Spectral studies on the calcium-binding properties of Mts1 protein and its interaction with target protein

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Abstract Two calcium-binding sites of the Mts1 protein, a member of S-100 protein family, were distinguished with the Fluo-3 fluorescent technique. The geometric mean of the apparent dissociation constant (K_d) for these two sites is 2.6 μM; the Hill coefficient (n_H) is 0.98. In the presence of a novel target protein p37, isolated from the mouse adenocarcinoma cell line CSML-100, Mts1 binds Ca^{2+} ions with higher affinity and with strong positive cooperativity ($K_d = 0.2$ μM, $n_H = 1.91$). Interaction of Mts1 with p37 is confirmed by the fluorescent probe 2-*p*-toluidinylnaphthalene-6-sulfonate (TNS). Reaction with TNS shows that p37 interacts with the hydrophobic site of Mts1 which is exposed due to the binding of Ca^{2+} ions.

Key words: S-100 protein; Calcium; Mts1; Fluo-3; Target protein

1. Introduction

Mts1 protein belongs to the family of S-100 proteins. This family consists of small Ca^{2+}-binding proteins which are expressed in different cell types and tissues [1]. Their biological function is still unclear. Studies on the expression of the *mts1* gene, which encodes the Mts1 protein, show a correlation between the presence of the Mts1 protein and metastatic phenotype of different tumorigenic cell lines [2]. The *mts1* gene is also expressed in several normal cell types such as lymphocytes, neutrophils, activated macrophages and some fibroblasts [3].

The ability of the S-100 proteins to bind two Ca^{2+} ions is due to the presence of two EF sites consisting of 12 amino acid residues each. This property of the S-100 proteins suggests their function as transducers or effectors of the calcium signal. Consequently, it is important to determine precisely the affinity of these proteins to Ca^{2+} ions. The most frequently used techniques are the radioligand method, in which $^{45}Ca^{2+}$ ions are used as labeled ligands, or the method based on measuring changes in intrinsic protein fluorescence induced by binding of metal cations. However, experiments performed using the first method give different values of the dissociation constant K_d for the same protein [4,5]. The results significantly depend on the experimental procedure, which includes separation of free and protein-bound forms of $^{45}Ca^{2+}$ that is the most difficult to reproduce. On the other hand, it is often impossible to get a well-recorded fluorescent signal in the case of intrinsic fluorescence registration.

Recently, the fluorescent Ca^{2+} chelators Quin-2, Indo-1, Fura-2 and Fluo-3 were proposed as indicators of concentrations of free Ca^{2+} in whole cells and in an extracellular medium [6]. These compounds change their spectral characteristic when binding Ca^{2+} ions.

Previously, we reported the results of our study of the Mts1 interaction with calcium by means of the chromophoric calcium chelator Fura-2 [7]. The procedure was based on the ability of Mts1 to bind calcium ions and competitively inhibit formation of Fura-2–Ca^{2+}. We found that Mts1 bound 2 mol Ca^{2+}/mol protein; the value of an apparent calcium dissociation constant was 2.4 μM. Since Fura-2 has a high affinity to calcium ions (dissociation constant K_d is ≈100–130 nM), the probe was completely saturated with Ca^{2+} when the concentration of the total calcium in medium exceeded 5 μM. Due to the lower affinity for Ca^{2+} of the second calcium-binding site, we could not determine the individual binding constants for both EF sites of Mts1 with a high precision.

In the present work, we determined the individual macroscopic calcium-binding constants for both Mts1 sites with the fluorescent probe Fluo-3 which is sensitive to calcium concentration fluctuations in a range wider than Fura-2.

It was suggested that physiological function of the S-100 proteins is mediated via the interaction with target proteins [1]. The apparent affinity of the S-100 proteins for calcium is greatly increased by addition of target proteins and peptides [1]. There are several proteins currently reported as possible targets for the Mts1 protein and its analogs in other species. These include heavy chain of non-muscle myosin [8], non-muscle tropomyosin [9] and 36-kDa MAP [10]. Here we report a possible new target, 37-kDa protein (p37), isolated from the mouse metastatic adenocarcinoma cell line CSML-100. These cells display highly metastatic behaviour and a high level of *mts1* gene expression. Interaction of p37 with Mts1 was confirmed by an increase of affinity of Mts1 to Ca^{2+} in complex with p37 and by the competition of p37 with the hydrophobic fluorescent probe 2-*p*-toluidinylnaphthalene-6-sulfonate (TNS) for the binding sites on the Mts1 protein.

2. Materials and methods

2.1. Isolation of Mts1 target proteins from cell lysates

Mouse metastatic adenocarcinoma CSML-100 [3] cells were cultured in a Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. Cells were incubated at 37°C in a humidified atmosphere containing 95% air/5% CO_2. When cells achieved 90% monolayer, they were removed from the flasks, collected and homogenised in 0.45 M sucrose, 2 mM EDTA, 2 mM EGTA, 4 mM ATP, 2 mM 2-mercaptoethanol, 50 mM imidazol-HCl (pH 7.5), 0.2 mM PMSF and centrifuged 4000×*g* at 4°C for 20 min. Supernatant was

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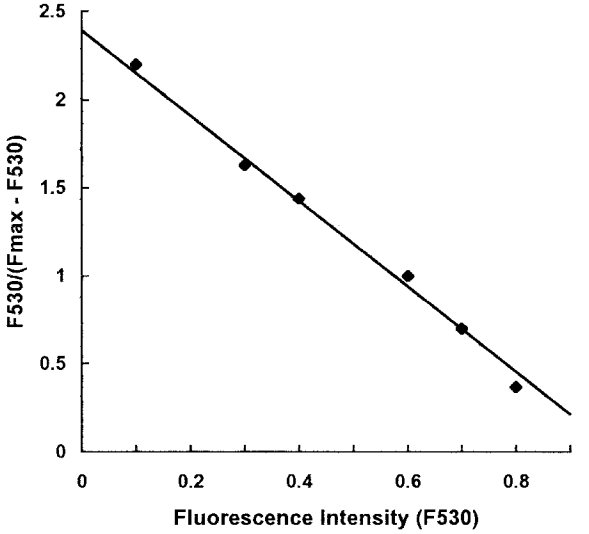

Fig. 1. Scatchard analysis of calcium binding to Fluo-3. Total concentration of calcium is 1 μM. The concentration of Fluo-3 ranged from 0.25 to 2.5 μM.

loaded on the column (1 ml) with Mts1–Sepharose 4B. Immobilisation of recombinant Mts1 on Sepharose was performed according to the manufacturer's (Pharmacia) instruction. Preparation and purification of recombinant Mts1 (recMts1) was carried out as described earlier [11]. The column was washed with 20 column volumes of 50 mM imidazol-HCl buffer (pH 7.5) containing 2 mM EDTA, 2 mM EGTA, and 0.15 M NaCl. Proteins or peptides bound on the column were eluted by 0.25 M triethylamine (pH 12.5) and dialysed against 10 mM imidazol-HCl (pH 7.5) and 0.1 M NaCl. The flow-through fraction was dialysed against the same buffer, $CaCl_2$ was added to 2 mM, and the fraction was loaded on the Mts1–Sepharose column equilibrated with 10 mM imidazol-HCl (pH 7.5), 0.1 M NaCl, and 2 mM $CaCl_2$. The column was washed with 20 volumes of this buffer and bound proteins were eluted by 20 mM EDTA. Protein-containing fractions were concentrated by the phenol-ether method [12] if needed and loaded on SDS-PAGE. After electrophoresis, proteins were transferred to the PVDF membrane (Costar) in the semi-dry transfer unit (Hoefer). The membrane was stained with Coomassie; separated proteins were cut out and eluted with 50 mM ammonium bicarbonate, 50% acetonitrile (pH 8.0) during 16 h at room temperature with mixing by inversion. Protein concentrations were determined by the Bradford method [13].

2.2. Calcium determination and calcium removal

The total calcium concentration was determined using the Perkin-Elmer 305B atomic absorption spectrophotometer. For removal of calcium, 10 mM EDTA and 10 mM EGTA were added to the proteins and then gel filtration on Sephadex G-25 was carried out. The protein-containing fractions were dialysed against deionised water. Total calcium in the protein preparations did not exceed 0.1 mol Ca^{2+}/mol protein.

2.3. Fluorescence measurements

2.3.1. Registration of fluorescence of Fluo-3. Fluo-3 was dissolved in 115 mM KCl, 20 mM NaCl, 10 mM K-MOPS (pH 7.2; buffer A). Fluorescence emission spectra were recorded using a Hitachi MPF-3 fluorimeter with excitation at 500 nm (bandwidth 5 nm) and emission scanned from 515 to 575 nm (bandwidth 10 nm). Spectra were recorded at 22°C in plastic cuvettes.

For performing of Scatchard analysis, the fluorescence of Fluo-3 probe was measured at zero calcium concentration (with 0.1 mM EDTA) and at saturating calcium concentration (1 mM $CaCl_2$) at 530 nm (F_{530}). Obtained rates were denoted as F_{min} and F_{max}, respectively.

The interaction of the fluorescent indicator with Ca^{2+} was mathematically described using the approach proposed by Olwin and Storm [14]. The macroscopic binding constants were calculated applying the SigmaPlot curve-fitting program.

2.3.2. TNS fluorescence registration. The fluorescence in probes, containing 5 μM Mts1, 0–18 μM target protein and 30 μM TNS in buffer A, was measured at an excitation wavelength 330 nm (bandwidth 5 nm). Spectra were recorded from 380 to 520 nm (bandwidth 8 nm).

3. Results and discussion

Previously, we determined the dissociation constant of the Mts1 protein with the fluorescent probe Fura-2 [7]. High affinity of this probe to calcium did not allow us to distinguish the individual dissociation constants of two different calcium-binding sites of the Mts1 protein.

Fluo-3 has a number of advantages: it is sensitive to calcium concentration in a wide range and almost lacks fluorescence in the calcium-free medium (quantum efficiency of fluorescence is 0.0051). For the complex of Fluo-3 with calcium, the quantum efficiency of fluorescence is 40 times higher [15]. In order to examine the Fluo-3–calcium interaction, a Scatchard analysis was performed (Fig. 1). The K_d value was 0.42 ± 0.03 μM, that is in good agreement with published results [15]. The concentration of contaminated Ca^{2+} ions in probes did not exceed 10 nM.

The binding of Ca^{2+} to Mts1 was determined with Fluo-3 and plotted as moles of Ca^{2+} bound per mole of Mts1 versus the free Ca^{2+} concentration (Fig. 2, line 1). The solid curve is an iterative fit to the Adair equation [16] for the data points presented. It had the best fit for a single class of two non-interacting binding sites yielding a K_d of 2.6 μM, where K_d is the geometric mean of macroscopic calcium dissociate constants for two EF sites, $K_d = \sqrt{K'_{Ca_1} \cdot K'_{Ca_2}}$. As noted by Linse et al. [17] and Porumb [18], the values of $K_1 \cdot K_2$ are determined more correctly than individual K_i values. The log interval of the free Ca^{2+} concentration between 10 and 90% saturation of Mts1 by Ca^{2+} can be used as an indicator of cooperativity. 10% saturation of Mts1 by Ca^{2+} was at $lg[Ca^{2+}] = -6.7$; 90% saturation was at $lg[Ca^{2+}] = -4.85$. The Hill coefficient (n_H) was estimated to be 0.98 showing that interaction of Mts1 with calcium lacked cooperativity. Similar absence of intersite calcium-binding cooperativity was observed for some other S-100 proteins [17,19].

The next step in our work was to isolate of a new Mts1

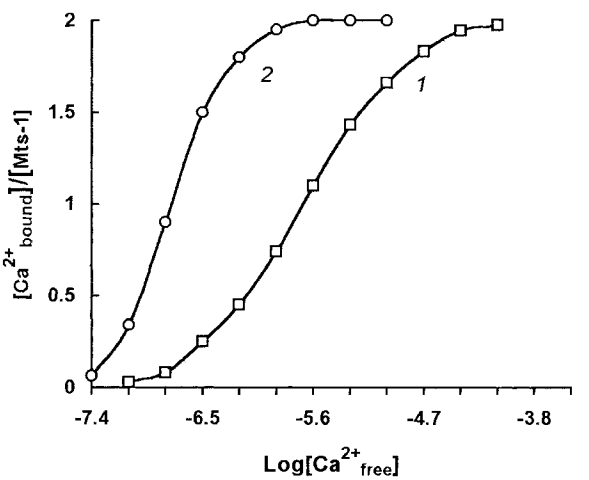

Fig. 2. Saturation curve for Ca^{2+} binding to Mts1 (1) and to the Mts1–p37 complex (2). Titrations were with 1 nmol of Fluo-3, 3.4 nmol of Mts1, 3.4 nmol of p37. The final sample volume was 1 ml.

target protein. For this purpose the column with immobilised recombinant Mts1 protein was prepared. Crude extract of CSML-100 cells was passed through the column with immobilised Mts1 protein. There were three proteins eluted from the column (p65, p37 and p32), but only one protein having a molecular mass of 37 kDa (p37) showed the ability to interact with Mts1 in further studies.

Mts1 in complex with p37 protein bound two Ca^{2+} ions with almost complete cooperativity with the Hill coefficient of 1.91 (Fig. 2, line 2), while p37 itself did not bind calcium at all (data not shown). 10% saturation of the complex by calcium was achieved at $lg[Ca^{2+}] = -7.15$, and 90% saturation at $lg[Ca^{2+}] = -6.2$. The apparent affinity of Mts1 to Ca^{2+} in complex with p37 became higher and the value of K_d was estimated as 0.2 μM.

It is well known that binding of ligands to protein leads to conformational changes of the protein (or to the stabilisation of a definite conformation of the protein). A widely used probe for studying conformational changes is TNS that is a fluorescent hydrophobic substance, the fluorescence of which is highly enhanced when it interacts with hydrophobic parts of the protein [20]. In the absence of proteins, the fluorescence of TNS is extremely low. In the presence of Mts1 without calcium, the fluorescence of TNS is enhanced 3.5-fold. In the presence of Mts1 with Ca^{2+} (concentration of calcium up to 0.5 mM), the TNS fluorescence is enhanced 8-fold (Fig. 3). For reference, Ca^{2+}-saturated form of calmodulin enhances the TNS fluorescence 10–30-fold, whereas only a 2-fold increase was obtained with calcium-free form of calmodulin [21]. The protein p37 with and without calcium did not affect the TNS fluorescence (data not shown). Our results suggest that Ca^{2+}-binding protein Mts1 has two types of hydrophobic regions: some of which are constantly accessible to TNS while others are accessible only in the presence of Ca^{2+}.

It has been suggested for S-100 and calmodulin-like proteins that calcium-dependent hydrophobic sites participate in interaction with target proteins [22]. The p37 protein decreased the TNS fluorescence of the Mts1–calcium complex almost to the level of fluorescence of Mts1 without Ca^{2+} (Fig. 4). In the absence of Ca^{2+}, p37 did not affect the TNS fluorescence of Mts1. Other proteins eluted from the Mts1-coupled column did not influence the TNS fluorescence of

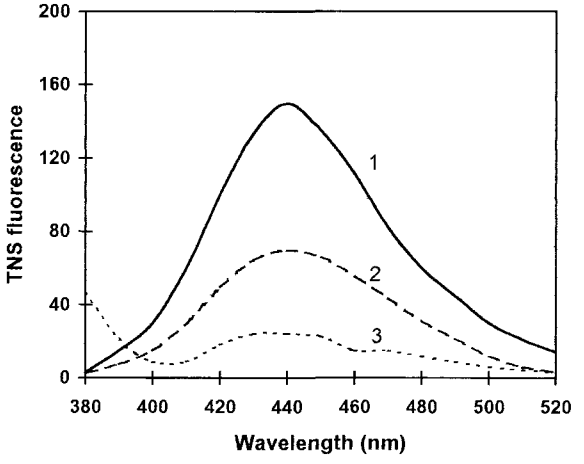

Fig. 3. Enhancement of TNS fluorescence in the presence of (2) Mts1 (5 μM) and (1) Mts1–Ca^{2+} complex (0.5 mM $CaCl_2$); (3) TNS fluorescence in the absence of protein.

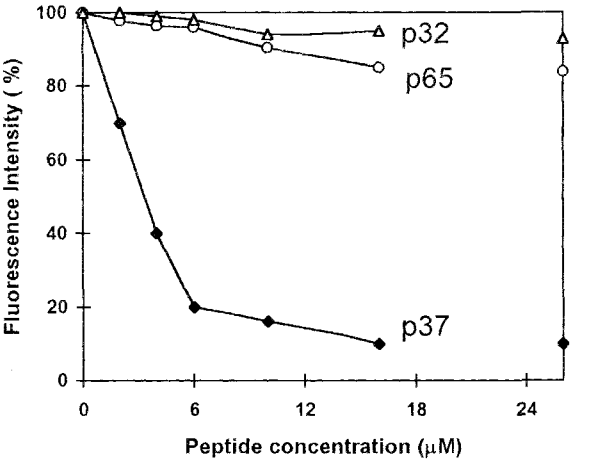

Fig. 4. Inhibition of Ca^{2+}-dependent TNS fluorescence enhancement of the Mts1 (5 μM) by different proteins. Zero of fluorescence intensity is the intensity of TNS fluorescence in the presence of Mts1 without Ca^{2+}, and intensity of the TNS fluorescence of Mts1–Ca^{2+} complex is expressed as 100% of fluorescence intensity.

Mts1 and Mts1–calcium complex. The most distinct inhibition was observed at the equimolar ratio of p37 and Mts1 (5 μM Mts1 and 6 μM p37). These results suggest the existence of competition between p37 and TNS for the same hydrophobic sites on Mts1. A similar phenomenon was described for some other proteins of the S-100 family and their target proteins [23,24]. We propose that p37 is a new target of Mts1. The molecular mass of p37 is close to that of tropomyosin; however, p37 does not interact with monoclonal antibodies to tropomyosin, recognising several isoforms of tropomyosin on Western (data not shown). The work to determine amino acid sequence of p37 protein is in progress.

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