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STRUCTURAL AND FUNCTIONAL ANALYSIS OF BIOPOLYMERS AND BIOPOLYMER COMPLEXES

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The Role of Calcium in the Conformational Changes of the Recombinant S100A8/S100A9[1]

N. Gheibi[a], H. Asghari[b], K. G. Chegini[c] *, M. Sahmani[c], and M. Moghadasi[b]

[a] *Cellular and molecular research center, Qazvin University of Medical Sciences, Qazvin, Iran*
[b] *Department of Biotechnology, Qazvin University of Medical Science, Qazvin, Iran*
[c] *Department of Clinical Biochemistry and Genetics, Qazvin University of Medical Science, Qazvin, Iran*
**e-mail: kgvand@ymail.com*

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Abstract—Calprotectin is a member of the EF-hand proteins, composed of two subunits, S100A8 (MRP8) and S100A9 (MRP14). These proteins are involved in important processes including cell signaling, regulation of inflammatory responses, cell cycle control, differentiation, regulation of ion channel activity and defense against microbial agents in a calcium dependent manner. In the present study, recombinant S100A8 and S100A9 were expressed in *E. coli* BL21 and then purified using Ni-NTA affinity chromatography. The structure of the S100A8/A9 complex in the presence and absence of calcium was assessed by circular dichroism and fluorescence spectroscopy. The intrinsic fluorescence emission spectra of the S100A8/A9 complex in the presence of calcium showed a reduction in fluorescence intensity, reflecting conformational changes within the protein with the exposure of aromatic residues to the protein surface. The far ultraviolet-circular dichroism spectra of the complex in the presence of calcium revealed minor changes in the regular secondary structure of the complex. Also, increased thermal stability of the S100A8/A9 complex in the presence of calcium was indicated.

Keywords: calprotectin, S100A8, S100A9, circular dichroism, thermal denaturation
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INTRODUCTION

Changes in the cytosolic calcium concentration regulate a variety of cellular processes [1]. These changes, act as a signal mediator, and the signal is transduced as activation or inactivation of Ca^{2+} binding proteins including a large family of proteins characterized by the EF-hand structural motif [2, 3]. S100 is a multigenic family of non-ubiquitous Ca^{2+}-modulated proteins of the EF-hand type expressed exclusively in vertebrates and implicated in intracellular and extracellular regulatory activities [4, 5]. S100A8 and S100A9 are members of the S100 family characterized by two calcium-binding sites with different affinity—a high affinity site at the C terminus (EF-hand II) and a low affinity site at the N terminus (EF-hand I) which are flanked by hydrophobic regions at either terminus and separated by a central hinge region [6]. They are specifically expressed in circulating neutrophils and early differentiation stages of monocytes, as well as in keratinocytes and epithelial cells under inflammatory conditions [7]. The elevated level of intracellular calcium also allows S100A8 and S100A9 to associate into non-covalent heterodimers and translocate from the cytosol to the cytoskeleton and the plasma membrane, thus mediating calcium signals by binding to other intracellular proteins [8]. The heterodimer of S100A8/A9 plays a crucial role in the regulation of inflammatory processes and the immune response, cell cycle control, differentiation, regulation of ion channel activity, apoptosis and defense against microbial agents [9–11]. Also increased serum levels of the heterodimer are observed in several human diseases such as cystic fibrosis, rheumatoid arthritis, acute inflammatory lesions, cardiomyophathy, and formation and deposition of amyloids in the ageing prostate and Alzheimer's disease [12, 13]. More recently, they were also detected in various human cancers [14, 15]. Calcium induces conformational changes in calprotectin which may allow its interaction with the target proteins [16]. These changes of conformation result in the exposure of the hydrophobic surface for interactions with other proteins [17–19].

The aim of this study focused on structural and thermodynamic characterization of S100A8 and S100A9 subunits and the role of calcium in their complexation to form a dimer protein with new conformational changes and thus its different role as calprotectin.

[1] The article is published in the original.

EXPERIMENTAL

Materials. Ni-NTA resin, Tris base, Acrylamide, Isopropyl β-D-1-thiogalactopyranoside (IPTG) and bacterial media (LB broth) were purchased from Sigma. Ampicillin was obtained from Roch, sodium dihydrogen phosphate (NaH_2PO_4), sodium chloride (NaCl), calcium chloride ($CaCl_2$), and all other reagents were purchased from Merck chemical co. and imidazole was purchased from applichem. Competent *E. coli* strain Bl21 (DE3) and the pET15b vectors containing an N-terminal His-Tag and the S100A8 or S100A9 coding sequence were obtained from Novagen. Other reagents were of analytical grade.

Expression and purification of proteins (His6-rhS100A8 and His6-rhS100A9). The pET15b expression vector, containing the coding region of S100A8 or S100A9 was used to express the recombinant human S100A8 (rhS100A8) or S100A9 (rhS100A9) in *E. coli* BL21 (DE3). Ampicillin was used as the selective marker for colonies carrying the recombinant vectors. Single colonies of S100A8 and S100A9 were separately cultured in Luria–Bertani medium containing 1% ampicillin with vigorous shaking until OD 600 reached to 0.5–0.6 at 37°C. 1 mM IPTG was added to the culture, and the induction was carried out at 37°C. Then the cells were harvested by centrifugation at 5000 *g* for 15 min. The cells were lysed with the lysis buffer (Glycerol 10%, HEPES 25 mM, NaCl 100 mM, pH 8) then sonicated and centrifuged at 16000 *g* for 30 min. The supernatant containing each of the soluble His-tagged subunits was purified by Ni^{+2}-NTA affinity chromatography. Then dialyzed against imidazole in buffer (NaCl 100 mM, NaH_2PO_4 25mM, pH 6.5) at 4°C for 12h, then the soluble protein was saved at −80°C. The concentrations of recombinant proteins were determined according to the Bradford method with bovine serum albumin as the standard [20]. Protein measurements were also accomplished using Nono Drop spectrophotometry.

CD spectroscopy. CD spectra were recorded from 195 to 260 nm in a 0.1 cm cuvette on a J815 spectropolarimeter (Jasco) which was used to obtain the CD data of regular secondary structures of r-S100A8/A9. The fraction of alpha-helix to beta-sheet was determined utilizing CD Deconvolution software. r-S100A8 and r-S100A9 were mixed 1 : 1 in equimolar concentrations and incubated with calcium chloride (1 mM) for 1 hour at room temperature forming the dimer protein. All CD spectra were obtained from protein samples dissolved in a 100 mM NaCl and 25 mM NaH_2PO_4 buffer at pH 6.5 with protein concentrations of about 15–25 μM for a path length of 0.1 cm. Spectra were corrected for the buffer and converted to molar CD per residue before analysis. The results were expressed as molar ellipticity (deg cm^2/dmol) considering the mean number of residues as 207 and an average molecular weight of 38 kDa for the S100A8/A9 complex. The molar ellipticity was determined as $[\theta] = \lambda[100(MRW) \times \theta_{obs}/(c \times l)]$, where θ_{obs} 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 light path in cm.

Fluorescence spectroscopy. Intrinsic fluorescence spectra of the purified protein samples were obtained using a Cary Eclips spectrophotometer (Varian, Salt Lake, Australia). Samples were excited at 280 nm and fluorescence intensities were collected from 300 to 450 nm. The spectra were obtained with 0.1 to 0.15 mg/mL protein samples dissolved in a 100 mM NaCl and 25 mM NaH_2PO_4 buffer at pH 6.5 at room temperature. The presence of a single tryptophan residue in both S100A8 and S100A9 allows to analyze these proteins by fluorescence spectroscopy.

Thermal denaturation studies. The effect of increasing temperature on the structural integrity of S100A8 and S100A9 subunits and their calcium induced complexes were studied by following the changes in the fluorescence emission intensity at 337 nm(λ max), in a range from 20 to 90°C, using a Cary Eclips spectrophotometer (Varian, Salt Lake, Australia). All spectra were obtained from protein samples dissolved in a 100 mM NaCl and 25 mM NaH_2PO_4 buffer at pH 6.5.

RESULTS

Expression and Purification

S100A8 and S100A9 were separately expressed as recombinant proteins in pET15b and *E. coli* BL21 (DE3) system using IPTG as the inducer. Protein expression was achieved after 4 hours of induction at 37°C and purification was accomplished at 150 mM imidazole. Expression and homogeneity of recombinant proteins were demonstrated by SDS-PAGE as two distinct bands indicated in Fig. 1.

Far UV-CD Studies of r-S100A8/A9 Complex

It has been proven that CD spectroscopy is 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 [21, 22]. Typically, the CD spectra of proteins are recorded in the far-UV region (180–250 nm) and the near-UV region (250–320 nm). An advantage of the CD technique in studies of proteins is that complementary structural information can be obtained from a number of spectral regions. The far-UV CD spectrum is directly related to the protein's secondary structure and near-UV CD is characteristic of the tertiary protein structure [23, 24]. Circular dichroism (CD) spectroscopy was carried out to show changes in the secondary structures of the recombinant S100A8/S100A9 when calcium ions are present. As shown in Fig. 2 and Table 1, the results indicate at a high degree of α-helix which is characteristic of EF- hand proteins [25]. Also the secondary

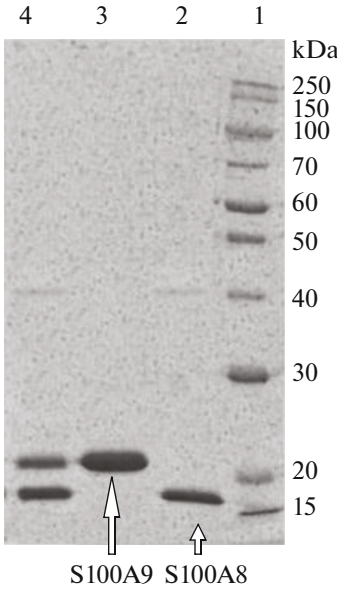

Fig. 1. SDS-PAGE of purified His6-tagged S100A8 and S100A9. Lane 1 contains the high molecular weight marker, Lanes 2 and 3 show purified r-S100A8 and r-S100A9 respectively. Lane 4 shows both subunits.

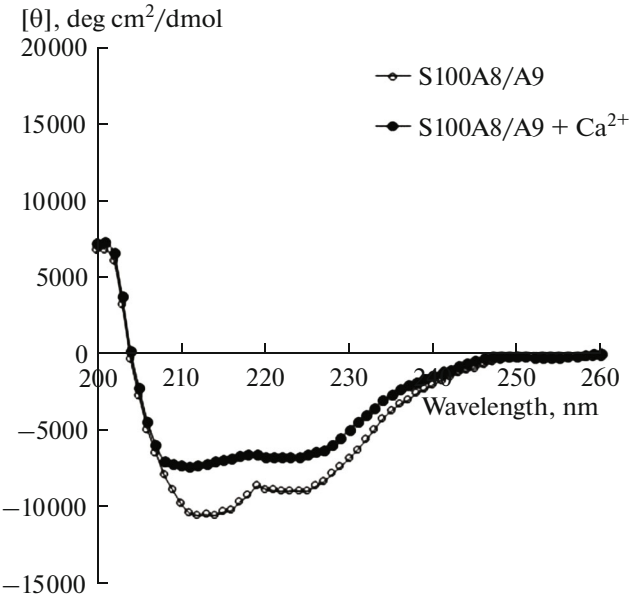

Fig. 2. Far UV circular dichroism spectrum for S100A/A9 complex in the absence (○) and presence of Ca^{2+} (●). The CD spectrum was obtained from a 0.2 mg/ml protein sample dissolved in a 100 mM NaCl, 25 mM NaH_2PO_4 buffer at pH 6.5 and 25°C.

structure was different in the presence of 1 mM calcium (Fig. 2 and Table 1). The CD data confirmed a reduction in α-helix content and an increase in β- and other structures of human S100A8/A9 complex in the presence of calcium (Fig. 2 and Table 1).

The Internal Fluorescence of S100A8 and S100A9 Subunits

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 fluorimetric measurements [26]. The fluorescence of a protein is caused by three intrinsic fluorophores present in the protein, i.e., the Trp, Tyr and Phe residues. Actually, the intrinsic fluorescence of many proteins is mainly contributed to by tryptophan [27]. There are several aromatic residues present in each subunit of the human calprotectin (Trp54, Tyr16, Tyr19, Tyr30, and Tyr54 in S100A8, and Trp88 and Tyr22 in S100A9 [28]. A significant reduction in the fluorescence intensity of the human S100A8/A9 complex was seen (Fig. 3), reflecting a conformational change within the protein, or quenching of fluorescence emission of the aromatic residues in the presence of calcium might indicate the exposure of aromatic residues to the solvent. Therefore, the emission spectra of human calprotectin in the presence of calcium suggest a change in the tertiary (or quaternary) structure and exposure of the hydrophobic groups of the protein to the solvent.

Table 1. Percentages of secondary structures in the S100A/A9 complex in the absence and presence of Ca^{2+}

	Secondary structures, %		
Group	α-sheet	β- structure	other structure
S100A8/A9	36.8 ± 2.4	14.7 ± 5.2	50.0 ± 0.8
S100A8/A9 + Ca^{2+}	28.6 ± 1. 6	19.7 ± 3.3	52.2 ± 2.6

Thermal Denaturation Analysis

Determination of the Gibbs free energy of denaturation (ΔG°), as a criterion of conformational stability of a globular protein, is based on the theory of two states as follows:

$$\text{Native (N)} \Leftrightarrow \text{Denatured (D)}.$$

This theory was developed by Pace et al [29, 30]. The process was described as a single denaturant-dependent step according to the two-step theory [31]. The denaturation process can be monitored based on the changes in the absorbance at 280 nm. Then the fraction of the denatured protein (Fd) as well as the equilibrium constant of the process (K) can be calculated using Equations 1 and 2, respectively:

$$\text{Fd} = (Y_N - Y_{obs})(Y_N - Y_D) - 1, \quad (1)$$

$$K = \text{Fd}(1 - \text{Fd}) - 1 = (Y_N - Y_{obs})(Y_{obs} - Y_D) - 1, \quad (2)$$

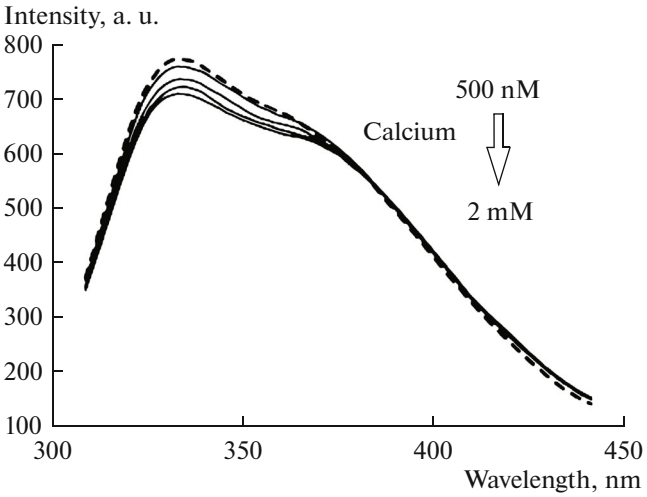

Fig. 3. Intrinsic fluorescence emission spectra of r-S100A8/A9 complex in the absence (– –) and presence of Ca^{2+}. The spectra were obtained from 0.15 mg/mL protein sample dissolved in a 100 mM NaCl, 25 mM NaH_2PO_4 buffer at pH 6.5 at room temperature.

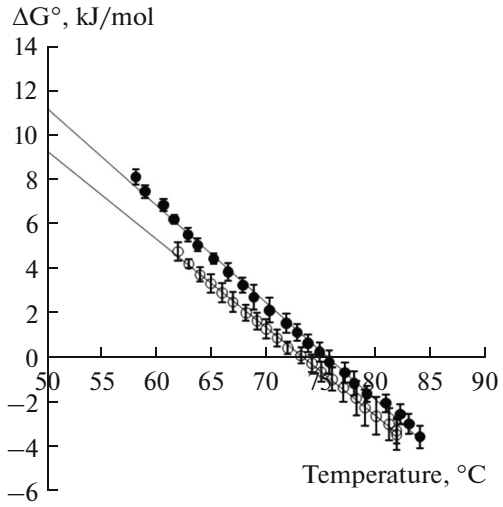

Fig. 4. Free energy changes for thermal denaturation of the S100A8/A9 complex in the absence (○) and presence of Ca^{2+} (●). The Gibbs free energy change ($\Delta G°$) was obtained by the equation $\Delta G°$ is the absolute temperature.

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

$$\Delta G° = -RT\ln K, \quad (3)$$

where R is the universal gas constant and T is the absolute temperature. By plotting $\Delta G°$ versus temperature, protein stability at any temperature, for example, at room temperature of 25°C ($\Delta G°$ 298), can be obtained [32]. Figure 4 shows the free energy changes versus temperature in the S100A8/A9 complex in the absence and presence of Ca^{2+}. The thermodynamic parameters are calculated and summarized in Table 2. The obtained results reveal that, thermal stability parameters (ΔG and T_m) of r-S100A8/A9 have increased in the presence of calcium (Figs. 4, 5).

The Gibbs free energy change ($\Delta G°$) was obtained using the equation $\Delta G° = -RT\ln K$, where R is the universal gas constant and T is the absolute temperature. By plotting $\Delta G°$ versus temperature, protein stability at any temperature, ($\Delta G°$), can be obtained.

DISCUSSION

In this study, the S100A8 and S100A9 subunits were expressed and purified successfully. They showed different secondary and tertiary structures in their sole forms, but their incubation with calcium induced the dimerization of the two subunits and changes in structural and the thermodynamic stability. Although studies support that each subunit may have its own individual functions, the general consensus is that the protein function is dependent on the heterodimer formation [28]. It is reported that homo-oligomeric forms of S100A8 and S100A9 are readily degraded by proteases, but the hetero-oligomeric S100A8/A9 complex displays a high resistance even against proteinase K degradation [33, 34]. Thermal denaturation of the dimer complex showed higher magnitudes of ΔG 25°C and Tm of the calcium-bound protein in comparison with its free form (Table 2). Binding to calcium is modulated by two EF-hands in each subunit. EF-hands have a helix-loop-helix structure and form stable structural domains in interacting pairs and typically undergo a conformational change upon Ca^{2+} binding [17]. There is a large change in the position of the helix HIII upon Ca^{2+}-binding [34]. The inter helical angle between helices HIII and HIV changes by 90° in S100B compared to the Ca^{2+}-free structure, opening the structure and exposing the residues required for target recognition and binding. This is also described as a change from the closed conformational state in the absence of Ca^{2+} to the open conformational state in its presence, as indicated for calmodulin and troponin C [17]. As indicated in this study, the calcium containing complex induced a conformational change in the dimer or an increase in the hydrophobic patch. As a result, two large hydrophobic surfaces are exposed for

Table 2. Thermodynamic parameters of thermal stability analysis of S100A8/A9 complex in the absence and presence of Ca^{2+}

Group	T_m, °C	ΔG^U, kJ/mol
S100A8/A9	73.4 ± 1.3	29 ± 1.2
S100A8/A9 + Ca^{2+}	75.6 ± 0.98	33 ± 0.2

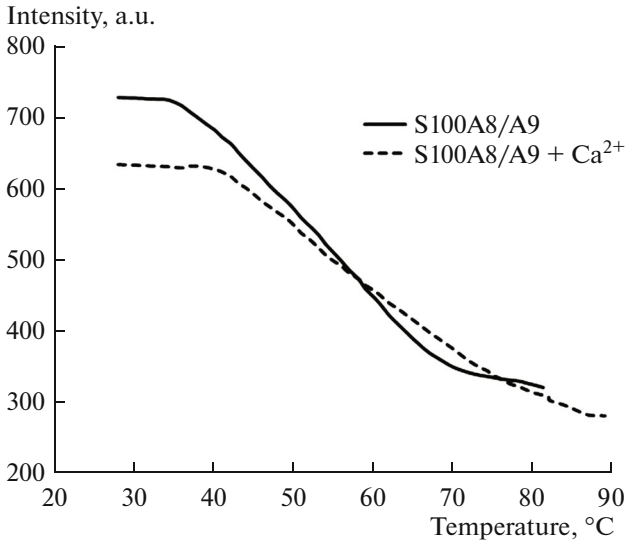

Fig. 5. Thermal denaturation profiles of S100A8 and S100A9 and the S100A8/A9 complex in the absence (–) and presence of Ca^{2+} (– –).

target interaction. The alteration of the molecular surface and the electrostatic potential provides several mechanistic explanations for how the calcium-binding domain could be involved in mediating resistance to bacterial invasion [10]. Recently a new property of S100A8 and S100A9 was discovered—their ability to self-assemble into highly heterogeneous amyloid complexes, encompassing both oligomeric species and highly stable fibrils able to grow and accumulate in the ageing prostate [12]. Our results demonstrated a minor shift in helical content transition in to β-sheet (data not shown). This was reported previously by Yousefi et al. as indicated in the mentioned study, this conformational change could induce calprotectin prone to aggregation at higher calcium concentrations [26]. Also the conformational transition, helix to β-sheet, is related to formation of amyloid plaques in some diseases like Alzheimer's, prion disease, aging prostate [13, 35, 36]. In prion disease, partial misfolding of PrP converts into a partially protease-resistant disease associated isoform, PrPc, which aggregates in the brain and forms deposits that are associated with the neurodegenerative changes [35].

It is identified that, the expression of S100A8 and S100A9 are enhanced in human prostate cancer. High intrinsic amyloid-forming capacity of the S100A8/A9 proteins may lead to their amyloid depositions in numerous ailments [12]. The calcification of amyloid deposits occurring in the prostate lead to their further stabilization, which is particularly important in the protease rich prostate fluid. Indeed, the mineral content of corpora amylacea indicates that calcification can be a regulating process. It is important to note that, S100A9 itself can serve as a promoter of the dystrophic calcification process as it has been shown in thermogenesis [37].

Our results revealed that, binding to calcium ions has an effect on secondary and tertiary structures of r-S100A8/A9 complex which might be necessary to obtain its biological function. Also thermal stability of the S100A8/A9 complex was elevated in the presence of calcium.

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