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Chaperone-like Activity of Tubulin

BINDING AND REACTIVATION OF UNFOLDED SUBSTRATE ENZYMES*

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The eukaryotic cytoskeletal protein tubulin is a heterodimer of two subunits, α and β, and is a building block unit of microtubules. In a previous communication we demonstrated that tubulin possesses chaperone-like activities by preventing the stress-induced aggregation of various proteins (Guha, S., Manna, T. K., Das, K. P., and Bhattacharyya, B. (1998) *J. Biol. Chem.* 273, 30077–30080). As an extension of this observation, we explored whether tubulin, like other known chaperones, also protected biological activity of proteins against thermal stress or increased the yields of active proteins during refolding from a denatured state. We show here that tubulin not only prevents the thermal aggregation of alcohol dehydrogenase and malic dehydrogenase but also protects them from loss of activity. We also show that tubulin prevents the aggregation of substrates during their refolding from a denatured state and forms a stable complex with denatured substrate. The activity of malic dehydrogenase, α-glucosidase, and lactate dehydrogenase during their refolding from urea or guanidium hydrochloride denatured states increased significantly in presence of tubulin compared with that without tubulin. These results suggest that tubulin, in addition to its role in mitosis, cell motility, and other cellular events, might be implicated in protein folding and protection from stress.

Molecular chaperones consist of several groups of proteins that suppress the aggregation of unstable intermediates of proteins and through well-coordinated interactions with the folding and transport machinery of the cell guide them to their correct fate *in vivo* (1–3). Thus, they are classes of polypeptide-binding proteins that are implicated in protein folding, protein targeting to membranes, protein renaturation, subcellular transport and degradation (2–5). The major classes of chaperones comprise GroEL, DnaK, heat shock protein 70 (HSP70),[1] HSP90, and the small HSPs. The key parameters measured *in vitro* that characterize a protein as a molecular chaperone include: (i) the ability to protect from aggregation during protein unfolding under stress conditions; (ii) the ability to suppress aggregation during protein refolding from a denatured state; and (iii) recovery of lost biological activity. Early work from this laboratory demonstrated that tubulin could efficiently suppress the thermal and chemically induced aggregation of several proteins in the unfolding pathway (6). In the present study, we show that tubulin, in a manner similar to that of some well known molecular chaperones (7–10), suppresses the aggregation of unfolded substrate proteins during refolding pathways, protects the enzymes from loss of activity during thermal stress, and increases the yield of biological activity of enzymes refolding from a fully denatured state. In the present study, model substrates taken for the refolding and reactivation studies are cytoplasmic malic dehydrogenase (MDH), lactate dehydrogenase, α-glucosidase, and alcohol dehydrogenase (ADH). Tubulin binds to the unstable conformers of these proteins during both unfolding and refolding, preventing their nonspecific aggregation. Activity yields of these refolding substrates are enhanced significantly in the presence of tubulin compared with those in its absence. Therefore, we propose that, in addition to their role in cell division, cell motility, and other cellular events, tubulin might assist in protein folding and renaturation in the cytoplasm.

EXPERIMENTAL PROCEDURES

Materials—PIPES, EGTA, GTP, ADH (EC 1.1.1.1), citrate synthase (CS; EC 4.1.3.7), MDH (EC 1.1.1.37), lactate dehydrogenase (EC 1.1.1.27), α-glucosidase, dimethylsulfoxide (DMSO), NADH, NAD, *p*-nitrophenyl α-D-glucopyranoside, and oxaloacetate were obtained from Sigma. All other reagents were of analytical grade. Fluorescine isothiocyanate (FITC) was from Molecular Probes. Sephacryl S-200 HR and Sephadex G-25 were from Amersham Pharmacia Biotech.

Purification of Tubulin and Its Estimation—Goat brain tubulin free from microtubule-associated proteins was prepared by two cycles of temperature-dependent polymerization and depolymerization (11) in a buffer consisting of 50 mM PIPES, pH 7.0, 1 mM EGTA, and 0.5 mM $MgCl_2$ in the presence of 1 mM GTP, followed by two more cycles in 1 M glutamate buffer, pH 7.0, and stored at −70 °C. The concentration of tubulin was determined by the method of Lowry *et al*. (12) using bovine serum albumin (BSA) as standard.

Thermal Aggregation of ADH and MDH and Assay of Enzymatic Activity—Aggregation of 5 μM ADH or MDH in 50 mM phosphate buffer, pH 7.0, with 100 mM NaCl was monitored in the absence or presence of tubulin by measuring the absorbance at 360 nm using a Shimadzu UV-160 spectrophotometer attached to a thermostatic cell holder assembly maintained at 50 °C through a circulating water bath from Neslab. Enzyme activity of ADH was determined by taking aliquots at different times from the assay mixture incubated at 50 °C. Enzyme activity of ADH was assayed in 100 mM phosphate buffer, pH 7.0, containing 0.2 mM NAD^+ and 1 mM ethanol in a final volume of 0.5 ml, and the rate of reduction of NAD^+ was monitored spectroscopically at 340 nm according to the method of Gnagliardi *et al*. (13). An activity assay of MDH was done using NADH and oxaloacetate as substrates (14).

Aggregation Assay of Enzymes Refolding from 6 M Guanidine Hydrochloride Solution—MDH (60 μM) and CS (15 μM) were denatured separately for 1 h in 6 M guanidine hydrochloride (GnHCl). The denatur-

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[1] The abbreviations used are: HSP, heat shock protein; ADH, alcohol dehydrogenase; MDH, malic dehydrogenase; CS, citrate synthase; PIPES, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); FITC, fluorescein isothiocyanate; GnHCl, guanidine hydrochloride; BSA, bovine serum albumin.

ation buffer for MDH was 100 mM sodium phosphate, pH 7.5, and 20 mM 2-mercaptoethanol, and that of CS was 100 mM Tris, pH 7.6, with 20 mM dithiothreitol. Refolding buffers were 100 mM phosphate, pH 7.1, 10 mM Mg-acetate, and 5 mM 2-mercaptoethanol for MDH and 100 mM Tris, pH 7.0, 10 mM $MgCl_2$, and 10 mM KCl for CS. An aggregation assay was performed at 25 °C by measuring the time dependence of light scattering with a Hitachi F4000 spectrofluorimeter with excitation and emission set at 500 nm. The spectral bandwidth was 1.5 nm for both channels.

Refolding Assay of Denatured Enzymes—All denaturation and renaturation reactions were carried out at 25 °C. Malic dehydrogenase was denatured at a concentration of 3 μM in 6 M GnHCl in a buffer containing 100 mM phosphate, pH 7.5, 10 mM EDTA, and 20 mM 2-mercaptoethanol for 1 h. A refolding experiment was initiated on dilution of the denatured enzyme 100-fold in 100 mM phosphate buffer, pH 7.2, with 10 mM Mg-acetate and 5 mM 2-mercaptoethanol. The enzyme concentration during refolding was 0.03 μM. Enzyme activity was assayed (14) at various refolding times. The extent of refolding was calculated by taking the ratio of the activity of the refolded enzyme to the activity of the same amount of native enzyme. Lactate dehydrogenase was denatured at a concentration of 5 μM in 6 M GnHCl for 1 h. Renaturation was initiated by 100-fold dilution in 20 mM Tris, pH 7.5, and 4 mM Mg-acetate. Activity of the enzyme was measured using NADH and sodium pyruvate as substrates (15). α-Glucosidase was denatured at a concentration of 5 μM in 50 mM PIPES, pH 7.5, containing 8 M urea. Refolding was initiated on 100-fold dilution in 50 mM PIPES, pH 7.0, and 10 mM Mg-acetate. Enzyme activity of α-glucosidase was measured as described (16).

FITC Labeling of MDH—The enzyme (80 μM) in 100 mM phosphate buffer, pH 7.5, containing 10 mM EDTA and 20 mM 2-mercaptoethanol was incubated at 4 °C with a 50-fold molar excess of FITC for 1 h. The labeled enzyme was separated from the free FITC by Sephadex G-25 gel filtration chromatography. FITC labeling did not change the enzyme activity or interfere with the denaturation and refolding of the enzyme. The amount of incorporated FITC was 1.2 mol/mol of MDH. Fluorescence measurement of FITC-labeled protein was done using 495 and 520 nm as the excitation and emission wavelengths, respectively.

Preparation of $\alpha_s\beta_s$-Tubulin—Digestion of tubulin (10 μM) with subtilisin was performed at 30 °C in 100 mM phosphate buffer, pH 7.0, in the presence of 1 mM GTP and 0.5 mM $MgCl_2$ (17). Subtilisin was taken in the ratio enzyme/tubulin of 1:100 (w/w). The reaction was terminated by the addition of 1% (w/v) phenylmethylsulfonyl fluoride in dimethylsulfoxide.

Size Exclusion Chromatography—For binding assay of tubulin with FITC-labeled MDH, a Sephacryl S-200 gel permeation column (33 × 1.2 cm) was used. The column was equilibrated in 100 mM phosphate buffer; pH 7.5. FITC-labeled MDH (3 μM) was denatured in 6 M GnHCl in a buffer containing 100 mM phosphate buffer, pH 7.5, 20 mM 2-mercaptoethanol, and 10 mM EDTA for 1 h; 10 μl of denatured FITC-labeled MDH was diluted 100-fold in 100 mM phosphate buffer, pH 7.0, containing 1 μM $\alpha\beta$-tubulin and allowed to refold for 30 min. 300 μl of refolded mixture was loaded in a gel filtration column. The elution profile was automatically recorded on a recorder connected to a UV detector containing a microflow cell.

FITC Fluorescence Measurement—FITC-labeled MDH was denatured in 6 M GnHCl in a buffer containing 100 mM phosphate, pH 7.5, 20 mM 2-mercaptoethanol, and 10 mM EDTA for 1 h; 10 μl of the denatured MDH was diluted 100-fold in 100 mM phosphate buffer, pH 7.0, containing 1 μM tubulin and allowed to refold for 30 min. This refolded mixture was spun through a Millipore microcentrifuge filter with an M_r 100,000 range at 4000 × *g* at room temperature, and it was concentrated severalfold. Fluorescence of both the retentate and the filtrate was measured in a Hitachi spectrofluorimeter at an excitation wavelength of 485 nm with bandwidths of 5 and 20 nm for excitation and emission, respectively. A similar experiment in the absence of tubulin was done as a control experiment. All the experiments were carried out at room temperature.

RESULTS

Tubulin Prevents the Thermal Aggregation and Protects the Activity of Substrate Enzymes—ADH was used as a model system to study the influence of tubulin on heat-induced loss of activity. We incubated ADH at 50 °C in the presence or absence of tubulin, and at different times of incubation, both aggregation and the enzyme activity were measured. As shown in Fig. 1*A*, ADH slowly loses its activity on incubation at this temperature (*trace 1′*). Loss of enzyme activity was accompanied by aggregation (*trace 1*). However, when the experiment was performed in the presence of tubulin, aggregation was prevented (*traces 2* and *3*), and the enzyme activity was protected (*traces 2′* and *3′*). The loss of enzyme activity was ~70% on incubation for 60 min at 50 °C. When ADH and tubulin at a molar ratio of 1:5 were co-incubated, the enzyme retained nearly 90% activity. In the control experiments, it was found that the addition of tubulin to native enzyme did not influence the specific activity of the enzyme. Complete suppression of aggregation and the protection of enzyme activity required ADH/tubulin at a molar ratio of 1:8 both in the absence and presence of ATP or GTP. When 50 μM BSA was used instead of tubulin, neither the inhibition of aggregation nor the protection of enzyme activity was observed (data not shown). These results suggest that tubulin recognizes and binds ADH that unfolds during thermal stress and protects it from irreversible aggregation and loss of enzymatic activity.

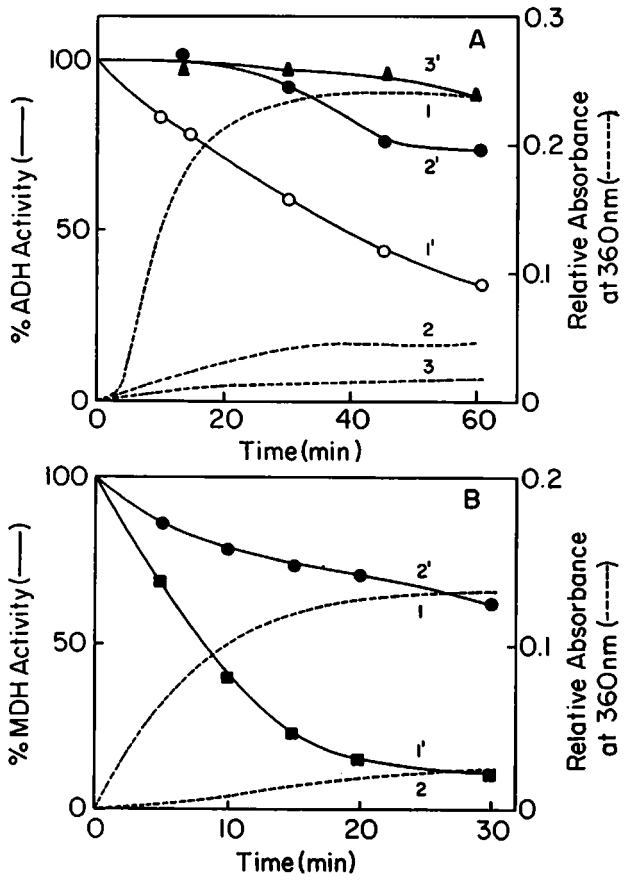

FIG. 1. **Effect of tubulin on thermal aggregation and loss of enzymatic activity of different substrate enzymes.** *A*, effect of tubulin on the aggregation and enzyme activity of ADH during its thermal denaturation at 50 °C. Aggregation profiles are shown by *dotted lines*, and changes of enzyme activity are shown by *solid lines*. *Traces 1* and *1′*, ADH (3 μM) alone; *traces 2* and *2′*, ADH (3 μM) with 6 μM tubulin; *traces 3* and *3′*, ADH (3 μM) with 15 μM tubulin. *B*, effect of tubulin on the aggregation and enzyme activity of MDH during its thermal denaturation at 45 °C. *Traces 1* and *1′*, MDH (3 μM) alone; *traces 2* and *2′*, MDH (3 μM) with 6 μM tubulin.

A very similar result was obtained with MDH (Fig. 1*B*). In this case, heating 3 μM MDH alone at 45 °C caused nearly 90% deactivation (Fig. 1*B*, *trace 1′*) in <30 min with concomitant aggregation (*trace 1*). In the presence of 6 μM tubulin, very little aggregation was observed (*trace 2*), and more than 60% activity was retained (*trace 2′*). These findings are similar to those obtained for many other chaperones (18–20).

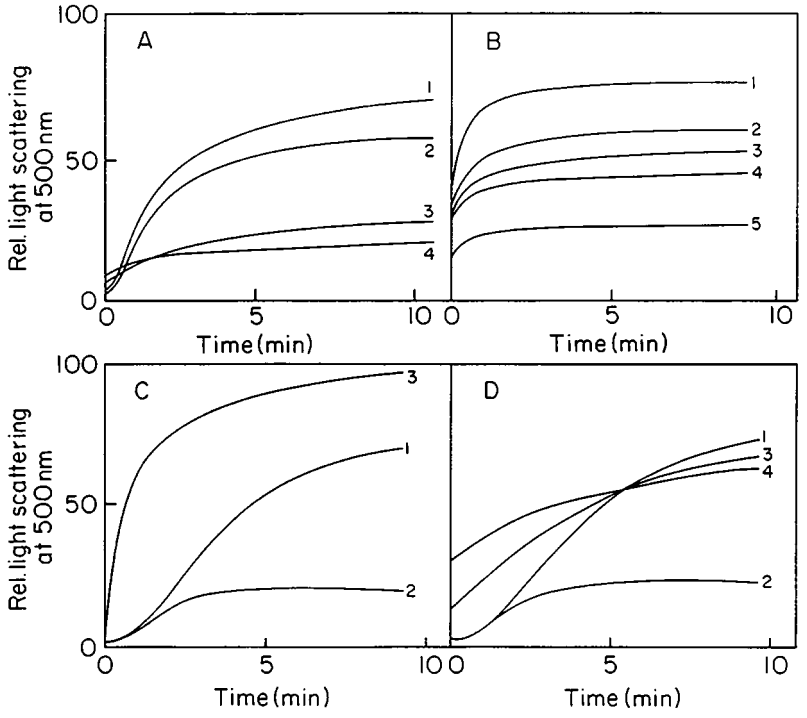

FIG. 2. **Effect of tubulin on the aggregation of different substrate enzymes during their time course of refolding.** *A*, light scattering in the time course of refolding of MDH (1 μM) in the presence of 1 μM (*trace 2*), 3 μM (*trace 3*), and 5 μM (*trace 4*) tubulin and in the absence of tubulin (*trace 1*). *B*, light scattering in the time course of refolding of CS (0.3 μM) in the presence of 1 μM (*trace 2*), 2 μM (*trace 3*), 3 μM (*trace 4*), and 5 μM (*trace 5*) tubulin and in the absence of tubulin (*trace 1*). *C*, effect of $\alpha_s\beta_s$-tubulin on the light scattering in the time course of refolding of MDH. *Trace 1*, 1 μM MDH alone; *trace 2*, with 5 μM $\alpha\beta$-tubulin; *trace 3*, with 5 μM $\alpha_s\beta_s$-tubulin. *D*, effect of tubulin added after various times of refolding of MDH on its aggregation. 5 μM tubulin was added after 10 s (*trace 3*) and 20 s (*trace 4*) of refolding. *Trace 1,* MDH (1 μM) alone; *trace 2,* in the presence of 5 μM of tubulin added before the start point of refolding. All the above experiments were carried out at room temperature.

Tubulin Suppresses Aggregation of Denatured Substrates during the Refolding Pathway—When MDH and CS are diluted 60-fold from their 6 M GnHCl solution, rapid aggregation takes place (Fig. 2, *A* and *B*). This aggregation is highly concentration-dependent. With 2 μM MDH, aggregation occurs with a half-life of <60 s (data not shown). With 30 nM MDH, aggregation is not detectable by 90° scattering measurement at 500 nm using a spectrofluorimeter. We therefore carried out the aggregation assay with 1 μM MDH. Fig. 2*A, trace 1*, refers to MDH alone at a concentration of 1 μM when it was allowed to refold spontaneously by diluting 60-folds in the refolding buffer. The aggregation was found to be suppressed significantly when diluting buffer contained increasing concentrations of tubulin (*traces 2–4*). With 5 μM tubulin, >80% aggregation was suppressed (*trace 4*).

In the case of CS (Fig. 2*B*), the concentration of enzyme in the refolding buffer was 0.3 μM. *Trace 1* refers to the aggregation of CS alone when diluted in refolding buffer. Addition of tubulin in refolding buffer decreased aggregation significantly. In the presence of 5 μM tubulin, >80% aggregation was suppressed (Fig. 2*B, trace 5*). These results indicate that tubulin recognizes and binds conformers of MDH and CS in the refolding pathway, preventing their aggregation. This finding is similar to data reported for the suppression of aggregation of substrates such as CS, rhodanese, and Rubisco during refolding by GroEL system (21–25).

We reported previously that cleavage of the flexible and negatively charged C-terminal tail of tubulin resulted in its loss of chaperone activity (6) as measured on the unfolding pathway. We tested the effectiveness of this C terminally truncated tubulin ($\alpha_s\beta_s$) in preventing aggregation of MDH during refolding. Results presented in Fig. 2*C* show that although 5 μM native tubulin in refolding buffer resulted in very little aggregation of 1 μM MDH (*trace 2*), when native tubulin was replaced by C-terminally cleaved tubulin ($\alpha_s\beta_s$), enhancement of aggregation instead of prevention was observed (*trace 3*).

In the above aggregation experiments (Fig. 2, *A* and *B*), tubulin was added to the refolding buffer before the unfolded substrate was added to it. If tubulin was added 10 or 20 s after the dilution of the GnHCl-denatured MDH, the suppression of aggregation was withdrawn almost completely (Fig. 2*D*). Because aggregation starts almost immediately on dilution, this experiment suggests that tubulin does not recognize aggregated species of substrate but binds to only nonaggregated states of the refolding intermediates. In fact, belated addition of tubulin to aggregating MDH during refolding enhanced the initial rate of aggregate formation (Fig. 2*D*, compare *traces 3* and *4* with *trace 2*).

Tubulin Remained Bound to Denatured MDH—During refolding, tubulin stably associates with the denatured malic dehydrogenase, and their complex could be detected by size exclusion chromatography with a Sephacryl S-200 column. Malic dehydrogenase was labeled with FITC to help quantitate the enzyme even at very low concentration that was used in these experiments. We checked that the activity of the enzyme remained unaltered after the labeling. Labeled MDH was denatured in 6 M GnHCl and then diluted 100-fold in the presence of tubulin and loaded into the column. Details of the experiments are described under "Experimental Procedures." The void volume of the column was 11.3 ml, and free tubulin and the FITC-labeled MDH were eluted at 23.8 and 27.3 ml, respectively. Fractions were scanned for the FITC fluorescence at 520 nm and for the absorbance measurement at 280 nm. The complex of MDH with tubulin was eluted out at 18.9 ml, which is between the void volume and the position for unbound tubulin (Fig. 3). Gel filtration experiments conducted with samples taken at various times of refolding showed no evidence for any dissociation of the complex. The presence of GTP or ATP in the refolding buffer had no effect on the dissociation of the complex (data not shown). A bound MDH-tubulin complex was also detected in refolding experiments followed by a Microcon (Amicon) centrifugation experiment, because FITC-labeled MDH was detected in the retentate (Fig. 4). Had it not been found, it would have escaped through repeated filtration through a 100-kDa membrane. Complex formation between tubulin and MDH is not due to FITC labeling, because the use of nonlabeled MDH showed an identical gel filtration profile. The complex between MDH and tubulin came at the same position (18.9 ml) as with FITC-labeled MDH. The protein peak eluted at 18.9 ml con-

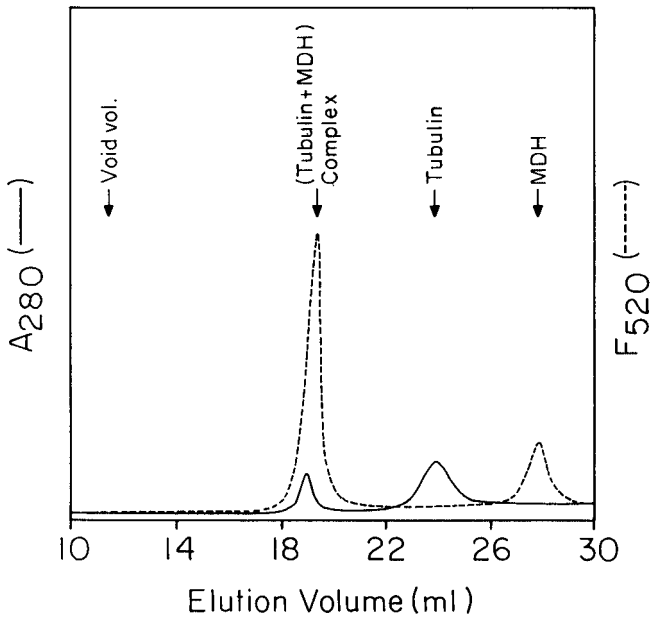

FIG. 3. **Gel filtration chromatography of MDH-tubulin complex.** Gel filtration chromatography of refolding mixtures of FITC-labeled MDH (0.03 μM) with tubulin (1 μM) was performed using a Sephacryl S-200 column. The FITC fluorescence intensity of the fractions collected from the above column is shown by the *dotted line*; the *solid line* refers to absorbance measurement at 280 nm.

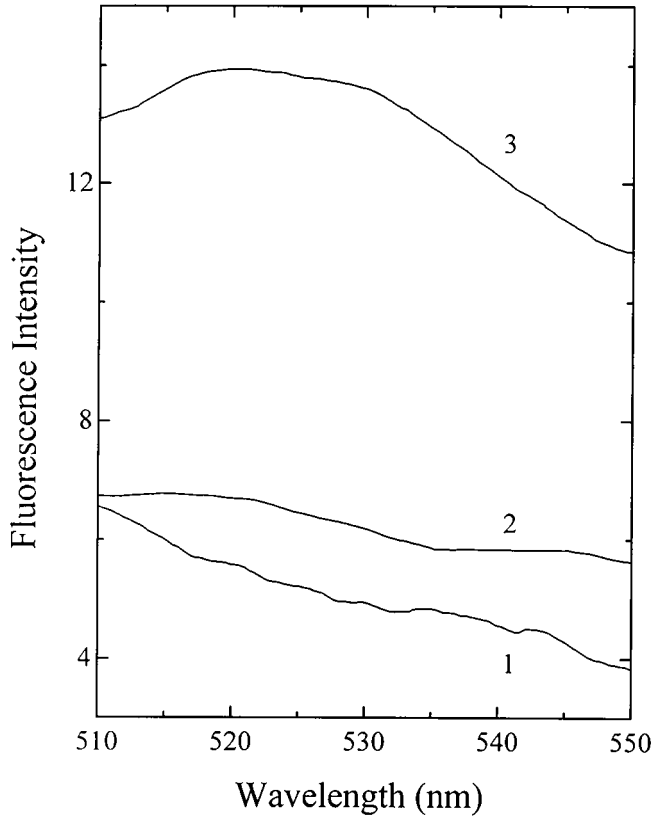

FIG. 4. **Measurement of FITC fluorescence of MDH (0.03 μM)-bound tubulin complexes.** The bound complex formed by FITC-labeled denatured MDH and tubulin was separated from the unbound species by passing the solution through a microcentrifuge filter of M_r 100,000 range. *Trace 2*, MDH alone in the retentate; *trace 3*, MDH with 1 μM tubulin in the retentate; *trace 1*, baseline of the buffer.

tains both MDH and tubulin, as observed on SDS-polyacrylamide gel electrophoresis analysis (data not shown).

Tubulin Promotes the Reactivation of Chemically Denatured

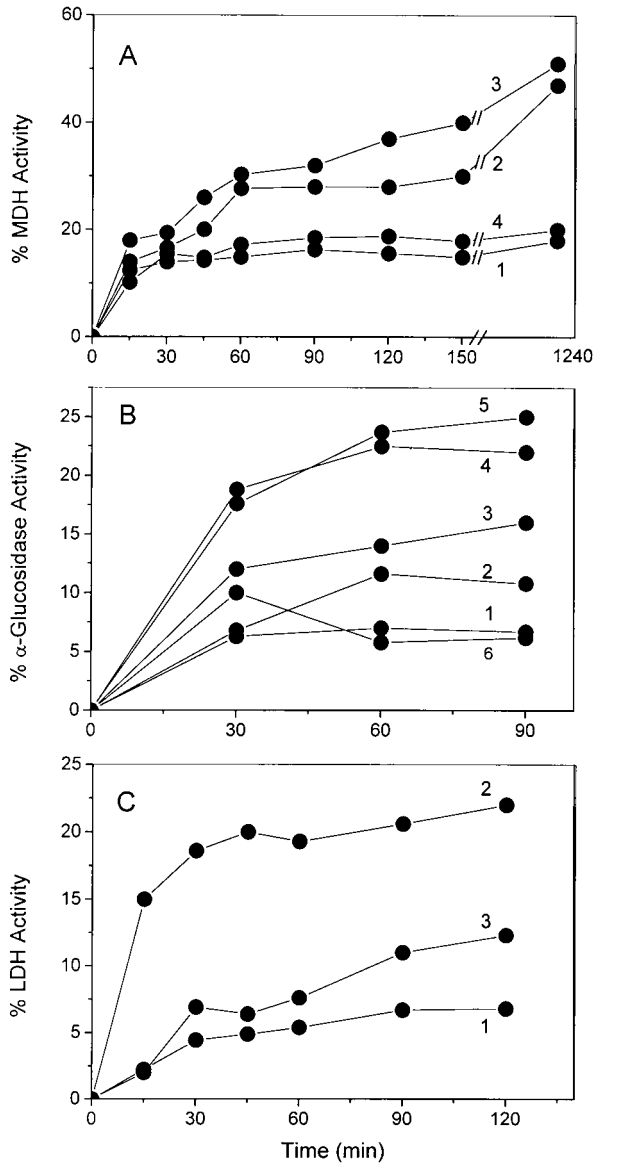

FIG. 5. **Influence of tubulin on the reactivation of different substrate enzymes.** *A*, time course of reactivation of MDH (0.03 μM) in the presence of 0.3 μM tubulin (*trace 2*), 1 μM tubulin (*trace 3*), 1 μM BSA (*trace 4*), and in the absence of tubulin (*trace 1*). *B*, time course of reactivation of α-glucosidase (0.05 μM) in the presence of 0.1 μM (*trace 2*), 0.2 μM (*trace 3*), 0.3 μM (*trace 4*), and 0.4 μM (*trace 5*) tubulin, 1 μM BSA (*trace 6*), and in the absence of tubulin (*trace 1*). *C*, time course of reactivation of lactate dehydrogenase (*LDH*, 0.05 μM) in the presence of 0.5 μM of tubulin (*trace 2*), 0.5 μM BSA (*trace 3*), and in the absence of tubulin (*trace 1*).

Substrate Proteins—Whether the binding by tubulin of refolding substrates from their fully unfolded state promotes its correct folding was investigated by measuring their biological activity after different periods of incubation in the refolding buffer containing tubulin. Under our experimental conditions (as described under "Experimental Procedures"), the refolding yields in the absence of tubulin were 16% for MDH, 6% for lactate dehydrogenase, and 6% for α-glucosidase (Fig. 5). When the same experiment was performed in the presence of tubulin with increasing concentrations, significant increases in yield up to 40% for MDH (Fig. 5*A*), 22% for lactate dehydrogenase (Fig. 5*B*), and 25% for α-glucosidase (Fig. 5*C*) were obtained. The activity yields were dependent on the time of refolding, and maximum activity was obtained in ~1.5 h. BSA facilitates reactivation of several proteins in a nonspecific way (26). How-

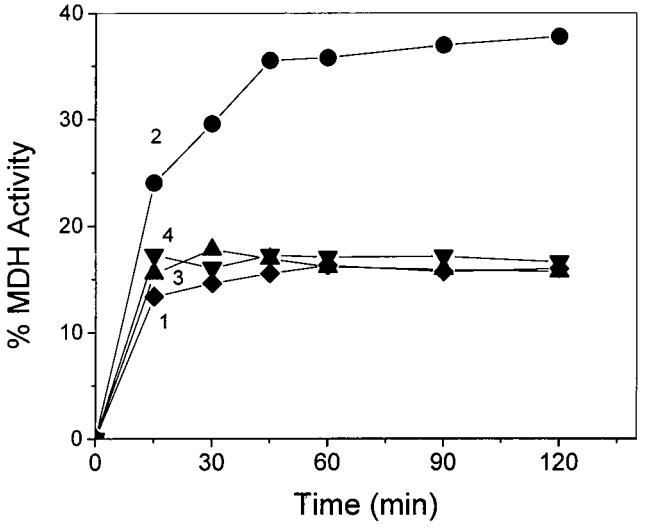

FIG. 6. **Effect of addition of tubulin at various times of refolding of denatured MDH.** 1 μM tubulin was added after 1 min (*trace 3*) and 3 min (*trace 4*) of refolding of denatured MDH (0.03 μM). *Trace 2,* reactivation in the presence of tubulin (1 μM) added before the start point of refolding; *trace 1*, extent of self-folding.

ever, our results show that BSA had an insignificant effect on the refolding of α-glucosidase, MDH, or lactate dehydrogenase. Activity yields of refolding enzymes assisted by the chaperone GroEL were reported to be enhanced (22–24, 27) on addition of ATP, GroES, or both. However, in the present case, additives such as GTP, ATP, and ADP were found to have no influence on the yield of active enzyme. The enhanced folding of MDH did not occur when tubulin was added at different time points after the start point of refolding (Fig. 6). Tubulin was added at 1 and 2 min after the dilution of the denatured MDH; in both cases, the extent of reactivation lay very close to the self-folding extent (Fig. 6, *traces 3* and *4*).

DISCUSSION

We have demonstrated here that tubulin not only prevents thermal or chemical stress-induced aggregation of proteins on their unfolding pathway but also inhibits protein aggregation caused by misfolding during refolding from a fully unfolded state. It binds to the unstable intermediates of the substrate proteins in their unfolding or refolding pathway and eventually through a relatively slow process guides the substrates to its correct folded state. The lost enzymatic activity is thus regained to a considerable extent compared with that without the tubulin. This behavior is very similar to some of the well-studied molecular chaperones.

Our results also show that the mechanism of this chaperone action is through preferential binding of the non-native or denatured states of substrate proteins (Figs. 3 and 4). This binding step is a key step and is common to all the known molecular chaperones (4). Tubulin is known to have distinct domains to interact with both hydrophilic and lipophilic moieties of other molecules (28, 29). Interaction of the exposed hydrophobic regions of unfolding or refolding substrates with those of tubulin reduces the chance of nonspecific hydrophobic association between substrate molecules. Self-aggregation of refolding substrates appears to be faster than the kinetics of tubulin-ssisted refolding (Figs. 2 and 5). It must be remembered that aggregation measured by the 90° scattering technique used here detects relatively large particles (30), which level off before 5 min (Fig. 2). Aggregation at the level of dimer or trimer starts almost instantly. Addition of tubulin just seconds after the initiation of refolding reactions leads to nearly complete loss of chaperone activity (Fig. 2*D*), indicating that tubulin acts only on the unaggregated substrates.

Although the general behavior of tubulin as a chaperone is similar to that of the well known chaperone GroEL in many ways, there are some notable differences. The activity yield of refolding substrates assisted by GroEL was in most cases ATP-dependent (21–25, 27, 31) and gave increased activity in the presence of ATP. In the present case, the *in vitro* activity yield was independent of GTP or ATP. It should be mentioned here that similar ATP-independent promotion of substrate protein folding has been reported earlier (32, 33) for other chaperones such as HSP90 and small HSPs. The role of ATP in the GroEL-assisted refolding is to dissociate the chaperone-substrate complex (2, 3), and the final refolded form is achieved by many cycles of binding and release of the substrate with the help of co-chaperone GroES and ATP (1–3). In the present case, we apparently failed to identify any dissociation of the complex by several means. Gel filtration data (Fig. 3) showed the presence of a bound stable complex. Repeated chromatography of the bound species (tubulin-substrate complex) on the same column showed no dissociation peak of the substrate, even in the presence of GTP and ATP (data not shown). Also, repeated concentration and dilution of the tubulin-MDH complex through a 100-kDa Microcon system showed no presence of free MDH (FITC-labeled) in the filtrate (Fig. 4). Thus our *in vitro* study reveals that the action of tubulin occurs through a substrate binding mechanism with apparently no active mechanism for its release. Nucleolar protein B23 has recently been reported to act as a molecular chaperone through a similar mechanism (34). However, as mentioned by us earlier (4), it should be pointed out that *in vivo* other chaperones, co-chaperones, and molecules may be involved in tandem in such a folding mechanism. Such a concerted mechanism has been accepted for GroEL chaperone function (2, 3). It should also be mentioned that such behavior as observed here is also consistent with the "marsupium" (kangaroo's bag) model (35), according to which the folding of the bound substrates continues within the complex without dissociation from the chaperone until folding is nearly complete. Early observations by others also support this model (27, 36).

We think that our findings have considerable physiological significance. Only a few specific molecular chaperones have been identified on eukaryotic cytosol. Some of them are known to mediate in the folding process of a very limited subset of proteins. For example, TRic is known to help fold tubulin and actin only but not other proteins (37–39). Although TRic is present in all eukaryotic cytosol, its abundance is quite low (2). It still remains an open question of how the majority of the proteins in the cytosol fold. The relatively large natural abundance of tubulin (~15%) in eukaryotic cytoskeleton and its ability to mediate in folding of various proteins throw new light on understanding this aspect. It is possible that other chaperones, not known yet, may also be involved in the folding sequence, but our results prove beyond doubt that tubulin plays considerable role in protein folding apart from its known role in mitosis and cell motility.

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