

# The effect of S-100a and S-100b proteins and $\text{Zn}^{2+}$ on the assembly of brain microtubule proteins in vitro

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Received 28 September 1983

The homologous proteins S-100a and S-100b affect the microtubule system in a distinctly different way in the presence of low molar ratios of  $\text{Zn}^{2+}$ . Assembly of brain microtubule proteins can be almost completely inhibited and rapid disassembly can be induced by low molar amounts of S-100b in the presence of low molar ratios [2–4] of  $\text{Zn}^{2+}$ . Higher molar ratios per S-100b (>4) potentiate the general  $\text{Zn}^{2+}$  effect, promoting the formation of sheets of microtubules. However, the effect of S-100a is quite different, no inhibition of assembly can be observed and the presence of S-100a seems to protect the microtubule proteins against the effect of  $\text{Zn}^{2+}$  by chelating the  $\text{Zn}^{2+}$  and decreasing the free metal-ion concentration. S-100a or S-100b cannot bind to the microtubule polymer-form, either in the absence or in the presence of  $\text{Zn}^{2+}$ .

*Microtubule assembly      Tubulin      S-100 protein      Calmodulin       $\text{Zn}^{2+}$  regulation*

## 1. INTRODUCTION

The cellular regulation of microtubule assembly and disassembly is unknown but divalent cations [1] may play an important role.  $\text{Mg}^{2+}$  is required for assembly of isolated microtubules and one  $\text{Mg}^{2+}$  is firmly bound to tubulin. On the other hand,  $\text{Ca}^{2+}$  in millimolar concentrations can induce disassembly and inhibit assembly. This effect is potentiated in the presence of  $\text{Ca}^{2+}$ -binding proteins like calmodulin, troponin and, as we recently reported, S-100 [2].

The brain S-100 protein is a mixture of two predominant components, the S-100a and S-100b proteins, which in bovine brain are present in almost equal amounts [3]. Both proteins are dimers of identical  $\beta\beta$  (S-100b)- or non-identical  $\alpha\beta$  (S-100a)-subunits. The  $\alpha$ - and the  $\beta$ -subunit have a molecular mass of about 10500 and a high (58%) sequence homology [4]. Both subunits also share the typical amino acid sequence associated

with a calcium-binding domain [5]. The biological activity of these proteins remains unknown. However, previous results [2] indicate that S-100 proteins regulate the calcium-induced microtubule disassembly in vitro.

It has recently been found that besides  $\text{Ca}^{2+}$  also  $\text{Zn}^{2+}$  can bind to the S-100a and to the S-100b protein and induce different conformational changes on both proteins [6]. It is well known that  $\text{Zn}^{2+}$  interferes with brain microtubules both in vitro [7] and in situ [8]. Tubulin has one high affinity site for  $\text{Zn}^{2+}$  [9].  $\text{Zn}^{2+}$  in micromolar concentrations interferes with the tubulin–tubulin association as tubulin no longer assembles into microtubules but into sheets [7,10] in which the protofilaments are aligned antiparallel. Moreover, low  $\text{Zn}^{2+}$  concentrations, less than 0.1 mM, stimulate self-assembly of tubulin in the absence of MAPs [11]. Although small amounts of zinc have also been detected in isolated microtubule proteins these are probably associated with the microtubule-associated proteins, MAPs [12].

As  $\text{Zn}^{2+}$  binds to S-100 proteins it was of interest to study the effect of  $\text{Zn}^{2+}$  on the microtubule system in the presence of the S-100 proteins. We here report that the S-100a and S-100b proteins induce quite different effects on the microtubule assembly in the presence of either micro- or millimolar  $\text{Zn}^{2+}$  concentrations.

## 2. MATERIALS AND METHODS

### 2.1. Protein preparation

Microtubule proteins and purified tubulin were prepared from bovine brain as in [13–15]. All assembly experiments were performed in assembly buffer: 100 mM piperazine-*N,N'*-bis(2-ethanesulphonic acid), 0.5 mM  $\text{MgSO}_4$ , 1 mM GTP, titrated to pH 6.8 with NaOH.

S-100a and S-100b protein were isolated from bovine brain by the technique in [3,16,17].

Calmodulin was isolated from bovine brain as in [3]. S-100 proteins and calmodulin were made calcium-free by trichloroacetic acid precipitation [18].

### 2.2. Protein concentration

The microtubule protein concentration was determined as in [13]. The concentration of S-100 was determined from  $E_{280} = 11500$  for S-100a, from 3400 for S-100b [6] and  $3300 \text{ M}^{-1} \cdot \text{cm}^{-1}$  for calmodulin [19].

### 2.3. Microtubule assembly and disassembly

Assembly was initiated by addition of concentrated microtubule proteins to S-100 protein and  $\text{ZnSO}_4$  in the assembly buffer at  $37^\circ\text{C}$  and was monitored continuously by the change in absorbance at 350 nm [13–15]. Negatively stained samples and embedded pellets of assembled microtubules were studied by electron microscopy, as in [15].

### 2.4. Immunological techniques

The concentration of S-100a and S-100b in the supernatants were determined by rocket immunoelectrophoresis [20].

## 3. RESULTS

### 3.1. *Zn(II)* protection by S-100a

The rate and extent of assembly of microtubule

proteins into microtubules were not affected by  $30 \mu\text{M}$  metal-free S-100a (fig.1A,a'), which is a 2.5-times molar excess over tubulin dimer. Furthermore, neither the rate nor the extent of assembly were affected (fig.1A,b',d') in the presence of a 2.5-times molar excess of S-100a in the presence of 120 or  $480 \mu\text{M}$   $\text{Zn}^{2+}$  (either a 4- or 16-times molar ratio of  $\text{Zn}^{2+}$  over S-100a). Electron micrographs of the formed assembly product showed the presence of perfect microtubules with 4- or 16-times excess of  $\text{Zn}^{2+}$  over S-100a, while with a 32-fold excess, opened-up microtubules and an indication for sheets could be seen (micrographs not shown). Microtubule proteins assembled in the presence of the latter concentration of  $\text{Zn}^{2+}$ , but in the absence of S-100a showed an increased turbidity and the electron micrographs revealed the presence of sheets, confirming previous findings [7]. When  $30 \mu\text{M}$  calmodulin was present with  $240 \mu\text{M}$   $\text{Zn}^{2+}$  no such protection as with S-100a could be observed. S-100a facilitates calcium-induced microtubule disassembly also in the presence of 4  $\text{Zn}^{2+}$ /S-100a, as addition of 1 mM  $\text{Ca}^{2+}$  still could induce a rapid and nearly complete disassembly of microtubules [2].

The concentration of S-100a in the supernatant after pelleting of the microtubules formed in the presence of S-100a and a 4-times excess of  $\text{Zn}^{2+}$  was determined by rocket immunoelectrophoresis [19]. The supernatant was found to contain the same concentration of S-100a as before microtubule assembly, indicating that S-100a is not bound to the microtubules.

The self-assembly of phosphocellulose-purified tubulin induced by low concentrations of  $\text{Zn}^{2+}$  [11] was inhibited by equimolar amounts of S-100a.

### 3.2. *Zn(II)* inhibition by S-100b

Microtubule assembly is nearly completely inhibited in the presence of S-100b and low molar ratios of  $\text{Zn}^{2+}$  concentration as both the rate of assembly and the extent of assembly are decreased (fig.1B,b'). The inhibition seems to be  $\text{Zn}^{2+}$ -dependent (fig.2). Only a slight inhibition is found in the absence of  $\text{Zn}^{2+}$  with increasing amounts of S-100b as shown in fig.2. The effect may be due to a contamination of S-100b protein by  $\text{Zn}^{2+}$  which was isolated by a  $\text{Zn}^{2+}$ -dependent affinity chromatography [17]. This is also indicated by the complete assembly obtained in the

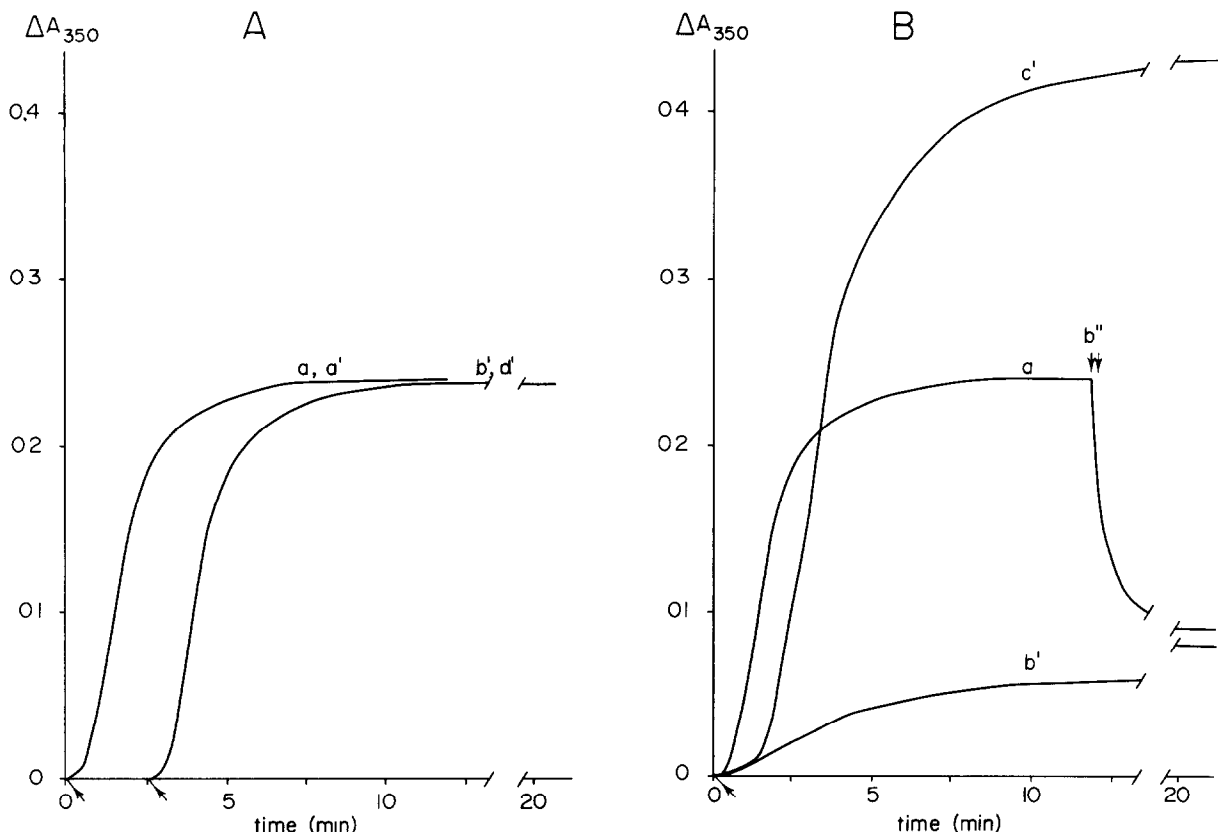


Fig.1. Microtubule assembly in the presence of  $\text{Zn}^{2+}$  and (A) S-100a and (B) S-100b protein. Microtubule proteins were assembled at  $37^\circ\text{C}$  in assembly buffer and monitored by the absorbance difference at 350 nm ( $\Delta A_{350}$ ), against time. The reference cell contained the same additions as the measuring cell but was kept at  $10^\circ\text{C}$ . At the arrow microtubule protein was added to the assembly buffer at  $37^\circ\text{C}$ . The microtubule protein concentration was 1.7 mg/ml of which the tubulin content was estimated to be 12  $\mu\text{M}$ . (A) In the primed (') tracings 30  $\mu\text{M}$  S-100a was present with: (a') 0  $\mu\text{M}$ , (b') 120  $\mu\text{M}$  and (d') 480  $\mu\text{M}$   $\text{Zn}^{2+}$ . Trace (a) was the control. (B) In the primed (') tracings 30  $\mu\text{M}$  S-100b was present with: (a') 0  $\mu\text{M}$ , (b') 120  $\mu\text{M}$  and (c') 240  $\mu\text{M}$   $\text{Zn}^{2+}$ . At the double arrow (b'') 30  $\mu\text{M}$  S-100b with 120  $\mu\text{M}$   $\text{Zn}^{2+}$  was added to the control (a).

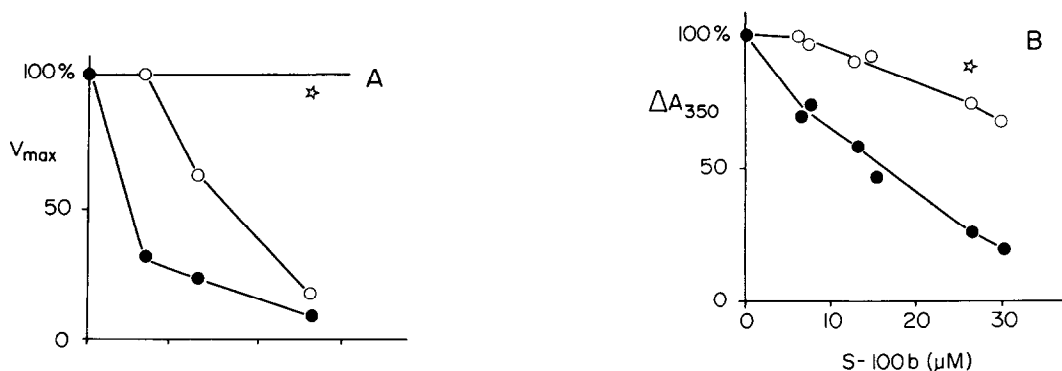


Fig.2. Assembly of microtubules in the presence of  $\text{Zn}^{2+}$  and S-100b. The conditions and microtubule protein concentration were as described in the legend to fig.1. (A) The maximal assembly rate ( $V_{\text{max}} = \Delta A_{350}/\text{min}$ ) is plotted against the added amount of S-100b; (B) the assembly level ( $\Delta A_{350}$ ) at steady state is plotted against the added amount of S-100b: (○) without  $\text{Zn}^{2+}$ ; (●) with 4  $\text{Zn}^{2+}$ /S-100b; (☆) with 1 mM EGTA.

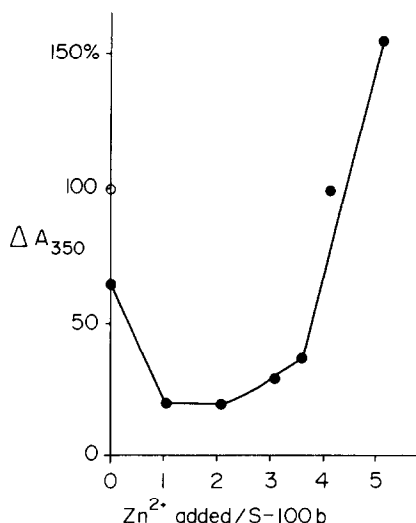


Fig.3. Assembly of microtubules in the presence of  $\text{Zn}^{2+}$  and S-100b. The assembly level ( $\Delta A_{350}$ ) at steady state is plotted against the added  $\text{Zn}^{2+}$ /S-100b. The concentration of S-100b was  $0 \mu\text{M}$  (○) and  $30 \mu\text{M}$  (●). The experimental conditions and microtubule protein concentration were as described in the legend to fig.1.

presence of S-100b when EGTA is added (fig.2 (\*)). As can be seen in fig.1B and fig.2 and 3, the rate and the extent of assembly decrease in the presence of S-100b and  $\text{Zn}^{2+}$  at an optimum effect, see fig.3, with 2–4  $\text{Zn}^{2+}$ /S-100b.

Furthermore, rapid disassembly of assembled microtubules is induced upon addition of S-100b with 2–4  $\text{Zn}^{2+}$  (fig.1B,b''). However, when this ratio is exceeded, microtubule assembly is enhanced (fig.1B,c' and 3) and electron micrographs show the formation of sheets of microtubules (not shown).

The determination of the concentration of S-100b in the supernatant of the microtubules and microtubule sheets formed in the presence of 0, respectively 6  $\text{Zn}^{2+}$  per S-100b showed that the S-100b concentration was about  $95 \pm 5\%$  of the value of the reference without microtubular protein. These results indicate that S-100b does not bind to the tubulin polymer.

#### 4. DISCUSSION

Although calmodulin and the S-100 proteins both have the amino acid sequences that could form the EF-hand [6] they exhibit different bin-

ding properties for divalent metal ions. Calmodulin binds 4  $\text{Ca}^{2+}$  per molecule with dissociation constants in the range from 4–18  $\mu\text{M}$  [18] and has low affinity for  $\text{Zn}^{2+}$  ( $K_d = 100 \mu\text{M}$ ) [21]. In contrast, the affinity of S-100 for  $\text{Zn}^{2+}$  is higher than for  $\text{Ca}^{2+}$ , with 4  $\text{Zn}^{2+}$  binding sites with dissociation constants in the range  $0.01 \mu\text{M}$  and at least 4 more  $\text{Zn}^{2+}$ -binding sites with lower affinity ( $1 \mu\text{M}$ ) [6]. Furthermore, there are significant differences in the  $\text{Zn}^{2+}$ -induced structural changes on the S-100a and S-100b proteins. S-100b becomes more hydrophobic in the presence of  $\text{Zn}^{2+}$ , while the hydrophobicity of S-100a is unaffected [6]. The present results show that an effect of  $\text{Zn}^{2+}$  and S-100 is found on the microtubule system with stoichiometric concentrations of  $\text{Zn}^{2+}$ , conditions under which calmodulin had no effect. S-100a and S-100b proteins have different effects on the microtubule disassembly. S-100a seems to protect the microtubules against the sheet-inducing effect of  $\text{Zn}^{2+}$  by decreasing the free  $\text{Zn}^{2+}$  concentration but not by binding to the microtubule proteins. Similar protective proteins have been observed in other tissues; e.g., metallothionein [22]. On the contrary, S-100b interacts with the microtubule proteins at low  $\text{Zn}^{2+}$  concentrations (2–4  $\text{Zn}^{2+}$ /S-100b), induces disassembly and inhibits microtubule formation (fig.1–3). However, at higher  $\text{Zn}^{2+}$  concentrations, i.e.,  $>4 \text{Zn}^{2+}$  per S-100b, the presence of S-100b enhances the general  $\text{Zn}^{2+}$  effect (fig.1B), inducing the formations of sheets. Thus, in the presence of the S-100b protein the inhibitory effect of  $\text{Zn}^{2+}$  on the microtubule assembly is within a narrow concentration range and may be correlated to specific  $\text{Zn}^{2+}$ -induced conformational changes of the S-100b protein. We have shown that the S-100 proteins are not incorporated in the microtubule polymer. However, this report confirms the interaction of the S-100 proteins with the microtubule system. It also demonstrates the different behaviour of the S-100a and S-100b proteins in the presence of  $\text{Zn}^{2+}$  which may suggest a different physiological role for both iso-proteins.

#### ACKNOWLEDGEMENTS

This study was supported by grants from the Naturvetenskapliga Forskningsrådet nos. 2535-110 (M.W.) and 2450-101 (J.D.) and from the CNRS,

INSERM CRL 80.3.007 (D.G.). We are indebted to Mrs Inger Holmquist for help with the electron microscopy.

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