dialysis.

**DISCUSSION**

Fig. 4 presents a summary of the sequence data for the two regions of β-tubulin (18) described above. The cleavage sites by CNBr (CB) and EP-GC (GC) are shown above the amino acid sequences with the presumptive carboxyl cleavage sites in parentheses. Our data agree (7) that β-Cys-12 is the primary reactive amino acid with E site nucleotide when tubulin is exposed to UV light. We, however, also find significant radiolabel bound to a second region of β-tubulin. The CNBr digestion data establish that the secondary site is within the β165–233 sequence, and the EP-GC data narrow the location of the reactive residue to the β206–224 sequence. The likely specific amino acid is β-Cys-211.

Little and Luduena (13) found that the cross-link formed by N,N′-ethylenebis(iodoacetamide) in β-tubulin depleted of E site nucleotide was between β-Cys-12 and either β-Cys-201 or β-Cys-211. Nogales et al. (3) recently presented a three-dimensional structure of tubulin in zinc-induced sheets and noted that the E site GDP was located between β-Cys-12 and β-Cys-211 and that it was closer to Cys-12 than to Cys-211. This is in accord with our observation of more extensive incorporation of radiolabel in Cys-12-containing peptides than Cys-211 peptides.

However, we have not actually demonstrated that the labeled position in either the radiolabeled CNBr peptide or the radiolabeled EP-GC peptide is β-Cys-211. In the microsequencing technique, it is the sliced PVDF membrane electroblot that is placed in the sequencing apparatus, and the Edman degradation performed on the membrane fragment, and generally fewer than 5–10 pmol of amino acid derivative is recovered at even the initial position of the sequenced peptide. The radiolabel in the EP-GC peptide (β206–224) is labile, even though the peptide is clearly bound by the boronate matrix. This suggests that the cross-linked peptide undergoes some sort of "depurination" reaction, with retention of the C2′-C3′-cis-diol responsible for binding of the peptide to the boronate column but loss of the radiolabeled C-8 atom in the guanine residue.

Although the thus far uncontrollable loss of radiolabel in the EP-GC β206–224 peptide probably accounts for the difference in our results from those of Shivanna et al. (7), it limits our confidence in assigning the position of the cross-link to Cys-211. Specific identification of all residues except β-Cys-211 in the EP-GC peptide β206–224 favors Cys-211 as being the site of the cross-link, but this requires that the peptide modification causing retention of the peptide on the boronate column is stable throughout the sequential Edman degradation. The ready loss of C-8 (the radiolabel) from the EP-GC peptide is worrisome in this regard, but the cross-link to the C2′-C3′-cis-diol segment appears to be more stable chemically. Because Cys residues are destroyed during the Edman procedure, failure to identify them cannot be taken as positive evidence that they are the site of the cross-link. As a minimum, it is unlikely that the photoreactions between Cys-12 and Cys-211 proceed by the same chemical mechanism (cf. Refs. 7 and 15). Thus it remains possible that another amino acid residue in the β206–224 peptide is cross-linked to the C2′-C3′-cis-diol derived from [8-14C]GDP and that the chemical bond is hydrolyzed during sequential Edman degradation, regenerating an unaltered amino acid residue.

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