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Purification and crystallization of the human EF-hand tumour suppressor protein S100A2

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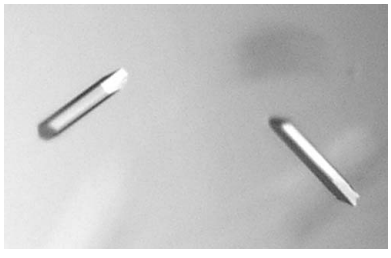

S100A2 is a Ca^{2+}-binding EF-hand protein that is mainly localized in the nucleus. There, it acts as a tumour suppressor by binding and activating p53. Wild-type S100A2 and a S100A2 variant lacking cysteines have been purified. CD spectroscopy showed that there are no changes in secondary-structure composition. The S100A2 mutant was crystallized in a calcium-free form. The crystals, with dimensions 30 × 30 × 70 µm, diffract to 1.7 Å and belong to space group $P2_12_12_1$, with unit-cell parameters a = 43.5, b = 57.8, c = 59.8 Å, $\alpha = \beta = \gamma = 90^\circ$. Preliminary analysis of the X-ray data indicates that there are two subunits per asymmetric unit.

1. Introduction

The S100 protein family constitutes the largest subgroup of EF-hand calcium-binding proteins. Recently, the 20th human member was identified (Marenholz *et al.*, 2004). They are small acidic proteins, with a molecular weight of 10–12 kDa, carrying a modified S100-specific EF-hand motif at the N-terminus (14 residues) followed by a classical C-terminal EF-hand motif (12 residues) linked by a hinge region. S100 proteins function as homodimers as well as heterodimers and show remarkable cell-specific and tissue-specific expression patterns, reflecting their involvement in widely different processes. These include cell-cycle regulation, cell growth, cell differentiation and motility (Heizmann *et al.*, 2002). Several human diseases, including chronic inflammation, cancer and neurodegenerative and cardiovascular disorders, are associated with the dysregulation of S100 proteins (Heizmann *et al.*, 2002). S100A2 is unique among the S100 proteins with regard to its predominant nuclear localization (Mandinova *et al.*, 1998). S100A2 was identified as a novel tumour suppressor in human mammary epithelial cells (Lee *et al.*, 1992) and recent studies have revealed that S100A2 binds p53 in a calcium-dependent manner (Mueller *et al.*, 2005). These findings directly link the tumour-suppressing activities of S100A2 with p53 and imply positive regulation of p53 through S100A2.

To date, there is no three-dimensional structure of S100A2 available. Multidimensional NMR studies revealed a molecular architecture similar to that of other S100 proteins (Randazzo *et al.*, 2001). It was shown that S100A2 exists in an equilibrium involving at least two isoforms, most probably owing to *cis–trans* isomerism of proline residues in the linker region and in the C-terminus (Randazzo *et al.*, 2001), which hampered the determination of the structure by NMR.

To date, high-resolution structural information from X-ray crystallography on the calcium-free form of S100 proteins is available only for S100A3 (Fritz *et al.*, 2002) and S100A6 (Otterbein *et al.*, 2002), whereas the majority of structures of S100 proteins represent the calcium-loaded form. To understand the molecular mechanism of the calcium-dependent conformational change in EF-hand proteins it is essential to characterize the three-dimensional structures of the calcium-free proteins. Here, we report an efficient method for expression and purification of recombinant human S100A2 similar to previously reported purification methods for other S100 proteins (Tarabykina *et al.*, 2000) and the crystallization and preliminary X-ray analysis of an S100A2 variant lacking cysteine residues.

2. Material and methods

2.1. Protein expression and purification

The cDNAs of S100A2wt and of S100A2-C2S-C21S-C86S-C93S (hereafter called S100A2ΔCys) in expression vector pMW162 (Stradal *et al.*, 2000) were a generous gift from Professor M. Gimona (Institute of Molecular Biology, Salzburg). *Eschericha coli* BL21(DE3) transformed with the corresponding plasmid was grown in 50 m*M* phosphate-buffered DYT medium pH 7.4 containing 0.2% glucose and 100 µg ml^{-1} ampicillin at 310 K. At OD_{600} = 0.6, expression was induced by the addition of 0.5 m*M* IPTG. After 3 h, cells were harvested by centrifugation at 6000*g* for 15 min. For protein purification, 10 g of cells were suspended in 20 ml 50 m*M* Tris–HCl, 5 m*M* $MgCl_2$ pH 7.6 with 0.5 m*M* PMSF. A few crystals of DNAseI were added and the cells were broken by two passages through a French press at 100 MPa. The crude extract was centrifuged at 100 000*g* for 1 h and the supernatant was diluted twofold with 50 m*M* Tris–HCl, 5 m*M* $CaCl_2$ pH 7.6. All following steps were carried out at 277 K. The supernatant was loaded onto a phenyl Sepharose Fast Flow column (100 ml, GE Healthcare) connected to an FPLC system. The column was equilibrated with 50 m*M* Tris–HCl, 5 m*M* $CaCl_2$ and washed with the same buffer until the absorption at 280 nm reached baseline. Bound S100A2 was eluted with 50 m*M* Tris–HCl, 10 m*M* EDTA pH 7.6. The protein was concentrated by ultrafiltration and loaded onto a Superdex 75 (26/60) column (GE Healthcare). Fractions containing S100A2wt or S100A2ΔCys were combined and the purity was controlled by SDS–PAGE and by the typical UV spectrum of S100A2. The proteins were concentrated to 20 mg ml^{-1} by ultrafiltration and aliquots were flash-frozen in liquid nitrogen. Protein concentration was determined using the calculated extinction coefficient ε_{278} = 2980 M^{-1} cm^{-1} (http://www.expasy.org/tools/#proteome). For oxidation kinetics, a sample of freshly reduced 200 µ*M* S100A2wt was incubated at room temperature and at several time points aliquots were removed and the cysteine thiol content was determined with DTNB in 50 m*M* Tris–HCl, 0.5 m*M* EDTA, 6 *M* GuHCl pH 8.3 using an extinction coefficient of 13 200 M^{-1} cm^{-1} per thiol (Riddles *et al.*, 1983). Native molecular weight was determined by size-exclusion chromatography. The column was calibrated with BSA (66 kDa), carboanhydrase (29 kDa), cytochrome *c* (12.4 kDa) and aprotinin (6.5 kDa) (Sigma) in the same buffer.

2.2. CD spectroscopy

Far-UV CD spectra of S100A2wt and S100A2ΔCys were recorded on a Jasco J-715 instrument. The main compartment of the instrument was flushed with dry nitrogen gas during the measurements. The spectra were recorded in 20 m*M* Tris–HCl, 5 m*M* $MgCl_2$ pH 7.6 in 0.10 and 0.01 cm quartz cells between 180 and 260 nm. The α-helical content was calculated with the program *CDNN* v.2.1 (Böhm *et al.*, 1992).

2.3. Protein crystallization and preliminary X-ray analysis

Prior to crystallization experiments, S100A2wt was incubated with an 80-fold excess of DTT for 2 h at 310 K to reduce all the cysteine residues. Metal ions were removed by the addition of 0.5 m*M* EDTA; both EDTA and DTT were removed by passage over an NAP5 desalting column (GE Healthcare) equilibrated with the chosen buffer. The protein concentration was adjusted to 10 mg ml^{-1}.

All crystallization experiments were carried out at 293 K. Crystallization experiments with S100A2wt were carried out under the exclusion of dioxygen in an anaerobic chamber filled with a 95% N_2/5% H_2 atmosphere (Coy Laboratory, Grass Lake, USA). For crystallization, 1 ml aliquots of crystallization solutions (JBS Screen Classic bulk 1–10, Jena Bioscience) were stored for at least four weeks in the anaerobic chamber in plastic vials until complete dioxygen to N_2/H_2 exchange was achieved. Dioxygen was removed from the S100A2 solution by passage through an NAP5 column equilibrated with dioxygen-free buffer. Crystallization of S100A2wt in the anaerobic chamber was performed by the hanging-drop vapour-diffusion method: 2 µl drops of protein solution (10 mg ml^{-1}) were mixed with 2 µl reservoir solution. Crystallization of S100A2ΔCys was achieved by robotic screening on the nanolitre scale using a nanodrop crystallization robot (Cartesian Technologies) and commercial crystallization buffers from Jena Bioscience (JBS Screen Classic bulk 1–10). For each crystallization buffer tested, a series of three drops was prepared by mixing 200 nl of the protein solution with three different volumes of the crystallization solution (100, 200 or 400 nl) and equilibrated by sitting-drop vapour diffusion against 100 µl of the crystallization solution in the reservoir. Final crystals of S100A2ΔCys were again grown by the hanging-drop vapour-diffusion method, mixing 2 µl drops of protein solution (10 mg ml^{-1}) with 2 µl reservoir solution and equilibrating against 500 µl reservoir solution (0.1 *M* sodium acetate, 10% 2-propanol, 35–40% PEG 4000). Data collection was carried out at the Swiss Light Source (Villigen, Switzerland) at beamline X06SA PX using a MAR 225 mosaic CCD detector (MAR Research). The diffraction data were processed with the *XDS* program package (Kabsch, 1993).

3. Results and discussion

The two-step purification protocol, which is similar to previous protocols for other S100 proteins (Tarabykina *et al.*, 2000), represents a gentle and fast method for the isolation of S100A2wt and S100A2ΔCys compared with the previously published protocol (Stradal *et al.*, 2000). Typical yields were 30 mg S100A2wt and up to 200 mg S100A2ΔCys from 2 l expression culture. In size-exclusion chromatography S100A2wt eluted as one major peak at a volume corresponding to a molecular weight of 22 kDa. The data show that the major fraction of S100A2wt formed the expected homodimer. Two further minor peaks eluting at smaller volumes also contained S100A2wt and represent a tetrameric and a larger multimeric species. Analysis of the free cysteine content of the fractions corresponding to

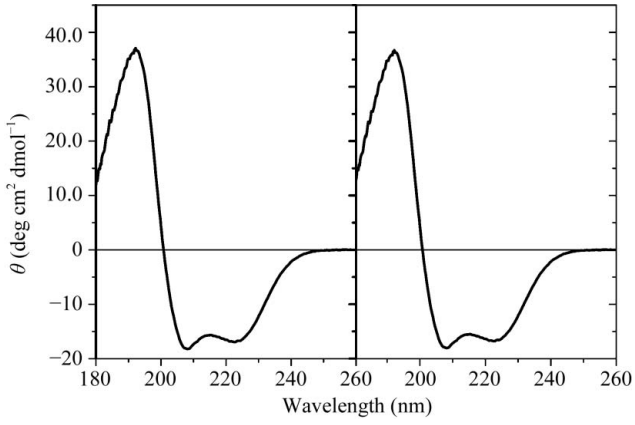

Figure 1
Secondary-structure analysis of S100A2wt and S100A2ΔCys by CD spectroscopy. (*a*) CD spectrum of S100A2wt. (*b*) CD spectrum of S100A2ΔCys. The spectra were recorded in 20 m*M* Tris–HCl, 5 m*M* $MgCl_2$ pH 7.6. Analysis of the spectra using *CDNN* revealed 51% α-helix, 9% β-sheet, 16% β-turn and 25% random coil for both proteins.

the homodimeric or multimeric forms of S100A2wt revealed that the dimeric protein has the correct content of eight cysteines per homodimer, whereas the high-molecular-weight species displayed a lower content. These results indicate that cysteine residues are involved in the formation of the high-molecular-weight species, most likely by the formation of intermolecular disulfide bonds. This finding was supported by the observation that in contrast to S100A2wt, S100A2ΔCys eluted in a single peak corresponding to the dimeric form.

The secondary-structure content of both proteins was analyzed by CD spectroscopy. The CD spectra of S100A2wt and S100A2ΔCys are virtually identical, exhibiting minima at 222 and 208 nm and a maximum at 192 nm typical of α-helical proteins (Fig. 1). Analysis of the spectra with *CDNN* v.2.1 (Böhm *et al.*, 1992) revealed a content of 51% α-helix, 9% β-sheet, 16% β-turn and 25% random coil for both proteins. Clearly, the cysteine-to-serine exchanges caused no major changes in the secondary-structure composition of the molecule.

The cysteine residues of S100A2wt are prone to oxidation, which leads to intermolecular and intramolecular disulfide formation, as shown by the analysis of high-molecular-weight fractions from size-exclusion chromatography. Analysis of the kinetics of the oxidation revealed that after 6 h at 300 K four out of eight cysteines per homodimer had reacted to form disulfides (Fig. 2). Thus, crystallization of S100A2wt in the presence of dioxygen is not feasible. Therefore, all crystallization experiments with S100A2wt were carried out with exclusion of dioxygen in an anaerobic chamber. After 5–10 d, small needles of S100A2wt were observed in 1.2 *M* sodium citrate, 0.1 *M* HEPES pH 8.0. However, several rounds of optimization trials did not result in larger crystals suitable for X-ray analysis. In order to overcome the problems of cysteine oxidation, we used a variant of S100A2 in which all cysteines had been replaced by serines (S100A2ΔCys). This enabled us to perform crystallization trials in the presence of dioxygen and robotic screening. The initial conditions found by robotic screening were refined in hanging-drop vapour-diffusion crystallization experiments. After 10 d, small crystals appeared which grew to dimensions of 0.3 × 0.075 × 0.05 mm at 293 K in 0.1 *M* sodium acetate, 10% 2-propanol, 35–40% PEG 4000 (Fig. 3). We tried to crystallize S100A2wt using similar conditions under the exclusion of dioxygen; however, these trials exclusively led to precipitation of the protein. Crystals of S100A2ΔCys were mounted in the crystallization buffer on nylon loops (Hampton Research) and were flash-frozen in the nitrogen cryostream (100 K).

Using synchrotron radiation, the crystals of S100A2ΔCys diffracted to 1.6 Å (Fig. 4) and a complete data set was recorded at 1.7 Å. Data statistics are given in Table 1. The space group was determined to be $P2_12_12_1$, with unit-cell parameters $a = 43.5$, $b = 57.8$, $c = 59.8$ Å, $\alpha = \beta = \gamma = 90^\circ$. Analysis of solvent content revealed that it is most likely that two subunits are present in the asymmetric unit. The solvent content of the asymmetric unit was calculated as 28.6%,

Table 1
X-ray data statistics.

Values in parentheses are for the outer resolution shell.

Space group	$P2_12_12_1$
Unit-cell parameters (Å, °)	$a = 43.5$, $b = 57.8$, $c = 59.8$, $\alpha = \beta = \gamma = 90$
Resolution (Å)	50–1.7 (1.9–1.7)
Wavelength (Å)	0.979
Completeness (%)	98.7 (98.1)
No. of unique reflections	31663 (8926)
R_{merge} (%)	10.4 (39.3)
$I/\sigma(I)$	12. 30 (3.47)
No. of molecules per ASU	2 subunits
Matthews coefficient (Å^3 Da^{-1})	1.72
Solvent content (%)	28.6

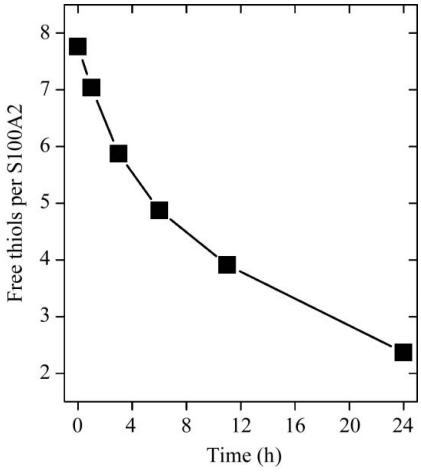

Figure 2
Oxidation kinetics of cysteines in S100A2wt.

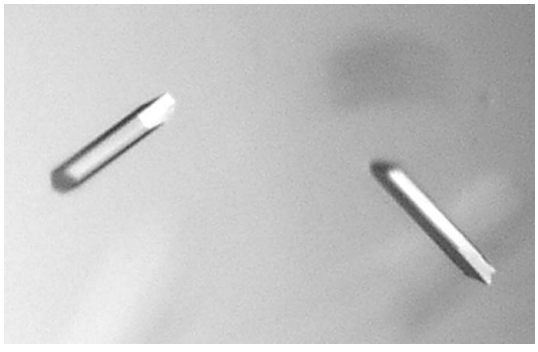

Figure 3
Crystals of recombinant human S100A2ΔCys. Crystal dimensions are 0.3 × 0.075 × 0.05 mm.

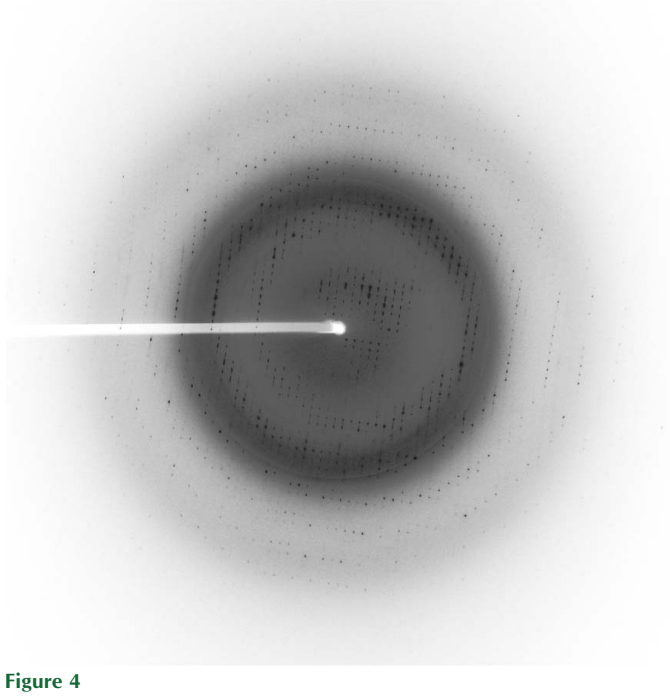

Figure 4
Typical diffraction pattern of a crystal of S100A2ΔCys.

with a Matthews coefficient of 1.72 Å^3 Da^{-1}. For molecular-replacement trials, a search model was created by threading the partial sequence of S100A2ΔCys (amino acids 5–93) on the crystal structure of calcium-free S100A3 (43% identical residues; PDB code 1kso; Fritz *et al.*, 2002) using *SwissPDBViewer* (Guex & Peitsch, 1997). In the resulting model of homodimeric S100A2ΔCys, only minor violations of side-chain distances were observed. After one round of energy minimization, the model containing 188 amino-acid residues was used without further modification. Molecular-replacement trials were carried out using the program *Phaser* v.1.3.1 (Storoni *et al.*, 2004; McCoy *et al.*, 2005), resulting in one solution (*Z* score of best solution 13.9; second solution *Z* score 10.9). The calculated electron densities appear to be suitable for model building and structure refinement. The ongoing analysis of the structure of S100A2 may provide further insights into the calcium-induced conformational change and recognition of target proteins such as p53.

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