

Investigation on the Surface Hydrophobicity and Aggregation Kinetics of Human Calprotectin in the Presence of Calcium

Reza Yousefi, Susan K. Ardestani*, Ali Akbar Saboury, Amina Kariminia[†], Madjid Zeinali and Mojtaba Amani

Institute of Biochemistry and Biophysics, University of Tehran, Tehran, Iran

[†]Immunology lab., Pasteur Institute of Iran, Tehran, Iran

Received 22 December 2004, Accepted 2 March 2005

Calcium and zinc binding protein, calprotectin is a multifunctional protein with broad spectrum antimicrobial and antitumoural activity. It was purified from human neutrophil, using a two-step ion exchange chromatography. Since surface hydrophobicity of calprotectin may be important in membrane anchoring, membrane penetration, subunits oligomerization and some biological roles of protein, in this study attempted to explore the effect of calcium in physiological range on the calprotectin lipophilicity. Incubation of human calprotectin (50 µg/ml) with different calcium concentrations showed that 1-anilino-8-naphthalene sulfonic acid (ANS) fluorescence intensity of the protein significantly elevates with calcium in a dose dependent manner, suggesting an increase in calprotectin surface hydrophobicity upon calcium binding. Our study also indicates that calcium at higher concentrations (6, 8 and 10 mM) induces aggregation of human calprotectin. Our finding demonstrates that the starting time and the rate constant of calprotectin aggregation depend on the calcium concentration.

Keywords: Aggregation, ANS-binding fluorescence, Calcium, Calprotectin, Hydrophobicity, Neutrophil

Introduction

Myeloid-related protein (MRP) 8 and MRP14 are two small anionic proteins, with zinc and calcium binding capacity; they belong to S-100 protein family and abundantly found in cytosolic fraction of neutrophils (Hessian *et al.*, 1993; Bella and Rossmann, 1999). They form a heterodimeric complex in a calcium dependent manner that called MRP8/14,

calprotectin or cystic fibrosis antigen (Steinbakk *et al.*, 1990; Murao *et al.*, 1998). Human MRP8 and MRP14 have molecular masses of 11 and 14 kDa with 93 and 114 amino acids, respectively. Each subunit of the protein is composed of two distinct calcium binding motifs (EF-hand) flanked by hydrophobic regions at either terminus and separated by a central hinge region (Ordink *et al.*, 1987; Hessian *et al.*, 1993).

Calprotectin shares properties with calmodulin but differ in molecular mass and it would be expected to exert a major biological effect by modifying the intracellular calcium level (Burgess *et al.*, 1980). High concentration of calprotectin in neutrophil acts as calcium sink and may be protecting cells from harmful effect of prolonged calcium elevation.

Calcium is a key secondary messenger during signal transduction and calprotectin may binds to free intracellular calcium and interfere with signal transduction (Heizmann and Hunziker, 1991).

Calprotectin is a multifunctional protein with broad spectrum antimicrobial and antitumoural effects (Steinbakk *et al.*, 1990; Satoru *et al.*, 1997) that is significantly elevated in the serum and body fluids of patients with cystic fibrosis (Dorin *et al.*, 1987) and inflammatory states such as rheumatoid arthritis (Berntzen *et al.*, 1991), Crohn's disease (Lawrance *et al.*, 2001) colorectal carcinoma (Tibble *et al.*, 2001), multiple sclerosis (Bogumil *et al.*, 1998) and human immunodeficiency virus (HIV) infection (Müller *et al.*, 1994). Also the large subunit of calprotectin is expressed in brain tissue of those suffering from the amyloid disease, Alzheimer (Akiyama *et al.*, 1994).

A variety of possible functions have been proposed for calprotectin such as the capacity for binding to poly unsaturated fatty acids (PUFAs) governed through calcium binding (Klempt *et al.*, 1997; Kerkhoff *et al.*, 1999). Investigators have shown that calprotectin inhibits the activity of casein kinases I and II (Murao *et al.*, 1989), two enzymes that mediate RNA polymerase (Stetler *et al.*, 1982; Rose *et al.*, 1983) and topoisomerase activity (Ackerman *et al.*, 1985).

Research also reveals that calcium binding induces

*To whom correspondence should be addressed.

Tel: 9821-6409517; Fax: 9821-6404680

E-mail: ardestani@ibb.ut.ac.ir

conformational changes in the molecule, complex formation, phosphorylation of MRP14 and translocation (Edgeworth *et al.*, 1989; Teigelkamp *et al.*, 1991; Roth *et al.*, 1993). Upon calcium binding, calprotectin translocates from the cytosol to the cytoskeleton and to the plasma membrane. As the protein kinase C (PKC) activates, this protein is released from human monocytes by a novel secretory pathway that is energy consuming and depends on the intact microtubule network (Roth *et al.*, 1993; Guignard *et al.*, 1996; Rammes *et al.*, 1997). Since the protein does not contain a membrane anchoring region or signal peptide for secretion, the mechanism by which calprotectin penetrates the plasma membrane and how this protein complex is anchored in the membrane is unclear (Muesch *et al.*, 1990). Hence surface hydrophobicity of calprotectin may be important in membrane anchoring, membrane penetration, subunits oligomerization and probably some biological roles of this protein.

In this study, we attempted to examine the effect of calcium concentration at physiological range on the human calprotectin lipophilicity. Also we investigated calprotectin aggregation at higher calcium concentrations. Aggregation can be associated with aberrant interaction with metal ions, changes in environmental conditions such as pH or temperature or chemical modification (oxidation and proteolysis), specific mutation and miss processing phenomena (Stefani and Dobson, 2003). Early aggregates have an intrinsic ability to impair fundamental cellular processes by interacting with cellular components (Bucciantini *et al.*, 2002). The presence of toxic aggregates inside or outside of cells can impair a number of cell functions that ultimately lead to cell death (Morishima *et al.*, 2001). The mechanism for calprotectin cell-death inducing activity is not precisely clear and calcium-induced calprotectin aggregation may be important in the cell-death inducing pathway of this protein.

Materials and Methods

Materials Dithiothreitol (DTT) and Lymphoprep were obtained from the Merck and Amersham Company, respectively. Fetal calf serum (FCS) was obtained from veterinary faculty at the University of Tehran. RPMI 1640 medium, Penicillin, Streptomycin and all other reagents and solvents were purchased from Sigma Chemical Co. (St. Louis, USA) and were at least of analytical grade. All solutions were made in double-distilled water.

Cell line K562 Chronic myelogenous leukemia cells were obtained from the cell bank of Pasteur Institute of 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 heparinated plastic bags and leukocytes isolation was performed by the dextran sedimentation in principle according to Skoog and Beck (Skoog and Beck, 1956). One unit of 500 ml heparinated blood was mixed

with 250 ml dextran T-500 (solution 6%). Sedimentation was allowed to proceed for 45 min at room temperature. The supernatant layer was harvested and leukocytes were spun down at 200 × g for 20 min at 4°C. Remaining red cells were lysed by the addition of ice-cold distilled water to the sediment and isotonicity was restored after 30 s by addition of phosphate-buffered saline (PBS). After being washed twice in PBS, granulocytes were separated from mononuclear cells by layering 3 ml suspensions on the top of 6 ml lymphoprep followed by centrifugation at 800 × g for 30 min at 20°C.

Granulocytes (neutrophils) were resuspended in PBS containing 0.2 M sucrose, 1 mM EDTA, 1 mM DTT and 0.5 mM phenyl methyl sulfonyl fluoride (PMSF). The cell suspension was sonicated for 30 s, five times by probe type sonicator (Model MK2-3.75, MSE, France). During this procedure, the cell container was kept in wet ice. After sonication the soluble fraction was separated from cell debris by centrifugation at 12000 × g for 10 min at 4°C and clear supernatant (crude neutrophil extract) was collected.

Purification of calprotectin Crude neutrophil extract was dialyzed against 25 mM Tris-HCl, pH 8.0, 1 mM EDTA, 1 mM DTT (Buffer I) and injected onto anion exchange column (Q-Sepharose) that has been pre-equilibrated with five column volumes of buffer I at a flow rate of 1 ml/min. Bound proteins were eluted from the column by the application of 0-0.5 M NaCl gradient in buffer I over 150 min, at 4°C. Anion exchange eluted fractions were analyzed by Tricine-SDS-PAGE (Gel 15%) under reducing conditions. To check growth inhibition of calprotectin-containing fractions, K562 cells were used as target.

These fractions were further dialyzed against 25 mM sodium acetate pH 4.5, 1 mM EDTA, 1 mM DTT (buffer II) and injected onto cation exchange column (SP-Sepharose) that pre-equilibrated with five column volumes of buffer II at flow rate of 1 ml/min. Bound proteins were eluted from the column by the application of 0-1.0 M NaCl gradient in buffer II over 100 min. At this stage MRP8 and MRP14 appeared to be essentially pure (>98%) with densitometric analysis of SDS-PAGE gel that visualized by Coomassie Blue staining.

Dialysis was performed in 1000 molecular weight cut-off dialysis tubing at all stages. The purified protein was aliquoted and stored at -70°C for the long term and at 4°C for short term use.

Electrophoresis For Tricine-SDS-PAGE, the method described by Schägger and von Jagow was used (Schägger and von Jagow, 1987). Samples were boiled in sample buffer with mercaptoethanol for 5 min and electrophoresed on poly-acrylamide gel (separating: 16.5% T, 3% C; Stacking: 4% T, 3% C) at 200V. Protein bands were visualized by Coomassie brilliant blue staining.

Protein assay The protein concentration was determined by using the Bradford reagent (Bio-Rad, Richmond, USA) with bovine serum albumin (BSA) as standard (Crestfield *et al.*, 1963).

Cell culture K562 cells were grown in RPMI with 10% heat-inactivated fetal calf serum (FCS), supplemented with 4 mM L-glutamine, 100 U penicillin, 100 µg/ml streptomycin at pH 7.4, in a humidified, 5% CO₂ incubator and temperature of 37°C. Harvested cells were seeded into 96-well plates (2 × 10⁴ cell/ml) and

proliferation curves for K562 were determined based on MTT assay.

Cell proliferation assay Relative cell number was measured using 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) assay as described by Mossman (Mossman, 1983).

MTT (Sigma catalog no M2128) was dissolved in PBS at a concentration of 5 mg/ml and solution was filtered through a 0.5 μ m filter, then it stored at 2-8°C for frequent use. Four hours before the end of incubations, 25 μ l of MTT solution (5 mg/ml in PBS) was added to each well containing fresh and cultured medium. The insoluble formazan produced was dissolved in a solution containing 10% SDS and 50% DMF (left for 2 h at 37°C in dark conditions) and the optical density (OD) was read against a reagent blank with multi well scanning spectrophotometer (ELISA reader, Model Multiskan MS) at a wavelength of 540 nm (OD540). The OD value of study groups was divided by the OD value of untreated groups and results presented as percentage of control groups (as 100%).

Turbidity measurements The turbidity of human calprotectin (50 μ g/ml) in 0.1 M ammonium acetate, at pH 7.0 and 25°C was monitored by measuring the light absorption at 420 nm, using a Cary spectrophotometer (Model Bio-100). The obtained data were fitted to equation (1), (Kurganov, 2002).

$$A = A_{\text{lim}}(1 - e^{-k(t-t_0)}) \quad (1)$$

Where A_{lim} is the limiting value of absorbance (A) at $t \rightarrow \infty$, k represents the first-order rate constant for aggregation reaction, t indicates time and t_0 represents aggregation starting time.

ANS-binding experiments 1-anilino-8-naphthalene sulfonic acid (ANS) was added to calprotectin solution. After incubation for 15 min, the fluorescence spectra were recorded at room temperature between 450 and 600 nm, using an excitation wavelength of 400 nm. All necessary back ground correction was made. The fluorescence measurements were made on Hitachi Spectrofluorimeter (Model MPF-4).

Statistical analysis Results were analyzed for statistical significance using two-tailed student's t-test. Changes were considered significant at $p < 0.05$.

Results and Discussion

Purification of human calprotectin Human calprotectin (MRP8/14) was purified from neutrophil cytosol, using a protocol devised for the rapid purification of large quantities of protein. Neutrophil cytosol containing a maximum of 35 mg of total protein was injected onto a Q-sepharose anion exchange column. MRP8 and MRP14 were eluted from the column at approximately 0.12 M, NaCl. Figures 1a and 1b show chromatogram and SDS-page profile of anion exchange column, respectively. By densitometric scanning of a coomassie blue-stained sample after SDS-PAGE, following Q-sepharose chromatography, the majority of MRP8 and MRP14 were observed to elute in a single peak (fractions a

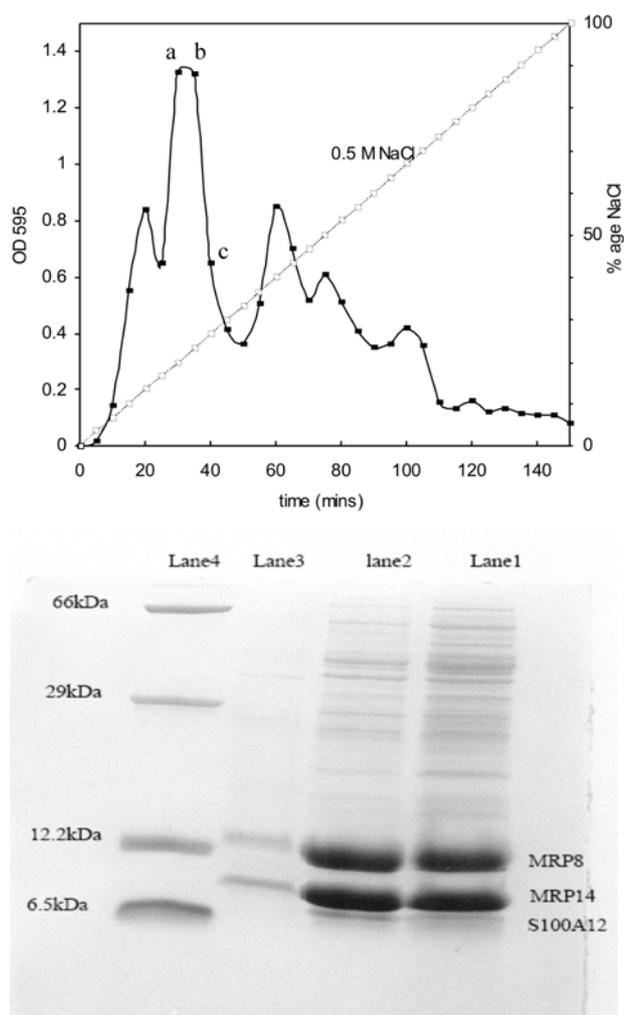


Fig. 1. (a) Neutrophil extract was dialyzed against 25 mM Tris-HCl pH 8.0, 1 mM EDTA, 1 mM DTT (buffer I), then loaded onto an anion exchange column (Q-Sepharose). Bound proteins were eluted by a linear NaCl gradient of 0-0.5 M in buffer I. The graph shows the 595-nm trace of eluted proteins by use of Bradford solution. Majority of MRP8 and MRP14 were eluted from the column at approximately 0.12 M NaCl. Fractions a, b and c were showed major growth inhibitory against K562. S100A12 also co eluted with calprotectin subunits from anion exchange column. (b) Active pooled fractions against K562 were subjected to tricine SDS-PAGE (15% gel) under reducing conditions and protein bands subsequently visualized by Coomassie Blue staining (lanes 1, 2 and 3 are equivalent to fractions a, b and c respectively). Molecular masses are indicated on the left (lane 4).

and b).

Similarly to recent reports (Vogl *et al.*, 1999) and as shown in Fig. 1b, S100A12 also co-eluted with calprotectin subunits from anion exchange column. S100A12 or MRP6 is a recently identified protein that exclusively expressed in neutrophils and it has close homology with both MRP8 and MRP14 (Robinson and Hogg, 2000).

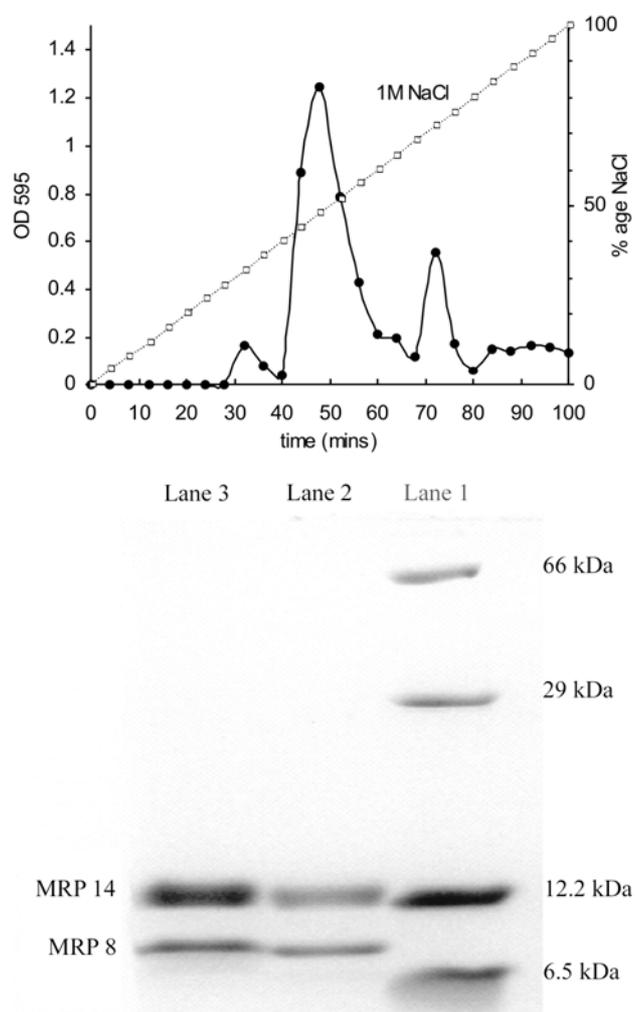


Fig. 2. (a) Calprotectin containing fractions a and b which were obtained from anion-exchange column, were dialyzed against 25 mM sodium acetate pH 4.5, 1 mM EDTA, 1 mM DTT, injected onto cation-exchange column (SP-Sepharose). Bound proteins were eluted from the column by the application of 0-1.0 M NaCl gradient in buffer II. The graph shows the 595-nm trace of eluted proteins by use of Bradford solution. (b) Purified fractions that had been obtained from cation exchange column were subjected to tricine SDS-PAGE (15% gel), under reducing conditions (Lane 2 and 3) and protein bands were detected by Coomassie Blue staining. Lane 1 indicates Molecular masses marker.

Fractions a and b from anion exchange column that showed major growth inhibitory activity against K562, were further applied to cation exchange column. MRP8 and MRP14 were eluted from the column at approximately 0.5 M NaCl (Fig. 2a). Figure 2b shows the SDS-page profile of purified sample of calprotectin.

Cell death inducing assay of human calprotectin Various concentrations of purified calprotectin were used to culture of tumor cell line K562 for 24 h, in order to examine its cell

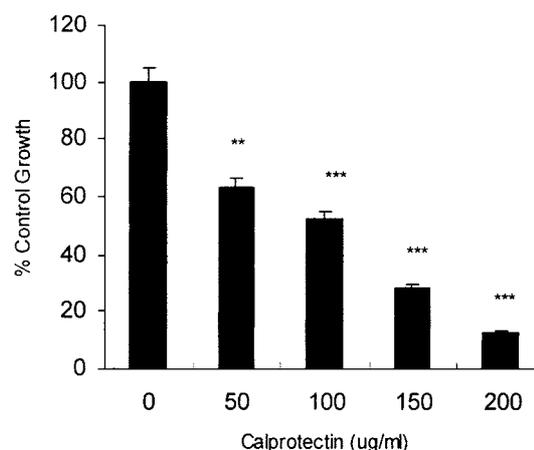


Fig. 3. K562 cells (2×10^4 cells/ml) were cultured with varying concentrations of calprotectin sample for 24 h and cell proliferation was assessed by MTT assay as described in materials and methods. Results (Cytotoxicity) are presented relative to cell growth under control conditions (absence of calprotectin) and expressed as percentage of control (as 100%). The vertical bars represent S.D. of triplicate determinations and asterisks indicate $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$ compared to control (absence of calprotectin).

death-inducing activity. As shown in Fig. 3, growth after 24 h was significantly reduced by all treatments ($p < 0.01$, $p < 0.001$, $p < 0.001$), indicating a dose dependent suppression on growing of K562 leukemia cell line.

ANS binding experiments and aggregation study of human calprotectin Previous investigations have revealed that the sequences at both NH_2 and COOH terminals of calprotectin subunits are relatively hydrophobic (Ordink *et al.*, 1987; Hessian *et al.*, 1993). An attractive hypothesis is that these areas of molecule are buried until calcium binding brings about the conformational changes cause their exposure making them potentially available for interaction with other molecules.

ANS, a hydrophobic dye, is useful for monitoring the existence of any exposed hydrophobic surface(s) in molecules (Wagner and MacDonald, 1998). In this study the ANS-binding fluorescence changes of human calprotectin were measured after incubating the protein (50 $\mu\text{g/ml}$) solution with 70 μM of ANS for 15 min, in 0.1 M ammonium acetate, at pH 7.0. ANS-fluorescence emission spectra were recorded at room temperature between 400 and 600 nm using an excitation wavelength of 400 nm. As shown in Fig. 4 after incubation of human calprotectin for 30 min with calcium concentrations in physiological range (1.0, 1.5, 2 mM) and measurement of ANS-binding fluorescence intensity, indicates a significant increase in emission spectra of calcium-incubated protein relative to the native state. Also, ANS-fluorescence of human calprotectin elevate with calcium in a dose-dependent manner.

ANS-fluorescence enhancement of calcium-incubated protein

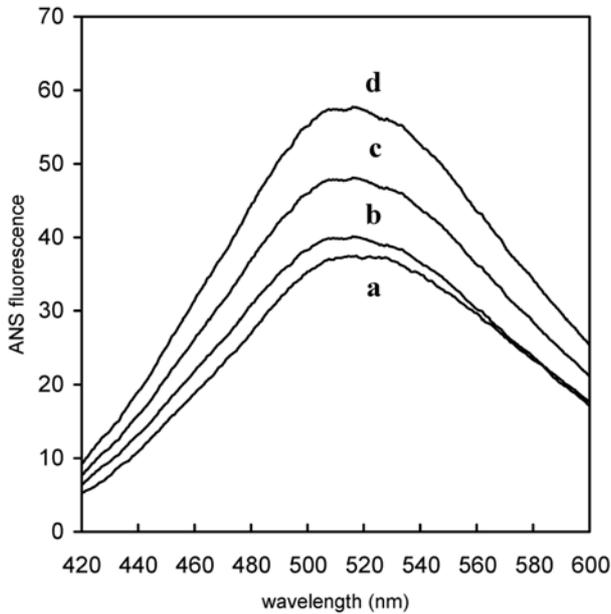


Fig. 4. The ANS fluorescence emission spectra of the human calprotectin (50 µg/ml) in the absence (a) and presence of 1 mM (b), 1.5 mM (c) and 2 mM (d) of calcium in ammonium acetate (0.1 M), at pH 7.0 and 25°C. The excitation wavelength was 400 nm.

relative to the native state indicates an increase in surface hydrophobicity of human calprotectin that take place during the interaction of protein with calcium. Hydrophobic regions of calprotectin that normally buried within the interior of the protein are probably exposed to aqueous environment upon calcium binding. Calprotectin does not have a signal peptide for membrane anchoring or secretion, but upon calcium binding and protein kinase C (PKC) activation, protein anchors in and secretes from the membrane respectively (Muesch *et al.*, 1990). Therefore an increase in lipophilicity of human calprotectin in the presence of calcium may be important in the membrane anchoring and membrane penetration of this protein.

Calcium binding induces conformational changes; increase surface hydrophobicity and also make calprotectin prone to aggregation. Aggregation is the interaction of unfolded protein molecules that are responsible for formation of agglomerates of irregular form as a result of incorrect protein-protein contacts. The aggregation experiments of human calprotectin at the presence of higher concentrations of calcium (6, 8 and 10 mM) were carried out in ammonium acetate, at pH 7.0 and room temperature and the turbidity was monitored by measuring the light absorption at 420 nm for 180 min. As shown in Fig. 5, calcium at concentration of 6 mM or higher induces the aggregation of human calprotectin. Calcium concentrations lower than 6 mM were also examined at 420 nm for 180 min, but we did not observe any increased absorbance. Previous investigations demonstrate that calprotectin has more thermal and structural stability at alkaline than acidic pH (Dale *et al.*, 1983). Hence,

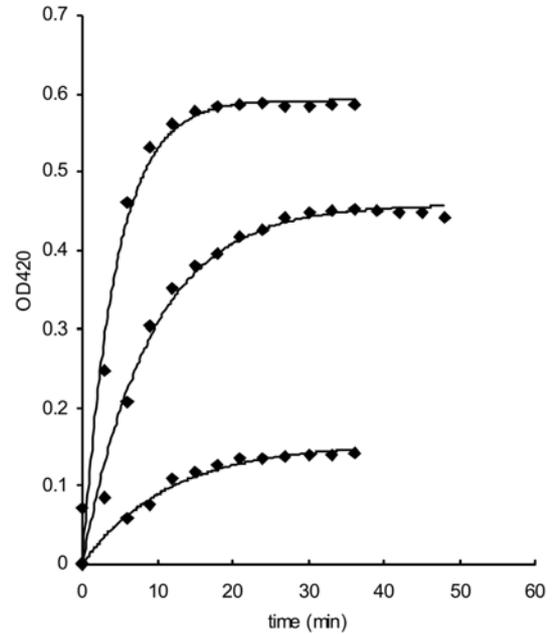


Fig. 5. Aggregation Kinetics of human calprotectin (50 µg/ml), at the presence 6 (a), 8 (b) and 10 mM (c) of Calcium in 0.1 M ammonium acetate at pH 7.0 and 25°C.

the aggregation experiment of human calprotectin was also carried out at pH 8.0 in the same buffer, indicating that calcium at concentrations 6, 8 and 10 mM (as mentioned above) could not induce calprotectin aggregation. This suggests that calprotectin stabilizing conditions such as increase in the pH has an important role in preventing calcium-induced human calprotectin aggregation.

The calcium binding proteins MRP8, MRP14, and S100A12 have been characterized in myeloid cells; however the relationship among these proteins remains unclear. S100A12 represents a major calcium-binding protein in granulocytes. Calcium binding level of S100A12 is approximately 18 times higher than that of MRP8 and comparable to MRP14 (Vogl *et al.*, 1999). In this study the semi-purified fractions that contain only S100 A12 as contamination were also used as target for the calcium induced aggregation. However, no aggregation was observed, even at the presence of 10 mM calcium. This finding shows that the presence of S100 A12, a major calcium binding protein, may reduce effective calcium concentration necessary for calprotectin aggregation.

The parameters of equation (1), obtained by fitting the experimental data are listed in Table 1. The starting time and the rate constant for aggregation depend on the calcium concentration. The appearance of a lag period at the initial part of the kinetics curve is probably due to the following reasons. First, aggregation is preceded by a stage of the unfolding of protein molecules, which makes unfolded protein prone to the aggregation. Second, an increment of absorbance becomes appreciable only after the appearance of sufficient

Table 1. Calprotectin aggregation parameters, calculated based on equation 1

[Ca ²⁺] mM	A _{lim}	k (min ⁻¹)	t ₀ (min)
6	0.153	0.087	81
8	0.564	0.109	36
10	0.592	0.225	15

aggregate in the solution (Kurganov, 2002).

A number of reports indicate that many proteins and peptides are able to aggregate into amyloid assemblies under suitable destabilizing conditions. The common hallmark of some pathological conditions such as Alzheimer, Parkinson, Cystic fibrosis, Type II diabetes and some forms of emphysema is the presence of proteinaceous deposits in the affected tissues and organs (Stefani and Dobson, 2003). It seems that calprotectin elevation in the brain, especially in conditions that make protein prone to aggregation may be important in Alzheimer disease, because it has been reported that calprotectin is expressed in brain tissue in this amyloid disease (Akiyama *et al.*, 1994).

It is not clear by which mechanism the calprotectin initiate signaling toward the cell death. It has been reported that early aggregates have an intrinsic ability to impair fundamental cellular processes by interacting with cellular membranes, causing oxidative stress and increases in free calcium that lead to impairment of the cell viability and eventually to cell death (Squire, 2001). It has been shown that antioxidants, such as tocopherol protect cells against aggregates toxicity (Zhang, 2001) and our previous studies also demonstrated that calprotectin cytotoxic effect against the cell lines MOLT4 and K562 has been reversed by α -tocopherol (the article has been submitted for publication).

We postulated that cell-death inducing activity of calprotectin may be governed through toxic pre-aggregation species. This is possible because calcium binding may lead to the formation of a toxic variant of calprotectin with sticky (hydrophobic) surface that interact with plasma membrane to kill cells.

Finally in calcium- and fatty acid binding capacity, also in death-inducing activity against tumor cells and bactericide effects, the milk protein α -lactalbumin shares similar properties to calprotectin (Stefani, 2004). Also it has been reported that acute phase isoforms of human serum amyloid A (SSAp), similar to calprotectin rises dramatically during inflammatory processes. Both α -lactalbumin and SSAp exhibit cytotoxic effects by forming toxic aggregation species (Stefani, 2004). These similarities led us to suppose that the death-inducing mechanism of calprotectin may be similar to those of SSAp and α -lactalbumin.

Acknowledgments This work was financially supported by grants from the Third World Academy of Science (TWAS) and the Research Council of University of Tehran. The authors would like to thanks Dr. F. C. Chilaka for proofreading of the manuscript.

References

- Ackerman, P., Glover, C. V. and Osheroff, N. (1985) Phosphorylation of DNA topoisomerase II by casein kinase II: modulation of eukaryotic topoisomerase II activity *in vitro*. *Proc. Natl. Acad. Sci. USA* **82**, 3164-3168.
- Akiyama, H., Ikeda, K., Katoh, M., McGeer, E. G. and McGeer, P. L. (1994) Expression of MRP14, 27E10, interferon-alpha and leukocyte common antigen by reactive microglia in postmortem human brain tissue. *J. Neuroimmunol.* **50**, 195-201.
- Bella, J. and Rossmann, M. G. (1999) Rhinoviruses and their ICAM receptors. *J. Struct. Biol.* **128**, 69-74.
- Bertzen, H. B., Olmez, ü., Fagerhol, M. K. and Munthe, E. (1991) The leukocyte protein L1 in plasma and synovial fluid from patients with rheumatoid arthritis and osteoarthritis. *Scand. J. Rheumatol.* **20**, 74-82.
- Bogumil, T., Rieckmann, P., Kubuschok, B., Felgenhauer, K. and Bruck, W. (1998) Serum levels of macrophage-derived protein MRP8/14 are elevated in active multiple sclerosis. *Neurosci. Lett.* **247**, 195-197.
- Bucciantin, M., Giannoni, E., Chiti, F., Baroni, F., Formigili, L., Zurdo, J., Taddei, N., Ramponi, G., Dobson, C. M. and Stefani, M. (2002) Inherent toxicity of aggregates implies a common mechanism for protein misfolding diseases. *Nature* **416**, 507-511.
- Burgess, W. H., Jemiolo, D. K. and Kretsinger, R. H. (1980) Interaction of calcium and calmodulin in the presence of sodium dodecyl sulfate. *Biochem. Biophys. Acta* **623**, 257-270.
- Crestfield, A. M., Moore, S. and Stein, W. H. (1963) The preparation and enzymatic hydrolysis of reduced and S-carboxymethylated proteins. *J. Biol. Chem.* **238**, 622-627.
- Dale, I., Fagerhole, M. K. and Naesgaard, I. (1983) Purification and partial characterization of a highly immunogenic human leukocyte protein L1 antigen. *Eur. J. Biochem.* **134**, 1-6.
- Dorin, J. R., Novak, M., Hill, R. E., Brock, D. J. H., Secher, D. S. and van Heynigen, V. (1987) A clue to the basic defect in cystic fibrosis from cloning the CF antigen gene. *Nature* **326**, 614-617.
- Edgeworth, J., Freemont, P. and Hogg N. (1989) Ionomycin regulated phosphorylation of the myeloid calcium binding protein P14. *Nature* **342**, 189-192.
- Guignard, F., Mael, J. and Market, M. (1996) Phosphorylation of myeloid related proteins MRP-14 and MRP-8 during human neutrophil activation. *Eur. J. Biochem.* **241**, 265-271.
- Heizmann, C. W. and Hunziker, W. (1991) Intracellular calcium binding protein: More sites than insights. *Trends Biochem. Sci.* **16**, 98-103.
- Hessian, P. A., Edgeworth, J. and Hogg, N. (1993) MRP8 and MRP14, two abundant Ca²⁺-binding proteins of neutrophils and monocytes. *J. Leuk. Biol.* **53**, 197-204.
- Kerkhoff, C., Klempt, M., Kaever, V. and Sorg, C. (1999) The two calcium-binding proteins, S100A8 and S100A9 are involved in the metabolism of arachidonic acid in human neutrophils. *J. Biol. Chem.* **274**, 32672-32679.
- Klempt, M., Melkonyan, H., Nacken, W., Wiemann, D., Holtkemper, U. and Sorg, C. (1997) The heterodimer of the Ca²⁺-binding proteins MRP-8 and MRP-14 binds arachidonic acid. *FEBS Letters* **408**, 81-84.
- Lawrance, I. C., Fiocchi, C. and Chackravarti, S. (2001)

- Ulcerative colitis and Crohns disease: distinctive gene expression profiles and novel susceptibility candidate genes. *Hum. Mol. Genet.* **10**, 445-456.
- Morishima, Y., Gotoh, Y., Zieg, J., Barrett, T., Takano, H., Flavell, R., Davis, R. J., Shirasaki, Y. and Greenberg, M. E. (2001) β -amyloid induces neuronal apoptosis via mechanism that involve the c-jun N-terminal kinase pathway and the induction of fas ligand. *J. Neurosci.* **21**, 7551-7560.
- Mossman, T. (1983) Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. *J. Immunol. Methods.* **65**, 55-63.
- Muesch, A., Hartmann, K., Rohde, A., Rubartello, R. and Rapoport, T. A. (1990) A novel pathway for secretory proteins. *Trends Biochem. Sci.* **15**, 82-86.
- Müller, F., Frøland, S. S., Aukrust, P. and Fagerhol, M. K. (1994) Elevated serum calprotectin levels in HIV-infected patients: The calprotectin response during ZDV treatment is associated with clinical events. *J. Acquir. Immune Defic. Syndr.* **7**, 931-939.
- Murao, S., Collart, F. R. and Huberman, E. (1998) A protein containing the cystic fibrosis antigen is an inhibitor of protein kinases. *J. Biol. Chem.* **262**, 8356-8360.
- Murao, S., Collart, F. R. and Huberman, E. (1989) A protein containing the cystic the fibrosis antigen is an inhibitor of protein kinase. *J. Biol. Chem.* **264**, 8356-8360.
- Ordink, K., Cerletti, N., Bruggen, J., Clerc, R. J., Tarcsay, L., Zwadlo, G., Gerhards, G., Shlegel, R. and Sorg, C. (1987) Two calcium-binding proteins in infiltrate macrophages of rheumatoid arthritis. *Nature* **330**, 80-82.
- Rammes, S., Kewitz, G., Versmold, H., Niggemann, B. and Rammes, A. (1997) Myeloid related protein (MRP)8 and MRP14, calcium binding proteins of the S100 family, are secreted by activated monocytes via a novel tubulin-dependent pathway. *Pediatr. Allergy Immunol.* **8**, 153-155.
- Robinson, M. J. and Hogg, N. (2000) A comparison of human S100A12 with MRP-14 (S100A9). *Biol. Biophys. Res. Comm.* **275**, 865-870.
- Roth, J., Burwinkel, F., VandenBos, C., Goebeler, M., Vollmer, E. and Sorg, C. (1993) MRP8 and MRP14, S-100-like proteins associated with myeloid differentiation, are translocated to plasma membrane and intermediate filaments in a calcium-dependent manner. *Blood* **82**, 1875-1883.
- Satoru, Y., Mikami, M., Kazusa, T. and Yamazaki, M. (1997) Growth inhibitory and apoptosis-inducing activities of calprotectin derived from inflammatory exudates cells or normal fibroblasts: regulation by metal ions. *J. Leukocyte Biol.* **61**, 50-57.
- Schagger, H. and von Jagow, G. (1987) Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. *Anal. Biochem.* **166**, 368-379.
- Skoog, W. and Beck, W. S. (1956) Studies on the fibrinogen, dextran and phytohemagglutinin methods of isolating leukocytes. *Blood* **11**, 436-454.
- Squier, T. C. (2001) Oxidative stress and protein aggregation during biological aging. *Exp. Gerontol.* **36**, 1539-1550.
- Stefani, M. and Dobson, C. M. (2003) Protein aggregation and protein toxicity: new insights into protein folding, misfolding diseases and biological evolution. *J. Mol. Med.* **81**, 678-699.
- Stefani, M. (2004) Protein misfolding and aggregation: new examples in medicine and biology of the dark side of the protein world. *Biochem. Biophys. Acta* **1739**, 5-25.
- Steinbakk, M., Naess-Andersen, C. F., Lingaas, E., Dale, I., Brandtzaeg, P. and Fagerhol, M. K. (1990) Antimicrobial actions of calcium binding leukocyte L₁ protein, calprotectin. *Lancet.* **336**, 763-765.
- Stetler, D. A. and Rose, K. M. (1982) Phosphorylation of deoxyribonucleic acid dependent RNA polymerase II by nuclear protein kinase NII: mechanism of enhanced ribonucleic acid synthesis. *Biochemistry* **21**, 3721-3728.
- Teigelkamp, S., Bhardwaj, R. S., Roth, J., Meinardus-Hager, G., Karas, M. and Sorg, C. (1991) Calcium independent complex assembly of the myeloid differentiation proteins MRP-8 and MRP-14. *J. Biol. Chem.* **266**, 13462-13467.
- Tibble, J., Sigthorsson, G., Foster, R., Sherwood, R., Fagerhol, M. and Bjarnason, I. (2001) Faecal calprotectin and faecal occult blood tests in the diagnosis of colorectal carcinoma and adenoma. *Gut. Sep* **49**, 402-408.
- Vogl, T., Propper, C., Hartmann, M., Strey, A., Strupat, K., Van den Bos, C., Sorg, C. and Roth, J. (1999) S100A12 is expressed exclusively by granulocytes and acts independently from MRP8 and MRP14. *J. Biol. Chem.* **274**, 25291-25296.
- Wagner, D. W. and MacDonald, P. J., (1998) The fluorescence enhancement of 1-anilinonaphtalene-8-sulfonate (ANS) by modified β -cyclodextrins. *J. Photochem. Photobiol. Chem.* **114**, 151-157.
- Zhang, L., Xing, G. Q., Barker, J. L., Chang, Y., Maric, D., Ma, W., Li, B-S. and Rubinow, D. R. (2001) α -lipoic acid protects rat cortical neurons against cell death induced by amyloid and hydrogen peroxide through the Akt signaling pathway. *Neurosci. Lett.* **312**, 125-128.