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Zinc binding reverses the calcium-induced arachidonic acid-binding capacity of the S100A8/A9 protein complex

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Abstract Analysis of the calcium-induced arachidonic acid (AA) binding to S100A8/A9 revealed that maximal AA binding was achieved at molar ratios of 1 mol S100A8 and 1 mol S100A9 and for values greater than 3 calciums per EF-hand. The AA binding capacity was not induced by the binding of other bivalent cations, such as Zn^{2+}, Cu^{2+}, and Mg^{2+}, to the protein complex. In contrast, the binding of AA was prevented by the addition of either Zn^{2+} or Cu^{2+} in the presence of calcium, whereas Mg^{2+} failed to abrogate the AA binding capacity. The inhibitory effect was not due to blocking the formation of S100A8/A9 as demonstrated by a protein-protein interaction assay. Fluorescence measurements gave evidence that both Zn^{2+} and Cu^{2+} induce different conformational changes thereby affecting the calcium-induced formation of the AA binding pocket within the protein complex. Due to the fact that the inhibitory effect of Zn^{2+} was present at physiological serum concentrations, it is assumed that released S100A8/A9 may carry AA at inflammatory lesions, but not within the blood compartment.

Key words: Arachidonic acid; Ca^{2+} binding protein; Ca^{2+}; Cu^{2+}; Fatty acid binding protein; Protein structure; Zn^{2+}

1. Introduction

The two myeloid-related proteins MRP-8 (S100A8) and MRP14 (S100A9) belong to the calcium binding proteins of the S100 family (for review see [1–4]). These proteins form heterodimeric complexes as well as higher order species, and they bind polyunsaturated fatty acids in a calcium-dependent manner [5–7]. Recently, we could demonstrate that the S100A8/A9 protein complex represents the exclusive AA binding capacity in the neutrophilic cytosol [7]. The proteins are expressed during myeloid differentiation, and are abundant in granulocytes and monocytes [8,9]. Elevated plasma levels of S100A8/A9 have been found in patients suffering from a number of inflammatory disorders including cystic fibrosis, rheumatoid arthritis, and chronic bronchitis [10–13]. The demonstration that S100A8/A9 is specifically secreted from human monocytes and neutrophils upon protein kinase C activation may indicate an extracellular role for S100A8/A9 [14,15]. The secreted S100A8/A9 from neutrophil-like HL-60 cells has been shown to carry the released arachidonic acid [7]. Although there are a number of hypotheses, the exact functions of the protein complex, especially in the extracellular milieu, remain unknown. Stimulatory effects on neutrophil adhesion have been described for S100A9 which are reversed by S100A8 [16]. However, the study fails to identify the cell type by which S100A9 is released independently from S100A8.

S100A8 and S100A9 are composed of two distinct helix-loop-helix motifs (EF-hand) flanked by hydrophobic regions at either terminus and separated by a central hinge region. The C-terminal EF-hand has a higher affinity for Ca^{2+} and encompasses 12 amino acids, whereas the N-terminal Ca^{2+} binding loop is formed by 14 amino acids. The large differences in the Ca^{2+} affinities of both EF-hands may indicate that they are heterobifunctional. In addition to the binding of Ca^{2+}, human S100A9 has also been shown to bind Zn^{2+}, and the antimicrobial activity of the secreted human S100A8/A9 may be due to this property [17]. The zinc binding also induces structural changes within the dimer [18]. However, the Zn^{2+} binding sites are apparently distinct and independent of the two Ca^{2+} binding domains [19]. Thus, different target binding domains may be designed that allow interaction with different target proteins. It has been assumed that in some cases the biological activity of S100 proteins is regulated by Zn^{2+} rather than by Ca^{2+} [20].

The present study was undertaken to analyze the effects of various bivalent cations, including Zn^{2+}, Cu^{2+}, and Mg^{2+}, on the binding of arachidonic acid (AA) to the S100A8/A9 protein complex. We could demonstrate that these cations failed to induce the AA binding capacity of the protein complex. However, both zinc and copper abrogated the AA binding to S100A8/A9 at physiological serum concentrations.

2. Materials and methods

2.1. Purification of S100A8 and S100A9 from human neutrophils

Human neutrophils were prepared from leukocyte-rich blood fractions ('buffy coat') according to Müller et al. [21]. S100A8 and S100A9 were purified as described by van den Bos et al. [22] with minor modifications. Prior to use, the proteins were rechromatographed by anion exchange chromatography using a UnoQ column (Bio-Rad, Munich, Germany).

2.2. Fatty acid binding assay

Binding of [^{3}H]AA to proteins was carried out as described earlier [7]. For the estimation of the molar ratio of S100A8 and S100A9 within the protein complex, in a total volume of 250 μl 1 nmol S100A8 was incubated in buffer containing 20 mM Tris-HCl, pH 7.4 and 0.01% (w/v) Triton X-100 together with 1 μM [^{3}H]AA and different molar ratios of S100A9 as indicated for 1 h at 37°C in the presence of 5 mM $CaCl_2$. For the determination of the molar ratio of calcium ions vs. the number of EF-hands, in a total volume of 1 ml 1 nmol S100A8/A9 was incubated with increasing concentrations of calcium as indicated and 1 μM [^{3}H]AA. The absolute calcium concentrations were measured by atomic adsorption spectrophotometry. For the analysis whether zinc induces AA binding capacity, 1 nmol S100A8/A9 was incubated with 1 μM [^{3}H]AA in the presence or

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Abbreviations: AA, arachidonic acid; FMLP, formylmethionylleucylphenylalanine; GST, glutathione *S*-transferase

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absence of either calcium, zinc or magnesium at concentrations as indicated in a total volume of 250 μl.

2.3. Recombinant expression of S100A8 and S100A9

Both human S100A8 and S100A9 cDNAs were used as templates to generate PCR fragments with suitable cloning ends. The PCR fragments were purified and cloned into pQE32 (Qiagen, Düsseldorf, Germany) or pGEX2T (Pharmacia, Freiburg, Germany) following standard protocols. Individual clones were analyzed by PCR and SDS-PAGE. Positive clones were grown up to $OD_{580} = 0.6$ and subsequently induced by 1 mM IPTG for 2 h. Cell pellets were resuspended in TBS, sonified, frozen and stored at −20°C. Histidine-tagged proteins were purified using the TALON affinity matrix (Clontech, Heidelberg, Germany). Briefly, cell pellets were resuspended in TBS, 8 M urea, insoluble material was removed by centrifugation, and the supernatant was incubated with the affinity matrix. Proteins were renatured on the column by washing with buffers containing decreasing urea concentrations. Finally, recombinant proteins were eluted with TBS containing 300 mM imidazole.

Glutathione *S*-transferase (GST)-S100A8 and GST-S100A9 fusion proteins were soluble in *Escherichia coli*, and were purified without denaturation. Glutathione-Sepharose (Pharmacia) was added to the cleared cell homogenate, washed and eluted with TBS containing 100 mM glutathione. Subsequently, glutathione was removed by dialysis and purity of the recombinant proteins was demonstrated by SDS-PAGE.

2.4. GST pull-down assay

GST fusion protein (10 μg) and 10 μg His-tagged S100A8 or S100A9 were incubated in TBS, 1% BSA buffer for 1 h at room temperature in a total volume of 100 μl. The glutathione-Sepharose was washed in TBS, 1% BSA three times and 25 μl of bed volume was added to the reaction. After 1 h incubation the glutathione-Sepharose was washed three times with 10 volumes of washing buffer (0.4 M NaCl; 20 mM Tris-HCl; 0.05% NP-40; pH 7.5). Finally, SDS sample buffer was added to the glutathione-Sepharose pellet, heated to 100°C, and analyzed by 15% SDS-PAGE.

2.5. Fluorescence measurements

Fluorescence spectra were recorded at 20°C with a spectrofluorometer (Spex, Model FluoroMax II, Instruments SA, Munich Germany) using protein concentrations of 5 μg/ml (0.208 μM). The excitation wavelength was 280 nm with a bandpass of 1 nm, and the emission scans were recorded between 300 and 400 nm with a bandpass of 4 nm and an integration time of 0.2 s. To avoid possible disulfide formation between S100A8 and S100A9, 1 mM DTT was added to the buffer (20 mM Tris, pH 7.5 at 20°C). All fluorescence spectra were buffer corrected. For titration of S100A8/9 with zinc and copper, the fully reduced complex was used in the absence of free reducing agents.

Ca^{2+}, Cu^{2+} and/or Zn^{2+} binding to the complex was monitored by the change in the intrinsic Trp and Tyr fluorescence. Calcium concentrations ranging from 0 to 20 μM, zinc and copper concentrations ranging from 0 to 50 μM were used for the titration. All samples were preequilibrated overnight prior to the fluorescence measurements. The absolute change of the fluorescence intensity at 342 nm and the change in the fluorescence maximum were plotted as a function of the Ca^{2+}, Cu^{2+} and/or Zn^{2+} concentration as indicated.

2.6. Protein determination

Determination of protein content was performed according to Smith et al. [23], using BSA as standard. The concentrations of the purified proteins were accurately determined using extinction coefficients of 0.998 for S100A8, 0.526 for S100A9 or 0.762 for S100A8/A9 at 280 nm.

3. Results and discussion

3.1. Binding of calcium to each EF-hand within S100A8/A9 is prerequisite for AA binding

It has been assumed that the N- and C-terminal EF-hands within the S100 proteins are heterobifunctional due to the large differences in their affinity towards calcium [20]. AA binding to S100A8/A9 has been demonstrated at calcium concentrations below 1 μM, the physiological range of activated neutrophils, as well as in the extracellular milieu [7], where the value for the calcium concentration is within the range of 1–2 mM. Therefore, we performed fatty acid binding assays with S100A8/A9 at a molar ratio of 1:1 in the presence of increasing calcium concentrations (Fig. 1). We found that the AA binding capacity of the protein complex increased with the number of calcium ions per EF-hand. Maximal AA binding was achieved for values greater than 3 calciums per EF-hand within the S100A8/A9 molecule. Excess calcium did not affect AA binding capacity of the protein complex. These results revealed that binding of one calcium ion to each of the four EF-hands present in the heterodimer is a prerequisite for the induction of the AA binding capacity.

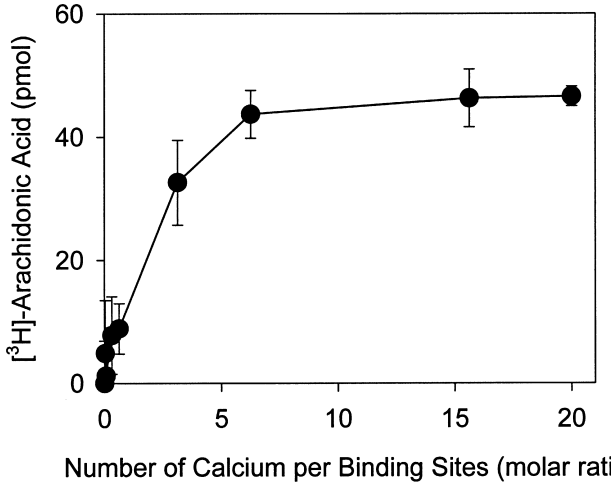

Fig. 1. Analysis of calcium ions vs. number of EF-hands within the protein complex. S100A8/A9 (24 μg) were incubated with increasing concentrations of calcium as indicated and 1 μM [^{3}H]AA in a total volume of 1 ml. The absolute calcium concentrations were measured by atomic adsorption spectrophotometry. The protein-bound fatty acids were separated from non-bound fatty acids using the Lipidex assay. The bars represent data from three independent experiments with duplicate determinations ± S.D.

3.2. S100A8/A9 complex consist of equal mols S100A8 and S100A9

The calculation of calcium ions per number of EF-hands is based on the assumption that the protein complex consists of equal mols of S100A8 and S100A9. Recently, two reports have been published by our group revealing that in addition to the heterodimer, a protein complex with a M_r of 48 kDa is formed upon calcium binding, consisting of two molecules S100A8 and two molecules S100A9 [7,24]. Both protein complexes are shown to display AA binding capacity [7]. In contrast, Roulin et al. [25] have reported that in human keratinocytes the protein complex is a trimer composed of two S100A8 and one S100A9 subunit. Therefore, we performed experiments using different molar ratios of S100A8 and S100A9 in the presence of calcium to induce protein complex formation followed by the determination of AA binding capacity. The S100A8 and S100A9 proteins were purified from human neutrophils and renatured as described in Section 2. The native proteins were incubated at different molar ratios in the presence of 5 mM calcium, and protein-bound AA was discriminated from the unbound fatty acid using the Lipidex assay. As shown in Fig. 2, the AA binding capacity of the formed protein complex was increased at molar ratios of S100A8 and S100A9 from 1:0.25 to 1:1. Maximal AA binding

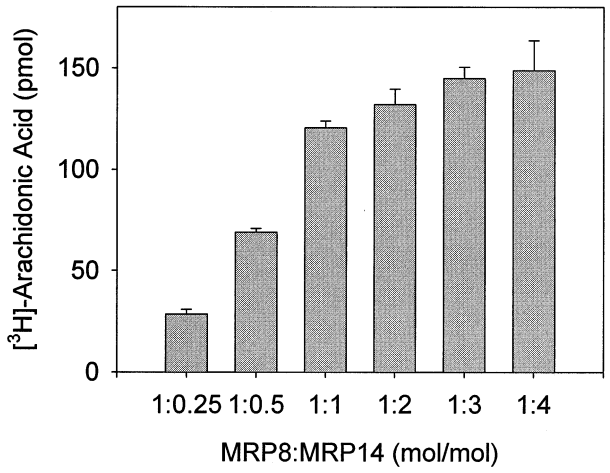

Fig. 2. Analysis of the molar ratio of S100A8 and S100A9 in the protein complex. The purified and renatured proteins were incubated at different molar ratios as indicated with 1 μM [^{3}H]AA for 1 h at 37°C in the presence of 5 mM $CaCl_2$. The protein-bound fatty acids were separated from non-bound fatty acids using the Lipidex assay. The bars represent data from three independent experiments with duplicate determinations ± S.D.

of the protein complex was achieved at a molar ratio of 1 mol S100A8 and 1 mol S100A9. An increase in the molar ratio of S100A8 and S100A9 from 1:1 to 1:4 did not enhance AA binding capacity of the protein complex. These findings confirmed the assumption that the protein complex, which is able to bind AA, consists of equal mols S100A9 and S100A8. This result is in agreement with other studies [24,26].

3.3. Zinc binding blocked AA binding capacity of S100A8/A9

The C-terminal end of S100A9 contained a His-x-x-x-His motif followed by an upstream Glu, which may represent the putative Zn^{2+} binding site. When present in an α-helix, this motif can bind Zn^{2+} with high affinity, as was found in a number of naturally occurring proteins [27]. Calcium as well as zinc binding have been shown to induce conformational changes within S100 proteins leading to the exposure of hydrophobic surfaces [18]. Therefore, we investigated whether zinc binding also induces the AA binding capacity of the S100A8/A9 protein complex. The protein complex did not display AA binding in the range from 1 μM to 1 mM zinc (data not shown). Then the fatty acid binding assay was performed with S100A8/A9 in the presence of 1 mM calcium and increasing concentrations of zinc. As shown in Fig. 3C, the AA binding capacity was reversed by increasing zinc concentrations. At 10 μM zinc the protein complex did not display any AA binding. This value for zinc is within the physiological range for serum. Control experiments were performed with S100A8/A9 and magnesium in the presence and absence of calcium. The addition of magnesium to S100A8/A9 failed to induce AA binding capacity (Fig. 3A). However, magnesium did not affect the AA binding capacity of the protein complex in the presence of calcium indicating that the observed effect was specific for Zn^{2+} (Fig. 3B).

It has been suggested that Zn^{2+} and Cu^{2+} share the same binding sites [28]. According to this suggestion, we found that Cu^{2+} failed to induce AA binding capacity of S100A8/A9 and reversed the AA binding to S100A8/A9 in a concentration-dependent manner (Fig. 3A,B). The K_i values for zinc and copper were 5 μM and 2 μM, respectively. The effects of zinc and copper were additive indicating that copper binds to the same site as zinc (data not shown).

3.4. Zinc does not influence the S100A8/A9 complex formation

It has been demonstrated that the AA binding was specific for the S100A8/A9 protein complex and the individual components were not able to bind AA. Thus, the reversing effect of Zn^{2+} upon AA binding to S100A8/A9 may be due to zinc-mediated conformational changes, which prevent protein-protein interaction. Therefore, an in vitro assay was established to investigate S100A8-S100A9 interaction in the presence of zinc. Both S100 proteins were recombinantly expressed each as histidine- and GST-tagged molecules and used in a GST pull-down assay in the presence and absence of zinc to monitor the capability of these proteins to associate. As shown in Fig. 4, zinc ions do not inhibit the protein complex formation of S100A8 and S100A9. Homodimers were not observed in

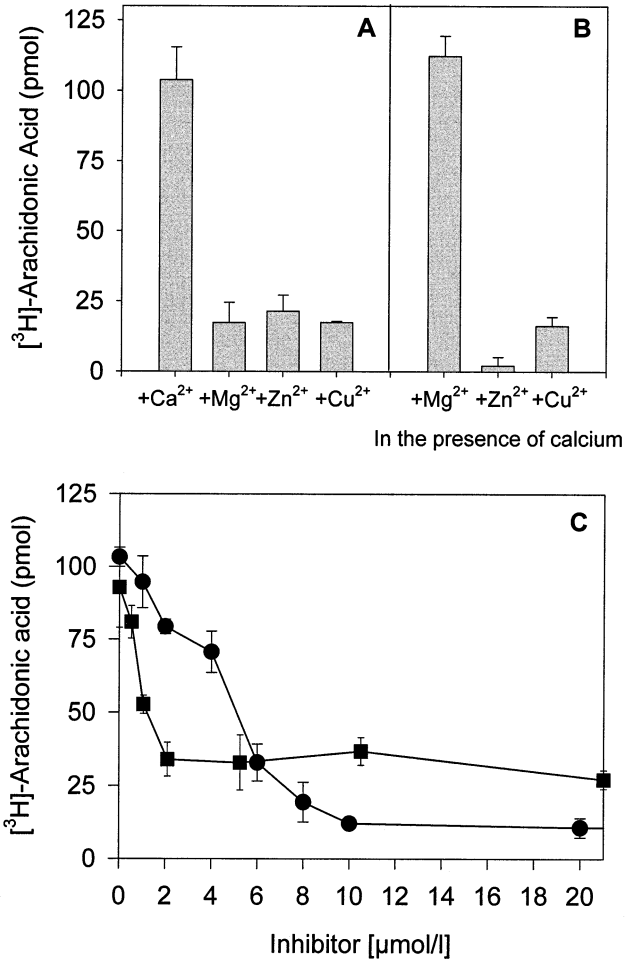

Fig. 3. Effect of zinc, copper, and magnesium upon AA binding capacity of S100A8/A9. S100A8/A9 (24 μg) was incubated with 1 μM [^{3}H]AA and zinc (100 μM), copper (100 μM), or magnesium (1 mM) in the absence (A) or presence (B) of 1 mM calcium. C: S100A8/A9 (24 μg) was incubated with 1 μM [^{3}H]AA in the presence of 1 mM calcium and increasing concentrations of zinc (●) or copper (■) as indicated. The protein-bound fatty acids were separated from non-bound fatty acids using the Lipidex assay. The bars represent data from three independent experiments with duplicate determinations ± S.D.

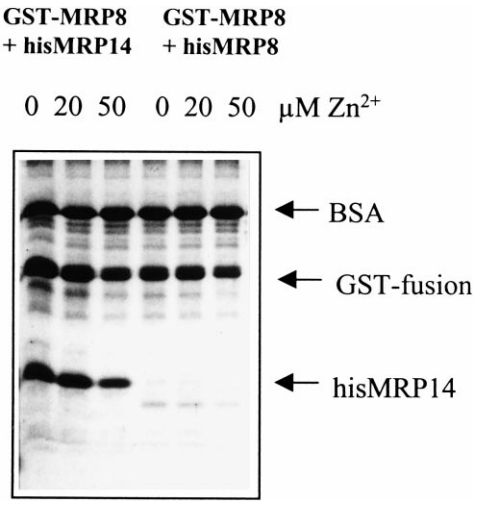

Fig. 4. S100A8/A9 (MRP8/14) complex formation is not inhibited by Zn ions. Prior to the addition of glutathione-Sepharose, GST fusion proteins were incubated with His-tagged proteins for 1 h at 4°C in the presence of 1 mM $CaCl_2$ and increasing concentrations of $ZnCl_2$ as indicated. The glutathione-Sepharose pellet was washed followed by the addition of SDS sample buffer. Then the suspension was heated to 100°C, and the proteins were analyzed by 15% SDS-PAGE.

this assay either in the absence or in the presence of zinc. GST alone bound neither S100A8 nor S100A9.

3.5. Bivalent cations induce conformational changes in S100A8/A9

Conformational changes within protein molecules after binding of ligands or after interaction with other proteins can be monitored by fluorimetric measurements. The fluorescence properties of proteins depend mainly on the presence of aromatic amino acids. There are several aromatic residues present in each human S100A8 (Trp^{54}, Tyr^{16}, Tyr^{19}, Tyr^{30}, and Tyr^{45}) and human S100A9 (Trp^{88} and Tyr^{22}). The Trp residues have been shown to be sensitive to the residues' immediate environment and to alter in a characteristic way when the local environment changes.

The addition of increasing Ca^{2+} concentrations to S100A8/A9 led to a strong shift of the fluorescence maximum from 342 to about 334 nm and to a decrease in the intensity of the emission spectrum from 20 000 to about 13 000 cps (Fig. 5A,C). This blue shift is typical for aromatic residues moving into a more hydrophobic environment. A similar behavior has been observed for several other S100 proteins (for review see [2,4]). Here, conformational changes within the homodimer occur upon Ca^{2+} binding leading to the exposure of hydrophobic surfaces. The addition of AA (0.2 μM) did not influence these structural changes (Fig. 5A,C).

The addition of Zn^{2+} to S100A8/A9 resulted into different conformational changes (Fig. 5B,D). At low Zn^{2+} concentrations both the fluorescence maximum and the fluorescence intensity were decreased to values comparable to the calcium-induced changes. However, at higher Zn^{2+} concentrations the fluorescence intensity was increased, but not the fluorescence maximum. The Zn^{2+}-induced changes were not altered by the simultaneous addition of 10 μM calcium in the presence of 0.2 μM AA. Surprisingly, the addition of Cu^{2+} to S100A8/A9 resulted into a significant decrease in the fluorescence intensity, but the fluorescence maximum was not altered (Fig. 5B,D). In accordance with Zn^{2+}, the Cu^{2+}-induced structural changes were not altered by the simultaneous addition of Ca^{2+} (data not shown). These observations might indicate stronger effects on the protein structure by the binding of Zn^{2+} and Cu^{2+} than of Ca^{2+}. From our data it is suggested that AA binds to S100A8/A9 when binding of the bivalent cation induces both a blue shift in the fluorescence maximum and a decrease in the fluorescence intensity. Therefore, one can assume that both Zn^{2+} and Cu^{2+} induce structural changes within the protein complex, which reverses the Ca^{2+}-induced formation of the AA binding site. Furthermore, we suggest that neither Zn^{2+} nor Cu^{2+} binds to the same binding site as Ca^{2+}, because otherwise the Ca^{2+}-induced changes should be reversed to apo-values. To date the three-dimensional structure of S100A8/A9 remains unknown. Thus, one cannot decide whether both Zn^{2+} and Cu^{2+} bind to the same binding site. However, the different fluorescence changes induced by the binding of both bivalent cations may indicate different binding sites. The analysis of the three-dimensional structure of S100A8/A9 is currently under investigation in our laboratory.

3.6. Ca^{2+} and Zn^{2+} binding allow interaction with different target proteins and cell surface binding sites

In the past several studies have dealt with the extracellular role of S100A8 and S100A9. For example, S100A8 and S100A9 were reported to have growth inhibitory activity upon cells [29], to have antimicrobial, cytostatic, and chemotactic activities [30–33] as well as stimulating neutrophil adhesion [16]. Recently, we could show that the S100A8/A9 pro-

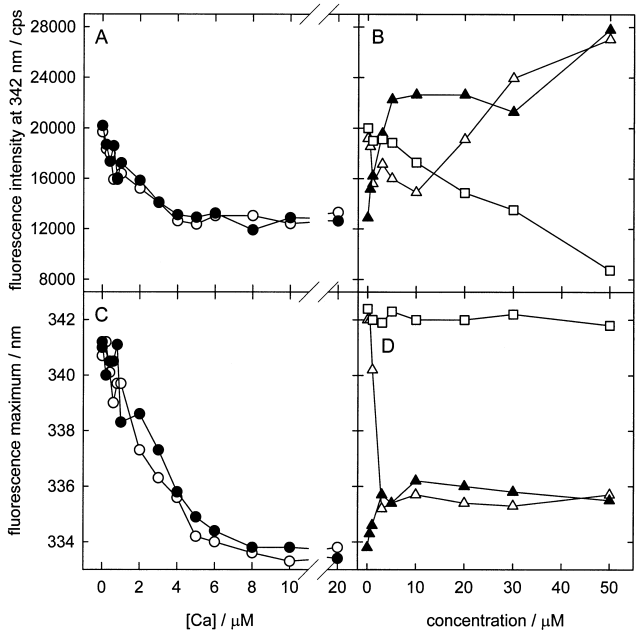

Fig. 5. Effect of zinc and calcium on Trp and Tyr fluorescence intensity at 342 nm and the fluorescence maximum. Variation of fluorescence intensity at 342 nm (A, B) and fluorescence maximum (C, D) of S100A8/A9 with increasing concentrations of bivalent cations in the absence and presence of 0.2 μM arachidonic acid as indicated. Spectra were taken at 20°C in 20 mM Tris, pH 7.5, 1 mM DTT at a protein concentration of 0.208 μM. ○, S100A8/A9 with increasing Ca^{2+} concentrations; ●, S100A8/A9 with 0.2 μM AA and increasing Ca^{2+} concentrations; △, S100A8/A9 with 0.2 μM AA and increasing Zn^{2+} concentrations; ▲, S100A8/A9 with 0.2 μM AA, 10 μM Ca^{2+} and increasing Zn^{2+} concentrations; □, S100A8/A9 with 0.2 μM AA and increasing Cu^{2+} concentrations.

tein complex is secreted from phorbol myristate acetate-stimulated neutrophil-like HL-60 cells which carry AA, thus assuming a modulatory role in inflammatory processes, probably as shuttle protein to transport AA to its target cells [7]. However, the precise function of S100A8/A9, particularly in the extracellular milieu, remains unclear. The present study represents the first report that binding of zinc as well as copper reversed the property of a S100 protein which has been induced by the binding of calcium. Both calcium and zinc binding induce conformational changes within the protein complex, and as shown in this study, zinc reversed the calcium-induced formation of an AA binding site. Therefore, it can be envisaged that different structural changes are induced by either calcium or zinc, thus allowing interactions with different target proteins as well as different cell surface binding sites. Thus, the variety of reported effects for the secreted S100A8/A9 may reflect that in some cases the biological activity of S100 proteins is regulated by Zn^{2+} and in other cases by Ca^{2+}.

It is likely that secretion of AA-S100A8/A9 complexes takes place at sites of inflammation, released by inflammatory cells targeted to such loci by a range of environmental cues. Physiological stimuli that induce S100A8/A9 secretion from human neutrophils are the chemoattractants FMLP and C5a [15] indicating that this mechanism is functional following receptor-dependent activation of cells. The serum concentration of Zn^{2+} is within the range 10–20 μM. The basic level of free intracellular Zn^{2+} is estimated within different tissue in the range of 0.024–0.5 nM [34–37]. However, the majority of Zn^{2+} is bound to proteins. Therefore, the Zn^{2+} concentrations within cells and tissue are assumed to be below 1 μM indicating that S100A8/A9 may bind AA in vivo. Consequently, the AA transport by S100A8/A9 may be limited by the concentration gradient of zinc between the bloodstream and the inflammatory lesion. This may represent another mechanism by which the property of a secreted protein is restricted to local environments. Recently, Harrison et al. [38] have demonstrated that oxidative modification affects the chemotactic activity of murine S100A8. The monomeric and chemotactic S100A8 are transformed into non-chemotactic S100A8 homodimers by hypochlorite oxidation-driven disulfide linkage. The oxidative modification of murine S100A8 is suggested by the authors to provide a mechanism limiting excess infiltration of leukocytes and terminating the progression of acute inflammation. However, performing analogous experiments with hypochlorite we could not observe any effect upon the fatty acid binding capacity of the S100A8/A9 protein complex [7].

Therefore, further investigations should try to identify target proteins binding to S100 proteins in a Ca^{2+}-independent but Zn^{2+}- and Cu^{2+}-dependent manner. In addition, they have to pinpoint the binding sites specific for S100A8 and S100A9 under consideration of the ion bound to the protein complex and the cells expressing the putative receptor(s).

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