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Crystallization and preliminary X-ray diffraction analysis of human calcium-binding protein S100A12

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S100A12, a member of the calgranulin family, isolated from human blood, has been crystallized by vapour diffusion in the presence of Ca^{2+}. Crystals belong to the space group $R3$ with unit-cell dimensions $a = b = 99.6$ $c = 64.2$ Å. There are two monomers per asymmetric unit, with a solvent content of 57.9%. The crystals diffract to at least 2.2 Å resolution and complete X-ray data have been collected to 2.5 Å on a conventional laboratory source.

1. Introduction and biological relevance

Human S100A12 has been described as a novel protein of the S100 family (Guignard *et al.*, 1995). The protein belongs to the calgranulin subfamily, with 70 and 78% sequence identity with calgranulin C from pig granulocytes (Dell'Angelica *et al.*, 1994) and CAAF1 protein from bovine amniotic fluid, respectively (Hitomi *et al.*, 1996). A significant feature of these three related proteins is the low sequence identity of their C-terminal regions (Ilg *et al.*, 1996). The gene coding for S100A12 is localized on human chromosome 1q21.2–22 between two other S100 proteins, A8 and A9 (Heizmann, 1996; Schäfer *et al.*, 1995). The three proteins are expressed in granulocytes and monocytes (Kerkhoff *et al.*, 1998), but are not found in other blood cells or in tissue macrophages.

The biological functions of S100A12 are not clear. Like a few other S100 family members, S100A12 possesses extracellular activities and triggers various types of cellular activation (Zimmer *et al.*, 1995). It has been shown that ligation of the RAGE cellular receptor with S100A12 mediates activation of endothelial cells, macrophages and lymphocytes, cells which are central to the inflammatory response (Hofmann *et al.*, 1999).

Involvement of S100A12 in exocytosis has also been proposed (Shishibori *et al.*, 1999). Recently, it was found that three distinct anti-allergy drugs, amlexanox, cromolyn and tranilast, which inhibit IgE-mediated degranulation of mast cells, bind to S100A12 (Shishibori *et al.*, 1999). These studies indicate that S100A12 may play a significant role in the process of degranulation, which remains a poorly understood aspect of exocytosis, involving a large number of genes and their products. Exocytosis events such as formation of secretory vesicles, their movement to the plasma membrane and docking and fusion with plasma membranes all require interactions between proteins and between lipids and proteins. Calcium ions and calcium-binding proteins are major players in exocytosis (Lang, 1999). Calmodulin, annexins, calcyclin (S100A6) and other members of the S100 family are among the candidates for essential roles in several steps of exocytosis (Drust & Creutz, 1988; Chen *et al.*, 1999; Okazaki *et al.*, 1994). For S100A12 and two other calgranulins, S100A8 and A9, translocation to the cellular membrane upon neutrophil activation has been demonstrated (Guignard *et al.*, 1995). The mechanism by which these proteins penetrate the cellular membrane and how they are anchored into the membrane remain unclear, since they have no transmembrane region. In addition, the lack of a leader sequence makes the mechanism of secretion unclear. One of the putative secretion pathways is a protein kinase C and microtubule-network-dependent pathway, as shown recently for S100A8 and S100A9 (Rammes *et al.*, 1997). However, S100A12 acts individually during calcium-dependent signalling, independent of MRP8 (S100A8), MRP14 (S100A9) and the heterodimer MRP8–MRP14 (Vogl *et al.*, 1999).

Understanding the structural basis of the function of S100 proteins requires three-dimensional analysis. Structures of S100B (Matsumura *et al.*, 1998), S100A7 (Brodersen *et al.*, 1998) and S100A10 (Réty *et al.*, 1999) are available and all members of the S100 family share common structural motifs such as dimerization and EF-hands, while individual members possess unique features: S100B undergoes global conformational changes in the presence of calcium, S100A10 forms covalent tetramers and S100A7 has a zinc-binding site (Brodersen *et al.*, 1999). S100A12 is a non-typical member of the S100 family. Alignment of S100A12 proteins from different species reveals very low sequence identity between their helices IV and C-termini when compared with other family members. Biochemical and structural data indicate that

Table 1
X-ray data statistics.

Resolution (Å)	No. of unique reflections	$\langle I/\sigma(I)\rangle$	$I/\sigma(I) > 3$ (%)	Completeness (%)	$R_{merge}(I)$ (%)
25.00–5.37	807	18.0	88.1	97.1	4.1
5.37–4.27	811	15.5	88.4	98.9	5.5
4.27–3.73	815	13.9	86.7	97.7	6.2
3.73–3.39	791	10.9	80.6	97.2	7.7
3.39–3.15	816	9.1	74.9	98.4	9.4
3.15–2.96	796	5.9	60.0	97.8	14.6
2.96–2.82	814	4.5	53.0	98.8	19.3
2.82–2.69	809	3.4	42.6	98.2	22.6
2.69–2.59	804	2.6	33.1	97.5	31.3
2.59–2.50	710	1.8	17.3	85.7	37.1
Overall	7973	9.4	62.5	96.7	8.5

† $R_{merge}(I)$ is defined as $100 \times \sum |I - \langle I\rangle| / \sum I$.

helix IV and the downstream amino-acid residues are very important for dimerization and target recognition and suggest they play significant roles in the function of S100 proteins. No structure of S100A12 has been published, although calgranulin C from pig granulocytes has been crystallized and X-ray diffraction data have been reported to 2.6 Å resolution (Nonato *et al.*, 1997).

2. Methods and results

2.1. Protein purification and analysis

240 ml of buffy coat was diluted with 120 ml PBS (without calcium and magnesium), 40 ml 10% dextran T500 in PBS and 1.6 ml 0.5 *M* EDTA (final concentration 2 m*M*). After 30 min incubation at room temperature, buffy coat was centrifuged for 5 min at 2500 rev min^{-1}. The pellets were pooled, resuspended with 45 ml of water and mixed well with 1.4 ml 5 *M* NaCl. The suspension was centrifuged for 10 min at 4000 rev min^{-1}. The pellet was resuspended in 400 ml of the supernatant from the first centrifugation (containing lymphocytes) and was centrifuged for 10 min at 4000 rev min^{-1}. The pellet was then resuspended in 20 ml 20 m*M* Tris pH 7.4, 150 m*M* NaCl, 10 m*M* EDTA, frozen at 193 K and then thawed and frozen again twice. A cocktail of proteinase inhibitors was added (100 µg ml^{-1} PMSF, 80 µg ml^{-1} benzamidine, 1 µg ml^{-1} bestatin, 1 µg ml^{-1} pepstatin, 1 µg ml^{-1} aprotinin) and the suspension was sonicated. After sonication, the supernatant was clarified by centrifugation at 16 000*g* for 30 min. $CaCl_2$ was added to the supernatant to 15 m*M* and the supernatant was incubated on ice for 10 min and centrifuged for 10 min at 5000 rev min^{-1}. The collected supernatant was absorbed on a phenyl–Sepharose column equilibrated with 20 m*M* Tris pH 7.5, 150 m*M* NaCl, 5 m*M* $CaCl_2$. Bound proteins were eluted with buffer containing 10 m*M* EDTA. Protein fractions were collected, concentrated and loaded onto a Resource Q10 column (1 ml, Pharmacia), equilibrated with 20 m*M* Tris–HCl buffer pH 7.7, at room temperature. Bound proteins were eluted with an NaCl gradient. Eluted proteins, mainly S100, were identified by MALDI mass-spectrometry. A protein with M_W = 10 443 Da, corresponding to S100A12, was eluted at about 160 m*M* NaCl in 20 m*M* Tris–HCl buffer pH 7.7. The high resolution of the Resource Q column allows us to obtain pure protein suitable for crystallization studies directly after anion-exchange chromatography without preparative isoelectrofocusing, as described by Van den Bos *et al.* (1998), or affinity chromatography (Yamashita *et al.*, 1999).

2.2. Crystallization

Crystallization of S100A12 was carried out by hanging-drop vapour diffusion. Prior to crystallization, the purified protein was concentrated using a 10K ultrafiltration membrane (Filtron) to 5–8 mg ml^{-1} in 10 m*M* Tris–HCl pH 7.5. 1.2 µl drops of protein solution were mixed with 0.4 µl of the reservoir solution and equilibrated against 1 ml of the reservoir solution. The reservoir solution contained 20–25% PEG 5K monomethyl ether (Brzozowski, 1993), 0.2 *M* $CaCl_2$, 0.1 *M* sodium cacodylate pH 6.5. Crystals grew in 1–2 d and reached their final size of 0.1 × 0.1 × 0.2 mm in about a week (Fig. 1). Crystals were obtained for both S100A12 isolated from human blood and the recombinant protein, but to date the recombinant protein crystals only diffract to 8 Å resolution.

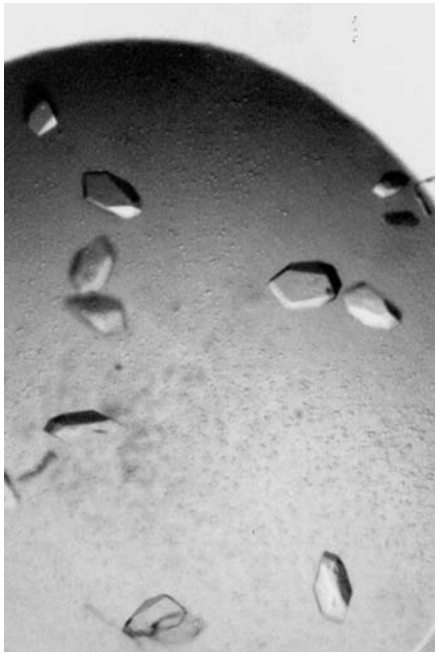

Figure 1
Crystals of native S100A12 protein from human neutrophils. Typical dimensions are about 0.1 × 0.1 × 0.2 mm.

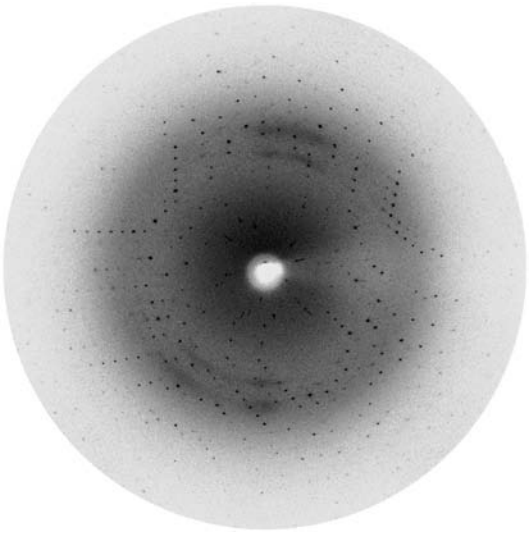

Figure 2
A 0.5° oscillation image of a typical partially twinned S100A12 crystal. The resolution at the edge of the diffraction pattern is 2.2 Å.

2.3. Crystallographic data collection

S100A12 crystals were characterized using Cu $K\alpha$ X-rays from a Rigaku RU-200 rotating-anode generator. The crystals were vitrified at 120 K using a cryocooling system (Oxford Cryosystems). The cryoprotectant solution contained 25% PEG 5K monomethyl ether, 0.2 *M* $CaCl_2$, 12.5% glycerol and 0.1 *M* sodium cacodylate pH 6.5.

Crystals belong to the space group *R*3, with unit-cell dimensions $a = b = 99.6$, $c = 64.2$ Å. Assuming two 10 443 Da monomers in the asymmetric unit, the specific volume V_M (Matthews, 1968) is 2.9 Å^3 Da^{-1} and the solvent content is 57.9%. The crystals diffract to at least 2.2 Å

(Fig. 2), but the best-diffracting crystals tend to be merohedral twins. Complete data have been collected to 2.5 Å resolution using an R-AXIS IIC imaging plate. Data were processed using the programs *DENZO* and *SCALEPACK* (Otwinowski & Minor, 1997). 17 318 individual measurements were reduced to 7973 unique reflections, giving an average redundancy for each reflection of 2.2. The X-ray data statistics are summarized in Table 1.

3. Future work

Structure determination using the molecular-replacement method is in progress using the structure of S100B (Matsumura *et al.*, 1998) as a starting model. It is proving difficult to collect data to higher resolution, because to date all the best-diffracting crystals have been twinned. We are continuing the search for non-twinned crystals by collecting partial data sets and analysing the cumulative intensity distribution. This is hindered by the small number of crystals that can be grown from the limited amounts of the non-recombinant human protein available. We therefore plan to 'de-twin' the data from a partially twinned crystal (Yeates & Fam, 1999). Knowledge of the 2.5 Å resolution structure will help in obtaining a good estimate of the degree of twinning. Knowledge of the three-dimensional structure of human S100A12 will play an essential part in locating the target-specific regions in different S100 proteins, in explaining the diversity of their biological functions and elucidating their molecular mechanisms.

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