**The GTP-binding Peptide of β-Tubulin**

**LOCALIZATION BY DIRECT PHOTOAFFINITY LABELING AND COMPARISON WITH NUCLEOTIDE-BINDING PROTEINS**

Klaus Linse and Eva-Maria Mandelkow†

From the Max-Planck-Unit for Structural Molecular Biology, DESY, Notkestraße 85, 18, D-2000 Hamburg 52, Federal Republic of Germany

The binding site of the guanine moiety of GTP on β-tubulin was located within the peptide consisting of residues 63–77, ALILDLEPGTMDSVR. The result was obtained using direct photoaffinity labeling, peptide sequencing, and limited proteolysis. Peptides were identified by end-labeling with a monoclonal antibody against β-tubulin whose epitope was located between 3 and 4 kDa from the C terminus. The sequence of the GTP-binding site is consistent with predictions from other GTP-binding proteins such as elongation factor Tu or ras p21.

The tubulin dimer is known to bind one nonexchangeable and one exchangeable GTP (GTP, and GTP, respectively, Jacobs et al., 1974). The binding and hydrolysis of GTP regulates microtubule assembly. Recently, proteins regulated by GTP have received much attention, particularly since some of them are oncogene products. Sequence homologies have been reported among GTP-binding proteins and other nucleotide-binding proteins (Wierenga and Hol, 1983; Sternberg and Taylor, 1984; Halliday, 1984; Leberman and Egner, 1984). When comparing α- and β-tubulin to other nucleotide-binding proteins, it appears that there are four regions of homology termed I, II, III, and IV in Fig. 1. Region I is the glycine cluster that is probably involved in a β-sheet and an α-helix near the phosphate-binding site; region II may be involved in the binding of the ribose, and region III is thought to be near the base of the nucleotide, as judged by homology with ATP- and dinucleotide-binding proteins (e.g. dehydrogenases, pp60^5^). However, there is another region IV that is thought to be near the base in a class of GTP-binding proteins, represented for example by EF-Tu or p21^TM^.

Thus, tubulin shows characteristics of two different classes of nucleotide-binding proteins, and an understanding of the nature of the GTP site might help to elucidate the structures of homologous proteins. In this paper, we describe the binding site of the base of the exchangeable nucleotide as determined by direct photoaffinity labeling of GTP, using a combination of approaches such as limited digestion of the native molecule, identification of peptides by end-labeling with monoclonal antibodies, and sequencing of the GTP-binding tryptic peptide. The binding site was found in the peptide comprising amino acid residues 63–77, consistent with region IV (Fig. 1) as an interaction site with the guanosine base. Preliminary reports of these studies have been published in abstract form (Linse and Mandelkow, 1986, 1987).

**MATERIALS AND METHODS**

**Protein Preparation**—Microtubule protein from porcine brain and phosphocellulose-purified tubulin were prepared as described (Mandelkow et al., 1985). The reassembly buffer was 0.1 M PIPES, pH 6.9, with a 1 mM concentration each of MgSO4, EGTA, GTP, and dithiothreitol. The protein was stored in liquid nitrogen until use.

**Photoaffinity Labeling**—Direct photoaffinity labeling of tubulin by [α-^32^P]GTP (Amersham, 1 mCi/ml, 3000 Ci/mmol) was performed at protein concentrations of 50–100 μM and concentrations of [α-^32^P]GTP around 0.1 μM, supplemented by equimolar concentrations of cold GTP. After preincubation for 5 min at 4 °C, the solution was irradiated by UV light of 254 nm (Super Pressure Hg Lamp HBO 100W/2, Osram, München) at a distance of 6–8 cm for 10–60 min. This amounts to an absorbed dose of about 0.025 milliwatt/cm^2^, well below the limit of radiation-induced disintegration of the protein (see Fig. 2A, and compare Zaremba et al., 1984). The integrity of the labeled protein was checked by SDS-PAGE and autoradiography.

**SDS-PAGE and Immunoblotting**—SDS-PAGE was performed following Laemmli (1970), with modifications (Mandelkow et al., 1985), using 0.5-mm thick microslab gels according to Matsudaira and Burgess (1978). Gels were either 12.5% acrylamide or had a linear gradient of 4–20% in the resolving gel and 4% acrylamide in the stacking gel. In some cases, a co-linear urea gradient from 1 to 8 M was used. Immunoblotting on nitrocellulose sheets was done following Bittner et al. (1980) with modifications (Kirchner and Mandelkow, 1989).

**Autoradiography**—Tubulin fragments labeled by [α-^32^P]GTP were detected by autoradiography from destained wet gels, dried gels, or nitrocellulose sheets, using Kodak X-Omat AR-5 film (exposure time 1–8 days).

**Limited Proteolysis and Sequencing**—PC-tubulin in column buffer (reassembly buffer without GTP) was digested at protein concentrations between 1 and 20 mg/ml. The following enzymes were used: α-chymotrypsin (Sigma), 15 °C, 30–60 min, enzyme:protein molar ratio 1:50 to 1:100; Staphylococcus aureus V8 protease (Miles), 15 °C, 30–60 min, enzyme:protein molar ratio 1:50 to 1:100 (with or without 1% SDS); subtilisin type VIII (Boehringer Mannheim), 15 °C, 30–60 min, enzyme:protein molar ratio 1:50 (the above proteases were stopped with 2 mM PMSF); clostripain (Sigma), 15 °C, 30–60 min, enzyme:protein molar ratio 1:500, activated with 2 mM CaCl2 and stopped with 2 mM PMSF, 2 mM EGTA, or 40 μg/ml α-macroglobulin. The 18-kDa fragment obtained by digestion with V8 protease was eluted from the SDS gel by diffusion (see Kirchner and Mandelkow, 1985). Sequencing was done with a gas phase Sequencer (Applied Biosystems).
GTP-binding Site of β-Tubulin

Isolation of GTP-binding Peptide—After photoaffinity labeling (60 min, up to 60% covalent incorporation of GTP), β-tubulin was split into its two major domains by limited chymotryptic digestion as above. The reassembly buffer was then changed to a column buffer of 20 mM PIPES, pH 6.9, with 4 M urea over a PD10 column (Pharmacia LKB Biotechnology Inc.), and the fragments were separated by FPLC using a Mono Q column (Pharmacia) and a gradient from 0 to 1 M NaCl. The protein was detected by a Beckman model min, up to 60% covalent incorporation of GTP), P-tubulin was split of 20 mM PIPES, pH 6.9, with 4 M urea over a PDL10 column and subjected to total tryptic digestion (37°C, protein:enzyme 1:50, 4 h). The peptides were separated by reversed phase HPLC (Beckman) using a C18 column (Bischoff) in 20 mM sodium phosphate, pH 6.9, and a gradient from 0 to 75% CH3CN and methanol (7:3, v/v). The radioactivity was detected by the Beckman model 170 detector and by scintillation counting (Tri-Carb, Hewlett-Packard). The radioactive activity was detected by the Beckman model 170 detector and by scintillation counting (Tri-Carb, Hewlett-Packard).

RESULTS

GTP Binds to β-Tubulin

Fig. 2A, and B show SDS gels of tubulin labeled with [α-32P]GTP and the corresponding autoradiograms. The label is found on β-tubulin (Fig. 2B, lane 2, top band). This shows that the exchangeable nucleotide is located on the β-subunit, in agreement with Geahlen and Haley (1977), Nath et al. (1985), and Hesse et al. (1985) but in contrast to other reports.

GTP Is Localized on the N-terminal Domain of β-Tubulin

The gel of Fig. 2B shows the results of photo-cross-linking of PC-tubulin with GTP and a subsequent brief digestion by chymotrypsin. This treatment cleaves β-tubulin at Tyr-281 into a large N-terminal (30 kDa) and a small C-terminal domain (20 kDa, Fig. 2B, lane 1). The autoradiogram (lane 2) shows that the label is incorporated into the 30-kDa N-terminal domain. This agrees with predictions derived from sequence homologies with nucleotide-binding proteins because all regions I-IV are located in the N-terminal domain, and with earlier reports (Fig. 1; cf. Mandelkow et al., 1985; Linse and Mandelkow, 1986; Nath and Himes, 1986).

The Base-binding Site of GTP, Is within 18 kDa of the N Terminus

PC-tubulin was labeled with GTP as before and then subjected to limited cleavage by V8 protease in 1% SDS. The gel shows various peptides (Fig. 3, lane 2). The lowest well-defined fragment showing radioactivity is at M, = 18,000 (lane 3). This band was excised and eluted from the gel, and 6 N-terminal residues were determined by gas-phase sequencing. They coincided with the N terminus of β-tubulin (MREIVH). This shows that the base binding is confined to the N-terminal 18-kDa region. This finding excludes the possibility that the base is cross-linked to region III.

Identification of Base-binding Peptides by Antibodies

The next step involved the identification of peptides by end-labeling with monoclonal antibodies.

C-terminal Labeling by a Monoclonal Antibody—In order to make use of the method, one requires a label for one of the ends of the polypeptide chain. As shown previously (Mandelkow et al., 1985), the region of highest antigenicity of both α- and β-tubulin is near their C termini. Several polyclonal and monoclonal antibodies were investigated. For our purposes, the most suitable antibody was the β-monoclonal antibody.
from Amersham. Its binding site was located between 3 and 5 kDa of the C terminus as follows.

1. The antibody reacts with the 20-kDa chymotryptic fragment of β-tubulin (Kirchner and Mandelkow, 1985) and thus is located on the C-terminal domain.

2. Limited proteolysis of native tubulin by subtilisin reveals two main cleavage sites of β-tubulin, one at about 5 kDa from the C terminus (see Serrano et al., 1984; Sackett et al., 1985), the other near the domain boundary. On SDS gels, one observes a 30-kDa band (N-terminal domain) and a 17-kDa band (C-terminal domain minus the 3-kDa C-terminal peptide, Fig. 4A, lane 2). The β-monoclonal antibody recognizes the 17-kDa fragment (Fig. 4A, lane 4). These results exclude the C-terminal 3 kDa as an antibody-binding site.

3. Limited proteolysis of native tubulin by V8 protease (without SDS) yields several fragments, among them those at $M_r = 49,000$ and 46,000 (Fig. 4B, lane 1). These carry the GTP label (lane 2) and thus represent derivatives of β-tubulin containing most of the molecule including the N-terminal domain (note that the V8 protease has no cleavage site near the N terminus, as shown above). However, the 49-kDa fragment does not react with the β-monoclonal antibody and therefore has lost approximately 4 kDa from the C terminus (these sizes are based on the observed $M_r = 53,000$ for β-tubulin, about 6% larger than the actual molecular weight).

Together, the subtilisin and V8 protease results show that the epitope lies between 3 and 4 kDa from the C terminus of β-tubulin. Therefore, for our purposes, the antibody can be used as a C-terminal end label to identify tubulin peptides (see diagram, Fig. 7).

**Limited Proteolysis by Clostripain**—The next step consisted of finding large proteolytic fragments that bind the C-terminal antibody label but not GTP. One such peptide is a 43-kDa fragment produced by limited proteolysis with clostripain (Fig. 5, lane 1). Fig. 5, lane 2, shows that the 43-kDa fragment binds the monoclonal antibody against β-tubulin, but not the labeled GTP (Fig. 5, lane 3). This means that the base-binding site must be located within the N-terminal 7–10 kDa.

**Isolation and Sequencing of GTP-binding Peptide**

In principle, the GTP-labeled peptides could be generated by tryptic digestion of tubulin dimers (PC-tubulin). However, in order to exclude ambiguities due to a mixture of α- and β-tubulin and to reduce overlap of peptides, we first chose to isolate the N-terminal chymotryptic GTP-binding fragment of β-tubulin described above, and then to use this for the identification of a tryptic peptide carrying the nucleotide. This fragment was obtained by FPLC using an ion exchange column (Fig. 6A, hatched peak) and identified by SDS-PAGE and autoradiography. This fraction was then subjected to total tryptic digestion, and the peptides were separated by HPLC on a reversed phase column. The elution was monitored by $A_{220}$ nm and radioactivity. The radioactive peptide peak (Fig. 6B, hatched) was then sequenced, yielding the sequence AILVDLEPGTMDSVR. This corresponds to residues 63–77 of β-tubulin; it agrees completely with one of the tryptic peptides expected from the N-terminal chymotryptic fragment. The GTP-binding residue itself could not be identified because of its lability during the sequencing procedure. (The fraction also contained a minor (15%) contamination by another peptide, FWEVISDEHGID... , corresponding to a peptide starting at residue 20.) We conclude that the photo-cross-linking of GTP occurs predominantly in region IV (Fig. 7) where tubulin shows homologies with other GTP-binding proteins.

**DISCUSSION**

**Comparison of Procedures for Locating the GTP-binding Site**—Direct photo-cross-linking of nucleotides has been used by several groups in order to identify the binding sites (e.g. ATP on myosin, Maruta and Korn, 1981; GTP on β-tubulin, Nath et al., 1985; NAD on diphtheria toxin, Carroll et al., 1985). This reaction usually involves decarboxylation and covalent binding of acid residues (Asp, Glu) to the base. In some cases, this causes the disruption of the bond between the base and the ribose (Carroll et al., 1985). In our studies, the GTP, appears to remain intact since the cross-linked base can be detected via the [α-32P]phosphate by autoradiography.

The combination of end-labeling by antibodies and specific enzymatic cleavage of the protein provides a convenient tool for identifying peptides of interest (Matsudaira et al., 1985). In order to use the method, we had to confirm that one of the antibodies tested bound near a terminus. It turned out that the epitope of the β-monoclonal antibody (Amersham) was located between 3 and 4 kDa of the C terminus.

The location of the GTP-binding region was carried out in several steps. The first is the demonstration that the exchangeable GTP binds to β-tubulin, in agreement with earlier reports (Nath et al., 1985; Geahlen and Haley, 1977, Hesse et al., 1985). The next question was to find out which of the two major domains of β-tubulin binds the exchangeable GTP. Chymotrypsin cleaves β-tubulin into a large N-terminal and a smaller C-terminal domain (mass = 30 and 20 kDa, Mandelkow et al., 1985). The large fragment binds the GTP, in agreement with the predictions (see below). However, since the homologies with other nucleotide-binding proteins suggested two possible regions of base binding, separated by about 170 amino acid residues, a further identification of the binding site was necessary.

In order to distinguish between the two choices, we searched for small peptides with GTP-binding activity and known position in the polypeptide chain. The example most suitable...
15208

GTP-binding Site of β-Tubulin

A further restriction is achieved by end labeling analysis with the monoclonal antibody against β-tubulin. Using a variety of proteases, we searched for the largest polypeptide that contains the C-terminal antibody-binding site, but not the radioactive label (base-binding site). A 43-kDa fragment produced by clostripain fulfilled this requirement. This implies that the base binding must occur within about 7–10 kDa of the N terminus (the uncertainty depends on whether the fragment still contains the C-terminal 3 kDa or not.

This result was confirmed by sequencing of a labeled trypsic peptide derived from the N-terminal domain of β-tubulin. It showed that residues 63–77 contained the cross-linked GTP, substantiating the earlier predictions. Thus, all the methods used here were consistent with one another.

Comparison with Other Studies on the GTP-binding Site of Tubulin—Despite some earlier ambiguities, there appears now to be a consensus that the binding site of the exchangeable GTP is on β-tubulin (Geahlen and Haley, 1977; Hesse et al., 1985), and, more specifically, on its N-terminal domain defined by chymotryptic cleavage (Nath and Himes, 1986; Linse and Mandelkow, 1986). However, there is a disagreement between our GTP-binding peptide and that of another recent study (Hesse et al., 1987) where the photo-cross-linked nucleotide was located between residues 155 and 162 of β-tubulin. This is not consistent with our sequencing results and with the restriction of the site to the N-terminal 7–10 kDa deduced from limited proteolysis and antibody end labeling. In order to reconcile their data with the predictions, Hesse et al. (1987) assume that the photo reaction takes place not at the purine but at the ribose moiety.

One could think of several reasons for the discrepancy. One is that Hesse et al. (1987) used total microtubule protein (including microtubule-associated proteins) for the photo-
cross-linking. This material is less homogeneous than the PC-purified tubulin used here; moreover, it is possible that microtubule-associated proteins induce a conformational change that alters the photoreaction. This would explain an earlier report by the same group where the nucleotide was found either on α- or β-tubulin, depending on the protein preparation used (Haley et al., 1983). Secondly, Hesse et al. (1987) used α/β-heterodimers as the starting material for tryptic digestion which results in a much larger number of peptides than the N-terminal fragment of β-tubulin used here; this increases the number of potential overlaps and the resulting uncertainties. For these reasons we think that peptide β63–77 is a more likely candidate for the photoreaction with the purine moiety. This agrees not only with the predictions (the homologies are in regions where the other GTP-binding proteins interact with the base), but also with other photo-cross-linking studies showing that the reaction usually involves the base and not the ribose (e.g. Maruta and Korn, 1981; Carroll et al., 1983). Nevertheless, one should bear in mind that a nucleotide-binding pocket is formed by different parts of the protein so that various ways of cross-linking cannot be ruled out. In particular, this could explain differences between direct photo-cross-linking of GTP and other studies employing a photoreactive azido derivative (cf. Geahlen and Haley, 1977; Haley et al., 1983).

Relationship with Homologous Proteins—Recently, two classes of nucleotide-binding proteins have been investigated with respect to their homologies. The first class contains several dehydrogenases and other nucleotide- and dinucleotide-binding proteins, including proteins related to p60Sos (Wierenga and Hol, 1983; Sternberg and Taylor, 1984). The second class contains the elongation factors, G-proteins, and proteins related to p21Cox (Leberman and Egner, 1984; Halliday, 1984).

β-Tubulin shares features of both classes. The homologies with the GTP- and dinucleotide-binding proteins are (see Fig. 1): I, a glycine-rich stretch between residues 132 and 148 (GGGTGSG); II, residues 180–183 (TVVE); III, residues 242–246 (LRFPG). (The sequences quoted are those of β-tubulin; the numbering of residues used from now on follows that obtained after optimal alignment of α- and β-tubulin, see Kraus et al., 1981.) Region I probably represents the turn between a β-strand and an α-helix as part of a nucleotide-binding motif (see Schulz and Schirmer, 1979) which is involved in the binding of the phosphates. Region II is thought to represent the end of another β-strand that could contribute to the binding of the ribose, whereas region III, the end of another β-strand, may be near the base. The sequences of both α- and β-tubulin are particularly comparable with the FAD-binding domain of glutathione reductase (Schulz et al., 1982). Note that the presumptive binding regions of phosphate, ribose, and base occur in the direction from the N to
the C terminus along the polypeptide chain.

The homologies with the GTP-binding proteins also include a glycine-rich region (residues 18–23 in EF-Tu, 10–15 in p21; Halliday, 1984; Leberman and Egner, 1984). Another region of homology thought to be involved in base-binding of EF Tu or p21 occurs in the vicinity of residues 60–70 of both tubulins (IV in Fig. 1). Thus, the homologies with different classes of proteins support two different base-binding regions. Note that all regions I–IV are highly conserved in the sequences of \( \beta \)-tubulin known thus far (Cleveland and Sullivan, 1985); moreover, they are conserved between \( \alpha \)- and \( \beta \)-tubulin. This would be expected if these regions had an important function such as GTP binding.

Only region IV is compatible with the results presented here. This result is notable in two respects. All the proteins mentioned here have their presumptive base-binding regions downstream from the phosphate-binding region by about 80–120 residues. This order appears to be reversed in tubulin since region IV is upstream from I. This apparent discrepancy need not be real; it may simply reflect our present poor understanding of GTP-binding pockets which must involve more than one stretch of the polypeptide chain. In fact, the conserved residues may not even be the ones directly responsible for hydrogen bonding to the nucleotide, a large part of which may be provided by the main chain rather than the residues (compare, for example, the details of the FAD-binding pocket in glutathione reductase, Schulz, 1982).

It is thus likely that the base-binding regions of the ATP- and GTP-binding proteins derived from homologies represent only one part of the respective polypeptide chain approaching the base; conversely, the homologous region III of tubulin may still be near the guanosine base without being directly photo-cross-linked. It is also possible that different parts of the protein take over the base-binding function in different conformational states, e.g. before and after the hydrolysis of the GTP. Note that region IV contains two acid groups in \( \beta \)-tubulin that could catalyze the photo-cross-linking reaction (Asp-69 or Glu-71), while there is none in region III.

Two GTP-binding proteins have thus far been solved by x-ray crystallography (EF-Tu, Jurnak, 1985; La Cour et al., 1985; ras p21, De Vos et al., 1988). The studies show that the sequence NCN (135–138 in EF-Tu) binds the base and suggest the same function for similar sequences in other GTP-binding proteins. If one aligns \( \beta \)-tubulin, EF-Tu, and ras p21 on the basis of the homologies between residues 60 and 70 of \( \beta \)-tubulin (Leberman and Egner, 1984), the sequence NCN (135–138) in EF-Tu would correspond to DLEP (69–72) in \( \beta \)-tubulin. The relationship between these sequences is not immediately obvious. However, both of them could provide hydrogen bonds to the base. If these stretches serve analogous functions, it means that the combination NCN is not necessary for base-binding, and, at any rate, this combination of residues does not occur in tubulin. The known structures also confirm the predictions that a cluster of small residues (such as Gly) forms the phosphate binding loop. Both in EF-Tu and p21, this region is upstream from the base-binding site, similar to the adenine nucleotide-binding proteins. Since this order is reversed in tubulin, it again reinforces the view that tubulin must be regarded as a class of its own with regard to GTP binding.

Finally, we note that the homologies discussed here are based on certain accepted procedures which include, among others, that sequences are read with defined orientations (i.e. from N to C terminus). Using these criteria, Dever et al. (1987) concluded that tubulin differed from other GTP-binding proteins, as discussed above. By contrast, Sternlicht et al. (1987) claim to have found the consensus sequences of Dever et al. (1987) in tubulin. They achieved this only by reading the sequence both forward and backward and by connecting sequence stretches with different orientations. It seems that the validity of this approach remains yet to be shown.

The study presented here was undertaken in order to learn more about the vicinity of the GTP-binding site of tubulin by comparison with other nucleotide-binding proteins. Considering the agreement between the predicted and observed GTP-binding site, it appears likely that the N-terminal GTP-binding domain adopts the general conformation of \( \alpha \)/\( \beta \)-proteins (central \( \beta \)-pleated sheet sandwiched between \( \alpha \)-helices, see Schulz and Schirmer, 1979); this would also be compatible with the x-ray pattern of microtubules. Further studies are underway to identify the location of the base-binding residue and to use the approaches of direct photo-cross-linking and antibody end-labeling to detect different conformational states of tubulin.

Acknowledgments—We thank Karin Kirchner for help with some of the digestion experiments and Eckhard Mandelkow for many discussions throughout this work. We are grateful especially to Prof. B. Wittmann-Liebold for providing the sequence data on the tryptic peptide.

REFERENCES

Kirchner, K., and Mandelkow, E. M. (1985) EMBO J. 4, 2397–2402

Downloaded from www.jbc.org by an authorized user on September 29, 2006