

Chaperone-like Activity of Tubulin*

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Tubulin, a ubiquitous protein of eukaryotic cytoskeleton, is a building block unit of microtubule. Although several cellular processes are known to be mediated through the tubulin-microtubule system, the participation of tubulin or microtubule in protein folding pathway has not yet been reported. Here we show that goat brain tubulin has some functions and features similar to many known molecular chaperones. Substoichiometric amounts of tubulin can suppress the non-thermal and thermal aggregation of a number of unrelated proteins such as insulin, equine liver alcohol dehydrogenase, and soluble eye lens proteins containing β - and γ -crystallins. This chaperone-like activity of tubulin becomes more pronounced as temperature increases. Aging of tubulin solution at 37 °C also enhances its chaperone-like activity. Tubulin loses its chaperone-like activity upon removal of its flexible hydrophilic C-terminal tail. These results suggest that both electrostatic and hydrophobic interactions are important in substrate binding by tubulin and that the negatively charged C-terminal tails play a crucial role for its chaperone-like activity.

Although it is the amino acid sequence of a protein that determines its final three-dimensional structure of the functional form, it is now well established that many proteins *in vivo* as well as *in vitro* fail to fold correctly in the active form without the aid from a class of proteins known as molecular chaperones (1–4). During the process of their synthesis, translocation, and even during normal functioning in the cell (particularly under stress conditions) proteins are often subjected to structural destabilization. Such conditions are likely to lead to an increase in the propensity for proteins to aggregate, leading to the ultimate death of the biopolymers. Chaperones rescue these unstable conformers by binding to them and eventually through a concerted steps of events, often involving appropriate co-factors and even other molecular chaperones, guide the substrate proteins to the correct folded structure (2, 3).

Molecular chaperones occur ubiquitously in both prokaryotic and eukaryotic cytosol, endoplasmic reticulum, mitochondria, archaeobacterial cytosol, and chloroplasts (2, 5). They comprise several protein families, which are structurally unrelated.

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These classes of proteins includes GroEL, BiP, GRP, TRAP, TRiC, DnaJ gene products, and heat shock proteins such as HSP40, HSP70, HSP90, etc. Molecular chaperones are now known to perform diverse functions not only helping substrate proteins to fold properly but also helping attain the correct fate of the protein *in vivo*, be it proper oligomeric assembly, transport to a particular subcellular compartment, or disposal of unwanted protein by degradation (1, 5). Different chaperones thus have different functions and they act sequentially (4). However, irrespective of their functions, their action requires a common step of binding the unstable non-native conformation of the substrate, preventing the off-pathway reaction that leads to protein aggregation.

The structural features of a protein that make it a chaperone are poorly understood. However, a close observation of the molecular chaperones known up to today reveals some interesting facts. They have a domain containing a bundle of hydrophobic residues suitably located at the surface of the protein enabling it to bind lipophilic substances such as 1-anilino-naphthalene-8-sulfonic acid (ANS) or 4,4'-dianilino-1,1'-binaphthyl-5,5-disulfonic acid, dipotassium salt (bis-ANS) (6–8). Most known chaperones, e.g. GroEL, DnaK, α -crystallin, etc., also have a flexible hydrophilic tail, the absence of which leads to loss of chaperone activity (9, 10). Because of these well separated hydrophilic and hydrophobic domains, many chaperones exist as a micelle-like large oligomeric form. GroEL exists as a 14-mer (9), and TriC is a ring complex having 8–9 subunits per ring (11, 12). Proposed oligomeric structures of α -crystallin that have chaperone-like activity (13) are based on micellar architecture (14, 15). The list of new chaperones discovered is increasing quite steadily.

Tubulin is a cytoskeleton protein that polymerizes to form microtubules. Folding of tubulin in cytoskeleton is assisted by the chaperone TCP-1 ring complex, also known as TRiC (16–18). Tubulin in general seems to have some of the essential characteristics of a chaperone protein such as surface hydrophobic pockets to bind lipophilic molecules (19, 20), a flexible hydrophilic tail especially rich in acidic residues (21), and a heterodimeric assembly of two subunits, α and β (M_r 50,000 each), which can further polymerize into microtubules. These features prompted us to test whether tubulin possesses any chaperone-like activity. The results presented in this communication reveal for the first time that tubulin has chaperone-like properties as it can prevent the aggregation of a number of proteins unrelated in structure or sequence.

EXPERIMENTAL PROCEDURES

Materials—PIPES,¹ EGTA, GTP, insulin, subtilisin, phenylmethyl-sulfonyl fluoride, dimethyl sulfoxide (Me₂SO), dithiothreitol (DTT), and alcohol dehydrogenase from equine liver were obtained from Sigma. All other reagents were of analytical grade.

Purification of Tubulin—Tubulin was isolated from goat brains by two cycles of GTP and temperature-dependent assembly and disassembly in buffer containing 50 mM PIPES, 1 mM EGTA, and 0.5 mM MgCl₂ (pH 7.0) followed by two further cycles in 1 M glutamate buffer (22). The purified tubulin freed from microtubule-associated proteins was extensively dialyzed against 10 mM phosphate buffer to remove any trace of glutamate and stored in aliquots at –70 °C until use. Protein concentrations were determined by the method of Lowry *et al.* (23) using bovine serum albumin as standard.

¹ The abbreviations used are: PIPES, 1,4-piperazinediethanesulfonic acid; DTT, dithiothreitol.

Preparation of Lens Crystallin Fractions—Freshly excised bovine eyes were obtained from a local slaughterhouse. The lenses were surgically removed and homogenized in 20 mM Tris-HCl (pH 7.6) containing 0.1 M NaCl and 0.02% (w/v) NaN_3 . A water-soluble fraction was obtained from the supernatant by centrifuging the homogenate at $27,000 \times g$ for 30 min at 4 °C. The supernatant was passed through a 100-kDa polysulfone membrane using an Amicon stirred cell. The filtrate thus collected was α -crystallin-depleted soluble lens protein, containing a mixture of γ -crystallin and low molecular weight β -crystallin and other minor constituents of soluble lens proteins.

Assay of Protein Aggregation—Insulin dissolved in a minimum volume of 0.02 M NaOH was diluted to the required concentration (0.3 mg/ml) in 100 mM phosphate buffer (pH 7.0). The reduction of insulin was initiated by adding 20 μl of 1 M DTT to 1 ml of the sample in the spectrophotometric cuvette, and the extent of aggregation of the insulin B chain was measured as a function of time at 25 °C by monitoring the apparent absorbance (scattering) at 360 nm in a Shimadzu UV-160 spectrophotometer. Thermally induced aggregation of alcohol dehydrogenase and soluble lens protein fractions were also measured in the same spectrophotometer using a thermostatic cell holder assembly maintained at constant temperature through a circulating water bath. Protein solution and buffer were mixed in the cuvette at room temperature and then placed in the thermostatic cell holder, and apparent absorbance was measured as function of time. For calculating the molar ratio of protein:tubulin resulting in nearly complete protection of aggregation, the molecular masses of tubulin, insulin, alcohol dehydrogenase, and α -crystallin-depleted soluble lens protein were taken as 100, 5.7, 80, and 60 kDa, respectively, of which the last one is an average value for the protein mixtures and not unambiguous (13).

Preparation of $\alpha_s\beta_s$ —Digestion of tubulin with subtilisin was performed at 30 °C in 100 mM phosphate buffer with 1 mM GTP. Subtilisin was taken in the ratio enzyme:protein = 1:100 (w/w). The reaction was terminated by the addition of 1% by volume of 1% (w/v) phenylmethylsulfonyl fluoride in Me_2SO .

RESULTS AND DISCUSSION

Insulin and alcohol dehydrogenase were used as substrates to study chaperone-like activity of many proteins including α -crystallin and HSP proteins. Aggregation of both insulin and alcohol dehydrogenase is suppressed when incubated in the presence of α -crystallin or HSP proteins (24, 25). Reduction of the disulfide bonds between the A and B chains of insulin with dithiothreitol rapidly leads to the aggregation of B chains. To investigate the function of goat brain tubulin, the aggregation of insulin (0.3 mg/ml) in 100 mM phosphate buffer (pH 7.0) was monitored at 25 °C in the absence and presence of varied amounts of goat brain tubulin. As shown in Fig. 1A, curve 1, insulin lost its native conformation upon cleavage of its disulfide bond at 25 °C. Denaturation was accompanied by aggregation. Bovine serum albumin had virtually no effect on the aggregation of insulin (data not shown). When the same experiment was performed in the presence of goat brain tubulin, aggregation was suppressed. At an insulin to tubulin weight ratio of 1:1, aggregation was significantly reduced (Fig. 1A, curve 2). At an insulin to tubulin weight ratio of 1:8, the aggregation was almost completely suppressed (Fig. 1A, curve 3). There was no evidence of any aggregation at a 1:10 weight ratio (data not shown). This complete prevention of aggregation corresponds to a stoichiometric ratio of 1:0.6 for insulin:tubulin. Thermal aggregation of alcohol dehydrogenase was carried out at 37 °C. Tubulin showed a pronounced effect on the prevention of aggregation of alcohol dehydrogenase, and at the alcohol dehydrogenase to tubulin weight ratio of 1:1 corresponding to a stoichiometric ratio of 1:0.8, 80% suppression of aggregation occurred (Fig. 1B, curve 3).

It is known that α -crystallin acts as a molecular chaperone on a native mixture of lens proteins (13). Thus the absence of α -crystallin from the total soluble protein fraction of lens homogenate (consisting of γ -crystallin and low molecular weight β -crystallin fractions and all other low molecular weight soluble components) caused thermal aggregation at 60 °C (13). Addition of α -crystallin to the soluble lens protein fraction

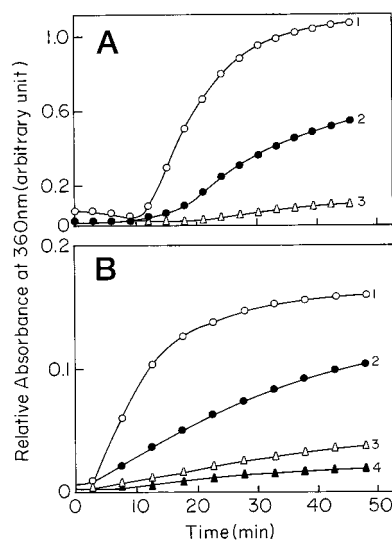


FIG. 1. Aggregation of insulin and alcohol dehydrogenase in the absence and presence of brain tubulin. A, aggregation of insulin (0.3 mg) B-chains was initiated using DTT in 100 mM phosphate buffer (pH 7.0) at 25 °C in a volume of 1 ml with: curve 1, only insulin; curve 2, plus 0.3 mg of tubulin; curve 3, plus 2.4 mg of tubulin. B, aggregation of alcohol dehydrogenase (0.4 mg/ml) in 50 mM phosphate buffer (pH 7.0) at 37 °C in a volume of 1.0 ml with the following conditions: curve 1, only alcohol dehydrogenase; curve 2, plus 0.2 mg of tubulin; curve 3, plus 0.4 mg of tubulin; curve 4, 2 mg of tubulin. Aggregation was monitored by measuring absorbance at 360 nm.

prevented the thermal aggregation. We have tested the effect of tubulin on the thermal aggregation of soluble lens proteins devoid of α -crystallin at 60 °C (Fig. 2). Tubulin showed a pronounced effect on the suppression of aggregation of soluble lens protein fraction even at a weight ratio of lens protein:tubulin as low as 1:0.2 (Fig. 2). Complete suppression of aggregation required the lens proteins:tubulin weight ratio of 1:0.5, corresponding to a stoichiometric ratio of 1:0.3.

Chaperone-like activity of many proteins become more pronounced at a higher temperature. The heat shock protein, HSP90, and α -crystallin bind more effectively to unfolded substrate proteins in the thermally modified form (7, 26–28). We have studied the prevention of thermal aggregation of insulin at 25 °C by tubulin, which was first incubated at 50 °C and then brought back to 25 °C. At an insulin:tubulin ratio of 1:1 (w/w) the prevention of aggregation by control insulin is 50% at 25 °C (Fig. 3A, curve 2). However, when tubulin preincubated at 50 °C was used in the same weight ratio, almost complete prevention of aggregation occurs (Fig. 3A, curve 3). We have also tested the effectiveness of tubulin preincubated alone at 37 °C for different periods of times (aged or thermally modified tubulin) to prevent the aggregation of alcohol dehydrogenase. Results of such an experiment are shown in Fig. 3B. Experiments were carried out at an insulin to tubulin weight ratio of 1:1 (molar ratio, \sim 1:0.06) at 25 °C. Tubulins used were native and 1, 2, 3, and 4 h aged at 37 °C. Although native tubulin inhibits 50% aggregation of insulin at 25 °C, prevention is much more with aged tubulin (Fig. 3B). Thermal treatment of tubulin and its aging at 37 °C are known to expose hydrophobic sites leading to enhanced binding of hydrophobic probes (19, 20). It is suggested that molecular chaperones suppress aggregation by providing appropriately placed hydrophobic surfaces to the denaturing protein substrates. Our observation of enhanced activity of thermally modified tubulin (Fig. 3B, inset) also indicates a direct correlation of chaperone-like activity with exposure of surface hydrophobic groups.

One of the characteristics of many known chaperones is the existence of a flexible tail region. GroEL is known to have

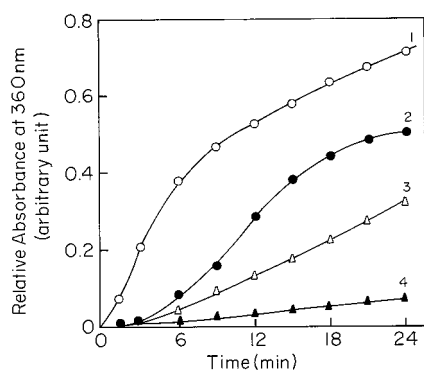


FIG. 2. **Prevention of aggregation of α -crystallin-depleted soluble lens proteins by tubulin.** Aggregation of total soluble fraction of bovine eye lens proteins at 60 °C after the removal of α -crystallin was done under the following conditions: total volume of assay mixture, 1.0 ml; curve 1, 0.3 mg of α -crystallin-depleted soluble eye lens protein; curve 2, plus 0.03 mg of tubulin; curve 3, plus 0.04 mg of tubulin; curve 4, plus 0.06 mg of tubulin.

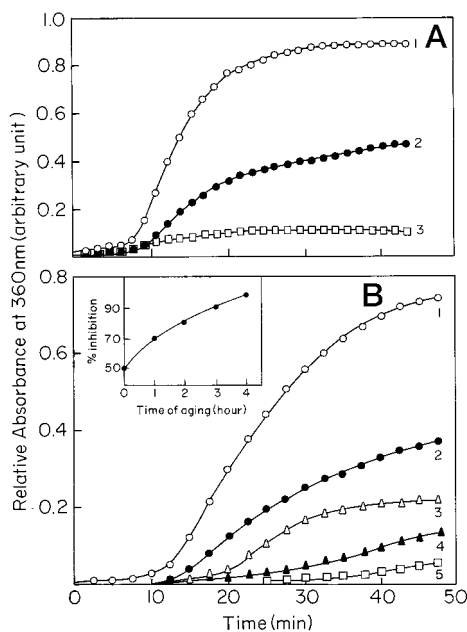


FIG. 3. **Effect of temperature on chaperone-like activity of tubulin.** A, aggregation of insulin in phosphate buffer (pH 7.0) with the following conditions: curve 1, insulin (0.3 mg) only at 25 °C; curve 2, plus 0.3 mg of tubulin at 25 °C; curve 3, plus 0.3 mg of tubulin preincubated first at 50 °C for 10 min and then cooled to 25 °C. The aggregation reaction was monitored at 25 °C. The total volume of the reaction mixture was 1.0 ml. B, chaperone-like activity of aged tubulin. Aggregation of insulin in phosphate buffer (pH 7.0) in a volume of 1.0 ml was done with the following conditions: curve 1, insulin (0.3 mg/ml) only; curve 2, plus 1-h aged tubulin; curve 3, plus 2-h aged tubulin; curve 4, plus 3-h aged tubulin; and curve 5, plus 5-h aged tubulin. In all cases, tubulin concentration was 0.3 mg/ml. Inset shows the relation between the time of aging and the aggregation inhibitory activity of tubulin.

flexible N- and C-terminal residues, which protrude in the central cavity and because of their flexibility are not resolved in the crystal structure (9). It was shown by NMR spectroscopy that α -crystallin and HSP25 have unstructured, flexible, and solvent-exposed C-terminal extensions (25). It was hypothesized that the polar and unstructured flexible C-terminal end plays a critical role in substrate-chaperone interactions and also functions as a solubilizer. In fact, mutation in the C-terminal end, deletion of 17 residues from the C-terminal end (10), or enzymatic truncation of the C terminus caused a marked reduction in chaperone-like activity of α -crystallin (29, 30). C termini of both α - and β -subunits of tubulin are rich in

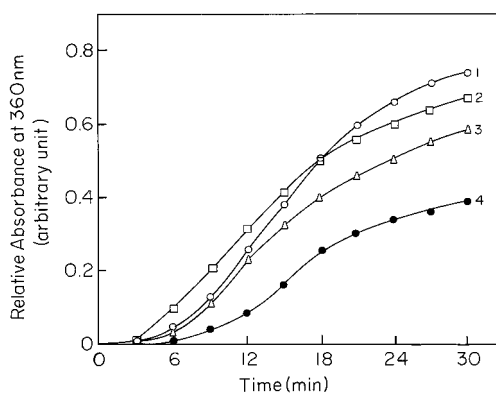


FIG. 4. **Effect of C termini on chaperone-like activities of tubulin.** Aggregation of insulin (0.3 mg) in the absence and presence of brain tubulin was monitored at 360 nm with the following conditions: curve 1, insulin (0.3 mg/ml) only; curve 2, plus 0.3 mg of subtilisin-cleaved tubulin (proteolyzed for 90 min); curve 3, plus 0.3 mg of subtilisin-cleaved tubulin (proteolyzed for 10 min); curve 4, plus 0.3 mg of $\alpha\beta$ -native tubulin.

acidic residues, especially glutamic acid (21). Because of the highest negative charge density, both termini are exposed to solvents, susceptible to proteolysis, and thought to be highly flexible (31, 32). Subtilisin can cleave both C termini when incubated with tubulin at 37 °C. Thus, the obvious question is does the removal of C termini of tubulin change its chaperone-like activity? The results of such an experiment are shown in Fig. 4. Tubulin lost its chaperone-like activity when the C termini of both subunits ($\alpha_s\beta_s$) are removed using subtilisin (Fig. 4). It is interesting to note that complete loss of chaperone-like activity needs removal of the C terminus from both the subunits. When the digestion is partial (Fig. 4, curve 3), tubulin retains some chaperone-like activity.

The functional significance of this finding is not clear at present. In eukaryotic cytosol, folding of proteins including tubulin and actin is mediated by the chaperonin TRiC (16–18). TRiC is known to be present in all eukaryotic cytosol (2). Although TRiC has a double cylindrical architecture very similar to that of GroEL, it is known to mediate the folding of only a very limited subset of proteins including tubulin and actin. This is in sharp contrast to the generally wide selectivity of GroEL (2). The relative abundance of TRiC in many cell types is quite low (5). There is also no evidence to date for the other abundant chaperones in the eukaryotic cytosol such as HSP90 to play any significant role in protein folding (2). It is therefore an open question as to how the majority of the proteins in the eukaryotic cytosol fold. Our finding of the chaperone-like activity of tubulin may have some role in the formation or maintenance of native conformation of cytosolic proteins. The answer to the question whether tubulin itself helps the proteins to fold or just holds the polypeptide temporarily before being taken up by TRiC or some other hitherto unknown chaperone to guide the substrates through folding awaits further study.

In summary, we have identified tubulin as having a chaperone-like function. Like GroEL, HSPs, small HSPs, and other members of the molecular chaperone family, tubulin is able to prevent the irreversible aggregation of proteins under heat shock as well as other conditions. Like other chaperones, its activity increases with a rise of temperature. Its activity is sensitive to the deletion of its flexible hydrophilic tail, a feature known also for other chaperones.

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