

# MAP2 prevents protein aggregation and facilitates reactivation of unfolded enzymes

## Implications for the chaperone-like activity of MAP2

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It is well established that in addition to its functional role in cell motility, cell division and intracellular transport, cytoskeletal protein tubulin also possesses significant chaperone-like activity. *In vitro* studies from our laboratory showed that dimeric tubulin can prevent stress induced aggregation of substrate proteins, can resist thermal deactivation of enzymes and can also refold enzymes from their fully denatured state [Manna, T., Sarkar, T., Poddar, A., Roychowdhury, M., Das, K.P. & Bhattacharyya, B. (2001) *J. Biol. Chem.* **276**, 39742–39747]. Negative charges of the C-termini of both subunits of tubulin are essential for this chaperone-like property as the deletion of only  $\beta$ -C-terminus or the binding of a 14-residue basic peptide P2 to the  $\alpha$ -C-terminus completely abolishes this property [Sarkar, T., Manna, T., Bhattacharyya, S., Mahapatra, P., Poddar, A., Roy, S., Pena, J., Solana, R., Tarazona, R. & Bhattacharyya, B. (2001) *Proteins Struct. Funct. Genet.* **44**, 262–269]. Based on these results, one would expect that the microtubular proteins (MTP, tubulin with microtubular-associated proteins, i.e. MAPs bound to the C-terminus) should not

possess any chaperone-like activity. To our surprise we noticed excellent chaperone-like activity of MTP. MTP prevents chemical and thermal aggregation of other proteins and can enhance the extent of refolding of fully unfolded substrate enzymes. Because MTP contains tubulin as well as several MAPs bound to the C-termini of tubulin, we fractionated and purified microtubular associated protein 2 (MAP2) and tau using phosphocellulose chromatography. Experiments with purified proteins demonstrated that it is the MAP2 of MTP that exhibits significant chaperone-like activity. This has been shown by the prevention of dithiothreitol-induced aggregation of insulin, thermal aggregation of alcohol dehydrogenase and regain of enzymatic activity during refolding of unfolded substrates. Tau, which shares a homologous C-terminal domain with MAP2, possesses no such activity.

**Keywords:** aggregation; C-termini; MTP; refolding; tubulin.

Tubulin is an essential cytoskeletal protein present in all eucaryotes as dimer as well as in the polymeric microtubule form. It plays a vital role in cell division, intracellular transport, maintaining cellular morphology and many other cellular events [1,2]. Many of these functions involve complex dynamic interactions [3–5] of microtubules with numerous microtubule-associated proteins (MAPs). MAPs are

believed to control regulation and distribution of tubulin in the cell. They also control the inherent dynamic instability of microtubules due to stochastic switching between growing and shrinking phases [6]. While one category of associated proteins such as katanin, Op18, XKCM1/XKIF2 destabilizes microtubule structure, the classical MAPs such as MAP2, tau, MAP4, etc., reduce microtubule dynamics and act as stabilizers [7]. *In vivo*, the structural and functional properties of microtubules are controlled by phosphorylation of MAPs [3]. MAPs also cross-link and bundle microtubules via their flexible N-terminal domain [7].

Recently, an important property of tubulin has been discovered from our laboratory [8]. *In vitro* experiments demonstrated that dimeric tubulin can act as a chaperone during both unfolding and refolding processes of other proteins [9]. Although, it is not clear what structural features make a protein to function as a chaperone, it is known that many of them possess distinct surface hydrophobic patches [10,11] in their folded structure and hydrophilic [12,13] stretches in the primary sequence. While the hydrophobic domains are involved in substrate binding, stretches of ionic residues are required to keep the complex in solution. Possession of hydrophobic patches on tubulin surface has

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**Abbreviations:** ADH, alcohol dehydrogenase; ANS, 1-anilinonaphthalene-8-sulfonic acid; GmHCl, guanidine hydrochloride; LDH, lactate dehydrogenase; MDH, malic dehydrogenase; MAPs, microtubular associated proteins; MTP, microtubular proteins; Pipes, piperazine-N,N'-bis(2-ethane sulfonic acid).

**Enzymes:** malic dehydrogenase (EC 1.1.1.37), alcohol dehydrogenase (EC 1.1.1.1), L-lactate dehydrogenase (EC 1.1.1.27).

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been well documented by ANS (1-anilinonaphthalene-8-sulfonic acid, potassium salt) and bis-ANS (4,4'-dianilino-1,1'-binaphthyl-5,5-disulfonic acid, dipotassium salt) binding studies [14–18]. Tubulin also possesses negatively charged C-terminal tails. We have recently shown that both the C-termini of  $\alpha\beta$ -tubulin are required for its chaperone-like activity [19]. Thus, the enzymatic deletion of either one or both C-termini or neutralization of negative charges through binding of specific basic peptides to one or both of the C-termini inactivates the chaperone-like function of tubulin. Although tubulin exists *in vivo* in an extremely complex form with numerous bound proteins, it is physiologically relevant to determine *in vitro* if the binding of MAPs to the C-terminal region of tubulin adversely affects the chaperone-like function of tubulin. The role of the individual MAPs in chaperone-like function also needs to be explored.

The results presented in this communication are interesting as MTP shows chaperone-like activity, both by prevention of aggregation as well as assisting the refolding of urea unfolded substrate enzymes. Experiments with different fractions of MTP indicate that the heat stable MAP2 prevents aggregation of insulin and assists refolding of unfolded proteins. Purified tau proteins neither prevent aggregation nor assist refolding. These results clearly demonstrated that MAP2 possesses chaperone-like activity.

## Materials and methods

### Materials

Pipes, EGTA, GTP, GDP, L-lactate dehydrogenase (LDH, EC 1.1.1.27), malic dehydrogenase (MDH, EC 1.1.1.37), alcohol dehydrogenase (ADH, EC 1.1.1.1), bovine serum albumin, NADH, NAD,  $\alpha$ -glucosidase,  $\alpha$ -D-glucopyranoside, oxaloacetate, insulin, subtilisin, dimethyl sulfoxide, phenylmethylsulfonyl fluoride and dithiothreitol were obtained from Sigma Chemical Co. (St. Louis, MO, USA). All other reagents were of analytical grade.

### Purification of microtubular proteins (MTP), tubulin, MAP2, MAP2c and Tau proteins

MTP was isolated from goat brain by two to four cycles of temperature-dependent assembly and disassembly processes [20] in 50 mM Pipes buffer (pH 7.0) containing 1 mM EGTA, 0.5 mM  $MgCl_2$  and 1 mM GTP. Purified MTP was either dialyzed against 20 mM phosphate buffer (pH 7.0) and stored at  $-70^\circ C$  or used directly in the experiment.

Tubulin was purified from MTP using two more cycles of temperature-dependent assembly and disassembly processes in 1 M glutamate buffer at pH 7.0 [21]. The purified tubulin, free from microtubule-associated proteins, was extensively dialyzed against 20 mM phosphate buffer (pH 7.0) to remove any trace of glutamate, and stored in aliquots at  $-70^\circ C$  until use.

MAP2 was isolated by heat treatment of MTP [22]. Heat stable MAPs (containing MAP2 and tau only) were further fractionated by phosphocellulose column to obtain the pure MAP2. Protein concentrations were determined by the method of Lowry *et al.* [23] using bovine serum albumin as standard.

pET 3a rMAP2c clone was transformed into *Escherichia coli* strain BL 21(DE 3). It was induced by isopropyl thio- $\beta$ -D-galactoside and the overexpressed protein was finally purified by SP ion-exchange chromatography [24]. The purity of the protein was verified by SDS/PAGE. Protein concentration was determined by the method of Lowry *et al.* [23].

Recombinant full-length tau polypeptides were expressed in BL21 (DE3) cells in *E. coli* using the pET vector expression system of Novagen (Madison, WI, USA). The coding sequence from the adult rat tau cDNA encoding the full-length 4R rat tau [25] was subcloned into the *NdeI* site of the pET-3c vector and introduced into BL21 (DE3) cells. For a more detailed method, see Panda *et al.* [26]. The purified tau protein was concentrated and the concentration was estimated by the method of Lowry *et al.* [23] and then stored at  $-70^\circ C$ .

### Preparation of $\alpha\beta_s$ and $\alpha_s\beta_s$

Digestion of tubulin with subtilisin was performed in 100 mM phosphate buffer (pH 7.0) containing 1 mM GTP following a published procedure [27,28]. Subtilisin was taken in the ratio enzyme/protein 1 : 100 (w/w). Digestion at  $4^\circ C$  yielded  $\alpha\beta_s$  and at  $30^\circ C$   $\alpha_s\beta_s$ . The reaction was terminated by adding 1% by volume of 1% (w/v) phenylmethylsulfonyl fluoride in dimethyl sulfoxide.

### Enzymatic digestion of MAP2

Trypsin digestion of MAP2 was carried out for 30 min at  $30^\circ C$  at enzyme to protein ratio of 1 : 100 (w/w) in 50 mM Pipes buffer (pH 7.0) containing 1 mM GTP. The reaction was terminated by adding three times the weight ratio of trypsin inhibitor in the same buffer.

### Assay of protein aggregation

Insulin was dissolved in minimum volume of 0.02 M NaOH and diluted to the required concentration with 100 mM phosphate buffer at pH 7.0. The reduction of insulin ( $0.3\text{ mg}\cdot\text{mL}^{-1}$ ) was initiated with 20 mM dithiothreitol in a spectrophotometer cuvette and the extent of aggregation of the insulin B chain was measured as a function of time at room temperature by monitoring the apparent absorbance (light scattering) at 360 nm in a Shimadzu UV-2401 PC spectrophotometer (Shimadzu Corporation, Kyoto, Japan). Thermally induced aggregation of ADH was measured in a Shimadzu UV-160 spectrophotometer attached with a thermostatic cell holder assembly maintained at constant temperature ( $45^\circ C$ ) through a circulating water bath from Neslab (Neslab Instruments Inc., Portsmouth, NH, USA). The molar masses of tubulin, insulin and ADH were taken as 100, 5.7 and 80 kDa, respectively.

### Refolding assay of denatured enzymes

LDH was denatured at a concentration of  $5\text{ }\mu\text{M}$  in phosphate buffer (pH 7.0) containing 6 M GmHCl for 1 h. Refolding was initiated by 100-fold dilution in 100 mM phosphate buffer (pH 7.0). The enzyme activity was measured using NADH and sodium pyruvate as substrates [29].

$\alpha$ -glucosidase was denatured at a concentration of 6  $\mu\text{M}$  in 50 mM Pipes (pH 7.5) containing 8 M urea. Renaturation was initiated on 100-fold dilution in 50 mM Pipes (pH 7.0) and 10 mM magnesium acetate. Enzyme activity of  $\alpha$ -glucosidase was measured as described by Kopetzki *et al.* [30]. MDH was denatured at a concentration of 3  $\mu\text{M}$  in 8 M urea in 100 mM phosphate buffer (pH 7.5), 10 mM EDTA and 20 mM 2-mercaptoethanol for 1 h. The refolding was initiated on 100-fold dilution of the denatured enzyme in 100 mM phosphate buffer (pH 7.2) with 10 mM magnesium acetate and 5 mM 2-mercaptoethanol. Enzyme activity was assayed at various times during refolding using NADH and oxaloacetate as substrates [31]. All denaturation and renaturation reactions were carried out at 25 °C. 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.

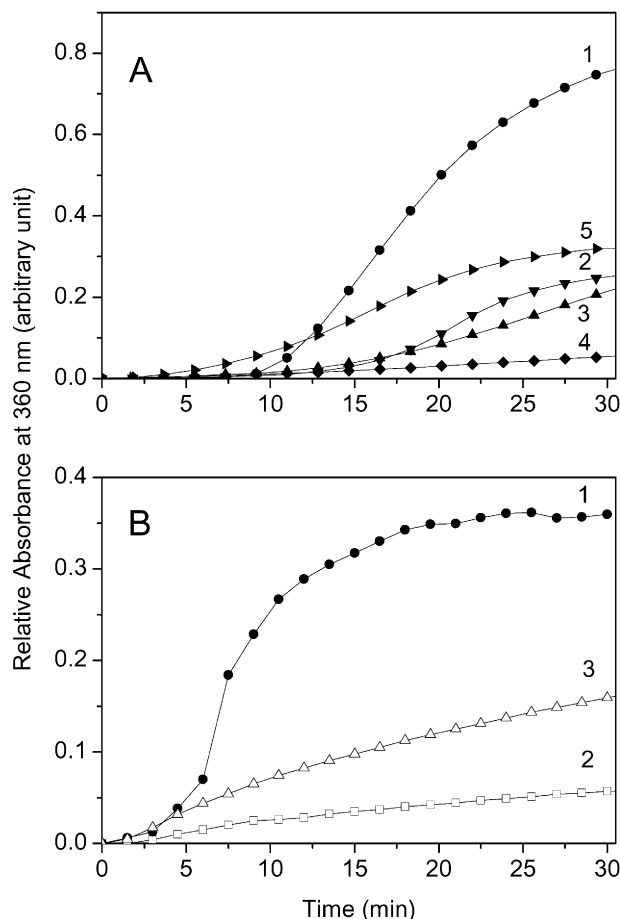
### Fluorescence experiments

The protein (0.2  $\text{mg}\cdot\text{mL}^{-1}$ ) was incubated with 10  $\mu\text{M}$  ANS in 20 mM phosphate buffer (pH 7.0) for 15 min. The fluorescence emission of the complex was scanned between 400 and 550 nm in a Hitachi F-3000 spectrofluorimeter (Tokyo, Japan) maintained at a constant temperature (25 °C) through a circulating water bath from Neslab. The excitation wavelength was 350 nm.

### Results

The existence of a flexible C-terminal tail has been reported as a characteristic structural feature of many known chaperones. Such a tail has been well documented in  $\alpha$ -crystallin, TRiC, thermosome and many other chaperones [32]. Deletion of the tail abolishes the chaperone function of these proteins. The importance of the acidic residue-rich tails of tubulin in its chaperone-like activity was demonstrated by the loss of its chaperone-like activity upon cleavage of its negatively charged C-termini by subtilisin digestion [8]. The role of the C-terminus of individual subunits has been reported recently [19]. Thus, we demonstrated that the digestion of  $\beta$ -C-terminus ( $\alpha\beta_s$ ) or C-terminus of both subunits ( $\alpha_s\beta_s$ ) or even the neutralization of negative charges through binding of a specific basic peptide (P2) to one or both C-termini inactivates tubulin chaperone-like function [19]. The results apparently implied that both C-termini of  $\alpha\beta$ -tubulin have to remain free for activation of its chaperone-like function.

If the negative charges of C-termini of tubulin are inevitable for its chaperone-like activity, then one expects that the microtubule proteins (MTP) containing tubulin with several basic microtubule associated proteins (MAPs) bound to its C-termini, should possess no or poor chaperone-like activity. The results obtained are quite surprising. Figure 1A shows the aggregation assay using dithiothreitol-induced aggregation of insulin at 25 °C. In all cases, 0.3  $\text{mg}\cdot\text{mL}^{-1}$  insulin was used and MTP concentrations were varied. Using the MTP to insulin ratios (w/w) of 0.55, 1.1 and 2.2, the inhibitions of insulin aggregation were observed to be 60, 70 and 90%, respectively (curves 2, 3 and 4). With pure tubulin near identical experimental conditions and with insulin to tubulin ratio of 1 : 1 (w/w), the



**Fig. 1. Insulin and ADH aggregation in the presence of MTP.** (A) Aggregation of insulin in presence of MTP: Curve 1 (●), insulin only; curve 2 (▼), insulin + MTP (0.165  $\text{mg}\cdot\text{mL}^{-1}$ ); curve 3 (▲), insulin + MTP (0.33  $\text{mg}\cdot\text{mL}^{-1}$ ); curve 4 (◆), insulin + MTP (0.66  $\text{mg}\cdot\text{mL}^{-1}$ ); curve 5 (►), insulin + tubulin (0.3  $\text{mg}\cdot\text{mL}^{-1}$ ). (B) Aggregation of alcohol dehydrogenase (ADH) in presence of MTP: Curve 1 (●), ADH only; curve 2 (□), ADH + MTP (0.22  $\text{mg}\cdot\text{mL}^{-1}$ ), curve 3 (△), ADH + tubulin (0.2  $\text{mg}\cdot\text{mL}^{-1}$ ). Insulin aggregation was initiated by adding 20 mM dithiothreitol, and the ADH aggregation was achieved by heating at 45 °C using a constant temperature water-circulating bath. The concentration of insulin and ADH were 0.3  $\text{mg}\cdot\text{mL}^{-1}$  and 0.4  $\text{mg}\cdot\text{mL}^{-1}$ , respectively.

inhibition of insulin aggregation is 60% (curve 5). These results indicate that the MTP possess chaperone-like activity. The effect of MTP on the thermal aggregation of alcohol dehydrogenase at 45 °C has been tested and the results are shown in Fig. 1B. At 1 : 0.55 (w/w) ratios of ADH to MTP, 80% prevention of ADH aggregation was observed (curve 2), whereas near identical conditions with pure tubulin prevention of ADH aggregation was only 65% (curve 3). Thus, MTP appears to be somewhat more potent compared to tubulin in preventing thermal and chemical aggregation of proteins.

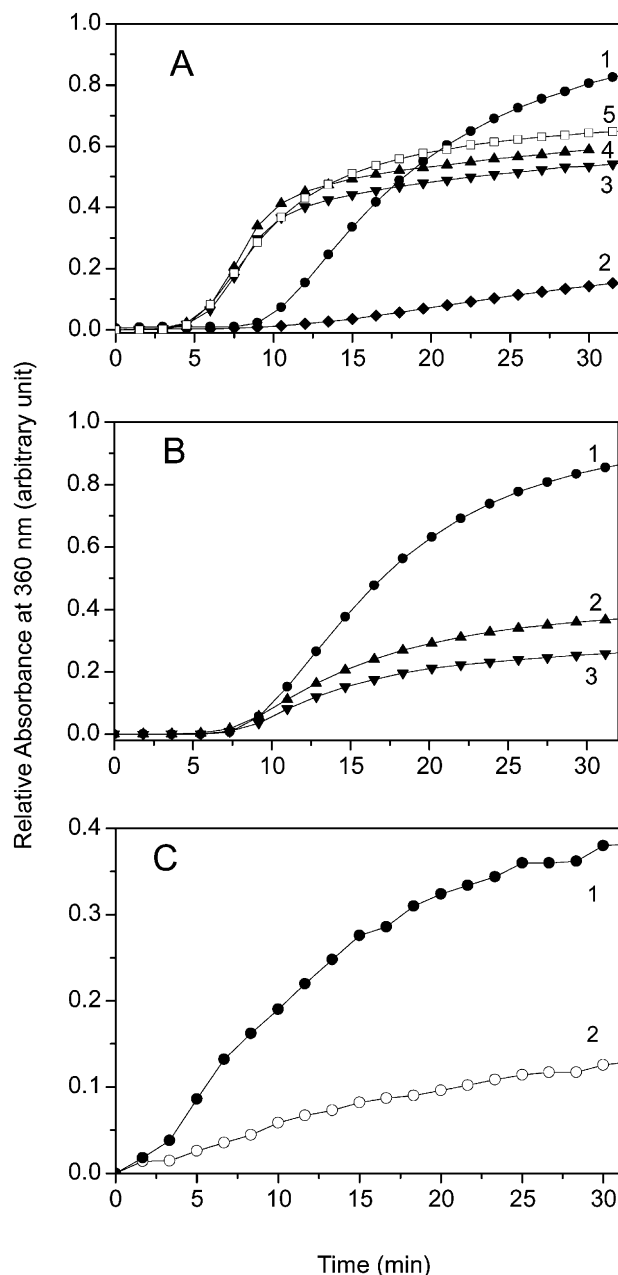
MTPs, used here for the aggregation assay, contain several proteins; approximately 80–85% tubulin and 15–20% MAPs [20,33]. MAPs are again a mixture of proteins containing predominantly MAP2 and tau with traces of MAP1, MAP4, etc. [33]. We checked the

chaperone-like activity of purified recombinant tau and tubulin-tau complex using dithiothreitol-induced insulin aggregation assay (Fig. 2A). Here, curve 1 is insulin alone and in the presence of 1 : 4 ratio (w/w) of insulin to tubulin the inhibition of insulin aggregation is about 80% (curve 2). Insulin aggregation is not affected by the presence of tau as shown in curve 5. However, if tubulin is pretreated with increasing concentration of tau and tubulin-tau complexes are tested for the chaperone-like activity, inhibition of insulin aggregation is gradually reduced (curves 3 and 4). More than 90% chaperone-like activity of tubulin is lost at tubulin/tau ratio 1 : 0.04 (w/w). These results indicate that the neutralization of negative charges of C-termini of tubulin is responsible for the above observation.

Another major constituent of MTP is MAP2. We purified MAP2 from the boiling fraction of MTP [22] where MAP1 precipitated. Figure 2B shows the results of investigation of chaperone-like activity of MAP2 using dithiothreitol-induced aggregation of insulin at 25 °C. Here, the inhibition of aggregation is about 70% in the presence of 0.1 mg·mL<sup>-1</sup> MAP2 (insulin/MAP2 1 : 0.33) (w/w) (curve 3). Identical results are obtained using the MAP2 purchased from Sigma Chemical Co., USA (data not shown). The effect of MAP2 on thermal aggregation of alcohol dehydrogenase at 45 °C has also been tested. Figure 2C shows that at 1 : 0.25 ratio (w/w) of ADH to MAP2, the prevention of aggregation is about 70% (curve 2). These results clearly demonstrate that the MAP2 itself possesses chaperone-like activity as well.

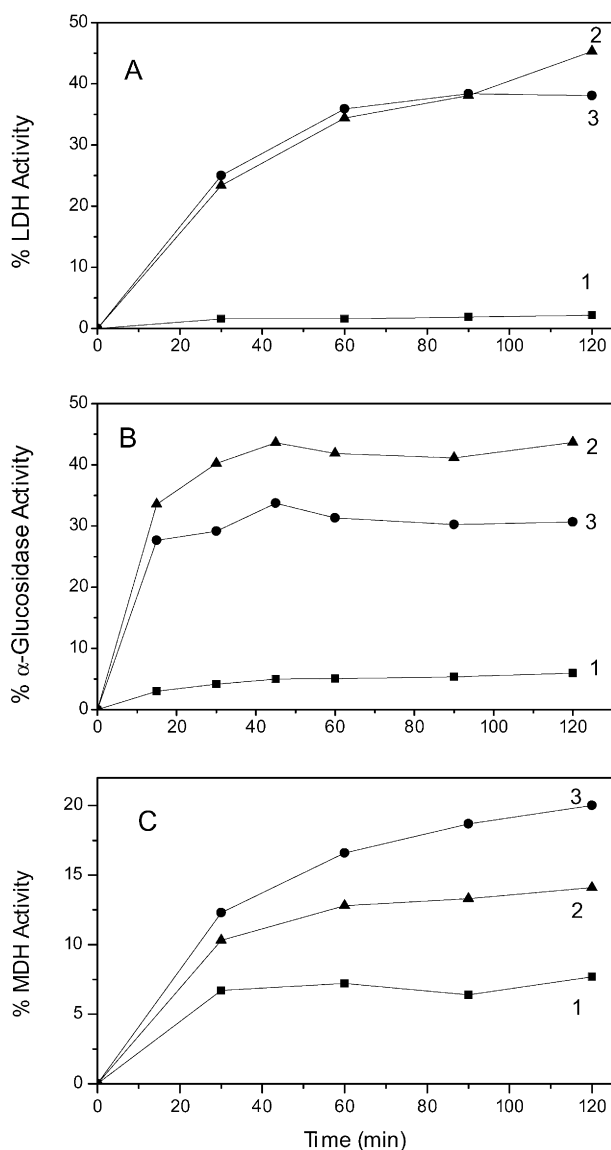
The key parameters measured *in vitro* that characterize a protein as a chaperone include: (a) its ability to protect a protein from aggregation during unfolding under stress conditions; and (b) recovery of lost biological activity during protein refolding from denatured state. Results presented in Fig. 2B,C demonstrated that MAP2 could efficiently suppress the thermal and chemically induced aggregation of several proteins in the unfolding pathway. Here, we show that, like some well known molecular chaperones including tubulin [9,34–36], MAP2 increases the yield of biological activity of refolded enzymes from its fully denatured states. In the present refolding study, the model substrate enzymes used were LDH,  $\alpha$ -glucosidase and MDH. Under the present experimental conditions (as described under Materials and methods), the refolding yields in the absence of MAP2 (i.e. self-refolding) were 3% for LDH, 6% for  $\alpha$ -glucosidase and 7% for MDH (Fig. 3A–C; curve 1). When the same experiments were carried out in the presence of MAP2, a significant increase in the enzyme activity with time was observed. Maximum yields achieved were 45, 43 and 14% for LDH (Fig. 3A, curve 2),  $\alpha$ -glucosidase (Fig. 3B, curve 2) and MDH (Fig. 3C, curve 2), respectively. Refolding data of these substrate enzymes in presence of tubulin (Fig. 3A–C; curve 3) have been included for the purpose of comparison. Both MAP2 and tubulin possess significant chaperone-like activity in respect of their ability to assist refolding. It was reported that bovine serum albumin could marginally reactivate some enzymes in a nonspecific way [37]. However, we observed that bovine serum albumin had no significant effect on the refolding yields of the enzymes used here (data not shown).

To know whether the intact MAP2 molecule is required to exhibit this unique property, trypsin-digested MAP2 was



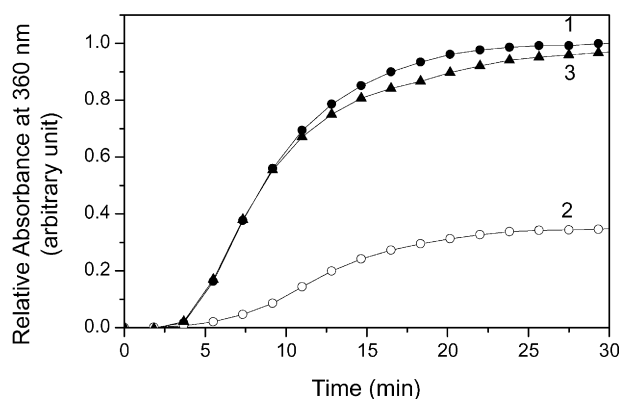
**Fig. 2.** Effect of tau protein, tau-bound tubulin and MAP2 on insulin aggregation, and of MAP2 on ADH aggregation. (A) Effect of tau protein and tau-bound tubulin on insulin aggregation: Curve 1 (●), insulin only; curve 2 (◆), insulin + 1.2 mg·mL<sup>-1</sup> tubulin; curve 3 (▼), insulin + (1.2 mg·mL<sup>-1</sup> tubulin + 0.03 mg·mL<sup>-1</sup> tau); curve 4 (▲), insulin + (1.2 mg·mL<sup>-1</sup> tubulin + 0.06 mg·mL<sup>-1</sup> tau); curve 5 (□), insulin + 0.12 mg·mL<sup>-1</sup> tau only (no tubulin). (B) Effect of MAP2 on insulin aggregation: Curve 1 (●), insulin only; curve 2 (▲), insulin + MAP2 (0.05 mg·mL<sup>-1</sup>); curve 3 (▼), insulin + MAP2 (0.1 mg·mL<sup>-1</sup>). (C) Effect of MAP2 on ADH aggregation: Curve 1 (●), ADH only; curve 2 (○), ADH + MAP2 (0.1 mg·mL<sup>-1</sup>). The concentration of insulin, ADH and other conditions were same as in Fig. 1.

tested for the chaperone-like activity using the insulin aggregation assay system. The results are shown in Fig. 4. Curve 1 represents the aggregation of insulin alone and



**Fig. 3.** Effect of MAP2 and tubulin on the reactivation of different substrate enzymes. (A) Time course of reactivation of  $0.05 \mu\text{M}$  LDH: Self-folding (trace 1, ■); in presence of  $0.05 \mu\text{M}$  MAP2 (trace 2, ▲); and in presence of  $0.4 \mu\text{M}$  tubulin (trace 3, ●). (B) Time course of reactivation of  $0.06 \mu\text{M}$   $\alpha$ -glucosidase: Self-folding (trace 1, ■); in presence of  $0.05 \mu\text{M}$  MAP2 (trace 2, ▲); and in presence of  $0.5 \mu\text{M}$  of tubulin (trace 3, ●); (C) Time course of reactivation of  $0.03 \mu\text{M}$  MDH: Self-folding (trace 1, ■); in presence of  $0.05 \mu\text{M}$  MAP2 (trace 2, ▲); and in presence of  $0.4 \mu\text{M}$  of tubulin (trace 3, ●). The refolding protocols of enzyme denaturation and refolding assays were described in 'Materials and methods'.

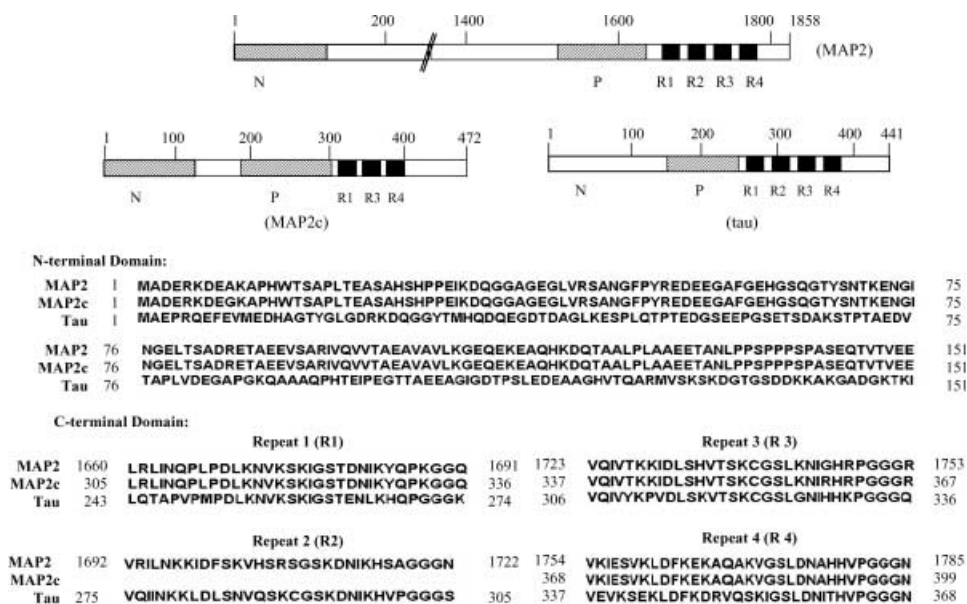
curve 2 shows insulin with undigested MAP2. About 70% of insulin aggregation is prevented when the insulin/MAP2 ratio is 1 : 0.33. Curve 3 depicts the result of insulin aggregation in presence of equal amount of digested MAP2. Digestion of MAP2 with trypsin totally abolished the chaperone-like activity. Therefore, this experiment reveals that none of the tryptic fragments meets the criteria for having this unique activity. Only intact MAP2 possesses both binding sequences as well as charged residues with



**Fig. 4.** Effect of trypsin digested MAP2 on insulin aggregation. Curve 1 (●), insulin only; curve 2 (□), insulin +  $0.1 \text{ mg}\cdot\text{mL}^{-1}$  MAP2; curve 3 (▲), insulin + trypsin digested MAP2 ( $0.1 \text{ mg}\cdot\text{mL}^{-1}$ ). Insulin concentration was  $0.3 \text{ mg}\cdot\text{mL}^{-1}$ .

regional segmental flexibility for solubilization requirements. It may be mentioned here that chaperone-like behavior of other flexible random coil protein  $\alpha$ <sub>s</sub>-casein is known in the literature [38]. Possession of high negative charges and structural flexibility were the hallmarks for  $\alpha$ <sub>s</sub>-casein's chaperone-like function. Segmental flexibility of a chaperone is important for the fast protein-protein recognition process [32] and may also play a big role in the dynamic solvation phenomena of the chaperone-substrate complex.

Both tau and MAP2 are heat-stable, flexible, noncompact protein molecules and possess no definite elements of secondary structure. Tau and MAP2 share a homologous C-terminal domain, composed of three or four microtubule binding repeats separated by inter-repeats (Fig. 5). Because tau possesses no chaperone-like activity, it is likely that the C-terminal domain does not play any significant role in the chaperone-like activity of MAP2. However, MAP2 is a high molecular mass ( $M_r \approx 280 \text{ kDa}$ ) protein that contains about 1850 amino acid residues whereas tau shares only about 120 residues of the C-terminus of MAP2 (Fig. 5). Therefore, the role of a large portion of the MAP2 remains unknown. To pinpoint the structural elements responsible for the observed chaperone-like activity, we used a low molecular mass isoform of MAP2 called MAP2c. Compared with low molecular mass MAP2c, the high molecular mass MAP2 sequence contains about 1350 additional amino acid residues inserted after residue 151 (Fig. 5). Another striking feature in MAP2c is the absence of second microtubule binding repeat due to alternative mRNA splicing (Fig. 5). We purified MAP2c and tested its behavior towards protein aggregation and refolding assay using two substrates ADH and MDH, respectively. The results are shown in Fig. 6. The thermal aggregation of ADH is prevented appreciably by MAP2c. About 80% aggregation is protected by 1 : 2.5 (w/w) ratio of ADH to MAP2c (Fig. 6A, curve 3). MAP2c also enhances the refolding of unfolded MDH as shown in Fig. 6B. In the presence of MAP2c ( $0.01 \text{ mg}\cdot\text{mL}^{-1}$ ), the refolding of MDH is increased to 12% (Fig. 6B, curve 2), whereas the self-refolding was only 5% (curve 1). Therefore, the above results demonstrated clearly that MAP2c, despite being significantly shorter than MAP2, is also able to manifest chaperone-like function, albeit to a lesser extent.



**Fig. 5. Sequence homology of MAP2, MAP2c and tau.** The N-terminal region (N), proline-rich region (P), four tubulin binding repeats in C-terminal region marked R1, R2, R3 and R4. The analysis was performed using sequences from NCBI protein data bank with accession numbers NP\_114034, AAB48097 and AAC04279, respectively.

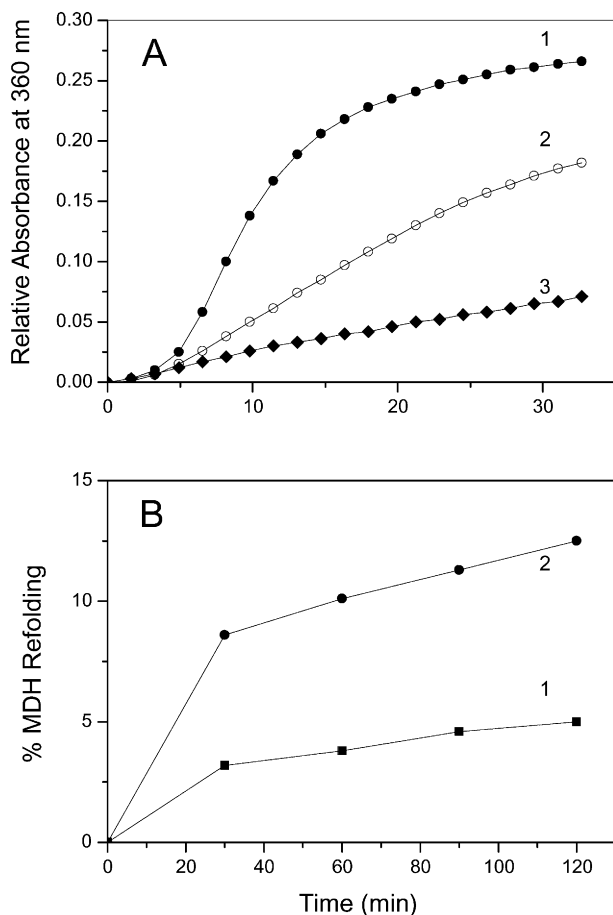
Co-existence of hydrophobic and hydrophilic domains has been shown to be an important characteristic of many chaperone proteins. It is believed that the stress-induced unstable conformer of the substrate proteins binds to the hydrophobic region of the chaperone, whereas the hydrophilic part helps in the solubilization of the chaperone-substrate complex. However, MAP2, an open-structured protein, is known to be predominantly hydrophilic in nature. We intended to examine whether MAP2 or MAP2c possess any concentrated patches of hydrophobic residues at all. The hydrophobic marker dye 1,8-ANS shows only a weak fluorescence when free in aqueous solutions. Its fluorescence is markedly increased in nonpolar environments such as hydrated hydrophobic surfaces in proteins and is accompanied by a blue shift in emission maximum. We incubated 10  $\mu\text{M}$  ANS with 0.2  $\text{mg}\cdot\text{mL}^{-1}$  of MAP2 for 15 min and then recorded the ANS fluorescence of the complex. The results are shown in Fig. 7. Interestingly, we observed that with 0.2  $\text{mg}\cdot\text{mL}^{-1}$  of MAP2 fluorescence intensity at 490 nm has increased almost three times (trace 2) compared with that of only ANS (trace 1). Furthermore, the emission maximum shows a significant blue shift from 515 nm to 475 nm. A similar experiment with MAP2c showed a two times enhancement of fluorescence intensity (trace 3) and an 8 nm blue shift of emission maximum. Also, the addition of urea to this incubated mixture caused rapid fall in the fluorescence intensity as well as the red shift of the emission maximum (data not shown). This result undoubtedly confirms the existence of significant hydrophobic patches in MAP2/MAP2c and also implicates the observed chaperone-like behaviour of this protein.

## Discussion

The neuronal microtubule associated proteins tau and MAP2/MAP2c bind tubulin through their C-terminus,

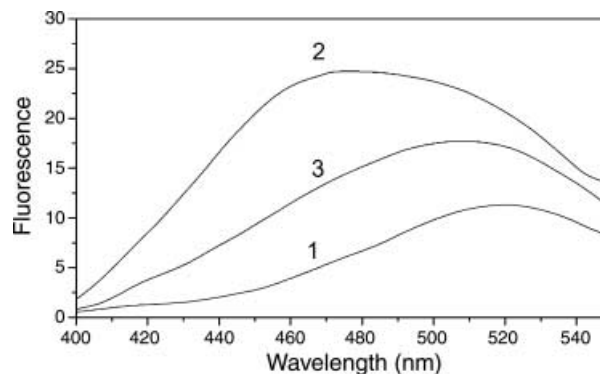
promote microtubule polymerization and stabilize microtubules. On the other hand, the precise role of the N-terminal domain of tau or MAP2 is not yet clear, although it is believed that the N-terminal domain acts as a projection domain which helps to maintain the distance between two microtubules. All these MAPs are predominantly extended random-coil structures and have little secondary structure. We found that except for tau, both MAP2 and MAP2c possess strong chaperone-like activity.

These MAPs are heat stable, without structure and have no clear-cut N- or C-terminal domain. Therefore, to compare these three MAPs, we have used the conventional nomenclature. Each protein can be divided into an N-terminal domain, followed by a proline-rich region and finally a C-terminal domain. To get a better insight of the observed chaperone-like activity we compared the amino acid sequences of three proteins using the BLAST 2 pairwise alignment program. A schematic representation of the sequence homology has been given in Fig. 5. The sequence analysis showed 66% similarities in their C-terminal domains. All of them had three to four repeated sequences of 18 amino acids separated by 13–14 residue inter-repeat sequences at their C-terminal region, which constitutes the tubulin-binding domain. Each of these repeats has more homology to the corresponding repeats of the other two than to the other repeats in the same protein. The proline-rich region is also common to them and exists just before the tubulin binding domain. Although the proline-rich region has similar amino acid composition, no significant sequence homology is observed in this region. However, when their N-terminal domains are compared it is observed that first 151 residues are identical in MAP2 and MAP2c but that of tau is very dissimilar (Fig. 5). It is also known that about 1350 amino acids residues of MAP2 after first 151 amino acids in N-terminal domain are spliced out in MAP2c. When one looks at the occurrence of the acidic amino acid



**Fig. 6.** Effect of MAP2c on aggregation and refolding. (A) ADH aggregation: Curve 1 (●), ADH only; curve 2 (○), ADH + MAP2c (0.4 mg·mL<sup>-1</sup>); curve 3 (◆), ADH + MAP2c (1.0 mg·mL<sup>-1</sup>). The concentration of insulin, ADH and other conditions were same as in Fig. 1. (B) Time course of reactivation of 0.03 μM MDH: Self-folding (trace 1, ■); in presence of 0.01 mg·mL<sup>-1</sup> MAP2c (trace 2, ●). The details of enzyme denaturation and refolding assays were described in Materials and methods.

patches in MAP2 and tau, it is noted that they contain 18.6 and 12.7% acidic amino acids, respectively, indicating both of them are predominantly basic in nature. In tau, the acidic amino acids are distributed all over the entire sequence. Only two patches of segregated acidic amino acids sequence (53–58, 104–107) are found in the N-terminal sequence of tau. In contrast, the N-terminal domain of MAP2 contains



**Fig. 7.** Fluorimetric study of ANS binding by MAP2 and MAP2c. Trace 1, ANS (10 μM) only; trace 2, ANS + MAP2 (0.2 mg·mL<sup>-1</sup>); trace 3, ANS + 0.2 mg·mL<sup>-1</sup> MAP2c. The excitation wavelength was 350 nm. The protein was incubated with 10 μM ANS for 15 min at 25 °C. The details are described in Materials and methods.

about 26 patches of acidic amino acids (Table 1). From the sequence analysis it is apparent that the presence and absence of acidic amino acid patches might also modulate the chaperone-like activity of the proteins. As all these MAPs have similar C-terminal domains, the differential behaviour of tau is likely due to its N-terminal domain. It seems that the identical part of MAP2 and MAP2c (first 151 amino acids) is crucial for the observed chaperone-like activity. As tau, which has a similar C-terminal domain to MAP2, does not display this property, it is apparent that only the C-terminal domain is unable to exhibit chaperone-like activity. It is also possible that the N-terminal domain works in conjunction with the C-terminal portion in the chaperone machinery of MAP2. Again MAP2c, despite lacking a significant portion of the N-terminal domain of MAP2, was also capable of exhibiting chaperone-like activity. However, MAP2c was less potent compare to MAP2 when their chaperone-like activities were compared (Figs 2C, 3C and 6A,B). It is noteworthy that the spliced out portion of MAP2 contains a large number of acidic amino acids and could be responsible for the lower potency of MAP2c. We also observed that the ANS binding capacity of MAP2 is higher than that of MAP2c (Fig. 7). Thus, the hydrophobic patches in the N-terminal region of MAP2, which are spliced out in MAP2c, are also likely to contribute in the chaperone-like function.

Brain MAP2, the most studied among of all the MAPs, is a thermo-stable, positively charged, filamentous and open

**Table 1.** Acidic amino acid patches in N-termini of MAP2, MAP2c and tau.

MAP2	MAP2c	tau
52–55 (E <sub>2</sub> D <sub>2</sub> E <sub>2</sub> E), 85–89 (E <sub>2</sub> T <sub>2</sub> A <sub>2</sub> E <sub>2</sub> E), 109–113 (E <sub>2</sub> Q <sub>2</sub> E <sub>2</sub> K <sub>2</sub> E), 150–152 (E <sub>2</sub> E <sub>2</sub> D <sub>2</sub> ), 179–183 (E <sub>2</sub> K <sub>2</sub> E <sub>2</sub> S <sub>2</sub> E), 217–219 (E <sub>2</sub> E <sub>2</sub> E <sub>2</sub> ), 294–300 (E <sub>2</sub> K <sub>2</sub> D <sub>2</sub> V <sub>2</sub> F <sub>2</sub> D <sub>2</sub> D), 369–373 (E <sub>2</sub> E <sub>2</sub> P <sub>2</sub> H <sub>2</sub> E), 409–413 (E <sub>2</sub> E <sub>2</sub> E <sub>2</sub> K <sub>2</sub> E), 437–446 (E <sub>2</sub> K <sub>2</sub> E <sub>2</sub> T <sub>2</sub> E <sub>2</sub> L <sub>2</sub> K <sub>2</sub> L <sub>2</sub> E <sub>2</sub> E), 467–469 (D <sub>2</sub> E <sub>2</sub> E), 762–770 (E <sub>2</sub> S <sub>2</sub> K <sub>2</sub> E <sub>2</sub> E <sub>2</sub> E <sub>2</sub> Q <sub>2</sub> I <sub>2</sub> E), 898–902 (E <sub>2</sub> G <sub>2</sub> T <sub>2</sub> D <sub>2</sub> D), 965–969 (E <sub>2</sub> K <sub>2</sub> S <sub>2</sub> E <sub>2</sub> E), 981–988 (E <sub>2</sub> E <sub>2</sub> A <sub>2</sub> G <sub>2</sub> D <sub>2</sub> E <sub>2</sub> I <sub>2</sub> E), 1024–1029 (E <sub>2</sub> I <sub>2</sub> A <sub>2</sub> E <sub>2</sub> V <sub>2</sub> E), 1128–1132 (E <sub>2</sub> E <sub>2</sub> S <sub>2</sub> Y <sub>2</sub> E), 1202–1206 (E <sub>2</sub> E <sub>2</sub> S <sub>2</sub> K <sub>2</sub> E), 1231–1240 (E <sub>2</sub> I <sub>2</sub> Q <sub>2</sub> S <sub>2</sub> E <sub>2</sub> E <sub>2</sub> E <sub>2</sub> E <sub>2</sub> I <sub>2</sub> E), 1267–1271 (E <sub>2</sub> E <sub>2</sub> F <sub>2</sub> V <sub>2</sub> E), 1288–1290 (E <sub>2</sub> D <sub>2</sub> D), 1327–1335 (D <sub>2</sub> E <sub>2</sub> E <sub>2</sub> E <sub>2</sub> F <sub>2</sub> E <sub>2</sub> V <sub>2</sub> E), 1355–1358 (E <sub>2</sub> R <sub>2</sub> E <sub>2</sub> E), 1370–1380 (D <sub>2</sub> D <sub>2</sub> Y <sub>2</sub> K <sub>2</sub> D <sub>2</sub> E <sub>2</sub> T <sub>2</sub> T <sub>2</sub> I <sub>2</sub> D <sub>2</sub> D), 1391–1396 (D <sub>2</sub> T <sub>2</sub> Q <sub>2</sub> D <sub>2</sub> D <sub>2</sub> D), 1410–1416 (E <sub>2</sub> E <sub>2</sub> K <sub>2</sub> A <sub>2</sub> E <sub>2</sub> K <sub>2</sub> E)	52–55 (E <sub>2</sub> D <sub>2</sub> E <sub>2</sub> E), 85–89 (E <sub>2</sub> T <sub>2</sub> A <sub>2</sub> E <sub>2</sub> E), 109–113 (E <sub>2</sub> Q <sub>2</sub> E <sub>2</sub> K <sub>2</sub> E)	53–58 (E <sub>2</sub> D <sub>2</sub> G <sub>2</sub> S <sub>2</sub> E <sub>2</sub> E), 104–107 (E <sub>2</sub> E <sub>2</sub> A <sub>2</sub> G <sub>2</sub> )

structured flexible molecule [33]. This is not the first time that a structureless protein has been reported to have chaperone-like activity. It has already been reported that  $\alpha$ -synuclein and mammalian milk protein  $\alpha_s$ -casein, both random coil proteins, exhibit chaperone-like activity [34,39]. The quest for chaperones within the eucaryotic cytosol is continuing to address the question as to how the majority of cytosolic proteins fold. With the exception of CCT and tubulin, no eucaryotic cytosolic chaperone has been identified to date [8,40]. Again, CCT has a very low abundance and, unlike GroEL, recognizes only a limited number of subsets of specific proteins [40–42]. Therefore it is quite likely that eucaryotic cytosol contains other chaperones, yet to be identified. In this context, our finding of the chaperone-like activity of tubulin and MAP2 may have important implications in the chaperone-aided protein-folding mechanism in the eucaryotic system.

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