with the experimental data because no isolated α-helices of any length were detected. The peptide, TM8, is an exception, having a negative free energy, and it seems to be unrealistically affected by fluctuations in the computed ΔG_h values.

We stress that the values in Table I represent gross estimates, and the uncertainty in each term can affect the experimental agreement. Our estimates of the hydrophobic interaction, ΔG_h, are virtually identical to those obtained using the buried surface area rule of Chothia (10), i.e., 25 cal of $\Delta G/1$ Å^2 of buried surface. We justify a dielectric constant of 50 because nearly all the atoms in these structures are exposed to water, although it would be appropriate to reduce the dielectric constant for the buried atoms in the supercoil. Most applications of the CHARMM program (5) have used much smaller dielectric constants, which would greatly increase the magnitude of ΔH_e and would eliminate the calculated stability of the supercoil at any length. Variation of the hydrogen bond energy differences within the range cited by Fersht (0.5–1.5 kcal/mol/bond) can shift the length-dependent transition in either direction, as can a different estimate of the configurational entropy, although our estimates correspond to values given by Privalov (11). Finally, using the highest suggested value for the translational entropy loss, 11 kcal/mol, (9) would not greatly affect the experimental agreement.

Our calculation of the energetics of supercoiled α-helices compares well with experimental data. However, the considerable calculational uncertainties make it possible to achieve the same agreement with experiment using different and compensating assumptions. Thus, further comparisons of experimental data with calculations are necessary to improve and refine the empirical free energy function.

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CONFORMATIONAL AND ASSEMBLY PROPERTIES OF NUCLEOTIDE-DEPLETED TUBULIN

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The reversible interaction of guanosine triphosphate (GTP) and guanosine diphosphate (GDP) with the exchangeable E-site of tubulin is central to mechanisms for GTP-supported microtubule assembly. High nucleotide affinities, with $K_a > 10^7$ M^{-1}, and protein instability hinder the study of nucleotide-depleted tubulin. We have, therefore, followed the removal of E-site GDP from microtubule (MT) protein and tubulin dimer with alkaline phosphatase, using HPLC to monitor nucleotide content, and near-ultraviolet (UV) circular dichroism (CD) to follow conformational properties.

We find that the conformational changes associated with nucleotide removal are reversible by adding GTP or GDP, and that GTP restores normal assembly. Most significantly, we show that pyrophosphate (3 mM) induces formation of microtubules from nucleotide-depleted tubulin in the absence of GTP, but does not restore the near-UV CD. We conclude that, contrary to the general view, e.g.

(1), E-site guanine nucleotide triphosphate is not essential for microtubule assembly, but that pyrophosphate can act specifically at the E-site to promote assembly.

RESULTS

Treatment of either microtubule (MT)-protein or tubulin dimer prepared by phosphocellulose chromatography (2) with alkaline phosphatase (AP : 10 IU/mg protein), causes hydrolysis of E-site GDP (3). After 25 min incubation at 20°C, hydrolysis removes 60% or 85% GDP from MT-protein or tubulin, whereas, within experimental limits, there is no loss of N-site GTP from either (unpublished results). The rate constant for loss of GDP from tubulin is 4×10^{-3} s^{-1}. The slower process with MT-protein is consistent with the slower exchange of nucleotide with tubulin in the presence of MAP (4).

After removal of hydrolysis products by G25 gel filtration, the nucleotide-depleted MT-protein, incubated with 1 mM GTP at 37°C, shows 90% assembly competence, as judged by the amplitude of the assembly-related turbidity. With longer AP-treatment, the assembly competence decreases progressively (5), presumably due to the increased instability of tubulin after loss of the E-site GDP.

Fig. 1 shows the effect of nucleotide removal on the CD spectrum of MT-protein. See references 2 and 6 for practical details. After addition of 3 mM pyrophosphate (to inhibit AP hydrolysis) and 50 μM GDP, the CD spectrum is intensified, being restored to 80% of its previous value. As control, pyrophosphate + GDP added to MT protein shows a 20% reduction in intensity, (*d*,*e*), attributed to oligomer dissociation (cf. reference 6). Adding pyrophosphate to nucleotide-depleted protein does not affect the CD spectrum. Thus addition of the GDP to the nucleotide-depleted MT protein effectively fully reverses the effect of hydrolysis of E-site GDP. The same reversal is found with GTP; no CD difference is detected at 20°C between tubulin (or MT-protein) with GTP or GDP.

Observation of the progressive decrease of CD intensity at 280 nm from 0–25 min gives rate constants $3.5 \pm 0.7 \times 10^{-3}$ s^{-1} and $1.7 \pm 0.3 \times 10^{-3}$ s^{-1} for AP-treated tubulin and MT-protein by fitting to a single exponential function. No corresponding changes could be observed in far-UV CD. Thus the loss of near-UV CD parallels the loss of E-site GDP.

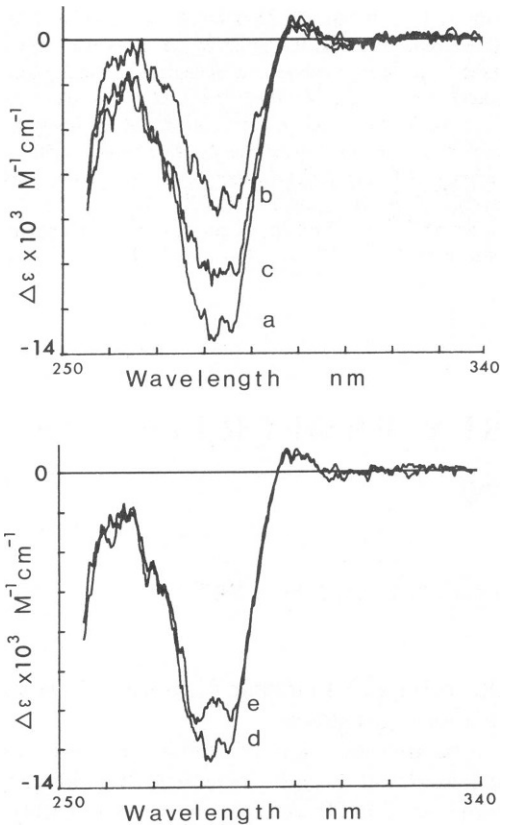

FIGURE 1 (*a*) and (*d*) Near-UV circular dichroism of bovine MT-protein, 1.2 mg/ml at 20°C, in MEM100 buffer (100 mM Mes, 0.1 mM EGTA, 0.5 mM Mg^{2+}, pH 6.5). Curve (*b*), following nucleotide removal with alkaline phosphatase (40 IU/ml; 45 min, 20°C). Curves (*c*), (*e*) after addition of 3 mM pyrophosphate and 50 μM GDP to samples in (*b*) and (*d*).

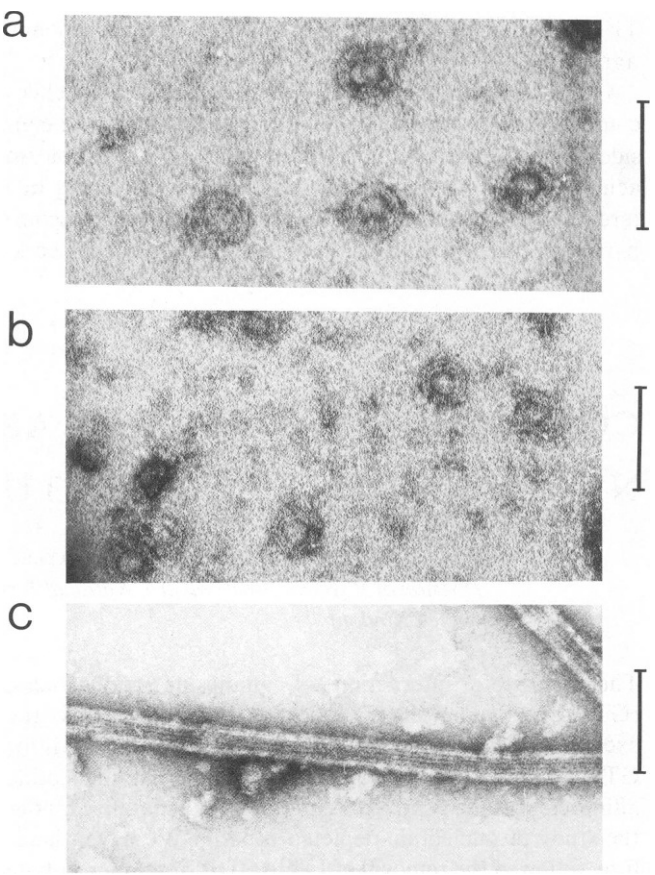

FIGURE 2 Electron micrographs of microtubule protein, negatively stained (as in reference 2). (*a*) MT-protein, 20°C, and (*b*) AP-treated MT-protein, 20°C, as in Fig. 1 *a* and *b*; (*c*) sample (*b*) assembled for 15 min at 37°C, with 3 mM pyrophosphate. The bar denotes 100 nm.

The difference CD (also observed with tubulin dimer) resembles that of the protein, with effects due to the aromatic chromophores; thus nucleotide dissociation causes a conformational change in the protein. Formally, it is possible that nucleotide interaction with a rigid site could generate a protein-based difference CD by strong coupling between the transition dipoles of the nucleotide and those of the aromatic residues. The expected exciton effects of such a mechanism are not observed. Additional evidence (proteolytic susceptibility, and protection by pyrophosphate and GDP) argues against a rigid conformation in the nucleotide-depleted protein.

The wavelength-range of the difference CD suggests predominant effects in tyrosine, as opposed to tryptophan residues, and this is borne out by the absence of any change in the fluorescence spectra. The 325 nm emission maximum of tubulin indicates strongly buried sites for probably all the tryptophan residues (four Trp per subunit). This is supported by the very low level of quenching attainable by external quenchers such as iodide. Both of these properties are largely preserved in the nucleotide-depleted protein. The results suggest that Trp-containing domains remain intact, but that tertiary structural changes occur, involving Tyr residues, when GDP dissociates.

Electron microscopy of the nucleotide-depleted MT-protein at 20°C shows the same ring-like oligomers as found in the control (Fig. 2 *a*, *b*). Sepharose-6B chromatography shows a similar distribution of tubulin between oligomer and dimer. At 37°C, amorphous aggregates are formed from nucleotide depleted MT-protein. Inclusion of 3 mM pyrophosphate, without added GTP, induces substantial formation of microtubules of normal morphology but of short length, indicating efficient nucleation (Fig. 2 *c*). These microtubules are cold, dissociable, and contain tubulin >50% depleted in E-site GDP. Under similar assembly conditions, AP-treated tubulin dimer behaves identically with 3 mM pyrophosphate on addition of MAP, and the microtubules formed are >85% depleted in E-site GDP (unpublished results). Thus, although pyrophosphate does not restore the CD typical of tubulin-GTP or tubulin-GDP, it is nonetheless effective in promoting microtubule assembly. This assembly involves incorporation of tubulin-GDP, probably in oligomeric form with MAP, consistent with the interpretation of biphasic kinetics of assembly of MT-protein (2).

DISCUSSION

These results show that whereas no conformational difference can be demonstrated by CD between reconstituted tubulin-(E)-GDP and tubulin-(E)-GTP under nonassembly conditions, removal of the (E)-nucleotide does cause specific protein conformational changes, which appear to involve tyrosine residues and the protein tertiary structure. These changes are not reversed by pyrophosphate ion, and we conclude that they are due to interactions involving the guanine moiety of GTP or GDP.

Nucleotide-depleted tubulin forms morphologically normal oligomeric species with MAP. Most significantly, it forms normal microtubules in the presence of pyrophosphate, and in the complete absence of any added GTP. This effect is apparently specific to pyrophosphate. These results imply that whereas occupancy of the guanine binding site causes conformational effects, binding at this site is not essential for assembly. We infer that the ability of pyrophosphate to promote assembly is due to its occupancy of the sites corresponding to the binding of the β–γ phosphate portion of GTP. Adoption of an assembly-competent state of tubulin dimer might therefore require no more than minor conformational readjustments from local electrostatic charge-neutralization, because of the simultaneous occupancy of two potentially adjacent positively charged sites by a negative bidentate ligand.

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MOLECULAR RECOGNITION IN MACROMOLECULES

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We are developing algorithms to model macromolecular assembly. Examples of assemblages exist at every level of complexity, ranging from individual proteins through protein:DNA complexes to ribosomal particles. Our initial studies have focused on the protein folding problem, but the approach is suited to multimolecular systems as well.