GTP Analogues Interact with the Tubulin Exchangeable Site during Assembly and upon Binding†

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ABSTRACT: The question of whether nonhydrolyzable nucleotide analogues and other nucleoside triphosphates support tubulin assembly was addressed. Tubulin which contained residual GTP at the exchangeable site polymerized in the absence of added GTP in the presence of DMSO or glycerol. After maximum absorbance was reached, disassembly occurred at a slow rate. When 0.5 mM GMPPCP, GMPPNP, or ATP was included in the assembly reaction, disassembly did not occur, and about 0.1 mol of these nucleotides per mole of tubulin was incorporated into the protein. When 5 mM nucleotide was used or alkaline phosphatase was included in the case of the nonhydrolyzable analogues, a greater amount of assembly occurred and about 0.7–0.8 mol of analogue was incorporated. The products of the assembly reaction were cold-labile microtubules and protofilament ribbons. After cold-depolymerization of the microtubules and ribbons, a second cycle of assembly produced some microtubules, but cold-stable amorphous polymers were the major product. In addition, when GTP at the exchangeable site was first removed by a cycle of assembly, followed by depolymerization, assembly in the presence of GMPPCP, GMPPNP, or ATP produced a mixture of microtubules and cold-stable polymers, both of which contained bound analogue. Incorporation of GMPPCP, GMPPNP, or ATP into polymerized tubulin always occurred at the expense of GDP at the exchangeable site, the content of which decreased correspondingly. Incubation of tubulin with 5 mM GMPPCP, GMPPNP, or ATP under nonassembly conditions also displaced GDP. Binding of GMPPCP was measured under conditions which produced the maximum removal of GDP and GTP from the exchangeable site, and a K_a of (5.3–7.1) × 10^4 M^{-1} was obtained. Studies with other nucleotides showed that assembly in the presence of 5 mM concentrations of ITP, UTP, and CTP led to microtubules and ribbons which did not depolymerize after reaching steady state. In the case of ITP, IDP was found in the polymerized protein, but no evidence for the incorporation of UTP, CTP, UDP, or CDP was obtained. The stabilization by the pyrimidine nucleotides was due to a low level of nucleoside-diphosphate kinase activity present in the tubulin preparation. From these studies, we conclude that several purine nucleoside triphosphates can bind to the exchangeable nucleotide site and, in the presence of microtubule seeds, tubulin containing these nucleotides participates in the elongation phase of polymerization. In the absence of microtubule seeds, polymerization under the conditions used leads to some microtubules, but the major product is an amorphous cold-stable polymer of tubulin. Tubulin with analogues at the exchangeable site, therefore, appears to have a reduced ability to participate in the nucleation phase of the microtubule polymerization process.

Tubulin, the principal protein of microtubules, is a heterodimer which has two guanine nucleotide binding sites (Weisenberg et al., 1968; Yanagisawa et al., 1968), one of which binds guanosine 5′-diphosphate (GDP)[1] or GTP reversibly and is situated in the β-subunit (Geahlen & Haley, 1979; Nath et al., 1985; Hesse et al., 1985). The second site contains a nonexchangeable GTP and is thought to be in the α-subunit. GTP bound to the β-subunit at the exchangeable site (E-site) is hydrolyzed when tubulin assembles into microtubules, and the resulting GDP becomes nonexchangeable in the microtubule wall.

The role GTP at the E-site plays in tubulin assembly and microtubule stability in vitro has been the subject of numerous investigations in recent years. Two important questions central to these topics are whether GTP hydrolysis is required for assembly, and, if so, whether it occurs concurrently with subunit addition. In regard to the second question, several reports indicated that hydrolysis and subunit addition are not tightly coupled and that during the elongation phase of assembly GTP can be found in the E-site of tubulin incorporated into the microtubules (Carlier & Pantaloni, 1981; Caplow et al., 1985), the amount of which depends on the concentration of tubulin used in the assembly reaction (Carlier et al., 1987a). However, other investigators showed that GTP hydrolysis occurred simultaneously with microtubule assembly (Geahlen & Haley, 1979; Hamel et al., 1982). More recent studies also did not provide evidence to support the proposal that hydrolysis and assembly are not coupled and indicated that, if GTP is found in the E-site during the growth of microtubules, it is

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[1] Abbreviations: GDP, guanosine 5′-diphosphate; GTP, guanosine 5′-triphosphate; ATP, adenosine 5′-triphosphate; GMPPCP, guanylyl 5′-(β,γ-methylenediphosphonate); GMPPNP, 5′-guanylyl imidodiphosphonate; Pipes, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; CTP, cytidine 5′-triphosphate; UTP, uridine 5′-triphosphate; ITP, inosine 5′-triphosphate; dTTP, thymidine 5′-triphosphate; MTP, microtubule protein; DMSO, dimethyl sulfoxide; SDS, sodium dodecyl sulfate; HPLC, high-pressure liquid chromatography.

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probably at the very ends, an amount which would be experimentally undetectable (Hamel et al., 1986a; Schilstra et al., 1987; O'Brien et al., 1987; Stewart et al., 1988).

One experimental approach to study the question of the requirement for GTP hydrolysis has been to use the nonhydrolyzable analogues GMPPCP and GMPPNP. Results of such investigations have been as contradictory as those related to the question of whether hydrolysis is coupled to subunit addition. Several studies done with microtubule protein (MTP) indicated that the analogues are capable of supporting assembly, and the authors concluded that GTP hydrolysis is not a requisite for subunit addition (Weisenberg et al., 1976; Arai & Kaziro, 1976; Penningroth & Kirschner, 1977; Karr et al., 1979; Terry & Purich, 1980; Kirsch & Yarbrough, 1981; Manser & Bayley, 1985). On the other hand, others have found that the analogues do not support assembly (Gaskin et al., 1974; Olmsted & Borisy, 1975; Maccioni & Seeds, 1982; O'Brien & Erickson, 1989). In the most recent work on this topic (O'Brien & Erickson, 1989), no evidence that the analogues bind to free tubulin could be found. In addition, although assembly did occur in the presence of the analogues, it was concluded that the assembly they observed, and that reported by others, was actually due to residual GTP bound to the E-site.

In this work, we show that the elongation phase in the assembly of tubulin in solutions containing DMSO, glycerol, or microtubule seeds is supported by GMPPCP, GMPPNP, and ATP and that these nucleotides prevent the disassembly of microtubules which occurs in the absence of GTP. Moreover, these nucleotides are found to be incorporated into polymerized tubulin at the expense of GDP. ITP also supports assembly and behaves like GTP in that it is hydrolyzed during the assembly reaction and IDP is incorporated into the polymers. CTP and UTP stabilize microtubules and protofilament ribbons at steady state, but we were unable to show their incorporation into polymerized tubulin. In addition, binding of GMPPCP to free tubulin is demonstrated, and a K_a of $(5.3–7.1) \times 10^4$ M^{-1} was determined, a value which is about 3 orders of magnitude lower than the K_a for GTP binding.

Experimental Procedures

Materials. Pipes was obtained from Research Organics. GTP, GMPPCP, GMPPNP, ATP, UTP, CTP, ITP, and dTTP were from Boehringer Mannheim. EGTA, glutaraldehyde, and bovine intestinal mucosa alkaline phosphatase (type VII-S) were from Sigma. Taxol was a gift from Dr. Matthew Suffness of the National Cancer Institute.

Preparation of Tubulin. MTP was purified from bovine brain by three cycles of assembly/disassembly according to the method of Shelanski et al. (1973). Tubulin was freed from microtubule-associated proteins by polymerization in a high ionic strength buffer containing 10% DMSO followed by chromatography on a phosphocellulose (Whatman P11)–Biogel P-10 piggyback column as described by Algaier and Himes (1988). The protein was eluted with 0.1 M Pipes buffer, pH 6.9, containing 1 mM EGTA and 1 mM $MgSO_4$(PEM buffer). The purified tubulin was drop-frozen in liquid nitrogen and stored at −80 °C. SDS gel electrophoresis showed the absence of microtubule-associated proteins.

Assembly Reactions. Assembly reactions were carried out in a Peltier temperature-controlled spectrophotometer at 37 °C in PEM buffer. In some cases, 10% DMSO or 25% glycerol was present. The assembly reaction was followed by measuring the apparent absorbance at 350 nm as a function of time.

Assembly in the presence of GTP analogues and 3 mg/mL of tubulin was done by three different protocols. In protocol 1, tubulin was incubated with analogue (usually 0.5 mM) in the presence and absence of alkaline phosphatase (4 units/mL) at 25 °C for 30 min before assembly was initiated by the addition of DMSO or glycerol and changing the temperature to 37 °C. In protocol 2, tubulin was first assembled in 10% DMSO and in the absence of added nucleotide and then depolymerized on ice for 15 min. The protein was then incubated with analogue at 25 °C for 30 min in the presence or absence of 4 units/mL alkaline phosphatase and then reassembled at 37 °C. In protocol 3, tubulin was polymerized in the absence of added nucleotide in 10% DMSO, analogues with or without 4 units/mL alkaline phosphatase were added after the absorbance reached a maximum value, and incubation was continued at 37 °C. GMP, the product of alkaline phosphatase hydrolysis of GDP and GTP, had no effect on the assembly reactions at the concentrations which would have been present in the assembly mixture.

The assembly of tubulin in the presence of analogues was also initiated by the addition of microtubule seeds. The nucleating seeds were prepared by three methods. Thrice-cycled MTP (4 mg/mL); tubulin (4 mg/mL) in the presence of 5 μM taxol, or tubulin in the presence of 10% DMSO were assembled at 37 °C in the presence of 0.25 mM GTP, and the microtubules were sheared by passing the solution several times through a 25-gauge syringe needle. A 40-μL aliquot of the solution was then added to 350 μL of 2 mg/mL tubulin which had been treated with 0.5 mM GMPPCP and alkaline phosphatase as described above (protocol 1) and had undergone one cycle of polymerization to remove GTP at the E-site. The tubulin–GMPPCP solution was prewarmed to 37 °C before the addition of seeds.

Electron Microscopy. For thin-sectioning samples, microtubules were first centrifuged at 37 °C for 4 min in a Beckman TL-100.3 rotor at 200000*g*. The pellet was rinsed carefully with warm 50 mM phosphate buffer, pH 6.8. The pellet was fixed with a 2% glutaraldehyde–2% tannic acid solution in phosphate buffer. Samples were postfixed in 1% OsO_4, dehydrated with acetone, and embedded in Araldite. Sections were collected on uncoated copper grids and stained first with 2% uranyl acetate and 0.3% lead citrate.

For negative staining, samples from an assembly reaction were diluted 15-fold in PEM buffer containing 0.25% glutaraldehyde. The samples were placed on Formvar- and carbon-coated copper grids (300 mesh), washed 3 times with H_2O, and stained with 2% uranyl acetate. Grids were viewed with a Philips 300 electron microscope.

To determine microtubule lengths, an aliquot of the assembly reaction mixture was diluted 25-fold into PEM buffer containing 0.25% glutaraldehyde, placed on carbon- and Formvar-coated grids of 100 and 300 mesh, and negatively stained as described above. Grids were examined and photographed in the scan mode at a magnification of 300×. Measurements of lengths were made from photographs using a Houston HiPad digitizing tablet and Sigma Scan (Jandell Scientific) software.

Nucleotide Analysis. To determine the nucleotide content of polymers assembled under different conditions, 0.35-mL samples were centrifuged through a sucrose cushion (2 mL of PEM buffer containing 50% sucrose) at 37 °C and 200000*g* for 12 min in a Beckman TL-100 ultracentrifuge. (For analysis of GMPPCP incorporation as a function of time of assembly, samples were centrifuged directly at 37 °C and 200000*g* for 2 min in the TL-100 ultracentrifuge.) Pellets were

washed with warm PEM buffer and dissolved in 0.35 mL of cold water. The protein was precipitated with 5% perchloric acid and the precipitate removed by centrifugation. The supernatant containing the nucleotides was treated with 9 μL of a 4 M potassium acetate–10 M KOH solution and the potassium perchlorate precipitate removed by centrifugation. Aliquots of the supernatant were applied to a Partisil SAX-10 anion-exchange HPLC column equilibrated with 0.1 M ammonium phosphate buffer, pH 4.5. Elution was performed with a linear gradient of 0.1–0.75 M ammonium phosphate buffer, pH 4.5. In some cases, an isocratic system was used with 0.4 M NaH_2PO_4–0.2 M NaCl as the elution buffer. Nucleotide concentrations were determined by comparison to a mixture of nucleotide standards eluted from the anion-exchange column. Areas of the nucleotide peaks were measured by the Sigma Scan computer program.

Determination of the Amount of Protein Assembled. To determine the amount of protein assembled, polymers were centrifuged in the TL-100 ultracentrifuge at 37 °C and 200000*g* either for 12 min through a 50% sucrose cushion or, when nucleotide analysis was not needed, directly for 4 min. No differences were observed in the two methods. The pellet was dissolved subsequently in cold PEM buffer and centrifuged at 4 °C and 27000*g* for 10 min. Protein concentration of the supernatant was determined by the method of Bradford (1976).

Binding of GMPPCP to Tubulin. The binding of GMPPCP to tubulin was determined under equilibrium conditions using an Amicon micropartition apparatus with a YMT ultrafiltration membrane. This system separates protein-bound ligands which remain in the retentate (top reservoir) from the free ligands which partition equally between the retentate and filtrate (lower reservoir).

Tubulin in PEM buffer was first incubated with 0.5 mM GDP at 4 °C for 30 min to replace all the GTP with GDP at the exchangeable site. The tubulin–GDP solution (1.0 mL) was centrifuged through Sephadex G-50 in PEM buffer in a 5.0-mL syringe to remove unbound nucleotide. Tubulin–GDP at 5–6 mg/mL was incubated with varying amounts of GMPPCP with or without 4 units/mL alkaline phosphatase present for 30 min at 25 °C.

Binding of the analogue to tubulin was also measured in the absence of Mg^{2+}. Tubulin was first assembled with 0.5 mM GTP and 10% DMSO and centrifuged at 200000*g* for 4 min at 37 °C. The pellet was dissolved in cold 0.1 M Pipes, pH 6.9, and the solution was centrifuged through Sephadex G-50 in the Pipes buffer. Alkaline phosphatase and different concentrations of GMPPCP were then added. Tubulin treated in this manner contained very little GDP (about 0.07 mol/mol of tubulin) and 0.94 mol of GTP. Thus, the E-site was essentially depleted of GDP and GTP. Binding was measured without Mg^{2+} and with 1 mM Mg^{2+} added back.

The sample (0.5 mL) was centrifuged through the micropartition apparatus for 3 min. Both filtrate and retentate were diluted 10–20-fold with the HPLC buffer, and the precipitated protein in the retentate was removed by centrifugation. The concentrations of GMPPCP in the retentate and filtrate portions were quantitated by HPLC as described above. Controls done without tubulin showed that GMPPCP partitioned equally between the filtrate and retentate. The addition of GMP, the product of alkaline phosphatase action on GTP and GDP, did not affect the results.

Results

Hydrolysis of Guanine Nucleotides by Alkaline Phosphatase. Because we used alkaline phosphatase to vacate the E-site (Purich & MacNeal, 1978), we first measured the rate at which the enzyme acts on GTP and GDP under the conditions of our experiments. Although the enzyme requires Zn^{2+} for maximum activity (Fernley, 1971), we found effective hydrolysis in the absence of added Zn^{2+} (the concentration added with the enzyme was 1 μM) and in the presence of EGTA. With 0.5 mM nucleotide, the hydrolysis rates in PEM buffer at room temperature were 10 μM/min for GDP and 8.5 μM/min for GTP. In the absence of Mg^{2+}, but with EGTA present, the rates were reduced only by about 30%. The addition of 10% DMSO reduced the rates by about 40%. To examine the effect of alkaline phosphatase on protein-bound nucleotide, tubulin (30 μM) in PEM buffer was treated with alkaline phosphatase under the conditions of our experiments, 30 min at 25 °C, followed by gel filtration. Before the treatment, the GTP and GDP contents were 1.21 and 0.82 mol/mol of tubulin, respectively. After the treatment, the GTP content was unchanged, but the GDP content decreased to 0.23 mol. Thus, alkaline phosphatase appears to remove GDP preferentially.

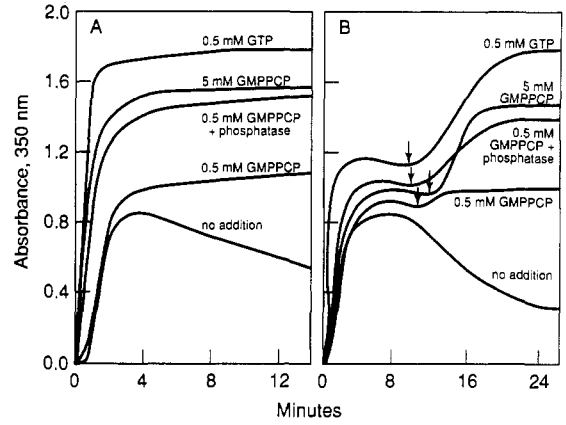

FIGURE 1: Tubulin assembly and microtubule stabilization in GMPPCP. (A) Assembly in the presence of GMPPCP. Tubulin (3 mg/mL) was preincubated with 5 mM GMPPCP or 0.5 mM GMPPCP with or without alkaline phosphatase (4 units/mL) and then assembled in PEM buffer, pH 6.9, and 10% DMSO at 37 °C. In control experiments, tubulin was incubated with or without 0.5 mM GTP before assembly. (B) GMPPCP added after assembly. Tubulin (3 mg/mL) was first assembled at 37 °C in 10% DMSO without added nucleotide. GMPPCP at 5 or 0.5 mM with or without 4 units/mL alkaline phosphatase was added after the absorbance reached the plateau value (at the arrow), and incubation was continued at 37 °C. The controls are the same as in (A). The experiments with 0.5 mM GTP and 5 mM GMPPCP were done with different tubulin preparations than the other experiments.

Assembly of Tubulin in the Presence of GTP Analogues. The results of an assembly experiment done in 10% DMSO at a tubulin concentration of 3 mg/mL after incubation with GMPPCP, in the presence or absence of alkaline phosphatase, are presented in Figure 1A. Similar results were obtained when GMPPNP (with or without alkaline phosphatase) or ATP (without alkaline phosphatase) were used. Tubulin assembled in the absence of added nucleotide, but after a peak of absorbance was reached, slow disassembly occurred, presumably due to the absence of GTP. On the other hand, in the presence of 0.5 mM GMPPCP, GMPPNP, or ATP, the absorbance did not decrease after reaching a maximum value. When alkaline phosphatase was present with GMPPCP or GMPPNP, or when the concentration of the analogue was 5 mM, the absorbance increased to a higher value and also remained constant. Examination of thin-section samples by electron microscopy showed that microtubules and protofilament ribbons were formed (Figure 2A–C). Similar products were obtained when tubulin was polymerized in the presence

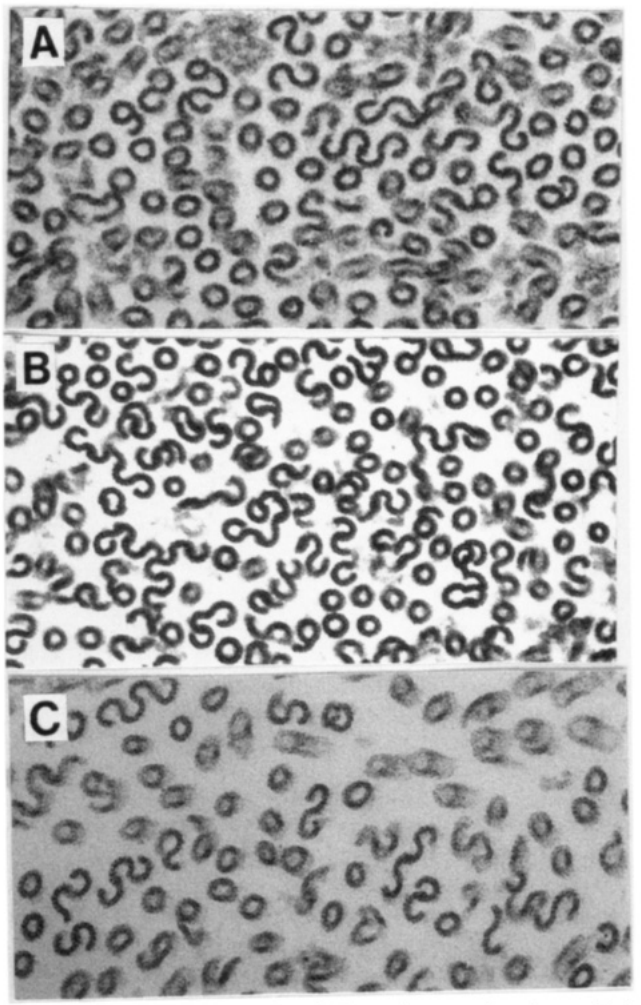

FIGURE 2: Electron micrographs of thin-section samples of assembled tubulin in the presence of analogues. Tubulin (3 mg/mL) was assembled with no added nucleotide (A), 0.5 mM GMPPCP and 4 units/mL alkaline phosphatase (B), and 5 mM GMPPCP (C). All samples were assembled in 10% DMSO.

of GMPPNP and ATP. In one experiment, microtubules formed in the absence of added nucleotide had a mean length of 6.1 μm while those formed in the presence of 0.5 mM GMPPCP and the phosphatase had a mean length of 3.9 μm. In all cases, a change to 5 °C after the plateau absorbance value was reached caused the absorbance to decrease to the value before assembly was initiated. In addition, no pellet was found after centrifugation of the depolymerized solution through sucrose in the cold. These results suggest the absence of any appreciable amount of nonspecific aggregates.

Samples were taken after the maximum absorbance was achieved and centrifuged through sucrose, and the pellets were examined for nucleotide content by HPLC after acid precipitation (Table I). Microtubules and protofilament ribbons which had been formed in the presence of 0.5 mM GMPPCP, GMPPNP, or ATP contained about 0.1 mol of these nucleotides per mole of tubulin. When 5 mM analogue was used, a 6–8-fold increase in the amount of nucleotide in the polymer was observed. Considerably more GMPPCP or GMPPNP was also incorporated into polymerized tubulin if alkaline phosphatase had been included in the incubation before assembly, undoubtedly because the enzyme hydrolyzes GDP released from tubulin. The percent of tubulin polymerized under these different conditions is also presented in Table I. In controls in which 0.5 or 5 mM GMPPCP was added to assembled tubulin immediately before centrifugation through sucrose, no analogue was found trapped in the pellet.

Table I: Nucleotide Content of Microtubules Formed in the Presence of GTP Analogues[a]

analogue added	nucleotide content (mol/mol of tubulin) GTP	GDP	analogue	% of tubulin polymerized
none	1.05	1.09		40
GMPPCP (0.5 mM)	1.09	0.89	0.10	47
GMPPCP (0.5 mM) + alkaline phosphatase	0.98	0.28	0.80	81
GMPPCP (5.0 mM)	1.00	0.24	0.81	75
GMPPNP (0.5 mM)	1.08	0.81	0.12	50
GMPPNP (0.5 mM) + alkaline phosphatase	0.96	0.36	0.67	73
GMPPNP (5.0 mM)	1.06	0.38	0.70	65
ATP (0.5 mM)	0.96	0.96	0.10	68
ATP (5.0 mM)	1.00	0.33	0.64	72
ITP (5.0 mM)	1.02	0.37	0.54 (IDP)	79

[a] Tubulin was assembled as described in Figure 1A. After 15 min, samples were centrifuged through sucrose, and the pellets were examined for nucleotide and protein content as described under Experimental Procedures.

Experiments were also done with ITP, CTP, UTP, and dTTP. At a 5 mM concentration, all except dTTP stabilized the polymers and prevented disassembly from occurring. With ITP present, the increase in absorbance was similar to that found with 0.5 mM GTP, and a significant amount of IDP was found in the polymers (Table I). However, nucleotide analysis failed to detect the incorporation of cytosine or uracil nucleotides into polymerized tubulin. At the end of an assembly reaction with 5 mM UTP, polymers were removed by centrifugation, the supernatant was passed through Sephadex G-50, and the eluted tubulin was examined for nucleotide content. The GDP and GTP contents were 0.72 and 1.32 mol/mol of tubulin, respectively. When no nucleotide was added, the respective values were 1.01 and 1.04. This result indicates the presence of nucleoside-diphosphate kinase activity. The presence of this enzyme activity was shown in an experiment in which 30 μM tubulin was incubated with 5 mM GDP and 5 mM UTP under nonassembly conditions for 30 min at 25 °C. Under these conditions, 2% of the GDP was converted to GTP. When tubulin was assembled in the presence of 5 mM ATP or 5 mM ITP, the unpolymerized tubulin contained 0.4–0.5 mol of GDP and 1.2–1.3 mol of GTP per mole of tubulin, the combined result of the enzyme activity and displacement of GDP by ATP or ITP. The GDP and GTP content of unpolymerized tubulin was 0.50 and 1.04 mol/mol of tubulin, respectively, when assembly was done in the presence of 5 mM GMPPCP.

In the case of GMPPCP-supported assembly in the presence of alkaline phosphatase, we examined the proportion of GMPPCP relative to GDP in polymerized tubulin during assembly and found that it increased during the elongation phase of the reaction. Samples were centrifuged directly for 2 min in a Beckman TL-100 centrifuge. Although the times are not accurate because of the time necessary to begin centrifugation, a sample taken at 2 min (30% of assembly completed) contained 0.4 mol of GDP and 0.6 mol of GMPPCP. After completion of assembly, these values were 0.2 and 0.9, respectively. This result is consistent with the concept that assembly at early stages is accompanied by hydrolysis of GTP at the E-site and that tubulin–GMPPCP adds to preexisting microtubules.

Stabilization of Microtubules after Addition of GMPPCP, GMPPNP, or ATP. In Figure 1A, it was seen that assembled tubulin, formed in the absence of added GTP, depolymerized

Table II: Nucleotide Content of Microtubules Stabilized after Assembly[a]

analogue added	nucleotide content (mol/mol of tubulin) GTP	GDP	analogue
none	0.90	1.0	
GMPPCP (0.5 mM)	1.03	0.81	0.10
GMPPCP (0.5 mM) + alkaline phosphatase	0.99	0.65	0.27
GMPPCP (5.0 mM)	1.08	0.75	0.26
GMPPNP (0.5 mM)	1.07	0.87	0.14
GMPPNP (0.5 mM) + alkaline phosphatase	1.09	0.79	0.29
GMPPNP (5.0 mM)	0.97	0.74	0.33
ATP (0.5 mM)	1.14	1.06	0.09
ATP (5.0 mM)	0.98	0.67	0.35

[a] Assembly of tubulin and the addition of nucleotides were done as described in Figure 1B. After a 15-min incubation with the analogue, samples were centrifuged through sucrose for nucleotide and protein determinations.

after the assembly was complete. Stabilization of the polymers could be achieved by the addition of nucleotides after the maximum extent of assembly was obtained, as shown for GMPPCP in Figure 1B. When alkaline phosphatase was also added or 5 mM GMPPCP was used, the absorbance and the amount of polymerized protein increased. The analogue content of the polymers after these treatments is presented in Table II. The results show an incorporation of the nucleotides which is associated with the increase in polymerization. The lower incorporation than obtained in the experiment in Figure 1A is expected because in the experiment described in Figure 1B, tubulin was first polymerized in the absence of analogue. The amount of incorporated GMPPCP remained constant during a 3-h incubation at steady state. Electron micrographs of samples taken after the additions were similar to those in Figure 2A–C.

Multiple Cycles of Assembly in the Presence of GMPPCP, GMPPNP, or ATP. When tubulin was assembled after incubation with 0.5 mM GMPPCP or GMPPNP and alkaline phosphatase, or with 5 mM analogue, the E-site was depleted of GTP, and most of the tubulin contained the analogue at the E-site (Table I). It was of interest to determine the assembly properties of such tubulin. This was done by examining whether multiple cycles of assembly and disassembly could be achieved. As shown in Figure 3, after cold disassembly of the polymers formed in the presence of GMPPCP and alkaline phosphatase followed by a return to 37 °C, the absorbance increased, but changed little when the temperature was lowered. A third cycle produced little further change. Examination of the second-cycle products in the electron microscope showed microtubules and clusters of dense amorphous-staining material in the sample at 37 °C, but only the amorphous product was present after cold-depolymerization. The relative contribution of microtubules and amorphous material in the second-cycle polymerization product was estimated by centrifuging a sample before and after cold treatment and determining the protein content in the pellets and supernatants. About 20% of the polymerized protein was depolymerized by the cold treatment. Addition of 3 mM $CaCl_2$ after the first polymerization in the presence of 0.5 mM GMPPCP and alkaline phosphatase caused complete depolymerization as measured by the decrease in absorbance. However, the second- and third-cycle polymers were unaffected by the addition of Ca^{2+} at 37 °C.

Nucleotide analysis of the polymers formed after each of the three cycles showed a gradual increase in the GMPPCP content of polymerized tubulin at the expense of bound GDP such that after the third cycle the nucleotide content per mole

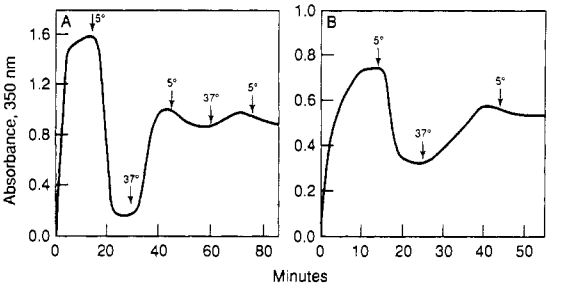

FIGURE 3: Cycles of microtubule assembly/disassembly in the presence of GMPPCP. Tubulin at 3 mg/mL was first treated with 0.5 mM GMPPCP and 4 units/mL alkaline phosphatase and then assembled at 37 °C in the presence of 10% DMSO (A) or 25% glycerol (B). After cold-depolymerization, additional warming and cooling cycles were carried out.

of tubulin was 1.03 GTP, 0.10 GDP, and 0.94 GMPPCP. A similar result was found when GMPPNP or when 5 mM analogues without alkaline phosphatase were used.

Assembly of Tubulin after Depletion of the E-Site GTP. In these experiments, tubulin was first assembled without added GTP and then depolymerized at 0 °C. The nucleotide content of the protein at this point showed that the GTP and GDP content were about 1 mol each/mol of tubulin. This protein was then incubated with 0.5 mM GMPPCP or GMPPNP with or without alkaline phosphatase or with 0.5 mM ATP at 25 °C before being assembled at 37 °C. The product of this assembly reaction with alkaline phosphatase present was a mixture of microtubules and cold-stable amorphous polymers. The products contained about 0.17–0.20 mol of analogue per tubulin dimer when the phosphatase was not used and 0.6–0.7 mol when the enzyme was present.

Assembly in the Presence of Glycerol. The assembly behavior of tubulin treated with GMPPCP and alkaline phosphatase was also observed when DMSO was replaced by glycerol (Figure 3B). In the first cycle, tubulin assembled into microtubules with a smaller proportion of ribbons than found in the DMSO-induced assembly. In the second cycle, cold-stable polymers as well as microtubules were produced. The polymer formed in the first warm cycle contained 0.50 mol of GDP and 0.51 mol of GMPPCP per mole of tubulin while in the second cycle, 0.47 mol of GDP and 0.55 mol of GMPPCP were present.

Assembly onto Microtubule Seeds. The data on the assembly of tubulin in the presence of GTP substitutes can be best explained by the following suggestion. The residual GTP at the E-site is responsible for the formation of seeds to which tubulin–analogue can add. When GTP at the E-site is depleted by one cycle of polymerization, subsequent polymerization of tubulin with analogue present leads primarily to nonmicrotubule, cold-stable polymers. This proposal was tested by adding small quantities of seeds to tubulin which had been pretreated with GMPPCP and alkaline phosphatase. Assembly was done in the absence of DMSO or glycerol. Figure 4 shows the results of an experiment done with seeds produced with taxol. No assembly was achieved unless both the seeds and either tubulin plus GTP or tubulin plus GMPPCP were present. The mean length of the microtubules increased from 5.9 μm (seeds) to 25.8 μm during the assembly with GMPPCP present. Upon cold treatment, the absorbance decreased 95% in the case of tubulin–GTP and 90% in the case of tubulin–GMPPCP. After centrifugation through sucrose, it was determined that the microtubules formed with seeds and tubulin–GMPPCP contained 0.40 mol of GDP, 0.57 mol of GMPPCP, and 1.05 mol of GTP. Similar results were ob-

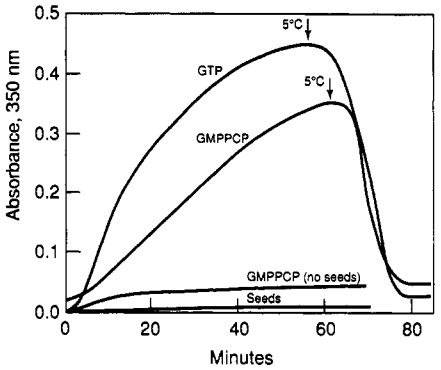

FIGURE 4: Assembly of tubulin onto microtubule seeds in the presence of GMPPCP. Tubulin (2 mg/mL) preincubated with 0.5 mM GMPPCP and 4 units/mL alkaline phosphatase was first depleted of GTP at the E-site by one cycle of assembly and then reassembled by addition of microtubule seeds at a w/w ratio of 0.2 to unpolymerized tubulin. The seeds were prepared by assembling 4 mg/mL tubulin with 0.25 mM GTP and 5 μM taxol, and the microtubules were sheared. As a control, tubulin (2 mg/mL) in the presence of 0.5 mM GTP was assembled onto the seeds. Tubulin–GMPPCP or microtubule seeds alone did not cause an increase in absorbance under the same conditions.

Table III: Depletion of GDP from the E-Site by GTP Analogues[a]

analogue added	nucleotide content (mol/mol of tubulin) GTP	GDP
none[b]	1.21	0.79
GMPPCP[b]	1.23	0.29
GMPPNP	1.30	0.32
ATP[b]	1.28	0.44
ITP[b]	1.24	0.32
UTP	1.32	0.67
CTP	1.27	0.74

[a] Tubulin (30 μM) was incubated with 5 mM analogue in 0.4 mL of PEM buffer at 25 °C for 30 min and then centrifuged through a 3.0-mL Sephadex G-50 column. [b] The mean value of two to three determinations.

tained when seeds were produced from tubulin in DMSO or from MTP.

Displacement of GDP from the E-Site by Nucleotides. Our data indicate that the purine nucleoside triphosphate substitutes bind to the E-site of tubulin. To substantiate this, tubulin was incubated under nonassembly conditions with 5 mM concentrations of the analogues and then passed through a gel filtration column. Table III presents the results of these experiments. After incubation with GMPPCP, GMPPNP, ATP, or ITP, the GDP content decreased, but there was little change in the GTP content, nor did tubulin contain bound analogue. Approximately the same amount of GDP displacement was achieved with a 0.5 mM concentration of the nonhydrolyzable analogues in the presence of alkaline phosphatase. After incubation with UTP or CTP, the total nucleotide content remained at 2 mol/mol of tubulin, and there was a slight increase in the GTP:GDP ratio, undoubtedly the result of nucleoside-diphosphate kinase activity.

Binding of GMPPCP to Tubulin. The results of the polymerization and GDP depletion experiments clearly indicate that purine nucleoside triphosphate analogues of GTP can be incorporated into the E-site of tubulin. However, the fact that the analogue was not found bound to tubulin after gel filtration suggests that the binding by the analogues is weak and/or freely reversible. Therefore, we studied binding under equilibrium conditions, using the micropartition method. Four different conditions were used, in the absence and presence

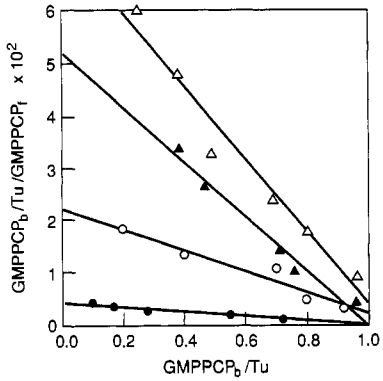

FIGURE 5: Binding of GMPPCP to tubulin. Tubulin (50–60 μM) was depleted of GTP at the E-site, and different concentrations of GMPPCP were added at 25 °C for 30 min in the absence (●) or presence (○) of alkaline phosphatase. In the other two cases, the tubulin (25 μM) solution was depleted of Mg^{2+}, and then GMPPCP and alkaline phosphatase were added at 25 °C for 30 min without (△) or with (▲) 1 mM Mg^{2+} added back.

of alkaline phosphatase in PEM buffer, and in the presence of alkaline phosphatase using Mg^{2+}-depleted buffer with or without Mg^{2+} added back before binding was measured. Binding was easily demonstrated, and the results in the form of a Scatchard plot are presented in Figure 5. The apparent K_a values were 4.2×10^3 M^{-1} (without alkaline phosphatase), 2.1×10^4 M^{-1} (with alkaline phosphatase), 7.1×10^4 M^{-1} (with alkaline phosphatase in the absence of Mg^{2+}), and 5.3×10^4 M^{-1} (with alkaline phosphatase in the absence of Mg^{2+} and Mg^{2+} added back before the binding was measured).

Discussion

The purpose of these studies was to resolve conflicting reports on whether GTP analogues support assembly and bind to the E-site on tubulin. The results presented in this work clearly show that three GTP substitutes, GMPPCP, GMPPNP, and ATP, promote the elongation phase in the assembly of pure tubulin, are incorporated into the E-site of polymerized tubulin, and bind to the E-site of unpolymerized tubulin. Our tubulin preparations usually contain 1.2–1.3 mol of GTP per mole of tubulin and 0.7–0.8 mol of GDP so that in 20–30% of the tubulin dimers, GTP is bound to the E-site. This tubulin assembles in 10% DMSO or in 4 M glycerol without the addition of more GTP, but the microtubules and ribbons depolymerize when the GTP is depleted (Figure 1). Such behavior of tubulin containing GTP at the E-site has been observed previously (Carlier et al., 1987b; Pirollet et al., 1987; O'Brien & Erickson, 1989). When the polymers prepared from tubulin in the absence of added GTP were cold-depolymerized, the resulting tubulin would not repolymerize under the conditions of our experiments, consistent with the view that tubulin–GDP either does not nucleate assembly or has a very high critical protein concentration (Weisenberg et al., 1976; Engelborghs & Van Houtte, 1981; Carlier & Pantaloni, 1982; Carlier et al., 1987a; O'Brien & Erickson, 1989). When 0.5 mM GMPPCP, GMPPNP, or ATP was included in the polymerization solution, disassembly did not occur upon depletion of the GTP [a finding also reported by O'Brien and Erickson (1989)], and a small amount (0.1 mol/mol of tubulin) of the analogue could be detected in the assembled tubulin. When tubulin was first treated with alkaline phosphatase and GMPPCP or GMPPNP, the GDP at the E-site, but not the GTP, was removed, and after assembly, the polymers contained about 0.7–0.8 mol of the analogue per tubulin dimer. A 5 mM concentration of GMPPCP, GMPPNP, or ATP used in the assembly reaction led to the incorporation of 0.6–0.8 mol of

nucleotide into polymerized tubulin. Finally, 5 mM concentrations of these nucleotides displaced an appreciable amount of GDP from tubulin. ITP also displaced GDP from tubulin, and assembly in the presence of 5 mM ITP was accompanied by the incorporation of IDP into the polymers, indicating that this purine nucleotide behaves in a fashion similar to GTP (Duanmu et al., 1986). Although we could not detect incorporation of CTP, UTP, or their hydrolysis products and these nucleotides did not displace GDP from the E-site, they did stabilize the polymers against depolymerization. With our HPLC analysis, we could have detected a content of 0.02 mol/mol of tubulin of these nucleotides. It is likely that stabilization by the pyrimidine nucleotides was caused by a low level of nucleoside-diphosphate kinase activity which constantly regenerates GTP at the E-site of unassembled tubulin. After cold-depolymerization of the polymers formed in the presence of the GTP substitutes, a second cycle of polymerization produced a mixture of microtubules and cold- and calcium-stable polymers.

Our interpretation of these results is that in the first cycle tubulin–GTP can serve to form microtubules (and ribbons) to which tubulin containing different purine nucleoside triphosphates bound to the E-site can add in an elongation process. In this first cycle, all of the GTP at the E-site is hydrolyzed so that in subsequent cycles only tubulin–analogue and tubulin–GDP are present. In this situation, tubulin–analogue polymerizes into a mixture of microtubules and cold-stable amorphous polymers, suggesting that tubulin–analogue has a reduced ability to participate in the nucleation process. Our hypothesis is supported by the experiment in which the GTP at the E-site was replaced by GDP by one cycle of polymerization, before the addition of alkaline phosphatase and GMPPCP. In this case, upon subsequent polymerization, microtubules and cold-stable structures were observed. On the other hand, when microtubule seeds were added to tubulin–GMPPCP, long microtubules were formed, indicating that the elongation reaction took place.

There are reports in the literature (Gaskin et al., 1974; Olmsted & Borisy, 1975; Maccioni & Seeds, 1982; O'Brien & Erickson, 1989) that the nonhydrolyzable analogues of GTP do not support tubulin assembly. However, in several of these cases, no effort was made to remove the E-site GDP or GTP. In most of those reports where assembly was achieved with the analogues, the E-site nucleotide was removed either by gel filtration (Weisenberg, 1976), with charcoal (Penningroth & Kirschner, 1977; Kirsch & Yarbrough, 1981), or with alkaline phosphatase (Karr et al., 1979; Terry & Purich, 1980). We determined an apparent K_a for GMPPCP binding of (5.3–7.1) $\times 10^4$ M^{-1} under the most optimal conditions. [This value is about 10% of that determined by Karr and Purich (1978) using MTP and a fluorometric assay.] In contrast to GTP (Correia et al., 1987), GMPPCP binding appears to be independent of Mg^{2+}. Compared to the values of (5–9) $\times 10^7$ M^{-1} for GTP binding and (1.2–2.7) $\times 10^7$ M^{-1} for GDP binding, both in the presence of Mg^{2+} (Zeeberg & Caplow, 1979; Fishback & Yarbrough, 1984; Correia et al., 1987), it is understandable why removing the E-site GDP or GTP would increase the efficiency of analogue binding and incorporation into polymerized tubulin. The importance of removing the E-site guanine nucleotide in nucleotide analogue studies has been addressed previously (Purich & Kristofferson, 1984).

In a recent study on this subject (O'Brien & Erickson, 1989), the investigators did not remove the E-site nucleotide and found no evidence for analogue binding or incorporation into microtubules, although they did find that the analogues prevented the microtubules from disassembly, implying some interaction with the microtubules. They proposed a separate "polymer binding site" with a low binding constant. Such a site had been proposed earlier by Maccioni and Seeds (1982). The reason O'Brien and Erickson could not detect binding by analogues is probably due to the fact that they measured depletion of GTP from tubulin with GTP bound to both the N- and E-sites after incubation with an 80-fold excess of the analogues. Considering that the GTP affinity is about 1000-fold higher than that of GMPPCP, only a 4% decrease in the total GTP content would have been expected. In one experiment, we also used tubulin which contained 1.81 mol of GTP and 0.12 mol of GDP per mole of tubulin. When this protein (30 μM) was incubated with 2.4 mM GMPPCP (80-fold excess) at 25 °C and desalted, the GTP content was 1.68 mol. However, when it was assembled with 2.4 mM GMPPCP present, the polymerized tubulin contained 1.10 mol of GTP, 0.65 mol of GDP, and 0.34 mol of GMPPCP. Binding studies done at 37 °C showed that the larger amount of analogue incorporated in the assembly experiment than GTP displaced in the binding experiment was due to a temperature effect. Removal of the GTP from the E-site was considerably more effective with alkaline phosphatase present. The GTP content was reduced to 1.33 mole after incubation with 0.5 mM GMPPCP and the enzyme, and after assembly under these conditions, the polymerized tubulin contained 0.88 mol of GMPPCP.

O'Brien and Erickson (1989) suggested that assembly observed by others in the presence of the guanine nonhydrolyzable analogues was actually due to residual GTP at the E-site. Our interpretation is in partial agreement with this, that some E-site GTP is necessary to initiate the formation of microtubules but that tubulin with analogue at the E-site can participate in the elongation phase, and microtubules containing almost exclusively analogue in the E-site can be formed. This is in agreement with an earlier study by Terry and Purich (1980) using MTP. Further, in the presence of DMSO or glycerol, tubulin–analogue alone assembles into cold-stable polymers. Our interpretation can explain why Maccioni and Seeds (1982) found that analogues supported MTP assembly if the charcoal treatment they used did not remove all of the GTP from the E-site but did not support assembly if all the E-site GTP had been removed. Since they did not have DMSO or glycerol in their assembly reactions, they could not have observed a polymerization in the latter case. Also, charcoal treatment of tubulin could have caused some denaturation of the protein, thereby affecting its assembly properties.

When we used ATP in the assembly reaction, we observed an amount of ATP incorporation essentially equal to that of GMPPCP and GMPPNP. Moreover, incubation of 5 mM ATP with tubulin displaced GDP from the E-site. Effects of ATP on the assembly of tubulin have been observed previously. In a series of studies by Zabrecky and Cole (1980, 1982, 1983), the effects of ATP on tubulin assembly and the production of ring aggregates and the labeling of tubulin by 8-azido-ATP were examined. These investigators concluded that ATP binds to a site distinct from the two guanine nucleotide sites. On the other hand, Duanmu et al. (1986) showed that ATP displaced GDP from the exchangeable site and proposed that the effect of ATP on assembly is due to a weak interaction at the E-site. We are in agreement with this latter conclusion. It is interesting that under the conditions used, ATP-supported tubulin assembly was not accompanied by ATP hydrolysis. To determine whether ATP hydrolysis occurred in the polymer

at a very slow rate, we examined the nucleotide content 90 min after initiation of assembly and found no evidence for the formation of ADP.

One puzzling point concerning the relationship between assembly (turbidity increase or amount of polymerized protein) and nucleotide incorporation is that the addition of alkaline phosphatase increased the incorporation of nonhydrolyzable analogue by 7–8-fold but the absorbance and amount of polymerized protein increased by a factor of 2 (Figure 1A, Table I). An explanation for this apparent discrepancy is that in the absence of alkaline phosphatase, tubulin–GDP is incorporated into the polymers. Evidence to support this is that in the absence of added nucleotide about 40% of the tubulin polymerized, even though only about 20% of the dimer contained GTP at the exchangeable site. In the presence of analogue and alkaline phosphatase, or 5 mM analogue, situations where tubulin–GDP is greatly decreased, if 80% of the protein were polymerized, the analogue content would be about 0.75 mol/mol of tubulin (6/8 of the protein would contain the analogue) with only a doubling in the amount of polymerization. The question of whether tubulin–GDP can elongate microtubules has not been completely resolved. Although some feel that it does not (Carlier et al., 1987a; Engelborghs & Van Houtte, 1981; Jameson & Caplow, 1980), evidence from several laboratories indicates that it does under some conditions (Karr et al., 1979; Bayley & Manser, 1985; Manser & Bayley, 1985; Hamel et al., 1986b; Carlier et al., 1987a; Roychowdhury & Gaskin, 1988).

Finally, it is interesting that incorporation of a small amount (0.1 mol/mol of tubulin) of purine nucleoside triphosphate in the E-site stabilizes microtubules at steady state in the absence of GTP. Such stabilization is consistent with some current views on the dynamic instability of microtubules in which it is proposed that a tubulin–GTP (or tubulin–nucleoside triphosphate) cap stabilizes microtubules from depolymerization (Carlier et al., 1984; Mitchison & Kirschner, 1984). Our results are also consistent with the finding that GMPPNP prevents microtubule disassembly during oscillations of assembly and disassembly (Pirollet et al., 1987), which occurs under some solution conditions (Carlier et al., 1987b; Pirollet et al., 1987; Mandelkow et al., 1988).

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Molecular Weight Determination of an Active Photosystem I Preparation from a Thermophilic Cyanobacterium, *Synechococcus elongatus*†

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ABSTRACT: An active photosystem I (PSI) complex was isolated from the thermophilic cyanobacterium *Synechococcus elongatus* by a procedure consisting of three steps: First, extraction of photosystem II from the thylakoids by a sulfobetaine detergent yields PSI-enriched membranes. Second, the latter are treated with Triton X-100 to extract PSI particles, which are further purified by preparative isoelectric focusing. Third, anion-exchange chromatography is used to remove contaminating phycobilisome polypeptides. The purified particles show three major bands in sodium dodecyl sulfate gel electrophoresis of apparent molecular mass of 110, 15, and 10 kDa. Charge separation was monitored by the kinetics of flash-induced absorption changes at 820 nm. A chlorophyll/P_{700} ratio of 60 was found. When the particles are stored at 4 °C, charge separation was stable for weeks. The molecular mass of the PSI particles, determined by measurement of zero-angle neutron scattering intensity, was 217 000 Da. The PSI particles thus consist of one heterodimer of the 60–80-kDa polypeptides and presumably one copy of the 15- and 10-kDa polypeptides, respectively.

In order to find out which polypeptides are indispensable for constitution of an active PSI[1] complex, preparations of isolated complexes are necessary. Purification schemes have been reported for detergent-solubilized photosystem I from chloroplasts of several higher plants (Vernon & Shaw, 1971; Shiozawa et al., 1974; Bengis & Nelson, 1975; Mullet et al., 1980; Argyroudi-Akoyunoglou & Thomou, 1981; Takabe et al., 1983; Vierling & Alberte, 1983; Bassi & Simpson, 1987) and algae (Nechustai & Nelson, 1981; Hiller et al., 1988) as well as from cyanobacteria (Newman & Sherman, 1978; Nakayama et al., 1979; Bishop & Öquist, 1980; Nechustai et al., 1983; Lundell et al., 1985; Ford, 1987; Ford et al., 1987) and a Chl *b* containing procaryote (Schuster et al., 1985).

For the fractionation of photosynthetic pigment–protein complexes, SDS is more efficient than nonionic detergents, allowing PSI to be purified in only one or two steps. It is, however, more aggressive and in several cases has stripped off the small molecular mass polypeptides of the PSI complex, resulting in a complex lacking the iron–sulfur complexes F_A and F_B that serve as secondary electron acceptors (Bengis & Nelson, 1977; Golbeck & Cornelius, 1986). Some of the procedures use SDS to purify PSI in polyacrylamide gels or in a density gradient (Bishop & Öquist, 1980; Argyroudi-Akoyonoglou & Thomou, 1981; Vierling & Alberte, 1983; Ford et al., 1987). In some of these studies the term CPI is used for a pigmented band containing the PSI polypeptides.

The other procedures use nonionic detergents in combination with centrifugational and chromatographic methods (Satoh & Butler, 1978; Boekema et al., 1987; Bruce & Malkin, 1988).

Density gradient fractionation is time consuming. In the present paper we describe a fast purification procedure that replaces the centrifugation step by isoelectric focusing. The second step is anion-exchange chromatography. Several milligrams of highly active PSI particles containing only four polypeptides and about 60 chlorophyll molecules can be prepared by this method within 2 days.

The polypeptide patterns of the PSI particles purified in various laboratories differ considerably. Nevertheless, all contain two large homologous polypeptides. Vierling and Alberte (1983) and Fish and Bogorad (1986) have reported two bands with apparent masses of 58 and 62 kDa, which in other studies were not resolved and migrated as a single band with an apparent molecular mass of up to 70 kDa (Lundell et al., 1985). Under mild conditions of solubilization and electrophoresis, these two polypeptides remain linked as a heterodimer containing chlorophyll, which migrates as a pigmented band at an apparent mass of approximately 110 kDa (Bruce & Malkin, 1988). This heterodimer contains the

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[1] Abbreviations: PSI, photosystem I; kDa, kilodalton; SB12, 3-(dodecyldimethylammonio)-1-propanesulfonate; SDS, sodium dodecyl sulfate; ET12H, 1-dodecylpropanediol-3-phosphocholine; ES12H, 1-dodecanoylpropanediol-3-phosphocholine; DM, *n*-dodecyl β-D-maltoside; IEF, isoelectric focusing; PEG, poly(ethylene glycol); SANS, small-angle neutron scattering; M_r, relative molecular mass; Chl, chlorophyll; cmc, critical micelle concentration; TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine.

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