Human Calprotectin: Effect of Calcium and Zinc on its Secondary and Tertiary Structures, and Role of pH in its Thermal Stability

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Abstract Calprotectin, a heterodimeric complex belonging to the S100 protein family, has been found predominantly in the cytosolic fraction of neutrophils. In the present study, human calprotectin was purified from neutrophils using two-step ion exchange chromatography. The purified protein was used for circular dichroism study and fluorescence analysis in the presence of calcium and zinc at physiological concentrations, as well as for assessment of its inhibitory activity on the K562 leukemia cell line. The thermal stability of the protein at pH 7.0 (physiological pH) and 8.0 (similar to intestinal pH) was also compared. The results of cell proliferation analysis revealed that human calprotectin initiated growth inhibition of the tumor cells in a dose-dependent manner. The intrinsic fluorescence emission spectra of human calprotectin (50 μg/ml) in the presence of calcium and zinc ions show a reduction in fluorescence intensity, reflecting a conformational change within the protein with exposure of aromatic residues to the protein surface that is important for the biological function of calprotectin. The far ultraviolet-circular dichroism spectra of human calprotectin in the presence of calcium and zinc ions at physiological concentrations show a decrease in the α-helical content of the protein and an increase in β- and other structures. Our results also show that increasing the pH level from 7.0 to 8.0 leads to a marked elevation in the thermal stability of human calprotectin, indicating a significant role for pH in the stability of calprotectin in the gut.

Keywords calprotectin; calcium; zinc; circular dichroism; thermal stability

The myeloid-related protein 8 (MRP8) and MRP14 are two small anionic proteins structurally related to the S100 protein family with zinc- and calcium-binding capacity [1]. The two myeloid-derived proteins are abundant in the cytosolic fraction of neutrophils and in lesser amount in monocytes [2]. They form a heterodimeric complex in a calcium-regulated process that has been called the calprotectin, MRP8/14, cystic fibrosis antigen, protein complex, Mac 387 and 27E10 antigen [3−5]. Human MRP8 (S100A8) and MRP14 (S100A9) have molecular masses of approximately 11 and 14 kDa and are composed of 93 and 114 amino acids, respectively [6]. The abundance of calprotectin in neutrophils and the calcium-binding capacity of this protein suggest a role in signal transduction [7].

Each subunit of this calcium-modulating signaling protein has two EF-hands (helix-loop-helix) that contain the calcium-binding site, flanked by hydrophobic regions at either terminus, and separated by a central hinge region [2]. Also, the affinity of calprotectin to calcium might be similar to that of calmodulin [8]. The zinc-binding capacity of calprotectin is not affected by calcium binding, because both chains of calprotectin contain distinct motifs (HEXXH motif) for binding to zinc. It was reported that the zinc-binding capacity of calprotectin was higher than that of other S100 proteins [3,9]. Several reports indicate that

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calprotectin has antimicrobial and apoptosis-inducing activities that are reversed by the addition of zinc [10–12]. Therefore, zinc might be a negative regulator, restricting the systemic cytotoxic effects of calprotectin, especially against normal cells. Zinc, as a ligand that modifies the function of calprotectin, might be an important target in the regulation of inflammatory reactions and might be an important factor that prevents tissue destruction where the concentration of calprotectin is dramatically increased in local inflammatory sites [13]. Calprotectin inhibits the activity of matrix metalloproteinases (MMPs) by sequestration of zinc. MMPs constitute a family of zinc-dependent enzymes with important roles in many normal biological processes, including wound healing, and pathological processes such as inflammation, cancer, and tissue destruction [14].

A previous study showed that calprotectin has a growth inhibitory effect on normal fibroblasts that regulate the repair of wound sites through cell growth and production of the material covering the intracellular matrix [11]. Calprotectin also binds to polyunsaturated fatty acids such as arachidonic acid in a calcium-dependent manner and probably has an important role in eicosanoid metabolism [15,16].

Therefore, this important calcium signal-inducing protein with pleiotropic function is a novel inflammatory mediator and it seems to be a novel player in wound repair [6,17].

Conformational changes occurring after calcium binding have been shown for a number of S100 proteins [18]. Nuclear magnetic resonance spectroscopy analysis revealed that calcium binding to the calprotectin heterodimer results in structural changes in the linker helix and second calcium binding loop regions [6,19]. These conformational changes lead to the exposure of the hydrophobic surface after calcium binding that might allow interaction with the target protein such as casein kinase, which is inhibited by calprotectin [20]. Inhibition of such kinase activity could also explain the cytostatic activity of calprotectin that played toward a variety of cell types [21]. However, fecal calprotectin has high stability and it has been recently proposed as a good clinical marker for colonic neoplasm and inflammation, with high diagnostic accuracy, in lower gastrointestinal tract [22].

Materials and Methods

Materials

Dithiothreitol (DTT) and Lymphoprep were obtained from Merck (Darmstadt, Germany) and Amersham (Piscataway, USA), respectively. Fetal calf serum (FCS) was obtained from the veterinary faculty at the University of Tehran (Tehran, Iran). RPMI 1640 medium, penicillin, streptomycin, calcium chloride (CaCl2), zinc chloride (ZnCl2), and all other reagents were purchased from Sigma Chemical Co. (St Louis, USA) and were of, at least, analytical grade. All solutions were prepared with double-distilled water.

Cell line

K562 chronic myelogenous leukemia cells were obtained from the cell bank of the Pasteur Institute of Iran (Tehran, Iran). These cells were maintained in RPMI 1640 medium supplemented with 10% FCS in a humidified incubator (37 °C and 5% CO2).

Neutrophil isolation and extraction

Fresh human blood was collected randomly from healthy donors into heparinized plastic bags and isolation of leukocytes was carried out by dextran sedimentation according to the method of Skoog and Beck [23]. One unit of 500 ml heparinized blood was mixed with 250 ml of 6% dextran T-500. Sedimentation was allowed to proceed for 45 min at room temperature. The supernatant was harvested and leukocytes were spun down at 200 g for 20 min at 4 °C. Residual red cells were lysed by the addition of ice-cold distilled water to the sediment and isotonicity was restored after 30 s by the addition of phosphate-buffered saline (PBS). After washing twice in PBS, granulocytes were separated from mononuclear cells by loading 3 ml suspension on the top of 6 ml Lymphoprep, followed by centrifugation at 800 g for 30 min at 20 °C.
Purification of calprotectin

Purification of human calprotectin was carried out as previously described [24]. Briefly, the crude neutrophil extract was dialyzed against buffer I (25 mM Tris-HCl, pH 8.0, 1 mM EDTA, and 1 mM DTT) then injected onto an anion exchange column (Q-Sepharose) that was pre-equilibrated with five column volumes of buffer I at a flow rate of 1 ml/min. Bound proteins were eluted from the column with 0–0.5 M NaCl gradient in buffer I over 150 min, at 4 °C. Anion exchange-eluted fractions were analyzed by tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; 15% gel) under reducing conditions. The growth inhibitory activities of calprotectin-containing fractions were checked using K562 cells as the target.

These fractions were further dialyzed against buffer II (25 mM sodium acetate, pH 4.5, 1 mM EDTA, and 1 mM DTT), then injected onto a cation exchange column (SP-Sepharose) that was pre-equilibrated with five column volumes of buffer II at a flow rate of 1 ml/min. Bound proteins were eluted from the column with 0–1.0 M NaCl gradient in buffer II over 100 min. At this stage MRP8 and MRP14 appeared to be essentially pure (>98%) by densitometric analysis of SDS-PAGE gels, visualized by Coomassie Brilliant Blue staining. During the purification procedure, in spite of extensive dialysis against the buffers that contained 1mM EDTA, and using inductively coupled plasma spectroscopy, it was shown that each calprotectin molecule contained two calcium ions, whereas the content of zinc ions in the protein was negligible. Thus, the experiments were carried out in the presence of excessive calcium ions.

Dialysis was carried out in 1000 Da cut-off dialysis tubing at all stages. The purified protein was aliquoted and stored at −70 °C or at 4 °C for short term.

Electrophoresis

For tricine-SDS-PAGE, the system described by Schägger and von Jagow [25] was used. Samples were boiled in a sample buffer with mercaptoethanol for 5 min and electrophoresed on polyacrylamide gel (separating gel: 16.5% T, 3% C; stacking gel: 4% T, 3% C) under 200 V. Protein bands were visualized by Coomassie Brilliant Blue staining.

Protein assay

The protein concentration was determined using Bradford reagent (Bio-Rad, South San Francisco, USA) with bovine serum albumin as the standard [26].

Cell culture

K562 cells were grown in RPMI 1640 medium (pH 7.4) with 10% heat-inactivated FCS, supplemented with 4 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin in a humidified 5% CO2 incubator at 37 °C. Harvested cells were seeded onto 96-well plates (2×104 cells/ml) and proliferation curves for K562 cells were determined using the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay.

Cell proliferation assay

The relative cell number was measured by MTT assay as described by Mossman [27]. MTT was dissolved in PBS to a concentration of 5 mg/ml and the solution was filtered through a 0.5 μm filter, then stored at 2–8 °C for frequent use. Four hours before the end of incubation, 25 μl MTT solutions (5 mg/ml) was added to each well containing fresh and cultured medium. The insoluble formazan produced was dissolved in a solution containing 10% SDS and 50% dimethylformamide (left for 2 h at 37 °C in the dark) and the optical density (OD) was read against a reagent blank with a multi-well scanning spectrophotometer (Multiskan, Helsinki, Finland) at 540 nm. The OD value of experimental groups was divided by the OD value of untreated control groups and presented as a percentage of the control groups (as 100%).

CD spectroscopy

The far ultraviolet (UV) region (180–250 nm) that corresponds to peptide bond absorption [28] was analyzed by a 215 AVIV spectropolarimeter (AVIV, Lakewood, USA) to give the content of regular secondary structures in human calprotectin.

Far UV-CD spectra were taken at a protein concentration of 0.2 mg/ml with 1 mm path length quartz cuvette. Protein solutions were prepared in 10 mM phosphate buffer at pH 7.0. The protein solutions were also incubated with calcium chloride (1 mM) and zinc chloride (10 μM) for at least 15 min to obtain the spectra of calcium- and zinc-incubated proteins. All spectra were collected from 200 to 260 nm and the background was corrected against the buffer blank. The results were expressed as molar ellipticity (deg·cm2/dmol) considering a mean residues number of 207 and an average molecular weight of 25 kDa for human calprotectin [6]. The molar ellipticity was determined as [θ]=100×(MRW)×θobs/(c×l), where θobs is the observed ellipticity in degrees at a given wavelength, c is the protein concentration in mg/ml and l is the length of the CD cell.

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of the light path in cm.

**Fluorescence spectroscopy**

Intrinsic fluorescence was measured by exciting the protein solution (50 μg/ml) with 1 cm path length cell at 280 nm in 10 mM phosphate buffer at pH 7.0 and emission spectra were recorded in the wavelength range of 300–450 nm at 25 ºC. Fluorescence measurements were carried out on an RF-5000 spectrofluorometer (Shimadzu, Tokyo, Japan) equipped with a 150 W xenon lamp and a DR-3 data recorder, and the excitation and emission slits were set at 5 and 10 nm, respectively.

**Thermal denaturation analysis of human calprotectin**

Thermal denaturation of human calprotectin (0.2 mg/ml) was carried out by following the absorbance at 280 nm, using a Cary spectrophotometer (Varian, Salt Lake, Australia). Before proceeding with the thermal denaturation experiments, the protein samples were dialyzed against Tris buffer (25 mM) at pH 7.0 and 8.0.

**Statistical analysis**

Results were analyzed for statistical significance using a two-tailed Student’s t-test. Changes were considered significant at $P<0.05$.

**Results**

**Cell death-inducing assay of human calprotectin**

In order to examine the cell death-inducing activity of calprotectin, various concentrations of the purified protein were used to culture tumor cell line K562 for 24 and 36 h. As shown in Fig. 1, the cell growth was significantly inhibited by all treatments, indicating a dose- and time-dependent suppression of growth of the K562 leukemia cell line. Treatment of this cell line with human calprotectin at concentrations of 50 μg/ml or above led to a marked decrease of cell proliferation as determined by MTT assay (Fig. 1).

Our study also showed that the growth inhibitory effect of 100 μg/ml human calprotectin was reversed by the coexistence of 10 μM zinc, indicating that the cytostatic effect of the purified sample was due to calprotectin (data not shown).

**Far UV-CD and fluorescence studies of human calprotectin**

CD has been proven to be an ideal technique for monitoring the transitional switch between regular secondary structures in proteins, which can occur as a result of changes in experimental parameters such as binding of ligands [28]. The far UV-CD spectra characterize the secondary structures of proteins due to the peptide bond absorption, and changes in this spectra usually reflect the major backbone changes in proteins [28,29].

As shown in Fig. 2 and Table 1, the far UV-CD spectra of the intact protein indicate a high degree of α-helix (approximately 49.0%), which is the characteristic of EF-hand proteins [7,19]. Far UV-CD analysis of this protein in the presence of 10 μM zinc and 1 mM calcium showed significant changes in the secondary structures (Fig. 2 and Table 1). In the presence of 1 mM calcium and 10 μM zinc (approximate physiological concentrations of both ions), a reduction in α-helix content and an increase in β- and other structures of human calprotectin were seen (Fig. 2 and Table 1).

Fluorescence spectroscopy is a useful technique to study the structure, dynamics, and binding properties of protein molecules in solution. Conformational changes within protein molecules after binding of ligands can be monitored by fluorometric measurements. The fluorescent properties of proteins depend mainly on the presence of aromatic amino acids. There are several aromatic residues present in each subunit of human calprotectin (Trp$^{22}$, Tyr$^{26}$, Tyr$^{19}$, Tyr$^{30}$, and Tyr$^{44}$ in MRP8, and Trp$^{58}$ and Tyr$^{22}$ in MRP14) [2,30]. When compared with the intact protein,
in the presence of zinc (10 μM), significant reductions in both fluorescence intensity and fluorescence maximum of human calprotectin were seen (Fig. 3), reflecting a conformational change within the protein or quenching of fluorescence emission of the aromatic residues. Decrease of the fluorescence intensity of human calprotectin in the presence of zinc might indicate the exposure of aromatic residues to the solvent. In contrast, in the presence of 1 mM calcium, a significant decrease in fluorescence intensity of the protein was seen, accompanied by a strong blue shift. This blue shift is typical for aromatic residues moving into a less hydrophobic environment. Therefore, the emission spectra of human calprotectin in the presence of calcium suggest a change in tertiary (or quaternary) structure and solvent exposure of hydrophobic groups of the protein.

Comparison between thermal stability of human calprotectin at different pH values

Previous studies revealed that calprotectin in stool, at room temperature, was stable for at least 3 days [22]. Fecal calprotectin correlates closely with colonic inflammation and it has recently been proposed as a good clinical marker of colonic neoplasm and inflammation with high diagnostic accuracy [22]. The clinical usefulness of calprotectin as an inflammatory marker led us to investigate thermal stability of human protein at physiological pH (pH 7.0) and at a pH near to that of the intestine (pH 8.0). Fig. 4 shows the absorption profiles of the protein at 280 nm versus temperature at pH 7.0 and 8.0. Determination of the Gibbs free energy of denaturation (ΔGº), as a criterion of conformational stability of a globular protein, is based on the theory of two states as follows:

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Native (N) ⇔ Denatured (D)

This theory was developed by Pace et al. [31–33]. The process was described as a single denaturant-dependent step according to the two-step theory [32]. The denaturation process can be monitored based on the change in the absorbance at 280 nm. Then the denatured fraction of the protein (F_d) as well as the equilibrium constant of the process (K) can be calculated using Equations 1 and 2, respectively:

\[ F_d = (Y_N - Y_{obs})/(Y_N - Y_d) \]

\[ K = F_d/(1 - F_d) = (Y_N - Y_{obs})(Y_{obs} - Y_d)/Y_d \]

where \( Y_{obs} \) is the observed variable parameter (e.g., absorbance value) and \( Y_N \) and \( Y_d \) are the values of \( Y \) characteristics of a fully native and denatured conformation, respectively. Then the Gibbs free energy change (\( \Delta G^o \)) can be obtained by Equation 3:

\[ \Delta G^o = -RT\ln K \]

where \( R \) is the universal gas constant and \( T \) is the absolute temperature. By plotting \( \Delta G^o \) versus temperature, the protein stability at any temperature, for example, at room temperature of 25 °C (\( \Delta G^o_{298} \)), can be obtained [34]. Fig. 5 shows the free energy changes versus temperature at both pH 7.0 and 8.0. The thermodynamics parameters of thermal denaturation processes of human calprotectin are calculated at 25 °C and 37 °C and summarized in Table 2. Our results indicate that an increase in pH from 7.0 to 8.0 markedly increases the thermal stability of human calprotectin at both 25 °C and 37 °C.

### Table 2 Thermodynamics parameters of thermal stability analysis of human calprotectin

<table>
<thead>
<tr>
<th>pH</th>
<th>( T_m ) (°C)</th>
<th>( \Delta G^o_{298} ) (kJ/mol)</th>
<th>( \Delta G^o_{37} ) (kJ/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.0</td>
<td>67.58±1.16</td>
<td>32.46±2.34</td>
<td>18.04±3.35</td>
</tr>
<tr>
<td>8.0</td>
<td>79.39±0.51</td>
<td>38.51±0.48</td>
<td>29.93±0.38</td>
</tr>
</tbody>
</table>

The Gibbs free energy change (\( \Delta G^o \)) was obtained using the equation \( \Delta G^o = -RT\ln K \), where \( R \) is the universal gas constant and \( T \) is the absolute temperature. By plotting \( \Delta G^o \) versus temperature, the protein stability at any temperature, for example, at room temperature of 25 °C (\( \Delta G^o_{298} \)), can be obtained.

### Discussion

As calprotectin concentrations in serum and local body fluid were reported to increase in various inflammatory diseases [17,35], our observations allow us to speculate that extracellular calprotectin in inflammatory sites might negatively influence the growth or survival state of other cells. It was reported that the synovial fluid concentration of calprotectin in some patients was higher than 100 μg/ml [35]. This concentration seems to be adequate for calprotectin to induce cell death, which might lead to tissue destruction in joints. High concentrations of calprotectin in local inflammatory sites might cause a delay in tissue repair and a deleterious effect on the inflamed tissues. When the local concentration of calprotectin is relatively high, neutralization of cell death–inducing activity of calprotectin by zinc, especially against normal fibroblasts, might be important in wound healing and, in the case of other cells, probably prevents tissue destruction. Zinc concentration in healthy human subjects is approximately 15 μM. More than half of serum zinc binds with albumin and amino acids and it is thought to be exchangeable with other ligands [36]. As 10 μM zinc is capable of neutralizing 100 μg/ml calprotectin, the systemic blood flow is an inhibitory milieu for the calprotectin to exert cell death–inducing activity against normal cells. However, it was reported that MMPs, a family of zinc-dependent enzymes that are important in wound healing, cancer, and tissue destruction, can be inhibited by calprotectin through sequestration of zinc [14]. Hence, calprotectin seems to be a novel player in cancer, inflammation, and wound repair. Zinc, as a ligand that modifies the function of calprotectin, might be an important goal in the regulation of inflammatory reactions, wound healing, and cancer.
The cell growth inhibitory activity of calprotectin has a zinc-reversible nature, and there are many reports that confirm this possibility that calprotectin can deprive nutrient zinc by chelating action from the medium to induce cell death [10–12]. Calcium, in contrast, was reported to have a significant role in the stability and aggregation of calprotectin [37,38]. In recent years there has been considerable interest in the ability of certain proteins to interconvert between different forms of secondary structures. Transition from \( \alpha \)-helix to \( \beta \)-structure appears to be physiologically important. It has been reported that the conformational switch from the soluble \( \alpha \)-helix to \( \beta \)-sheet structures to formation of amyloid structures [39,40]. The \( \alpha \)-helix to \( \beta \)-sheet conformational transition has been shown successfully in Alzheimer’s AB peptide [39,40]. Those have also been found to occur in many other instances such as transmission of the conformational changes in insulin [26], the conformational switch of the prion protein [41], modulation of inhibitory activity of plasminogen activators, and the activation of elongation factor Tu by a conformational switch [35]. It is now believed that the ability to form amyloid structures is not an unusual feature of the small number of proteins associated with amyloid diseases but is instead a general property of the polypeptide chain [42]. The larger subunit of calprotectin (MRP14) was found to be expressed in brain tissue of patients with Alzheimer’s disease [39]. In a previous study we showed that calcium at higher concentrations made calprotectin prone to aggregation [38].

Even though calcium and zinc did not noticeably affect the transition from \( \alpha \)-helix to \( \beta \)-structures in human calprotectin, it can be suggested that elevation of the calprotectin subunit in the brains of Alzheimer’s patients might be associated with the pathology of this disease, especially when environmental conditions make the protein prone to aggregation.

The changes in the structure and movement of aromatic residues to the protein surface after calcium binding might be important in some biological functions of the protein, such as inhibition of casein kinase and even the cell death-inducing activity of this protein [41]. The calprotectin heterodimer, but not the single subunit, has high calcium-dependent affinity for arachidonic acid that is reversed by the addition of zinc [43]. The docking of two subunits during calcium binding probably creates an asymmetric hydrophobic fatty acid binding site that locates at the interface between the subunits [15,16]. Fluorescence measurements gave evidence that zinc induced different conformational changes, thereby affecting calcium-induced formation of the amino acid-binding pocket with the protein complex. Movement of aromatic residues from a more hydrophobic environment to the surface of the protein after interaction with calcium is in accordance with the propensity of calcium to increase surface hydrophobicity and the propensity of the protein for aggregation.

Conclusions

The changes in secondary and tertiary structures of human calprotectin after calcium and zinc binding probably influenced the biological functions of this protein. The marked elevation in thermal stability of human calprotectin when the pH level was increased from 7.0 to 8.0 showed a significant role for pH in the stability of calprotectin in the gut, where it was already seen to have fair stability and proposed to be a useful clinical marker in the diagnosis of inflammatory conditions of the digestive tract.

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