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AFFINITY LABELING OF THE EXCHANGEABLE NUCLEOTIDE BINDING SITE IN PLATELET TUBULIN

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SUMMARY: The exchangeable nucleotide binding site of platelet tubulin was labeled with [^{14}C]p-fluorosulfonyl benzoylguanosine (FSBG). FSBG promoted polymerization of tubulin but depolymerization did not occur in the presence of this nucleoside analogue. GTP was able to block FSBG binding to tubulin. [^{14}C]Iodoacetamide-treated tubulin which was first reacted with FSBG was digested with trypsin. The resultant peptides were analyzed by reverse phase high pressure liquid chromatography. One FSBG-labeled peptide could be identified both by its radioactivity and the characteristic UV absorbance spectrum associated with it. This may represent the exchangeable nucleotide site. A second peptide with a distinct nucleotide absorbance peak was found both in FSBG-treated and untreated tubulin preparations. This evidence is suggestive of the non-exchangeable nucleotide binding site.

The role of nucleotides in the polymerization of microtubule protein and the stabilization of tubulin continues to be a subject of great interest. While there remain questions on the exact function of the two molecules of guanine nucleotides associated with the tubulin heterodimer, there is no uncertainty about the exchangeability of the nucleotide at one of the binding sites and the unexchangeable ligation of GTP at the second (1). Identification of the nucleotide binding sites would be an important step in clarifying their function in microtubules. Studies of this nature require nucleotide analogues that preferable bind covalently to their binding sites on tubulin. A growing number of such compounds are available (2,3) some of which are photoactivatable while others are irreversibly bound through electrophilic interaction. Fluorosulfonyl benzoyl guanosine (FSBG) is a compound that utilized the latter mechanism

Abbreviations: FSBG, p-fluorosulfonyl benzoylguanosine; PIPES, piperazine-N,N'-bis (2-ethane sulfonic acid).

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to bind to tubulin. [^{14}C]labeled FSBG was prepared and then used to study the nucleotide binding site. This compound as several other guanine nucleotide analogues supports polymerization of tubulin. In addition, FSBG has an absorption spectrum which is quite different from that of peptides and thus facilitates its measurement and detection.

I have used this analogue, whose binding could be inhibited by GTP, to identify the exchangeable nucleotide binding site on platelet tubulin. This site could be localized to a specific tryptic peptide which was separated by high performance liquid chromatography. In addition, a second nucleotide absorbance peak was seen both in FSBG-treated and untreated tubulin which may represent the nonexchangeable nucleotide binding site.

MATERIALS AND METHODS

Synthesis of p-Fluorosulfonyl [^{14}C]Benzoyl-5-Guanosine

The method of Esch and Allison (4) was followed for the synthesis of [^{14}C]p-fluorosulfonyl benzoyl chloride starting with carboxy-p-amino [^{14}C]benzoic acid. The latter was obtained fron ICN; Irvine, CA (spec. act. 56 mCi/mmol). The guanosine ester of p-fluorosulfonyl benzoic acid was prepared essentially according to the method described by Pal et al. (3) using guanosine instead of adenosine. All reaction products were checked by thin layer chromatography. FSBG had R_fs of 0.13 and 0.25 when the silica gel TLC plates were developed with isopropyl ether:acetone: H_2O (65:20:15). Radiochromatograph scanning revealed high radioactivity in both bands. After elution with methanol identical UV absorption spectra were obtained. Maxima were found at 253 and 275 nm. For these studies I used the band with R_f 0.13 as it could be shown to be 5'-p-fluorosulfonyl benzoyl guanosine. The other band was a structural isomer.

Preparation of Platelet Tubulin

Microtubule protein was isolated from human platelets according to a previously published method (5) using temperature-dependent polymerization and depolymerization. Two cycle microtubule protein was depolymerized at 4 ° C and then freed of exchangeable nucleotide by the method of Maccioni et al. (6). It was then repolymerized in the presence of FSBG. The protein was dialyzed exhaustively against 0.1 M piperazine-N, N'-bis (2-ethane sulfonic acid) (PIPES) buffer, pH 6.9 containing 2 mM $MgSO_4$ and 8 mM EDTA. Protein concentration was determined by the method of Lowry et al. (7) using bovine serum albumin as standard.

Isolation of Covalently Modified Peptide

Platelet microtubule protein isolated by 3 cycles of temperature-dependent polymerization-depolymerization was dialyzed under an atmosphere of nitrogen against several changes of 8 M urea in 0.35 M Tris/HCl containing 3 mM EDTA, pH 8.5 and 0.12 M mercaptoethanol. After concentrating tubulin to approximately 2-3 mg/ml by ultrafiltration,

the protein solution was treated with 6.25 μCi [^{14}C]iodoacetamide per ml (spec. act. 23.6 μCi/mmol) and nonradioactive iodoacetamide whose concentration was adjusted to 2.7 mM. After 60 min at 25 ℃ the reaction was stopped by dialysis against distilled water. This solution was lyophilized and then redissolved in distilled H_2O (1 ml) at pH 8.8. To this solution was added ammonium bicarbonate at a final concentration of 25 mM and tosylphenylalanyl chloromethyl ketone treated trypsin (10 μg/ml microtubule protein. After proteolytic digestion for 4 hr at 37 ℃, the pH of the mixture was decreased to 2 and the mixture lyophilized. The residue was dissolved in 0.01 M ammonium acetate, pH 6.07, passed through a 0.2 μ Nylon filter (Rainin Instrument Co., Woburn, MA) and an aliquot corresponding to 4-6 nmols of tubulin injected into a Bondapak C18 (Waters Associates, Inc. Milford, MA) high pressure liquid chromatograph column. The peptides were eluted by the following program: 1) 0.01 M ammonium acetate, pH 6.07 (buffer A); 2) 40% acetonitrile in 0.01 M ammonium acetate buffer, pH 6.07 (buffer B). Starting with 100% buffer A, the program proceeds to 50% buffer B in 50 min, then to 100% buffer B in another 80 min. The optical absorbance was measured at 220 nm and individual peaks were scanned from 210 - 320 nm. Major peaks were collected directly into scintillation vials to which Aquasol (New England Nuclear, Boston, MA) was added and then counted. Contamination by microtubule associated proteins (MAPs) was determined by sodium docecyl sulfate (SDS) polyacrylamide gel electrophoresis. Quantitative evaluation of the amount of contaminants by densitometric scanning of the Coomassie blue stained gels showed that 3 cycle tubulin contained $\leq$ 15% MAPs.

The modified peptide was identified by scanning the optical absorbance in the wavelength range from 210-320 nm as the peak eluted from the HPLC column.

Measurement of Tubulin Assembly

Microtubule protein dissolved in PIPES buffer, pH 6.9 containing 2 mM $MgSO_4$ and 8 mM EGTA at a concentration of $\geq$ 1 mg/ml was polymerized at 37 ℃ either with 1 mM GTP or 1 mM FSBG in the medium. Reassembly was monitored by measuring the change in optical absorbance at 580 nm which was recorded as a function of time. Disassembly of microtubules was evaluated by measuring the reversibility of the optical changes on cooling the solutions to 4 ℃.

Polyacrylamide Gel Electrophoresis

To separate α- and β-tubulin SDS-urea polyacrylamide gel electrophoresis was utilized essentially as described by Eipper (8). Regular disc gel electrophoresis was performed on 7.5% polyacrylamide gels (5).

RESULTS

[^{14}C]FSBG was able to bind to tubulin. The β-subunit was the primary site of attachment as demonstrated by SDS-urea polyacrylamide gel electrophoresis (Fig. 1). After exhaustive dialysis of tubulin against 1-1.5 mM FSBG the molar ratio of the analogue was 0.62-0.68 for the β -subunit. At this ratio tubulin appeared to be saturated. The α-tubulin band isolated by gel electrophoresis showed a molar ratio of 0.14. GTP in concentrations ranging from 0.2 to 1.0 mM

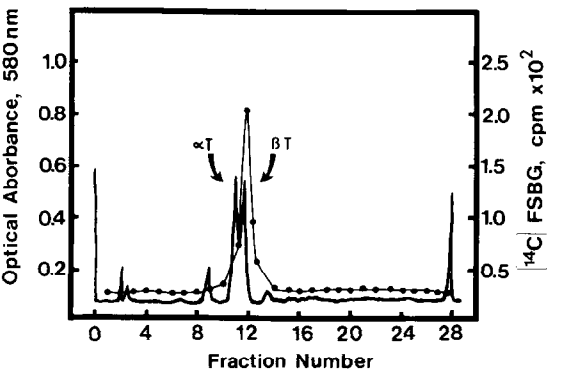

Figure 1. SDS-urea polyacrylamide gel electrophoresis of platelets microtubule protein treated with [^{14}C]FSBG. The Coomassie blue stained gel was scanned at 580 nm (solid line), dried, sliced and counted for [^{14}C]activity (dots).

effectively inhibited the binding of equimolar FSBG by tubulin. In this concentration range incorporation of FSBG was reduced by 74-81%.

Assembly and disassembly of FSBG-treated tubulin was tested (Fig. 2). FSBG-tubulin polymerized without delay and produced the same maximal deviation from the baseline as normal GTP-tubulin. FSBG-tubulin was unable to depolymerize when exposed to 4 °C.

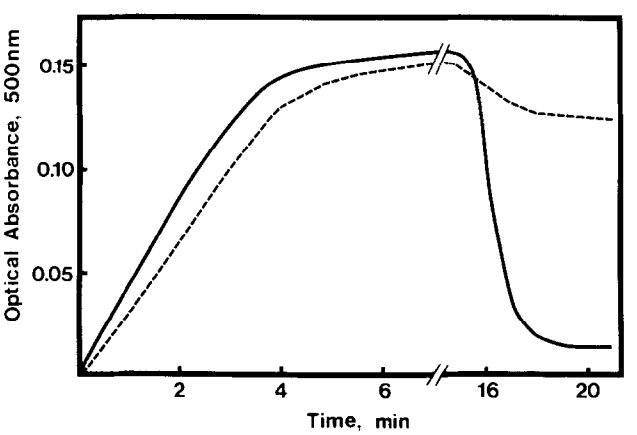

Figure 2. Tubulin assembly measured by turbidimetric assay. Platelet tubulin was isolated by 2 cycles of temperature-dependent polymerization and depolymerization and resuspended in PIPES buffer containing 1 mM FSBG. After exposure to the nucleoside derivative for 60 min at 10 °C the temperature of the tubulin solution was raised to 37 °C and the optical absorbance changes at 580 nm recorded as a function of time (interrupted line). Controls (solid line) were exposed to dimethylsulfoxide (the solvent for FSBG) equal in volume to the FSBG solution added and were polymerized in the presence of GTP at 37 °C. After complete assembly, the temperature was lowered to 4 °C and the resultant changes in optical absorbance recorded.

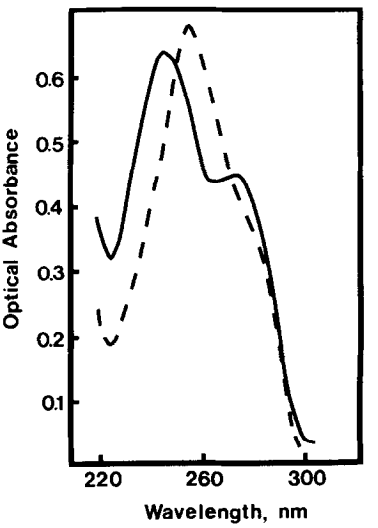

Figure 3. Absorbance spectra of FSB (solid line) and FSBG (interrupted line).

The absorbance spectrum of FSBG-tubulin is shown in Figure 3. Maxima were found at 254 and 274 nm. FSB for comparison had absorbance maxima at 245 and 274.

The tryptic peptides of microtubule protein were analyzed by high pressure liquid chromatography (Fig. 4). [^{14}C]-iodoacetamide-alkylated protein showed a large number of radioactive peptides. There were 16 major peaks of radioactivity. Comparing their radioactivity, it can be

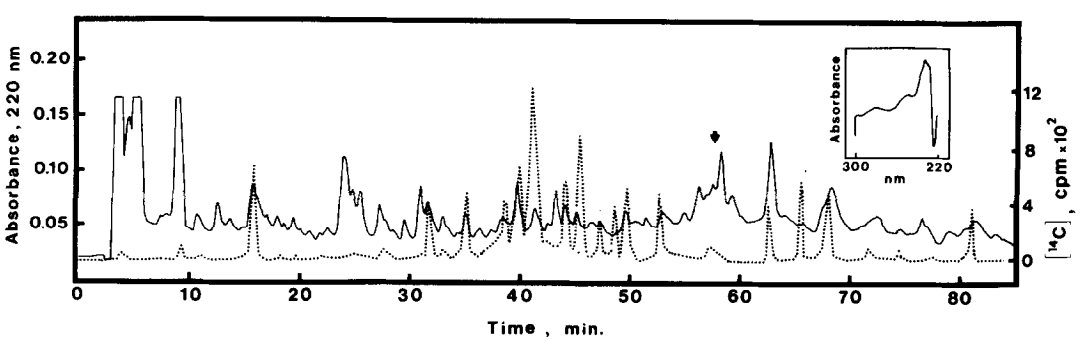

Figure 4. Soluble tryptic peptides of 3 cycle [^{14}C]carboxamidomethylated platelet tubulin isolated by reverse phase HPLC. Optical absorbance was measured at 220 nm (solid line). Each peptide peak was scanned from 220-320 nm as they eluted from the column and was collected into a liquid scintillation vial for measurement of radioactivity (dotted curve). Details of the procedure are given under METHODS. The arrow indicates the peptide modified by FSBG. The inset shows the absorbance scan of the FSBG-peptide.

recognized that the peptide eluting at 41.9 min had approximately 3 times the radioactive counts of the majority of the other labeled peptides. In addition, there were 4 other peaks that had counts approximately double those of the prevailing type. This distribution of radioactivity correlates well with the tryptic peptides that can be predicted from the known amino acid sequence of α- and β tubuion (9,10).

Most of the absorbance scans of the peaks as they eluted from the HPLC column revealed the typical spectra of peptides with a steep descent in the 200-220 nm range, a trough in the 240-250 nm range, and another peak at 270-280 nm corresponding to the aromatic amino acids. Two distinct peaks had additional maxima in the 252-255 nm area. When [^{14}C]FSBG was used, radioactivity was found to be associated with the peptide which eluted at 58.3 min and showed an absorbance peak at 252 nm. Free FSBG, GTP or GDP eluted very early (within the first 5 min), far ahead of this peptide. Unbound nucleotides were found with each separation of peptides. A second nucleotide peak was always recognizable at 12.4 min irrespective of whether the tubulin preparation was pretreated with FSBG or not. The absorbance maximum in the nucleotide area was at 254 min.

DISCUSSION

Fluorosulfonyl benzoyl nucleotide has been used as an effective affinity label for a series of different nucleotide binding enzymes (3,11,12). In the present study convincing evidence for preferential binding of FSBG to the exchangeable nucleotide binding site of tubulin was obtained. Both the inhibition by GTP and the association of [^{14}C] FSBG with the β-subunit of tubulin as demonstrated by SDS-urea polyacrylamide gel electrophoresis provide strong support for this hypothesis. It is not surprising that FSBG can promote microtubule assembly. A number of different nonhydrolyzable analogues of GTP have been found to do this (13). FSBG provided a convenient affinity label that was

easily synthesized. Bound to tubulin, FSBG had a characteristic absorbance peak in the UV region.

Maccioni and Seeds (14) recently reported that 2 different hydrolyzable GTP analogues, an azidobenzoyl and a periodate oxidation product of GTP bound to both α- and β-subunits of lamb brain tubulin. Competitive displacement by GTP and support of polymerization by the analogues could be shown.

The alkylation of free SH groups with [^{14}C]iodoacetamide reaffirmed the previously described abundance of free thiols in tubulin (15). We estimated a total of 20 cysteinyl residues. These findings are in good agreement with the published sequence of tubulin showing 8 SH groups in the β-subunit and 12 in the α-monomer (9,10). One tryptic peptide had 3,4 had 2 and 9 had 1 SH group. FSBG was found to bind to the tryptic peptide that eluted at 58.3 min. It had an absorbance maximum at 252 nm and was clearly far removed from the position where free GTP or GDP eluted. This result was reproducible and the absorbance peak was quite distinct from that of aromatic amino acids.

The consistent presence of an absorbance peak at 254 nm in the peptide with retention time of 12.4 min both in tubulin preparations which were treated with FSBG as well as those which were not, suggests that this represents the peptide containing the nonexchangeable nucleotide binding site. This was clearly a different nucleotide from that eluting at 58.3 min. There were no radioactive counts associated with that peak when [^{14}C]-FSBG-treated tubulin was analyzed.

I have not yet identified the exact location of the peptides and the amino acids that bind the nucleotide at the exchangeable and non-exchangeable binding sites. Such studies are in progress now.

ACKNOWLEDGEMENTS

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