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NMR STRUCTURE NOTE

NMR structure note: the structure of human calcium-bound S100A11

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Biological context

Calcium is a necessary nutrient required in many important biological processes (Jaiswal 2001). The S100 proteins belong to a large family of the EF-hand calcium-binding proteins regulating a wide range of important cellular processes via protein–protein interactions (Schafer and Heizmann 1996). Calcium interactions with the EF-hand motifs result in a conformation change for target protein binding by the exposure of hydrophobic regions in S100 proteins (Smith and Shaw 1998). Most S100 proteins adopt a native conformation of homodimer via non-covalent interactions for their functions (Skelton et al. 1994). S100A11, also known as S100C, is a protein with potential functional roles of tumor promoter or tumor suppressor found in many tissues, including placenta, heart, lung, kidney, skeletal muscle and liver, and raised in several tumors (Inada et al. 1999; Rehman et al. 2004; Ohuchida et al. 2006). The S100A11 protein possesses multiple functions upon binding to different target proteins including annexin A1, annexin A2, the ATPase Rad54B, p53 and RAGE (He et al. 2009; Leclerc et al. 2009; Rezvanpour and Shaw 2009). RAGE (Receptor for Advanced Glycation Endproducts) is a multiple-ligand receptor on cell surface involving in several pathological diseases such as rheumatoid arthritis, cardiovascular diseases, diabetic neuropathy and Alzheimer's disease (Leclerc and Heizmann 2011). RAGE is a membrane protein composing of an extracellular N-terminal moiety and cytosolic C-terminal domain connected by a transmembrane helix (Fritz 2011). The human RAGE receptor possesses three N-terminal immunoglobulin domains, including V-domain, C1-domain and C2-domain, required for ligand recognitions (Koch et al. 2010). So far, many of the S100 proteins have been reported binding to the RAGE receptor (Leclerc et al. 2009; Fritz 2011; Yammani 2012). However, it's still not clear whether the S100A11 protein possesses specific-interaction with the RAGE V-domain. In the present study, we have accomplished the ^{1}H, ^{15}N and ^{13}C resonance assignments and the structure determination of calcium-bound human S100A11 dimer using a variety of NMR techniques. Furthermore, the interactions between S100A11 and the RAGE V-domain have been studied at the molecular level.

Methods and results

Protein expression and purification

The recombinant S100A11 have been constructed into vector pET20b and expressed as protein containing 105 amino acids without tag in *E. coli* BL21-CodonPlus. Isotopically labeled S100A11 proteins were prepared by growing the cells in M9 minimal media, containing $^{15}NH_4Cl$, either with $^{13}C_6$-D-glucose or $^{12}C_6$-D-glucose. 1.0 mM of Isopropylthio-β-D-galactoside (IPTG) was utilized for the induction of protein expression when the OD_{600} of culture reached 0.6–0.8 at 37 °C. The culture with additional 20 h of incubation with 200 rpm shaking at 25 °C was harvested by centrifugation at 6,000 rpm and 4 °C for 20 min. The cell pellets were resuspended by pH 8.0 sample-buffer (containing 25 mM Tris and 100 mM

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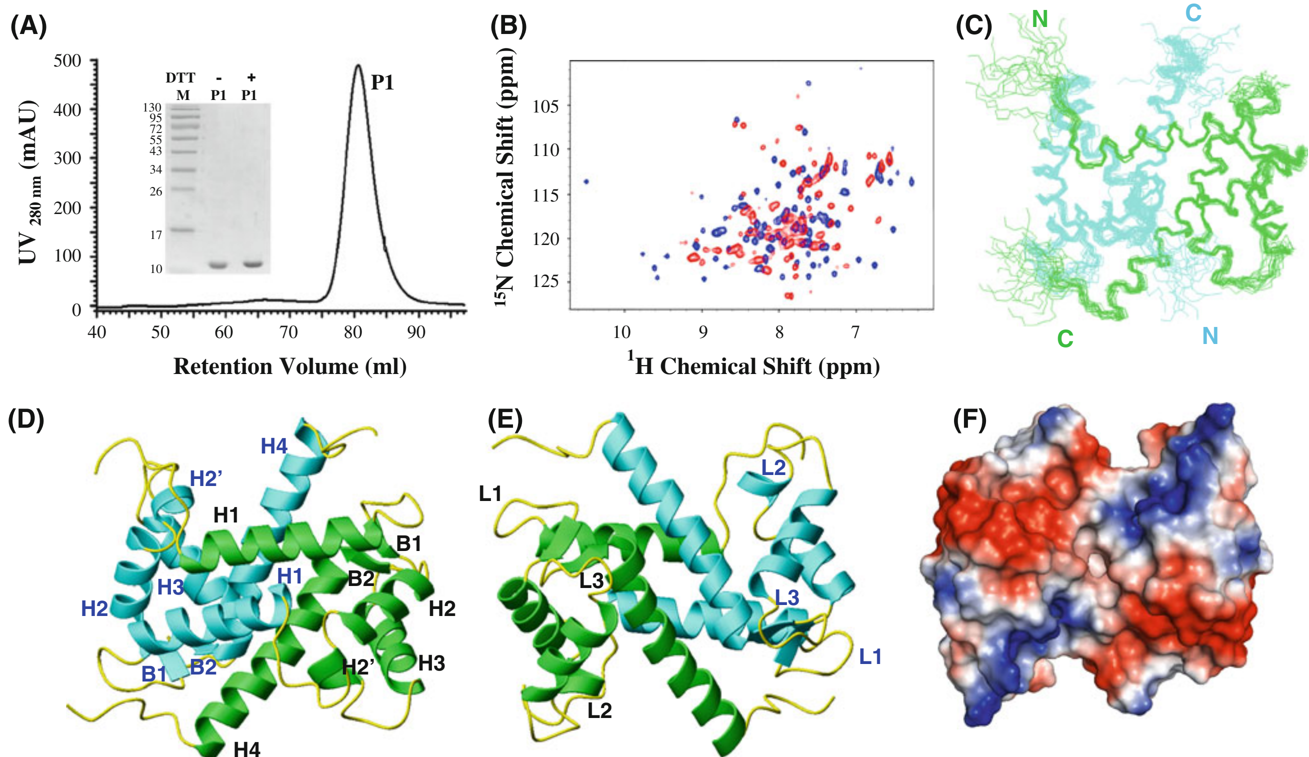

Fig. 1 **a** Size-exclusion chromatography of human calcium-bound S100A11 using a HiLoad 16/60 Superdex 75 column on an AKTA FPLC system (GE Healthcare) at pH7.5 and 25 °C. The *insert figure* shows the SDS-PAGE of elution peak P1 in the absence (−) and presence (+) of dithiothreitol (DTT). The results indicate that human holo S100A11 was eluted at a molecular size corresponding to a dimer without intermolecular disulfide bond. **b** ^{1}H-^{15}N HSQC spectra of 1 mM of the recombinant human calcium-bound (*blue*) and apo-form (*red*) S100 A11 acquired at pH7.5 and 25 °C. The spectrum of calcium-free S100A11 protein was obtained in the presence of 5 mM EDTA. **c** Overlay of backbone traces for the ensembles of 20 lowest-energy NMR structures of human holo S100A11 dimer (*green* and *cyan*). The N- and C-terminal ends are indicated as N and C, respectively. **d** Ribbon representation of the tertiary structure of human holo S100A11 dimer. The secondary structure elements of monomers in *green* and *cyan* are labeled in *black* and *blue*, respectively. **e** A 180° rotation of the view in (**d**). L1, L3 and L2 are loops connecting H1-H2, H3-H4 and EF-hand motifs, respectively. The same color codes shown in (**d**) are used. **f** Surface charge distribution of human calcium-bound S100A11 dimer shown in the same orientation as (**e**). The surfaces with negative charge, positive charge and hydrophobic are colored in *red*, *blue* and *white*, respectively

NaCl) with 5 mM $CaCl_2$. The cell lysis was performed by using a French press. After 30 min of centrifugation at 16,000 rpm and 4 °C, the S100A11 protein expressed in soluble form was loaded onto a Phenyl-Sepharose column (GE). The unwanted proteins were washing by pH 7.5 sample-buffers with decreasing concentration of $CaCl_2$ (5–1 mM). Elution of target protein was carried out using pH 7.5 sample-buffer containing 5 mM EDTA. The protein with ~90 % purity was exchanged against pH 7.5 sample-buffer with 5 mM $CaCl_2$ and further purified through a HiLoad 16/60 Superdex 75 column. The purity and identity of human S100A11 were checked by SDS-PAGE and confirmed by mass spectrometry.

NMR spectroscopy

All NMR experiments were acquired at 25 °C on Varian NMR 700 MHz or Bruker Avance 800 MHz spectrometers equipped with triple resonance cryogenic probes. 1 mM of ^{15}N and $^{15}N/^{13}C$-labeled protein samples were prepared in 25 mM Tris buffer (pH 7.5), 100 mM NaCl, 5 mM $CaCl_2$ and 10 % D_2O. Samples for the measurement of intermolecular NOEs were prepared by refolding of denatured $^{15}N/^{13}C$-labeled and unlabeled S100A11 mixture at 1:1 molar ratio. The combined information obtained from ^{1}H-^{15}N HSQC, ^{1}H-^{13}C HSQC, HNCA, HN(CO)CA, HNCO, HN(CA)CO, CBCA(CO)NH, HNCACB, ^{15}N-edited NOESY-HSQC, ^{13}C-edited NOESY-HSQC, HCCH-TOCSY, HCCH-COSY, HCC(CO)NH, CC(CO)NH and HBHA(CO)NH spectra were utilized for the resonance assignments of protein backbone and side chains. All 3D NOE experiments were acquired with a mixing time of 120 ms. NMR data were processed using software VnmrJ/Topspin and analyzed by software SPARKY (T. D. Goddard and D. G. Kneller, SPARKY 3.114, University of California, San Francisco).

Table 1 Structural statistics of human calcium-bound S100A11

Number of NMR restraints	
Intraresidual NOEs ($\|i–j = 0\|$)	691
Sequential NOEs ($\|i–j = 1\|$)	468
Short–range NOEs ($\|3 \geq i–j \geq 2\|$)	294
Medium–range NOEs ($\|5 \geq i–j \geq 4\|$)	126
Long–range NOEs ($\|i–j > 5\|$)	238
Intermolecular NOEs	182
Hydrogen bonds	29
Dihedral angles	166
CNS energies (kcal mol^{-1})	
E_{total}	−7,176.72 ± 246.57
E_{bond}	81.95 ± 6.27
E_{angle}	345.61 ± 20.86
E_{impr}	607.86 ± 91.24
E_{dihed}	1,054.18 ± 13.01
E_{vdw}	−658.12 ± 35.27
E_{elec}	−8,608.19 ± 163.65
R.M.S.D. from experimental constraints	
Distances (Å)	0.034 ± 0.002
Dihedral angles (deg.)	0.45 ± 0.12
Backbone in structured region[a] (Å)	0.35 ± 0.06
Heavy atoms in structured region[a] (Å)	0.97 ± 0.11
Backbone in whole protein (Å)	1.59 ± 0.42
Heavy atoms in whole protein (Å)	2.18 ± 0.35
Ramachandran plot (%)	
Most favored region	81.9
Additionally allowed	17.0
Generously allowed	1.1
Disallowed	0.0

[a] Residues in the structured regions: 9–26, 33–35, 36–46, 48–53, 57–67, 74–76, 77–98

Structure calculation

The NOE distance restraints for structure determination of dimeric protein were extracted from ^{15}N-edited NOESY-HSQC, ^{13}C-edited NOESY-HSQC and ^{13}C F1-filtered, F3-edited NOESY-HSQC spectra. The angle restraints of protein backbone including phi (ϕ) and psi (ψ) torsion angles were empirically predicted using the TALOS software program (Cornilescu et al. 1999). Hydrogen-bond restraints derived from the prediction of protein secondary structural elements using software CSI (Wishart and Sykes 1994) were introduced as a pair of distance restraints. Families of homodimeric structures were calculated by a simulated annealing (SA) approach and followed by a refinement procedure using software ARIA 2.3 with C2 symmetry parameters (Rieping et al. 2007). Non-bonded interactions were calculated by PARALLHDG force field. Torsion angle dynamics (TAD) was employed for the energy minimization in structure calculation. The initial-folded structures of dimeric protein were generated based on manually-assigned intramolecular and intermolecular NOEs. The unambiguous and ambiguous NOE restraints derived from outputs of previous ARIA run were analyzed and utilized as inputs for the next structure calculation. Lower ambiguous cutoff parameters and slow-cooling standard SA protocols were used in refinement steps of structure calculations. The structures with lowest overall energy were further calculated followed by water refinement in ARIA. The quality of calculated conformers was checked by analyzing the violations using MOLMOL and PROCHECK software programs (Koradi et al. 1996; Laskowski et al. 1996).

Structure determination of S100A11

The recombinant human S100A11 protein exists as a dimer in solution (Fig. 1a). The ^{15}N-^{1}H HSQC spectra of human S100A11 at pH 7.5 contain a single set of protein resonance with well-dispersed peaks, indicates that the recombinant protein is homodimeric and well-structured (blue peaks in Fig. 1b). Addition of 5 mM EDTA resulted in a significant change of ^{15}N-^{1}H signals (red peaks in Fig. 1b), indicating that the purified human S100A11 protein (blue signals in Fig. 1b) is in its calcium-bound form. The ^{1}H, ^{15}N and ^{13}C resonances of human holo S100A11 at pH 7.5 and 25 °C have been completely assigned and deposited in the BMRB under accession number 18425.

Based on the distance geometry calculation and energy minimization using ARIA software, the dimeric solution structure of human calcium-bound S100A11 was calculated with 3,202 experimental and empirical NMR restraints, including 3,007 NOE distance restraints, 29 hydrogen-bond distance restraints and 166 dihedral angle restraints. Figure 1c shows the superimposition of backbone traces of an ensemble of 20 structures with lowest energies selected from a set of 200 structures, revealing a good agreement with NMR restraints. A summary of structure statistics for the best 20 conformers of human holo S100A11 dimer is given in Table 1. The root-mean-square-deviation (RMSD) values of these structures were estimated to be 0.35 ± 0.06 and 1.59 ± 0.42 Å for the backbone atoms in structured regions and full length protein, respectively. The large RMSD of the full length protein is mainly due to the poorly defined N- and C-terminal regions in human holo S100A11 dimer. The Ramachandran plot analysis indicated that 81.9 % of residues are in the most favored region, 17 % in the additionally allowed region, 1.1 % in the generously allowed region and 0.0 % of residues are in the disallowed regions. The NMR restraints and coordinates of 20 best structures of human

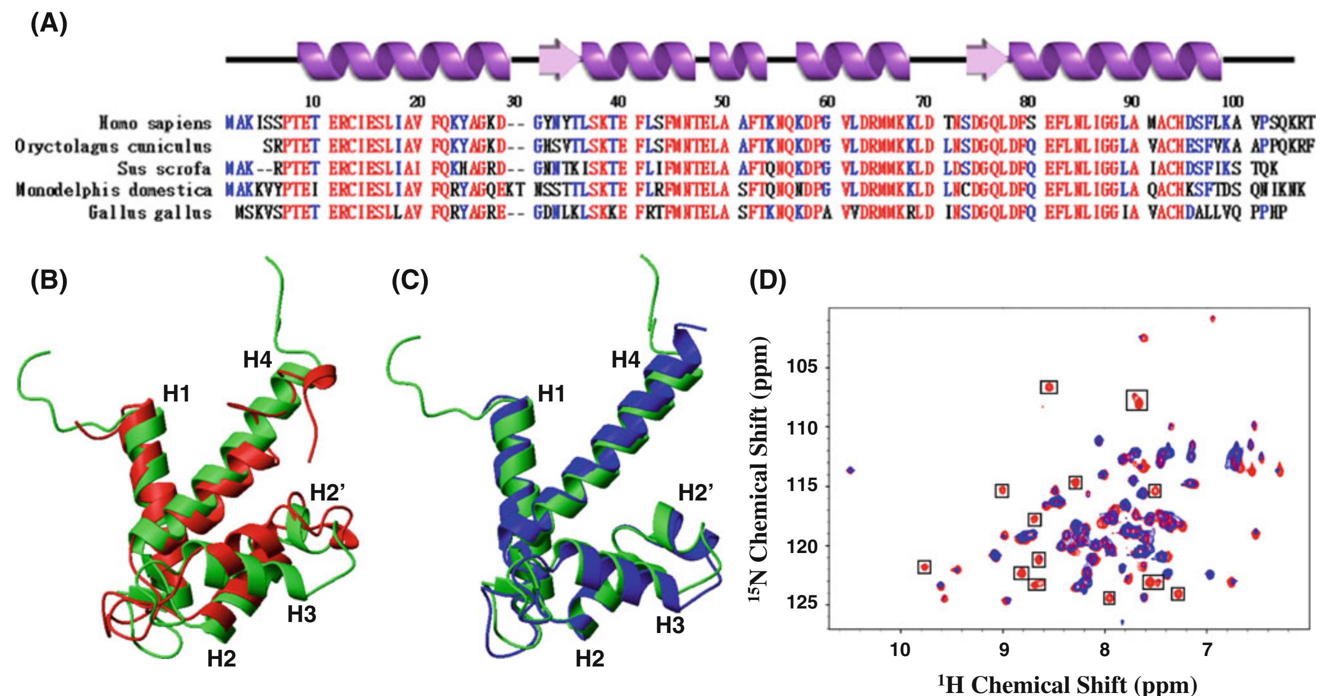

Fig. 2 **a** Sequence alignments of proteins in S100A11 family. The identities/similarities of S100A11 are scaled from 89 %/94 % to 74 %/84 % with 0–2 % gaps. *Arrows and spirals* at the top of the figure represent the secondary structural elements in human calcium-bound S100A11. **b** Structure comparison of human holo- (*green*) and rabbit apo- (*red*, PDB ID: 1NSH) S100A11 monomer. **c** Overlapping structures of human holo- (*green*) and pig holo- (*blue*, PDB ID:1QLS) S100A11 monomer. The secondary structure elements in human holo-S100A11 (*green*) are labeled. **d** ^{1}H-^{15}N HSQC spectra of human calcium-bound S100A11 titrated without (*red*) and with 1:1 molar ratio of unlabeled human RAGE V-domain (*blue*). The line broadening residues in S100A11 were boxed, indicating those residues interacted with RAGE V-domain molecule when complex is formed

calcium-bound S100A11 dimer have been deposited in Protein Data Bank (PDB ID: 2LUC).

Overview of the structure of human calcium-bound S100A11 protein

The NMR solution structure of human holo-S100A11 dimer reveals that each monomer comprises two β-strands (B1, a.a. 33–35; and B2, a.a. 74–76) and five α-helixes (H1, a.a. 9–26; H2, a.a. 36–46; H2′, a.a. 48–53; H3, a.a. 57–67; and H4, a.a. 77–98) in a H1-B1-H2-H2′-H3-B2-H4 topology. Two EF-hand motifs (helix-loop-helix), including H1-B1-H2 and H3-B2-H4, are connected by helix H2′/loop L2 (Fig. 1d, e). B1 and B2 are short β-strands forming an anti-parallel β-sheet between two EF-hand motifs. Residues near L1 and L3 possess highly negatively charged surfaces for specific calcium interactions (Fig. 1e, f). The sequence comparison of S100A11 proteins using Multalin (Corpet 1988) shows that residues close to L1 and L3, including Asp^{28}, Glu^{38}, Asp^{68}, Asp^{72}, Asp^{76} and Glu^{79} in human S100A11, are well-conserved as calcium ligands (Fig. 2a). Interestingly, residues showing poor similarity in the N- and C-terminal regions of S100A11 proteins possess a flexible unstructured property found in the structure family of human S100A11 (Fig. 2a, 1b).

The human S100A11 protein is highly homologous to rabbit (oryctolagus cuniculus) and pig (sus scrofa) S100A11 with 89 and 81 % identity, respectively (Fig. 2a). Compare the S100A11 monomer structure of human calcium-bound form (Fig. 2b, c, green color) with that of rabbit apo-form (Dempsey et al. 2003) (PDB ID: 2NSH; Fig. 2b, red color) and pig calcium-S100A11-annexin I complex (Rety et al. 2000) (PDB ID: 1QLS; Fig. 2c, blue color), the RMSD values of full length proteins are 2.247 and 1.372 Å, respectively. The results suggest that the S100A11 proteins adopt a similar structural fold for functions, however, calcium-S100A11 interactions extend the length of C-terminal helix H4 and induce a conformational change, enlarging the angle between helix H3 and H4 (Fig. 2b), and result in an exposure of residues essential for ligand/annexin I binding of S100A11 (Fig. 2c). Interestingly, annexin I interactions further promotes an open structure of helix H2′, H3 and H4 in the S100A11 protein binding to calcium (Fig. 2c).

Results of the structural analysis using PDBsum (Laskowski 2001) indicate that 39 residues are involved in the dimer interface of human calcium-loaded S100A11 protein, encompassing 14 hydrogen bonds and 468 non-bonded contacts, with an interface area of 1,824 Å^2. Nevertheless, unlike observations in the structure of calcium-S100A11-annexin I complex (Rety et al. 2000),

analyses of SDS-PAGE (Fig. 1a) and cysteine ^{13}C C_α/C_β chemical shifts (63.6/26.5 ppm and 62.5/27 ppm for Cys^{13} and Cys^{91}, respectively) (Sharma and Rajarathnam 2000) reveal that there is no disulfide bond found in calcium-bound S100A11 structure for protein dimerization.

Protein interaction of S100A11 with the RAGE V-domain

RAGE receptor is a membrane protein containing multiple domains on cell surface related to several pathological diseases. The interactions of RAGE with S100A11 have been reported, however, not at molecular level. The recombinant V-domain of human RAGE (a.a. 24–129) containing four extra N-terminal residues (GSHM) was prepared in pH7.5 sample-buffer with 5 mM $CaCl_2$ for the interaction studies (data not shown). Figure 2d reveals that addition of the unlabeled RAGE V-domain to the ^{15}N-labeled holo S100A11 protein at 1:1 molar ratio results in a significant peak broadening in the ^{15}N-^{1}H HSQC spectra. The corresponding residues mapped onto our solved structure reveal that most residues showing line broadening are located on helixes H2′ and H4, similar to the surfaces observed in the pig holo-S100A11 protein binding to annexin I (Rety et al. 2000), suggesting that a specific interaction was found between the human RAGE V-domain and human S100A11 in the presence of calcium, which undergoes an intermediate exchange binding kinetic on the NMR timescale. Our results for the first time report that V-domain is indispensable for the functions of RAGE receptor in complex with calcium-bound S100A11 protein.

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