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Direct Photoaffinity Labeling of Tubulin with Guanosine 5′-Triphosphate†

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ABSTRACT: Irradiation of tubulin in the presence of [^{3}H]GTP or [^{3}H]GDP at 254 nm led to the covalent incorporation of nucleotide into the protein. The specific nature of the labeling was shown in the following manner: with tubulin depleted of exchangeable nucleotide, the amount of labeling increased to a plateau value as the [^{3}H]GTP concentration was increased, with saturation being reached at a ratio of approximately 1.5; the same amount of labeling was obtained with GTP/tubulin ratios of 1 and 100; [^{3}H]GMP was not incorporated into the dimer, nor did GMP inhibit the incorporation of [^{3}H]GTP; [^{3}H]ATP was not incorporated; [^{3}H]GTP incorporation did not occur into denatured tubulin or into serum albumin. When [α-^{32}P]GTP was used in the irradiation experiments, sodium dodecyl sulfate–polyacrylamide gel electrophoresis of the carboxymethylated protein demonstrated that the incorporated label was associated with the β subunit. The radiation treatment did cause changes in the tubulin molecule resulting in a decrease in assembly competence and in sulfhydryl groups, but these effects were minimized when a large excess of GTP was present during irradiation. Labeling of tubulin in the assembled state was much less than that observed in the free state.

The principal protein component of microtubules (MTs),[1] tubulin, is a dimer of M_r 100 000. The dimer, which consists of nonidentical monomers, α and β, contains 2 mol of guanine nucleotide, one at a readily exchangeable site and the other at a nonexchangeable site (Weisenberg et al., 1968). Upon polymerization of tubulin to form MTs, the exchangeable nucleotide is hydrolyzed to GDP (Kobayashi, 1975; Weisenberg et al., 1976), although under some conditions hydrolysis is not coincident with assembly (Carlier & Pantaloni, 1981). In addition, assembly can occur in the presence of nonhydrolyzable GTP analogues (Weisenberg et al., 1976; Arai & Kaziro, 1976). At the present time the role GTP plays in the polymerization process is unclear.

To localize and characterize the exchangeable GTP site, use has been made of photoaffinity analogues of GTP, such as 8-azido-GTP (Geahlen & Haley, 1979) and 3′-(*p*-azidobenzoyl)-GTP (Maccioni & Seeds, 1983) as well as the periodate oxidation product of GTP 2-[(guanylformyl)methoxy]-3,3,3-triphosphopropanal (Maccioni & Seeds, 1983; Kirsch & Yarbrough, 1981). Geahlen & Haley (1979) concluded that the 8-azido-GTP binds to the β subunit, although significant nonspecific binding to the α subunit also occurred. More recently, Haley et al. (1983) reported that the α subunit was labeled exclusively by this photoaffinity analogue. Maccioni & Seeds (1983) found that their analogues bound equally well to the α and β subunits but the binding was

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[1] Abbreviations: Mes, 2-(*N*-morpholino)ethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; Pipes, 1,4-piperazinediethanesulfonic acid; MTs, microtubules; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); DTT, dithiothreitol; Me_2SO, dimethyl sulfoxide; PED buffer, 100 mM Pipes, 1 mM EGTA, and 1 mM DTT, pH 6.9; PEMD buffer, PED buffer containing 1 mM $MgSO_4$; PEI, poly(ethylenimine).

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saturable and competitive with GTP, indicating that it was due to a specific interaction at the exchangeable GTP site. On the other hand, Kirsch & Yarbrough (1981) concluded that labeling with the periodate-oxidized GTP was nonspecific on the basis that they found no competition with GTP. Although the analogues described above can be useful for identification of the amino acid sequence at the exchangeable GTP binding site, their use has lead to conflicting results.

Direct photoaffinity labeling has the advantage of covalently cross-linking the natural substrate or ligand to its binding site in proteins by means of irradiation (Yue & Schimmel, 1977; Carroll et al., 1980; Caras et al., 1983; Goldman et al., 1983; Muhn & Hucho, 1983). With this method proteins are cross-linked to their natural ligands under the direct action of ultraviolet light, without the introduction of affinity labels on either of the reactants. In this paper we report the successful covalent cross-linking of GTP and GDP to tubulin by the action of ultraviolet light and describe studies on some of the characteristics of this interaction. The ultimate aim of this work is to identify the exchangeable nucleotide binding site.

Experimental Procedures

Materials. GTP, EGTA, Mes, sodium dodecyl sulfate, Tris, and DTT were products of Sigma Chemical Co. Pipes was obtained from Research Organics Inc. 5,5′-Dithiobis(2-nitrobenzoic acid) was purchased from Pierce Chemical Co. [^{3}H]GTP, [^{3}H]GDP, and [α-^{32}P]GTP were purchased from ICN Pharmaceuticals Inc., and [^{3}H]GMP was from Moravek Biochemicals Inc. Iodoacetic acid was from Eastman Organic Chemicals. Acrylaide and Gel bond for polyacrylamide gel electrophoresis were purchased from FMC Corp. All other reagents were of analytical grades.

Preparation of Tubulin. Bovine brain tubulin was prepared by the assembly–disassembly procedure of Shelanski et al. (1973). After two cycles of assembly–disassembly, the protein was suspended in a buffer containing 100 mM Mes, 0.5 mM $MgSO_4$, 1 mM EGTA, and 2 M glycerol, pH 6.5, at a protein concentration of 10–15 mg/mL and dialyzed against the same buffer for 4 h at 0 °C. The solution was then added dropwise to liquid nitrogen (Detrich & Williams, 1978) and the resulting pellets were stored at −70 °C. Further purification was done to obtain the tubulin dimer. Protein solution pellets were thawed, and an equal volume of 0.8 M Pipes–20% Me_2SO, pH 6.9, was added. The protein was then polymerized at 37 °C in the presence of 0.5 mM GTP. This procedure results in tubulin largely depleted of associated proteins (Himes et al., 1977). The remaining associated proteins and buffer components were removed by passing the polymerized protein through a 10 × 1.2 cm column of phosphocellulose (Whatman P-11) that was placed on top of a Bio-Gel P-10 column (25 × 1.2 cm). The column had been washed and equilibrated with PED buffer (100 mM Pipes, pH 6.9, containing 1 mM EGTA and 1 mM DTT), and the same buffer was used to elute tubulin. In some cases, 1 mM $MgSO_4$ was included in the buffer (PEMD buffer). The fractions containing tubulin were pooled, concentrated, frozen dropwise in liquid nitrogen, and stored at −70 °C.

Tubulin depleted of nucleotide at the exchangeable site was prepared by charcoal treatment. Tubulin (5 mg/mL) in PEMD buffer was treated for 20 min at 5 °C with a 10-fold (w/w) excess of bovine serum albumin saturated Norit charcoal (Sandoval et al., 1977). In some cases, a second treatment was performed. Charcoal was removed by two centrifugations at 40000g, and protein and bound nucleotide contents were determined in the supernatant. In several experiments, the amount of nucleotide remaining at the exchangeable site after charcoal treatment ranged from 0 to 0.3; i.e., total bound nucleotide released by $HClO_4$ was 1.0–1.3.

UV-Induced Cross-Linking. In a typical experiment, 0.5 mL of solution containing tubulin and radiolabeled nucleotide in a 4.3 × 4.3 × 1 cm plastic weighing boat (Fisher Scientific) was placed on ice. Irradiation was performed by exposing the solution from a distance of 6 cm to short-wavelength radiation from a RPR 253.7-nm lamp equipped with a 2 mm thick Vycor filter, at a dosage of 600–700 $\mu W/cm^2$ as measured with a Blak-Ray J-225 ultraviolet intensity meter. UV irradiation at 37 °C was performed in an incubator. The solution was placed in a plastic dish inside a Vycor tubing closed at both ends to prevent evaporation. The degree of covalent labeling was determined from the amount of radioactive nucleotide found in the protein after precipitation with 10% $HClO_4$. The precipitated protein was washed several times with 5% $HClO_4$ and H_2O until radioactivity was no longer found in the supernatant. The final precipitate was dissolved in 0.1 N NaOH, and the protein and radioactivity contents were determined. Irradiation did not affect the spectral characteristics of GTP or its mobility on PEI–cellulose TLC plates.

Assembly Reactions. Assembly reactions were carried out in the presence of 10% Me_2SO at 37 °C in PEMD buffer containing GTP as indicated. The reaction was followed by measuring the apparent absorbance at 350 nm as a function of time.

Protein, Nucleotide, and Sulfhydryl Determinations. Protein concentrations were determined by the method of Bradford (1976) or from the A_{275} value with a ϵ value of 1.13 mg^{-1} mL cm^{-1}. Noncovalently bound nucleotide was determined by precipitating the protein with 10% $HClO_4$ and measuring the absorbance of the supernatant at 254 nm with a molar extinction coefficient of 12 400 M^{-1} cm^{-1}. The free sulfhydryl content of tubulin was estimated with 5,5′-dithiobis(2-nitrobenzoic acid) (Ellman, 1959) by using a molar extinction coefficient of 13 600 M^{-1} cm^{-1} at 412 nm.

Electron Microscopy. Samples were negatively stained on carbon-coated grids with 2% uranyl acetate and viewed in a Philips 300 electron microscope.

SDS–Polyacrylamide Gel Electrophoresis. After covalent incorporation of [α-^{32}P]GTP into tubulin, excess unbound GTP was removed by 10% $HClO_4$ precipitation followed by several 5% $HClO_4$ and H_2O washes of the pellet. The pellet was dissolved in a small known volume of 100 mM Tris-HCl buffer, pH 8.0, containing 6 M guanidine hydrochloride, and carboxymethylated under reducing conditions as described by Allen (1981). The solution was then dialyzed overnight against 100 mM NH_4HCO_3 at 5 °C. SDS electrophoresis on slab gels was performed according to a modified procedure of Laemmli (1970) where an acrylamide–acrylaide stock solution (30%:1%) was used and the gel was cast against a polyacrylamide FMC Corp. Gel bond to provide easy handling of the polymerized gel. The subunits were separated with 7.5% acrylamide in the separating gel and 3% in the stacking gel. The gels were stained with Coomassie blue and dried by standard procedures. For autoradiography after electrophoresis, the dried gel was exposed for 3 days to a preflashed Kodak XRP5 X-ray film in a casette with intensifying screens, type "regular". To determine the distribution of label on the autoradiograph, scans were made of the negatives on a ISCO Model VA-5 monitor with an attached scanner Model 1310 set at 580 nm. In a few experiments, the distribution of label was determined by removing the stained gel slices containing the α and β subunits and digesting them in a solution containing 30% H_2O_2 and

Table I: Covalent Labeling of Tubulin with [^{3}H]GTP[a]

irradiation time (min)	mol of [^{3}H]GTP/mol of tubulin	
	b	*c*
0	0	0
10	0.05	0.08
30	0.20	0.33
45	0.22	0.37

[a] Tubulin (5 mg/mL) in PED buffer was incubated with a 100 M excess of [^{3}H]GTP (5000 cpm/nmol). After incubation in ice for 60 min, the mixture was placed in a plastic weighing boat on ice and irradiated as described under Experimental Procedures. Aliquots were removed at the times shown, and the amount of covalently bound GTP was determined. [b] The amount of covalently bound GTP was based on total tubulin present. [c] The calculations took into account the fact that the tubulin contained 0.6 mol of [^{3}H]GTP/mol of tubulin.

Table II: Specificity of Labeling[a]

protein	labeled nucleotide	incorporation (mol/mol of tubulin)
2.3 μM tubulin	0.23 mM GTP	0.19
4.5 μM tubulin	0.45 mM GTP	0.20
45 μM tubulin	45 μM GTP[b]	0.20
45 μM tubulin	4.5 mM GTP	0.20
45 μM tubulin	4.5 mM ATP	0.01
45 μM tubulin	4.5 mM GDP	0.19
22.5 μM tubulin	22.5 μM GTP[b] + 225 μM GMP (unlabeled)	0.20
22.5 μM tubulin	22.5 μM GTP[b] + 2250 μM GMP (unlabeled)	0.16
45 μM tubulin	450 μM GTP + 4500 μM GMP (unlabeled)	0.22
45 μM tubulin	4500 μM GMP + 45 μM GTP (unlabeled)[b]	0.01
40 μM tubulin, urea denatured	4.5 mM GTP	0.01
45 μM serum albumin	4.5 mM GTP	0.02

[a] Photo-induced cross-linking was performed as described under Table I. [b] No excess GTP was added. The amount shown refers to the amount present in the exchangeable site.

70% $HClO_4$ (9:15 v/v) at 65 °C for 6 h (Czarnecki et al., 1982) before counting.

Results

UV-Induced Cross-Linking of [^{3}H]GTP to Tubulin. The solution conditions for the cross-linking experiments include the presence of 1 mM DTT and a large excess of GTP. The presence of the DTT and GTP retard denaturation of the protein by the radiation as will be described below. Irradiation as a function of time led to an apparent maximum incorporation of 0.2 mol of GTP/mol of dimer in 30 min (Table I). The same molar ratio was incorporated over a 20-fold protein concentration range (see Table II). In separate experiments gel filtration was used to determine the amount of [^{3}H]GTP that actually had exchanged into tubulin used for the cross-linking experiments. The average value was 0.6 for a number of experiments. Using this figure, 33% of the [^{3}H]GTP that exchanged into the dimer became covalently incorporated upon irradiation (Table I). Increasing the time of irradiation beyond 30 min did not result in significantly greater amounts of covalent labeling but did result in the formation of cross-linked protein aggregates and cleaved protein products having molecular masses of less than 55 kDa, as determined by SDS–PAGE (not shown). The conclusion that the labeling was covalent is based on the stability of the label to $HClO_4$ precipitation, urea denaturation, and boiling in SDS.

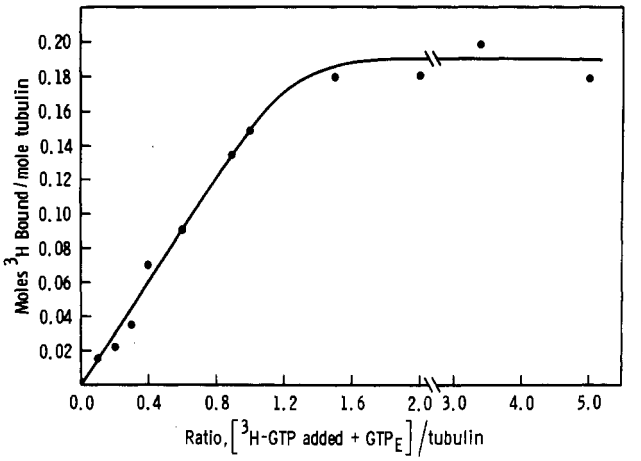

FIGURE 1: Photolabeling of charcoal-treated tubulin as a function of increasing [^{3}H]GTP concentration. A 2 mg/mL solution of charcoal-treated tubulin in PEMD buffer was incubated for 30 min at 5 °C with increasing amounts of [^{3}H]GTP (400–550 cpm/pmol) to yield varying ratios of GTP (remaining E site nucleotide + added GTP) to tubulin. Irradiation and the determination of the amount of covalently bound ^{3}H per mole of tubulin were done as described under Experimental Procedures. The specific activity of [^{3}H]GTP used was corrected for the residual E site GTP present in tubulin. The results are the average values of four separate experiments.

To determine whether the covalent labeling was due to a specific interaction of GTP with tubulin, several experiments were done. Nonspecific labeling results in a nonsaturable incorporation of label. When we examined the dependence of the amount of labeling on GTP concentration, we found that it was independent of the amount of excess GTP present (Table II). Although the exchangeable site nucleotide does exchange with nucleotide in the medium, it is tightly bound and remains bound after gel filtration. When we removed excess GTP by gel filtration before irradiation, we observed the same amount of cross-linking as that obtained when a 100-fold excess of GTP was present. If nonspecific labeling were occurring, it would be expected to increase with the addition of a 100-fold excess of GTP. Although this experiment strongly suggests the absence of nonspecific labeling, the lowest concentration of GTP present was equivalent to the amount of tubulin present since the exchangeable nucleotide is not removed by gel filtration. To determine whether the amount of labeling approaches a constant value as the binding site becomes saturated, nucleotide was removed from the exchangeable site by charcoal treatment and UV irradiation was done in the presence of increasing concentrations of [^{3}H]GTP. The results in Figure 1 show that covalent incorporation increased with increasing nucleotide concentration and a plateau value was reached at a GTP to tubulin ratio of about 1.2–1.5. Saturation would not be expected if nonspecific labeling were responsible for the incorporation of isotope.

The results of experiments with GMP also argue against nonspecific labeling (Table II). (GMP does not bind strongly to the exchangeable site.) If the labeling were caused by a UV-induced formation of an amino acid residue free radical not at the GTP binding site, which then reacts with the guanine nucleotide, GMP should produce the same amount of labeling as GTP. However, a 100-fold excess of [^{3}H]GMP over tubulin did not result in significant incorporation of label. Nor did GMP inhibit the cross-linking of [^{3}H]GTP. Finally, specificity was demonstrated by the lack of incporation of ATP into tubulin and the lack of GTP incorporation into serum albumin and urea-denatured tubulin (Table II). Photo-induced cross-linking with [^{3}H]GDP produced the same amount of

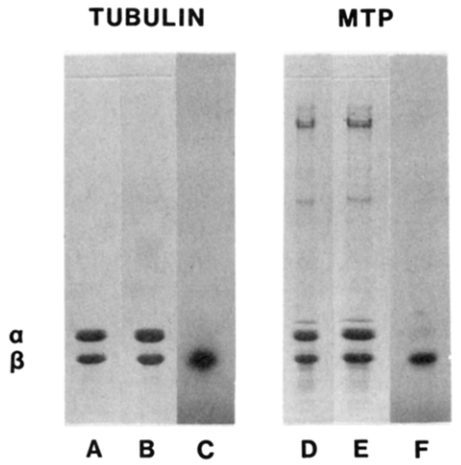

FIGURE 2: SDS–polyacrylamide gel electrophoresis and autoradiography of carboxymethylated tubulin–[α-^{32}P]GTP after irradiation. Tubulin and twice-cycled microtubule protein were UV irradiated in the presence of [α-^{32}P]GTP and prepared for SDS–PAGE and autoradiography as described under Experimental Procedures. (A and D) Coomassie-stained sample without UV irradiation; (B and E) Coomassie-stained sample after 30-min UV irradiation; (C and F) autoradiograph of 30-min UV-irradiated samples.

nucleotide incorporation as obtained with [^{3}H]GTP. This is expected since GDP and GTP bind with similar affinities (Zeeberg & Caplow, 1979).

After irradiation for 30 min in the presence of a 100-fold excess of [^{3}H]GTP, the protein was assembled in the presence of 10% Me_2SO and centrifuged, and the amount of covalent labeling was determined. Before assembly, it was determined that 0.21 mol of nucleotide was incorporated per dimer. After assembly, the MTs contained only 0.03 mol of label per dimer while in the unassembled portion this figure was 0.16. It is concluded, therefore, that tubulin which contains the cross-linked nucleotide is not capable of self-assembly.

Cross-Linking Occurs in the β Subunit. To localize the site of cross-linking, tubulin was irradiated in the presence of [α-^{32}P]GTP followed by SDS–PAGE of the carboxymethylated protein and by autoradiography (Figure 2). Clearly, the β subunit contains all but a trace of the label. Incubation of tubulin with [α-^{32}P]GTP under similar conditions without irradiation followed by SDS–PAGE and autoradiography revealed no incorporation of the label. Densitometric scans of the autoradiographs and digestion of gel slices showed that at least 95% of the label was in the β subunit. The results of experiments involving irradiation of a microtubule protein preparation are also shown in Figure 2. The presence of microtubule-associated proteins in such preparations does not change the labeling pattern.

Effect of UV-Induced Cross-Linking on GTP Exchange. To further support the conclusion that the cross-linking was indeed occurring at the exchangeable nucleotide binding site, we determined the effect of prior irradiation of tubulin, containing unlabeled GTP, on the subsequent exchange with [^{3}H]GTP. Tubulin was irradiated in the absence of excess GTP and in the presence of a 100 M excess of GTP for 30 min followed by incubation with [^{3}H]GTP and gel filtration. In the absence of excess GTP, the irradiation treatment caused a decrease in the amount of GTP that could be exchanged from 0.6 to 0.19 mol/mol of tubulin (68% reduction). When excess GTP was present during the irradiation, however, the value decreased from 0.57 to 0.37 (35%).

Effect of Irradiation on GTP Content of Tubulin. Treatment with UV radiation can cause structural alterations in the protein as well as nucleotide cross-linking. Major alterations

Table III: Loss of Bound Nucleotide after Irradiation[a]

irradiation time (min)	GTP added (mM)	nucleotide content (mol/mol of tubulin) protein fraction[b]	nucleotide fraction[c]
0	0	2.01	0
30	0	1.27	0.45
0	4.5	1.97	ND[d]
30	4.5	1.79	ND

[a] Tubulin (5 mg/mL) in PED buffer received either no or 30-min irradiation, and 0.45 mL was passed through a Bio-Gel P-6 DG column (1.2 × 12 cm). The fractions containing protein and excess of nucleotide, determined from absorbance measurements, were pooled. [b] The protein was precipitated by adding an equal volume of 20% $HClO_4$ and the nucleotide content of the supernatant determined from the A_{254} value. [c] The fractions containing nucleotide were lyophilized and dissolved in 1 mL of H_2O, and the A_{252} value was determined. A correction was made for buffer that had been treated in the same manner because DTT contributes to the absorbance. The values listed are the total nucleotide in this fraction divided by the amount of tubulin placed on the column. [d] ND, not determined. The presence of a large excess of nucleotide during the irradiation obviated this determination.

Table IV: Effect of Irradiation on the Free Sulfhydryl Content of Tubulin[a]

buffer additions	mol of SH/mol of tubulin 0 min	10 min	30 min
none	19.6	17.5	12.0
1 mM DTT	20.4	19.6	18.1
1 mM DTT and 4.5 mM GTP	19.5	19.2	19.2

[a] Tubulin at 5 mg/mL in 100 mM Pipes, pH 6.9, containing 1 mM EGTA was irradiated for the times shown; then, an aliquot was withdrawn and passed through a Bio-Gel P-6DG desalting column (1.2 × 12 cm). The protein fractions were pooled, and the free sulfhydryl content was determined as described under Experimental Procedures.

were evident by SDS–PAGE after periods of irradiation for more than 30 min as described above. To examine for conformational changes that could result in the release of bound nucleotide from the exchangeable or nonexchangeable sites, we measured the nucleotide content after irradiation. The experiment was done under two conditions, in the absence and presence of excess GTP during the irradiation (Table III). After irradiation, the protein was separated from unbound nucleotide by gel filtration and examined for bound-nucleotide content. When excess GTP was present, the decrease in nucleotide released upon addition of $HClO_4$ (0.18 mol/mol of tubulin) can be accounted for solely by the amount of cross-linking that occurred. When excess GTP was not present, the decrease in nucleotide released by $HClO_4$ was much larger. This was due to the fact that 0.45 mol of nucleotide/mol of tubulin dissociated from the dimer during irradiation, probably as a result of structural changes in the protein.

Effect of Irradiation on Tubulin Sulfhydryl Content and Assembly Competence. One way by which radiation can bring about structural changes in the tubulin dimer is by causing oxidation of sulfhydryl groups. Indeed, it has been shown that tubulin sulfhydryl groups are oxidized as a result of ionizing radiation (Zaremba & Irwin, 1981) and ultraviolet radiation (Zaremba et al., 1984). We examined the irradiation under different solution conditions: in the absence of added DTT or GTP, in the presence of DTT, and in the presence of DTT and GTP (Table IV). From the results, it is clear that tubulin sulfhydryls are sensitive to the irradiation treatment; a loss of approximately 40% of the sulfhydryls occurred within 30 min in the absence of DTT and GTP. DTT significantly protected the sulfhydryls, reducing the loss to 10% in the same time period. In the presence of both DTT and a 100-fold

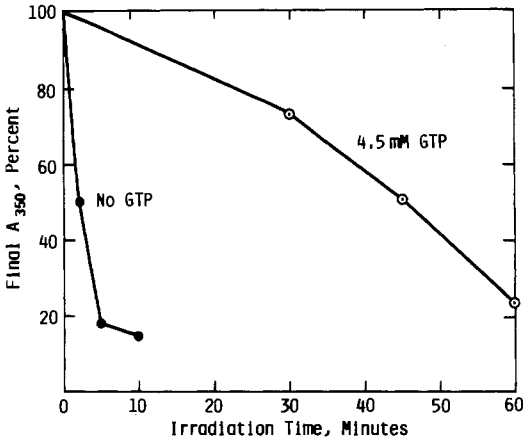

FIGURE 3: Effect of UV irradiation on the self-assembly of tubulin. Tubulin (45 μM) in PEMD buffer containing either no added GTP or 4.5 mM GTP was irradiated as described under Experimental Procedures for different periods of time. Aliquots were taken and assembly was performed at 1.5 mg/mL tubulin concentration in 10% Me_2SO at 37 °C. The final concentrations of GTP during assembly were 0.5 mM for the sample irradiated in the absence of GTP and 2.0 mM for the sample irradiated in the presence of GTP. The maximum A_{350} values for each irradiated aliquot are expressed as a percentage of the value of the control (no irradiation).

excess of GTP, no detectable sulfhydryl loss occurred. The latter conditions are those chosen for most of the labeling studies conducted in this work.

Alterations in tubulin structure and sulfhydryl loss would be expected to result in decreased assembly competence of the protein. We measured assembly in 10% Me_2SO after irradiation, and the results are presented in Figure 3. Electron microscopy showed that MTs were the only polymerized product present, and the absorbance decreased to zero upon cooling the sample on ice, indicating the absence of nonspecific aggregated products. In the absence of a large excess of GTP, almost complete inhibition of assembly occurred within 5 min. However, the rate of inactivation was greatly reduced when 4.5 mM GTP was present.

Labeling of Preformed Microtubules. It is of interest to know whether labeling of the tubulin dimer differs when it is incorporated into the wall of a MT. To examine this possibility, we irradiated a sample of tubulin that had been preassembled at 37 °C in the presence of 10% Me_2SO. In other experiments, it was demonstrated that 10% Me_2SO did not affect the amount of labeling at 4 °C. The MTs were separated from the dimer by centrifugation, and the amount of covalently bound nucleotide in each fraction was determined. The content of cross-linked nucleotide in free tubulin (0.07 mol) was 7 times greater than that in polymerized tubulin (0.01). The lower incorporation of label into the dimer than that found in the previous experiments (Tables I and II) was probably due to some denaturation of the protein at 37 °C. In separate experiments, labeling of the dimer in the absence of Me_2SO (nonassembling conditions) was also found to be lower than that at 0 °C. Moreover, in the absence of radiation, some loss in assembly competence was observed after prolonged incubation at 37 °C, indicating denaturation of the protein.

Discussion

The advantage of the direct photoaffinity labeling method over the use of photoaffinity or other chemically reactive analogues is that the natural ligand is cross-linked to the protein. This increases the probability that the interaction with the protein is at the ligand binding site and is therefore specific and reduces the probability of conformational changes at the binding site, which the protein may undergo to accommodate a modified ligand. Our results indicate that the covalent incorporation of nucleotide was highly specific; For example, when tubulin depleted of the exchangeable nucleotide was used with low and variable concentrations of added GTP, a saturation curve was obtained (Figure 2). The ratio of GTP to tubulin that produced saturation is the ratio expected considering the reported dissociation constant of Tu–GTP of 22 nM (Zeeberg & Caplow, 1979). Even the presence of a 100-fold excess of nucleotide over the tubulin–nucleotide complex did not increase the amount of label incorporated, and essentially all of the label was found in the β subunit. The absence of labeling by GMP and ATP is further support of the specific nature of the labeling. With the use of analogues, significant incorporation also occurred in the α subunit (Geahlen & Haley, 1979; Maccioni & Seeds, 1983; Kirsch & Yarbrough, 1981). Results with 8-azido-GTP first supported the conclusion that labeling of the α subunit was of a nonspecific nature and labeling of the β subunit was due to an interaction at the exchangeable nucleotide site (Geahlen & Haley, 1979), but later results demonstrated labeling exclusively in the α subunit (Haley et al., 1983). In the first case, tubulin containing microtubule-associated proteins was used, and in the second case, tubulin free of these proteins was used.

The conditions we chose for the irradiation treatment minimized damage to the protein. When tubulin in the absence of added nucleotide was irradiated, the assembly competence was almost completely destroyed within 5 min, reduction in free sulfhydryl content occurred, there was a 68% decrease in the quantity of GTP that could exchange into the exchangeable site, and there was a loss in nucleotide from the exchangeable site. The presence of a large excess of GTP prevented the loss in sulfhydryl groups and nucleotide and decreased the loss in assembly competence and GTP exchange activity. This effect is probably due to the absorbance of harmful radiation by the nucleotide solutions. Under this condition, approximately 0.2 mol of nucleotide became incorporated into the protein, determined by measuring either incorporation of label or decrease in the amount of nucleotide released by $HClO_4$ precipitation. Moreover, the amount of nucleotide that could subsequently exchange into tubulin was decreased by the same amount. The fact that approximately the same amount of cross-linking is achieved when measured by incorporation of labeled nucleotide (exchangeable site) or by reduction in nucleotide released on treatment with $HClO_4$ (exchangeable and nonexchangeable sites) indicates that the nucleotide at the nonexchangeable site is not being covalently incorporated into the protein. This may mean that the conformations around the two sites are not identical.

Prior to irradiation, [^{3}H]GTP was exchanged into the exchangeable site. After exchange, the dimer contained 0.6 mol of [^{3}H]GTP. Why it is not possible to exchange in one nucleotide per tubulin dimer is not clear, but other investigators have also noted this fact (Correia & Williams, 1983). One possibility is that two populations of dimers exist in solution, either different states of the dimer (conformational or chemical) or a mixture of dimer and aggregates. On the basis of the amount of covalent incorporation that occurred, it can be proposed that nucleotides at 40% of the exchangeable sites (which do not exchange with nucleotide in the medium) do not become covalently incorporated as a result of irradiation treatment. If this were not so, the amount calculated from that not released by $HClO_4$ would be higher than that cal-

culated from label incorporation. An assumption that one-third of the GTP that exchanged into the dimer became covalently incorporated can explain the data. The fact that all of the GTP could not eventually be incorporated is probably due to structural changes in the protein that occur during irradiation. This was indicated by the continued loss of assembly competence over a 60-min irradiation period (Figure 3). It has been noted (Merrill et al., 1984) that the efficiency of cross-linking by direct photoaffinity labeling is usually less than 25%. It is interesting that labeling of tubulin assembled into MTs was very much reduced (essentially zero), suggesting that the exchangeable nucleotide site in the MT is somehow different from that in the free dimer and may be more like the nonexchangeable site, which apparently did not become cross-linked in the dimer.

Zabrecky & Cole (1982) have demonstrated that ATP binds weakly to tubulin and by using 8-azido-ATP (Zabrecky & Cole, 1983) showed that both the α and β subunits become labeled with the photoaffinity analogue. It is unclear from their reported results what the labeling stoichiometry was, but we did not find evidence of labeling by [^{3}H]ATP (Table II) with the direct photoaffinity labeling technique. This may reflect differences in the two photochemical processes.

Although the direct photoaffinity labeling technique has been used to covalently link nucleotides to a number of proteins and nucleic acids to proteins (Smith, 1976), very little is known about the photochemistry of these reactions and the nature of the adducts formed (Shetlar, 1980). Indirect evidence from photo-induced reactions of purines with alcohols (Steinmaus et al., 1971) suggests that threonine and serine may be able to form adducts with guanine at the 8-position. In the case of actual cross-linking studies, it has been suggested that a glycine is involved in the labeling of Ile-tRNA synthetase with ATP (Yu & Schimmel, 1977), an isoleucine residue of ribonuclease A is cross-linked to pUp (Havron & Sperling, 1977), glycine, alanine, and tyrosine residues are involved in cross-linking DNA to alcohol dehydrogenase and salmine (Toth & Dose, 1976), and a phenylalanine residue participates in the cross-linking of single-stranded DNA to DNA binding proteins (Merrill, 1984). Because the amino acid sequence of tubulin is known, it should be possible to determine what residue(s) in tubulin reacts with GTP. Our studies indicate that the direct photoaffinity labeling technique should be useful for the determination of the amino acid sequence around the exchangeable nucleotide site.

Registry No. GTP, 86-01-1; GDP, 146-91-8.

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