Solution Structure of the Soluble Receptor for Advanced Glycation End Products (sRAGE)*

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The receptor for advanced glycation end products (RAGE) is a multiligand cell surface receptor involved in various human diseases, as it binds to numerous molecules and proteins that modulate the activity of other proteins. Elucidating the three-dimensional structure of this receptor is therefore most important for understanding its function during activation and cellular signaling. The major alternative splice product of RAGE comprises its extracellular region that occurs as a soluble protein (sRAGE). Although the structures of sRAGE domains were available, their assembly into the functional full-length protein remained unknown. We observed that the protein has concentration-dependent oligomerization behavior, and this is also mediated by the presence of Ca\(^{2+}\) ions. Moreover, using synchrotron small angle x-ray scattering, the solution structure of human sRAGE was determined in the monomeric and dimeric forms. The model for the monomer displays a J-like shape; whereas the dimer is elongated and forms through association of two N-terminal domains.

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Solution Structure of sRAGE

this can be related to signal transduction (16). Studies monitoring RAGE intracellular trafficking show that internalization of the complex receptor-ligand is essential to produce a cellular activation in response to AGEs (17). RAGE is also involved in triggering neurodegeneration in familial amyloidotic polyneuropathy, a neurodegenerative disorder characterized by extracellular deposition of transthyretin fibrils in different tissues, particularly in the peripheral nervous system. RAGE acts as a selective cell surface acceptor site for aggregated transthyretin, triggering inflammatory and oxidative stress pathways (14). Overproduction of RAGE and sRAGE was also described in patients with Alzheimer disease near the Aβ aggregates in the brain. The binding of soluble Aβ to sRAGE inhibits further aggregation, whereas membrane-bound RAGE-Aβ interaction elicits activation of NF-κB transcription factor promoting sustained chronic neuroinflammation (18). Interestingly, distinct regions of RAGE are involved in Aβ-induced cellular and neuronal toxicity, depending on the aggregation state (19).

RAGE interacts with several S100 proteins that are tissue- and cell type-specific (20). Binding of S100 to their target proteins is in most cases calcium-dependent and modulates their activity through RAGE. There is evidence that different S100 proteins may interact with distinct domains of RAGE (21). The most recent studies show that RAGE forms homodimers at the plasma membrane, a process potentiated by S100B and AGEs, which is essential for ligand recognition and signal transduction (22).

The three-dimensional structure of the independent V domain of sRAGE was determined by NMR, revealing that the overall surface of the molecule is positively charged and assumes a structure similar to those of other immunoglobulin V-type domains (23) (PDB code 2E5E). However, in the V domain of sRAGE, there are two distinct regions different from conventional immunoglobulin structures, namely an additional α-helix and the lack of two β-strands present in the conventional V domain. Moreover, on the surface of the V domain of sRAGE there is a distinguishable area where positive charges are densely localized to form a cationic center (23). Recently, two RAGE-associated crystal structures were released, namely the VC1 domain (24) (PDB code 3CJJ) and its complex with maltose (25) (PDB code 3O3U). Accordingly, VC1 was found to form a bent elongated structure with an angle of 145° between the two Ig domains (24). The interface between domains involves residues that are strictly conserved and form interdomain hydrogen bonds and hydrophobic interactions. The C1 domain has a unique topology, distinct from other Ig fold C domains, because it contains two additional β-strands (24). The NMR structures of the independent C2 domain (PDB code 2ENS) were also released. The structural organization of the entire sRAGE has remained unknown, although this is most important for a better understanding of how it is activated and functions in cellular signaling. In this study we determined the solution structure of sRAGE using small angle x-ray scattering (SAXS), and we investigated the effect of Ca²⁺ ions over the protein stability and oligomerization behavior.

EXPERIMENTAL PROCEDURES

Protein Expression and Purification—The human sRAGE, with an N-terminal fusion Trx-His₆-S tag separated by an enterokinase cleavage site in the pET32b expression vector (Novagen), was overexpressed in Escherichia coli strain Origami-(DE3) (Novagen). Cells were grown at 37 °C to a OD₆₀₀ ~0.8, adjusted to 20 °C for 30 min, induced with 0.5 mM isopropyl 1-thio-β-D-galactopyranoside, and allowed to express overnight. Cells were lysed at 4 °C in 20 mM Tris-HCl, 20 mM imidazole, 300 mM NaCl at pH 8.0 in the presence of lysozyme (50 μg/ml), followed by sonication (4 min with a 50% duty cycle). Lysates were centrifuged (25,000 × g, 20 min); supernatants were filtered and loaded onto a 5-ml HisTrap HP column (GE Healthcare) previously equilibrated with buffer A (20 mM imidazole, 300 mM NaCl, 20 mM Tris-HCl, pH 8.0). After a washing step with 5% buffer B (300 mM imidazole, 300 mM NaCl, 20 mM Tris-HCl, pH 8.0), the fusion protein (Trx-His₆-S-sRAGE) was eluted with a 6-column volume linear gradient to buffer B. Fractions containing His-tagged sRAGE were dialyzed against buffer A followed by buffer C (150 mM NaCl, 20 mM Tris-HCl, pH 8.0). Following dialysis, the Trx-His₆-S tag was removed by enterokinase (Invitrogen) cleavage (0.01 unit per mg of fusion protein) incubated at 20 °C for 18 h. After digestion, enterokinase was removed from the fusion protein digestion by EK-Away resin (Invitrogen) and dialyzed against buffer D (150 mM NaCl, 20 mM Na₂HPO₄, 300 mM NaHPO₄, pH 6.0). This was followed by separation of sRAGE from the fusion tag over a HiPrep 26/60 Sephacryl S-100 column (GE Healthcare) using buffer D. sRAGE was further purified on a Mono S column (GE Healthcare) using buffer D and an 18-column volume linear gradient from 0.15 to 1 M NaCl. Fractions containing sRAGE were dialyzed against buffer C and concentrated to 10 mg/ml. Protein concentration was determined by absorption at 280 nm using a theoretical 39,620 M⁻¹ cm⁻¹ molar extinction coefficient.

Dynamic Light Scattering (DLS) Analysis—DLS measures time-dependent fluctuations in the scattering intensity arising from particles undergoing random Brownian motion. They are measured across very short time intervals to produce a correlation curve, from which the particle diffusion coefficient and subsequently the particle size can be obtained. For monodisperse samples, the correlation curve can be fitted to a single exponential form (26), and the hydrodynamic radius (Rₘ) is calculated from the diffusion coefficient (D) using the Stokes-Einstein Equation 1, where k is the Boltzmann constant; T is the temperature, and η is the medium viscosity (26).

\[ Rₘ = kT/6πηD \]  

(Eq. 1)

By definition, the DLS measured radius is the radius of a hypothetical hard sphere that diffuses with the same speed as the particle under examination. The obtained hydrodynamic radius is an average value, weighted by particle scattering intensity. The size distribution obtained by DLS is a plot of the relative intensity of light scattered by particles in various size classes and is therefore known as an intensity size distribution. If the plot shows one peak with a substantial tail or more than one peak, the intensity size distribution must be converted to a volume size distribution for a more realistic view of the data,
considering the importance of the tail or a second peak (35). The polydispersity is the relative standard deviation and describes the width of the particle size distribution. A sample is considered monodisperse if the polydispersity is less than 20%; it is medium dispersed if this value is in the range of 20–30%; and it is polydispersed for values above 30%. Molecular size measurements were carried out in a Zeta sizer Nano Zs DLS system (Malvern Instruments) (27). sRAGE samples with concentrations of 1, 1.5, 3, 6, 7.5, 10, and 14 mg/ml in buffer C (20 mM Tris-HCl, 150 mM NaCl, pH 8.0) or buffer C + 40 mM CaCl₂ were centrifuged at 100,000 rpm for 30 min at 4 °C in an Airfuge® air-driven ultracentrifuge (Beckman Coulter). Then in a 45-μl DTS 2112 cuvette, three independent measurements were obtained at 20 °C for each sample. All data were then analyzed using DTS (nano) 6.01, the software for the instrument (27). The melting point of sRAGE (1 mg/ml) both in the presence and absence of 40 mM CaCl₂ was also studied by DLS. Temperature-dependent (from 20 to 65 °C) size measurements were obtained employing a 1 °C incremental temperature ramp and a 2-min equilibrium time at each point.

**Analytical Size-exclusion Chromatography**—For analytical size-exclusion chromatography, a Superose 12 10/300 column (GE Healthcare) was equilibrated with buffer C or buffer C + 40 mM CaCl₂ at 0.5 ml/min. The column was calibrated with protein standards of known Stokes radius (∙nm) as follows: ribonuclease (1.64), chymotrypsinogen A (2.09), ovalbumin (3.05), albumin (3.55), aldolase (4.81), and blue dextran 2000 (Amer sham Biosciences). The \( K_{av} \) parameter was determined according to the equation \( K_{av} = (V_e - V_o)/(V_t - V_o) \), where \( V_e \) represents the elution volume, \( V_o \) the void volume of the column, and \( V_t \) the total bed volume. Stokes radius (\( a \)) for the experimental data were calculated using: \((\log K_{av})^{1/2} = \alpha \) \( a \).

**Isokinetic Rate Zonal Ultracentrifugation**—For isokinetic rate zonal ultracentrifugation, two continuous gradients of 5–20% sucrose (Merck) were prepared in buffer C and buffer C + 40 mM CaCl₂. sRAGE and standard proteins of known sedimentation coefficient (\( S \)), ovalbumin (3.5), albumin (4.6), and ribonuclease (2.0), were applied on the top of gradients. Tubes were ultracentrifuged for 30 h and 30 min at 39,000 rpm in an Optima™ L-80 XP ultracentrifuge (Beckman Coulter) equipped with a Beckman SW41 Ti rotor at 4 °C. After ultracentrifugation, each gradient was fractioned, and each protein was precipitated in 10% trichloroacetic acid and analyzed by 12.5% SDS-PAGE. The gel was stained with Coomassie Blue, and protein distribution was analyzed by densitometry. sRAGE native molecular mass (M) and frictional ratio (\( f/f_o \)) in both conditions, in the presence and absence of 40 mM CaCl₂, were calculated according to the following: \( M = 6\pi\eta N As/(1 - \nu p) \) and \( f/f_o = a/(3\nu M/4\pi N)^{1/3} \), where \( \eta \) is the viscosity of the medium; \( N \) is the Avogadro’s constant, \( a \) is the Stokes radius, \( s \) is the sedimentation coefficient, \( \nu \) is the partial specific volume of the protein, and \( \rho \) is the density of the medium (28). Partial specific volume for sRAGE was estimated to be 0.7334 ml/g based on the protein’s amino acid composition using the method of Cohn and Edsall (29) and the program SEDNTERP, version 1.08.

**SAXS Measurements**—Solution x-ray scattering data were collected at X33 beamline (30) of the European Molecular Biology Laboratory (EMBL) in the storage ring DORIS III of the Deutsches Elektronen Synchrotron (Hamburg, Germany). The range of the momentum transfer \( q \) in the measurement was 0.1–5.0 nm⁻¹ \((q = 4\pi\sin(\theta)/\lambda, \) where \( 2\theta \) is the scattering angle and \( \lambda = 0.154 \) nm is the x-ray wavelength). Additional measurements were also obtained at the beamline cSAXS of Swiss Light Source (Villigen, Switzerland), with similar experimental parameters. sRAGE solutions with and without Ca²⁺ (40 mM CaCl₂) were measured, with protein concentrations varying from 0.9 to 15.2 mg/ml. The overall parameters, namely the forward scattering intensity \( I(0) \), radius of gyration \( R_g \), and maximum particle diameter \( D_{max} \) were obtained from the scattering patterns with programs PRIMUS and GNOM from the ATSAS program suite (31). Molecular mass was determined from the \( I(0) \) value by using bovine serum albumin (BSA) as a calibration standard (32).

The tertiary structure of sRAGE was modeled with the program BUNCH (33). A model combining the high resolution structures of the VC1 (24) and C2 (PDB code 2ENS) domains with the flexible linker and tail regions (residues 211–216 and 303–319, respectively) was constructed. The relative position and orientation of the VC1 and C2 domains and the conformation of the flexible regions was determined by minimizing the discrepancy \( \chi^2 \) between the scattering curve calculated from the model and experimental data. Modeling runs were done using a monomeric model, a dimeric model with an enforced \( C_2 \) symmetry and a tetramer, which was a dimer of the dimers obtained from dimer modeling.

**Thermal Shift Assay**—The thermal shift (Thermofluor) assays were done using SYPRO Orange, an environmentally sensitive fluorescence dye. The SYPRO Orange dye (Invitrogen) is delivered as a 5000 × solution in 100% (v/v) DMSO. The dye was first diluted into one of the two buffer solutions (buffer C or C + 40 mM CaCl₂) before adding the protein, to prevent damage of the protein because of interaction with high concentrations of DMSO. Solutions of 25 μl (1.8 and 0.9 mg/ml) of sRAGE in two buffer conditions (buffer C and C + 40 mM CaCl₂) and 25 μl (10 ×) of SYPRO Orange (in corresponding buffers) were added to a white 96-well PCR plate (Bio-Rad). Thus, the final concentration of sRAGE was 0.9 and 0.45 mg/ml, respectively, and the SYPRO Orange dye was diluted to 2.5 and 5×. For both conditions (with and without CaCl₂), four replicates were prepared. Six replicates were prepared for the two controls without protein: SYPRO Orange (2.5 or 5×) and DMSO (0.05 or 0.1%) in buffer C or C + 40 mM CaCl₂. The PCR plate was sealed with Optical-Quality Sealing Tape (Bio-Rad) and centrifuged at 1500 rpm for 1 min to remove bubbles. The thermal shift assay was then performed in an iCycler iQ5 Multicolor Real Time PCR detection system (Bio-Rad) running the following protocol: heating from 30 to 65 °C with a 30-s hold time every 0.5 °C, followed by a fluorescence reading using Cy3 dye filter (excitation/emission, 545/585). The melting curves were analyzed using the CFX Manager software (Bio-Rad) which calculates the \( T_m \) from the maximum value of the first derivative curve of the melting curve. In the same conditions, similar Thermofluor assays were performed with 1 and 2 mg/ml samples of hen egg white lysozyme (Sigma), a control protein that does not specifically bind Ca²⁺.
Fluorescence Spectroscopy—The intrinsic tryptophan and ANS fluorescence assays were performed in a Horiba Fluoro-max-5 fluorimeter. Both assays were carried out with three replicates of 20 μM of sRAGE in the presence and absence of 40 mM of CaCl₂. Fluorescence emission spectra of tryptophans of sRAGE were measured in the 300–450-nm interval using 295 nm as the excitation wavelength (4 nm excitation and emission slits, 0.1-s integration time).

ANS fluorescence assay was carried out with 300 μM ANS, and the emission spectra was measured in the 400–700-nm interval using 385 nm as the excitation wavelength for ANS (5 nm excitation and emission slits, 0.1-s integration time).

RESULTS
sRAGE Homogeneity State—The protocol used for expression and purification of the recombinant protein provided 12–18 mg/liters of 95% pure sRAGE. The homogeneity of the obtained protein was assessed by DLS. Because the data obtained from our measurements (size distributions by intensity) show a substantial tail, especially at higher concentrations (see Fig. 1A), the plots of the size distribution by intensity were converted to volume distribution (see Fig. 1B) for a more accurate interpretation of the data. The RH values were therefore determined from the volume distributions (see Tables 1 and 2). The DLS data obtained at different sRAGE concentrations showed one major population (see Fig. 1A); however, the high polydispersity values (30–34%) indicate a broad size range within this population, suggesting the presence of multiple species. Interestingly, this high polydispersity (%Pd) value decreased dramatically at high concentrations of sRAGE (10 mg/ml), when CaCl₂ was added to the protein sample (see Table 1). Titration of the protein with CaCl₂ showed that the %Pd of sRAGE (10 mg/ml) reached its lowest value (18%) in the presence of 40 mM CaCl₂ (see Table 1). Thus sRAGE (10 mg/ml) in presence of 40 mM CaCl₂ becomes monodisperse, and the RH size of the molecule decreases (see Fig. 1A, B, C, and Table 1). The effect of 40 mM CaCl₂ was further studied at different sRAGE concentrations. The RH values for all studied concentrations in the presence and absence of CaCl₂ were determined from the volume distributions (see Table 2). Moreover, these values were used to estimate the molecular weight (assuming a globular protein) at different concentrations (see Table 2), using an empirical calibration graph developed by Malvern Instruments available in the Nano software (27). The results show that at low protein concentrations (1 and 1.5 mg/ml), the sRAGE molecule in the absence and presence of 40 mM CaCl₂ has the same size (RH = 2.8 nm). This value corresponds to an apparent molecular mass of 37 kDa, which is consistent with sRAGE monomer (34 kDa). Although for these low concentrations the size distributions by volume show the same narrow size range in both conditions (see Fig. 1D), a high %Pd

![Distribution of hydrodynamic radii from DLS spectrum of sRAGE in the presence and absence of 40 mM CaCl₂ at (A and B) high, 10 mg/ml, and (C and D) low, 1 mg/ml, protein concentration.](image-url)

**TABLE 1**

| Concentration of CaCl₂ (mM) | RH (nm) | %Pd |
|----------------------------|---------|-----|
| 0                          | 5.84    | 33.6|
| 2                          | 5.05    | 28.5|
| 9                          | 4.36    | 23.0|
| 20                         | 3.90    | 20.8|
| 40                         | 3.76    | 18.0|
| 80                         | 3.76    | 22.8|

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value (30%) was obtained in the absence of CaCl₂, which decreased (22–25%) in the presence of 40 mM CaCl₂ (see Table 2). Here, the high flexibility of sRAGE monomer, and not the presence of different size species, is probably responsible for the high %Pd in the absence of CaCl₂. Increasing protein concentrations (3, 6, 7.5, and 10 mg/ml) in the absence of CaCl₂ led to higher size values and consequently higher apparent molecular weights for sRAGE (see Table 2). These data indicate that different oligomeric species appear when protein concentration is increased and suggest a concentration-dependent oligomerization of sRAGE (above 1.5 mg/ml). The high %Pd values (33–34%) and the broad size range (see Fig. 1A) at these concentrations, in the absence of CaCl₂, are consistent with these results. 10 mg/ml is the highest protein concentration that we could achieve in the absence of CaCl₂, and above this concentration, we observed protein precipitation. The oligomerization observed above 1.5 mg/ml protein concentrations is prevented or reduced in the presence of 40 mM CaCl₂, as indicated by the lower %Pd and %Pd values (see Table 2). Thus, in the presence of CaCl₂, higher oligomeric species (dimers) appear only above 3 mg/ml protein concentration. The DLS data show that at 10 and 14 mg/ml concentrations, in the presence of 40 mM CaCl₂, sRAGE forms almost only dimers (really low %Pd value, see Table 2). The 6 and 7.5 mg/ml sRAGE sample in the presence of CaCl₂ is probably a mixture of monomers and dimers (see the %Pd and M_app values in Table 2). In the case of 14 mg/ml sRAGE sample, the CaCl₂ was added before protein concentration.

To investigate further the hydrodynamic properties and association state of sRAGE, we used a method specially designed for determination of the molecular mass of nonglobular proteins (28), which combines Stokes radius (obtained from gel filtration experiments) and sedimentation coefficients (derived from density gradient centrifugations). The Stokes radius (Rₛ) and sedimentation coefficient (s) of sRAGE at a low protein concentrations (below 1 mg/ml) were determined in the absence (2.81 nm and 2.19 S) and presence (3.48 nm and 1.79 S) at 40 mM CaCl₂. These parameters were used to calculate the molecular mass of the native protein, which was estimated to be 26.42 kDa in the presence and 26.10 kDa in the absence of 40 mM CaCl₂. Thus, the molecular weight of sRAGE in both conditions is consistent with a monomer (34 kDa), considering the errors associated with the evaluation of the molecular weight using this procedure. Moreover, from the frictional ratio values obtained in the absence (f/f₀ = 1.3) and in the presence of 40 mM CaCl₂ (f/f₀ = 1.6), we inferred that the protein shape becomes more elongated with the addition of CaCl₂.

Small Angle X-ray Scattering—Table 3 shows the basic SAXS parameters determined for sRAGE in the presence and absence of 40 mM CaCl₂ (see supplemental Fig. 1). The apparent molecular weight and size of sRAGE increase with the concentration of the sample (see Fig. 2 and Table 3), indicating concentration-dependent oligomerization of the protein. At low concentrations (1.2 mg/ml and below), the apparent molecular weight and size of sRAGE matches the monomer (34 kDa), and when the protein concentration increases, the observed size grows to values matching the higher oligomeric species. In the presence of 40 mM CaCl₂, sRAGE remains monomeric at concentrations up to 5.2 mg/ml, and above this concentration the observed size corresponds to dimeric species (see Fig. 2 and Table 3). In Fig. 2, the apparent molecular weights determined by SAXS and DLS are presented in the same graph as a function of protein con-

### TABLE 2

| Concentration mg/ml | %Pd sRAGE | %Pd sRAGE + CaCl₂ | %Pd sRAGE | %Pd sRAGE + CaCl₂ |
|---------------------|-----------|------------------|-----------|------------------|
| 1                   | 30.0      | 25               | 2.80      | 2.80             |
| 1.5                 | 29.7      | 22               | 2.80      | 2.80             |
| 3                   | 33.2      | 28               | 3.25      | 3.25             |
| 6                   | 34.2      | 25               | 4.36      | 4.36             |
| 7.5                 | 34.8      | 26               | 5.05      | 5.05             |
| 10                  | 33.6      | 18               | 5.84      | 5.84             |
| 14                  |           | 22               |           |                  |

### TABLE 3

Overall SAXS parameters of sRAGE in the presence and absence of 40 mM CaCl₂

| Concentration mg/ml | M_w sRAGE | M_w sRAGE + CaCl₂ | D_max sRAGE | D_max sRAGE + CaCl₂ | R_g sRAGE | R_g sRAGE + CaCl₂ |
|---------------------|-----------|------------------|-------------|------------------|-----------|------------------|
| 0.9                 | 29.0      | 22.0             | 13.0        | 13.0             | 4.0       | 4.0              |
| 1.2                 | 35.0      | 22.0             | 13.5        | 13.5             | 4.2       | 4.2              |
| 2.4                 | 49.0      | 33.0             | 15.9        | 15.9             | 4.8       | 4.8              |
| 3.7                 | 33.6      | 22.0             | 13.0        | 13.0             | 4.05      | 4.05             |
| 4.4                 | 34.7      | 22.0             | 13.0        | 13.0             | 4.04      | 4.04             |
| 5.2                 | 104.0     | 35.0             | 23.5        | 23.5             | 4.30      | 4.30             |
| 6.5                 | 35.9      | 22.0             | 14.5        | 14.5             | 4.39      | 4.39             |
| 8.4                 | 43.6      | 22.0             | 15.0        | 15.0             | 4.54      | 4.54             |
| 10.5                | 58.0      | 22.0             | 16.2        | 16.2             | 4.90      | 4.90             |
| 11.7                | 47.3      | 15.0             | 15.0        | 15.0             | 4.73      | 4.73             |
| 15.2                | 50.2      | 22.0             |           |                  |           |                  |

The following abbreviations are used: M_w, apparent molecular mass; D_max, maximum particle diameter; and R_g, radius of gyration obtained from the p(r) function.
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Concentration dependence of apparent molecular weight of sRAGE, determined by SAXS (○, ◦) and DLS (□, □), in the presence (●, ●) and absence (●, ◦) of 40 mM CaCl₂. The horizontal dashed line shows the molecular mass of the monomeric sRAGE, 34 kDa.

The scattering curves from the samples with an apparent molecular mass corresponding to monomeric and dimeric species were modeled by BUNCH using a combined rigid body-dummy residue chain fitting. All curves were modeled assuming monomeric and dimeric models, as described under “Experimental Procedures.” The results confirmed the conclusions obtained from the basic scattering parameters. Lower χ² values (i.e. better fits) were obtained when the oligomeric state of the model (monomer or dimer) matched the oligomeric state obtained from apparent molecular weight. The best χ² values obtained for each sample were within the 1.0–1.6 range, showing that the models reproduced well the features in the experimental scattering curves. Fig. 3 shows the SAXS data and model fits for the monomer and the dimer.

The models obtained from repeated modeling runs against the monomeric sRAGE, both with and without Ca²⁺, displayed a similar J-like shape (see Fig. 4A), within the experimental resolution (about 2 nm). The dimensions of the monomer are 12 × 7 × 4 nm, and the long axis of the C2 domain was tilted by ~80° with respect to the long axis of the V and C1 domains.

Elongated dimer models were obtained from sRAGE + CaCl₂ scattering data measured at the protein concentrations above 5.2 mg/ml. It was evident from the data that the length of the dimer was equivalent to ~5 immunoglobulin-like domains. Because of the low resolution of the scattering data, it was not possible to determine the exact location of the binding contact, and it could be located between any of the two end domains, either V or C2. However, in a recent work it was demonstrated that the interaction between RAGE molecules occurs via the V domain and also that dimerization is essential for RAGE-mediated signal transduction (22). SPR experiments showed that sRAGE and the RAGE V peptide both bind RAGE in a concentration-dependent way (22). Using the available information, the sRAGE dimer was modeled assuming that the contact surface was at the interface between two V domains (see Fig. 4B). The dimer has a length of 20 nm, in agreement with approxi-
mate length of five immunoglobulin-like domains. The $\chi^2$ between experimental SAXS intensity and intensity calculated from the model is 1.2, showing that the model is in good agreement with the experimental data (see Fig. 3).

In the absence of Ca$^{2+}$ ions, and for protein concentrations above 5.2 mg/ml, sRAGE was best fitted with a tetramer model built from two dimers, showing again an elongated form with overlapping end domains. These models produced $\chi^2$ values varying between 1.7 and 2.3, showing incomplete fitting, most probably because of the presence of several oligomeric states at these conditions.

The set of scattering patterns from sRAGE in the presence of Ca$^{2+}$ at various concentrations was also analyzed using computed scattering curves from monomer, dimer, and tetramer models. The experimental intensity was represented by a linear combination of model curves, and volume fractions of the components were calculated with the program OLIGOMER (34). This combination of models reproduced the experimental data well ($\chi^2<2.3$) and also when applied to an independent data set over a wide concentration range (2.4–15.2 mg/ml), which was not used to derive the models ($\chi^2<5.2$). In this independent concentration series, the tetramer volume fraction was found to be 0 in all concentrations, and a linear increase of the dimer volume fraction as a function of sRAGE concentration was observed (supplemental Fig. 2).

**Conformational Stability of sRAGE Monomer**—The fluorescence-based thermal shift assay was used to check the stability effect of CaCl$_2$ over the sRAGE monomer. Results from DLS and SAXS showed that the sRAGE is in a monomeric form at low concentrations. Therefore, the Thermofluor assays were performed at protein concentrations as low as 0.45 and 0.9 mg/ml, in the presence and absence of 40 mM CaCl$_2$ using 2.5$\times$ and 5.0$\times$ concentrated SYPRO Orange dye. The results showed an increase of 4.5°C in the melting point of sRAGE monomer, for both proteins and SYPRO Orange concentrations, in the presence of 40 mM CaCl$_2$, which changes from 46.5 to 51.0°C (see Fig. 5). The Thermofluor assay performed with hen egg white lysozyme, in the same buffer conditions of sRAGE, shows an increase of only 1.5°C in the protein melting point, in the presence of 40 mM CaCl$_2$ (supplemental Fig. 3).

The effect of Ca$^{2+}$ on the thermal stability of sRAGE monomer was further tested using DLS. In fact, the denaturation of a protein under the influence of heat is visible in light scattering studies due to a significant increase in both size and scattering intensity. The melting point ($T_m$) is the marked point where both the size and the intensity start to increase significantly (35). The melting point data measured by DLS at low (1 mg/ml) protein concentration shows an increase of 4.0°C (from 43.5 to 47.5°C) for the temperature associated with the denaturation of sRAGE monomer in the presence of CaCl$_2$ (supplemental Fig. 4). This shift of 4.0°C measured by DLS is consistent with the shift of 4.5°C obtained with Thermofluor assays. These results support that Ca$^{2+}$ stabilizes the sRAGE monomeric form.

To probe for possible conformational modifications of sRAGE monomer in the presence of CaCl$_2$, intrinsic tryptophan fluorescence was used. The data were measured for sRAGE monomer (0.7 mg/ml) in the presence and absence of 40 mM CaCl$_2$. The results show an increase of 25% in fluorescence intensity in the presence of 40 mM CaCl$_2$ (supplemental Fig. 5), indicating the existence of a conformational alteration. To test if these alterations resulted in an increase in the hydrophobicity of the protein surface, the binding of the hydrophobic probe ANS to the sRAGE monomer was monitored by fluorescence spectroscopy. However, no changes in ANS fluorescence emission were observed for the sRAGE monomer (0.7 mg/ml) when 40 mM CaCl$_2$ was added to the protein solution (data not shown).

**DISCUSSION**

sRAGE is composed of three extracellular immunoglobulin-like domains (one V (variable) and two C (constant) domains; C1 and C2). It is known that the C2 domain of sRAGE has significant rotational freedom in relation to the VC1-integrated structural unit (8, 24). In fact, the purified monomeric (1 mg/ml) sRAGE presented a high polydispersity (30%) when studied using DLS, and this value decreased (25%) in the presence of 40 mM CaCl$_2$, which is consistent with the existence of an independent domain, whose orientation in relation to the VC1-interacting domains varies and in the presence of Ca$^{2+}$ becomes more tightly bound to the protein. The high level of polydispersity of sRAGE in the absence of CaCl$_2$ for all the
tested protein concentrations is consistent with a lack of structural stability and a tendency to aggregate. The stabilization effect of Ca\(^{2+}\) was further confirmed by measuring the melting point of the protein using DLS and thermal shift assay, in the presence and absence of Ca\(^{2+}\).

RAGE interacts with structurally different ligands, and this may happen in the monomeric form or through the oligomerization (16) or dimerization (22) of the receptor on the cell surface. The exact molecular mechanisms involved in each interaction are still unknown. DLS and SAXS experiments show a concentration-dependent oligomerization of sRAGE (above 1 mg/ml, higher oligomeric species appear in addition to the monomers). The oligomerization is significantly reduced by the addition of CaCl\(_2\) to the protein. The oligomerization state can be followed by fitting the form factors obtained by SAXS. Thus sRAGE in the presence of 40 mM CaCl\(_2\) is a monomer up to a 5 mg/ml protein concentration, and at higher concentrations the dimer/monomer ratio increases so that at 10 mg/ml sRAGE with 40 mM CaCl\(_2\) is mostly dimeric. This observation is consistent with the DLS results of sRAGE at high (10 mg/ml) concentration, showing a dramatic decrease of polydispersity (from 33.6 to 18%) and size (from 5.84 to 3.76 nm) in the presence of 40 mM CaCl\(_2\). The SAXS data measured at the concentrations up to 5.0 mg/ml with CaCl\(_2\) and 1.0 mg/ml without it allowed us to build a low resolution model for the sRAGE monomer, revealing that it has a J-shape with the angle between the long axes of VC1 and C2 units at around 80° (see Fig. 4A).

Despite the fact that the entire linker region between VC1 and C2 units (amino acids 211–216) was allowed to be flexible during the modeling, the hinge-like movement between the two subunits appears rather restricted. The C-terminal (tail) region connected to the C2 domain shows a larger variability in the modeling and probably does not have a specific fold in solution. The limited accuracy of the data due to the low concentrations of protein that had to be used in the absence of CaCl\(_2\), to avoid aggregation, did not allow for a meaningful analysis of the effect introduced by the positive charges of Ca\(^{2+}\) on the shape of the protein. However, we obtained biochemical evidence that the monomer becomes more elongated in the presence of Ca\(^{2+}\) and shows an increase in tryptophan fluorescence, revealing a conformational alteration corresponding to the exposure of one or more of these amino acids to the solvent. It is known that immunoglobulin-like domains have a sandwich structure with two \(\beta\)-sheets linked by a disulfide bridge and a tryptophan packed against the disulfide bond. The three-dimensional model of sRAGE (see Fig. 4A) clearly shows the predominant features for the three domains as follows: the V domain has a disulfide bond established between Cys-38 and Cys-99, and Trp-51 is packed against it; the C1 domain contains a disulfide bond between Cys-144 and Cys-208, with Trp-157 associated with it, and in the C2 domain, Cys-229 and Cys-271 are covalently linked with Trp-241 associated with the disulfide bond. Because sRAGE has six tryptophans (Trp-51, Trp-61, Trp-72, Trp-157, Trp-230, and Trp-241), we conclude that this difference in fluorescence observed upon de-addition of Ca\(^{2+}\) results from alterations occurring close to Trp-61, Trp-72, and/or Trp-230, because the other tryptophans belong to the core of the molecule. Although Trp-61 and Trp-72 are in the V domain and located on the surface of the V domain, Trp-230 lies in the linker region between the two C domains. It is therefore conceivable that in the elongated monomer the exposure of Trp-230 to the solvent is different, thereby increasing the fluorescence.

Recently, it was shown in vivo that RAGE forms homodimers, via V domain, on the plasma membrane, and this is an important step in receptor signaling following ligand binding (22). We also observed sRAGE dimerization in vitro, although it should be emphasized that in our experiments the protein concentrations were at least 1000-fold higher than the natural concentration of sRAGE in serum. We were able to construct a model for the dimeric sRAGE in solution. The overall shape is elongated, with a length of five Ig-like domains, revealing pronounced dimerization interface over the entire length of the V domain. Fig. 4B shows the sRAGE homodimer, and the side chains of amino acids involved in AGEs and S100B binding are highlighted.

The described cationic center of the V domain, consisting of Lys-39, Lys-43, Lys-44, Arg-48, Lys-52, Arg-98, Lys-104, Arg-
107, and Lys-110, was checked in relation to AGE binding activities (23). Although the binding was inhibited for six of the constructed mutants (K43A, K44A, R48A, K52A, R98A, and R104A), no large differences were detected for the other three (K39A, K107A, and K110A) (23). In Fig. 4B, the mutations leading to inhibition of complex formation, with AGEs, are deciphered in red, and the other mutations are presented in green. Interestingly, the red amino acids and in particular those corresponding to the triple mutant K43A/K44A/R104A, which abolishes AGE binding almost completely (23), are clearly located on the surface of our sRAGE dimeric model and is presented in brown (residues 54–67) of the V domain (25). This region is also located on the contact surface between the monomers or in the vicinity of amino acids, which are more important for AGE binding (red amino acids in Fig. 4B).

Previous isothermal calorimetry and deletion experiments indicate that the recognition of S100B by RAGE is dependent on residues in a loop (residues 54–67) of the V domain (25). This region is also located on the surface of our sRAGE dimeric model and is presented in brown in Fig. 4B.

Recently, it was also shown that both sRAGE and V peptide are capable of binding to intact RAGE, and it was found that these interactions inhibit RAGE homodimerization and signaling (22). These results indicated that RAGE signaling can be prevented by sRAGE not only through competitive ligand binding but also through blockade of intrinsic RAGE homodimerization (22). Based on the structure of sRAGE homodimer in solution, we envisage that interaction of sRAGE with RAGE will occur through the V domain as presented in Fig. 6. Moreover, this model indicates how the intact RAGE homodimer will form on the surface of the membrane (see Fig. 6). Although a different model for the interaction sRAGE/RAGE has been proposed (24), we have now structural data for the sRAGE dimer in solution, which indicates a different interaction between the V domains in the dimer.

In this work, we were able to improve our knowledge about the structural organization of sRAGE, in its monomeric and dimeric forms, and to demonstrate the importance of Ca2+ ions over the protein oligomerization behavior. Based on these models, it is now possible to design drugs that inhibit RAGE homodimerization, which is essential for signal transduction. Further work to analyze the interaction of sRAGE with the ligands and the intact RAGE will proceed. We plan to determine the three-dimensional structure of complexes formed by sRAGE and its natural ligands, and by using site-directed mutagenesis, we will delineate potential therapeutic strategies against the relevant diseases.

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