

# Tau Protein Binds to Microtubules through A Flexible Array of Distributed Weak Sites

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**Abstract.** Tau protein plays a role in the extension and maintenance of neuronal processes through a direct association with microtubules. To characterize the nature of this association, we have synthesized a collection of tau protein fragments and studied their binding properties. The relatively weak affinity of tau protein for microtubules ( $\sim 10^{-7}$  M) is concentrated in a large region containing three or four 18 amino acid repeated binding elements. These are separated by apparently flexible but less conserved linker sequences of

13–14 amino acids that do not bind. Within the repeats, the binding energy for microtubules is delocalized and derives from a series of weak interactions contributed by small groups of amino acids. These unusual characteristics suggest tau protein can assume multiple conformations and can pivot and perhaps migrate on the surface of the microtubule. The flexible structure of the tau protein binding interaction may allow it to be easily displaced from the microtubule lattice and may have important consequences for its function.

**T**AU protein is a microtubule-associated protein present in brain and other neuronal tissues (Binder et al., 1985; Drubin et al., 1986; Weingarten et al., 1975). It is found in the axonal microtubules of mature neurons (Binder et al., 1985) and in the axonlike elongated neurite processes synthesized by differentiating neurons in culture (Drubin et al., 1986; Ferreira et al., 1989; Kosik and Finch, 1987; Peng et al., 1986). Tau protein also accumulates in non-physiologic aggregates within the characteristic neurofibrillary tangles of Alzheimer's disease (Grundke-Iqbal et al., 1986; Kosik et al., 1986; Nukina and Ihara, 1986; Wischik, 1988; Wood, 1986).

Axons are filled with parallel arrays of densely packed microtubules (Peters and Vaughn, 1967) that act as a scaffolding that supports the extended structure of the process (Daniels, 1975; Seeds et al., 1970; Yamada et al., 1970). Axonal microtubules also function as the track along which organelle transport occurs (Schnapp et al., 1985; Vale et al., 1985). One way in which tau protein is likely to function in neurons is by stabilizing the microtubules. In support of this idea, tau protein microinjected into fibroblast cells (which do not normally contain tau protein) binds to cytoplasmic microtubules and stabilizes them against depolymerization by the drug nocodazole (Drubin et al., 1986). Another way in which tau protein may function is by cross-linking parallel microtubules. Expression of tau protein by transfection into fibroblast cells induces microtubule bundling which is consistent with a role for tau protein in microtubule cross-linking (Kanai et al., 1989). It is not known, however, whether this bundling is physiologic or an artifact of over-expression.

When isolated from adult brain tissue and examined by one-dimensional electrophoresis, tau protein is a mixture

of six related phosphopeptides with apparent molecular weights of between 50 and 70 kD (Cleveland et al., 1977b; Himmler, 1989; Goedert and Jakes, 1990). Phosphatase treatment decreases the number of resolvable forms to four (Baudier et al., 1987). On the basis of recent molecular studies, the remaining heterogeneity is likely to be because of differential mRNA splicing of a single gene (Himmler, 1989). Structurally, tau protein is rod shaped according to both hydrodynamic data (Cleveland et al., 1977a) and EM (Hirokawa et al., 1988). In addition, the morphologies of tau paracrystals viewed in the electron microscope indicate tau protein is a flexible and elastic molecule (Lichtenberg et al., 1988) that becomes stiffer and more extended with increasing phosphorylation (Hagedstedt et al., 1989).

The sequences of several tau isoforms show a common structural motif consisting of a series of 18 amino acid long sequences repeated three or four times (repeats) separated by less conserved stretches of 13 or 14 amino acids (linkers) (Goedert et al., 1989; Himmler et al., 1989; Lee et al., 1988). Since tau is an extended molecule, it is attractive to speculate that the multiple repeats are multiple tubulin-binding sites on the microtubule lattice. Consistent with this idea, tau peptides containing repeats have been shown to bind microtubules in vitro (Himmler et al., 1989; Lee et al., 1989). Furthermore, a single 18 amino acid tau repeat is sufficient to stimulate microtubule assembly from solutions of purified tubulin (Ennulat et al., 1989). These repeats are found in other (Aizawa et al., 1989; Lewis et al., 1988), but not all (Noble et al., 1989) microtubule-associated proteins and may define a family of proteins whose members bind microtubules via the same sequence motif.

Tau isoforms with different numbers of repeats are synthesized preferentially at different stages of brain development.

More specifically, the three repeat tau forms predominate in the fetal brain while four repeat forms are characteristic of the adult (Goedert et al., 1989; Kosik et al., 1989). Regulation of repeat number could have functional significance in that adult tau forms have been shown to be more efficient than fetal tau forms in promoting microtubule assembly in vitro (Goedert and Jakes, 1990).

Although it is known that tau protein fragments that contain repeats are able to bind microtubules, the binding domain of tau has not been rigorously characterized. We do not know, for example, which sequences (repeats, linkers, or sequences outside the repeat domain) bind to microtubules and which may regulate tau affinity. If multiple-binding units exist, we will want to know whether they bind cooperatively or independently. Finally, we do not know the structure of tau protein when it binds to microtubules or whether the tau molecule even adopts a rigid-binding geometry.

To understand the interaction of tau protein with microtubules, we have synthesized a collection of tau protein fragments in vitro and measured the strength of their binding. We have defined several different classes of tau protein subdomains which each have distinct roles in binding. In addition, we have shown the binding regions to tau protein are flexible and that their binding energy is distributed in a very diffuse manner. This type of unstructured and distributed binding interaction has not been well studied by x-ray crystallography but it may be very important in the interaction of many proteins and peptides of cell biological interest. We will discuss ways in which these features of tau protein may be necessary to fulfill its specialized functions.

## Materials and Methods

### Purification and Quantitation of Tubulin

Bovine brain tubulin was purified by two polymerization/depolymerization cycles followed by phosphocellulose chromatography (Mitchison and Kirschner, 1984). Quantitation was by Amidoschwarz (Schaffner and Weissman, 1973) and Bradford (Bradford, 1976) assays using a tubulin protein standard. Tubulin was stored in frozen aliquots and thawed once only, just before use. To prepare the tubulin protein standard used for quantitation, phosphocellulose-purified tubulin was separated from unbound GTP by gel filtration chromatography and quantitated by measuring the OD<sub>274</sub> in 6 M guanidine-hydrochloride ( $\epsilon_{274} = 1.15$ ) (Lee et al., 1973). Tubulin molarities are expressed as moles tubulin dimer. Microtubule molarities are given in units of moles polymerized tubulin dimer.

### Purification of Tau Protein

Tau protein was purified from bovine brain microtubule protein using phosphocellulose and hydroxyapatite chromatography (Cleveland et al., 1977b). Quantitation was accomplished by measuring the OD<sub>278</sub> in BRB-80 (80 mM K Pipes, 6.8, 1 mM MgCl<sub>2</sub>, 1 mM EGTA) supplemented with 1 mM DTT ( $\epsilon_{278} = 0.29$ ) (Cleveland et al., 1977a).

### Plasmid Constructions for In Vitro Transcription

The SP6 vector used in these experiments was derived from pSP64T (gift of D. Melton, Harvard University, Boston, MA) as follows. The BglII site separating the 5' and 3' untranslated Xenopus  $\beta$ -globin sequences in pSP64T was converted to an NcoI site and the G in the -3 position was converted to an A (gift of B. Hansen, University California, San Francisco, CA). In addition, the 200-bp BglII-SalI fragment containing the 3' untranslated globin sequences was replaced by a polylinker containing EcoRI, HindIII, and ClaI restriction sites. Tau subclones were constructed as follows: p110: the 450 bp SmaI-RsaI pBT 43-12 fragment was ligated to the 3-kb NcoI-EcoRV vector fragment after the NcoI site had been end filled. p132: a FokI-ClaI fragment encoding pBT 43-12 residues 314 to 323 was substituted

for the 220-bp FokI-ClaI fragment of p110. The substitution preserved the parental amino acid sequence and introduced four new restriction sites (AvaI, NsiI, SmaI, and BglI). p138: the 1160-bp SfiI-BamHI fragment of p147 encoding pBT 43-12 residues 152-431 was ligated with an NcoI-SfiI adaptor and the 3-kb NcoI-BamHI fragment from p110. p144: a BalI-Hinfi adaptor encoding pBT 43-12 residues 325 and 326 was substituted for the 44-bp BalI-Hinfi p110 fragment. p147: a 1.7-kb NdeI-BamHI fragment encoding the complete pBT 43-12 sequence was excised from bovine tau *Escherichia coli* expression plasmid pNde 43-12 (kind gift of D. Drechsel, UCSF). This fragment was ligated into the 3-kb NcoI/BamHI p110 vector fragment using an NcoI/NdeI adaptor. p154: an oligonucleotide encoding the amino acid sequence GSQVEVKSEKLDKFDG was inserted into the BalI site of p110 interrupting residue 324 of pBT 43-12. p156: an oligonucleotide encoding residues VTSVQIVYKPVVDGTK was inserted into the BstEII site of p110 between residues 306 and 307 of pBT 43-12. p166: an oligonucleotide encoding residues TGSQVEVKSEKLDKFDG was inserted into the BalI site of p154 interrupting the distal most residue (G) encoded by the oligonucleotide used to construct p154. p167: the 500-bp Thal/BamHI fragment of p138 was ligated with an NcoI/Thal synthetic fragment encoding residues GSKKIETHKLTFR and a 3-kb NcoI/BamHI vector fragment. The finished construct encodes pBT 43-12 residues 358 to the carboxy terminus 430. p175: the 3.3 kb SmaI-EcoRI p138 vector fragment was ligated with the 1.8-kb EcoRI-SmaI fragment obtained from the "A" murine tau cDNA clone (Lee et al., 1988; and see Fig. 7).

### Synthesis and Quantitation of <sup>35</sup>S-labeled Tau Proteins

Capped transcripts were generated by incubating 1  $\mu$ g of linearized SP6 plasmid template with 6 U SP6 RNA polymerase (Boehringer Mannheim Biochemicals, Indianapolis, IN) for 1 h at 40°C in a 10  $\mu$ l reaction containing 40  $\mu$ M Tris-HCl (7.5), 6 mM MgCl<sub>2</sub>, 2 mM spermidine, 10 mM NaCl, 10 mM DTT, 100  $\mu$ g/ml BSA, 0.05 mM GTP, 0.5 mM ATP, CTP, UTP, and GpppG cap analog. Transcribed RNA was purified by extraction with phenol-chloroform, precipitated with ethanol, and translated for 2 h at 25°C in a 50  $\mu$ l wheat germ extract system (Promega Biotech, Madison WI) supplemented with <sup>35</sup>[S]methionine (Amersham Corp., Arlington Heights, IL). Translated protein in wheat germ extract was precleared by ultracentrifugation and added directly to the binding reaction.

Under these conditions, ~0.5 to 1.0 ng/ $\mu$ l tau protein was translated. These values were calculated by measuring the total (radioactive and cold) methionine concentrations in these translation reactions and also by determining numbers of pmoles of <sup>35</sup>[S]methionine incorporated into each of three translated tau protein fragments 2 to 16 kD in size. Methionine pool size was determined by adding increasing amounts of cold methionine to a series of parallel translation reactions which each contained the same tracer amounts of <sup>35</sup>[S]methionine. The size of the methionine pool was taken to be equal to that concentration of exogenous methionine which reduced the rate of <sup>35</sup>[S]methionine incorporation into translated protein by 50%.

### Microtubule-binding Assay

Taxol microtubules were prepared from purified bovine brain tubulin that was precleared of aggregates by ultracentrifugation (100,000 RPM, 10 min, 4°C, TL 100 rotor; Beckman Instruments, Inc., Palo Alto, CA) immediately before use. Taxol (gift of Ven L. Narayanan, National Cancer Institute) was sequentially added to 0.1, 1.0, and 10  $\mu$ M at 5-min intervals to a stock solution of either 17 or 40 mg/ml (0.4 or 0.17 mM) tubulin incubating at 37°C in reassembly buffer BRB-80 (80 mM K Pipes (6.8), 1 mM MgCl<sub>2</sub>, 1 mM EGTA) supplemented with 1 mM GTP, 10  $\mu$ M taxol and 100  $\mu$ g/ml BSA. The polymerized solutions were very viscous and so repeated micropipetting and tube flicking was used to ensure any subsequent additions were distributed homogeneously. Tubules were diluted as necessary into BRB-80 at 37°C containing 1 mM GTP, 10  $\mu$ M taxol, and 100  $\mu$ g/ml BSA. Tau protein samples were precleared as above and incubated with taxol microtubules for 10 min at 37°C. The mixture was divided into 50  $\mu$ l aliquots which were each ultracentrifuged through 80  $\mu$ l of a 50% sucrose cushion made up in BRB-80 supplemented with 1 mM GTP, 10  $\mu$ M taxol, and 100  $\mu$ g/ml BSA using the preclearing centrifugation conditions at 37°C. Pellets (already resuspended at 4°C in BRB-80 and 100  $\mu$ g/ml BSA) and supernatants were precipitated with 10% TCA and electrophoresed. Samples containing tau proteins or fragments greater than 15 kD were examined on 5-15% polyacrylamide gradient gels. Very small (2 to 10 kD) tau protein fragments were resolved on 27.5% borate-acrylamide gels (Maurer and Allen, 1972). Purified tau protein was detected by immunoblotting. Protein

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----- 1 -----|-----
MAEPRQEFDMEDHAQGGDYTLQDQEGDMDPGLKESPLQTP
      10          20          30          40
----- 2 -----|----- 3 -----
ADDGSEEPGSGSETSDAKSTPTAEDATAPLVDEGAPGEQAAA
      50          60          70          80
----- 4 -----|-----
QAPAEIPEGTAAEEAGIGDTSNLEDQAAGHVQTARMVSKG
      90          100         110         120
----- 5 -----|----- 7 -----
KDGTPDDKTKGADGKPGTKIATPRGAAPPQGKQANAT
      130         140         150         160
-----
RIPAKTTPPKTSPGESGKSGDRSGYSSPGSPGTPGSRSR
      170         180         190         200
-----
TPSLPTPTREPKKVAVVRTPPKSPSAAKSRLQAAPGMP
      210         220         230         240
-----
DLKNVSKKIGSTGNLKHQPPGGKVQIINKKLDLSNVOSKC
      250         260         270         280
----- 11 -----|-----
GSKDNIKHVPPGGSVQIVYKPVDSLKVTSKCGSLGNLHHK
      290         300         310         320
----- 12 -----|-----
PGGGQVEVKSEKLDKDRVQSKIGSLDNITHVPPGGNKKI
      330         340         350         360
----- 13 -----|-----
ETHKLTFRENAKAKTDHGAEIVYKSPVVSVDTSRHLNSV
      370         380         390         400
-----
SSTGSIDMVDSPQLATLADVEVSASLAKQGL
      410         420         430

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**Figure 1.** Deduced amino acid sequence of bovine tau clone pBT 43-12. Plasmid pBT 43-12 is a full-length tau clone constructed by joining the 5' portion of bovine cDNA clone pBT-43 to the 3' portion of pBT-12 via a StyI site present within a region of sequence overlap (Himmler et al., 1989). The amino acid sequence predicted from the long open reading frame is shown in one letter code. Exon numbers and boundaries are positioned above corresponding residues. Amino acids are numbered beneath the sequence with the initiating methionine marked as position 1. The four 18 amino acid repeats are underlined.

was transferred to a nitrocellulose filter at 300 mA for 3 h in 20 mM Tris-base, 190 mM glycine, 0.1% sodium dodecyl sulfate, and 25% methanol. The filter was blocked in 4% BSA and probed with a 1:200 dilution of affinity purified rabbit antibody 7A-5 (gift of D. Drechsel, UCSF). This antibody was raised against a synthetic peptide homologous to the first bovine tau exon, MAEPRQEFDMEDHAQGGDYTLQDQEGDMDPGLK (gift of D. Martin, Genentech Inc., San Francisco, CA). Immunoreactive bands were detected by autoradiography using <sup>125</sup>I-protein A (ICN Radiochemicals, Irvine, CA). <sup>35</sup>S-labeled tau proteins and fragments were detected by autoradiography of gels fixed and dried without enhancement. Band intensities were quantitated using a video densitometer (model 620; Bio-Rad Laboratories, Richmond, CA) and regression analysis was accomplished with Enz Fitter data analysis software for the IBM PC.

## Results

### Assaying the Affinity of Tau Protein for Microtubules

To study the capacity of various subdomains of tau protein to bind to microtubules, we expressed fragments of tau protein in a wheat germ extract and measured the affinity of the labeled tau protein subdomains for microtubules stabilized with the drug taxol. The tau protein isoform selected for these studies is an ~45-kD protein containing exons 1, 2, 3,

4, 5, 7, 9, 10, 11, 12, and 13 of the tau gene (Fig. 1). It is the product of the bovine tau cDNA plasmid pBT 43-12 (Himmler et al., 1989). When translated in vitro, it comigrates with the slowest migrating of the four major isoforms seen in dephosphorylated preparations of tau protein isolated from bovine brain (Himmler, 1989). As expected, the expressed protein reacts with specific antisera directed against either exon 2, 3, or 12 (data not shown).

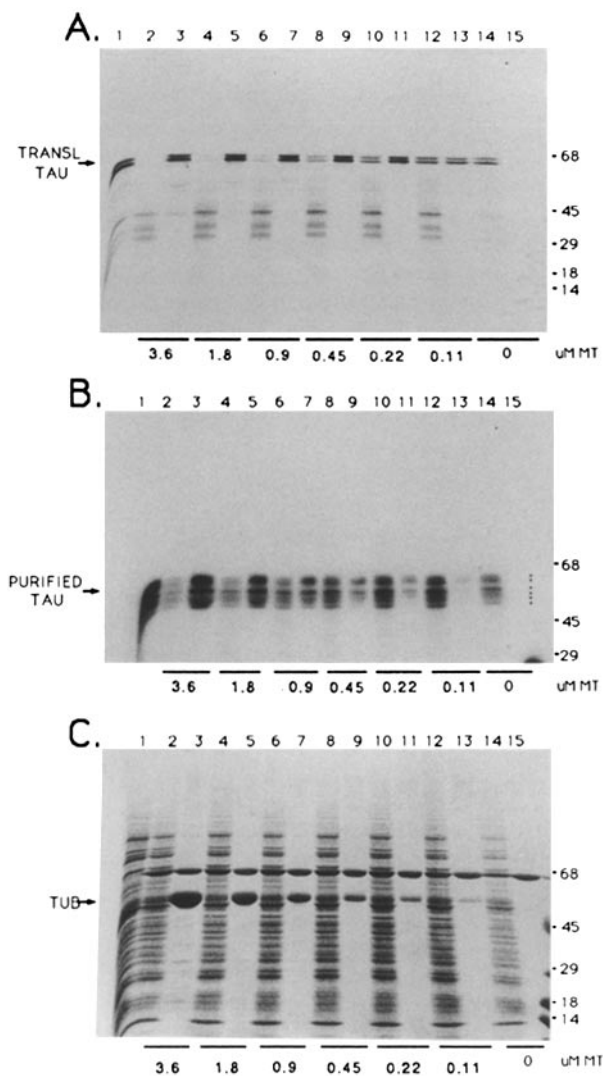
When full-length 43-12 plasmid is used as a transcriptional template, the principal translation products migrate as a doublet of apparent molecular weights 61 and 62 kD. The 61-kD product is severalfold more abundant (Fig. 2A). As will be discussed later, both members of the doublet show similar microtubule affinities. Although the predicted molecular mass from the 43-12 cDNA sequence is only 44.4 kD (Himmler et al., 1989), the observed molecular weights are consistent with the abnormally slow migration of tau proteins on SDS gels in general. The doublet is most likely because of secondary translational initiations at either or both of the methionines at positions 11 and 18 in addition to the expected site at residue 1. Each of the secondary sites as well as the position 1 methionine are located within good translational contexts (Kozak, 1987). The doublet is not vector or polymerase specific, since it is also seen when pBT 43-12 is transcribed off the T7 promoter of the Promega Bluescript vector (Promega Biotec) and translated in reticulocyte lysate (data not shown). The minor lower molecular weight translation products occur only in the presence of added RNA and are probably either tau protein degradation products or the result of premature chain terminations. They bind microtubules weakly, if at all, and do not interfere with the analysis.

To assay the affinity of translated tau protein for microtubules, the translation mixture was diluted with various concentrations of taxol-stabilized microtubules. Tau protein bound to microtubules was separated from unbound tau protein by rapidly sedimenting the microtubules through a sucrose cushion in a fixed angle rotor. Fractionated tau proteins were quantitated by autoradiography and densitometry after gel electrophoresis (Fig. 2).

In designing this assay, there were a number of potential caveats to address. First, we needed to suppress the microtubule depolymerization that would occur naturally in the dilute solutions used. We found that by including the drug taxol in all assay solutions we could vary the microtubule concentration from 200 to 0.1 μM tubulin and retain >90% of the added tubulin in polymer form (data not shown). Taxol promotes microtubule assembly and stabilizes formed microtubules (Schiff et al., 1979; Kumar, 1981; Schiff and Horwitz, 1980). It does not compete for microtubule binding against any of the known MAPs and appears to have a binding site which is completely distinct from that of tau protein (Vallee, 1982).

Secondly, to avoid having the complication of a "parking problem" (insufficient microtubule polymer so that tau protein molecules interfere with each other on binding) (McGhee and von Hippel, 1974), the molar ratio of tau protein to tubulin was never allowed to exceed 1:20, well below the saturation density of tau protein which is about 1 mole tau protein to 5 moles tubulin in polymer (Cleveland et al., 1977b; Hirokawa et al., 1988).

Thirdly, it is essential that tau protein does not disassociate from microtubules during centrifugation; it apparently does



**Figure 2.** Comparative microtubule affinities of recombinant vs purified tau proteins. Full-length tau mRNA (transcribed in vitro from p147 linearized at BamHI in the vector polylinker) was translated in a wheat germ system containing  $^{35}\text{S}$  methionine. Approximately 12 ng ( $2.4 \times 10^{-9}$  M) of translated protein were mixed with 120 ng ( $2.4 \times 10^{-8}$  M) of bovine brain tau protein and incubated with 40  $\mu\text{g}$  tubulin (lanes 2 and 3), 20  $\mu\text{g}$  (lanes 4 and 5), 10  $\mu\text{g}$  (lanes 6 and 7), 5  $\mu\text{g}$  (lanes 8 and 9), 2.5  $\mu\text{g}$  (lanes 10 and 11), 1.25  $\mu\text{g}$  (lanes 12 and 13), or 0  $\mu\text{g}$  (lanes 14 and 15) in a total volume of 112  $\mu\text{l}$ . Bound and unbound fractions were separated by ultracentrifugation. Equivalent relative amounts of each fraction and one half that amount of the control (nonmicrotubule) unbound fraction (lane 14) were electrophoresed with an equivalent relative amount of the unincubated tau mixture on duplicate 5–15% polyacrylamide gradient gels. For each gel: lane 1 is unincubated tau protein mixture, lanes 2, 4, 6, 8, 10, 12, and 14 are unbound (*soluble*) fractions, lanes 3, 5, 7, 9, 11, 13, and 15 are bound (*pelleted*) fractions. One gel was stained with Coomassie blue, dried, and exposed to film to show translated tau protein (A). The other, to show purified tau protein, was transferred to nitrocellulose, incubated with affinity purified 7A5 anti-tau  $1^\circ$ , and  $^{125}\text{I}$ -Protein A  $2^\circ$  and exposed through an extra sheet of film to completely block the  $^{35}\text{S}$  signal (B). This gel was electrophoresed an additional hour at 25 mA to resolve the six tau isoforms indicated with dots to the right of lane 15. C is the Coomassie-stained gel used to generate A. The microtubule concentration used in each reaction is given beneath each supernatant/pellet couple. Migration positions of translated

not. In the experiment shown in Fig. 2, all of the tau protein added to each binding reaction is distributed between the resulting supernatant and pellet fractions (e.g., Fig. 2 A, lanes 12, 13, and 14); tau protein is not lost in the cushion and appears stably bound to microtubules during centrifugation. In further support of this contention, tau protein was not detectable when the cushion was examined directly (data not shown).

Given the rapid reversibility of tau protein binding in solution (see below) its apparent stability during sedimentation is surprising. The effect is not because of the sucrose in the cushion since tau protein also binds reversibly in 50% sucrose solutions (data not shown) and is probably a consequence of using a fixed angle rotor for the separation step. In a spinning rotor of this configuration, the tau protein-microtubule complexes will be rapidly concentrated as they are first moved quickly through the buffer and pelleted onto the side of the tube. They will be further concentrated as the run continues and they are moved down the side of the tube into the sucrose cushion and then towards the tube bottom. The very high concentrations of tubulin expected within pelleted microtubules such as these should greatly inhibit tau protein disassociation.

Fourthly, we ensured that tau protein was in binding equilibrium with microtubules in the assay by varying the equilibration time. For this, three reactions were set up: a full-length synthetic tau protein was incubated with 0.05  $\mu\text{M}$  polymerized tubulin, a four repeat tau protein fragment was incubated with 0.5  $\mu\text{M}$  polymerized tubulin, and a two repeat tau protein fragment was incubated with 175  $\mu\text{M}$  polymerized tubulin. When incubation times for each reaction were varied from 2, 10, 30, to 90 min, the fraction tau bound remained constant (data not shown). We also determined that tau protein binding is freely reversible. When tau protein is first equilibrated with 6.25  $\mu\text{M}$  microtubules and subsequently diluted 10-fold, the fraction tau protein bound is decreased within 5 min to the level observed with 0.625  $\mu\text{M}$  microtubules (data not shown).

Finally, it was important to show that the translated tau protein was both released from ribosomes and was also no longer coupled to amino-acyl tRNAs, since either of these could affect the binding measurement. In cases where the mRNA contains a termination codon, release should occur naturally. In cases where the mRNA terminus was specified by a restriction cut in the plasmid template, complete polypeptides might still be associated with the ribosomal complex. After two hours of translation in a wheat germ extract, 87% of full-length nascent tau protein was spontaneously released and could be recovered in postribosomal supernatants (data not shown). The soluble tau protein is not precipitated by cetyltrimethylammonium bromide (data not shown) which is considered good evidence that it is no longer bound to amino acyl tRNA (Gilmore and Blobel, 1985). To ensure we were assaying the affinity of free tau protein only, each translation reaction was ultracentrifuged immediately before incubation with microtubules.

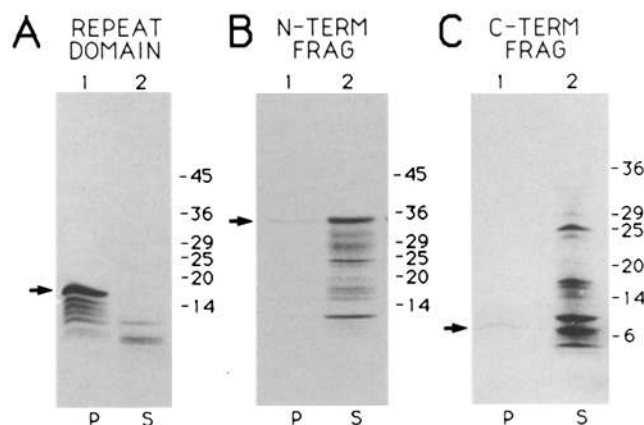
tau protein, purified brain tau protein, and tubulin are indicated to the right of the appropriate gels. Molecular weights of standards to the right of each gel are in kilodaltons.

**Tau Protein Expressed In Vitro Binds Microtubules with A Slightly Higher Affinity than Tau Protein Isolated from Brain**

We compared the microtubule-binding affinities of synthetic tau protein with naturally expressed tau protein purified from brain. Purified tau protein was obtained from recycled bovine brain microtubules using column chromatography rather than by more rapid but also harsher methods involving boiling (Herzog and Weber, 1978) or perchloric acid (Baudier et al., 1987). Full-length synthetic tau protein was translated in vitro, labeled with  $^{35}\text{S}$ methionine, mixed with tau protein purified from bovine brain, and then incubated with taxol stabilized microtubules. Free and bound tau proteins were separated by centrifugation and electrophoresed on duplicate gels. One gel was stained, destained, dried, and autoradiographed to visualize synthetic tau protein (Fig. 2 A); the second gel was Western blotted with affinity purified tau antibody to detect the purified tau protein (Fig. 2 B); Fig. 2 C is the Coomassie-stained gel used to generate the autoradiograph shown in Fig. 2 A. In both cases, the fraction tau protein bound to microtubules increased as the concentration of microtubules increased.

We can express the affinity of tau protein for microtubules as the concentration of polymerized tubulin required to bind half the total added tau protein. Before we present an analysis of the saturation curve, we will refer to this value as the apparent  $K_d$ . For tau protein synthesized in vitro, the apparent  $K_d$  is  $0.11 \mu\text{M}$  polymerized tubulin, since the  $0.11 \mu\text{M}$  supernatant (Fig. 2 A, lane 12) contains the same amount of tau protein as the pellet (lane 13), which is equivalent to half the concentration of added tau protein (lane 14). The bovine brain tau protein preparation resolves into six isoforms on a 5–15% polyacrylamide gradient gel (Fig. 2 B) with no contaminants visible on a Coomassie-stained gel (data not shown). The isoforms have similar but not identical affinities. The two higher molecular weight forms have an apparent  $K_d$  of  $\sim 0.45 \mu\text{M}$  (compare Fig. 2 B, lanes 8, 9, and 14) while the four lower molecular weight forms have apparent  $K_d$ s of  $0.9 \mu\text{M}$  (compare Fig. 2 B, lanes 6, 7, and 14). Thus, the purified tau protein species that corresponds to the synthetic tau protein binds microtubules with fourfold lower affinity than the synthetic tau protein. The reproducibly weaker affinity of purified tau protein could be a consequence of its partial denaturation during purification and/or specific types of posttranslational modifications such as phosphorylation that may occur in vivo but are not duplicated in the wheat germ extract. Phosphorylation, in particular, has been associated with a decrease in microtubule affinity for tau protein and other MAPs (Burns et al., 1984; Jameson et al., 1980; Lindwall and Cole, 1984; Murthy and Flavin, 1983).

An analysis of binding data of the type shown in Fig. 2 demonstrates that the association of synthetic tau protein with microtubules is specific, saturable, and inhibitable by purified tau protein. Tau protein does not pellet in the absence of microtubules (Fig. 2, A and B; lane 15). Also, out of the complicated mixture of proteins in the translation reaction, only tau protein binds (compare the supernatants and pellets in Fig. 2, A and B with those in Fig. 2 C). Increasing amounts of purified tau protein will completely block the binding of synthetic tau (data not shown).

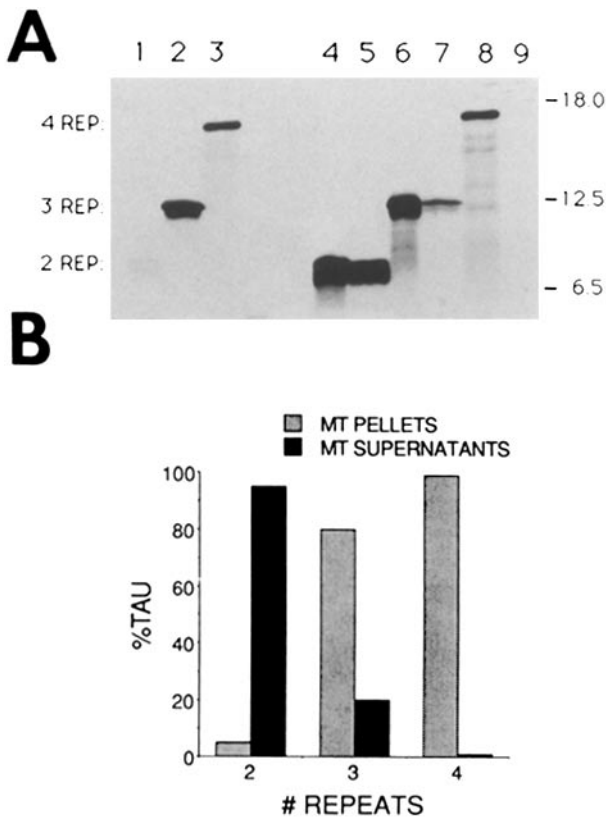


**Figure 3.** Binding of tau protein domains to microtubules. Capped transcripts were generated from three linearized plasmid templates and translated in wheat germ containing  $^{35}\text{S}$ methionine. Approximately 20 ng ( $1.5 \times 10^{-8}$  M) of each translation product was incubated with 525  $\mu\text{g}$  ( $1.75 \times 10^{-4}$  M) taxol microtubules. Bound and unbound tau protein fractions were separated and electrophoresed through 5–15% acrylamide gradient gels (A, B, or C) and autoradiographed. For each gel, bound fractions are shown in lane 1, unbound fractions in lane 2. (A) Repeat domain. The template was p110 linearized at the *Tha*I site and encodes the tau protein sequence from amino acid residue 237 to 367. (B) Amino terminal domain. The template was p147 linearized at *Apa*I and encodes the tau protein sequence from residue 1 to 237. (C) Carboxy terminal domain. The template was p167 linearized within the vector poly-linker and encodes tau protein sequence from residue 358 to the carboxy terminus at 431. Molecular weight markers are given to the right of each gel in kilodaltons.

To establish whether tau protein binds principally to the ends or sides of microtubules, we compared the affinities of tau protein for two populations of microtubules with different length distributions and consequently, different concentrations of ends. Tau protein translated in vitro was incubated either with tubulin polymers averaging  $16.2 \pm 8 \mu\text{m}$  in length (by darkfield microscopy) or with microtubules that were sheered to an average length of  $5.2 \pm 2.5 \mu\text{m}$ . The fraction tau protein bound was dependent only on the concentration of polymerized tubulin and was unaffected by the change in the relative number of tubule ends (data not shown) suggesting tau protein binds primarily to the lateral surfaces of microtubules.

**The Strongest Microtubule Affinity Is in the Repeat Domain of Tau Protein**

Previous reports (Himmler et al., 1989; Lee et al., 1989) have shown the repeat domain of tau protein is able to bind microtubules but these initial reports did not carefully examine whether other domains also affect binding. To address this issue, three different tau protein fragments were synthesized in vitro: (a) the tau protein repeat domain (residues 237 to 367); (b) the amino terminal half of tau protein (residues 1–237); and (c) the carboxy terminal nonrepeat domain of tau protein (residues 358 to 430). Assays for the binding of these constructs are shown in Fig. 3. Polymerized tubulin at a concentration of  $150 \mu\text{M}$  is sufficient to bind virtually all of the repeat domain fragment (Fig. 3 A, lane 1 vs lane 2) demonstrating the apparent  $K_d$  for this fragment is



**Figure 4.** Increasing the number of repeats increases the relative affinity of tau protein for microtubules. (A) Microtubule binding reactions of tau protein subfragments containing 2, 3, or 4 repeats. Approximately 24 ng ( $1.2 \times 10^{-8}$  M) of in vitro translated  $^{35}$ S-methionine-labeled protein were incubated with 60  $\mu$ g ( $1.5 \times 10^{-5}$  M) taxol stabilized microtubules. Bound and unbound tau protein fractions were separated, electrophoresed through a 27.5% tris-borate polyacrylamide gel and autoradiographed. (Lane 1) Microtubule pellet. (Lane 5) Microtubule supernatant from a binding reaction with the two repeat tau protein fragment extending from amino acid 237 to 297 and specified by p110 linearized at AccI. (Lane 4) Unprocessed translation reaction. (Lane 2) Microtubule pellet. (Lane 7) Microtubule supernatant from a binding reaction with the three repeat tau protein fragment extending from amino acid 237 to 343 and specified by p110 linearized at AvaII. (Lane 6) Translation reaction. (Lane 3) Microtubule pellet. (Lane 9) Microtubule supernatant from a reaction containing the four repeat tau protein fragment extending from amino acid residue 237 to 382 and specified by p110 linearized at the ClaI polylinker site downstream of the tau insert. (Lane 8) Translation reaction. Molecular weight markers in kilodaltons are shown at right. Number of repeats within each fragment is given to the left of the corresponding band. (B) Histogram. Relative amounts of bound and unbound tau protein fragments from microtubule reactions in A were quantitated using densitometry. The number of repeats contained within each tau protein fragment is shown beneath the corresponding pellet/supernatant pair.

significantly less than this microtubule concentration. By contrast, the amino and carboxy terminal nonrepeat domains bind very poorly, even at this high concentration of microtubules: Fig. 3 B, lane 1 vs lane 2 and Fig. 3 C, lane 1 vs lane 2. Assuming the binding of the two latter fragments is described by a simple binding algorithm, we can extrapolate from the percent fragment bound and estimate their  $K_d$ s are

>1.5 mM. From these results, we conclude the repeat domain contributes most of the binding energy for microtubules.

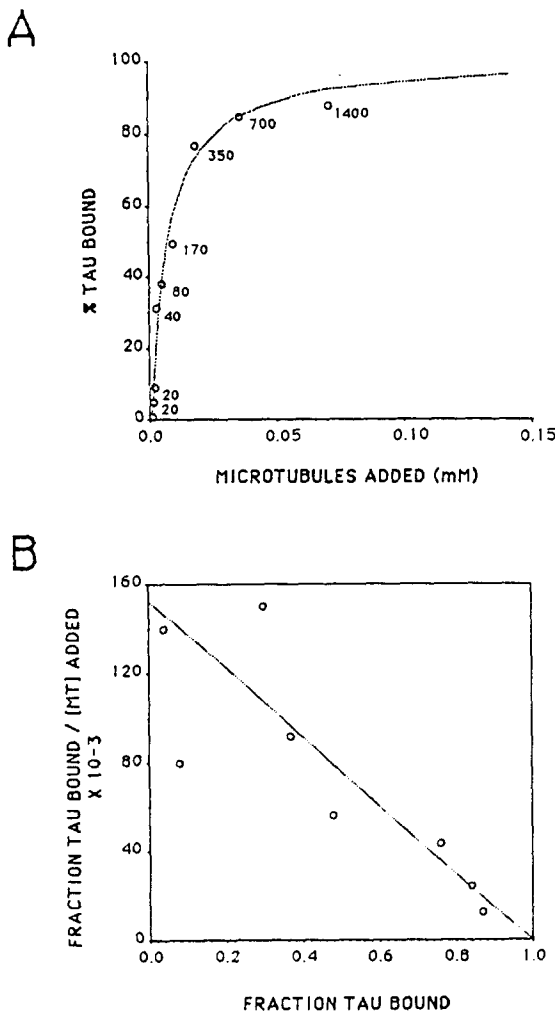
#### **Affinity of Tau Protein for Microtubules Increases with the Number of Repeats**

Since the repeat domain of tau protein seems to be responsible for most of the microtubule-binding energy, we measured how changes in the number of repeats affect the affinity of tau protein for microtubules. A series of labeled tau protein fragments containing two, three, and four repeats were synthesized in vitro from a full-length tau transcriptional template by linearizing it at various restriction sites. Each protein fragment was incubated with microtubules as shown in Fig. 4, A and B. A tau protein fragment containing only two repeats binds relatively weakly with only 5% co-pelleting with 15  $\mu$ M polymerized tubulin. Including an additional repeat increases fragment affinity such that 80% is bound by 15  $\mu$ M polymerized tubulin. Adding a fourth repeat raises fragment affinity such that virtually 100% is bound by 15  $\mu$ M polymerized tubulin. From these results, it is clear that the strength of the microtubule/tau protein interaction is increased by increasing the number of repeats.

#### **Tau Protein Binding to Microtubules Is Homogeneous, Reversible and Not Cooperative**

Complete binding curves extending to near saturation provide information about the strength of binding, the heterogeneity in binding sites and the independence of binding sites. For a simple binding curve, the disassociation constant,  $K_d$  is the midpoint of a hyperbolic saturation curve.

To generate accurate tau protein binding data, we chose to study a tau protein fragment extending from amino acid residue 152 to 297 and containing the first two tau protein repeats. Its apparent  $K_d$  fell in the middle of the range of microtubule concentrations that are technically optimal for these experiments. This fragment was synthesized in vitro and equivalent amounts were incubated with a series of dilutions of stabilized microtubules. In analyzing this binding isotherm, we consider the tau protein fragment, which is at a fixed and low concentration, to be the analog of the macromolecule in a typical binding experiment and the microtubule, composed of identical binding elements, to be the analog of the ligand, whose concentration is varied in the experiment. The resulting saturation curve for this fragment (Fig. 5, A and B, where it is shown as a Scatchard plot) fits the simple theoretical binding curve that describes the behavior of a macromolecule with a single binding site or single class of noninteracting sites with a disassociation constant,  $K_d$ , of  $6.5 \times 10^{-6}$  M and a SE of 19%. In this series of binding measurements no more than 3% of the total tubulin binding sites are ever occupied. This estimate conservatively assumes the tau protein concentration is 0.1  $\mu$ M or less (estimated from the specific activity of the labeled methionine in the translation mixture) and that the binding stoichiometry of tau protein/tubulin is no less than the saturation stoichiometry of 1:5 (Cleveland et al., 1977b; Hirokawa et al., 1988). A similarly precise fit to a single-site binding function was seen for full-length tau protein when binding data for the two highest molecular weight isoforms of purified tau protein (Fig. 2 B) were quantitated by densitometry and



**Figure 5.** Data analysis of the microtubule-binding function of a tau protein fragment containing two repeats. An  $^{35}\text{S}$ -labeled tau protein fragment extending from amino acid 152 to 297 (encoded by p138 linearized at AccI) was synthesized in vitro and incubated with various concentrations of microtubules. Bound and unbound tau protein fractions were separated by ultracentrifugation, electrophoresed through a 5–15% acrylamide gradient gel, autoradiographed, and quantitated by densitometry. (A) Binding curve. Percent tau protein bound for each microtubule reaction was determined by densitometry. Experimental data points are indicated by open circles. To the right of each point is the molar ratio of tubulin monomer to tau protein used in the reaction. The curve shown is the best fit theoretical binding function for this data calculated by regression analysis and describes the behavior of a macromolecule with a single binding site. ( $K_d = 0.65 \times 10^{-5}\text{M}$  with a standard error of  $1.25 \times 10^{-6}\text{M}$ ) (B) Scatchard plot of the data shown in A. Line shown is the Scatchard transformation of the theoretical function shown in A.

plotted as both hyperbolic and Scatchard functions (data not shown). The  $K_d$  for each was  $3.1 \times 10^{-7}\text{M}$  with a standard error of 15%.

The close fit of both sets of experimental data points to this type of theoretical curve demonstrates several properties of the tau protein/microtubule association. First, tau protein behaves as though it contains a single tubulin-binding domain. Secondly, there is no evidence for cooperative effects. When a tau molecule binds to a microtubule at a low ratio

of tau protein to microtubule binding sites, it does not promote or inhibit the binding of additional tau molecules. Cooperative effects between tau molecules could occur at high saturation, but here the analysis of the data would be complicated by the “parking problem” (McGhee and von Hippel, 1974).

### *Contribution of the Individual Repeats to the Binding Affinity of Tau Protein*

The contribution of each binding domain to the affinity of the complete tau molecule can be inferred by measuring and comparing the affinities of larger fragments containing different numbers of repeats, assuming there is no cooperativity within the repeat domain. The correctness of this assumption can be verified by measuring the binding energy of a specific repeat in different tau protein contexts. It is important to realize that we are not comparing calculated binding energies to those of free repeats. Individual repeats bind too weakly to measure (data not shown) and the results with the free domains would not necessarily be comparable for theoretical reasons elaborated in the Discussion.

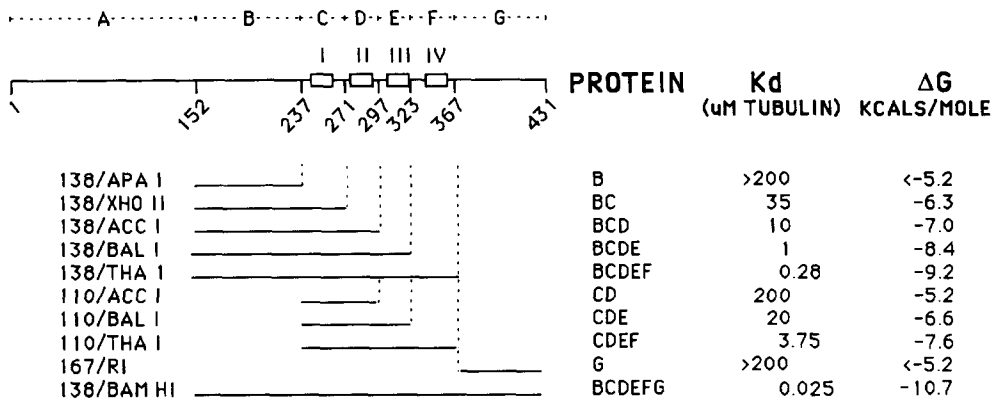
Fig. 6 summarizes the measured binding affinities of various domains of the tau molecule. In each case the  $K_d$  was measured by varying the polymerized tubulin concentration and interpolating the concentration of half maximal tau protein binding. The affinity of the entire repeat domain and flanking sequences extending from residue 152 to residue 431 (fragment BCDEFG) was experimentally found to be  $2.5 \times 10^{-8}\text{M}$ , corresponding to a free energy of  $-10.7\text{ kcal/mole}$  at  $37^\circ\text{C}$ . This was 4.4 times greater than the affinity of full-length tau protein, and as shown below indicates that the  $\text{NH}_2$ -terminal domain weakens tau protein affinity.

The affinity contributed by the second tau protein repeat (domain D) could be calculated from the data in Fig. 6 by subtracting the binding energy of the BC protein ( $-6.3\text{ kcal/mole}$ ,  $K_d = 35\ \mu\text{M}$ ) from the binding energy of the BCD protein ( $-7.0\text{ kcal/mole}$ ,  $K_d = 10\ \mu\text{M}$ ) giving an energy difference of  $-0.7\text{ kcal/mole}$  which is recorded in Table I.

The binding energy contributed by repeat 4 (domain F) could be calculated in two ways. By subtracting the binding energy of the CDE protein ( $-6.6\text{ kcal/mole}$ ) from that of the CDEF protein ( $-7.6\text{ kcal/mole}$ ) a binding energy of  $-1.0\text{ kcal/mole}$  is obtained. Alternatively, subtracting the binding energy of the BCDE protein ( $-8.4\text{ kcal/mole}$ ) from that of the BCDEF protein ( $-9.2\text{ kcal/mole}$ ) gives a binding energy of  $-0.8\text{ kcal/mole}$ . The average,  $-0.9\text{ kcal/mole}$ , is shown in Table I.

The binding energy contributed by repeat 3 (domain E) could also be calculated in two independent ways. The fragments compared were either the CD and CDE or BCD and BCDE proteins. The calculated free energy for repeat 3 based on either set of fragments was  $-1.4\text{ kcal/mole}$ .

For repeat 1 (domain C) the affinity of domain B is too weak to measure experimentally and so a somewhat different strategy was used. By comparing the measured  $K_d$ s of pairs of tau protein fragments which differ only in the presence or absence of domain B, we can calculate that domain B increases the affinity of neighboring repeat fragments an average of 18 times. For example, the BCD protein binds 20



**Figure 6.** Binding affinities of tau protein fragments containing different numbers or repeats. The top line is a diagram to scale of full-length 43-12 tau protein (431 amino acids). Each repeat is boxed with the appropriate number (I, II, III, or IV) indicated above each. The amino acid residue numbers and locations shown beneath map correspond to beginnings or ends of tau protein fragments positioned below. For each fragment, the plasmid and linearization site used to generate the *in vitro* transcriptional template are noted to the right. Protein names shown to the left of each construct diagram reflect the tau protein sequences within each protein according to the letter code above the p110 diagram.  $K_d$  values of  $^{35}\text{S}$ -labeled translation products were measured as described in Materials and Methods and are expressed in micromolar units of polymerized tubulin. Corresponding binding energies in kcal/mole are given under the heading  $\Delta G$  calculated at 37°C.

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times tighter than the CD protein, the BCDE protein binds 20 times tighter than the CDE protein, and the BCDEF protein binds 13 times tighter than the CDEF protein. On the strength of this correlation, it seems reasonable to assume that the B domain increases the affinity of the BC protein to a similar extent; the affinity of the C domain alone would then be  $\sim 0.7$  mM ( $\Delta G = -4.4$  kcal/mole). According to these calculations, the first repeat with its flanking sequences (residues 237 to 271) has an affinity approximately 100 times greater than repeats 2, 3, or 4.

As mentioned above, the validity of these calculations rests on the testable assumption that different tau protein repeats bind independently of each other. The reasons why this assumption might be valid for a flexible molecule like tau and not for more rigid molecules (Jencks, 1981) is elaborated in the Discussion. To determine whether repeat binding is independent or cooperative, we summed the four calculated repeat binding energies and compared the sum to the binding energy of the repeat domain measured experimentally. If repeats bind independently of each other, the sum of their calculated binding energies should be equal to the binding energy of the repeat domain determined experimentally. If repeats bind in a cooperative manner, deletion of a single repeat would have a large effect on binding and the sum of the calculated repeat energies would be measurably greater than the binding energy measured experimentally for the complete domain. As shown in Table I the sum of the individual repeat binding energies (C+D+E+F) is  $-7.4$  kcal/mole, a value that agrees very well with the  $\Delta G$  of  $-7.6$  kcal/mole

**Table I. Calculated Intrinsic Binding Energies for Individual Repeats**

Repeat	Boundaries	$\Delta G$
1	237-271	-4.4
2	271-297	-0.7
3	297-323	-1.4
4	323-367	-0.9

Relative binding energies for each repeat were calculated from the measurements given in Fig. 6 as described in the Results section and are listed here in kcal/mole under the heading  $\Delta G$ .

experimentally determined for the complete repeat domain (protein CDEF) specified by the p110/Tha I plasmid (Fig. 6). This close agreement between the theoretical and measured values implies the absence of cooperative effects among the repeat units.

As a test of the relevance of these calculations to the repeat energies within whole tau protein, the affinity of the repeat domain from a tau isoform missing the second repeat was compared to the affinity of the repeat domain from a four repeat form of tau protein. For this experiment the COOH-terminal *Sma*I/*Eco*RI fragment of mouse tau cDNA "A" clone (Lee et al., 1988) which contains only three repeats was substituted for the analogous *Sma*I/*Eco*RI fragment of the p138 bovine tau expression plasmid which contains four repeats. The resulting bovine-murine hybrid is referred to as p175. p175 is missing the second 18 amino acid repeat and the linker that precedes it in p138 but is otherwise virtually identical to the bovine sequence it replaced (Fig. 7 A). The affinities of the *in vitro* translation products specified by p175/Tha I and p138/Tha I were measured according to procedures described above. Binding curves for each are displayed in Fig. 7 B. The  $K_d$  for the three repeat tau protein domain is  $\sim 0.75$   $\mu\text{M}$  polymerized tubulin corresponding to a binding energy of  $-8.6$  kcal/mole. The  $K_d$  for the four repeat tau protein domain is  $\sim 0.15$   $\mu\text{M}$  polymerized tubulin corresponding to a binding energy of  $-9.6$  kcal/mole. The difference in binding energies between the two forms of tau protein is  $\sim -1.0$  kcal/mole. This is in very close agreement with the calculated free energy contribution of repeat 2,  $-0.7$  kcal/mole (see Table I).

### Fine Structure Analysis of the Repeat Domain

We have seen that the affinity of tau protein for microtubules is mainly distributed over the repeat domain in several independent-binding regions. Within these regions, previous experiments gave no information regarding the distribution of binding energy and in particular, whether repeats and linkers have distinguishable contributions. To investigate the detailed functional features of repeats and linkers, the binding constants for a series of eight tau protein fragments were determined as described in Materials and Methods. Each fragment initiates just before the first repeat at amino acid



A

p138/THA I:  
 p175/THA I:

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      160      170      180      190
  MGQKGGANATRIPAKTTPTPKTSPGESGKSGDRSGYSSPG
  MGQKGGANATRIPAKTTPTPKTSPGESGKSGDRSGYSSPG

      200      210      220      230
  SPGTPGSRSRTPSLPTPPTRPKKVAVVRTPPKSPSAAKS
  SPGTPGSRSRTPSLPTPPTRPKKVAVVRTPPKSPSASKS
      160      170

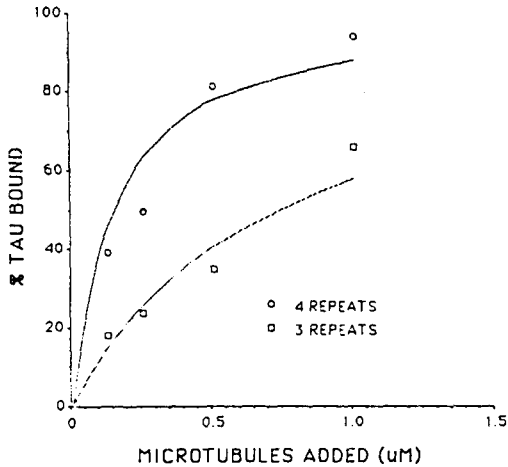
      240      250      260      270
  RLQAAPGPHPLDKNVYKSKIGSTENLKHOPGGGKQVQIINKK
  RLQTAAPVPHPLDKNVYKSKIGSTENLKHOPGGGK-----
      180      190      200

      280      290      300      310
  LDLSNVOSKCGSKDNKHKVPGGGSVQIVYKFPVDLSKVTSK
  -----VQIVYKFPVDLSKVTSK
      210      220

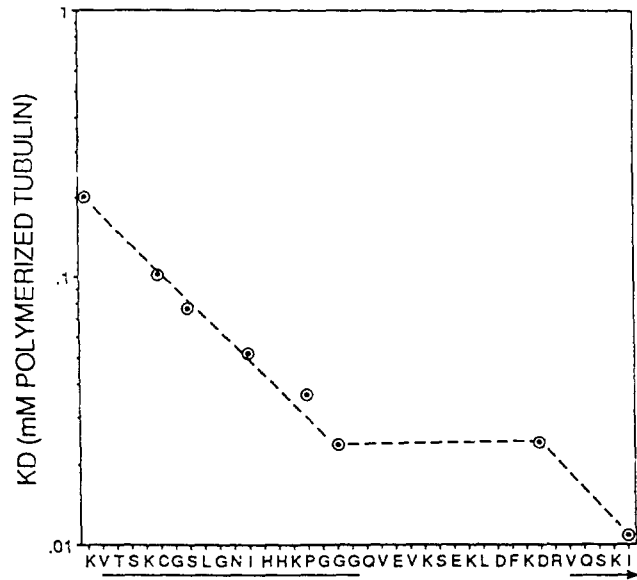
      320      330      340      350
  CGSLGNIHHKPGGGQVEVKSEKLDKDFKDRVQSKIGSLDNIT
  CGSLGNIHHKPGGGQVEVKSEKLDKDFKDRVQSKIGSLDNIT
      230      240      250      260

      360
  HVPGGGKNIETHKLTFF
  -----
  HVPGGGKNIETHKLTFF
      270
  
```

B



**Figure 7.** Comparative microtubule affinities of tau protein fragments containing either three or four repeats. (A) Amino acid sequences of p138 and p175 fragments used to direct in vitro transcription/translation reactions. Plasmid p138/ThaI (p138 linearized at a ThaI site) has four repeats and contains the bovine pBT 43-12 fragment encoding amino acid 152 to 367 (see Fig. 1). Plasmid p175/ThaI is a three repeat bovine/murine hybrid tau which contains the pBT 43-12 fragment encoding amino acids 152 through 209 (see Fig. 1) joined to the murine cDNA "A" fragment (Lee et al., 1988) encoding murine tau amino acids 152 through 278. Sequences are displayed in one letter code and the numbering scheme is that of the parent bovine or murine sequence. Asterisks denote amino acid homologies between the murine portion of p175 and the corresponding bovine sequence in p138. Repeat domains are underlined. (B) Binding curves for p138/ThaI and p175/ThaI tau protein fragments. Plasmids p138 and p175, linearized at ThaI, were used as templates to generate <sup>35</sup>[S]methionine-labeled tau protein fragments which were incubated with various concentrations of taxol-stabilized microtubules. Bound and unbound tau protein fractions were separated, electrophoresed on 5–15% acrylamide gradient



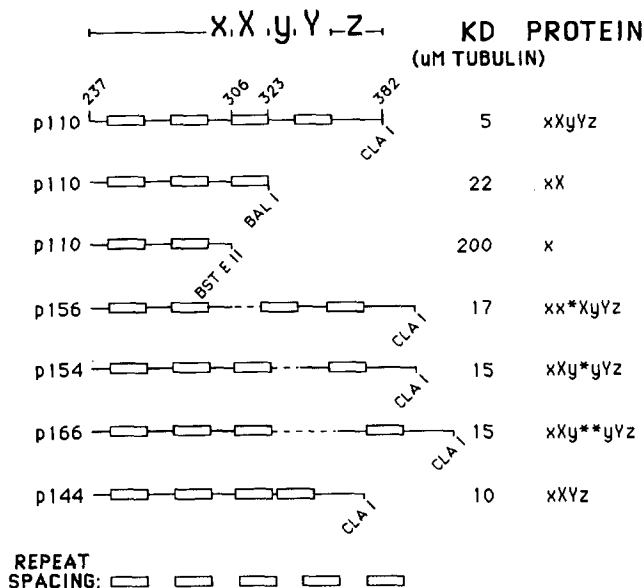
C-TERMINAL AA OF TAU FRAGMENT ASSAYED

**Figure 8.** Fine structure analysis of bovine tau protein repeat domain: linker regions do not contribute to binding affinity. Binding constants to taxol microtubules were determined for a collection of eight <sup>35</sup>[S]methionine-labeled tau protein fragments translated in vitro. Fragments each initiate at amino acid 237 and terminate at the various residues between 306 and 345 shown beneath each data point on the X-axis. Underlined amino acids indicate the relative positions of the third and beginning of the fourth repeats. Linearized plasmid templates and the fragments of tau protein they encode are as follows: p110/BstEII: 237 to 306; p110/FokI: 237 to 311; p132/AvaI: 237 to 313; p132/NsiI: 237 to 317; p132/SmaI: 237 to 321; p132/BalI: 237 to 322; p154/BalI: 237 to 337; p110/AvaII: 237 to 343. Amino acid numbering scheme refers to that of pBT 43-12 given in Fig. 1.

residue 237 and terminates at different points within the third or fourth repeat. The results of the measured  $K_d$  are displayed graphically at a function of distance through the sequence in Fig. 8.

There are two clear conclusions from these data. First, the repeat domain contributes to binding, while the linker region does not. Specifically, tau fragment affinity is increased ninefold when 17 amino acids completing the third repeat are added but does not increase at all when the subsequent 13 linker amino acids are added. It then increases another 2.5 times when the first seven amino acids from the fourth repeat are added. Secondly, tau fragment affinity increases linearly as amino acids are added within the third repeat. The linearity of this relationship suggests that individual or small groups of repeat amino acids bind independently with respect to neighboring sequences and contribute roughly similar amounts of binding energy ( $\sim 0.08$  kcal/mole/amino acid) to the tau protein-microtubule interaction.

gels, and autoradiographed. The percent tau protein bound was calculated by densitometry and plotted. The best fit theoretical binding function for each data set was calculated by regression analysis and plotted. These functions describe the behavior of molecules with a single ligand binding domain and ligand affinities of either 0.15  $\mu$ M (p138/ThaI data) or 0.75  $\mu$ M (p175/ThaI).



**Figure 9.**  $K_d$  determinations of tau protein fragments with expanded or contracted linker regions. The upper map is a scale diagram of the bovine tau protein repeat domain (amino acids 237 to 382) encoded by p110. Locations of repeats are shown by open boxes. Amino acid residue numbers above the diagram refer to Fig. 1 and correspond to the beginnings, ends or sites of disruption for each of the construct proteins diagrammed below. The  $K_d$  for each tau protein fragment was measured as described in Materials and Methods and is shown to the right of the appropriate diagram. Template plasmids are shown at left with the restriction sites used for linearization indicated beneath the right edge of each diagram. Plasmids p156, p154, p166, and p144 are derivatives of p110 which differ from the parent plasmid only by the insertion or deletion of sequences encoding linker amino acids in the positions shown by the dashed lines. Solid lines are sequences in common with p110. Repeat spacing is given by positions of bars at the bottom of the figure. Protein names reflect the tau protein sequences within each protein according to the letter code shown above the p110 diagram. Insertions are named with the letter of the p110 segment they most resemble followed by one or two asterisks to show they are distinct. Plasmid derivations relative to p110 are as follows: p156: VTSVQIVYKPVDTGK inserted between residues 306 and 307; p154: SQVEVKSEKLDKFDG inserted between residues 324 and 325; p166: SQVEVKSEKLDKFDGTGSQVEVKSEKLDKFDG inserted between 324 and 325; p144: amino acids between 325 and 338 deleted.

### The Spacing Between Repeats

The linker sequences in both tau protein and MAP-2 each consist of either 13 or 14 amino acids. Since the linkers are more constant in size than in sequence, and do not seem to bind to microtubules, it is tempting to speculate that they are involved in positioning and spacing repeat units. To test the importance of spacing, a series of repeat domain derivatives were made such that the linker regions were either expanded with extra linker sequences or contracted (Fig. 9). Aside from the altered linker regions, these constructions are identical to the fragment extending from residue 237 to 382, containing the repeat domains. To facilitate a comparison of these constructs, we have named each protein according to the regions of the repeat domain encoded (Fig. 9). By this nomenclature, the complete fragment is named xXyYz. Inserted sequences are named for the tau protein sequence

from which they were derived as explained in the figure legend. In each binding assay, we measured the  $K_d$ s of the full-length fragment and a fragment terminating just before the site of linker as controls.

The dissociation constants of various constructs with different linker lengths are displayed in Fig. 9. Increasing the number of linker amino acids between repeats 2 and 3 from 13 to 28 amino acids (xx\*xYyZ) produced an affinity of 17  $\mu$ M ( $\Delta G = -6.7$  kcal/mole). This is only a modest (3.5-fold) decrease relative to the unaltered domain xXyYz (5  $\mu$ M,  $\Delta G = -7.4$  kcal/mole) but is a marked (11-fold) increase over the 200  $\mu$ M ( $\Delta G = -5.2$  kcal/mole) affinity expected if the two normally spaced pairs of repeats were sterically prohibited from binding simultaneously. The expected affinity in the latter situation was calculated from data in Table I and Fig. 9 and largely reflects contributions from the first two tau protein repeats since together they possess over 50 times the affinity of the third and fourth repeats together. In a similar experiment, increasing the linker length between repeats 3 and 4 from 14 to 29 amino acids (xXy\*yYz) decreases repeat affinity threefold but again, this altered domain has a greater affinity (15  $\mu$ M;  $\Delta G = -6.8$  kcal/mole) than that expected for a domain where the first three repeats are unable to bind simultaneously with the fourth (22  $\mu$ M;  $\Delta G = -6.5$  kcal/mole).

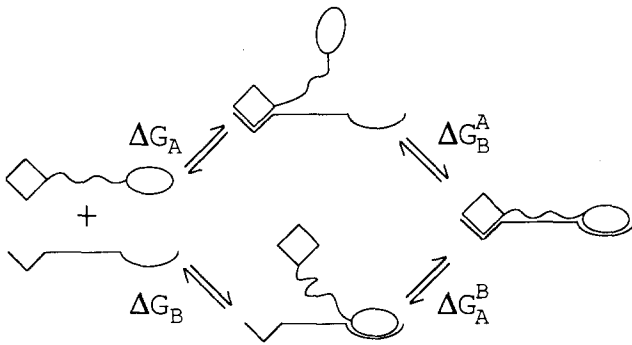
In another experiment, 12 of the 14 linker amino acids between the third and fourth repeats were deleted (xXYz). Although the loss of this linker produced a twofold decrease in tau protein fragment affinity (10  $\mu$ M;  $\Delta G = -7.0$  kcal/mole) the construct protein still binds more tightly than a control fragment with only three repeats (22  $\mu$ M;  $\Delta G = -6.5$  kcal/mole).

In a final linker experiment, the linker region between repeats 3 and 4 was expanded to a total of 46 amino acids (the length of two linkers and a repeat). Depending on the structure of the linker, an extension of this length might be expected to position the last repeat at a distance corresponding to the next tau protein binding site on the microtubule. This construct (xXy\*\*yYz) has the same affinity as the parent molecule (xXy\*yYz) showing that the exceptionally expanded linker does not position the fourth repeat to a greater precision than the shorter linker, nor does it additionally interfere with the simultaneous binding of all four repeats.

These results show that either expanding or eliminating linker amino acids interferes with but does not prevent all four repeats from binding as a single unit. The linkers then, seem to play a surprisingly small role in optimizing the repeat positions and do not appear to be the rigid spacing elements one might have expected.

### Discussion

We have used an accurate binding assay for microtubule-associated proteins along with in vitro translation of specific protein subfragments to gather structural information about the microtubule binding interaction. In this simple assay, the concentration of stable microtubules was varied and the concentration of tau protein was held fixed and the levels of tau protein were kept well below saturation for the microtubule lattice to avoid any binding interference from overlapping sites. The drug taxol was used to suppress microtubule dynamics at low tubulin concentrations. The rapid sedimenta-



**Figure 10.** Energy diagram showing pathways by which a molecule with two tau protein repeats can interact with the microtubule lattice. The surface of the lattice with tau receptors is represented by a straight line connecting a half-diamond and a half-sphere shape. Each shape represents a binding site for a single tau repeat. The complete diamond and sphere shapes joined by a wavy line represent two adjacent tau repeats joined by a flexible linker.  $\Delta G_A$ ,  $\Delta G_B$ ,  $\Delta G_{AB}$ , and  $\Delta G_{BA}$  are free energies of binding.

tion of equilibrated tau protein-microtubule complexes in a fixed angle rotor effectively blocked complex disassociation and allowed the accurate determination of tau protein concentrations at equilibrium. That a true equilibrium was achieved in these experiments is demonstrated by the establishment of the same equilibrium value from both directions.

Tau protein binds to microtubules primarily through a 130 amino acid repeat region (amino acids 237 to 367) that is subdivided into four repeated binding units that are separated from each other by three nonbinding linker units. Each repeat binds weakly and appears to be unstructured. These unusual characteristics are suggested by the finding, shown in Fig. 8, that the amount of binding energy possessed by a tau protein fragment with an incomplete repeat domain increased linearly as repeat amino acids were added to its distal end. The average increase in binding energy was only  $-0.08$  kcal/mole ( $0.336$  kJ/mole) per residue added. Such weak interactions suggest van der Waals or highly shielded ionic forces are being exerted within the tau repeats. The uniform dispersion of binding energy along the entire repeat suggests the repeats are flexible. There is no discontinuity in tau protein affinity that would be expected if the repeat was assuming a rigid and defined structure. Although this analysis was not carried out to the resolution of individual amino acids, we can say that groups no larger than two to four amino acids in size are contributing weak binding energies as independent units.

We have shown that the individual domains bind non-cooperatively, i.e., the sum total of the binding free energy for the repeat domain is equal to the sum of the free energies of interactions of each repeat. In general this is not to be expected for rigid molecules as discussed by Jencks (1981) and Erickson (1989). The reason is shown in Fig. 10, which is taken from Jencks (1981). In general,  $\Delta G_A \neq \Delta G_A^0$  and  $\Delta G_B \neq \Delta G_B^0$  because the molecule is already constrained and immobilized in a favorable configuration. It is possible that  $\Delta G_A^0$  could be less favorable than  $\Delta G_A$ , but in general, immobilization of the first domain to bind would limit the translation and rotation of the second domain and facilitate binding. This can be expressed in a slightly different manner (Jencks, 1981). For the binding of any domain the free

energy of interaction  $\Delta G$  is composed of two parts:  $\Delta G = \Delta G_i - T\Delta S_{\text{config}}$ , where  $\Delta G_i$  is the intrinsic free energy of interaction and is independent of the rotational and translational entropy lost in binding and  $T\Delta S_{\text{config}}$  represents the loss of free energy because of the loss of entropy from the immobilization of the rotation and translation of the domain on binding. Considering a two domain molecule AB, we calculated in our experiments the binding of the B domain by subtracting the free energy of binding of the A domain alone,  $\Delta G_A$ , from the free energy of binding of the AB domain,  $\Delta G_{AB}$ . In general this would not be proper (Jencks, 1981), especially for rigid molecules. Each independent binding measurement would include a value for the loss of configurational entropy; yet for a rigid molecule this entropy could only be lost once. The second domain should bind much more effectively, since it was already positioned in the proper configuration. For flexible molecules like tau the situation is apt to be very different.

Continuing the argument, if the molecule were rigid,  $\Delta G_A$  would have a  $T\Delta S_{\text{config}}$  identical to that for  $\Delta G_{AB}$  and such a calculation should give us  $\Delta G_i$  for each domain. In the end when we sum  $\Delta G_i$  for each domain, it should give us a value that was larger in magnitude (more negative) than the experimentally measured value of  $\Delta G_{AB}$  for the entire binding molecule because the measured value would have to have a contribution of  $-T\Delta S_{\text{config}}$  because of immobilization of the molecule on the lattice. Tau is clearly not a rigid molecule so that the binding of any new domain even when part of the molecule is already bound would also require a translational and rotational entropy loss and would therefore have a  $-T\Delta S_{\text{config}}$  because of immobilization of a previously flexible part of the molecule. It is not clear how much the previous binding of one domain constrains the binding of the next domain. If it does not at all, then each domain would contribute  $\Delta G_i - T\Delta S_{\text{config}}$  and the total configurational entropy of tau would be the sum of the configuration entropies of the individual domains. Efforts to estimate the configurational entropies for rigid molecules have not been very successful. Early theories estimated values as high as 35 kcal/mole (see discussion in Erickson, 1989), but they underestimated the flexibility of the molecules in the bound state. Recently, Erickson (1989) estimated 7 kcal/mole of translational and rotational free energy loss to configurational entropy. However, the best experimental data on bidentate binding of antibody molecules suggests that the true value is either much lower than 7 kcal, or compensated for by unfavorable steric problems (see discussion in Erickson, 1989). Thus, even Erickson's 7 kcal estimate is unsupported for rigid molecules and certainly an overestimate for more flexible molecules. Tethering on a flexible linker may make a very small contribution to the configurational entropy and each domain would bind independently. This explains in part why the binding of each domain is so weak. In addition to the intrinsic interaction, each domain must overcome rotational and translational entropy not very different from that of free domains.

Although the repeats are similar in structure, each repeat has a different affinity for the microtubule lattice (Table I). The first tau protein repeat binds 75 to 200 times more tightly than the other repeats (Table I). This repeat does not have a distinctive net charge and the molecular basis for its higher affinity is unknown. The effect could be caused by sequences flanking the repeat or by particular amino acids

within the repeat. Flanking sequence effects would have to be very local since the high affinity of the first repeat is retained when only 16 of the neighboring amino acids remain appended (Fig. 6). Although the sequence of the first tau repeat is similar to the other repeats, it and the first repeats of both MAP-2 (Lewis et al., 1988) and the 190-kD adrenal MAP (Aizawa et al., 1989) possess characteristic amino acids that might generate the high affinity. If particular residues are important, they are not confined to a single localized cluster. Tau protein fragments initiating after the first six residues of repeat 1 (omitting a characteristic basic residue at position 246; Fig. 1) lost only a portion (31%) of the binding energy associated with the first repeat. The 12 remaining repeat 1 residues still possessed over 10 times the microtubule affinity of any other repeat (data not shown).

Although the repeat domain clearly contributes the largest increment of microtubule binding energy ( $\Delta G = -7.6$  kcal/mole) sequences outside the domain also have measurable effects on binding. For example appending amino acids 152 to 237 onto the proximal side of a cluster of either 2, 3, or 4 repeats increases fragment affinity 10 to 20 times ( $\Delta G = -1.6$  to  $-1.8$  kcal/mole; see Fig. 6). In addition, attaching the tau protein sequence that extends from amino acid 367 to the tau carboxy-terminus (amino acid 431) to the distal side of the repeat domain produces an additional 10-fold increase in affinity ( $-1.5$  kcal/mole). Similarly, in experiments where fragments of MAP-2 were expressed in cultured cells, a qualitative enhancement of microtubule binding was seen by immunofluorescence when the repeat domain was flanked by either its proximal 53 or distal 73 amino acids (Lewis et al., 1989); the repeat domain and flanking sequences are from regions of MAP-2 that share a high degree of homology with tau protein (Lewis et al., 1988).

In contrast to the increased microtubule affinity associated with the COOH-terminal and central tau protein sequences described above, sequences within the amino terminal half of tau were found to decrease affinity. The COOH-terminal half of tau (amino acids 152 to 431) binds with a  $K_d$  of  $2.5 \times 10^{-8}$  M while full-length tau protein translated in vitro (amino acids 1 to 431) binds with a  $K_d$  of only  $1.1 \times 10^{-7}$  M. This affinity difference suggests the amino terminal half of tau protein could act as a regulatory domain to modulate the affinity of the binding domain.

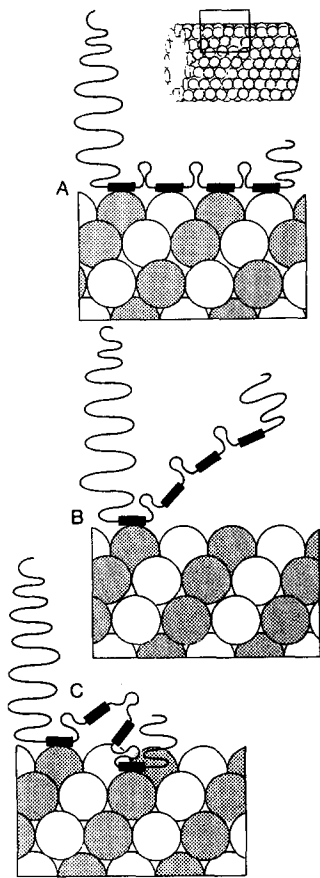
Linker sequences also influence tau protein affinity although they do not bind microtubules directly. The absence of inherent linker affinity for microtubules is shown in a sensitive experiment where restoration of the natural linker to a fragment which previously terminated near the end of a repeat did not increase fragment affinity (Fig. 8). A small but measurable role for linkers in optimizing repeat position is suggested by the threefold loss of affinity caused by doubling the length of the linker (Fig. 9) and the twofold loss of affinity caused by eliminating a repeat. These effects are surprisingly small and suggest that the linkers are flexible and do not constrain the tau structure very much. In addition, strong but slightly diminished binding continues to occur after deletion of the linker; the construct protein missing the linker between repeats 3 and 4 has over twice the affinity of a control tau protein containing only three repeats (Fig. 9). One interpretation of this last result is that there are several, nearly equivalent, configurations for tau binding to the microtubule lattice. Alternatively, the tau binding sites on microtubules

could be spaced sufficiently close together so that two adjacent tau repeats could span them without a linker. In this latter view, the linker would have little effect on the distance between tau repeats. This entire set of results suggests that while the linkers play some role in optimizing repeat binding, their influence on tau protein affinity is small compared to the repeats. Furthermore, since each of the four repeats is able to contribute binding energy in construct proteins with altered linker lengths, the linkers must not be rigid spacing elements; instead they appear to have some flexibility.

The apparent flexibility of the repeat domain and the lack of cooperative repeat binding is consistent with sequence analyses of tau protein which predict little, if any secondary structure within this region and within tau protein in general (Lee et al., 1988). It is also consistent with the flexibility and elasticity seen in populations of tau paracrystals (Lichtenberg et al., 1988) and in preparations of purified tau protein bound to microtubules and visualized in the electron microscope (Hirokawa et al., 1988). The structural basis for this flexibility is unknown but may be related to unusually high concentrations of proline and glycine in tau protein (Lee et al., 1988).

In summary, the microtubule-binding region of tau protein is very large and has a complex substructure. When tau protein binds, the higher relative affinity of the first tau repeat suggests it could anchor tau protein to the microtubule while the more weakly interacting remaining repeats rapidly release about once per millisecond (assuming diffusion limited on rates) and then rebind in different combinations and in different positions as diagrammed in Fig. 11. The saturating molar ratios measured for tau protein binding to polymerized tubulin (1:5) (Cleveland et al., 1977*b*; Hirokawa et al., 1988) and for tau protein binding a peptide fragment from the tau binding region of tubulin (1:4) (Maccioni et al., 1988) indicate each tau protein repeat binds to a separate tubulin monomer. Theoretically, a single repeat with a relaxed structure should be able to span not only the 4-nm distance between sites in the same protofilament but the 5-nm distance between sites in adjacent protofilaments as well. In this way, tau protein could assume a variety of bound positions on the microtubule lattice.

Although dispersed, noncooperative and unstructured binding interactions may seem unusual, they may be very common in cell biology. To date, our most detailed structural information has been obtained from x-ray crystallography, which has concentrated on easily crystallizable and generally rigid structures. Thus, the protein and DNA, protein and other protein, or protein and ligand interactions studied so far are probably of structures with only a few stable conformations. Several important interactions in biology may not conform to such models. For example, the HLA molecule binds a large variety of different peptides (Buus et al., 1987) in one binding pocket (Bjorkman et al., 1987*a,b*). Similarly, the binding of signal sequences to the SRP shows no rigid sequence specificity (von Heijne, 1985), although the SRP molecule is likely to recognize some general features of signal peptides. A similar situation may exist for the binding of mitochondrial import signal to its receptor (von Heijne, 1986; Allison and Schatz, 1986), the binding of lipoproteins to the scavenger receptor (Brown and Goldstein, 1983) and the binding of a variety of partially unfolded proteins to the



**Figure 11.** Diagram showing how tau protein might pivot on the microtubule lattice. In the upper right is a side view of a short section of a microtubule made up of 13 protofilaments.  $\alpha$  and  $\beta$  tubulin subunits are shown as gray or white spheres, respectively. The framed area is expanded and shown in A–C with tau protein in three different sequential conformations. In each case, the tau molecule is oriented with its amino terminus at the left. The locations of the four repeats are indicated by filled boxes with repeat I to the left and repeat IV to the right. Linkers are represented by the hairpin structures separating the repeats. (A) Tau with all four repeats bound to the microtubule lattice. (B) Repeats II through IV are transiently unbound and tau is tethered through repeat I only. (C) Repeat IV has rebound in a new position.

chaperonins (Rothman, 1989). What is known about all these interactions is that although they may have certain structural features in common, the allowable sequences are so divergent that it is unlikely that they have a specific rigid conformation. Like tau protein binding to the microtubule lattice these molecules may be flexible and have several weak and highly dispersed binding sites.

The reason for the flexible and weak multiple interactions of tau protein with microtubules is unknown but we may speculate that these properties allow tau to regulate microtubule dynamics in the neuron. Tau protein has been shown to stabilize microtubules in vivo (Drubin and Kirschner, 1986). In addition, Drechsel and Gelfand (unpublished results) have found that in tau protein-stabilized microtubule arrays, all microtubules are able to incorporate biotinylated tubulin along their entire lengths. This indicates tau protein can stabilize microtubules without completely suppressing intrinsic polymer dynamics. The putative ability of tau protein to migrate even small distances on the microtubule lattice may help explain how tau protein can function in this manner. It is reasonable to think that a migrating tau protein could transiently bind and stabilize random polymer subunits and thus slow or limit microtubule shrinking phases. Under these conditions, any transiently unbound tubulin subunits at the ends of tubules could still undergo depolymerization. Additionally, one might imagine that a tau molecule tethered near a polymer end could strongly stimulate the recovery of shrinking ends by increasing the local concentration of tubulin monomer and thus the rate of monomer addition. The unusual characteristics of tau binding could also

explain how tau could saturate and stabilize axonal microtubules (Drubin et al., 1985) while retaining sufficient lattice mobility so as not to impede the transient interaction of motor proteins needed for the axoplasmic transport of organelles. Such a function for a microtubule-associated protein may be critical to the ability of the kinetochore to bind and migrate on the ends of microtubules (Mitchison, 1988). Finally, a flexible binding domain is well suited to accommodate the curved and coiled geometries seen in intermediate stages of microtubule polymerization in vitro (Penningroth et al., 1976) or the sharp bending of microtubules seen in growth cones in vivo (E. Tanaka, S. Reinsch, J. Sabry, and L. Evans, unpublished observations). If these unusual properties of tau protein are modified by phosphorylation or are changed by other posttranslational events in disease we might predict important consequences for fundamental neuronal processes such as transport and growth.

We wish to thank a reviewer for urging a more complete discussion of the thermodynamics of bindings, Ken Dill for helpful discussions of configurational entropy, and S. Harrison and D. Agard for helpful discussions about molecular structure. We are also grateful to T. Hyman for help with darkfield microscopy and to T. Mitchison for a critical reading of the manuscript. We particularly thank D. Drechsel for many valuable suggestions and for his generosity in sharing and in helping to generate many of the tau protein reagents.

This work was supported by grant GM26875 from the National Institute of General Medical Sciences.

Received for publication 27 March 1991 and in revised form 21 June 1991.

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