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Purification, crystallization and preliminary X-ray diffraction studies on human Ca^{2+}-binding protein S100B

Thorsten Ostendorp,[a,b] Claus W. Heizmann,[b] Peter M. H. Kroneck[a] and Günter Fritz[a]*

[a]Fachbereich Biologie, Universität Konstanz, Postfach M665, Universitätsstrasse 10, 78467 Konstanz, Germany, and [b]Division of Clinical Chemistry and Biochemistry, Department of Pediatrics, University of Zürich, Steinwiesstrasse 75, 8032 Zürich, Switzerland

Correspondence e-mail: guenter.fritz@uni-konstanz.de

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S100B, a Ca^{2+}-binding protein, acts intracellularly as a Ca^{2+}-signalling protein but is also secreted to the extracellular space, acting in a cytokine-like manner through its receptor RAGE. Recombinant human S100B has been purified and crystallized in the Ca^{2+}-bound state. Size-exclusion chromatography indicates that S100B can exist as a dimer and as a multimer in solution. Crystals of S100B diffract to 1.9 Å and belong to space group $P2_1$, with unit-cell parameters $a = 63.4$, $b = 81.6$, $c = 71.5$ Å, $\alpha = 90$, $\beta = 107$, $\gamma = 90^\circ$. Preliminary analysis of the X-ray data indicate that there are four homodimers per asymmetric unit.

1. Introduction

S100B belongs to the S100 protein family, the largest subgroup within the EF-hand protein superfamily (Marenholz *et al.*, 2004). S100 proteins are small acidic proteins of 10–12 kDa containing two distinct EF-hand motifs connected by a hinge region and function as homodimers and heterodimers (Fritz & Heizmann, 2004). The C-terminal Ca^{2+}-binding site is composed of 12 amino acids and is homologous to the classical Ca^{2+}-binding loop common to all EF-hand proteins. The N-terminal Ca^{2+}-binding site comprises 14 amino acids and is characteristic for the S100 proteins. In humans, 20 different S100 proteins are known to date, which show cell-specific and tissue-specific expression patterns, reflecting specific biological functions: S100 proteins are involved in cell-cycle regulation, cell growth, cell differentiation and motility. Altered expression levels of S100 proteins are associated with diseases such as neurodegeneration, cancer, cardiomyopathy and chronic inflammation (Heizmann *et al.*, 2002). S100B is mainly expressed in the brain and involved in brain development, learning and memory function. Intracellularly, S100B acts as Ca^{2+}-signalling protein and binds Ca^{2+}-dependently to regulatory proteins such as p53 (Rustandi *et al.*, 2000) and Ndr-kinase (Bhattacharya *et al.*, 2003). Surprisingly, S100B was also found in the extracellular space. It has been shown that glioblastoma cells secrete S100B upon stimulus (Davey *et al.*, 2001) and that extracellular S100B acts in a cytokine-like manner through the receptor for advanced glycation end products (RAGE; Huttunen *et al.*, 2000). It was proposed that not the homodimeric but a multimeric form of S100B promotes its extracellular function (Selinfreund *et al.*, 1991). The structures reported so far for S100B revealed a homodimeric form of the protein which might reflect the intracellular state of S100B (for an overview, see Fritz & Heizmann, 2004). However, no structural information is so far available for a multimeric form of S100B. Furthermore, only the structure of Ca^{2+}-loaded bovine S100B has been determined by X-ray crystallography, whereas structures of Ca^{2+}-loaded S100B from rat and human have been determined by NMR spectroscopy. Although the sequences of the S100B proteins differ only in one or two amino-acid positions, the structural alignments yield rather large r.m.s.d.s of the C^α positions of between 2.2 and 3.4 Å. In view of the extracellular function, the putative different form of extracellular S100B and the deviations of structures available so far, S100B requires further structural characterization. Here, we report an optimized expression and purification protocol for human recombinant S100B, crystallization and preliminary X-ray analysis of S100B crystals.

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2. Material and methods

2.1. Protein expression and purification

The cDNA for human S100B was cloned into expression vector pGEMEX and S100B was expressed in *Escherichia coli* BL21(DE3). For expression, bacterial cells were grown at 310 K in 50 m*M* phosphate-buffered DYT medium pH 7.4 containing 0.2% glucose. Expression was induced by the addition of 1 m*M* IPTG at an optical density $OD_{600\,nm}$ of 0.6 and the culture was grown for 6 h. Cells were harvested by centrifugation at 4000*g* for 20 min. For protein purification, 15 g of cells were typically suspended in 30 ml 50 m*M* Tris–HCl, 5 m*M* $MgCl_2$ pH 7.6. A few crystals of DNAse I were added and cells were broken by two passages through a French press at 100 MPa. After cell breakage, 0.5 m*M* PMSF was added and the crude extract was centrifuged at 100 000*g* for 1 h. The supernatant was diluted with 50 m*M* Tris–HCl pH 7.6 and 2 m*M* $CaCl_2$ was added. The diluted supernatant was then loaded onto a phenyl-Sepharose column (100 ml volume; Amersham, GE-Healthcare) equilibrated with 50 m*M* Tris–HCl, 2 m*M* $CaCl_2$ pH 7.6. The column was washed with 20 volumes of the same buffer and bound S100B protein was eluted with 50 m*M* Tris–HCl, 5 m*M* EDTA pH 7.6. The obtained protein was concentrated by ultrafiltration and loaded onto a Superdex 75 column (Amersham, GE-Healthcare) equilibrated with 20 m*M* Tris–HCl, 150 m*M* NaCl pH 7.6. The collected protein was pure as judged by SDS–PAGE and by the typical UV spectrum of S100B. The protein was concentrated to 20–30 mg ml^{-1} and aliquots were flash-frozen in liquid nitrogen and stored at 203 K. Protein concentration was determined using the extinction coefficient $\varepsilon_{278\,nm}$ = 1520 M^{-1} cm^{-1}.

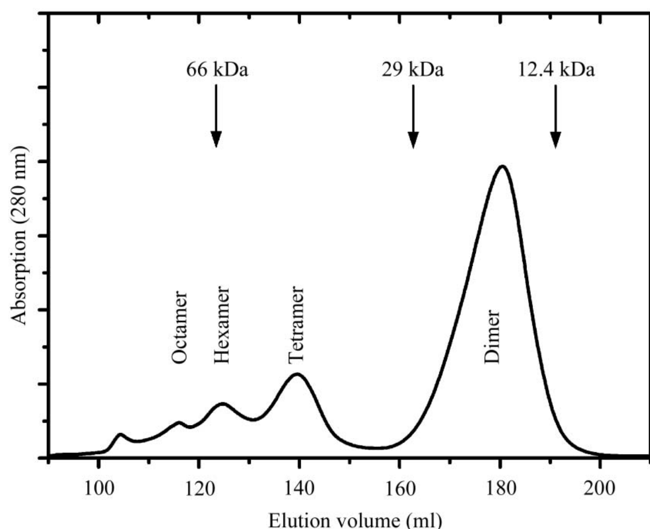

Figure 1
Elution profile of recombinant human S100B from a Superdex 75 size-exclusion chromatography column (Amersham, GE-Healthcare). The elution volumes of marker proteins are indicated by arrows.

Figure 2
Crystals of human recombinant S100B. Dimensions are 0.4 × 0.2 × 0.2 mm.

2.2. Protein crystallization and preliminary X-ray analysis

Prior crystallization buffer was changed to 10 m*M* Tris–HCl pH 7.6 using an NAP-5 column (Amersham) and the protein concentration was adjusted to 20 mg ml^{-1}. Crystallization of S100B was performed by sitting-drop vapour diffusion. 2 µl drops of protein solution were mixed with 2 µl reservoir solution (18–20% PEG 400, 0.2 *M* $CaCl_2$, 0.1 *M* HEPES pH 7.2) and equilibrated against 500 µl reservoir solution at 289 K. Data from the S100B crystals were collected in-house at 100 K using a rotating-anode Cu *Kα* X-ray source equipped with a MAR345 detector. The crystals were soaked for 5 s in cryo-protectant solution containing 30% PEG 400, 0.02 *M* $CaCl_2$, 0.1 *M* HEPES pH 7.2 and immediately flash-frozen in the cryo-nitrogen stream. The diffraction data obtained were processed with the program package *XDS* (Kabsch, 1988). The self-rotation function was calculated with *GLRF* included in the *REPLACE* program package (Tong & Rossmann, 1997)

3. Results and discussion

The expression levels of S100B in *E. coli* could be improved by the use of phosphate-buffered DYT instead of LB medium. The expression phase could be extended to 6 h instead of the standard 3 h, which gave about four times the amount of protein compared with standard expression. A two-step purification protocol was developed which typically yields 150 mg S100B from 15 g wet weight of cells. Since only fully folded S100B undergoes a conformational change

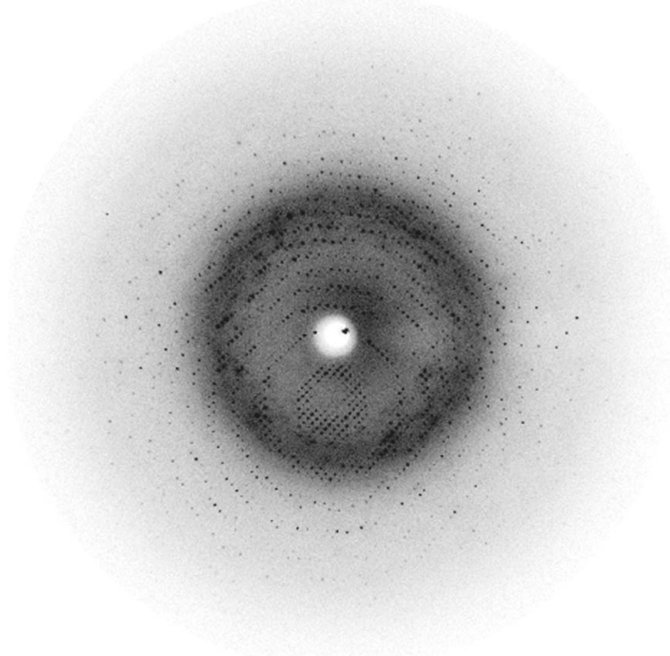

Figure 3
Typical diffraction pattern of a crystal of S100B.

Table 1
X-ray data statistics.

Space group	$P2_1$
Unit-cell parameters (Å, °)	a = 63.4, b = 81.6, c = 71.5, α = 90, β = 107, γ = 90
Resolution (Å)	50–1.9 (2.0–1.9)
Completeness (%)	99.0 (94.1)
No. of unique reflections	54400 (7334)
$R_{\rm merge}$ (%)	7.8 (31.5)
$I/\sigma(I)$	15.8 (4.9)
No. of molecules per asymmetric unit	8
Matthews coefficient (Å^3 Da^{-1})	2.07 (8 molecules perAU)
Solvent content (%)	40.5 (8 molecules per AU)

upon Ca^{2+} binding, the Ca^{2+}-dependent purification on phenyl-Sepharose yields only functional S100B molecules. S100B eluted from the Superdex 75 column in four major peaks corresponding to the molecular weights of dimeric, tetrameric, hexameric and octameric S100B, of which the peak corresponding to the homodimer contained about 80% of the total protein (Fig. 1). Only S100B from the dimer fraction was used for crystallization trials. Initially, plate-shaped crystals were obtained using a PEG 4000-based screen. Since bovine and human S100B differ by only three residues in the amino-acid sequence, we also tried to obtain crystals of human S100B by applying the crystallization conditions for bovine Ca^{2+}-loaded S100B. However, no crystals of human S100B were obtained under these conditions. Further crystallization trials at 289, 291 and 293 K revealed a large influence of temperature on the crystallization, in which the best and largest crystals were obtained at 289 K. In optimized crystallization trials S100B crystals grew within 10 d to typical dimensions of 0.4 × 0.2 × 0.2 mm (Fig. 2). Using home-source Cu $K\alpha$ X-rays the crystals diffracted to 1.9 Å (Fig. 3) and a complete data set was recorded. Statistics are given in Table 1. The space group was determined to be $P2_1$, with unit-cell parameters a = 63.4, b = 81.6, c = 71.5 Å, α = 90, β = 107, γ = 90°. The unit-cell parameters indicated the presence of more than one homodimeric S100B per asymmetric unit or an unusual high solvent content. The self-rotation function showed five peaks in the κ = 180° section with intensities between 26 and 39% of the intensity of the origin peak, indicating the presence of several twofold non-crystallographic axes (Fig. 4). Initial molecular-replacement trials using the structure of one subunit of bovine S100B (PDB code 1mho) as a search model gave no clear peak as a result of the rotation-function analysis. Trials using the structure of the S100B homodimer as a search model improved the results. Several approaches using *CNS* (Brünger *et al.*, 1998) or *AMoRe* (Navaza, 1994) finally showed that there were four equivalent peaks. The calculated electron-density maps are suitable for model building and refinement and clearly show the presence of four homodimers in the asymmetric unit. The solvent content was calculated to be 41%, with a Matthews coefficient of 2.1 Å^3 Da^{-1}. Although the results from size-exclusion chromatography (SEC) show the presence of multimeric S100B species, it remains unclear whether the packing of S100B in the crystal could reflect a multimeric state in solution. Since it has been proposed that the cytokine-like action of S100B arises from intermolecular disulfide bonds (Winningham-Major *et al.*, 1989), we analyzed S100B from dissolved crystals and from the high-molecular-weight fractions from SEC by non-reducing SDS–PAGE. However, no disulfide bonds were detected, indicating that S100B forms multimeric species without oxidation. The ongoing analysis of the structure of S100B might contribute to our understanding of how S100B forms such multimeric structures.

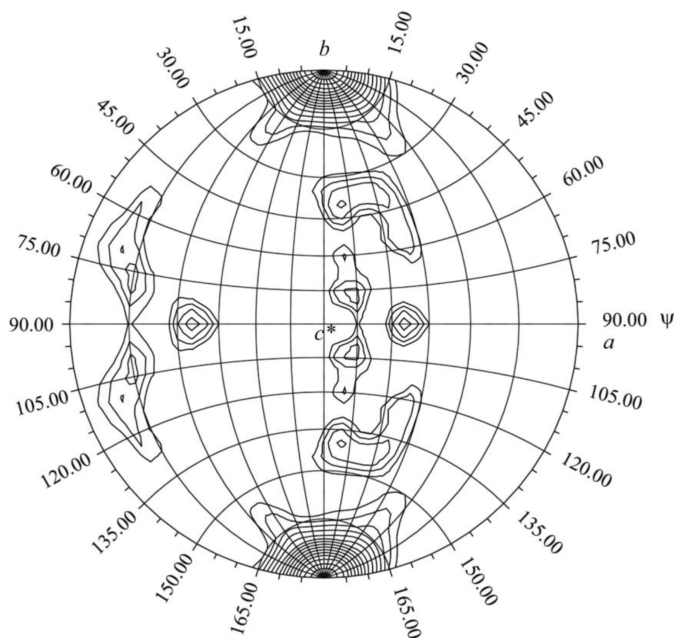

Figure 4
Projection of the κ = 180° section from the self-rotation function calculated using a resolution range of 12–5 Å and a integration radius of 15 Å. The circumference of the circle corresponds to φ = 0° and increases to the centre of the circle. Contour levels are plotted for all peaks greater than 20% of the origin peak in intervals of 5%.

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